Université de Montréal

ÉTUDE DE L'EXPRESSION DU GÈNE DE
L'ANGIOTENSINOGÈNE DANS UNE LIGNÉE CELLULAIRE
DE TUBULE PROXIMAL DE REIN DE RAT (IRPTC)

Par Shao-Ling Zhang

Département des Sciences Biomédicales
Faculté de Médecine

Mémoire présenté à la faculté des études
supérieures en vue de l'obtention
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Ce mémoire intitulé

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Présenté par:

Shao-ling Zhang

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Présidente-rapporteuse: Dr. Michele Gagnan

Directeur de recherche: Dr. John S.D. Chan

Codirecteur: Dr. Janos G. Filep

Membre du jury: Dr. Richard Beliveau

Mémoire accepté le:.........................

Love is the strongest and most fulfilling emotion possible.

This thesis is dedicated to my parents, my lovely daughter, and most especially, my dear husband.

Special thanks also to my relatives who helped me take care of my daughter.

Thank you for all of your moral support.

Résumé

De façon à étudier l'effet de fortes concentrations de glucose et d'insuline sur l'expression du gène de l'angiotensinogène (ANG) de rat au niveau du rein, des cellules tubulaires proximales immortalisées (IRPTC) ont été cultivées en monocouche. Les niveaux d'expression de la protéine ANG de rat et de son ARNm au niveau des IRPTC furent quantifiés par des analyses de radioimmunoessais spécifiques pour la protéine ANG de rat (RIA-rANG) ainsi que par des réactions de polymérisation en chaîne inversées (RT-PCR). De fortes concentrations de D(+)-glucose (25mM) ont permis une augmentation marquée de l'expression de l'IR-rANG et de son ARMm dans les IRPTC aussi bien que les niveaux intracellulaires de sorbitol, fructose, diacylglycérol (DAG) et de l'activité de la protéine kinase C (PKC). Cet effet stimulateur du D(+)-glucose (25mM) fut bloqué par un inhibiteur de l'aldose réductase, le Tolrestat. Des inhibiteurs de la PKC ont également inhibé l'effet stimulateur du D(+)-glucose (25mM) sur l'expression de l'IR-rANG dans les IRPTCs. L'addition de phorbol 12-myristat 13-acétate (PMA) a potentialisé l'effet stimulateur du D(+)-glucose (25mM) sur l'expression de l'IR-rANG dans les IRPTCs tout en bloquant l'effet inhibiteur du Tolrestat.

Par contre, l'insuline a inhibé l'effet stimulateur de fortes concentrations de glucose (25mM) et du PMA sur la synthèse de la protéine ANG ainsi que sur l'expression du gène ANG dans les IRPTCs. Cette action inhibitrice de l'insuline sur l'expression du gène ANG fut bloquée par le PD98059 (un inhibiteur des "mitogen-activated protein kinase kinases (MEK)) mais non par la Wortmannin (un inhibiteur de la phosphatidylinositol-3-kinase). Le PD98059 fut efficace dans l'inhibition de la phosphorylation de la protéine MEK1/2 et MAPK p44/42 dans les IRPTCs stimulées par l'insuline. Finalement, l'effet inhibiteur de l'insuline sur l'hypertrophie des IRPTCs fut annulé par le PD98059.

En conclusion, ces études démontrent que l'effet stimulateur de fortes concentrations de glucose (25mM) sur l'expression du gène ANG au niveau des IRPTC est médié, au moins en partie, par la synthèse *de novo* du DAG, un activateur de la voie de transduction de signal de la PKC. De son coté,

l'insuline prévient l'effet stimulateur de fortes concentrations de glucose sur l'expression du gène ANG rénal et de l'hypertrophie dans les IRPTCs, au moins en partie, via la voie de transduction de signal de la MAPK kinase (MEK). Ces études soulèvent la possibilité que l'expression rénale du gène ANG peut être stimulée par une hyperglycémie *in vivo*. Conséquemment, l'augmentation de la formation locale de l'ANG II rénale contribue à la remodélisation rénale (i.e. l'hypertrophie rénale dans le diabète précose). Une thérapie à base d'insuline pourrait dès lors atténuer cet événement en inhibant l'activation du système renin-angiotensin du rein.

ABSTRACT

To investigate the effect of high levels of glucose and insulin on the expression of the rat angiotensinogen (ANG) gene in the kidney, immortalized proximal tubular cells (IRPTC) were cultured in monolayer. The levels of expression of rat ANG and ANG mRNA in the IRPTC were quantified by a specific radioimmunoassay for rat ANG (RIA-rANG) and by a reverse transcription-polymerase chain reaction (RT-PCR) assay. High levels of D(+)-glucose (25mM) markedly increased the expression of IR-rANG and ANG mRNA in IRPTC as well as intracellular levels of sorbitol, fructose, diacylglycerol (DAG) and protein kinase C (PKC) activity. These stimulatory effect of D(+)-glucose (25mM) were blocked by an inhibitor of aldose reductase, Tolrestat. PKC inhibitors also inhibited the stimulitory effect of D(+)-glucose (25mM) on the expression of the IR-rANG in IRPTCs. The addition of phorbol 12-myristat 13- acetate (PMA) further enhanced the stimulatory effect of D(+)-glucose (25mM) on the expression of the IR-rANG in IRPTCs and blocked the inhibitory effect of Tolrestat.

In contrast, insulin inhibited the stimulatory effect of a high level of glucose (25mM) and PMA on the expression of ANG and the expression of the ANG gene in IRPTC. This inhibitory action of insulin on the ANG gene expression was blocked by PD98059 (an inhibitor of mitogen-activated protein kinase kinase (MEK)) but not by Wortmannin (an inhibitor of phosphatidylinositol-3-kinase). PD98059 was effective in inhibiting the phosphorylation of MEK1/2 and MAPK p44/42 in IRPTC stimulated by insulin. Finally, the inhibitory effect of insulin on the hypertrophy of IRPTC was revesed by PD98059.

In conclusion, these studies demonstrate that the stimulatory effect of high levels of glucose (25mM) on the expression of the ANG gene in IRPTCs is mediated, at least in part, via the *de novo* synthesis of DAG, an activator of PKC signal transduction pathway. Insulin prevents the stimulatory effect of high levels of glucose on the expression of the renal ANG gene and hypertrophy in IRPTCs, at least in part, via the MAPK kinase (MEK) signal transduction pathway. These studies raise the possibility that the expression of the renal ANG gene may be stimulated in hyperglycemia *in vivo*. Consequently, the

increased local formation of renal Ang II contribute to renal remodeling (i.e. renal hypertrophy in early diabetes). Insulin therapy may therefore attenuate this event by inhibiting the activation of local renal RAS.

Acknowledgments

I would like to express my deepest gratitude to my supervisor, Dr. John S.D. Chan, for the opportunity he has provided for me to study in the Laboratory of Molecular Nephrology and Endocrinology, and for his helpful academic direction, patient methodological advice, strict training, and generous help in all aspects.

I am also greatly indebted to my co-director, Dr. Janos G. Filep for the very kind academic direction and generous help in all aspects.

I would like to thank all members of the Research Center, Maisonneuve-Rosemont Hospital, and all members of our laboratory for the great convenience provided and the warm atmosphere created in the Center.

I would like to thank Dr. Kenneth D. Roberts in proof-reading my thesis.

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II. RESULTS (Unpublished Results)

List of Abbreviations

ACE	Angiotensin converting enzyme
ACE I/D	ACE Insertion/ Deletion
AGCF1	ANG core promotor binding factor 1
AGCE1	ANG core promotor element 1
AGE	Advanced glycosylation end-products
ANG	Angiotensinogen
Ang I	Angiotensin I
Ang II	Angiotensin II
Ang III	Angiotensin III
AT1	Angiotensin II type I receptor
AT2	Angiotensin II type II receptor
BB rat	Biobreeding spontaneously rat
CRE	cAMP responsive element
CREB	cAMP responsive element binding protein
DAG	1,2-diacylglycerol
DEX	Dexamethasone
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle's Medium
DN	Diabetic nephropathy
ECM	Extracellular matrix
ERE	Estrogen responsive element
ERK	Extracellular responsive kinase
ESRF	End stage renal failure
GAP	GTPase activating protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFR	Glomerular filtration rate
GH	Growth hormone
GLUT1	Glucose transporter 1
GRE	Glucocorticoid responsive element
H-7	1-(5-isoquinilinylsulfonyl)-2-methylpiperazine
IDDM	Insulin-dependent diabetic mellitus
IGF	Insulin-like growth factor
IP3	Inositol 1,4,5-triphosphate

IRPTC	Immortalized rat proximal tubular cell
IRS	Insulin receptor substrate
JG	Juxtaglomerular
JUK	c-Jun N-terminal kinase
kDa	KiloDaltons
mRNA	Messenger RNA
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
MBP	Maltose binding protein
MEK	Mitogen activated protein kinase kinase
NE	Norepinephrine
NIDDM	Non-insulin-dependent diabetic mellitus
NO	Nitric Oxide
OK	Opossum kidney
PCR	Polymerase chain reaction
PEPCK	Phosphoenolpyruvate carboxykinase
PI	Phosphatidyl inositol
PI3	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMA	12-O-tetradecanoylphorbol-13-acetate
PS	Phosphatidylserine
rANG	Rat angiotensinogen
RAS	Renin-angiotensin system
Ras	ras oncogene
RIA	Radioimmunoassay
ROS	Reactive oxygen species
Rsk	p90S6 kinase
SAPK	Stress-activated protein kinase
SHR	Spontaneously hypertensive rat
SOS	Son of sevenless
TGFß	Transforming growth factor-ß
THM	Tsukuba hypertensive mice
TRE	Thyroid hormone responsive element
VEGF	Vascular endothelial growth factor

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To whom it may concern

We, the undersigned, agree and consent that Madame Shao-Ling Zhang can use the following articles in her M.Sc. thesis entitled "Études de l'expression du gène de l'angiotensinogène dans une lignée cellulaire de tubule proximal de rein de rat (IRPTC)" to be submitted to the Faculté des études supérieures, Université de Montréal.

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I. INTRODUCTION

1. DIABETIC NEPHROPATHY

1.1 Statistics of Diabetic Nephropathy (DN) in North America

Nephropathy is a major cause of morbidity and mortality in diabetes mellitus (1-2). It is one of the most serious long term complications in diabetic patients.

Approximately 20% to 40% of the patients with insulin-dependent diabetes mellitus (IDDM) suffer this complication, compared to only 10% to 20% of those with non-insulin-dependent diabetes mellitus (NIDDM). Both groups are nearly equal in contribution to the total number of diabetic patients reaching end stage renal failure (ESRF). In total, it is estimated that 30% to 50% of diabetic patients in Canada and in the USA will eventually develop chronic renal failure (3).

1.2 Characteristics of Diabetic Nephropathy

The pathophysiological evolution and clinical characteristics of DN is similar in both IDDM and NIDDM. The early features of diabetic nephropathy include intraglomerular hyperfusion, micro-albuminuria, hypertrophy of glomerular and tubular structure as well as the thickening of glomerular and tubular basement membranes. During progression of the disease,accumulation of extra-cellular matrix components in the glomerular mesangium increases and eventually blocks or obliterates the glomerular capillary causing a decline in the glomerular filtration rate (GFR) (4-6).Furthermore, the development of tubulo-interstitial fibrosis and renal arteriosclerosis is often observed during progression of the disease (7-8).

Clinical DN (i.e. persistent proteinuria>0.5g/24h or >300 mg albumin/24h) is rare during the first 10 years of diabetes. Thereafter the incidence increases to a maximum of 2-3% per year after 13-20 years of duration. Although as many as 25%-50% of IDDM patients initially have increased kidney size and GFR, worsening of proteinuria and hypertension is accompanied by a progressive decline in GFR. Approximately 50% of patients with NIDDM may have modest

proteinuria and hypertension initially, but only 10% to 20% of NIDDM patients will develop ESRF(86).

2. The molecular mechanism(s) of the pathogenesis of DN

Multiple factors, including hemodynamic alterations (glomerular hyperfiltration and intrarenal hypertension), hyperglycemia, activation of the renin angiotensin system (RAS), genetic predisposition and various growth factors have been implicated in the pathogenesis of DN. However, the molecular mechanism(s) of action of these factors in the pathogenesis of nephropathy is far from being completely understood (9-11).

The follwing is a brief review of our current knowledge of the factors that might contribute to the pathogenesis of DN.

2.1 Genetics and DN

Several studies have suggested that DN may be determined by genetic factors (12). First, familial clustering of DN (13-15) , as well as an association between predisposition to essential hypertension and the development of DN has been observed (16-19). Second, studies of renal structure in sibling pairs in which both pairs had IDDM for at least 10 years showed glomerular basement membrane thickening, mesangial volume fraction, and an increase in peripheral capillary surface density that were highly correlated. Third, polymorphism in the 5'-end of the aldose reductase gene is strongly associated with the development of DN in both IDDM and NIDDM patients (20-21). Fourth, genetic variations in the RAS have been examined mostly for their linkage to DN. DNA polymorphism M235T and T174M in the angiotensinogen gene associated with essential hypertension and may contribute to the risk of DN in IDDM and NIDDM (22-24). An Insertion/Deletion (I/D) polymorphism of the Angiotensin I Converting Enzyme (ACE) gene can affect GFR and produce DN in IDDM (25-26). IDDM patients with II genotype have higher GFR and effective renal plasma flow during normoglycemia than those within the ID or DD genotypes (27). However, many investigators are in support of the theories that ACE I/D polymorphism can affect the development and progression of DN (28-29). Finally, there is no gene linkage between AT1 receptor polymorphism and hypertension with the exception of one

report which showed that AT1 (A1166C) polymorphism is related to essential hypertension in a French population (30).

2.2 The role of glomerular hemodynamic factors in DN

Glomerular hyperfusion and hyperfiltration can be observed in the early stage of diabetes in IDDM patients long before a major decrease in GFR, with increments in renal plasma flow and blood pressure. Prospective studies in IDDM patients have demonstrated paralled increases in blood pressure and in urinary albumin excretion rate (31).

The pathogenesis of diabetic hyperfiltration is multifactorial. Defective autoregulation at the afferent arteriole is a major defect, which has been demonstrated both experimentally (32) and clinically (33). Many mediators have been proposed to be involved in this effect (Table 1) (34).

Table1. Potential mediators of diabetic hyperfiltration

Hyperglycemia/insulinopenia

Extracellular fluid volume expansion

Blunted tubulo-glomerular feedback

Advanced glycosylation end products

Impaired afferent arteriolar voltage-gated calcium channels

Atrial natriuretic peptide

Endothelial-derived relaxing factor (NO)

vasodilator prostaglandins

Increased kallikrein activity and enhanced kinin production

Increased plasma ketone bodies, organic acids

Increased plasma glucagon levels

Increased plasma growth hormone levels

Increased insulin-like growth factors-1

Altered responsiveness to catecholamines and angiotensin II

Abnormalities in calcium metabolism

Abnormal myo-inositol metabolism

Tissue hyoxia and abnormalities in local vasoregulatory factors

---

2.3 Growth factors and DN

Cytokines and growth factors have also been implicated as causative agents to contributors to the renal and vascular hemodynamic, metabolic and structural changes in DN as shown in Table 2 (35).

Table2. Biological functions of growth factors that may be relevant to Diabetic Nephropathy

---

- Hemodynamic effects:
    ___Increase in RBF and GFR: IGFI
    ___Decrease in RBF and GFR: PDGF
- Cell hypertrophy: TGFß, IGFI
- Apoptosis: TNF α,, TGFß
- Basement matrix expansion: TGFß, IGFI, PDGF
- Hypercellularity: PDGF, FGF
- Inhibition or potentiation of insulin receptor signaling: TNF α,, IGFI
- Modulation of glucose transporter(s)/enzymes involved in glucose metabolism: PDGF

In addition, glomerular hypertension and vasoactive hormones such as angiotensin II (Ang II) and endothelin-1, could enhance the expression of growth factors such as PDGF and TGFß (36-38). Interleukin-1 (IL-1) and IL-6 have been implicated in the development of glomerular sclerosis (39). Futhermore, nitric oxide (NO) production is enhanced early in the case of DN and may contribute to renal hyperfusion and glomerular hyperfiltration (40).

2.4 Advanced Glycation End-products (AGEs) and DN

Chronic hyperglycemia can lead to the formation of AGEs in diabetes. Many studies have confirmed an increase in AGEs in the diabetic kidney (41) and retina (42).

It is likely that AGEs binding to their receptors can induce production of a range of cytokines which mediate important pathological changes involved in

tissue remodeling including cell adhesion such as VCAM-1 (43), extracellular matrix accumulation such as TGFß1 (44), vascular permeability such as vascular endothelial growth factor(VEGF) (45), and cell proliferation such as PDGF (46) as shown in Figure.1.

Glucose

↓

AGEs

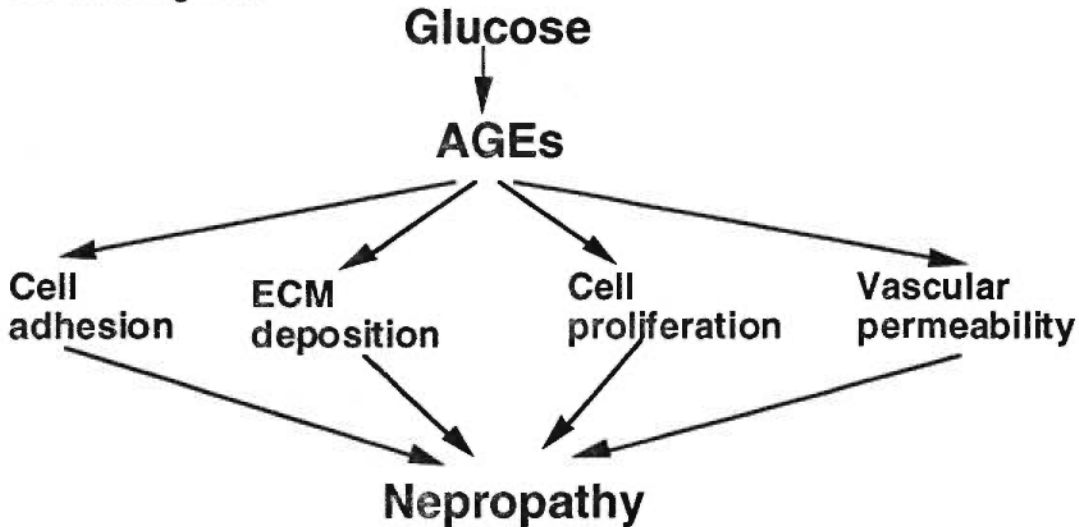| Cell adhesion | ECM deposition | Cell proliferation | Vascular permeability |

Nepropathy

Figure 1. AGEs contributes to the development of nephropathy.

2.5 Renin angiotensin system (RAS)-ACE inhibitors and DN

It is well established that the RAS plays an important role in the development of renal complications. AngII exerts hemodynamic as well as non-hemodynamic effects on renal cells that may contribute to the progression of DN.

Not only is hypertension a risk factor for the development of DN, but it also contributes adversely to the rate of decline in GFR if it is poorly controlled. ACE inhibitors can reduce systemic hypertension, normalize intraglomerular capillary pressure, and attenuate an increased glomerular capillary permeability (40). ACE inhibitors reduce myofibroblast formation through the downregulated expression of PDGF in the tubules (47). ACE inhibitors diminish the urinary albumin excretion rate, postpone and may even prevent progression to clinical overt nephropathy (48). Nevertheless, within the last ten years convincing clinical evidence has been accumulated to indicate that, at least in some settings, ACE inhibitors are uniquely effective over other antihypertensive agents in attenuating the progressive deterioration of DN (49), suggesting that the RAS plays an important role in the pathogenesis of DN. The following is a review of our current knowledge concerning the biochemistry and physiology of the RAS.

3. RENIN ANGIOTENSIN SYSTEM (RAS) AND DN`

3.1 RAS Cascade

The RAS cascade is shown in Figure 2

THE RENIN-ANGIOTENSIN SYSTEM

RENIN

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
ANGIOTENSINOGEN ASP-ARG-VAL-TYR-ILE-HIS-PRO-PHE-HIS-LEU-VAL-ILE-HIS-ASN
(LEU)

452 AMINO ACID RESIDUES GYLCOPROTEIN MW=50-60 000

ANGIOTENSIN CONVERTING ENZYME

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
ANGIOTENSIN I ASP-ARG-VAL-TYR-ILE-HIS-PRO-PHE-HIS-LEU

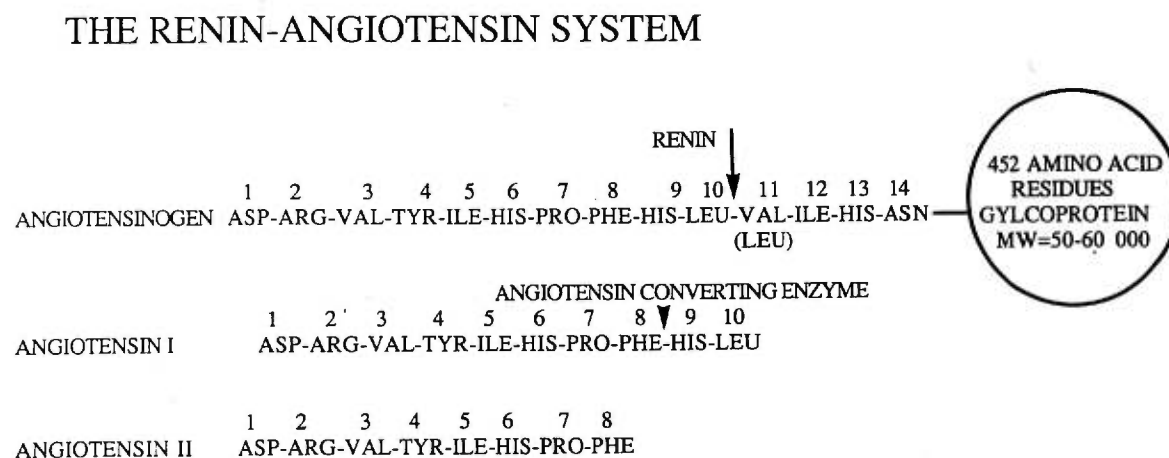| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
ANGIOTENSIN II ASP-ARG-VAL-TYR-ILE-HIS-PRO-PHE

Figure 2. RAS cascade. The angiotensinogen (ANG) is predominantly synthesized in the liver and secreted into the circulation. ANG is then cleaved by plasma renin to release angiotensin I (Ang I), which is subsequently converted into Ang II by the angiotensin converting enzyme (ACE). Ang II is the biologically active component of the RAS.

3.2 Angiotensinogen (ANG) gene

3.2.1 Structure of the ANG gene

ANG is a glycoprotein consisting of 452 amino acid residues (approximately 62,000 daltons) with the AngII sequence occurring at its amino terminal portion (51-52). The ANG gene is approximately 11.8 kilobase pairs long, consisting of 5 exons separated by 4 introns (53-54) (Figure 3).
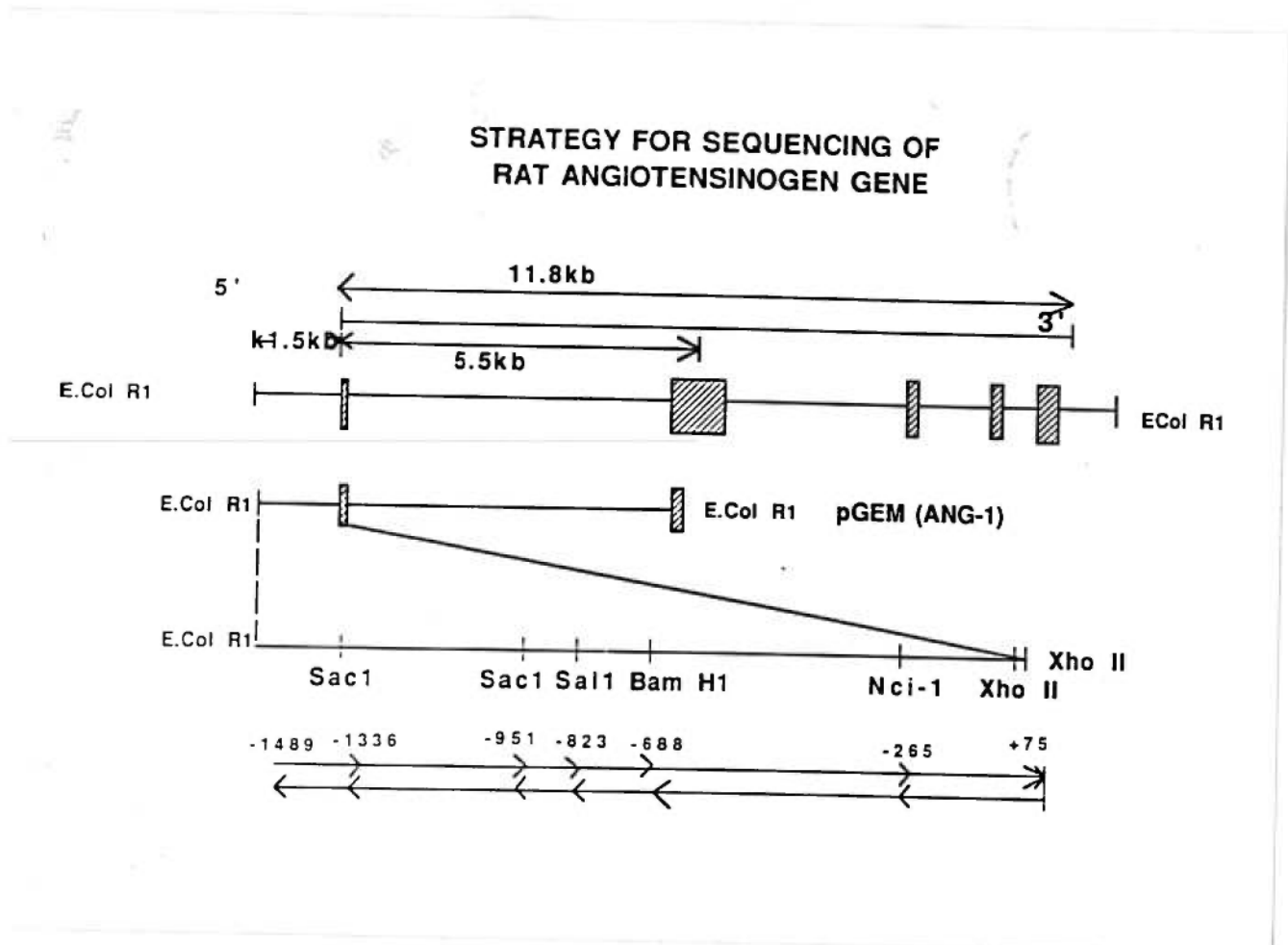
STRATEGY FOR SEQUENCING OF RAT ANGIOTENSINOGEN GENE

Figure 3. Structure and strategy for sequencing of the rat angiotensinogen gene (adapted from Chan et al Pediatr Nephrol 4: 429-435,1990)

The ANG gene is a single copy and has been mapped to chromosomes 19, 8 and 1 for the rat, mouse and human, respectively (55-57). The DNA sequence of 5'-flanking regulatory portion of the rat ANG gene(up to 1498 nucleotides upstream of the start site of transcription (57)) has been reported by our laboratory. Studies in our laboratory have demonstrated that this 5'-flanking regulatory region is responsive to the addition of thyroid hormone, dexamethasone, 8-Bromo-cAMP, forskolin, phorbol ester (PMA) and catecholamines(i.e. dopamine, ß- and α_2-adrenergic receptor agonists) in opossum kidney (OK) proximal tubular cells (58-66), indicating that the active thyroid hormone responsive element (TRE), cAMP-responsive element (CRE) and glucocorticoid responsive elements (GREs) are localized in the 5'-flanking region of the rat ANG gene. These regulatory elements have been mapped as shown in Figure 4.

Figure 4. Nucleotide sequence of the 5'-flanking region of the rat angiotensinogen gene (adapted from Chan et al Pediatr Nephrol 4: 429-435,1990)

The human ANG gene is 1,455 nucleotides long and codes for a 485-amino acid protein and located on chromosome 1q42-3. The ANG gene contains five exons and four introns, which span 13kb. There are two potential ATG sites, and the second exon codes for the first 252 amino acids (59%) of the mature protein. Mature ANG contains 452 amino acid residues: the first ten amino acids correspond to angiotensin I (AngI) and the larger portion corresponds to des (Ang I) gene (53). The human ANG gene structure is shown in Figure 5 .
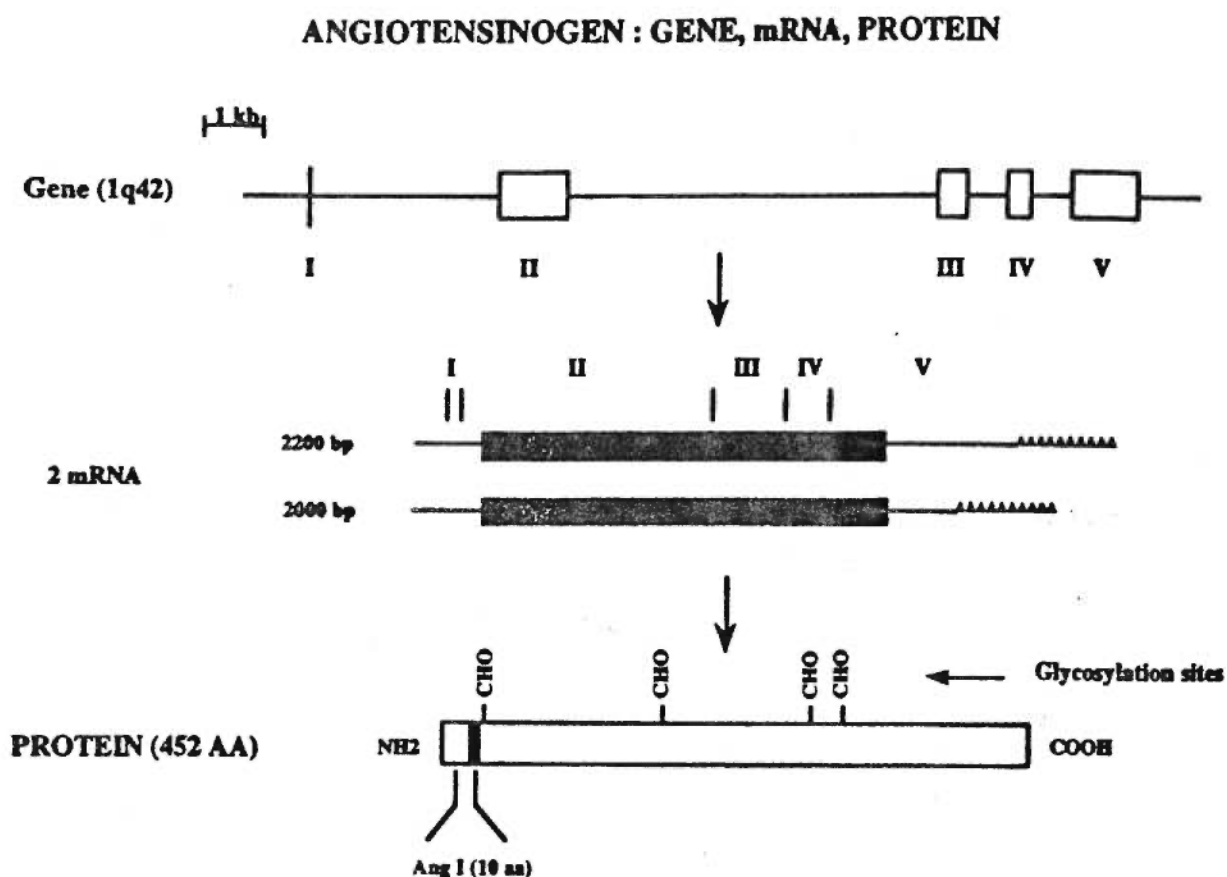
Figure 5. Structure of the human angiotensinogen gene, mRNA and protein

There are many tentative regulatory sequences, including glucocorticoid (N-130/N-125 and N-675/N-670), estrogen (N-337/N-324), acute phase (N-278/N-270), cAMP (N-839/N-833), and heat shock (N-574/N-561) responsive elements in the 5'-flanking region of the human ANG gene (88) (Figure 6). Recent studies indicate that cis- and trans elements regulate the ANG gene (89). Furthermore, the core promotor region of the human ANG gene in HepG2 cells has been analyzed by electrophoretic mobility shift assay which revealed that an ubiquitously expressed nuclear factor (ANG core promotor binding factor1, AGCF1) bound to a region between positions -25 to -1, AGCE1 (ANG core promotor element1), located between the TATA box and the transcription intiation site. AGCE1 appears to play a major role in activating ANG transcription, in particular by the downstream core elements. This region is probably more complex with several nuclear factors binding to its 5'-or 3'-side (90), which may be important for the general rate of transcription initiation and also for determining the pattern of ANG gene expression.

Figure 6. Nucleotide sequence of the 5'-regulatory sequence of human ANG gene.
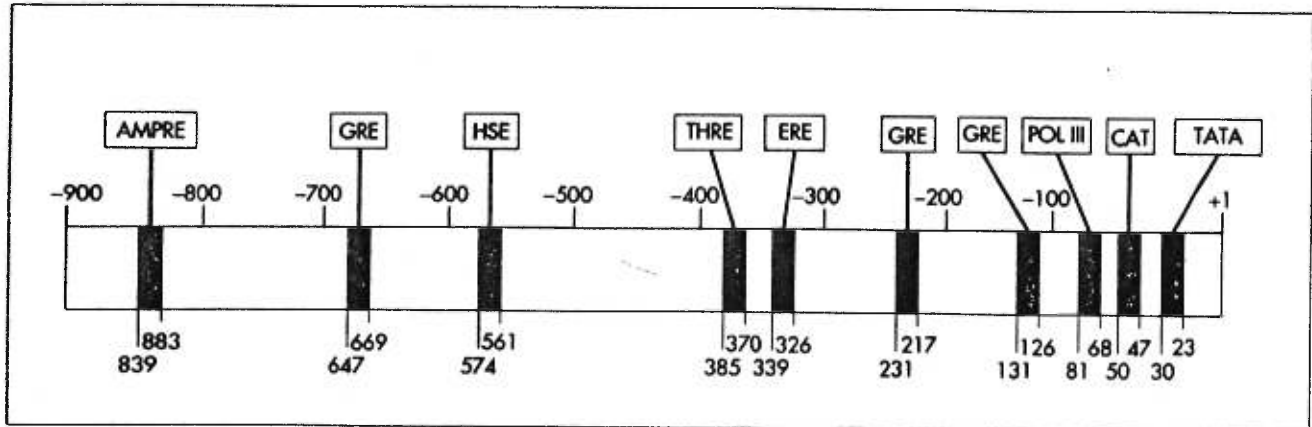
Fig. 6   Hypothetical organization of the regulatory region of the human angiotensinogen gene. The 900 base-pair DNA sequence, located upstream from the cap site (+1) contains sequences potentially interacting with proteins initiating, modulating, or regulating the transcription of the gene. These sequences, essentially called responsive elements, are from the right to the left: the TATA box (TATA), the CAT box (CAT), the consensus sequence for RNA polymerase III promoters (POLIII), glucocorticoid responsive elements (GRE), estrogen responsive element (ERE), thyroid hormone responsive element (THRE), heat-shock protein responsive element (HSE), and cAMP responsive element (AMPRE). Positions of the first and last nucleotide of each element are indicated below the schematic representation of the human angiotensinogen gene 5' flanking region.

3.2.2 ANG gene genetic linkage to DN

More recently, genetic linkage studies in humans showed that the ANG gene locus is associated with a predisposition for the development of hypertension in different populations (67-73).

The most important DNA polymorphisms of the ANG gene are M235T (Met change to Thr at position 235 of mature ANG) and T174M (Thr change to Met at position 174) which are in complete linkage disequilibrium. M235T is more frequently reported in essential hypertensives (23,24,74) than T174M (22, 75). The ANG M235T-allele is associated with hypertension. This association increased with a positive family history and more severe hypertension in Whites (91) and in the Chilean population (92). The genotype C/C or allele C of M235T is a risk factor for hypertension (76). Recent studies have reported that there is no evidence for a linkage of the ANG gene to hypertension among Chinese (77) or in a European study (93) but a linkage has been found in the Taiwanese population (76).

3.3 Renin gene

3.3.1 Structure of the renin gene

Renin, produced in the juxtaglomerular (JG) cells of the kidney, is released into the circulation and mediates the first step of angiotensin formation in plasma by cleaving the decapeptide angiotensin I (Ang I) from the prohormone angiotensinogen. The molecular weight of this glycoprotein is approximately 37 to 40 kDa (78).

Humans and rats have a single renin locus but some strains of mice have a duplication of the renin gene (99-100). The human renin gene has been located to the long arm of chromosome 1,in the q32 region using a GT microsatellite repeat at the renin locus(76% heterozygosity) (94). The renin gene (Ren1 and Ren2) is located on chromosome 1 in the mouse (Figure 7.) and on chromosome 13 in the rat (90). The mice and rat renin gene contains nine exons separated by eight intervening sequences, whereas the human renin gene contains ten exons separated by nine introns (95). The exon pairs (exon 2 with

exon6, exon 3 with exon 7, etc.) are neccessary to form symmetrical lobes in the model of renin (96). The renin gene of the three species spans 12kb approximately. In its 5'-flanking region, major control elements are present which include promoters and enhancers as well as regulatory elements such as estrogen and glucocorticoid receptors, and a cis-acting cAMP-responsive element (CRE) (79). The combined action of these elements will result in tissue and cell-specific gene expression (80-81). The promoter of the rat renin gene contains two TATA boxes which are selected specifically as transcription starting sites in different tissues, while the renin genes of all other species have a classical CAAT box (97-98).

Figure 7. The structure of mouse renin gene
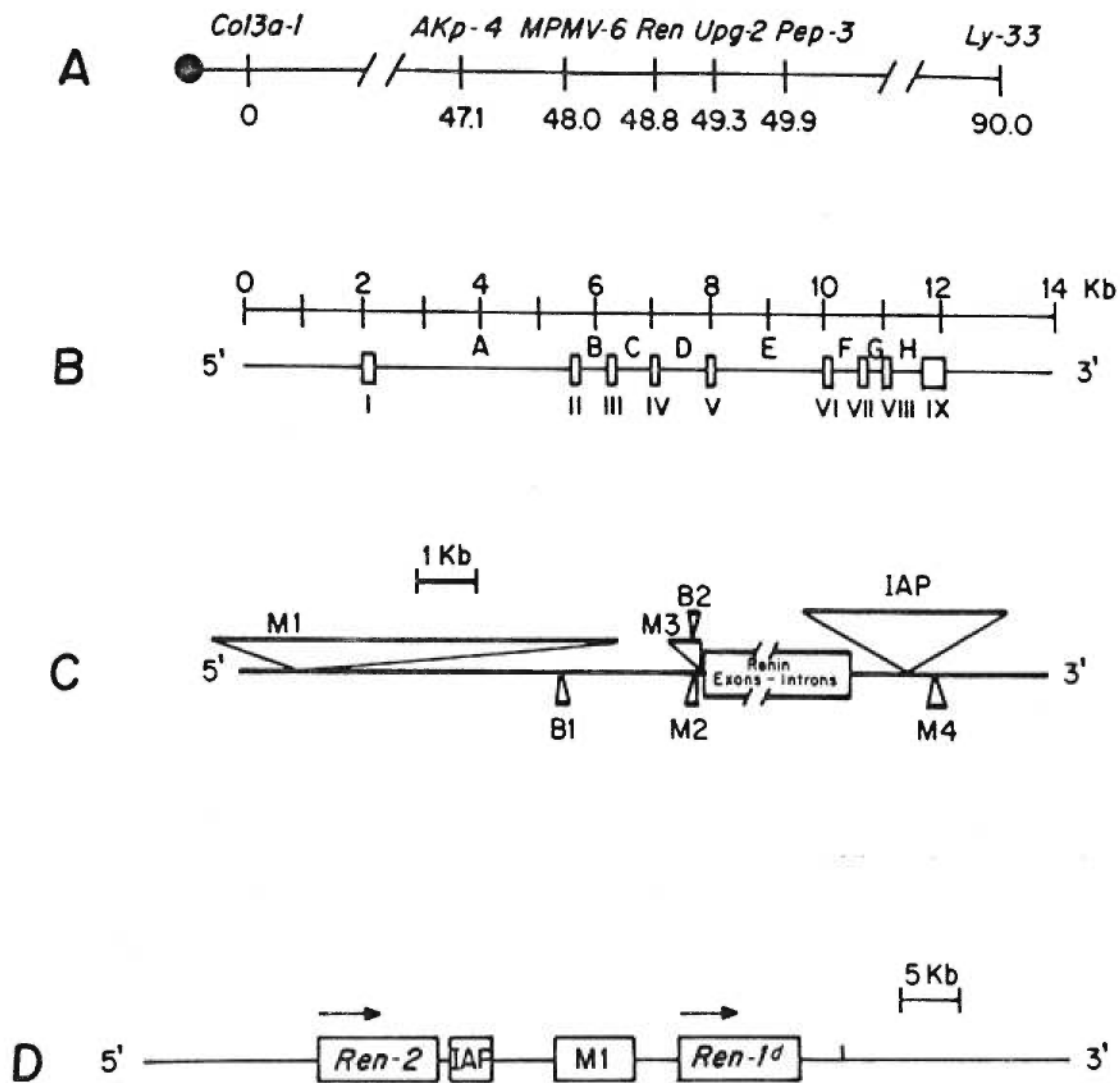
FIGURE 7. (A) Composite linkage map of mouse chromosome 1 illustrating the *Ren* locus and the placement of the closest known flanking loci in cM relative to the centromere. The two loci on the centromeric side of the renin locus *(Ren)* are alkaline phosphatase 4 *(AKp-4)* and modified polytropic murine leukemia virus-6 *(MPMV-6)*. The two loci distal to the *Ren* locus are urinary pepsinogen 2 *(Upg-2)* and peptidase 3 *(Pep-3)*. The collagen-3-α-1 *(Col3a-1)* locus is most proximal to the centromere while lymphocyte antigen-33 *(Ly-33)* is most distal. (Adapted from Seldin et al.[96]) (B) Structure of the transcribed region of murine renin genes. This diagram illustrates the approximate arrangement and size of the exons (roman numerals) and introns (capital letters) of the mouse renin genes. (C) Location of murine insertional elements. This representation illustrates the approximate size and location of the insertional elements relative to the murine renin coding sequences. Refer to Table 2 for gene-specific insertions. (D) The renin locus containing the duplicate gene. This schematic illustrates the placement of the duplicated gene relative to the progenitor gene. Arrows indicate direction of transcription.

3.3.2 Renin and DN

There is no genetic linkage of the gene locus of renin associated with DN (82). However, there is a evidence indicating that the sodium-renin product is increased in patients with DN, compared with diabetic patients without complications and non-diabetic controls (83). Furthermore, an elevation of circulating inactive renin is common and appears to be a marker for microvascular complications (84) and total serum renin may predict the subsequent development of incipient nephropathy in IDDM patients (85).

3.4 ACE gene

3.4.1 ACE gene structure

ACE is a Zn-containing dipeptidyl carboxypeptidase which catalyzes production of the vasoactive peptide AngII from its precursor, Ang I. The human gene for ACE spans approximately 21 kb on chromosome 17q23 and includes 26 exons ranging in size from 88 to 481 bp, encoding for a 4020-bp cDNA (101-103). The structure of the human ACE gene is shown on Figure 8. Two positive transcriptional elements, a putative cAMP response element (CRE) at -55 and a TATA box at -32 have been identified in the promoter of mouse testis ACE (105).The first polymorphism found in this gene was a 287-bp insertion/deletion (I/D) located in intron 16, and the D allele of this polymorphism was associated with higher serum ACE levels (104). Another five polymorphisms (1Dde, 2Rsa-1, 2Rsa-2, 4Alu-1, and 4Alu-2) were reported in the ACE gene (106).

Figure 8. The structure of the human ACE gene
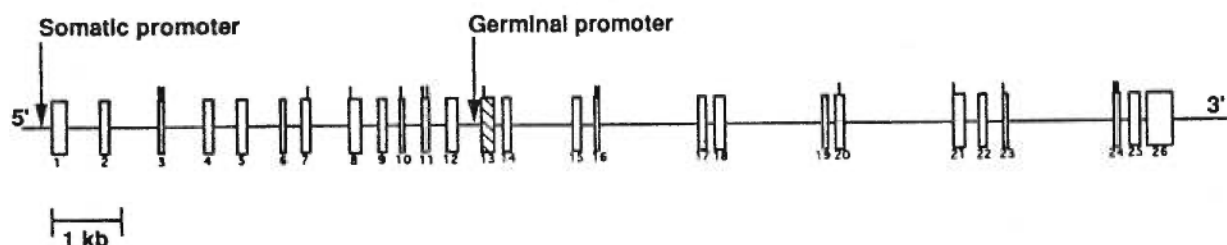
FIGURE 8. Structure of the human ACE gene and presence of two alternate promoters. Location of the 26 numbered exons (vertical bars). Exon 13 (open bar) is specific to the testicular ACE mRNA. The two promoters are indicated by vertical arrows. Vertical bars above the exon boxes indicate the location of the cysteine residues. (Adapted from Hubert, C. et al., *J. Biol. Chem.*, 266, 15377, 1991.)

3.4.2 ACE gene genetic linkage to DN

As mentioned above, the role of ACE I/D polymorphisms in the predisposition to DN in both IDDM and NIDDM patients remains controversial. The following is a brief summary of the most recent studies (1997-1998) concering the relationship of DN with ACE I/D polymorphism.

Many positive studies have shown that the ACE gene is a sex-specific candidate gene for the development of hypertension and that there is an association of the ACE DD genotype with increased diastolic blood pressure in men but not in women (107). The interaction of ACE I/D and M235T ANG polymorphism can contribute to essential hypertension (108). The ACE DD genotype is independently associated with the presence of DN and may be potentially used as a marker for NIDDM patients at risk for developing DN (109). Hypertensive albuminuric IDDM patients with the II genotype are particularly susceptible to the commonly advocated renoprotective treament (110). However, a number of negative reports argue that there is no association between the ACE I/D polymorphisms and DN in IDDM and NIDDM patients (111-114). However ACE D allele prevalence was higher among hypertensive patients with a parental history of myocardial infarction and stroke incidence before 60 years of age (114).

3.5. Angiotensin II receptors(AT1 and AT2)

3.5.1 Biological function and distribution of AT1 and AT2

Two distinct types of cell-surface angiotensin II receptors (AT1 and AT2) have been defined and cloned (115). Both subclasses of AngII receptors have seven transmembrane domains typical of G protein-coupled receptors. AT1 and AT2 receptors share only 30% sequence homology and have distinctive functional properies and cell-signaling mechanisms following stimulation by Ang II (116). AT1 receptors mediate all of the known functions of angiotensin II in regulating salt and fluid homeostasis (115). In the kidney, all actions of AngII on hemodynamic and tubular function are thought to be mediated via the AT1 receptors, including afferent and efferent arteriolar vasoconstriction, decreased glomerular filtration rate (GFR) (136) and renal blood flow, and stimulation of sodium and fluid reasorption in the proximal tubules (117-120,137). AT1

receptors are thought also to mediate growth and differentiation in the kidney (121). There are two AT1 subtypes in rodents. In situ hybridization and reverse transcription-polymerase chain reaction analyses showed their level of expression to be regulated differently in various tissues: AT1a is the principal receptor in the blood vessels, brain, kidney, lung, liver, adrenal gland and fetal pituitary. AT1b predominates in the adult pituitary and is expressed only in specific regions of the adrenal gland (zona glomerulosa) and kidney (glomeruli) (122). The biological function of the AT2 receptors is largely unknown (115,120,123-125). Recent studies have suggested that AT2 receptors mediate cell-signaling pathways related to the inhibition of cell growth and differentiation (126-128). AT2 is involved in apoptosis (138-139) and accelerated fibrosis and collagen deposition occurs in the renal interstitium (140). During fetal life, the AT2 receptor gene is expressed predominantly in areas of active mesenchymal differentiation, but the mRNA expression levels decrease rapidly and disappear within a few days after birth (129-134). In the adult, the mRNA of AT2 receptors has been detected in the adrenal gland, heart, and brain (115,130,135). AT2 receptor mRNA is expressed in the fetal and neonatal rat kidney but disappears after the neonatal period and is not expressed in the normal adult (130-133).

3.5.2 Angiotensin II receptors (AT1 and AT2) gene

3.5.2.1 AT1 and AT2 gene structure

The first two AT1 cDNAs were obtained in the rat and bovine by expression cloning (140-141). It consists of a polypeptide chain of 359 amino acids . In the rat, the AT1a is composed of four exons and three introns and is greater than 84kb in size (142). The third exon contains the entire coding sequence, whereas exons 1 and 2 encode 5'-untranslated sequences and exon 4 encodes an additional 1 kb of the 3'-untranslated sequence. Thus, the 2.3kb AT1a mRNA is identified in all tissues expressing AT1. The AT1b gene is larger than 15kb and has a three exon structure with two introns (143) and encodes mRNA transcripts that are approximately 2.4 kb in size. The two rat AT1 genes are localized on different chromosomes; the AT1a gene is on chromosome 17 and the AT1b on chromosome 2 (144). The single human AT1 gene has an more complex stucture because four exons upstream of the coding exon have been identified (145-146). Exons 2,3 and 4 are alternatively spliced and potentially

result in the production of at least eight different mRNA species. RT-PCR analysis has shown that mRNAs containing exons 1 and 5 or 1, 2 and 5 are the most abundant in all tissues examined, but mRNAs containing exon 3 spliced to exon 5 represent up to one-third of the transcript and potentially encode an AT1 receptor having an amino terminal extension of 32-35 amino acids. The human AT1 gene is 47kb in size and is localized on the chromosome 3 band q22 (147). The human and mouse AT2 gene are approximately 5kb in size and are composed of three exons (148-149). Comparison of the gene structures for the rat (r) AT1a and AT1b, human(h) AT1 and AT2 receptors are shown in Figure 9.
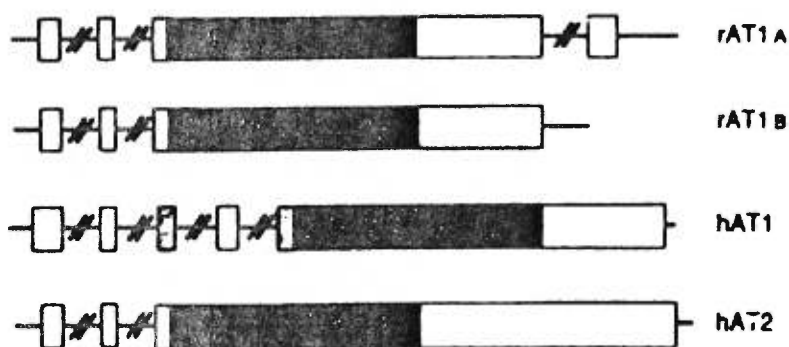
Fig. 9 Comparison of the gene structures for the rat (r) $AT1_A$ and $AT1_B$, human (h) AT1 and AT2 receptors. Exons are represented by white boxes, and black lines represent the introns that separate the exons. The coding sequences are shown in grey. Transcripts of the human gene that contain exon 3 spliced to exon 5 encode a longer receptor isoform that has an amino-terminal extension. This additional sequence is indicated in light grey. These data were assembled from References 35–40 and 42.

Comparison of the cis-acting regulatory elements contained in the 5'-flanking regions of the rat (r) AT1a and AT1b and human (h) AT1 and AT2 receptor genes as shown in Figure 10. The rat AT1a gene has a classical "TATA" box (142), whereas the AT1b (143) and probably the human AT1 (146) genes are TATA-less genes. All three genes have numerous enhancer sequences, such as proximal SP1 binding sites (142,143,145,146,150). The human AT1 or rat AT1a genes have liver-specific sequences (HP1, ANF, HNF5 and LFA1) and pituitary-

specific elements(Pit1) and are expressed in these tissues (142,146,150). However, the rat AT1b gene has liver-specific but no pituitary-specific elements, which is surprising because rat AT1b is expressed in the adult pituitary but not in the liver (143). Furthermore, several cis-acting regulatory elements such as cAMP (CRE), glucocorticoid (GRE), estrogen (ERE) and growth factors (AP1 and AP2) have been identified in both the rat AT1a and the human AT1 genes (142,150).
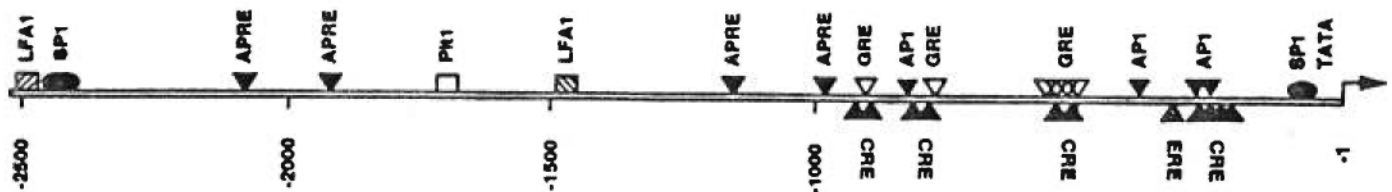
at AT1$_A$

it AT1$_B$
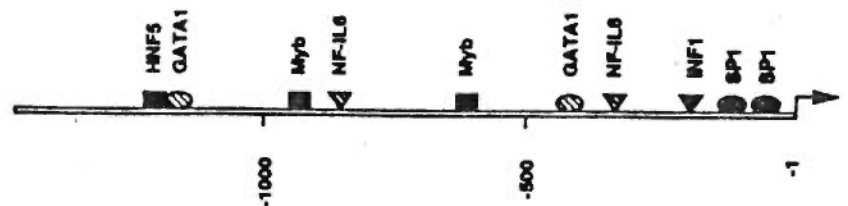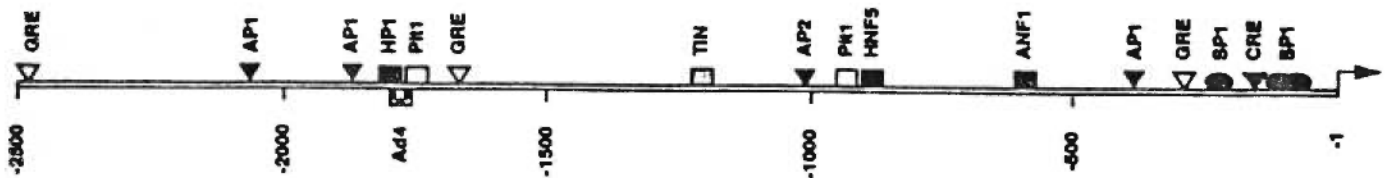
uman AT1

uman AT2

Comparison of the *cis*-acting regulatory elements contained in the 5′-flanking regions of the rat (r) AT1$_A$ and AT1$_B$ and human (h) AT1 AT2 receptor genes. These *cis*-acting regulatory sequences are designated by different symbols according to their potential roles: enhancer . tissue-specific element (□) or extracellular signal response element (△) and their names. Their relative locations with respect to the putative scription start site of each gene are indicated. These data were assembled from References 35 to 40. 42 and 70.

3.5.2.2 AngII receptors (AT1 and AT2) genetic linkage to DN

There is no genetic linkage between AT1 receptor polymorphism and hypertension with the exception of one report where it was shown that AT1 (A1166C) polymorphism is related to essential hypertension in a French population (30).

3.6 Importance of the ANG gene in kidney function

3.6.1 ANG gene " knock-out" mice

Recent advances in genetic engineering enabled us to seek a definitive answer to the physiological role of RAS. An ANG gene knockout ($Atg^{-/-}$) mouse strain was generated by gene targeting to obtain a model for examining RAS regulation mechanisms. Homozygous mutant mice completely lacked ANG and angiotensin peptides, and therefore lacked a functional RAS. $Atg^{-/-}$ mice exhibited chronic hypotension, increased expression of renal renin, and abnormal renal morphology (167-169). Recent studies have shown that chronic volume depletion by dietary salt restriction causes a marked decrease in GFR in $Atg^{-/-}$ mice but no change in GFR in $Atg^{+/+}$ mice (170). $Atg^{-/-}$ mice can increase urine output and decrease urine osmolality compared with wild-type mice ($Atg^{+/+}$) when provided free access to water. On the other hand, urinary excretion of aldosterone in $Atg^{-/-}$ mice is lower than the nomal range (171). A genetic deficiency of ANG produced an impaired urine concentrating ability and tubulointerstitial lesions, indicating the critical role of ANG in developing normal tubular function and constriction.

3.6.2 Transgenic mice

The role of ANG in blood pressure regulation can also be explored by creating transgenic mice that overproduce ANG. Kimura et al.(172) generated transgenic mice which contained the entire rat ANG gene including 1.6 kb of 5'-flanking sequence. All transgenic mice developed hypertension, especially the male mice. It is found that ANG was overproduced in the liver and the brain. Other transgenic mice have been generated by Ohkubo et al.(173) who introduced the rat renin gene, the rat ANG gene, or both into mice. Although

many transgenic animals clearly demonstrated that overexpression of the renin and ANG genes as well as increase blood pressure, their relevance to the pathogenesis of human hypertension, however is questionable. A recent study using Tsukuba hypertensive mice (THM) indicated that RAS plays a significant role in cardiac hypertrophy and renal disorders found in THM (174).

3.7 Role of Ang II in DN

Angiotensin II exerts many hemodynamic as well as non- hemodynamic effects on the renal cells that could contribute to the progression of DN (Table 3) (40).

Table3. Effects of AngII that may contribute to Diabetic Nephropathy

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Hemodynamic effects
•Induction of systemic vasoconstriction and hypertension
•Increase in glomerular afferent and efferent arteriolar resistance.
•Increase in glomerular capillary pressure
•Reduction in filtration surface area
•Increase in glomerular capillary permeability
•Constriction of vasa recta with subsequent reduction in medullary blood flow

Transport effects
•Stimulation of proximal sodium, bicarbonate, and water reabsorption
•Stimulation of proximal tubular glucose transport

Metabolic effects
•Stimulation of ammoniagenesis
•Stimulation of gluconeogenesis

Immunomodulatory effects
•Stimulation of macrophages/monocytes migration

Growth stimulatory effects on extracellular matrix production
•Induction of hypertrophy and/ or proliferation
•Stimulation of collagen and fibronectin synthesis
•Inhibition of protein and extracellular matrix turnover

3.8 Biological effects of angiotensin

Ang II produces arteriolar constriction and a rise in systolic and diastolic blood pressure (175). Ang II induces hypertrophy of cultured rat aortic smooth muscle cells (176) and activates the expression of proto-oncogenes and the collagen gene in vascular smooth muscle and kidney proximal tubular cells, respectively (177-179). Ang II is one of the most potent stimulators of aldosterone production by the zona glomerulosa of the adrenal gland (180). Aldosterone is known to enhance renal distal tubular sodium reabsorption which contributes to the pathogenesis of hypertension (181).

In addition, AngII has multiple effects on the kidney. Ang II inhibits renin release (182). It is a potent renal vasoconstrictor (183) and causes dose-dependent changes in intrarenal blood flow distribution (184) and has marked effects on the glomerular filtration rate (GFR) (185-186). Ang II has been shown to participate in cardiovascular homeostasis by potent regulation of transepithelial sodium transport in renal tubular cells.

Evidence that Ang II directly affects proximal tubule transport was demonstrated by Harris and Young as early as 1977 (187) who showed that the microvascular injection of Ang II increased the steady-state sodium concentration gradient in stationary fluid droplets within the late proximal convoluted tubule of the rat. Later studies by Schuster and co-workers (188-189) demonstrated that low doses of Ang II (10^{-11} and 10^{-10} M) increased sodium and water absorption in the microperfused, subcortical rabbit proximal tubule. Higher Ang II concentrations (10^{-8} and 10^{-6}M) inhibited sodium transport. Subsequently, studies by various laboratories (190-193) have confirmed the observations of Harris and Young (187) and Shuster et al (188-189) . In particular, studies by Cogan and co-workers (190-193) have shown that the infusion of Ang II at a subpressor systemic concentrations within the physiological range of 10^{-12} to 10^{-11} M increases water reabsorption by renal proximal tubules and the infusion of saralasin (Ang II antagonist) depresses water reabsorption. These studies showed that the direct tubular effects of Ang II are independent of changes in renal or systemic hemodynamics. More recent studies by Chan and co-workers (204) and Harris and Navar (205) have further shown that Ang II has a biphasic effect on the luminal side of proximal tubules. Low doses of Ang II stimulate bicarbonate and volume reabsorption with maximal stimulation at 10^{-11} M while high doses of Ang II inhibit with a maximal inhibition observed at 10^{-6}M. The stimulatory effect of Ang II is diminished by amiloride. Thus, the above studies

demonstrate unequivocally that Ang II directly affects the epithelial cell transport in the proximal tubule. The major biological effects of Ang II are shown in Figure 11.

MAJOR BIOLOGICAL EFFECTS OF THE ANGIOTENSIN II

(1) INCREASES BLOOD PRESSURE
   BY DIRECT VASOCONSTRICTION
   OF PERIPHERAL RESISTANT VESSELS

(2) INCREASES SECRETION OF
   ALDOSTERONE FROM ADRENAL
   GLAND

(3) REDUCTION OF RENAL BLOOD FLOW
   AND GLOMERULAR FILTRATION RATE

(4) INCREASES SODIUM REABSORPTION
   IN PROXIMAL TUBULAR CELLS OF THE
   KIDNEY
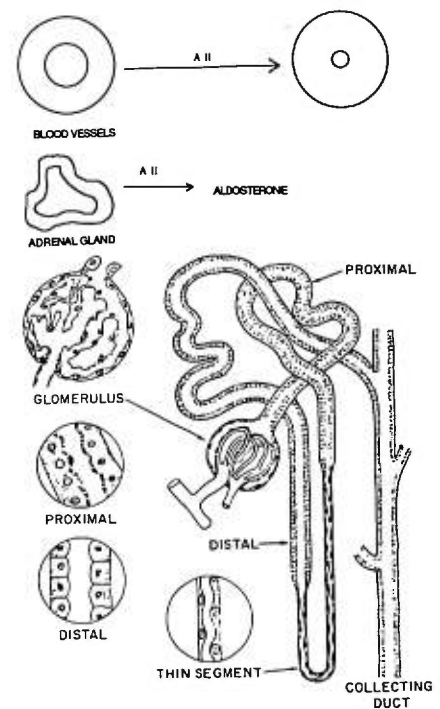
Figure 11. Major biological effects of Angiotensin II

3.9 Molecular mechanism(s) of action of AngII

The mechanisms by which Ang II acts on the epithelial cells of proximal tubules have received particular attention during the last several years. Ang II can affect epithelial cell function directly, by occupancy of membrane receptors , or indirectly, by activating those presynaptic receptors that control catecholamine release from nerves having terminal contacts on the cell. Proximal tubular cells have specific Ang II receptors (206-209) and are richly innervated by adrenergic nerves (210). Thus, Ang II can potentially affect the tubular function by either one or both mechanisms. It has been shown, however, that denervation of the renal nerve markedly reduces sodium chloride reabsorption in the proximal tubules (181, 211-212) while infusion of Ang II can still increase bicarbonate reabsorption (181). Thus, these studies demonstrated that Ang II predominantly affects sodium bicarbonate transport in the proximal tubule via epithelial cell receptors, whereas changes in sodium chloride absorption occurs predominantly via presynaptic receptors on renal sympathetic nerves (181).

The Ang II receptor binding studies by Brown and Douglas (191-192) and Cox et al (193) have demonstrated that Ang II receptors are present on both the basolateral and brush border membranes. The Kd was 2.2×10^{-9}M for the basolateral membrane and 9.5×10^{-9}M for the brush membrane. The functional importance of these receptors has been further evaluated by Liu and Cogan (192) who showed that the signaling transport responses evoked by Ang II binding to the luminal receptor are only 30-50% of those observed when Ang II occupies the basolateral receptors after intravenous infusion of Ang II when the luminal perfusate is devoid of Ang II. Thus, AngII is somewhat novel in being able to elicit a change in transepithelial transport from both the luminal and basolateral membrane of the cell.

Post-receptor events that mediate AngII-induced epithelial transport are not clearly understood. Ang II does not stimulate adenylate cyclase to produce cAMP; rather Ang II-induced inhibition of adenylate cyclase has been demonstrated in the proximal tubule (182, 213-214). Reduction of cAMP in the proximal tubular cells stimulates bicarbonate transport because cAMP inhibits Na^+-H^+ antiporter activity on the luminal membrane (215-216).

A second signal-transduction pathway which may be activated by Ang II is a phosphatidyl-inositol breakdown with subsequent increase in protein kinase C and intracellular IP3 and calcium concentration (204, 217-218). Protein kinase C

stimulates Na^+-H^+ antiporter activity in proximal tubular cells (219-220). However, recent studies in Brunette's lab (321) demonstrated that PMA ($10^{-7}M$) inhibits Na^+-H^+ exchanger in the luminal membrane of proximal tubule.Liu and Cogan (221) have shown that protein kinase C is inactivated by chronic exposure to high levels of phorbol ester (PMA) and sphingosine, transport stimulation by Ang II is attenuated by approximately 30%, suggesting that the protein kinase C pathway contributes approximately 1/3 of the effect of AngII. Moreover, the studies showed that the pathway of protein kinase C activation does not interfere with cAMP levels in the proximal tubule, suggesting that the protein kinase C pathway may act independently of the cAMP pathway to mediate the effect to modify epithelial transport remains unclear. Moreover, the molecular mechanism by which high doses of Ang II inhibit the transport of bicarbonate is still not understood. Indeed, more experiments along these lines are definitely required to elucidate the molecular mechanisms of action of Ang II in the proximal tubule.

3.10 Regulation of expression of the intrarenal RAS genes

Since 1990, with the discovery that the ANG mRNA is expressed in the rat proximal tubules (151-152), studies have been focused on the regulation of expression of the RAS in the kidney. Studies have shown that sodium depletion in rats(fed a low salt diet) increases renal renin mRNA, ANG mRNA and renal AT1a-receptor mRNA levels, decreases renal AT1b mRNA levels and has no effect on the hepatic ANG mRNA levels (153-154). These studies indicate that there is a tissue-specific regulation of expression of the ANG gene in the liver and kidney in response to varying sodium balance.

Various hormones also modulate the expression of renal ANG mRNA. Studies by Ellison et al (155)have shown that the administration of androgen restores the renal ANG mRNA and renin mRNA in ovariectomized rats. Studies by Campbell and Habener (156)demonstrated that rat ANG mRNA in the kidney, liver and many extrahepatic tissues is increased by treatment with a combination of dexamethasone (DEX) and triiodothyronine and ethinyl estradiol in vivo. Studies in our laboratory have shown that DEX and thyroid hormone, L-T3, stimulate the expression of the ANG gene, in a dose-dependent manner in OK cells (59-60). The addition of estrogen, testosterone or progesterone had no

effect (59-60). Moreover, recent studies in our laboratory showed that DEX stimulated the expression and secretion of the ANG from IRPTC. In contrast, the administration of DEX diminished the level of AT1b-receptor mRNA in vivo but not AT1a-receptor mRNA (159).

Our laboratory has also demonstrated that the addition of isoproterenol (β-adrenoceptor agonist) and iodoclonidine (α2-adrenoceptor agonist) stimulated the expression of the ANG gene via protein kinase A (PKA) and protein kinase C (PKC) signal transduction pathways, respectively (61-63). DEX and isoproterenol acts synergistically to stimulate the expression of ANG gene in both rat proximal tubular cells and OK cells. Moreover, the addtion of isoproterenol enhances the stimulatory effect of cAMP-responsive element binding protein (CREB) on the expression of ANG gene in OK cells (62,65)via the cAMP-response element (CRE) in the 5'-flanking region of the rat ANG gene (164).

Our more recent studies have demonstrated that norepinephrine (NE) stimulates the expression of the ANG gene in OK cells in a dose-dependent manner (66). The effect of NE is mediated via either the β1-adrenoceptor or α2-adrenoceptor or a " cross-talk" between both receptors. Our studies were confirmed by the in vivo studies of Nakamura and Johns (166) who reported that low levels of renal nerve stimulation decrease sodium excretion and increase the level of ANG mRNA but not renin mRNA in the rat kidney in vivo. Administration of the β1-adrenoceptor antagonist, atenolol, blocked the effect of renal nerve stimulation. These studies demonstrate a functional relationship between NE (or renal nerve stimulation) and the activation of the ANG gene in the kidney.

4. GLUCOSE AND ANG GENE EXPRESSION

4.1 Effect of hyperglycemia on the expression of the intrarenal RAS genes in vivo

Conflicting results have been obtained from different groups concerning the effect of high glucose levels on the intrarenal RAS genes in vivo. Correa-Rotter et al (222) found that the renal renin protein and renin mRNA expression were not different in diabetic and normal animals, but the renal and liver ANG mRNA levels were lower in the diabetic group. Kalinyak et al (223) found no significant difference in the expression of renal renin and ANG mRNA in rats two weeks after the induction of diabetes compared to controls. On the other hand,

Jaffa et al (224) reported a 50% decrease in renal renin mRNA in rats 3 weeks after the induction of diabetes compared to controls. In contrast, Anderson et al (225) reported a small increase in renal renin and ANG gene expression in rats 6 to 8 weeks after the induction of diabetes. In adult Biobreeding (BB) spontaneously diabetic rats, an increase and a decrease in renin mRNA was observed at 2 months and 12 months of diabetes, respectively (226). At present, the reason for the inconsistencies between the different studies is not known. One possible explanation may be that the different groups used rats at various time periods after the induction of diabetes by streptozotocin.

One unexpected observation in diabetic rats is that the density of Ang II receptors and its mRNA in the glomerulus and in the proximal tubules were reduced after the induction of diabetes (227-229), thus demonstrating that high levels of glucose directly down-regulate the expression of the AT1-receptor mRNA. The molecular mechanism(s) of down-regulation of renal proximal tubular AT1-receptor expression in diabetic rats is not known. One possibility may be that the increase in intrarenal AngII induced by hyperglycemia may down-regulates the expression of AT1-receptor mRNA. The physiological significance of down regulation of AT1-receptor expression in the proximal tubules of diabetic animals is unknown.

4.2 Effect of hyperglycemia on mesangial cells and proximal tubular cell function in vitro

Studies have shown that the addition of a high level of glucose plus PMA or TGFß phosphorylated the nuclear transcription factor, CREB, in rat mesangial cells(244). In cultured murine proximal tubular cells, high glucose (i.e.25mM) or Ang II(i.e.10^{-8} M) stimulates cellular hypertrophy and increases the expression of TGFß and collagen type I and type IV (245-251). Moreover, Ang II and high levels of glucose upregulate the AT1-receptor mRNA in rabbit proximal tubular cells(252-253). Studies in our laboratory have shown that high levels of glucose stimulate the expression of rat and human ANG gene promoter activity in OK cells (Appendix II) as well as the expression of the rat ANG gene in IRPTC (Appendix V). The effect of high glucose on the expression of the rat ANG gene is blocked in the presence of inhibitors of PKC (i.e. H-7 and staurosprine) and Tolrestat(an inhibitor of aldose reductase). Also, our studies suggest that hyperglycemia enhances the expression of the renal ANG gene, at least in part,

via the polyol and PKC pathways. These studies suggest that there might be a functional relationship between hyperglycemia, the expression of renal ANG and the induction of hypertrophy of renal proximal tubular cells. However, at present, there is no conclusive evidence to support the hypothesis that the hypertrophy of proximal tubular cells is mediated via the elevation of intrarenal Ang II in vivo and in vitro.

4.3 Hyperglycemia in the development of DN

The Diabetes Control and Complication Trial recently reported that the strict maintenance of euglycemia by intensive insulin treament can prevent the development and progression of DN (230), suggesting that the adverse effects of hyperglycemia on metablic pathways can cause chronic complications in diabetes such as kidney disease. The importance of hyperglycemia in the development of DN is supported by the results of Heilig et al.(231) who have found that the overexpression of glucose transporter1 (GLUT1) in glomerular masangial cells enhanced the production of extracellular matrix components which can contribute to mesangial expansion and finally glomerulosclerosis, even under normal glucose levels. Multiple biochemical mechanisms have been proposed to explain the adverse effects of hyperglycemia. Activation of the diacylglycerol (DAG)-protein kinase C (PKC) pathway (232-233), enhanced polyol pathway related to myo-inositol depletion (234), altered redox state (235), overproduction of advanced glycation products (236), and enhanced growth factor and cytokine production (237-238) have all been proposed as potential cellular mechanisms by which hyperglycemia induces the chronic complications observed in diabetic patients.

4.4. The mechanism(s) of glucose effect.

Glucose enters the mesangial cells by GLUT1 transport and excess glucose is metabolized through pathways that interact with kinase/ phosphatase signaling cascades. In recent research, three major hypothesized effects of glucose are mentioned (1). Advanced glycosylation end-products (AGEs) as mentioned above. (2).Reactive Oxygen species (ROS) act as signal transduction messengers to regulate gene expression through transcription factors such as

NFkB and activator protein (AP)-1 (254). (3). DAG-PKC pathway and the polyol pathway. The following is a brief review DAG-PKC and polyol pathway.

4.4.1 DAG-PKC pathway

4.4.1.1. PKC family

Protein kinase C (PKC) is a family of protein kinases that undergoes translocation from one intracellular compartment to another when activated by neurotransmiters, hormones, and growth factors. Most members of this family depend for their activation on phosphatidylserine (PS), diacylglycerol (DAG) and, to different extent, on calcium and other lipid second messengers (255-256). The PKC family of isozymes can be divided into at least three subfamilies based on their homology and sensitivity to activators (257). (1) Conventional PKCs, such as α, β_I, β_{II} and γ contain four homologous domains (C1, C2, C3 and C4). These isoforms require calcium, phospholipid, as well as DAG or phorbol ester for activity. (2) Novel PKCs, such as δ, ε, η (L in humans) and , have a similar domain structure to the cPKCs except that the C2 domain lacks certain key oxygens and does not bind calcium. The novel PKCs are thus activated by phospholipid and DAG (or phorbol ester) but do not require calcium. (3) Atypical PKCs, such as ζ, ι and λ, contain no C2 domain and one, instead of two, cysteine-rich zinc finger sequences in the C1 domain , and also require phospholipid for activation but are not sensitive to calcium, DAG, or phorbol ester. The PKC family is shown in Figure 12.

Conventional isoforms: α, β–I, β–II, γ

Novel isoforms: δ, ε, η (L), θ, μ

Atypical isoforms: ζ, ι, λ

Figure 2: Classification of protein kinase C isoforms.
The Protein kinase C family can be divided into three groups based on domain structure and activation characteristics. Conventional PKCs are calcium, DAG, and phorbol ester-dependent, novel PKCs are calcium-independent yet DAG and phorbol ester-dependent, and atypical PKCs are calcium, DAG, and phorbol ester-independent.

4.4.1.2. DAG-PKC pathway activation

The functional link between high glucose and PKC activation may be via the *de novo* synthesis of membrane-associated diacylglycerol (DAG) (274). DAG acts as the hydrophobic product of the phosphodiesterase-mediated cleavage of inositol phospholipids which is thought to have a second messenger function through activation of PKC (268). DAG can be generated either by de novo synthesis or by stimulated breakdown of phospholipids by PLC (269-270). DAG is generated by hydrolysis of phosphatidyl inositol (PI) which also generates inositol 1,4,5-triphosphate (IP3), which in turn, releases intracellular Ca^{2+} or phosph-atidylcholine (271-272). Moreover, DAG is the major regulating activator of PKC which is a serine and threonine kinase implicated in cell growth, proliferation, contraction, and hormone-receptor interactions as well as tumorigenesis and diabetic complications.

PKC activation can increase vascular permeability, extracellular matrix synthesis, contractility, leukocyte attachment, cell growth and angiogenesis (259-260). High levels of glucose can increase both DAG and PKC levels in vascular cells or tissue including renal glomeruli, retina, and aorta (261-264). Recently, the possible activation of DAG-PKC has also been reported in the liver and skeletal muscle of insulin-resistant diabetic animals, suggesting that the activation of the DAG-PKC signal transduction pathway by hyperglycemia may also induce insulin resistance in those tissues. Another important biochemical change induced by the DAG-PKC activation is the inhibition of Na^+-K^+ ATPase, an integral component of the sodium pump, which is involved in the maintenance of cellular integrity and functions such as contractility, growth, and differentiation (265)

In the kidney, the activation of the PKC-DAG pathway in the glomeruli of diabetic rats and cultured mesangial cells exposed to high levels of glucose (261, 263, 266). Multiple PKC isoforms are activated in each vascular tissue of diabetic animal models. Among them PKC ß isoforms appear to be most consistently increased. Zhou et al (267) reported that mesangial cell filamentous actin (F-actin) disassembly by high glucose levels is likely mediated through DAG-sensitive PKC isoforms, including PKC-δ, and that Tolrestat (an inhibitor of aldose reductase) can reduce this effect. Our studies showed that high levels of glucose can stimulate the expression of the ANG gene, increase total PKC, DAG activity and also increase intracellular sorbitol levels in immortalized rat proximal tubular cells (IRPTC) (Appendix V) and OK cells (Appendix II) in vitro. Tolrestat

can also inhibit high levels of glucose effect. These studies demonstrate that the effect of glucose on the expression of the ANG gene is mediated via the *de novo* synthesis of DAG and the activited PKC signal transduction pathway. However, we do not know exactly which PKC isoforms are involved in the effect of high glucose levels in these cell lines.

4.4.2. The polyol pathway.

Activation of the polyol pathway, also termed as the sorbitol pathway, has long been suspected to be responsible for certain diabetic complications including cataract, neuropathy, retinopathy and nephropathy (273). The polyol pathway consists of the reduction of glucose to sorbitol by aldose reductase, followed by the oxidation of sorbitol to fructose by sorbitol dehydrogenase with coproduction of NADH. Aldose reductase has a low affinity for glucose, and under normal conditions very little glucose is metabolized by this pathway.

Chronic hyperglycemia leads to sorbitol accumulation in a variety of tissues such as peripheral neurons, lense and renal tubuli (278). The subsequent synthesis of fructose from sorbitol generates NADH which is then available for the conversion of the glycolytic intermediate glycerol-3-phosphate (G-3-P) into dihydroxyacetone phosphate (DHAP), leading to the *de novo* synthesis of DAG (275). DAG in turn, may activate several PKC isoforms. The polyol pathway is shown in Figure 13. Recent studies have shown that the polyol pathway mediates high glucose-induced collagen and fibronectin synthesis in the proximal tubule (276, 292). An aldose reductase inhibitor prevents a glucose-induced increase in TGFβ and PKC activity in cultured human mesangial cells (277). However, some studies have shown that cells may counterregulate inositol depletion (279-280). Thus, it is not generally agreed that the increase in intracellular sorbitol is the cause of the impaired function of affected tissues in diabetes. Treatment of diabetic rats for six months with the aldose reductase inhibitor Tolrestat, resulted only a slight reduction in the urinary albumin excretion rate, indicating that other mechanisms are operating in diabetic nephropathy.
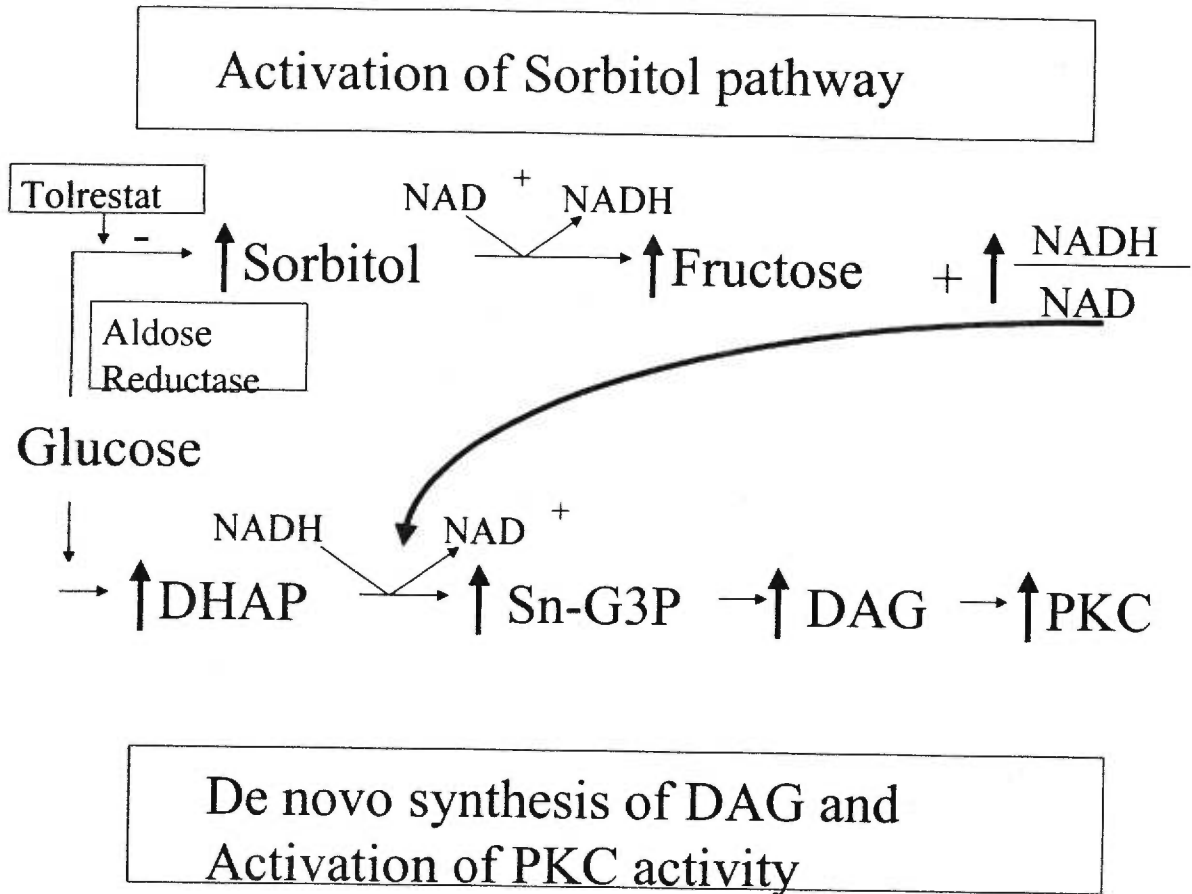
Figure 13. The polyol pathway

4.4.3. Growth factor effect-emphasize on TGFß effect

High levels of glucose can stimulate the synthesis of matrix components like fibronectin, laminin and collagen IV either by direct action or via formation of AGE products. The increased matrix sythesis is probably mediated by cytokines. In particular, transforming growth factor ß (TGFß) is a multifunctional cytokine in the regulation of extracellular matrix production in DN (237-238). TGFß is unique among the cytokines in (i) stimulating the synthesis of matrix, (ii) inhibiting matrix degradation. (iii) stimulating mesangial cell proliferation. (iv)inhibiting proximal

tubular cell proliferation. TGFß can stimulate the production of extracellular matrix such as Type IV collagen, fibronectin and laminin in cultured mesangial cells and epithelial cells (239-240). Increases in gene and protein expression of TGFß were found in the glomeruli of diabetic animals as well as of human diabetics (241-243), suggesting that overexpression of TGFß might contribute to the development of mesangial expansion in diabetic nephropathy.

5. INSULIN

5.1 Insulin action

The principal role of insulin is to control plasma glucose concentration by stimulating glucose transport into muscle and adipose cells, as well as by reducing glucose output from the liver(281). These effects of insulin occur through activation of effectors such as glucose transporters and glycogen synthase, or through regulation of the amount of specific protein participants in metabolic pathways (282). In addition, insulin is a major hormonal regulator of lipid metabolism, inhibits lypolysis and stimulates fatty acid synthesis (283). It also stimulates protein synthesis by affecting amino acid transport and the initiation of translation (284). Furthermore, insulin promotes cell growth and differentiation of specific cells and tissues.

Insulin action at the cellular level can be viewed as existing in three levels (Figure 14). Level 1 is the initiation of insulin action and depends upon insulin binding to and stimulating its receptor tyrosine kinase, which results in tyrosine phosphorylation of the receptor and intracellular substrates. Level 2 actions are the intermediate signals in the insulin cascade and involve a number of serine kinases, such as MAP and S6 kinases, as well as lipid kinases, such as phosphatidylinositol (PI)3-kinase. At level 3 are the final biological effectors of insulin signaling. These are the enzymes and transports required for insulin's effects on glucose, lipid and protein metablism (285).
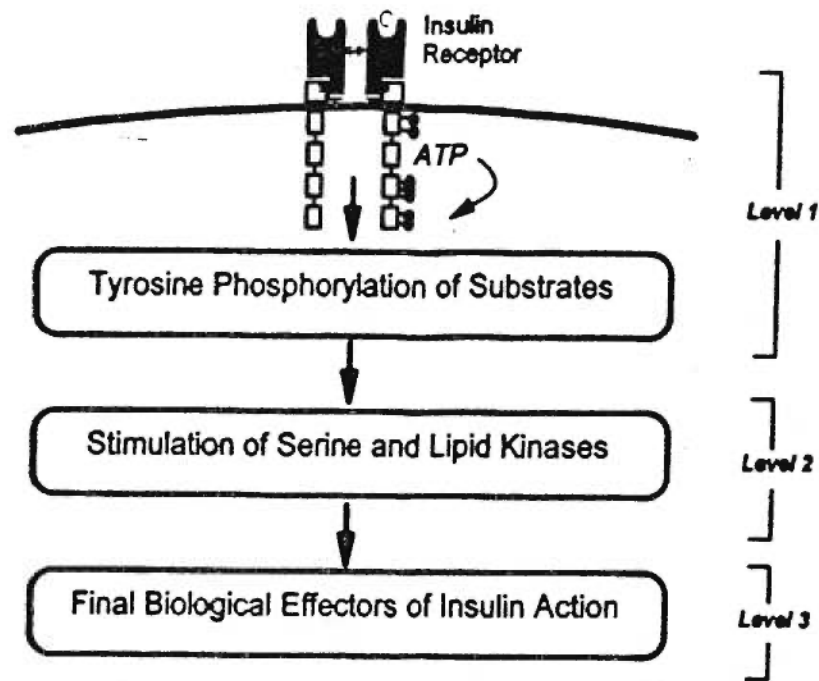
Figure 14: Three levels of insulin actions (adapted from reference 285).

5.2 The insulin receptor structure

The receptor is a transmembrane glycoprotein complex with a molecular weight of about 460 kDa (286). The receptor consists of two 135 kDa α-subunits that are entirely extracellular and two 95 kDa ß-subunits with an extracellular domain, a transmembrane domain and a large intracellular domain (285). Both α- and ß-subunits are derived from a single chain proreceptor encoded by a single gene. In humans, the insulin receptor gene is located on the short arm of chromosome 19 (287). In the mature receptor, the α- and ß-subunits are linked by disulfide bonds to form a tetramer with a ß-α-α-ß structure (286, 288). Following insulin binding to the α-subunit, the tyrosine kinase activity of the ß-subunit is activated, resulting in autophosphorylation of the receptor on at least six sites. Together with the kinase domain, these define the four subdomains of the intracellular portion of the ß-subunit: the juxtamembrane region which contains tyrosine phosphorylation site 960, the ATP binding region, the regulatory region which contains three tyrosine sites of phosphorylation, and the C-terminal receptor domain which contains two tyrosine phosphorylation sites (285). The structure of insulin receptor is shown in Figure 15.
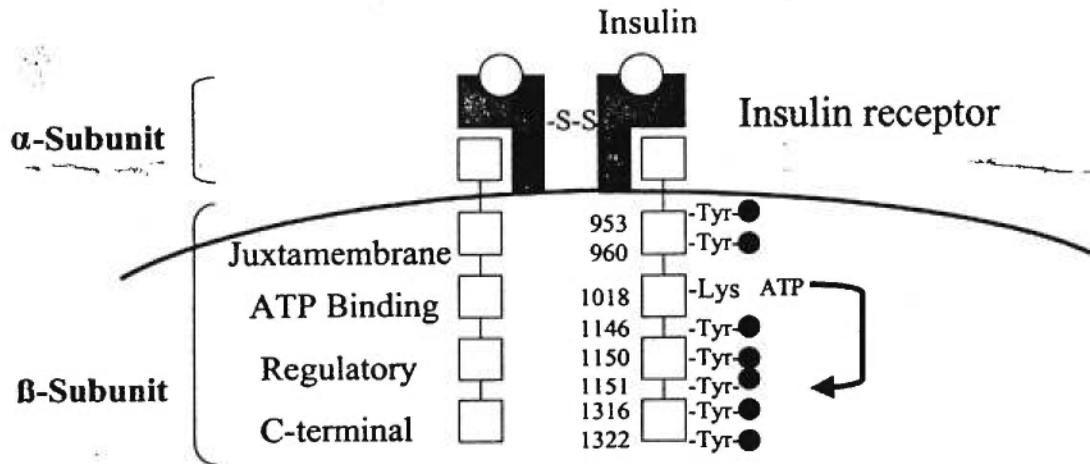
Figure 15. Insulin receptor structure. The insulin receptor consists of an α-subunit which is entirely extracellular and a ß-subunit with an extracellular domain, a transmembrane domain and a large intracellular domain. Following insulin binding to the α-subunit, the tyrosine kinase activity the ß-subunit is activated, resulting in autophosphorylation of the receptor on at least six sites.

5.3 Potential insulin signal transduction pathway

Current understanding of the signal transduction pathways that underlie insulin's major physiological actions is incomplete. The binding of insulin to its cell surface receptors activates their intrinsic tyrosine kinase activity, leading to receptor autophosphorylation of cytosolic proteins, known as insulin receptor substrates (IRSs), which serve as adapters in intracellular signaling (290). IRS-1, the predominant and most throughly characterized IRS, binds a variety of signaling molecules when specific tyrosines are phosphorylated, including the regulatory subunit of PI3, Shc-1, and Grb2 (290-291). Interaction of these IRS-associated molecules initiates signaling cascades leading to the activation of a variety of protein kinases, including MAPK, protein kinase B, protein kinase C, glycogen synthase kinase-3, pp90rsk II, and p70S6 kinase. All of these kinases have been implicated in one or more of the growth or metabolic effects attributed to insulin (293).

The following information is concerned with protein ser/thr kinase cascades as signalling intermediates, and their status as participants in insulin regulation of energy metabolism. (1) Ras-MAPK pathway which is the most well-

characterized insulin regulatory pathway. (2). PI3 kinase pathway which include elements indispensible for insulin regulation of glucose transport, glycogen and cAMP metabolism. PKB/ cAkt is activated by the lipid products of PI3-kinase and this requires dimerization through its PH domain. (3). ERK pathway (i.e. such as the SAPK/JNK pathway) and Nck/cdc42-regulated PAKs(homologs of the yeast Ste 20) as participants in the cellular response to insulin (289). The current knowledge of insulin action on cells is shown in Figure 16.
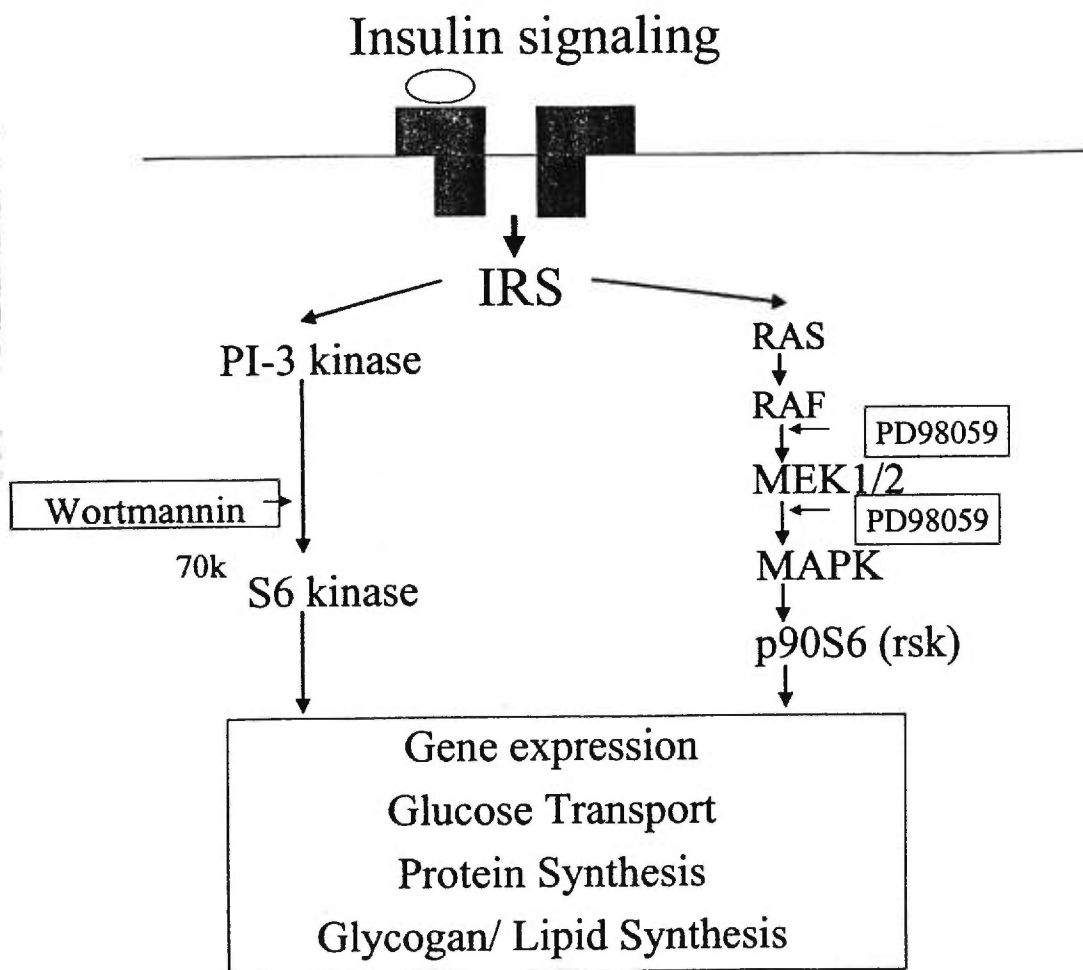
Figure 16. Potential insulin signal transduction pathways

5.3.1 Ras-MAP kinase cascade

5.3.1.1 Ras pathway

The Ras pathway is activated by a number of growth factors, hormones, and cytokines. In fact, activating mutants of Ras are found in nearly 10% of all human tumors and are associated with greater than 80% of all colon and pancreatic cancers (298).

Ras (p21^{ras}) family consists of four related GTP binding proteins termed H-Ras, K-Ras, N-Ras and R-Ras which are important regulators of cell growth and metablism (299). Following insulin stimulation, Ras is converted from the GDP-bound form to the GTP-bound form by the presence of a guanine nucleotide exchange factor called SOS (son of sevenless). When insulin signaling is reversed, Ras GTP is reconverted to Ras GDP in the presence of a GTPase activating protein (GAP). The activated form of Ras, i.e. the GTP-bound form, is responsible for activation of the Raf-MAP kinase cascade (Figure 17.) (294-296).
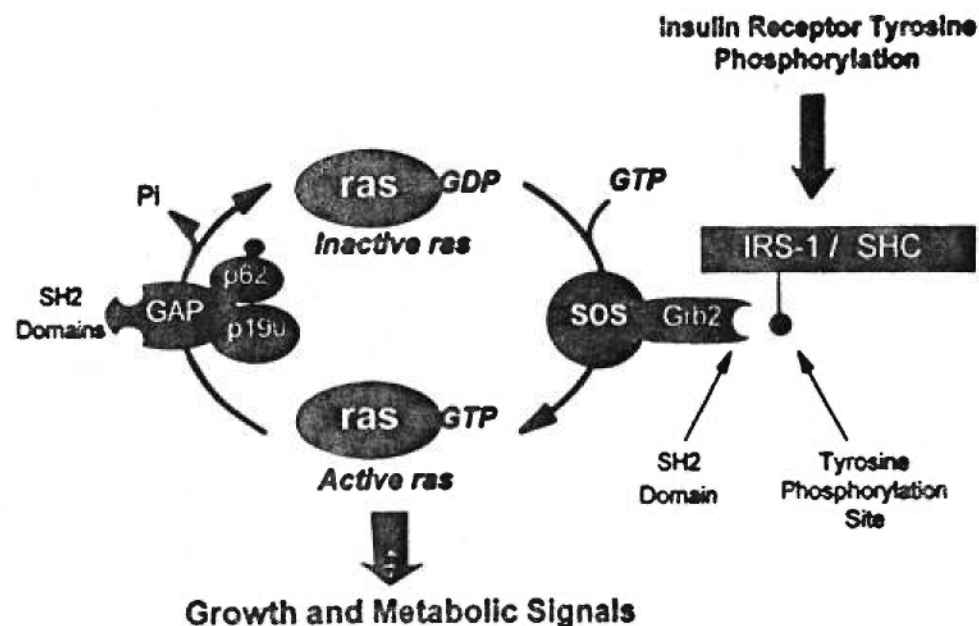
Figure 17: Ras pathway

5.3.1.2 Mitogen-activating protein kinase (MAPK) pathway

The three well-characterised sub-families of the mitogen-activated protein kinase (MAPK) superfamily are the extracellularly responsive kinases (ERKs), the c-Jun N-terminal kinases (JNKs) which are also known as stress-activated protein kinases (SAPKs), and the P38-MAPKs (297).

Activation of Ras then results in the stimulation of a cascade of serine/ threonine phosphorylation involving Raf-1 kinase, MAP kinase kinase (also termed MEK), MAPK and p90 S6 kinase (also called Rsk) (295-296). The Rsk then phosphorylates and activates several transcription factors which regulate gene expression. Rsk have three isoforms (Rsk1, Rsk2, Rsk3) and each one encodes two complete protein (ser/ thr) kinase catalytic domains which appears to contain the major site of MAPK phosphorylation, but at least for Rsk2, is uncertain (289). Phosphorylation by Rsk also activates the protein (PPG-1) which can dephosphorylate and activate three enzymes in the glycogen synthetic pathway: glycogen synthetase, phosphorylase kinase and glycogen phosphorylase (295). Thus, one can envisage an 11-step process in which Ras forms the critical link between the insulin receptor and glycogen synthesis.

Studies with dominant negative mutants of Ras or SOS have revealed that the Ras-MAPK cascade is important in the stimulatory effect of insulin on cell growth and DNA synthesis (312-313). Furthermore, introduction of IRS-1 antisense RNA or antibodies to IRS-1 into cells, or a point mutation in the Grb2 binding site of IRS-1 attenuate the effect of insulin on DNA synthesis (314-315), indicating that IRS-1-Grb2 complex plays a major role in the mitogenic signalling pathway of insulin. In contrast, metabolic actions of insulin, such as stimulation of glucose uptake and activation of glycogen synthase were not affected by dominant negative mutant of Ras or SOS or by a synthetic inhibitor of MEK (313, 316-318), indicating that the Ras-MAPK pathway does not contribute to the major metabolic actions of insulin. However, the exact mechanisms of regulating the Ras-MAPK pathway are not clear. Thus, the Ras-MAP-rsk pathway may or not play a role in the metabolic actions of insulin, this pathway is probably most important in the control of transcription factor activity and mitogenesis (296,300-301).

5.3.2 The PI3 kinase pathway.

The first downstream molecule that was shown to be associated with IRS-1 is PI3-kinase (302). PI3-kinase consists of two subunits, a regulatory subunit of molecular weight 85kDa (p85) and a catalytic subunit with a molecular weight of 110 kDa (p110). The p85 subunit contains one SH3 domain and two SH2 domains, the latter of which associates with tyrosine-phosphorylated IRS-1 (303-304). It also contains a bcr homology region with proline-rich sequences, although the roles of these regions in insulin signal transduction are unknown. The catalytic activity of p110 is enhanced by the binding of p85 (305), which occurs through the NH2 terminal region of p110 and the region between the two SH2 domains p85 (306-307). Recently, an additional regulatory subunit has been reported of PI3-kinase termed p55^{PIK} which also contains two SH2 domains and a binding site for p110, but it does not possess SH3 or bcr homology domains. Functional differences between the regulatory subunits of PI3-kinase are still under investigation (308). Although the exact mechanisms remain unknown, activation of PI3-kinase appears to be a critical upstream step for insulin of Glut4 glucose transporter translocation and glucose uptake, as well as stimulation of some enzymes involved in protein synthesis, such as pp70 S6 kinase (285).

The roles of PI3-kinase in insulin signal transduction have been investigated by two approaches by using a constructed mutant p85 termed Δp85 and inhibitors(Wortmannin and Ly294002). Insulin-induced glucose transport as well as translocation of GLUT1 were markedly attenuated by Δp85 (309) and two PI3 inhibitors (310-311). Wortmannin-or Ly294002 sensitive effects of insulin including antilipolysis, phosphorylation and activation of cAMP phosphodiesterase, stimulation of fatty acid synthesis, activation of acetyl CoA carboxylase and activation of glycogen synthase, inhibition of glycogen synthase kinase 3ß (GSK3ß) activity, stimulation of protein synthesis, phosphorylation of eukaryotic translation initiation factor 4E (eIF4E) and eIF4E binding protein (4E-BP1)/ phosphorylated heat-and acid-stable protein regulated by insulin (PHAS-I), stimulation of the transcription of specific genes such as that encoding phosphoenolpyruvate carboxykinase (PEPCK), regulation of the cytoskeleton and activation of the serine-threonine kinases, p70kinase and Akt (308). Moreover, although several evidences indicate that insulin activated PI3-kinase phosphorylates lipids that can activate protein kinase B (PKB) and Ca^{2+}/DAG insensitive forms of protein kinase C (PKC) but the roles of PKB and PKC in

insulin action are presently under intensive investigation (289). Agati JM et al (283) reported that insulin inhibition of PKA-induced PEPCK expression does not require MAPK activation but does require the activation of PI3-kinase, although this signal is not transmitted through the PKB or PKC pathways and suggested that, an as yet uncharacterized target of PI3-kinase, mediates insulin inhibition of cAMP-induced PEPCK gene transcription or that alternate pathways may exist.

5.3.3 Other ERK-based protein kinase cascades

Based on the general working sequence MEKK-MEK-ERK, other ERK-based protein kinase cascades such as the SAPKs(also known as JNKs) and p38 pathways have been identified. MAPK-activated protein kinase2 (MAPKAPK2) is preferentially phosphorylated by p38-MAPK and transcription factor c-Jun is a substrate for SAPKs/JNKs, although both can be phosphorylated by ERKs in vitro(297). The mechanism(s) of insulin action on these pathways is still not clearly defined.

5.4 Insulin and the development of DN

The pathophysiology of diabetes mellitus in all of its forms is ultimately due to a deficiency in insulin signaling. In Type1 diabetes, this is a result of a deficiency in insulin itself, whereas in Type 2 and most secondary forms of diabetes, the primary defect is the resistance to insulin action at target cell level. Insulin therapy of patients with IDDM delays the onset and slows the progression of nephropathy (319-320). Studies in diabetic rats have shown that normalization of blood glucose by insulin reverses the established glomerular hyperfiltration, renal hypertrophy and extracellular matrix protein synthesis (161-162). The mechanism(s) of the beneficial effects of insulin treatment, however, is not completely understood.

6. OBJECTIVE OF THE PRESENT STUDIES

The objectives of the present studies were to explore (1): The molecular mechanism(s) of action of high levels of glucose on the expression of the rANG mRNA and protein in immortalized rat proximal tubular cells (IRPTC) .(2) The

mechanism(s) of insulin action on the expression of the rANG gene and hypertrophy in IRPTC .

We hypothesized that : (1) The high levels of glucose stimulate the expression of the ANG gene in IRPTC via the *de novo* synthesis of DAG (polyol pathway) and protein kinase C (PKC) pathways ; (2) Insulin inhibits ANG gene expression and hypertrophy in IRPTC, at least in part, via the MAPK signal transduction pathway .

III. RESULTS

Appendix I: Wang LS, Lei CL, **Zhang SL**, Roberts KD, Tang SS, Ingelfinger JR, Chan JSD: Synergistic effect of dexamethasone and isoproterenol on the expression of angiotensinogen in immortalized rat proximal tubular cells. *Kidney Int.* 53: 287-295, 1998.

Appendix II: Wang TT, Wu XH, **Zhang SL**, Chan JSD: Effect of glucose on the expression of the angiotensinogen gene in opossum kidney cells. *Kidney Int.* 53: 312-319, 1998.

Appendix III : Wang TT, Wu XH, **Zhang SL**, Chan JSD: Molecular mechanism(s) of action of norepinephrine on the expression of the angiotensinogen gene in opossum kidney cells.*Kidney Int.* 54: 785-789, 1998.

Appendix IV : Wang TT, Chen X, Wu XH, **Zhang SL**, Chan JSD: Molecular mechanism(s) of action of isoproterenol on the expression of the angiotensinogen gene in opossum kidney proximal tubular cells. *Kidney Int* 55: in press, 1999.

Appendix V : Zhang SL, Filep JG, Hohman TC, Tang SS, Ingelfinger JR, Chan JSD: Molecular mechanism(s) of glucose action on angiotensinogen gene expression in rat proximal tubular cells. *Kidney Int.* 55: 454-464, 1999.

Appendix VI : Zhang SL, Chen X, Filep JG, Tang SS, Ingelfinger JR, Chan JSD: Insulin inhibits angiotensinogen gene expression via the mitogen-activated protein kinase (MAPK) pathway in rat proximal tubular cells. *Endocrinology* (Acceptance pending), 1999.

IV. DISCUSSION

Studies in our laboratory have reported that ANG protein is secreted from IRPTC as measured by a specific radioimmunoassay for rat ANG (RIA-rANG) (Appendix I) providing evidence that intrarenal Ang II is indeed derived from ANG synthesized by the renal proximal tubular cells.

The RIA-rANG was developed by employing rabbit antiserum against the purified recombinant rat ANG. The purified rANG from plasma and the iodinated rANG were used as the hormone standard and tracer, respectively. The RIA is specific for rat ANG and it has no cross-reactivity with other pituitary hormones or other rat plasma proteins. The sensitivity of the RIA is approximately 2 ng of rANG. By employing this RIA, the levels of IR-rANG in the culture media of IRPTC were detected in the range of 2 to 5 ng/ml/24 hr/10^6 cells.

The limiting step in the use of this RIA is the amount of purified ANG that could be isolated from rat plasma as well as its purity. Rat plasma ANG was purified by a three-step method by passing sequentially through two IgG-affinity column chromatographies (Appendix I). In our hands, from 15 ml of rat plasma, we were able to purify 100-200 ug of ANG. The purified rANG displayed at least two major bands with apparent molecular weights of 56 and 62 kDa as analyzed by a 6 to 15% linear gradient SDS-PAGE as shown in Figure 2A (Appendix I). By Western Blot analysis, both molecular weight species of purified rat ANG (56 and 62 kDa) interacted well with the antiserum against the purified MBP-rANG fusion protein (Figure 2B, Appendix I). The presence of two molecular forms of rat plasma ANG is in good agreement with the studies of Hilganfeldt and Hackenthal (197), who isolated two forms of rANG (apparent molecular weights 56.6 and 60 kDa) from the plasma of nephrectomized rats.

A faint IR-rANG band with an apparent molecular weight of 62 kDa was also observed in rat plasma samples (Figure 2B, Appendix I). We were surprised that our rabbit antiserum displayed weak cross-reactivity with the purified human ANG (hANG), despite the fact that there is at least 60% homology in the amino acid sequence between the rat and human rANG(198-199). At present, we do not have an explanation for this observation. One possible explanation may be that the tertiary structure of rat ANG and human ANG might play an important role in determining the antigenicity of the protein. Indeed, more experiments are warranted to clarify this observation.

Our RIA for rANG displayed no cross reactivity with rat albumin, rat pituitary hormones (that is, rGH, rTSH, rLH and rPRL) and plasma samples from the dog, rat, hamster and human (Figure 3, Appendix I), indicating that the RIA is specific for ANG and is also species specific. On the other hand, serial dilution of rat and mouse plasma samples (Figure 3, Appendix I) and culture media of the rat IRPTC and H4-IIE cells (rat hepatoma cells) (Figure 4, Appendix I) displayed a parallelism with the rANG standards demonstrating that the RIA is appropriate and feasible to quantify the levels of IR-rANG in rat and mouse plasma and in the culture media of IRPTC and H4-IIE cells.

To further confirm that ANG is secreted from the IRPTC into the culture media, I have performed Western Blot analysis on the concentrated media collected from the IRPTC cultured in low (5 mM) and in high (25 mM) glucose DMEM. My results (Figure 18, unpublished results) showed that the rat ANG was detected as a 62 kDa molecular species. Exposure of IRPTC to a high concentration of glucose (25 mM) stimulated the expression of IR-rANG at least 1.5-fold higher ($p \leq 0.05$) than the IRPTC incubated in a low (5 mM) glucose medium (Figure 18, unpublished results). Most interestingly, Northern blot analysis of the ANG mRNA in IRPTC revealed that the level of expression of the ANG mRNA in IRPTC cultured in a high glucose (25 mM) medium was also at least 1.5 fold higher than IRPTC cultured in a low (5 mM) glucose medium (Figure 19, unpublished results). These results demonstrate a relationship between hyperglycemia and the expression of the ANG gene in IRPTC.
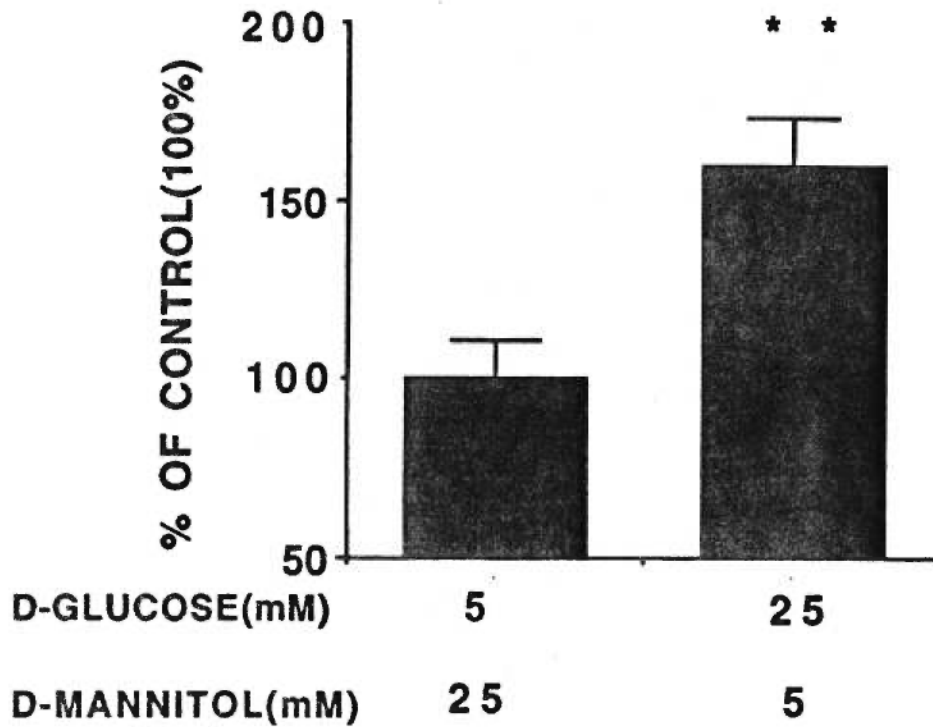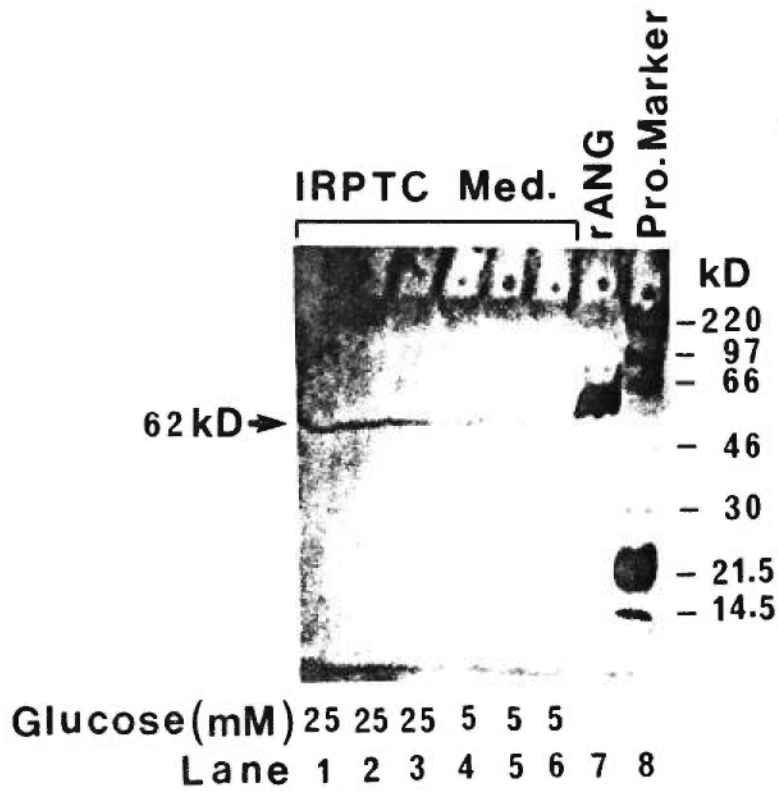
Figure 18: Western Blot analysis of the culture medium which were concentrated by centricon-30. The methods see Appendix I
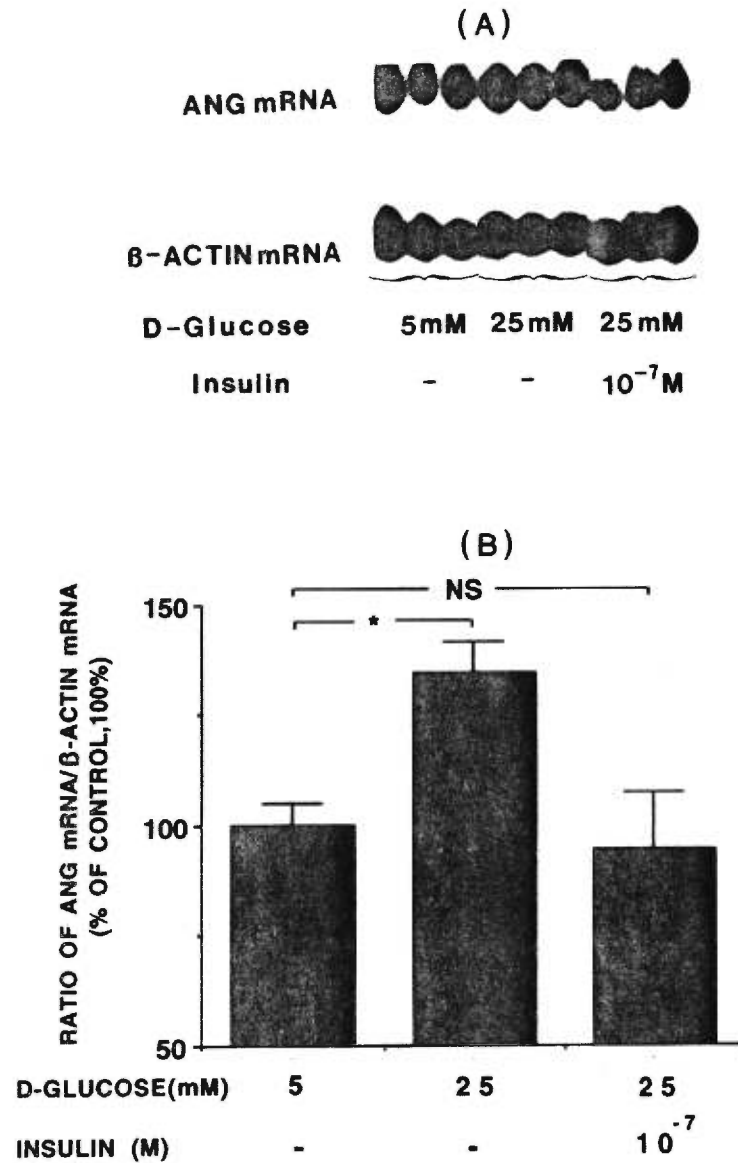
Figure 19: Effect of glucose and insulin on the expression of rat angiotensinogen mRNA in IRPTCs. The methods see Appendix VI.

Very few studies have demonstrated the effect of high glucose levels on the expression of RAS genes in renal proximal tubular cells in vitro. In the studies of heterologous opossum kidney (OK) cells, we showed that the addition of high levels of glucose stimulate the expression of the fusion gene, pOGH (ANG N-1498/+18) in OK27 cells in a dose-dependent manner (Figure 1, Appendix II). Moreover, the stimulatory effect of a high glucose level is time-dependent (Figure 2, Appendix II). The highest stimulatory effect of glucose was observed after two days of incubation (Figure 2 Appendix II). The stimulatory effect of glucose was diminished with more than 2 days of incubation. We also found that the increased promoter activity of pOGH (rANG-1498/+18) was blocked by H-7 and staurosporine but not by Rp-cAMP (an inhibitor of protein kinase AI and II) (Figure 6, Appendix II), suggesting that the effect of glucose on the expression of the ANG gene was mediated via the PKC signal transduction pathway.

A novel finding of our studies is that the exposure of IRPTCs to high concentrations of D(+)-glucose, stimulated the expression of the ANG in a concentration dependent manner (Figure 1, Appendix V). We did not observe any stimulation of the expression of ANG by L-glucose, D-mannitol or 2-deoxy-D-glucose (Figure 2, Appendix V), indicating that the effect of high D(+)-glucose levels on the expression of ANG in IRPTCs is independent of changes in osmolarity and is probably mediated via the metabolism of D(+)-glucose.

Our studies also demonstrated that the inhibition of aldose reductase with Tolrestat blocked the stimulatory effect of 25 mM D(+)-glucose on the expression of the ANG in IRPTCs (Figure 3, Appendix V). Furthermore, the addition of staurosporine (Figure 4A, Appendix V) and H-7 (Figure 4B, Appendix V) also blocked the stimulatory effect of high D(+)-glucose on the expression of ANG in IRPTC, suggesting that the effect of high levels of glucose is mediated, at least in part, via the polyol pathway as Tilton et al have suggested (201). Activation of the polyol pathway results in stimulation of *de novo* synthesis of DAG, which subsequently could stimulate the PKC activity in IRPTCs. The involvement of PKC in the expression of ANG in IRPTCs is further supported by the observation that the addition of PMA stimulated the expression of ANG in IRPTCs (Figure 5, Appendix V). Consistent with these findings, the stimulatory effect of PMA was abolished after a 24-hour pre-incubation of IRPTCs with 5 mM D(+)-glucose in the presence of 10^{-5} M PMA (Figure 6B, Appendix V) compared to the control cells without the pre-incubation with PMA (Figure 6B, Appendix V). These results are consistent with the notion that the prolonged exposure to PMA will down-

regulate the PKC activity and protein expression levels(202). The observation that PMA abolished the inhibitory effect of Tolrestat in the expression of ANG in IRPTCs (Figure 7, Appendix V) suggests that direct activation of PKC activity by PMA could stimulate the ANG gene expression independently of the polyol pathway. These studies also suggest that the PKC activation occurs downstream of the polyol pathway stimulated by high levels of glucose.

To examine whether the effect of glucose could be mediated via *de novo* synthesis of DAG and subsequent activation of PKC activity, we assayed the cellular levels of sorbitol. fructose, DAG, and PKC activity in IRPTC exposed to high glucose in the presence or absence of Tolrestat. Indeed, culture of IRPTCs in 25 mM D(+)-glucose dramatically increased the intracellular level of sorbitol and fructose (Figure 8, Appendix V) and markedly increased the levels of DAG (Figure 9, Appendix V) and membrane PKC activity (Figure 10, Appendix V) compared with IRPTCs cultured in medium containing 5 mM glucose. Tolrestat (10^{-4} M) blocked the increase in sorbitol, fructose and DAG levels and PKC activity stimulated by 25 mM D(+)-glucose (Figure 8,9, and 10, Appendix V). These data provide additional support to the notion that the effect of high levels of glucose (25 mM) on the expression of the ANG in IRPTCs is mediated, at least in part, via the *de novo* synthesis of DAG and the activation of PKC activity. Apparently, more experiments are warranted to determine the isoform(s) of PKC involved. Cellular myoinostitol levels were also quantified in IRPTCs exposed to 25 mM glucose in the presence or absence of Tolrestat (Figure 8, Appendix V). Myoinositol levels are considerably decreased in some tissues from diabetic animals. These observations have been interpreted as evidence for a decreased phosphoinositide turnover and decreased DAG production, possibly leading to decreased PKC activity (203). Our studies are in agreement with that of Ziyadeh et al (194), however, the cellular myoinositol levels in IRPTCs were unaffected by glucose or Tolrestat.

The effect of glucose on ANG appears to occur at the mRNA level. Indeed, exposure of IRPTCs to a high glucose concentration (25 mM) stimulated the expression of the ANG mRNA by two fold (P≤0.05) compared to levels in control cells (that is, 5 mM glucose; Figure 11, Appendix V). Tolrestat (10^{-4}M) completely blocked the stimulatory effect of 25 mM D(+)-glucose. Currently, it is uncertain whether glucose could increase the transcription or might affect the stability of the ANG mRNA in IRPTCs. Our results in OK27 cells, however, had shown that a high level of glucose stimulated the expression of pOGH (rANG N-

1498/+18) in OK27 cells (Figure 1, Appendix II), indicating that the effect of high glucose is mediated at least in part, via the transcriptional levels. Nevertheless, more studies are warranted to investigate these observations.

In these studies, we have not quantified the levels of Ang II in the medium to demonstrate a parallel increase in ANG and Ang II in the medium stimulated by high glucose levels. Studies by Tang et al (195), however, have shown that the Ang II is present in the incubation medium of IRPTCs. Clearly, more experiments are needed to correlate the levels of expression of the ANG and Ang II in the medium.

In summary, our studies showed that high glucose levels directly stimulate the expression of the ANG gene in IRPTCs. We have further demonstrated that the stimulatory effect of high glucose was blocked by Tolrestat, staurosporine, and H-7, implicating the involvement of both the polyol pathway and PKC signal transduction pathway in the overexpression of renal ANG gene under hyperglycemic conditions (Figure 23). These results suggest that inhibition of aldose reductase and PKC might be useful agents for the prevention and / or attenuation of glucose-induced ANG gene expression and consequently development of diabetic nephropathy. However, it remains to be investigated whether aldose reductase inhibitors may be beneficial in the long term for the treatment of diabetic nephropathy.
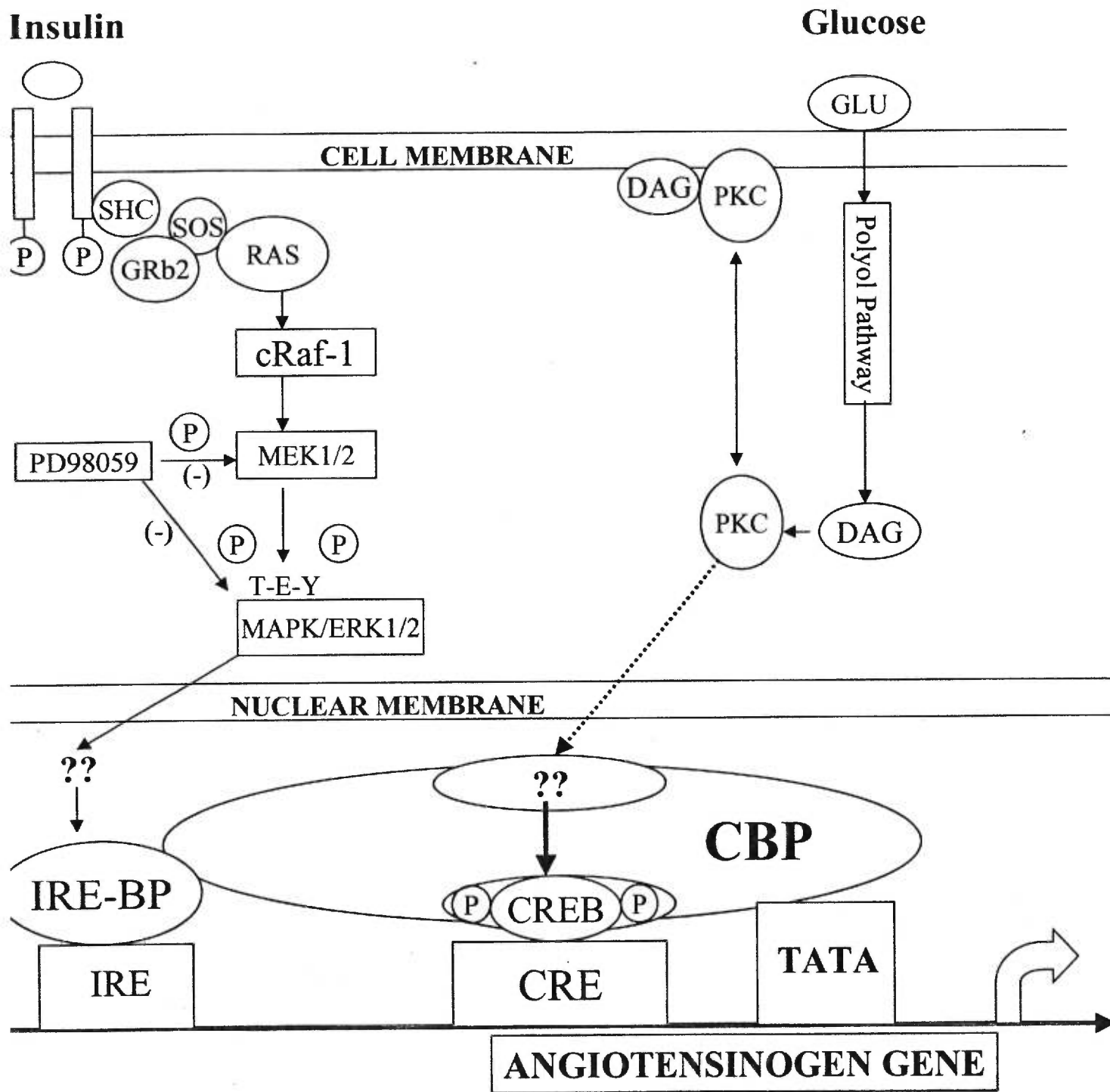
Figure 23: Molecular model for glucose and inulin action on the expression of rat angiotensinogen (ANG) gene.

Studies by Chang and Perlman (196) have shown that insulin attenuates the expression of the ANG mRNA in rat hepatoma cells in vitro. More recently, Aubert et al (160) also demonstrated that insulin down-regulates ANG gene expression and ANG secretion in cultured adipose tissue. Consistent with these results, we have observed that insulin inhibited the stimulatory effect of glucose on the expression of IR-rANG in a dose-dependent manner (Figure 1A, Appendix VI). These results together with those of Chang and Pedman (196) and Aubert et al (160), suggest that insulin may downregulate ANG gene expression. We did not observe any significant inhibition of the expression of IR-rANG in IRPTC treated with various concentration of IGF-I and IGF-II (Figure 1B, Appendix VI). These results suggest that the inhibitory action of insulin on the expression of IR-rANG in IRPTC is specific for insulin and mediated via the insulin receptor.

Our present studies show that PMA (10^{-7}M) stimulated the expression of IR-rANG in IRPTC incubated in either a low (5 mM) glucose medium (Figure 2A, Appendix VI) or in a high (25 mM) glucose medium (Figure 2B, Appendix VI), supporting the hypothesis that the effect of high glucose levels on the expression of the ANG gene is mediated via the protein kinase C pathway (Appendix II and V). Indeed, the involvement of PKC in modulating the expression of ANG in IRPTC is confirmed by our recent studies where it was reported that the stimulatory effect of high level (25 mM) glucose on the expression of rat ANG gene in OK cells (Appendix II) and IRPTC (Appendix V) is blocked by inhibitors of PKC (i.e., staurosporine and H-7). Insulin blocked the stimulatory effect of PMA on the expression of IR-rANG in IRPTC in a dose-dependent manner (Figure 2A and 2B, Appendix VI). While the overnight incubation of IRPTC with a high dose of PMA (10^{-5}M) did not abolish the inhibitory effect of insulin on the expression of IR-rANG it did abolish the stimulatory effect of a lower dose of PMA (10^{-7}M) (Figure 3, Appendix VI). Although we do not understand the molecular mechanism(s) of the opposing effect of PMA and insulin on the expression of IR-rANG in IRPTC, our observation raises the possibility that the inhibitory effect of insulin on the expression of the ANG gene may be mediated downstream of the PKC signal transduction pathway or mediated via other signal transduction pathway.

It is interesting to note that PD98059 (an inhibitor of MEK (163)) at 10^{-5}M or greater concentrations enhanced the stimulatory action of 25 mM glucose (Figure 4A, Appendix VI) and 25 mM glucose plus PMA (Figure 4B, Appendix VI) on the expression of the rANG. These results indicate that the stimulatory effect

of high levels of glucose and PMA on the expression of ANG gene may be enhanced by inhibition of the MEK signal transduction pathway. Indeed, our results show that PD98059 blocked the stimulatory effect of insulin in a dose-dependent manner (Figure 5A, Appendix VI),whereas Wortmannin (an inhibitor of phosphatidylinositol-3-kinase (PI_3) activity (165)) had no effect (Figure 5B, Appendix VI). These data are consistent with the notion that the inhibitory effect of insulin is mediated, at least in part, via the MEK pathway.

The effect of glucose and insulin on ANG gene expression appears to occur at the mRNA level. Exposure of IRPTC to a high glucose concentration (25 mM) significantly ($P \leq 0.05$) stimulated the expression of the ANG mRNA (i.e, an increase of 2-fold) compared to expression in control cells (cultured in 5 mM glucose medium) (Figure 6, Appendix VI). Insulin ($10^{-7}M$) completely blocked the stimulatory effect of 25 mM glucose. PD98059 reversed the inhibitory effect of insulin. At present, it is uncertain whether insulin decreases ANG mRNA levels at the transcriptional level or affects the stability of the ANG mRNA in IRPTC. Our preliminary studies (Figure 20, unpublished results), however, showed that insulin inhibits the expression of the pOGH (rANG N-1498/+18) in OK 27 cells. These studies suggest that the inhibitory effect of insulin occurs, at least in part, via the transcriptional level. Nevertheless, additional work is warranted along this line.
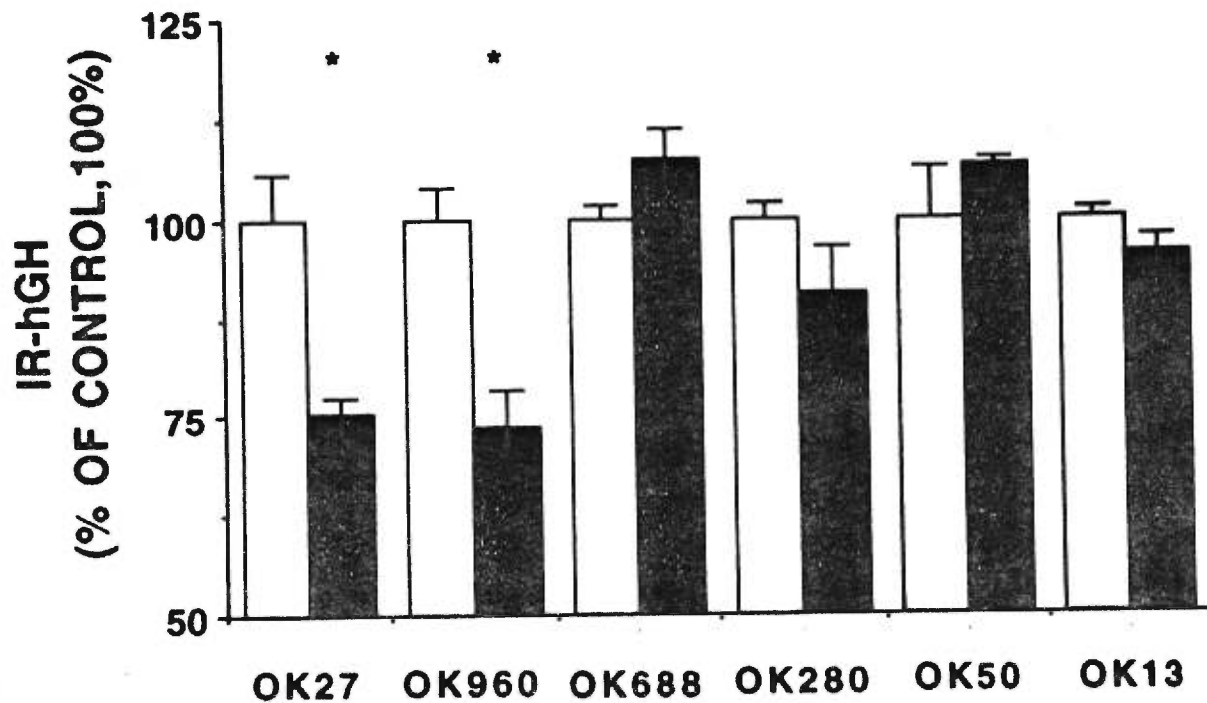
Figure ❽: Inhibitory effect of insulin on the expression of fusion genes in OK cells.
Method: OK 27, OK 960, OK 688, OK 280, OK 53 and OK 13 are stable cell lines with pOGH (rANG N-1498/+18) , pOGH (rANG N-960/+18), pOGH (rANG N-688/+18), pOGH (rANG N-280/+18), pOGH (rANG N-53/+18) and pTKGH stably integrated into their genomes, respectively. These cells were incubated in 25 mM glucose medium in the absence or presence of insulin (10^{-7} M) for 24 hours. Then, media were collected and assayed for the levels of immunoreactive human growth hormone (IR-hGH) secreted into the media. The blank bar on the left (i.e. control) represents the cells incubated in the absence of insulin. The solid bar on the right represents the cells incubated in the presence of insulin (10^{-7} M).

Figure 20:

Our studies also showed that the incubation of IRPTC in 25 mM glucose induced cellular hypertrophy (Figure 21, unpublished results) and increased the cellular protein content (Figure22, unpublished results). Such results are consistent with other studies showing that high glucose media (i.e. ≥25 mM) may induce cellular hypertrophy in murine proximal tubular cells (258). Our data demonstrated that insulin is effective to attenuate or block the hypertrophy of renal proximal tubular cells in a high glucose media.
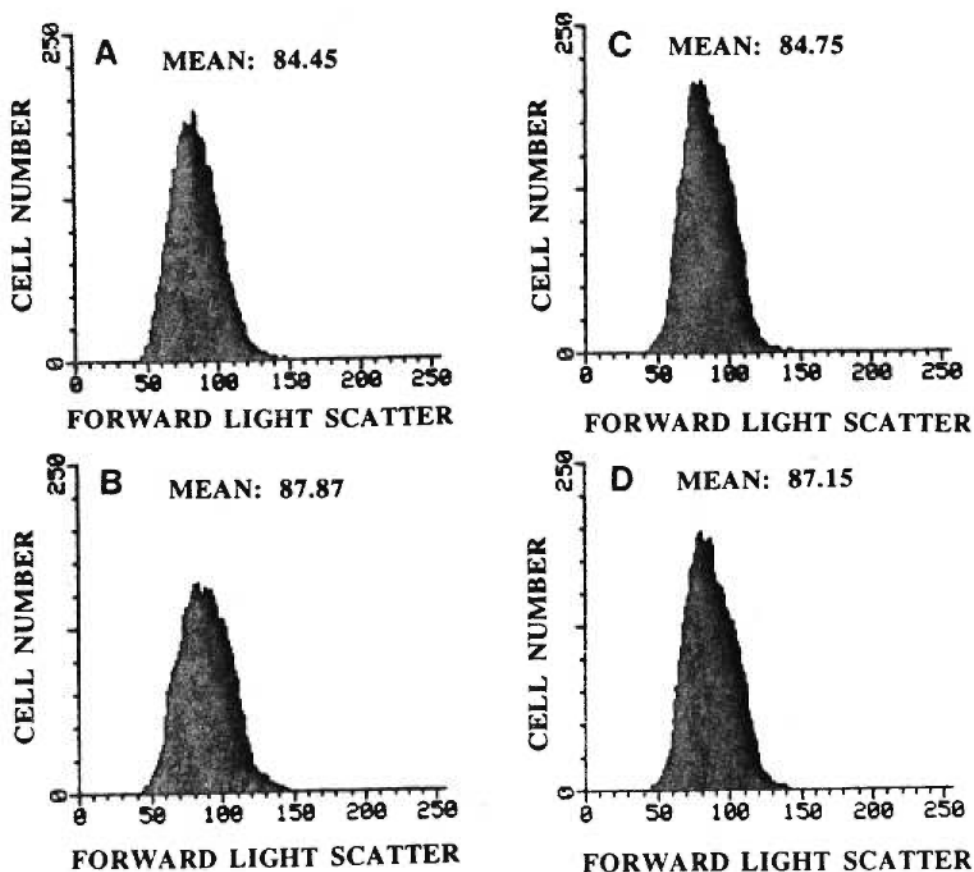
Figure 21: Effect of glucose and insulin on cellular hypertrophy in IRPTC. After a 48 hour incubation in media containing 5mM glucose (A), 25 mM glucose (B), 25 mM glucose plus insulin (10^{-7}M) (C), and 25 mM glucose plus 10^{-7}M insulin and 10^{-7}M PD98059 (D), IRPTC were analyzed by flow cytometry. DNA was stained with propidium iodide to determine the cell viability (not Shown). Forward light scatter was expressed in arbitrary units after computer analysis. Righward shift of the plot on the X-axis indicates an increase in celluar diameter. Plots are representative for three expriments.
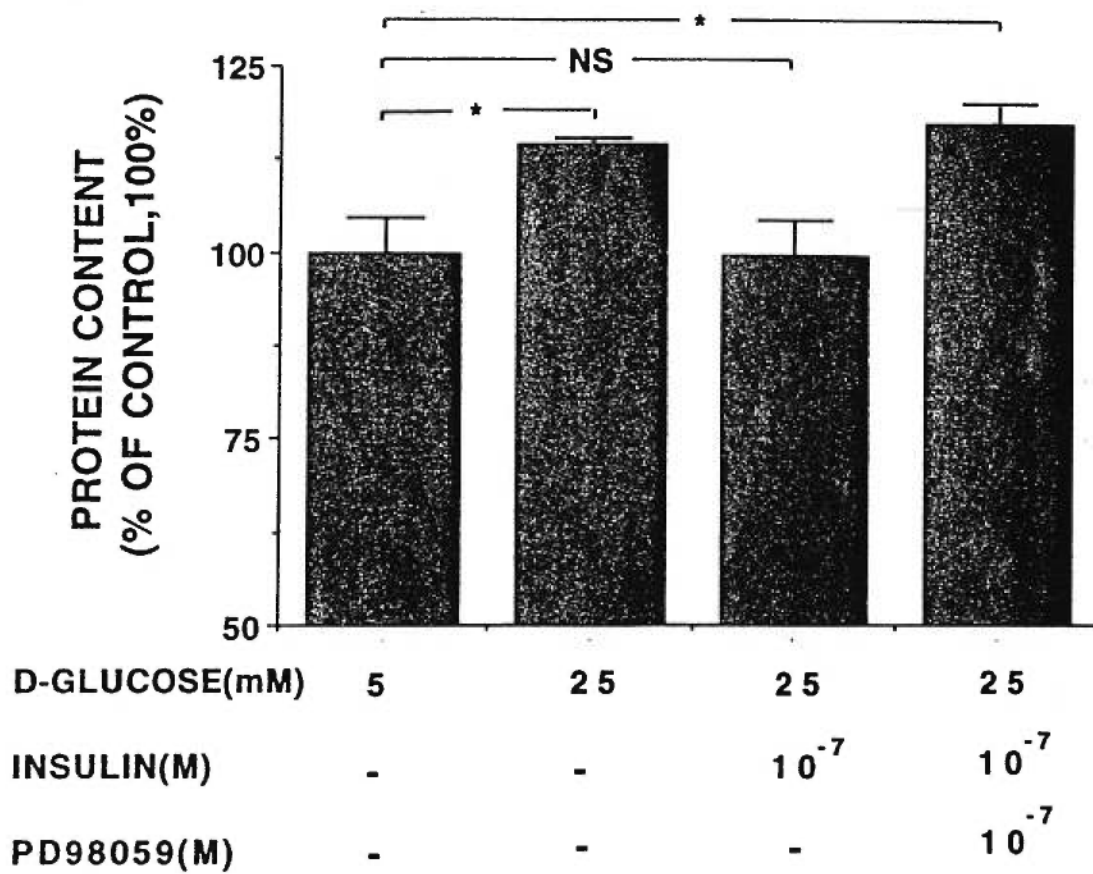
Figure 22: Total cellular protein content in IRPTC as determined by a modified method of Markwell (322) and expressed per 10 [6] cells. Cells were incubated for 48 hours in the presence of 5 mM glucose, 25 mM glucose, 25 mM glucose plus insulin (10-7M), and 25 mM glucose plus 10-7M insulin and 10-7M PD98059 . Cells were then harvested and analyzed for total cellular protein content. The content of cellular protein in IRPTC incubated in 5 mM glucose represents the control level (i.e. 52.65 ± 2.98 ug protein per 10 [6] cells). Each point represents the mean ± S.D. of three determinations (*P≤ 0.05). Similar results were obtained in two other experiments.

At present, we do not understand the exact molecular mechanism(s) of high glucose levels on the expression of the ANG gene in IRPTC. One possibility may be that high glucose levels may stimulate *de novo* synthesis of diacylglycerol (DAG) from metabolized glucose via the polyol pathway as suggested by Tilton et al (201). This leads to an increase in protein kinase C activity. Indeed, our studies (Appendix V) showed that high levels of glucose increase the cellular levels of DAG and PKC activity in IRPTC. Once PKC is activated, it may phosphorylate the 43 kDa cAMP-response element binding protein (CREB) or CREB-like nuclear protein(s), since the 43 kDa CREB contains the role of phosphorylation by protein kinase C (87). Moreover, recent studies by Kreisberg et al (157) have shown that PMA and high glucose levels stimulate the phosphorylation of the 43 kDa-CREB. Phosphorylated CREB then binds to the cAMP-responsive element (CRE) of the rat ANG gene (TGACGTAC, nucleotides N-795 to N-788) (58) and subsequently, stimulates the expression of the rat ANG gene. This possibility is supported by our studies which demonstrated that transient transfection of 43 kDa-CREB into OK cells stimulates the expression of rat ANG gene promoter activity (158) and that ANG-CRE binds with the 43 kDa-CREB (Appendix III/IV).

Similarly, we do not understand the exact molecular mechanism(s) of action of the inhibitory effect of insulin on the expression of ANG gene in IRPTC. One possibility might be that insulin activates the MAP kinase signal transduction pathway as shown in Figure 7 (Appendix VI) and induce the phosphorylation or expression of certain protein(s). The insulin-induced protein(s) then suppresses the ANG gene via a yet undefined pathway. Alternatively, the inhibitory effect of insulin may be mediated, at least in part, via an insulin-responsive element (IRE) in the 5'-flanking region of the ANG gene. We have not yet identified the IRE in the rat ANG gene. Studies along these lines, however, are underway in our laboratory to explore these possibilities.

Interestingly, Wortmannin failed to block the inhibitory effect of insulin on the expression of the ANG in IRPTC (Figure 5, Appendix VI). Therefore, it is unlikely that the inhibitory action of insulin on the expression of the rat ANG gene is mediated via the PI-3 kinase signal transduction pathway.

In summary, our studies showed that insulin inhibits the stimulatory effect of high levels of glucose on the expression of the ANG gene and hypertrophy in IRPTC. The stimulatory effect of the insulin appears to be mediated , at least in part, via the MAPK kinase signal transduction pathway (Figure 23). These

studies raise the possibility that the expression of the renal ANG gene may be stimulated in hyperglycemic states in vivo. Consequently, the increased local formation of renal Ang II may contribute to renal remodeling (i.e, renal hypertrophy in early diabetes). Insulin therapy may therefore attenuate this event by inhibiting the activation of local renal RAS.

V. CONCLUSIONS

1. We have demonstrated that D(+)-glucose stimulates the expression of ANG in IRPTC in a dose-dependent manner and the effect of D(+)-glucose is specific because D-mannitol, L-glucose and 2-deoxy-glucose had no effect.

2. The stimulatory effect of glucose appears to be mediated, at least in part, via the *de novo* synthesis of DAG (polyol pathway) and subsequently activates the PKC signal transduction pathway.

3. Tolrestat (an inhibitor of aldose reductase) blocks the stimulatory effect of high levels of glucose on the expression of the ANG mRNA in IRPTC.

4. Insulin also inhibits the stimulatory effect of high levels of glucose on the expression of ANG and ANG mRNA in IRPTC.

5. IGF-I and IGF-II had no effect on the expression of the ANG in IRPTC.

6. The inhibitory effect of insulin appears to be mediated, at least in part, via the MAPK kinase signal pathway and independent of PKC signal transduction pathway.

7. High levels glucose (25 mM) stimulate hypertrophy in IRPTC.

8. The hypertrophic effect of glucose is inhibited by insulin.

9. These studies demonstrate that high levels of glucose stimulate while insulin inhibits the expression of the ANG gene and hypertrophy in IRPTC, underlying the importance of beginning insulin therapy as soon as possible in diabetic patients.

VI. FUTURE DIRECTIONS AND EXPERIMENTS

Our studies clearly demonstrated that high levels of glucose stimulated the expression of the ANG gene in IRPTC, at least in part, via the PKC signal transduction pathway. Insulin inhibits the stimulatory effect of high levels of glucose on the expression of the ANG gene, at least in part, via the MAPK kinase signal transduction pathway. The molecular mechanism(s) of glucose and insulin regulation of ANG gene expression, however, remain(s) undefined. The objective of my future studies (Ph.D. program Sept. 1999 to August 2002) aim to define the molecular mechanism(s) of glucose and insulin regulation of ANG gene expression in IRPTC and to investigate the role(s) of renal ANG in the development of hypertrophy in IRPTC. The specific aims are :

(1). To Identify the specific PKC(s) that may be involved in the expression of the ANG gene in IRPTC.

(2). To establish the functional relationship between PKC activation, phosphorylation of the expression cAMP-responsive element binding protein (CREB) and the expression of the ANG gene in IRPTC.

(3). To investigate whether the inhibitory effect of insulin is mediated, at least in part, via the activation of immediate early genes, i.e. c-Fos and c-Jun. The activation of c-Fos and c-Jun subsequently block the ANG gene expression via the interaction with CREB (i.e., protein/ protein interaction).

(4). To investigate the effect of high levels of glucose on the expression of extracellular matrix proteins (ECMP) and the hypertrophy of IRPTC without the endogenous ANG gene.

Methods:

Aim 1: IRPTC will be incubated in low (5 mM) or high (25 mM) glucose DMEM at various time periods. Then, the IRPTC will be harvested and separated into cytosol and membrane fractions. The expression of various fraction(s) of PKC will be identified by Western blots using specific antibodies against the

different isoforms of PKC. The PKC activity will be determined by its enzymatic activity. Furthermore, antisense oligonucleotides(s) against the specific isozyme(s) of PKC will be transiently transfected into PKC and the stimulatory effect of high levels of glucose on the expression of the ANG gene will be demonstrated accordingly. These studies will demonstrate the specific PKC isozyme(s) that are activated by high levels of glucose as well as the translocation of PKC isozyme(s) on the expression of the ANG gene in IRPTC.

Aim 2: To demonstrate that the effect of PKC activation is mediated, at least in part, via the phosphorylation of CREB, IRPTC will be stablly transfected with the plasmid containing the 43 kDa-CREB cDNA in corrected and reversed orientation. The effect of high levels of glucose with or without PMA (10^{-7}M) on the phosphorylation of CREB and the expression of the ANG gene will be determined by Western blots using rabbit polyclonal antibodies against the non-phosphorylated and phosphorylated CREB and RT-PCR for rat ANG mRNA, respectively. These studies will demonstrate whether the stimulatory effect of high levels of glucose on the expression of the ANG gene is mediated via the phosphorylation of CREB by specific isozyme(s) of PKC.

Aim 3: To investigate whether the inhibitory effect of insulin on the expression of the ANG gene in IRPTC is mediated via the expression of c-Fos / c-Jun, IRPTC will be incubated in 5 mM or 25 mM DMEM in the presence or absence of insulin (10^{-7}M) for various time periods. The effect of insulin on the expression of c-Fos / c-Jun and mRNA of c-Fos / c-Jun will be determined by Western blots using specific polyclonal antibodies against the c-Fos/ c-Jun and by RT-PCR for c-Fos/ c-Jun mRNA. The effect of insulin on the phosphorylation of ELK-1, p90 Rsk and c-Jun will also be determined by Western Blots using specific antibodies against the phosphorylated ELK-1, p90 Rsk and c-Jun. Furthermore, antisense oligonucleotides against the c-Fos/ c-Jun will be transiently transfected into IRPTC, the effect of insulin on the expression of the ANG gene will be determined. These studies will demonstrate whether the inhibitory effect of insulin on the expression of the ANG gene is mediated via the expression of the c-Fos/ c-Jun.

Aim 4: To demonstrate that the stimulatory effect of high levels of glucose on the expression of transforming growth factor ß1 (TGFß1) and ECMP genes

and hypertrophy of IRPTC is mediated, at least in part, via the expression of the ANG gene consequently on the activation of renal RAS, IRPTC will be stably transfected with the plasmid containing the rat ANG cDNA in corrected or reversed orientation. The effect of high levels of glucose on the expression of proteins and mRNA of ANG, TGFß1 and ECMP will be determined by Western blots and RT-PCR. The effect on cellular hypertrophy will be determined by flow cytometry, cellular protein content and incorporation of ^{3}H-leucine into proteins. Moreover, the amount of immunoreactive Ang II in the cultured media will be determined by a specific RIA for Ang II to correlate the hypertrophic effect. These studies will demonstrate unequivocally whether the stimulatory effect of high levels of glucose on the expression of genes of TGFß1 and ECMP and hypertrophy of IRPTC is mediated, at least in part, via the expression of the ANG gene and the activation of the local RAS.

In summary, the above studies should demonstrate, at least partially, the molecular mechanism(s) of high levels of glucose and insulin on the expression of the ANG gene and the role(s) of the ANG and RAS in the induction of hypertrophy in IRPTC. These studies will provide important information concerning the pathogenesis of nephropathy in diabetes and the molecular mechanism(s) of insulin therapy.

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VIII. APPENDIX

Appendix I: Wang LS, Lei CL, **Zhang SL**, Roberts KD, Tang SS, Ingelfinger JR, Chan JSD: Synergistic effect of dexamethasone and isoproterenol on the expression of angiotensinogen in immortalized rat proximal tubular cells.*Kidney Int.* 53: 287-295, 1998.

ynergistic effect of dexamethasone and isoproterenol on the xpression of angiotensinogen in immortalized rat proximal ıbular cells

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iynergistic effect of dexamethasone and isoproterenol on angiotensino-ı in immortalized rat proximal tubular cells. To investigate whether the ıression of angiotensinogen (ANG) in rat kidney proximal tubules is nulated by dexamethasone and isoproterenol, immortalized rat proxi-l tubular cells (IRPTC) were cultured in a monolayer. Immunoreactive ANG (IR-rANG) in the culture medium was measured by a specific ioimmunoassay (RIA) for rANG. This RIA was developed by employ-rabbit antiserum against the purified recombinant rat ANG (rANG). ɛ purified rANG from plasma and the iodinated rANG were used as the mone standard and tracer, respectively. The RIA is specific for rat G and it has no cross-reactivity with other pituitary hormone prepa-ons or other rat plasma proteins. The sensitivity of detection of the ١ is approximately 2 ng of rANG. The levels of IR-rANG in the culture ıia of IRPTC ranged from 2 to 5 ng/ml/24 hr/10^6 cells. The addition of amethasone (10^{-13} to 10^{-5} M) stimulated the expression and secretion ANG from IRPTC in a dose-dependent manner, whereas the addition soproterenol alone had no effect. However, a combination of both amethasone and isoproterenol synergistically stimulated the expres-ı and secretion of rANG by IRPTC. The synergistic effect of dexa-hasone and isoproterenol was blocked by the presence of RU 486 (a :ocorticoid receptor antagonist) or propranolol (β-adrenoceptor block-
These studies suggest that the addition of dexamethasone and ıroterenol acts synergistically to stimulate the expression and secretion ١NG protein in rat proximal tubules *in vivo.*

ıngiotensin II (Ang II), an octapeptide derived from the cursor protein angiotensinogen (ANG), exerts a dose-depen-t biphasic effect on proximal tubular sodium reabsorption ɜ] and induces growth of the maturing kidney [4]. This study cates that Ang II may play an important role in the control of :trolyte reabsorption in the kidney and in maintaining renal nerular growth.

hysiological studies have shown that the levels of Ang I and ɡ II in the luminal fluid in rat proximal tubules are as high as ⁹ M, whereas the levels of plasma Ang I or Ang II are less than

10^{-12} M [5, 6], suggesting that high levels of Ang I and Ang II in the renal tubular fluid exceed those that can be accounted for from the circulation (glomerular filtrate). Furthermore, ANG mRNA has been localized in rat proximal tubules by the techniques of *in situ* hybridization [7] and polymerase chain reaction [8]. Recent studies have also shown that the mRNAs for the components of RAS, including ANG, renin, angiotensin-converting enzyme and angiotensin-II receptor (AT_1-receptor) are expressed in murine (mouse and rat) immortalized proximal tubular cell lines [9–11]. We [12, 13] have also demonstrated that the ANG mRNA is expressed in opossum kidney (OK) proximal tubular cells. All these studies provide evidence that the intrarenal Ang II is probably derived from the ANG that is synthesized in renal proximal tubular cells. To our knowledge, until now there has been no report on the expression and secretion of the ANG protein from the proximal tubular cells. Hence, experimental evidence to demonstrate that the ANG protein is expressed and secreted from the proximal tubular cells is definitely warranted.

The objective of the present studies was to develop a specific radioimmunoassay (RIA) for rANG by employing rabbit antiserum against the purified recombinant rat ANG (rANG). This RIA-rANG was used to quantify the level of ANG protein expressed and secreted from the immortalized rat proximal tubular cells (IRPTC) *in vitro.* We also investigated whether the expression of the ANG could be modulated in the presence of dexamethasone and isoproterenol (IRPTC) *in vitro.*

Our studies showed that the total amount of IR-rANG in the cell extract of IRPTC was less than 5% of that detected in the medium. The addition of dexamethasone alone stimulated the expression and secretion of ANG in IRPTC in a dose-dependent manner, whereas isoproterenol alone had no effect. Furthermore, a combination of both dexamethasone and isoproterenol synergistically stimulated the expression of ANG in IRPTC. This stimulatory effect of dexamethasone and isoproterenol could be blocked by the presence of RU 486 (an antagonist of glucocorticoid) or propranolol (a non-selective β-adrenoceptor blocker). Our present results confirm the recent report of Loghman-Adham [11] who showed that the addition of dexamethasone (10^{-6} M) stimulated the expression of mouse ANG as measured by indirect radioimmunoassay in immortalized murine proximal tubular cells.

THODS

terials

All of the hormone preparations listed below, with the exception of human ANG (hANG) and rat albumin, were kindly plied by the NIAMDD of the National Institutes of Health AMDD-hGH-I-1 AFP-4793B; NIH, Bethesda, MD, USA), rat wth hormone (rGH; NIADDK-rGH-RP-2, 0.6 IU/mg), rat roid stimulatory hormone (rTSH; NIADDK-rTSH-RP-2, P-5153B), rat luteinizing hormone (rLH; NIADDK-rLH- -2, AFP-566C), rat prolactin (rPRL; NIADDK-rPRL-RP-3, P-4459B). Human angiotensinogen and rat albumin were ·chased from Calbiochem-Novabiochem Corporation (La Jolla, ., USA) and Sigma Chemicals (St. Louis, MO, USA), respec- :ly.

R(-)-isoproterenol(+)bitartrate salt (a β-adrenoceptor ago- t), S(-)-propranolol hydrochloride (an inhibitor of β_1 and β_2 ·enoceptors) were purchased from Research Biochemicals Inc. BI, Natick, MA, USA). Dexamethasone was purchased from ma Chemicals. RU 486 (an antagonist of glucocorticoid) was a : provided by Dr. Alain Bélanger (Laval University, Quebec, nada).

Na^{125}I was purchased from Dupont, New England Nuclear EN, Boston, MA, USA).

rification of recombinant rat angiotensinogen from bacteria
d rat plasma

The plasmid containing the full-length of rat ANG cDNA, ;EM (rANG cDNA) was obtained from Dr. Joel F. Habener [GH, Boston, MA, USA). The rat ANG cDNA was inserted o a bacterial expression vector, pMalc (New England Biolabs, verly, MA, USA) at the *Eco*RI polylinker site and then .nsformed the *E. coli* TB-1 cells (New England Biolabs). The oression of the fusion protein [Maltose-binding protein (MBP) ;ed with rat ANG (MBP-rANG)] in TB-1 cells was induced by dition of 2.5 mM isopropylthiogalactoside (IPTG) into the lture-medium and incubated for four hours. The bacteria were :n harvested and the MBP-rANG fusion proteins were purified)m the bacterial extracts by amylose resin affinity column romatography according to the protocol supplied by the man- acturer (New England Biolabs). The purified MBP-rANG fu-)n proteins were used to immunize New Zealand white rabbits 'harles River Inc., St. Constant, Quebec, Canada) according to e procedures we described previously for ovine placental lacto- n [14].

The rabbit polyclonal antibodies (IgG) against the MBP-rANG sion protein were purified from the rabbit antiserum by protein affinity chromatography according to the protocol supplied by 1armacia Inc. The purified IgGs were coupled to CN-activated :pharose 4B and then used to set up the IgG-affinity column 'harmacia Inc.) for purification of rANG from rat plasma.

Rat plasma ANG was purified by a three step method by issing sequentially through the IgG-affinity column chromatog- phy, a Blue-Sepharose column (purchased from Pharmacia .c.) and finally, another IgG-affinity column.

haracterization of MBP-rANG fusion protein and purified rat
asma angiotensinogen

The purified MBP-rANG fusion protein and the purified rat asma ANG preparations were analyzed by a linear gradient of 6 to 15% polyacrylamide gel electrophoresis containing sodium dodecyl sulfate (PAGE-SDS) [15]. The protein standard markers (Rainbow protein markers) were purchased from Amersham Canada Ltd. (Oakville, Ontario, Canada).

Western blot analysis was performed to analyze the purified rat plasma ANG, rat plasma proteins and the concentrated culture media by employing the rabbit polyclonal antibodies against the MBP-rANG fusion protein, anti-rabbit horseraddish peroxidase (HRP; Bio-Rad Laboratories, Richmond, CA, USA) conjugates and the avidin-HRP conjugates according to the protocol of the supplier (Bio-Rad).

Radioimmunoassay for rat angiotensinogen

The radioimmunoassay (RIA) procedure was performed according to the procedures we described previously for ovine placental lactogen (oPL) by employing a double-antibody precipitation [14]. The purified rat plasma ANG was used for both iodination and as a hormone standard. The limit of sensitivity of the assay was 2 ng/ml. The inter- and intra-assay coefficients of variation were 9% ($N = 10$) and 14% ($N = 10$), respectively.

Cell culture

Immortalized rat proximal tubular cells (IRPTC; #93-p-2-1) were obtained by the transfection of rat proximal tubular cells with the origin-defective SV 40, as previously described [10]. The characteristics of the IRPTC have been reported previously [10]. Briefly, these cells express the mRNA and protein of ANG, renin, angiotensin-converting enzyme and angiotensin-II receptor [10].

The rat hepatoma (H-4-II-E, CRL-1548) cells were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). This cell line expresses the ANG mRNA and ANG and has been used extensively by various investigators [16, 17].

The IRPTC and H-4-II-E cells were grown in 100×20 mm plastic Petri dishes (Gibco, Burlington, Ontario, Canada) in Dulbecco's modified Eagle medium (DMEM; pH 7.45), supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml of streptomycin. The cells were grown in a humidified atmosphere in 95% air—5% CO_2 at 37°C. For subculturing, cells were trypsinized (0.05% trypsin and EDTA) and plated at 2.5×10^4 cells/cm^2 in 100×20 mm Petri dishes.

Basal expression and secretion of immunoreactive rat
angiotensinogen in immortalized rat proximal tubular cells

The IRPTC were plated at a density of 5×10^6 cells per 100×20 mm plastic Petri dish (Gibco) in DMEM, pH 7.45, supplemented with 10% FBS and incubated until 90% confluent. Then, the cells were incubated for 24 hours in medium containing 1% depleted FBS (dFBS). At the end of the incubation period, media and cells were harvested and kept at $-20°$ until the assay for IR-rANG. The cells were extracted with 0.1 M NH$_4$ HCO$_3$ after sonication. After gentle agitation overnight at 4°C, cell extracts were centrifuged at $15,000 \times$ g for 10 minutes at 4°C to remove the precipitate. The protein content was measured according to the protocol of the Bio-Rad protein kit. The number of cells were counted with a hemocytometer.

The total amount of IR-rANG present was corrected by the volume of medium and cellular extract and expressed as total nanograms of IR-rANG.

To analyze the molecular species of IR-rANG, the media were concentrated by Centricon-10 (Amicon Inc., Beverly, MA, USA) to less than 5% of the original volume before Western blotting. The cellular extracts were analyzed by Western blotting without concentration by Centricon-10.

The depleted fetal bovine serum (dFBS) was prepared by incubation with 1% activated charcoal and 1% AG 1×8 ion exchange resin (Bio-Rad) for 16 hours or more at room temperature as described by Samuels, Stanley and Shapiro [18]. This procedure removed endogenous steroid and thyroid hormones from the FBS as demonstrated by Samuels et al [18].

Effect of dexamethasone or isoproterenol on the expression and secretion of angiotensinogen by immortalized rat proximal tubule cells

The IRPTC were plated at a density of 1 to 2×10^6 cells/well in six-well plates and incubated overnight in DMEM containing 1% depleted FBS. Then, cell growth was arrested by incubation in serum-free medium for 24 hours. Subsequently, various concentrations of dexamethasone (10^{-13} to 10^{-5} M) or isoproterenol (10^{-15} to 10^{-7} M) were added to the culture medium containing 1% dFBS. At the end of the incubation period, media were collected and kept at $-20°C$ until assay for IR-rANG.

Synergistic effect of isoproterenol and dexamethasone on the expression and secretion of angiotensinogen

The IRPTC were plated at a density of 1 to 2×10^6 cells/well in six-well plates and incubated overnight in DMEM containing 1% dFBS. Then cell growth was arrested by incubation in serum-free medium for 24 hours. Subsequently, various concentrations of isoproterenol (10^{-15} to 10^{-7} M) were added to the culture medium containing 1% dFBS and incubated for 24 hours in the absence or presence of 10^{-6} M dexamethasone.

Inhibitory effect of RU 486 or propranolol on the expression and secretion of angiotensinogen in immortalized rat proximal tubule cells in the presence of dexamethasone and isoproterenol

To investigate whether RU 486 or propranolol could block the effect of dexamethasone and isoproterenol on the expression of ANG in IRPTC, RU 486 (10^{-5} M) or propranolol (10^{-5} M) were co-cultured with dexamethasone (10^{-6} M) and isoproterenol (10^{-7} M) for 24 hours. At the end of the incubation period, the media were collected and kept at $-20°C$ until assay.

Statistical analysis

The experiments were performed at least three times in triplicate. The data were analyzed with the Student's t-test or ANOVA analysis. A probability level of $P \leq 0.05$ was regarded as significant.

RESULTS

Purification and characterization of recombinant rat angiotensinogen from the bacteria and rat plasma

Figure 1 shows the analysis of the MBP-rANG fusion proteins by 6 to 15% polyacrylamide gel electrophoresis containing sodium dodecyl sulfate (PAGE-SDS). Three major bands with apparent molecular weights of 46, 69 and 97 kDa for MBP-rANG were observed. The 46 kDa molecular species was the induced maltose-
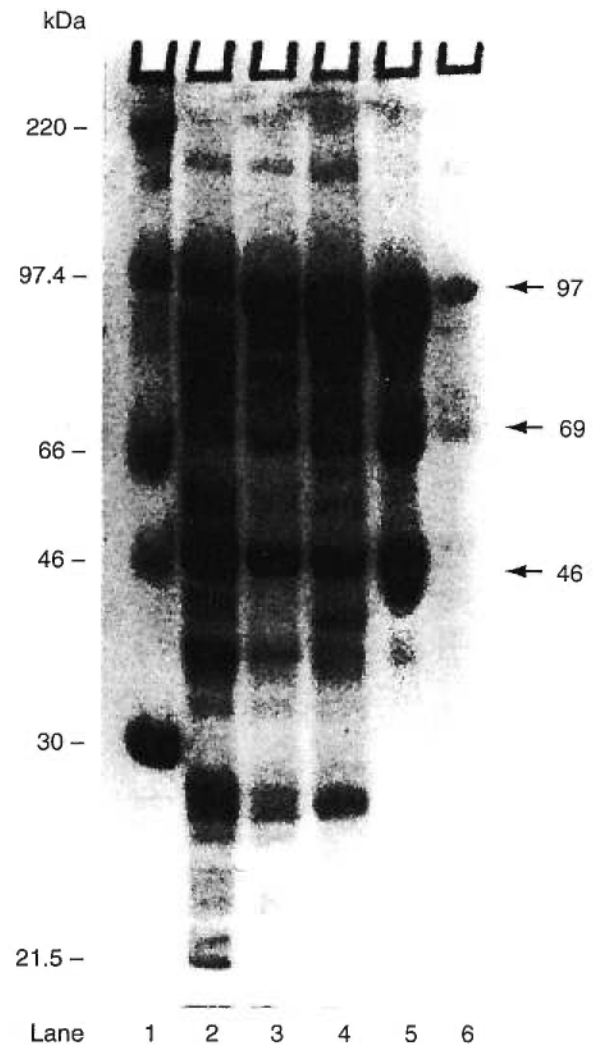

Fig. 1. Linear gradient 6 to 15% polyacrylamide gel electrophoresis containing sodium dodecyl sulfate (PAGE-SDS) analysis of the maltose-binding protein—rat ANG (MBP-rANG) fusion protein from the bacterial culture. Lane 1 (Amersham's Rainbow markers), lane 2 [crude bacterial extract without induction by isopropylthiogalactoside (IPTG), 150 μg], lane 3 (crude bacterial extract after induction by IPTG, 150 μg), lane 4 (crude bacterial extract after 10 to 50% ammonium sulfate precipitation, 150 μg), lane 5 (purified MBP-rANG after amylose resin column chromatography, 150 μg) and lane 6 (purified MBP-rANG after the amylose resin column chromatography, 15 μg).

binding protein as suggested by the supplier (New England Biolabs). The 97 kDa molecular species was the molecular weight of deglycosylated rANG (that is, 51 kDa) fused with the MBP (46 kDa), whereas the 69 kDa molecular species was probably the partially degraded MBP-rANG fusion proteins. The mixture of these three major species was used to immunize rabbits to raise polyclonal antibodies.

Rat plasma ANG was purified by three consecutive chromatographic steps including an IgG-affinity column, a Blue-Sepharose column and a second IgG-affinity column. It is evident that at least two major molecular species with apparent molecular weights of approximately 56 and 62 kDa were observed for the purified rat plasma ANG preparation (lane 3, Fig. 2A). Their
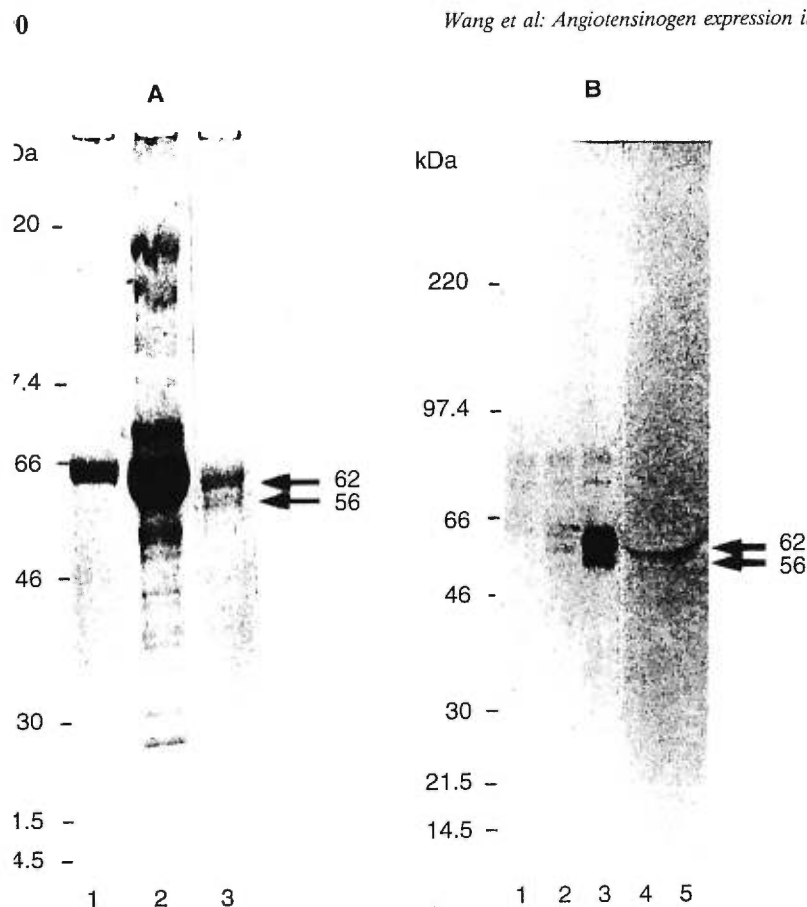
Fig. 2. Linear gradient 6 to 15% PAGE-SDS analysis (*A*) and Western blot analysis (*B*) of the purified rat plasma angiotensinogen (ANG). (A) Six to 15% PAGE-SDS analysis, lane 1 (rat albumin, 10 μg)), lane 2 (rat plasma, 10 μg) and lane 3 (purified rat plasma ANG after two passes through the IgG-affinity column and one pass through the Blue-Sepharose column, 10 μg). (B) Western blot analysis, lane 1 (bovine serum albumin, 10 μg), lane 2 (human ANG 10 μg), lane 3 (purified rat plasma ANG, 10 μg), lane 4 (5 μl of rat plasma) and lane 5 (10 μl of rat plasma). The apparent molecular weight of Amersham's Rainbow markers are indicated on the left of each panel. The apparent molecular weights of rat ANG are indicated on the right of each panel.

olecular weights were consistent with those reported in the :erature.

Western blot analysis (Fig. 2B) shows that the antiserum against ıe MBP-rANG fusion protein interacted with the purified rANG om rat plasma and the ANG in the rat plasma (lanes 3, 4 and 5), ıt did not interact with the purified bovine serum albumin (lane ı, human ANG (lane 2) and other proteins in the rat plasma. .gain, both forms of rat ANG (that is, 56 and 62 kDa) interacted ell with the antiserum against the MBP-rANG fusion protein. hese studies demonstrate that the antiserum against MBP-\NG is specific for rat ANG and has no cross-reactivity with ovine serum albumin or other rat plasma proteins, suggesting ıat the antiserum is suitable to develop the radioimmunoassay ır rANG.

adioimmunoassay for rat angiotensinogen

Figure 3 shows the typical standard curve for the radioimmu-oassay of rat ANG (RIA-rANG) by employing the rabbit ntiserum against the MBP-rANG fusion proteins. The purified ıt plasma ANG and the iodinated rANG were used as hormone :andard and tracer, respectively. The lower limit of sensitivity of ıe RIA was approximately 2 ng of rANG. It has no cross-:activity with either rat albumin or rat pituitary hormone prep-rations (rGH, rTSH, rLH and rPRL) or plasma samples of the og, cat, hamster and human. Serial dilutions of rat and mouse lasma samples, however, displayed a parallelism with the purified ıt ANG standard. Similarly, serial dilutions of the culture media om IRPTC and the rat hepatoma (H-4-II-E) cells displayed a arallelism with the rat ANG standard (Fig. 4). On the other

hand, minimal cross-reactivity (or non-parallelism) was observed for the culture medium containing 1% dFBS. These studies demonstrate that the RIA is specific for rANG and is suitable to quantify the level of IR-rANG in the rat or mouse plasma and in the culture medium of IRPTC and H-4-II-E cells.

Basal expression of the immunoreactive rat angiotensinogen in immortalized rat proximal tubule cells
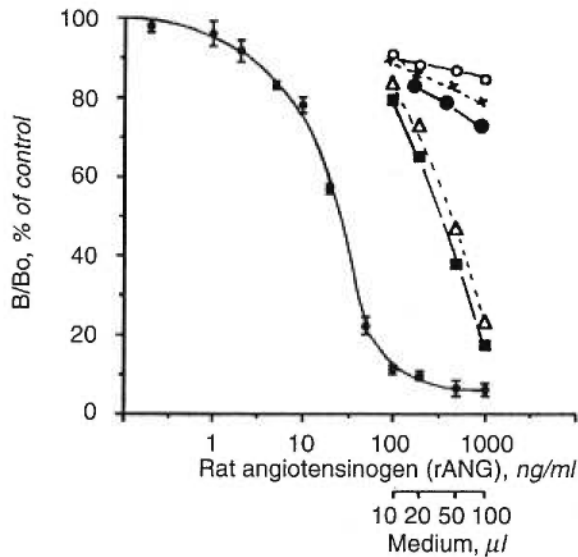
Table 1 compares the levels of the IR-rANG in the media and cell extracts of IRPTC that were incubated for 24 hours in the medium containing 1% dFBS. It is obvious that the total amount of IR-rANG in the cell extract was less than 5% (range 2 to 3%) of that detected in the medium. These studies indicate that the rANG expressed in the IRPTC is not stored within these cells but is secreted constitutively by these cells. Hence, these data indicate that the level of IR-rANG measured in the culture medium is a good indication of the level of ANG expressed in these cells.

Figure 5 shows the Western blot analysis of the IR-rANG in IRPTC culture medium and cell extract by employing antibodies against the rat ANG-MBP fusion protein. The IRPTC culture medium was concentrated by Centricon-10. Two major molecular species of rANG were apparent in the IRPTC cell culture medium, one major band with a molecular weight of 62 kDa, and one minor band with a molecular weight of 56 kDa. On the other hand, the 56 kDa protein was the major species in IRPTC cell extract. These studies demonstrate that the 62 kDa rANG is the major secretory form of the rANG from the IRPTC.
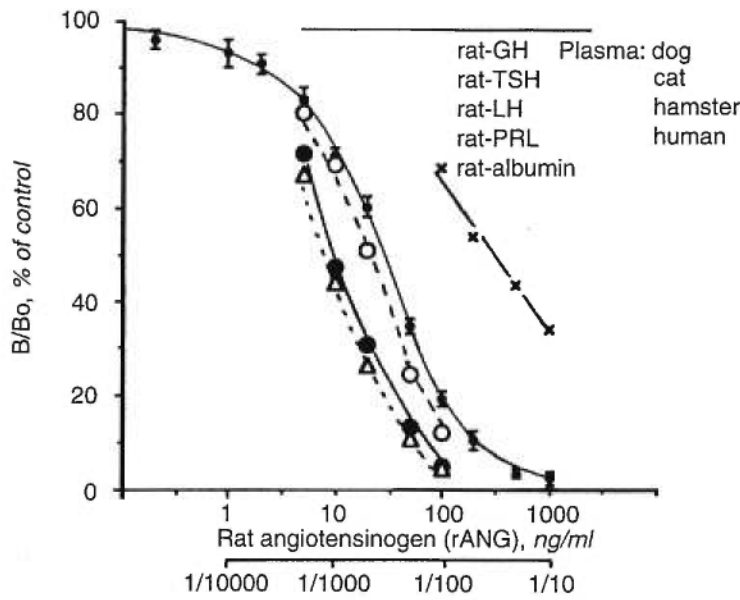
Fig. 3. Radioimmunoassay for rat angiotensinogen (RIA-rANG) by employing rabbit polyclonal antibodies against the Maltose-binding protein-rat angiotensinogen (MBP-rANG) fusion protein. Iodinated purified rat ANG and rat ANG were used as tracer and standard, respectively. Plasma samples from the rat (●, △ and ○) and mouse (X) were serially diluted (that is, dilution in 1:10 to 1:10,000) in RIA-buffer and assayed. Serial dilutions of rat and mouse plasma samples displayed a parallelism with the purified rat ANG standard. No cross-reactivity was observed with rat pituitary hormone preparations and plasma samples from the dog, cat, hamster and human.

Table 1. Comparison of levels of immunoreactive rat angiotensinogen (IR-rANG) in 10 ml of culture medium and corresponding cell extract of immortalized rat proximal tubule cells (IRPTC; $N = 3$)

Total content	IRPTC
Culture medium ng	42.61 ± 8.12
Cell extract ng	0.93 ± 0.05
Ratio of IR-rANG *medium/cell extract*	$46 = 1$

Similar results were obtained from three independent experiments.

On the other hand, the addition of isoproterenol (10^{-15} to 10^{-7} M) alone had no significant stimulatory effect on the expression of IR-rANG in IRPTC (Fig. 7). Similar results were observed from five independent experiments.

These studies demonstrate that the addition of dexamethasone alone stimulated the expression of IR-rANG in IRPTC whereas the addition of isoproterenol alone had no effect.

Synergistic effect of dexamethasone and isoproterenol on the expression of the immunoreactive rat angiotensinogen in immortalized rat proximal tubule cells

Figure 8 shows that the addition of isoproterenol (10^{-13} to 10^{-7} M) enhanced the stimulatory effect of dexamethasone (10^{-6} M) on the expression of the IR-rANG in IRPTC. The maximal enhancing effect of isoproterenol was observed at 10^{-9} to 10^{-7} M. Similar results were obtained from three other experiments.

Inhibitory effect of RU 486 or propranolol on the expression of immunoreactive rat angiotensinogen in immortalized rat proximal tubule cells stimulated by dexamethasone and/or isoproterenol

Figure 9 shows that the addition of RU 486 (10^{-5} M) completely blocked the stimulatory effect of dexamethasone (10^{-6} M) on the expression of IR-rANG in IRPTC. These studies demonstrate that the effect of dexamethasone on the expression of IR-rANG in IRPTC is mediated via the glucocorticoid receptor.

Fig. 4. Cross-reactivity of the culture media in the radioimmunoassay-rat angiotensinogen (RIA-rANG) culture media, with or without prior concentration by Centricon-10, were serially diluted in RIA buffer before assay (○, DMEM + 1% dFBS; X, IRPTC medium without concentration; ●, IRPTC medium with dexamethasone stimulation; ■, IRPTC medium with 20-fold concentration by Centricen-10; △, H-4-II-E medium without concentration).

Effect of dexamethasone or isoproterenol on the expression of angiotensinogen in immortalized rat proximal tubule cells

Figure 6 shows that the addition of dexamethasone (10^{-13} to 10^{-5} M) stimulated the expression of immunoreactive-rANG (IR-rANG) in IRPTC in a dose-dependent manner. The maximal and one-half maximal stimulation of the expression of the IR-rANG were found with 10^{-5} M and 10^{-9} M of dexamethasone, respectively. The addition of dexamethasone (10^{-5} M) stimulated the expression of the IR-rANG by 2.5-fold ($P \leq 0.01$) in IRPTC.

Western blot analysis of the culture medium and cell extract of talized rat proximal tubule cells (IRPTC). The culture medium was trated 20-fold before Western blotting, and 100 μg or 200 μg of trated medium or cell extract was applied onto each lane. The nt molecular weight of Amersham's Rainbow markers are indicated left, and the apparent molecular weights of rat ANG are indicated right.

ire 10 shows that the addition of propranolol (10^{-5} M) lly blocked the synergistic effect of dexamethasone and terenol on the expression of the IR-rANG in IRPTC. ver, the addition of RU 486 completely blocked the ;istic effect of dexamethasone and isoproterenol. These s demonstrate that the synergistic effect of dexamethasone soproterenol on the expression of IR-rANG in IRPTC es the presence of both the activated glucocorticoid recep- d the β-adrenoceptors.

USSION

re are two major advantages for utilizing the bacterial sion system to isolate recombinant protein for immuniza- First, the bacterial expression will yield a sufficient amount on protein for routine immunization and the fusion gene is purified from the bacterial culture. Secondly, the foreign ns expressed by the bacteria are usually non-glycosylated. non-glycosylated proteins are useful to raise antibodies t the protein backbone. Indeed, we were able to purify ximately 300 to 500 μg of MBP-rANG fusion protein from ter of bacterial culture. This was sufficient to enable us to nize three rabbits at least three times.

: purified MBP-rANG fusion protein preparation consisted least three major bands (that is, 46, 69 and 97 kDa in ent molecular weight; Fig. 1). The 97 kDa MBP-rANG ;ents the intact rANG fused with MBP (the apparent ular weights of non-glycosylated rANG and the induced protein are 51 kDa and 46 kDa, respectively). No attempts,
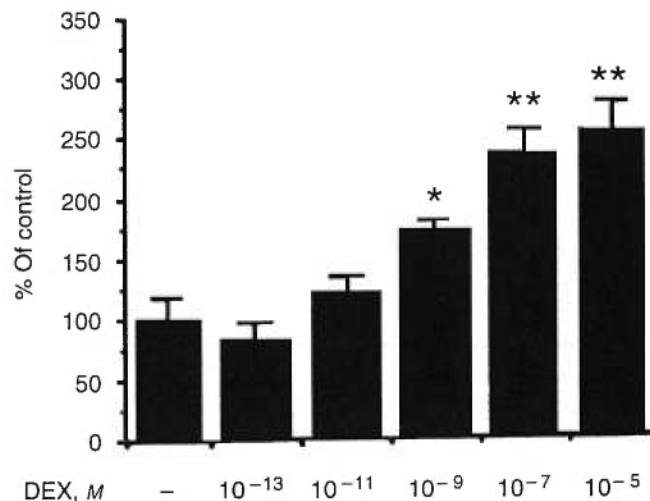
Fig. 6. Effect of dexamethasone on the expression of immunoreactive rat angiotensinogen (IR-rANG) in immortalized rat proximal tubule cells (IRPTC). Cells were incubated for up to 24 hours in the presence of various concentrations of dexamethasone. Media were harvested after 24 hours of incubation and assayed for IR-rANG. The concentration of IR-rANG in the absence of dexamethasone represents the control (2.25 ± 0.49 ng/ml). Each data point represents the mean ± sp of at least three determinations (*$P \le 0.05$ and **$P \le 0.01$).
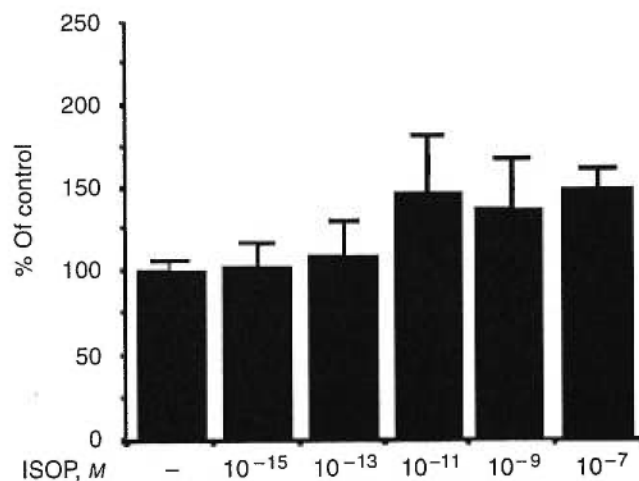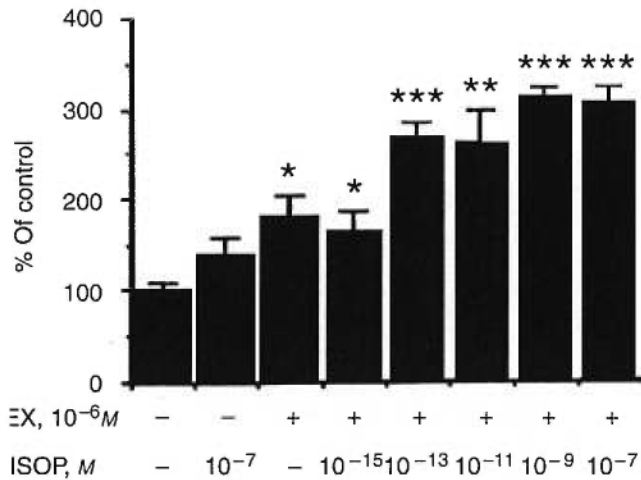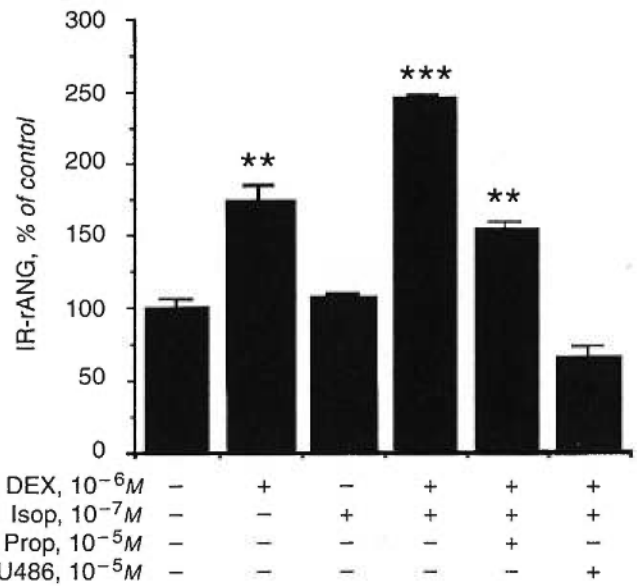

Fig. 7. Effect of isoproterenol on the expression of immunoreactive rat angiotensinogen (IR-rANG) in immortalized rat proximal tubule cells (IRPTC). Cells were incubated for up to 24 hours in the presence of various concentrations of isoproterenol. Media were then harvested and assayed for IR-rANG. The concentration of IR-rANG in the absence of isoproterenol represents the control (that is, 2.88 ± 0.17 ng/ml). Each data point represents the mean ± sp of at least three determinations.

however, were made to separate the 97 kDa MBP-rANG fusion protein more completely from other proteins. We used similar preparations for immunization of the rabbits.

Our purified rANG from rat plasma displayed at least two major bands with apparent molecular weights of 56 and 62 kDa as analyzed by a 6 to 15% linear gradient PAGE-SDS (Fig. 2A). By Western blot analysis, both molecular species of the purified rat plasma ANG (56 and 62 kDa) interacted well with the antiserum against the purified MBP-rANG fusion protein (Fig. 2B). The

8. Effect of isoproterenol on the expression of immunoreactive rat angiotensinogen (IR-rANG) in immortalized rat proximal tubule cells (IRPTC) in the presence of dexamethasone (10^{-6} M). Cells were incubated for 24 hours in the presence of various concentrations of isoproterenol and 10^{-6} M dexamethasone. Media were then harvested and assayed for IR-rANG. The concentration of IR-rANG in the absence of isoproterenol and dexamethasone represents the control (that is, 4.16 ± 0.29 ng/ml). Each point represents the mean ± SD of at least three determinations (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$).

Fig. 10. The inhibitory effect of RU 486 or propranolol on the expression of immunoreactive rat angiotensinogen (IR-rANG) in immortalized rat proximal tubule cells (IRPTC) stimulated by a combination of dexamethasone and isoproterenol. The cells were incubated for 24 hours in the presence of a combination of both dexamethasone (10^{-6} M) and isoproterenol (10^{-7} M) and in the absence or presence of RU 486 (10^{-5} M) or propranolol (10^{-5} M). Media were then harvested and assayed for IR-rANG. The concentration of IR-rANG in the absence of dexamethasone and isoproterenol represents the control (that is, 6.05 ± 0.27 ng/ml). Each point represents the mean ± SD of at least three determinations (**$P \leq 0.01$ and ***$P \leq 0.005$).
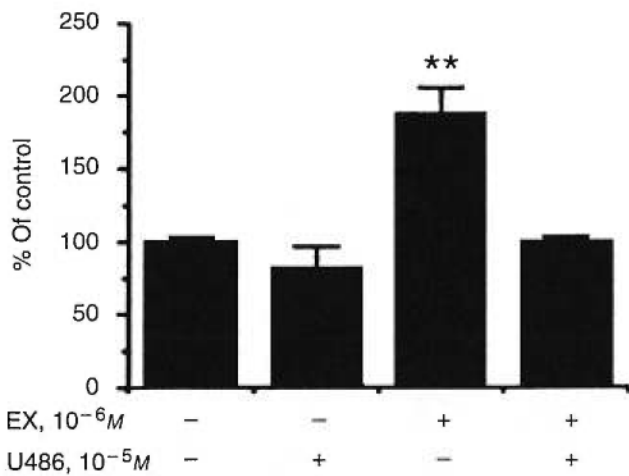
9. The inhibitory effect of RU 486 on the expression of immunoreactive rat angiotensinogen (IR-rANG) in immortalized rat proximal tubule cells (IRPTC) stimulated by dexamethasone. Cells were incubated 24 hours in the presence of dexamethasone (10^{-6} M) with or without addition of RU 486 (10^{-5} M) (an antagonist of glucocorticoid). Media were then harvested and assayed for immunoreactive human angiotensinogen (IR-hANG). The concentrations of IR-rANG in the absence of dexamethasone and RU 486 represents the control (that is, 4.72 ± 0.28 ng/ml). Each point represents mean ± SD of at least three determinations ($P \leq 0.01$).

sence of two molecular forms of rat plasma ANG is in good agreement with the studies of Hilgenfeldt and Hackenthal [19], who isolated two forms of rANG (apparent molecular weights 5 and 60 kDa) from the plasma of nephrectomized rats. A faint IR-rANG band with an apparent molecular weight of 62 kDa was also observed in rat plasma samples (Fig. 2B). We were surprised that our rabbit antiserum displayed weak cross-reactivity

with the purified human ANG (hANG), despite the fact that there is at least 60% homology in the amino acid sequence between the rat and human ANG [20, 21]. At present, we do not have an explanation for this observation. One possible explanation may be that the tertiary structure of the rat ANG and human ANG might play an important role in determining the antigenicity of the protein. Indeed, more experiments are warranted to clarify this observation.

Our RIA for rANG displayed no cross-reactivity with rat albumin, rat pituitary hormones (that is, rGH, rTSH, rLH and rPRL) and plasma samples from the dog, cat, hamster and human (Fig. 4), indicating that the RIA is not only specific for ANG but is also species-specific. On the other hand, serial dilutions of rat and mouse plasma samples (Fig. 3) and culture media of the IRPTC and H-4-II-E cells (Fig. 3) displayed a parallelism with the rANG standard, demonstrating that the RIA is appropriate and feasible to quantify the levels of IR-rANG in rat and mouse plasma and in the culture media of IRPTC and H-4-II-E cells.

The amount of total IR-rANG in the cell extract was less than 3% of that detected in the medium (Table 1). These studies indicate that the ANG expressed in IRPTC is not stored in the cells, but is constitutively secreted by these cells. Hence, the level of IR-rANG in the medium is a good estimation of the level of rANG expressed in these cells.

It is interesting that the larger molecular species of rANG (62 kDa) was the predominant form in the medium, whereas the smaller molecular species of ANG (56 kDa) was the predominant form in the cell extract (Fig. 5). One possible explanation for the

ifference in apparent molecular weight might be due to the level f glycosylation during the process of maturation and secretion. ructural studies of the ANG protein have indicated that there 'e three potential glycosylation sites in the rANG [22]. Never-ieless, more experiments are warranted to confirm this possibil-y.

Our present studies showed that the addition of dexamethasone imulated the expression of the ANG in IRPTC in a dose-ependent manner (10^{-9} to 10^{-5} M; Fig. 6). These concentrations f dexamethasone are within the physiological concentrations of asma cortisol in humans (that is, normal morning plasma level, }.9 μg/dl or 3.75 × 10^{-7} M) [23]. These studies confirm the udies of Loghman-Adham et al [11], who reported that the ldition of dexamethasone (10^{-6} M) stimulated the expression of ouse ANG in immortalized mouse proximal tubular cells. Our 'esent results also confirm our previous studies in which the ldition of dexamethasone stimulated the expression of ANG-AT fusion gene, pOCAT (ANG N-1498/+18) containing the -flanking regulatory region of the rat ANG gene fused with a iding sequence of chloramphenicol acetyl transferase (CAT) as e reporter, in a dose-dependent manner in OK cells [24, 25].

We were surprised to observe that isoproterenol alone had no gnificant stimulatory effect on the expression of the rANG in lPTC culture (Fig. 7). Our present studies are in disagreement ith our previous studies that the addition of isoproterenol or Bromo-cAMP alone directly stimulated the expression of the NG-GH fusion gene, pOGH (ANG N-1498/+18) containing the -flanking region of the rat ANG gene fused with the human owth hormone gene as reporter, in OK cells [26]. One likely :planation for this discrepancy may be that the IRPTC behave fferently from natural proximal tubular cells. Another possibility that the IRPTC express a low level of β-adrenoceptors. Indeed, ₂ often observed a slight increase in the expression of the ANG IRPTC at 10^{-11} to 10^{-7} M isoproterenol (approximately 1.4- to 5-fold) compared to controls (Fig. 7). However, the increase was it statistically significant from the controls. More experiments e warranted concerning the identification and quantification of adrenoceptors in the IRPTC.

On the other hand, the addition of both dexamethasone and pproterenol acted synergistically to stimulate the expression of e ANG gene in IRPTC (Fig. 8). These studies confirm our evious studies that dexamethasone and isoproterenol or β_1-lrenoceptor activation act synergistically to enhance the expres-m of the ANG gene in OK cells [25].

RU 486 is an antagonist of glucocorticoid and competes with ucocorticoid for binding to the glucocorticoid receptors [27]. ur present studies showed that incubation of IRPTC with RU i6 blocked the stimulatory effect of dexamethasone as shown in gure 9. These studies demonstrate that the effect of dexameth-one on ANG expression is mediated via the glucocorticoid ceptor complex. Similarly, the addition of propranolol blocked e enhancing effect of isoproterenol on the ANG expression in !PTC when stimulated by dexamethasone and isoproterenol 'ig. 10). These studies indicate that the enhancing effect of pproterenol on ANG expression is mediated via the β-adreno-ptors.

At present, the exact molecular mechanism(s) for the synergis-: effect of isoproterenol and dexamethasone on the expression the ANG in IRPTC is not known. One possible explanation ₃y be that the addition of dexamethasone stimulates the expres-

sion of β_1-adrenoceptors in IRPTC, since a glucocorticoid respon-sive element (GRE) has been identified in the 5'-flanking region of the β_1-adrenoceptor [28]. We have observed, however, that the pre-incubation of IRPTC with dexamethasone (10^{-11} to 10^{-5} M) alone for 24 hours did not display any synergism in the presence of isoproterenol (unpublished results). These studies suggest that the synergistic effect of dexamethasone and isoproterenol on the expression of ANG is probably not mediated via the increased expression of the β_1-adrenoceptor in the IRPTC *per se*, but may be mediated via the interaction of the activated glucocorticoid receptor(s) with some undefined nuclear transcription factor, that is, cAMP-responsive element binding protein, since the studies of Imai et al [29] have shown that the activated glucocorticoid receptor interacted with the cAMP-responsive element binding protein *in vitro*. Studies are underway in our laboratory to elucidate the molecular mechanism(s) of the synergistic effect of isoproterenol and dexamethasone on the expression of the ANG gene in the kidney proximal tubular cells.

In summary, we have developed a specific radioimmunoassay (RIA) for recombinant angiotensinogen (rANG). By employing this RIA, we demonstrated that dexamethasone stimulated the expression and secretion of ANG by immortalized rat proximal tubule cells (IRPTC). Isoproterenol alone had no effect. How-ever, a combination of both dexamethasone and isoproterenol acted synergistically to stimulate the expression and secretion of the rANG by IRPTC. The addition of RU 486 or propranolol inhibited the synergistic effect of dexamethasone and isoprotere-nol in IRPTC. These results suggest that the high levels of dexamethasone may act synergistically with β_1-adrenoceptor ac-tivation in the proximal tubular cells to enhance the expression of the renal ANG. The local formation of renal Ang II might then modulate the physiology of the renal proximal tubular cells, including sodium and fluid reabsorption [1–3]. Hence, the local intrarenal renin-angiotensin system may play an important role in the development of hypertension.

ACKNOWLEDGMENTS

APPENDIX

ANG, angiotensinogen; Ang II, angiotensin II; AT_1-receptor, angioten-sin II receptor; CAT, chloramphenicol acetyl transferase; dFBS, depleted fetal bovine serum; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; hANG, human angiotensinogen; HRP, horseradish peroxidase; IgG, rabbit polyclonal antibodies; IPTG, isopropylthiogalac-toside; IRPTC, immortalized rat proximal tubule cells; IR-rANG, immu-noreactive rat angiotensinogen; MBP, Maltose-binding protein; MBP-rANG, Maltose-binding protein fused with rat angiotensinogen; OK, opossum kidney; oPL, ovine placental lactogen; PAGE-SDS, polyacryl-amide gel electrophoresis containing sodium dodecyl sulfate; pOCat,

angiotensinogen-chloramphenicol acetyl transferase fusion gene; pOGH, angiotensin-growth hormone fusion gene; rANG, rat angiotensinogen; rGH, rat growth hormone; RIA, radioimmunoassay; RIA-rANG, radioimmunoassay of rat angiotensinogen; rLH, rat leutinizing hormone; rPRL, rat prolactin; rTSH, rat thyroid stimulatory hormone.

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Appendix II

Effect of glucose on the expression of the angiotensinogen gene in opossum kidney cells

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Effect of glucose on the expression of the angiotensinogen gene in opossum kidney cells. To investigate whether D(+)-glucose has a stimulatory effect on the expression of the angiotensinogen (Ang) gene in opossum kidney (OK) cells, we used OK cells with a fusion gene containing various lengths of the 5'-flanking regulatory sequence of the rat Ang gene fused with the human growth hormone (hGH) gene as a reporter, stably integrated into their genomes. The level of expression of the fusion gene was quantified by the amount of immunoreactive-human growth hormone (IR-hGH) secreted into the medium. The addition of D(+)-glucose stimulated the expression of pOGH (Ang N-1498/+18) in OK 27 cells in a dose-dependent manner (5 to 25 mM), whereas the addition of D-mannitol, L-glucose and 2-deoxy-D-glucose (25 mM) had no effect. The stimulatory effect of D(+)-glucose (25 mM) was blocked by the presence of staurosporine or H7 (an inhibitor of protein kinase C) or U73122 (an inhibitor of phospholipase C and A$_2$) but not blocked by the presence of Rp-cAMP (an inhibitor of cAMP-dependent protein kinase A). The addition of D(+)-glucose (25 mM) also stimulated the expression of pOGH (Ang N-960/+18) and pOGH (Ang N-688/+18) in OK 960 and OK 688 cells, respectively. It had no stimulatory effect, however, on the expression of pOGH (Ang N-280/+18) and pOGH (Ang N-35/+18) in OK 280 and OK 35 cells, respectively. The addition of D(+)-glucose also had no effect on the expression of pTKGH in OK 13 cells, an OK cell line, into which had been stably integrated a fusion gene, pTKGH containing the promoter/enhancer DNA sequence of the viral thymidine-kinase (TK) gene fused with a human growth hormone gene as a reporter. These studies demonstrate that the stimulatory effect of high D(+)-glucose concentration (25 mM) on the expression of the angiotensinogen-growth hormone fusion genes in OK cells is mediated via the 5'-flanking region of the angiotensinogen gene and the protein kinase C signal transduction pathway. Our data indicate that a high glucose concentration may activate the renin-angiotensin system in the renal proximal tubular cells.

Studies have shown that the mRNA components of the renin-angiotensin system (RAS), including angiotensinogen (Ang), renin, angiotensin-converting enzyme (ACE) and angiotensin-II receptor (AT$_1$-receptor) are expressed in murine (mouse and rat) immortalized proximal tubular cell lines [1–4]. More recently, we have reported that the Ang protein is secreted from rat immortalized proximal tubular cells as measured by a specific radioimmunoassay for rat Ang [5]. We [6] as well as Ingelfinger et al [7] have also demonstrated that the Ang mRNA is expressed in

opossum kidney (OK) proximal tubular cells. These studies indicate that the intrarenal angiotensin II (Ang II) is probably derived from the Ang that is synthesized within the renal proximal tubular cells.

We have previously reported that isoproterenol and iodoclonidine stimulate the expression of the Ang gene in OK cells *in vitro* in a dose-dependent manner [8, 9]. The effect of isoproterenol is mediated via the β_1-adrenoceptor and cAMP-dependent protein kinase A (PKA) pathway [8], whereas the effect of iodoclonidine is mediated via the α_2-adrenoceptor and protein kinase C (PKC) pathway [9]. Our studies confirm the reports of Nakamura and Johns [10] that low levels of renal nerve stimulation increase the Ang mRNA levels in the rat kidney *in vivo*. Our studies and those of Nakamura and Johns [8–10] together indicate the presence of a functional relationship between the renal sympathetic nervous system and the activation of local intrarenal RAS. Thus, the local formation of renal Ang II may play an important role in the physiology of the renal proximal tubular cells (that is, sodium and fluid reabsorption [11–14]).

Hyperglycemia is an important etiologic factor in the development of diabetic nephropathy [15]. However, the molecular mechanisms for the development of nephropathy in diabetes are not completely understood. Studies have shown that the incubation of mesangial cells in a high glucose medium increases cellular proliferation, extracellular matrix protein synthesis and the expression of mRNA of extracellular matrix proteins in mesangial cells [16–19]. Experimental evidence also indicates that the effect of elevated glucose concentration is probably mediated via the activation of the protein kinase C pathway in mesangial cells [20–24]. Furthermore, tubular basement membrane thickening, accumulation of glycogen droplets in proximal tubular epithelium and tubulointerstitial fibrosis are often observed in diabetic patients [25]. These studies indicate that the elevated glucose concentration may directly or indirectly be responsible for the development of diabetic nephropathy.

We have previously demonstrated that the addition of phorbol 12-myristate 13-acetate (PMA, a protein kinase C stimulator) has a stimulatory effect on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells [9], indicating that the PKC signal transduction pathway plays a regulatory role on the expression of the Ang gene in OK cells. In the present studies, we investigated the possible effect of glucose on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells. Our results showed that the expression of pOGH (Ang N-1498/+18) fusion gene in OK 27

; stimulated by high concentrations (25 mM) of D(+)-
:, but not by D-mannitol, L-glucose or 2-deoxy-D-glucose.
rmore, the addition of staurosporine or H-7 (an inhibitor of
ı kinase C) and U73122 (an inhibitor of phospholipase C
,) blocked the stimulatory effect of glucose. Finally, the
n of high glucose (25 mM) had a stimulatory effect on the
iion of pOGH (Ang N-960/+18) and pOGH (Ang N-688/
ı OK 960 and OK 688 cells, respectively. The addition of
: had no effect, however, on the expression of pOGH (Ang
+18), pOGH (Ang N-35/+18) and pTKGH in OK 280, OK
OK 13 cells, respectively.

ODS

)-glucose, L-glucose, D-mannitol and 2-deoxy-D-glucose
urchased from Sigma Chemicals (St. Louis, MO, USA).
porine (an inhibitor of protein kinase C), H-7 (an inhibitor
ein kinase C), U73122 (an inhibitor of phospholipase C and
ı Rp-cAMP (an inhibitor of the cAMP-dependent protein
AI and II) were purchased from Research Biochemicals
BI, Natick, MA, USA).
plasmid, pRSV-Neo, containing the coding sequence for
cin (Neo) with the Rous Sarcoma Virus (RSV) enhancer/
er sequence fused in the 5'-end of the Neomycin gene was
from Dr. Teresa Wang (Dept. of Pathology, Stanford
iity, Stanford, CA, USA). The plasmid, pTKGH, contain-
thymidine kinase (TK) enhancer/promoter sequence fused
5'-end of the hGH gene was purchased from the Nichols
e of Diagnostics (La Jolla, CA, USA).
·adioimmunoassay kit for hGH (RIA-hGH) was a gift from
)K, NIH, USA. The RIA procedure has been described
sly [26]. NIAMDD-hGH-I-1 (AFP-4793 B) was used for
lination and as a hormone standard. The limit of sensitiv-
ie assay was 0.1 ng/ml. The inter- and intra-assay coeffi-
f variation were 10% (n-10) and 12% (n-10), respectively.
'I was purchased from Dupont, New England Nuclear
Boston, MA, USA). Calcium chloride was purchased from
krodt, Inc. (Montreal, Quebec, Canada), Geneticin (G
as purchased from Bethesda Research Laboratories
BRL, Burlington, Ontario, Canada). Other molecular
grade reagents were obtained either from Sigma Chemi-
bco-BRL, Boehringer-Mannheim, Pharmacia Inc. (Baie
Quebec, Canada), or Promega-Fisher, Inc. (Montreal,
, Canada).

iction of fusion genes

method of construction of the Ang-GH fusion genes,
(Ang N-1498/+18) and pOGH (Ang N-35/+18), has been
:d previously [26]. To construct pOGH (Ang N-960/+18)
GH (Ang N-280/+18), we simply transferred the DNA
its, Ang N-960/+18 and Ang N-280/+18, from the plas-
)CAT (Ang N-960/+18) and pOCAT (Ang N-280/+18)
pectively, into the pOGH vector.

ture

)possum kidney (OK) proximal tubular cell line was
l from the American Tissue Culture Collection (ATCC,
e, MD, USA). This cell line is derived from the kidney of
: American opossum and retains several properties of
l tubular epithelial cells in culture [28, 29] and expresses

a low level of Ang mRNA [6, 7]. The culture conditions of OK
cells have been described previously [8, 9, 30].

Opossum kidney cell stable transformants

Opossum kidney 27 and OK 13 cells are stable transformants
with pOGH (Ang N-1498/+18) and pTKGH integrated into OK
cellular genomes, respectively. The characteristics of these cells
have been previously reported [8, 9]. Briefly, OK 27 and OK 13
cells that had passed through at least three repetitions of limiting
dilution and continued to secrete high levels of immunoreactive-
hGH (IR-hGH) after three months in the presence of G418 were
considered to be stable clones.

By similar procedures, we have obtained stable transformants
OK 960, OK 688, OK 280 and OK 35 with pOGH (Ang
N-960/+18), pOGH (Ang N-688/+18), pOGH (Ang N-280/+18)
and pOGH (Ang N-35/+18) integrated into OK cellular genomes,
respectively. The expression of the Ang-GH fusion genes in these
cells was time-dependent. The levels of IR-hGH in cellular
extracts were consistently less than 5% of those found in the
culture media, suggesting that renal Ang is not stored in the cell.

**Effect of D(+)-glucose on the expression of angiotensinogen-
growth hormone fusion genes in opossum kidney 27 cell stable
transformants**

Opossum kidney 27 cells were plated at a density of 1 to 2 × 10⁵
cells/well in six-well plates and incubated overnight in DMEM
containing 10% FBS. Cell growth was then arrested by incubation
in serum-free and a low D(+)-glucose concentration (5 mM)
medium for 24 hours. Subsequently, various concentrations of
D(+)-glucose (final concentration 5 to 25 mM) were added to the
culture medium containing 1% depleted fetal bovine serum
(dFBS) and incubated for 24 hours. Since glucose changes the
tonicity of the media, the media were supplemented with D-
mannitol until the final concentration was reached equivalent 30
mM (that is, 5 mM D(+)-glucose plus D-mannitol until equivalent
30 mM, etc.). At the end of the incubation period, media were
collected and kept at −20°C until assayed for IR-hGH.

To determine the specificity of D(+)-glucose, 5 or 25 mM of
L-glucose or D-mannitol or 2-deoxy-glucose was added to the
culture medium and incubated for 24 hours. Then, the media were
collected and kept at −20°C until assay for IR-hGH.

To compare the inhibitory effect of staurosporine, H-7, U73122
and Rp-cAMP on the expression of pOGH (Ang N-1498/+18) in
OK 27 cells, various concentrations (10⁻¹³ to 10⁻⁷ M) of the
inhibitors were co-cultured with the D(+)-glucose (25 mM) for 24
hours. At the end of the incubation period, media were collected
and kept at −20°C until assay for IR-hGH.

The depleted FBS was prepared by incubation with 1% acti-
vated charcoal and 1% AG 1 × 8 ion-exchange resin (Bio-Rad
Laboratories, Richmond, CA, USA) for 16 hours or more at room
temperature as described by Samuels, Stanley and Shapiro [31].
This procedure removed endogenous steroid and thyroid hor-
mones from the FBS as demonstrated by Samuels et al [31].

Statistical analysis

The experiments were performed at least three to four times in
triplicate. The data were analyzed with Student's t-test or analysis
of variance (ANOVA). A probability level of $P \le 0.05$ was
regarded as significant.
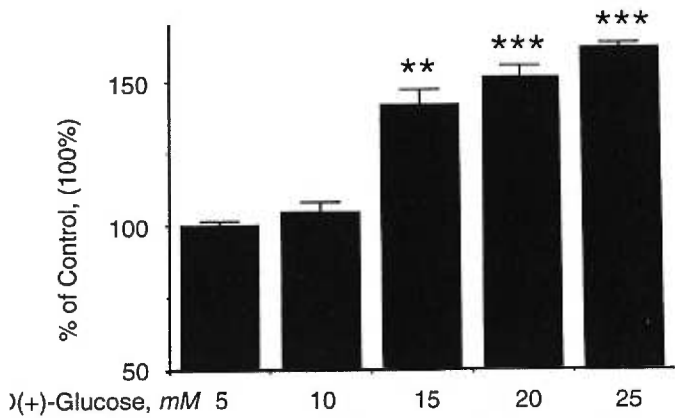
Fig. 1. Effect of D(+)-glucose on the expression of pOGH (Ang N-1498/+18) in opossum kidney (OK) 27 cells. Cells were incubated for up to 24 hours in the presence of various concentrations of D(+)-glucose. Media were collected after 24 hours of incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the medium containing low D(+)-glucose (5 mM) (that is, 1.43 ± .1 ng/ml) is considered as the control level. Each point represents the mean ± SD of at least three dishes (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq$ .005). Experiments were repeated three times.

RESULTS

Effect of D(+)-glucose on the expression of angiotensinogen-growth hormone fusion gene in opossum kidney cell stable transformants

Figure 1 shows the expression of the pOGH (Ang N-1498/+18) in OK 27 cells in the presence of various concentrations (5 to 25 mM) of D(+)-glucose after 24 hours of incubation. A dose-dependent relationship between D(+)-glucose concentrations and the stimulation of expression of pOGH (Ang N-1498/+18) was observed at 5 to 25 mM. The maximal stimulation of expression of the pOGH (Ang N-1498/+18) was found with 25 mM of D(+)-glucose.

Figure 2 shows the expression of the pOGH (Ang N-1498/+18) in OK 27 cells in the presence of 5 mM or 25 mM D(+)-glucose at different time periods. The maximal expression of the pOGH (Ang N-1498/+18) with 25 mM D(+)-glucose was found after two days of incubation. The stimulatory effect of 25 mM D(+)-glucose was significantly reduced following three days of incubation. There was no significant difference between the stimulatory effect observed with 25 mM D(+)-glucose at four days of incubation compared to the 5 mM D(+)-glucose. For subsequent studies, we routinely performed the experiments following 24 hours of incubation in the presence of low or high glucose levels.

Figure 3 shows that the effect of the addition of 5 mM or 25 mM D(+)-glucose on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells when OK 27 cells were pre-incubated with 25 mM D(+)-glucose (Fig. 3A) or 5 mM (D+)-glucose in the presence of 10^{-5} M phorbol 12-myristate, 13-acetate (PMA) (Fig. 3B) for 24 hours. It is apparent that the pre-incubation with 25 mM of D(+)-glucose or 10^{-5} M PMA for 24 hours abolished the stimulatory effect of 25 mM of D(+)-glucose compared to the 5 mM D(+)-glucose level.

Figure 4 compares the effect of the addition of 5 mM or 25 mM of D(+)-glucose, D-mannitol, L-glucose or 2-deoxy-D-glucose on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells
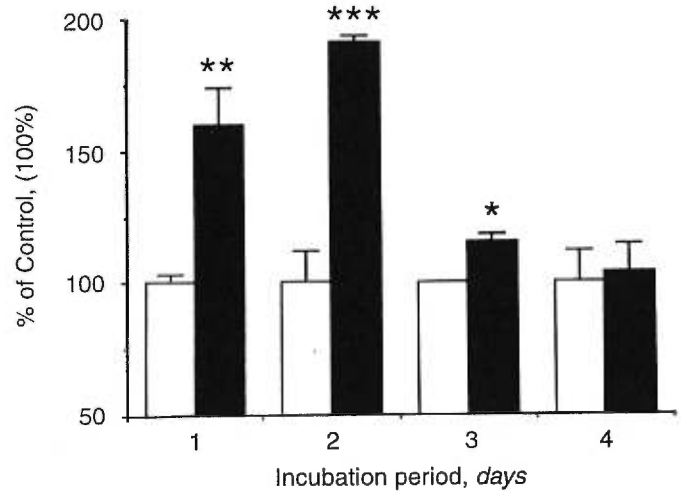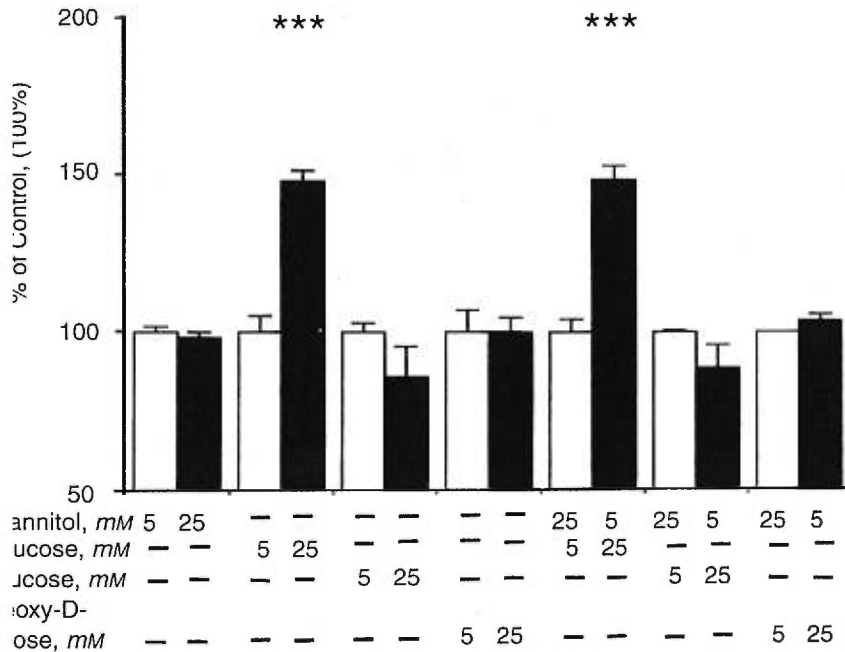

Fig. 2. Effect of incubation period on the expression of pOGH (Ang N-1498/+18) in opossum kidney (OK) 27 cells. Cells were incubated for one to four days in the presence of 5 mM or 25 mM of D(+)-glucose. Media were harvested after various periods of incubation and assayed for IR-hGH. The concentration of IR-hGH in the medium containing low D(+)-glucose (5 mM) (that is, Day 1, 1.32 ± 0.1 ng/ml; Day 2, 1.61 ± 0.15 ng/ml; Day 3, 1.86 ± 0.15 ng/ml; Day 5, 1.91 ± 0.1 ng/ml) is considered as the control level. Each point represents the mean ± SD of at least three dishes (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from two other experiments.
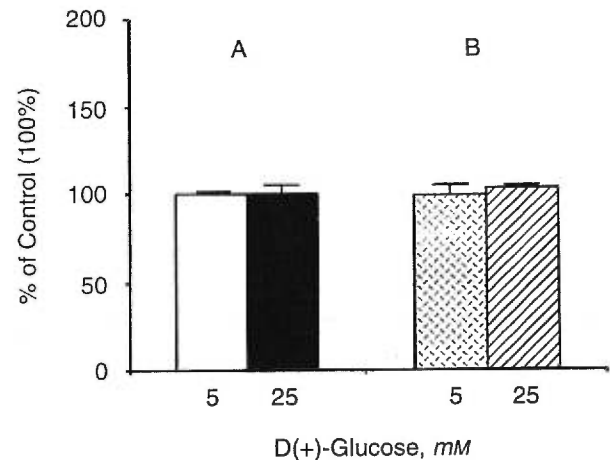

Fig. 3. Effect of the addition of D(+)-glucose on the expression of the pOGH (Ang N-1498/+18) in opossum kidney (OK) 27 cells when the cells were pre-incubated with a high level of glucose or phorbol 12-myristate 13-acetate (PMA). Cells were incubated for 24 hours with 25 mM D(+)-glucose (A) or 5 mM D(+)-glucose in the presence of 10^{-5} M PMA (B). Then, the media were replaced with the fresh media containing 5 mM or 25 mM D(+)-glucose and incubated further for 24 hours. Subsequently, the media were havested and assayed for immunoreactive-human growth hormone (IR-hGH). The concentration of IR-hGH in the medium containing 5 mM D(+)-glucose in A or B (that is, 1.80 ± 0.1 ng/ml or 1.51 ± 0.2 ng/ml) are expressed as 100% (control). Each point represents the mean ± SD of at least three dishes (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.005$). Similar results were obtained from two other experiments.

after 24 hours of incubation with or without the supplementation of D-mannitol. In contrast to D(+)-glucose, the addition of 25 mM D-mannitol, L-glucose or 2-deoxy-D-glucose had no significant

Fig. 4. Effect of D(+)-glucose, D-mannitol, L-glucose and 2-deoxy-D-glucose on the expression of pOGH (Ang N-1498/+18) in opossum kidney (OK) 27 cells. Cells were incubated for up to 24 hours in the presence of low (5 mM) or high (25 mM) of D(+)-glucose, D-mannitol, L-glucose or 2-deoxy-D-glucose with or without supplementation of D-mannitol. Media were harvested after 24 hours of incubation and assayed for immunoreactive-human growth hormone (IR-hGH). The concentration of IR-hGH in the medium containing 5 mM D(+)-glucose (4.71 ± 0.35 ng/ml), 5 mM D-mannitol (2.53 ± 0.05 ng/ml), or 5 mM L-glucose (3.32 ± 0.06 ng/ml), 5 mM 2-deoxy-D-glucose (1.42 ± 0.17 ng/ml), 5 mM D(+)-glucose plu 25 mM mannitol (2.84 ± 0.10 ng/ml), 5 mM L-glucose plus 25 mM mannitol (2.58 ± 0.02 ng/ml) or 5 mM 2-deoxy-D-glucose plus 25 mM mannitol (1.61 ± 0.01 ng/ml) are considered as the control level. Each point represents the mean ± SD of at least three dishes (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from two other experiments.
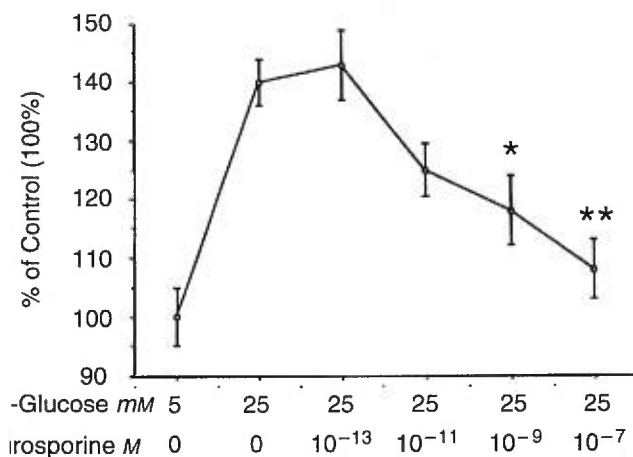
5. Inhibitory effect of staurosporine on the expression of pOGH N-1498/+18) in OK 27 cells in the presence of 25 mM D(+)-glucose. were incubated for 24 hours in the presence of 5 mM or 25 mM -glucose. Media were harvested and assayed for the level of IR-hGH. evels of IR-hGH in the medium containing low D(+)-glucose (5 mM) is, 1.69 ± 0.15 ng/ml) are expressed as 100% (control). The inhibitory of staurosporine is compared with cells that were incubated in 25 mM -glucose (without the presence of staurosporine). Each point represents ean ± SD of at least three dishes (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq$ l. Similar results were obtained from another experiment.

t on the expression of the pOGH (Ang N-1498/+18) in OK lls compared to those at 5 mM.

t of staurosporine, H-7, U73122 or Rp-cAMP on the ssion of pOGH (Ang N-1498/-18) in OK 27 cells in the nce of D(+)-glucose

gure 5 shows that the addition of staurosporine (10^{-13} to M) inhibited the stimulatory effect of D(+)-glucose (25 mM) e expression of the pOGH (Ang N-1498/+18) in OK 27 cells

in a dose-dependent manner. The effective inhibitory dose for inhibition of the stimulated expression (25 mM D(+)-glucose) of the pOGH (Ang N-1498/+18) was at 10^{-9} M staurosporine ($P \leq 0.05$). At 10^{-7} M staurosporine, the stimulatory effect of D(+)-glucose (25 mM) on the expression of the pOGH (Ang N-1498/+18) was completely abolished.

Similarly, the addition of H-7 or U73122 (10^{-7} M) completely inhibited the stimulatory effect of D(+)-glucose on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells (Fig. 6). On the other hand, the addition of Rp-cAMP (10^{-7} M) had no inhibitory effect on the expression of pOGH (Ang N-1498/+18) stimulated by D(+)-glucose (25 mM; Fig. 6).
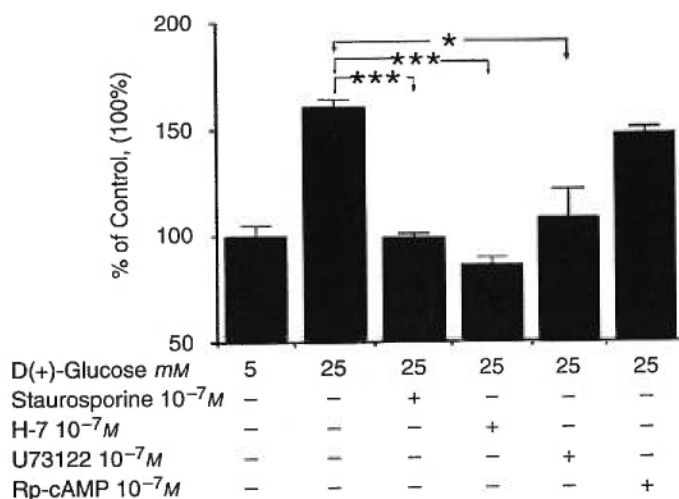
Effect of D(+)-glucose on the expression of the angiotensinogen-growth hormone fusion genes and pTKGH in opossum kidney cells

Figure 7 shows that the addition of 25 mM D(+)-glucose stimulated the expression of pOGH (Ang N-1498/+18), pOGH (Ang N-960/+18) and pOGH (Ang N-688/+18) in OK 27, OK 960 and OK 688 cells compared to the 5 mM D(+)-glucose, respectively. The addition of 25 mM D(+)-glucose had no stimulatory effect on the expression of pOGH (Ang N-280/+18) and pOGH (Ang N-35/-18) in OK 280 and OK 35 cells compared to the 5 mM D(+)-glucose, respectively.
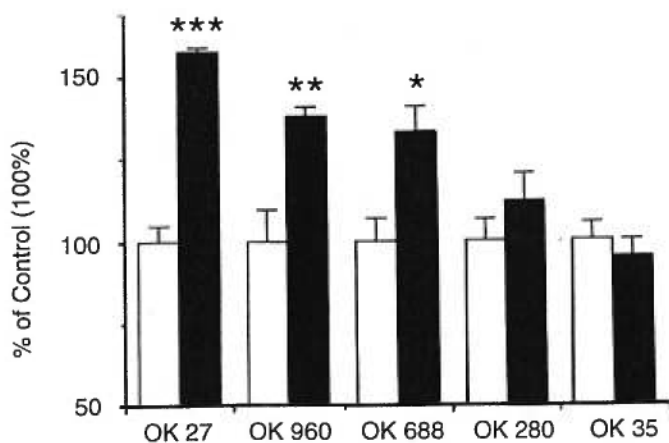
Figure 8 shows that the addition of D(+)-glucose (5 to 25 mM) had no stimulatory effect on the expression of the pTKGH in OK 13 cells.

DISCUSSION

Studies *in vitro* on cultured murine proximal tubular cells in a high glucose-containing medium (that is, ≥ 25 mM) showed that the high glucose levels stimulated the hypertrophy of the proximal tubular cells [32]. It appears that the hypertrophic effect of high glucose is mediated via the autocrine induction of transforming growth factor (TGF-β) [33]. These observations were confirmed
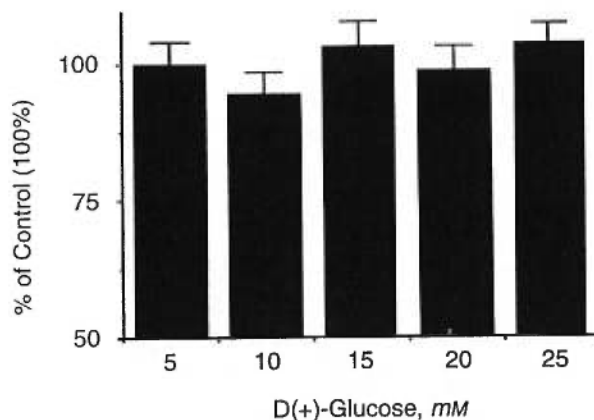
Fig. 6. Inhibitory effect of staurosporine, H-7, U73122 or Rp-cAMP on the expression of pOGH (Ang N-1498/+18) in OK 27 cells stimulated by 25 mM D(+)-glucose. Cells were incubated for 24 hours in the presence of 5 mM or 25 mM D(+)-glucose plus 10^{-7} M of staurosporine or H-7 or U73122 or Rp-cAMP. Media were harvested and assayed for the level of immunoreactive-human growth hormone (IR-hGH). Levels of IR-hGH in the medium containing the low D(+)-glucose (5 mM) (that is, 3.72 ± 0.18 ng/ml) in the absence of staurosporine, H-7, U73122 or Rp-cAMP are expressed as 100% (control). The inhibitory effect of staurosporine, H-7, U73122 or Rp-cAMP is compared with cells that were stimulated by 25 mM D(+)-glucose. Each point represents the mean ± SD of at least three dishes (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from two other experiments.

Fig. 8. Effect of D(+)-glucose on the expression of pTKGH in opossum kidney (OK) 13 cells. Cells were incubated for up to 24 hours in the presence of various concentrations of D(+)-glucose. Media were harvested after 24 hours of incubation and assayed for immunoreactive-human growth hormone (IR-hGH). The concentration of IR-hGH in the medium containing low glucose (5 mM) (that is, 1.19 ± 0.08 ng/ml) is considered as the control level. Each point represents the mean ± SD of at least three dishes (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from two other experiments.

Fig. 7. Effect of D(+)-glucose on the expression of angiotensinogen-growth hormone (Ang-GH) fusion genes in opossum kidney (OK) cells. Cells were incubated for up to 24 hours in the presence of 5 mM or 25 mM D(+)-glucose. Media were harvested after 24 hours of incubation and assayed for IR-hGH. The concentration of IR-hGH in the medium containing low glucose (5 mM) (that is, OK 27 cells, 1.42 ± 0.08 ng/ml; OK 960, 1.69 ± 0.1 ng/ml; OK 688, 1.43 ± 0.1 ng/ml; OK 280, 1.28 ± 0.1 ng/ml, OK 35, 1.60 ± 0.12 ng/ml) is considered as the control level. Each point represents the mean ± SD of at least three dishes (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from two other experiments.

by *in vivo* studies, where it was found that the renal hypertrophy is also associated with the increased renal expression of the TGF-β in spontaneously diabetic Bio-Breeding (BB) rats and non-obese diabetic rats [34].

Studies *in vitro* also showed that the addition of Ang II stimulates the hypertrophy of mouse proximal tubular cells (MCT) and LLC-PK₁ (porcine proximal tubular cells) [2, 35–37]. The hypertrophic effect of Ang II also depends on the autocrine induction of TGF-β gene expression [38, 39]. However, studies by Wolf et al [40] demonstrated that the Ang II-mediated hypertrophy of proximal tubular cells occurs only in the presence of high concentrations of glucose. Thus, it appears that Ang II has an additive effect on the hypertrophy of the murine proximal tubular cells only in the presence of high glucose levels in the medium.

Conflicting results have been reported concerning the expression of the renin and Ang genes in the kidney in experimental diabetes mellitus. For example, studies of Kaylinyak et al [41] showed that there is no significant difference in renal renin and Ang mRNA levels in rats two weeks after the induction of diabetes compared with controls. Studies of Correa-Rotler, Hostetter and Rosenberg [42], however, reported that the levels of renal and liver Ang mRNA are lower in the diabetic group. In contrast, the studies of Everett et al [43] showed that there is an increase in immunoreactive Ang in the renal proximal tubules while there is no significant increase in renal proximal ANG mRNA in rats 4 to 8 months after induction of diabetes. The studies of Anderson, Jung and Ingelfinger [44] demonstrated that a small but significant increase is observed in renal renin and Ang mRNA expression in rats six to eight weeks after induction of diabetes with the administration of streptozotocin. Thus, until now it has been uncertain whether high glucose levels may modulate the expression of the renal Ang gene during the course of diabetes mellitus.

Our present studies showed that the addition of D(+)-glucose stimulated the expression of the fusion gene, pOGH (Ang N-1498/+18) in OK 27 cells in a dose-dependent manner (Fig. 1). Moreover, the stimulatory effect of a high D(+)-glucose level is time-dependent (Fig. 2). The highest stimulatory effect of D(+)-glucose was observed during two days of incubation (Fig. 2). The stimulatory effect was diminished with greater than two days of

ation. We had measured the levels of glucose in the medium :ious times of incubation (unpublished results). Our results ed that the levels of glucose were 0.1 mM and 15.7 mM after days of incubation. These studies indicate that the lack of latory effect by the high level of glucose following four days cubation was not due to the depletion of glucose in the um. We have also performed experiments by changing the ledia after 48 hours of incubation with fresh media (unpub-l results). The addition of fresh media did not displayed any latory effect of 25 mM D(+)-glucose on the expression of the H (Ang N-1498/+18) in OK 27 cells compared to the ion of 5 mM D(+)-glucose. At present, we do not understand onger incubation periods (that is, > two days) diminished or shed the stimulatory effect of high D(+)-glucose on the ssion of the pOGH (Ang N-1498/+18) in OK 27 cells. One ole explanation may be that the prolonged exposure of OK to high D(+)-glucose may desensitize the protein kinase C l transduction pathway. Indeed, our results (Fig. 3) showed the 24 hours pre-incubation of OK 27 with 25 mM D(+)-se (Fig. 3A) or 5 mM D(+)-glucose in the presence of 10^{-5} ırbol 12-myristate 13-acetate (PMA) (Fig. 3B) abolished the latory effect of high D(+)-glucose (25 mM) on the expres-of the fusion gene in OK 27 cells. Nevertheless, more 'iments are warranted to clarify these observations. We did ɔbserve any significant stimulation of the pOGH (Ang ϶8/+18) by L-glucose, D-mannitol or 2-deoxy-D-glucose 4). These studies indicate that the effect of high D(+)-se levels on the expression of the pOGH (Ang N-1498/+18) ⟨ 27 cells is probably mediated via the metabolized products '+)-glucose to stimulate the PKC pathway.

ır present studies showed that the addition of staurosporine ıhibitor of protein kinase C) blocked the stimulatory effect of)-glucose on the expression of pOGH (Ang N-1498/+18) in !7 cells in a dose-dependent manner (Fig. 5). Furthermore, ddition of H-7 (an inhibitor of protein kinase C) or U73122 ıhibitor of phospholipase C and A₂) 10^{-7} M also completely ed the stimulatory effect of high D(+)-glucose levels on the ssion of the pOGH (Ang N-1498/+18) in OK 27 cells (Fig. 'hese data support the hypothesis that the effect of high)-glucose levels on the expression of the Ang gene is medi-via the protein kinase C pathway and not via the protein e A pathway, since Rp-cAMP (an inhibitor of cAMP-ndent protein kinase A) did not inhibit the effect of high)-glucose levels on the expression of the pOGH (Ang N-1498/ in OK 27 cells (Fig. 6). Indeed, the involvement of PKC on xpression of the Ang gene in OK cells are confirmed by our ous studies where it was reported that the addition of PMA ilates the expression of the pOGH (Ang N-1498/+18) in OK ills [9]. This stimulatory effect of PMA is blocked in the :nce of staurosporine [9].

ır data show that the addition of high glucose (25 mM) ilated the expression of pOGH (Ang N-1498/+18), pOGH N-960/+18) and pOGH (Ang N-688/+18) in OK 27, OK 960 ƆK 688 cells, respectively (Fig. 7). The addition of high levels ucose, however, had no effect on the expression of pOGH N-280/+18) and pOGH (Ang N-35/+18) in OK 280 and OK ills, respectively. These studies indicate that the glucose-ɔnsive element is probably localized within nucleotides 98 to N-280 in the 5'-flanking region of the rat Ang gene. At :nt, we have not identified the precise DNA sequence of the

glucose-responsive element in the 5'-flanking region of the rat Ang gene. Studies are underway in our laboratory to identify the putative glucose-responsive element in the rat angiotensinogen gene.

Opossum kidney 13 is a cell line into which has been stably integrated a fusion gene: pTKGH containing the promoter/ enhancer DNA sequence of the viral thymidine kinase gene fused with the human growth hormone gene as a reporter. Therefore, we used OK 13 cells as control cells to examine the effect of D(+)-glucose. We did not observe any significant stimulation of expression of the pTKGH by D(+)-glucose at various concentrations (5 to 25 mM) in OK 13 cells (Fig. 8). These data demonstrate that the promoter/enhancer DNA sequence of the TK gene is not responsive to the addition of D(+)-glucose. On the other hand, our studies demonstrated that the effect of D(+)-glucose on the expression of pOGH (Ang N-1498/+18) in OK 27 cells is gene-specific and is mediated via the 5'-flanking regulatory sequences of the rat Ang gene and not mediated via the DNA sequence of the hGH reporter gene.

At present, we do not understand the exact molecular mechanism(s) of D(+)-glucose and protein kinase C on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells. One possibility may be that the D(+)-glucose may stimulate the *de novo* synthesis of diacylglycerol (DAG) from the metabolized glucose via the polyol pathway which then increases the protein kinase C activity [45]. Surprisingly, our studies also showed that the addition of U73122 completely blocked the stimulatory effect of D(+)-glucose (Fig. 6). Since U73122 is an inhibitor of phospholipase C and A₂, these studies suggest that the glucose may indirectly increase the phospholipase C activity in OK cells by some undefined mechanism(s). Indeed, studies are underway in our laboratory to explore this possibility.

Once the PKC is activated, it is possible that the protein kinase C may phosphorylate the cAMP-responsive element binding protein (CREB) or CREB-like nuclear protein(s), since CREB contains the site of phosphorylation by protein kinase C [46] and recent studies by Kreisberg et al [47] have shown that PMA and high glucose levels stimulate the phosphorylation of CREB. The phosphorylated CREB then binds to the putative cAMP-responsive element (CRE) of the rat Ang gene (TGACGTAC, nucleotides N-795 to N-788) [26] and subsequently enhances the expression of the Ang gene. This possibility is supported by our recent studies [48] whereby the cloned CREB is able to stimulate directly the expression of the pOGH (Ang N-1498/+18) in OK 27 cells. Nevertheless, more studies are warranted to elucidate the molecular mechanism(s) of D(+)-glucose and PKC activation and the expression of the Ang gene in OK cells.

In summary, our studies show that the high D(+)-glucose levels directly stimulate the expression of the Ang-GH fusion genes in OK cells. The stimulatory effect of high D(+)-glucose concentrations was blocked by the presence of staurosporine, H-7 and U73122. Our studies suggest that the expression of the renal Ang gene may be stimulated during hyperglycemia *in vivo*. The local formation of renal Ang II might then modulate the physiology of the renal proximal tubular cells (that is, sodium and fluid reabsorption, as well as the induction of the hypertrophy of the proximal tubular cells). Thus, local renal RAS might play a significant role in the development of diabetic nephropathy.

APPENDIX

Abbreviations used in this article are: ACE, angiotensin converting enzyme; Ang, angiotensinogen; Ang II, angiotensin II; ANOVA, analysis of variance; MCT, mouse proximal tubular cells; AT_1-receptor, angioten-sin II receptor; CRE, cAMP-responsive element; CREB, cAMP-respon-sive element binding protein; DAG, diacylglycerol; dFRS, depleted fetal bovine serum; hGH, human growth hormone; IR-hGH, immunoreactive-human growth hormone; LLC-PK_1, porcine proximal tubular cells; Neo, Neomycin; OK, opossum kidney; PKA, protein kinase A; pOGH (Ang N-1498/+18) and pOGH (Ang N-35/+18), angiotensinogen-growth hor-mone fusion genes; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RAS, renin-angiotensin system; RIA-hGH, radioimmunoassay for human growth hormone; Rp-cAMP, an inhibitor of cAMP-dependent protein kinase A; RSV, Rous Sarcoma Virus; TGF-β, transforming growth factor; TK, thymidine kinase.

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Molecular mechanism(s) of action of norepinephrine on the expression of the angiotensinogen gene in opossum kidney cells

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Molecular mechanism(s) of action of norepinephrine on the expression of the angiotensinogen gene in opossum kidney cells.

Background. Norepinephrine (NE) is the major endogenous neurotransmitter of the renal sympathetic nerves interacting with both the α- and β-adrenoceptors in the renal proximal tubules. We have previously reported that isoproterenol and iodoclonidine stimulate the expression of the angiotensinogen (ANG) gene in opossum kidney (OK) proximal tubular cells via the β_1-adrenoceptor and α_2-adrenoceptor, respectively. We hypothesized that NE may interact with the β- and/or α_2-adrenoceptors to stimulate the expression of the ANG gene in OK cells.

Methods. The fusion genes containing the various lengths of the 5'-flanking regulatory sequence of the rat ANG gene fused with a human growth hormone (hGH) gene as a reporter were stably transfected into the OK cells. The stimulatory effect of NE on the expression of the fusion genes was evaluated by the amount of immunoreactive hGH (IR-hGH) secreted into the culture medium.

Results. The addition of NE stimulated the expression of the fusion gene, pOGH (ANG N-1498/+18) in a dose-dependent manner. The stimulatory effect of NE was inhibited in the presence of propranolol, atenolol, Rp-cAMP, yohimbine, staurosporine, H-7 and U73122 but not in the presence of ICI 118,551 and prazosin. The addition of a combination of isoproterenol and iodoclonidine synergistically stimulated the expression of pOGH (ANG N-1498/+18) as compared to the addition of isoproterenol and iodoclonidine alone. Furthermore, the addition of NE, forskolin, 8-Br-cAMP or phorbol 12-myristate (PMA) stimulated the expression of pOGH (ANG N-806/-779/-53/+18), a fusion gene containing the putative cAMP responsive element (CRE, ANG N-806/-779) upstream of the ANG promoter (ANG N-53/+18) in OK 95 cells, but had no effect on the expression of fusion genes containing the mutant of the CRE.

Conclusion. These studies demonstrate that the stimulatory effect of NE on the expression of the ANG gene in OK cells may be mediated via both the β_1- and α_2-adrenoceptors and via the CRE (ANG N-806/-779) in the 5'flanking region of rat ANG gene.

The existence of an intrarenal renin-angiotensin system (RAS) has now been generally accepted [1, 2]. Angio-

tensinogen (ANG) mRNA has been localized in rat proximal tubules by the techniques of *in situ* hybridization [3] and polymerase chain reaction (PCR) [4]. Recent studies have also shown that the mRNA components of RAS, including ANG, renin, angiotensin-converting enzyme and angiotensin II receptor (AT_1-receptor) are expressed in murine (mouse and rat) immortalized proximal tubular cell lines [5, 6]. We [7] as well as Ingelfinger et al [8] have also demonstrated that the ANG mRNA is expressed in opossum kidney (OK) proximal tubular cells. Thus, these studies demonstrate that the intrarenal angiotensin II (Ang II) is probably derived from the ANG that is synthesized by the renal proximal tubular cells.

We have previously reported that isoproterenol and iodoclonidine stimulate the expression of the ANG gene in OK cells [9, 10]. The effect of isoproterenol is mediated via the β_1-adrenoceptor and cAMP-dependent protein kinase A (PKA) pathway [9], whereas the effect of iodoclonidine is mediated via the α_2-adrenoceptor and protein kinase C (PKC) pathway [10]. Our studies confirm the reports of Nakamura and Johns [11] that low levels of renal nerve stimulation increase the ANG mRNA levels in the rat kidney *in vivo*. Furthermore, our studies [9, 10] and those of Nakamura and Johns [11] together indicate the presence of a functional relationship between the renal sympathetic nervous system and the activation of local intrarenal RAS. Thus, the local formation of renal Ang II may play an important role in the physiology of the renal proximal tubular cells (that is, sodium and fluid reabsorption) [12–15].

Norepinephrine (NE) is the major endogenous neurotransmitter of the renal sympathetic nerves [16, 17] and it interacts with both the α- and β-adrenoceptors in the renal proximal tubules [18–21]. Thus, the objective of the present studies was to investigate whether NE modulates the expression of the ANG gene in OK cells. Our studies showed that addition of NE stimulates the expression of the ANG gene via both β_1-and α_2-adrenoceptors and their respective PKA and PKC pathways. Moreover, we demonstrated that the effect of NE on the expression of the ANG

is mediated via the putative cAMP-responsive ele-
(CRE) in the 5′-flanking region of the ANG gene.

IODS

ials

: plasmid, pRSV-Neo, containing the coding se-
e for Neomycin (Neo) with the Rous Sarcoma Virus
) enhancer/promoter sequence fused in the 5′-end of
:omycin gene was a gift from Dr. Teresa Wang (Dept.
thology, Stanford University, Stanford, CA, USA).
)lasmid, pTKGH, containing the thymidine kinase
enhancer/promoter sequence fused to the 5′-end of
;H gene was purchased from the Nichols Institute of
ostics (La Jolla, CA, USA).
: radioimmunoassay kit for hGH (RIA-hGH) was a
)m NIADDK (NIH, Bethesda, MD, USA). The RIA
lure has been described previously [22]. NIAMDD-
I-1 (AFP-4793 B) was used for both iodination and as
none standard. The limit of sensitivity of the assay
.1 ng/ml. The inter- and intra-assay coefficients of
ion were 10% ($N = 10$) and 12% ($N = 10$), respec-

iimbine hydrochloride (α_2-adrenoceptor antagonist),
)clonidine hydrochloride (α_2-adrenoceptor agonist),
;in hydrochloride (α_1-adrenoceptor antagonist),
·isoproterenol (+)-bitartrate salt (β-adrenoceptor ag-
, atenolol (β_1-adrenoceptor antagonist), ICI 118,551
lrenoceptor antagonist), phorbol 12-myristate 13-ac-
(PMA, a stimulator of protein kinase C), staurospor-
n inhibitor of protein kinase C), H-7 (an inhibitor of
n kinase C), U73122 (an inhibitor of phospholipase C
·2), S(−)-propranolol hydrochloride (an inhibitor of
d β_2-adrenoceptors), 8-bromo-cAMP, forskolin, Rp-
′ (an inhibitor of the cAMP-dependent protein kinase
d II) were all purchased from Research Biochemicals
RBI, Natick, MA, USA). (−)Arterenol bitartrate
)inephrine) was purchased from Sigma Chemicals
)uis, MO, USA).
;onucleotides for the putative CRE of the rat ANG
(ANG-CRE), N-806 to N-779 (5′ AAG AGA TTA
GAC GTA CTG GAT GCA A 3′) [22], mutant 1
(5′ AAG AGA TTA CTT GAC TTA CTG GAT
A 3′), mutant 2 (M2) (5′ AAG AGA TTA CTT GAA
CTG GAT GCA A 3′) and mutant 3 (M3) (5′ AAG
TTA CTT ATA TTA CTG GAT GCA A 3′) were
:sized by Biosynthesis, Inc. (Lewisville, TX, USA).
^{25}I was purchased from Dupont, New England Nu-
(NEN, Boston, MA, USA). Calcium chloride was
:ased from Mallinckrodt, Inc. (Montreal, Quebec,
la); Geneticin (G 418) was purchased from Bethesda
rch Laboratories (Gibco-BRL, Burlington, Ontario,
la). Other molecular biology grade reagents were
:ed either from Sigma Chemicals, Gibco-BRL, Boeh-
-Mannheim (Dorval, Quebec, Canada), Pharmacia

Inc. (Baie d'Urfe, Quebec, Canada) or Promega-Fisher,
Inc. (Montreal, Quebec, Canada).

Construction of fusion genes

The method of construction of the ANG-GH fusion
genes, pOGH (ANG N-1498/+18), pOGH (ANG N-960/
+18), pOGH (ANG N-688/+18), pOGH (ANG N-280/
+18) and pOGH (ANG N-53/+18) has been described
previously [22]. To construct the fusion genes, pOGH
(ANG N-806/-779/-53/+18), and its mutants (that is, M_1,
M_2, and M_3), the double-strand DNA fragment (ANG
N-806/-779) with the *Hin*dIII enzyme restriction site on
both the 5′ and 3′-ends was inserted upstream of the
minimal promoter of rat ANG gene (ANG N-53/+18) in
the pOGH (ANG N-53/+18) [22] that had been previously
digested with the restriction enzyme *Hin*dIII and alkaline
phophatase.

The sequence and orientation for the fusion genes were
confirmed by dideoxy sequencing with SP6 primer (Pro-
mega-Fisher, Inc.) and restriction enzyme digestion map-
ping.

Cell culture

The opossum kidney (OK) proximal tubular cell line was
obtained from the American Tissue Culture Collection
(ATCC; Rockville, MD, USA). This cell line is derived
from the kidney of a female American opossum and retains
several properties of proximal tubular epithelial cells in
culture [23, 24] and expresses a low level of ANG mRNA
[7, 8]. The culture conditions of the OK cells have been
described previously [9, 10, 25].

OK cell stable transformants

OK 27, OK 960, OK 688, OK 280, OK 53 and OK 13 cells
are stable transformants with pOGH (ANG N-1498/+18),
pOGH (ANG N-960/+18), pOGH (ANG N-688/+18),
pOGH (ANG N-280/+18), pOGH (ANG N-53/+18) and
pTKGH integrated into OK cellular genomes, respectively.
The method of obtaining these transformants had been
previously reported [9, 10]. Briefly, the ANG-GH fusion
gene and the plasmid pRSV-Neo were co-transfected (20
mg) each into OK cells utilizing calcium phosphate-medi-
ated endocytosis. The stable transformants were selected by
growing the cells in the presence of G418 (Geneticin;
Gibco, Inc.). OK 95, OK 95/M1, OK 95/M2, OK 95/M3
cells are stable transformants with pOGH (ANG N-806/-
779/N-53/+18) or its mutants and pRSV-Neo fusion genes
co-integrated into OK cellular genomes. The method for
the selection of OK cell stable transformants with the high
expression of the fusion gene was identical to the method
described previously for OK 27 cells [9, 10].

Effect of norepinephrine on the expression of the ANG-GH fusion gene in OK cell stable transformants

OK cell stable transformants were plated at a density of
1 to 2 × 10^5 cells/well in six-well plates and incubated

overnight in DMEM containing 10% FBS. Then, cell growth was arrested by incubation in serum-free medium for 24 hours. Subsequently, various concentrations of NE (10^{-13} to 10^{-5} M) were added to the culture medium containing 1% resin and charcoal-treated FBS and incubated for 24 hours. At the end of the incubation period, media were collected and kept at $-20°C$ until assayed for IR-hGH.

To compare the inhibitory effect of propranolol, atenolol, ICI 118,551, yohimbine, prazosin, Rp-cAMP, staurosporine, H-7 and U73122 on the expression of ANG-GH fusion gene in OK cell transformants, various concentrations (10^{-13} to 10^{-7} M) of the antagonists or inhibitors were added in the presence of NE (10^{-9} M) for 24 hours. At the end of the incubation period, media were collected and kept at $-20°C$ until assay for IR-hGH.

To compare the effect of NE on the expression of various fusion genes in OK 960, OK 688, OK 280, OK 53 and OK 13 cells, NE (10^{-9} M) was added to the culture medium containing 1% resin and charcoal-treated FBS and incubated for 24 hours. At the end of the incubation period, media were collected and kept at $-20°C$ until assayed for IR-hGH.

The resin and charcoal-treated FBS was prepared by incubation with 1% activated charcoal and 1% AG 1 × 8 ion-exchange resin (Bio-Rad Laboratories, Richmond, CA, USA) for 16 hours or more at room temperature as described by Samuels, Stanley and Shapiro [26]. This procedure removed endogenous steroid and thyroid hormones from the FBS as demonstrated by Samuels et al [26].

Statistical analysis

The experiments were performed at least three to four times in triplicate. The data were analyzed with Student's *t*-test or analysis of variance (ANOVA). A probability level of $P \leq 0.05$ was regarded as significant.

RESULTS

Effect of norepinephrine on the expression of ANG-GH fusion genes in OK cell stable transformants

Figure 1 shows the expression of the pOGH (ANG N-1498/+18) in OK 27 cells in the presence of various concentrations (10^{-13} to 10^{-5} M) of NE. A dose-dependent relationship between NE concentrations and the stimulation of expression of pOGH (ANG N-1498/+18) was observed for NE at 10^{-13} M to 10^{-7} M. The maximal stimulation of expression of the pOGH (ANG N-1498/+18) was found with 10^{-9} M to 10^{-7} M for NE, whereas the addition of concentrations greater than 10^{-7} M (that is, 10^{-5} M) of norepinephrine had no effect.

Figure 2 shows the effect of NE (10^{-9} M) or isoproterenol (10^{-9} M) or iodoclonidine (10^{-9} M) on the expression of the pOGH (ANG N-1498/+18) in OK 27 cells without (Fig. 2A) or with (Fig. 2B) the pre-incubation with a high
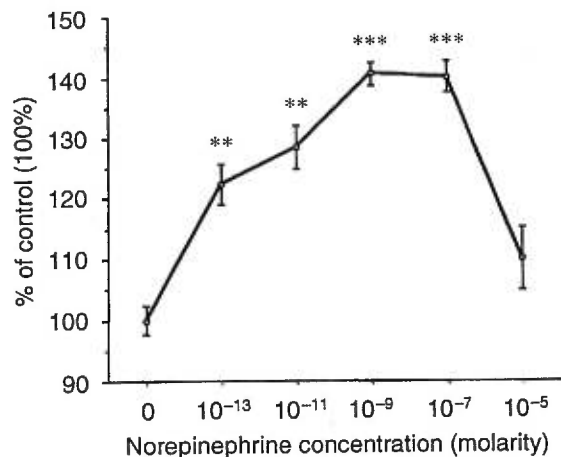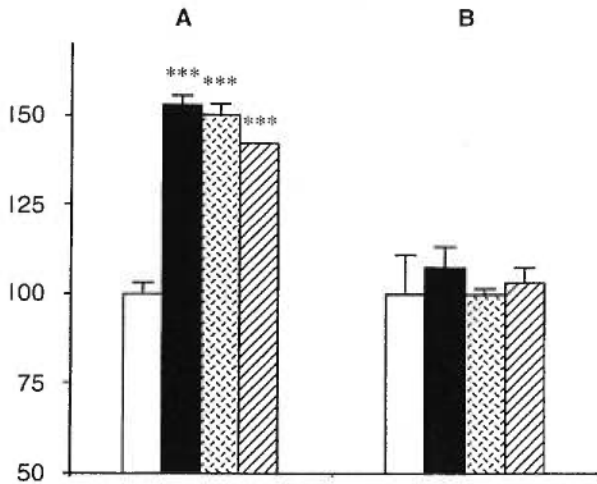

Fig. 1. **Effect of norepinephrine on the expression of pOGH (ANG N-1498/+18) in OK 27 cells.** Cells were incubated for up to 24 hours in the presence of various concentrations of norepinephrine (10^{-13} to 10^{-5} M). Media were harvested after 24 hours of incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentrations of IR-hGH in the absence of norepinephrine represents the control level (that is, 1.65 ± 0.03 ng/ml of IR-hGH). Each point represents the mean $\pm$ SD of three determinations (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from three other experiments.

concentration of NE (10^{-5} M) for 24 hours. It is apparent that the pre-incubation of OK 27 cells with NE (10^{-5} M) abolished the stimulatory effect of NE, isoproterenol or iodoclonidine on the expression of the pOGH (ANG N-1498/+18; Fig. 2B). These studies suggest that the pre-incubation with high concentrations of NE (10^{-5} M) will desensitize or down-regulate the β- and α_2-adrenoceptors in OK 27 cells.

Figure 3 shows that addition of either propranolol or yohimbine inhibits the stimulatory effect of NE on the expression of the pOGH (ANG N-1498/+18) in OK 27 cells in a dose-dependent manner. The effective dose for the inhibition of the stimulated expression (by norepinephrine) of the pOGH (ANG N-1498/+18) was found with 10^{-11} M of propranolol ($P \leq 0.05$) and 10^{-9} M yohimbine ($P \leq 0.05$). Yohimbine or propranolol at 10^{-7} M completely inhibited the stimulatory effect of NE ($P \leq 0.01$). These studies suggest that the stimulatory effect of NE is mediated via both β_1-adrenoceptor and α_2-adrenoceptor.

The inhibitory effect of various adrenoceptor antagonists on the expression of the pOGH (ANG N-1498/+18) in OK 27 cells stimulated by NE (10^{-9} M) is shown in Figure 4. Propranolol, atenolol and yohimbine at 10^{-7} M completely inhibited the stimulatory effect of NE ($P \leq 0.01$), whereas ICI 118,551 and prazosin had no effect. The studies further confirm that the effect of NE is mediated via both β_1-adrenoceptor and α_2-adrenoceptor.

Figure 5 shows that addition of Rp-cAMP, staurosporine or U73122 inhibits the stimulatory effect of NE on the expression of pOGH (ANG N-1498/+18) in OK 27 cells in a dose-dependent manner. The maximal and half-maximal inhibition of the stimulated expression of the pOGH (ANG
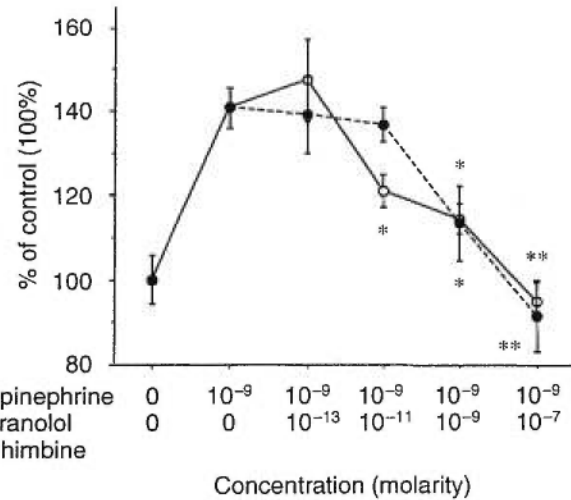
. **Effect of norepinephrine, isoproterenol and iodoclonidine on the** sion of the pOGH (ANG N-1498/+18) in OK 27 cells when the cells pre-incubated with a high level of norepinephrine. Cells were cubated for 24 hours without (*A*) or with (*B*) 10^{-5} M norepinephrine. the media were replaced with the fresh media containing 10^{-9} M inephrine or 10^{-9} M isoproterenol or 10^{-9} M iodoclonidine and ted further for 24 hours. Subsequently, the media were harvested sayed for IR-hGH. The concentration of IR-hGH in the medium t the addition of drugs in A or B (that is, 2.5 ± 0.1 ng/ml or $4.0 \pm$ /ml) is expressed as 100% (control). Each point represents the $\pm$ SD of three dishes (*$P \le 0.05$, **$P \le 0.01$ and ***$P \le 0.005$). ls are: (□) control medium without the addition of drugs; (■) m in the presence of 10^{-9} M norepinephrine; (▨) medium in the ce of 10^{-9} M isoproterenol; (▧) medium in the presence of 10^{-9} M onidine. Similar results were obtained from two other experiments.

Fig. 3. Inhibitory effect of propranolol (β-adrenoceptor antagonist) or yohimbine (α_2-adrenoceptor antagonist) on the expression of pOGH (ANG N-1498/+18) in OK 27 cells stimulated by norepinephrine. The cells were incubated for up to 24 hours in the presence of norepinephrine (10^{-9} M) and various concentrations of propranolol (10^{-13} to 10^{-7} M) or yohimbine (10^{-13} to 10^{-7} M). Media were harvested and assayed for the level of IR-hGH. The concentration of IR-hGH in the absence of NE or propranolol or yohimbine represents the control level (1.53 ± 0.06 ng/ml of IR-hGH). The inhibitory effect of propranolol or yohimbine was compared to cells that were incubated with 10^{-9} M norepinephrine. Results are expressed as the mean $\pm$ SD of three determinations (*$P \le 0.05$, **$P \le 0.01$ and ***$P \le 0.005$). Symbols are: (○) propranolol; (●) yohimbine. Experiments were repeated three times.

98/+18) was found with 10^{-7} M ($P \le 0.01$) and 10^{-11} ≤ 0.05) Rp-cAMP. These studies indicate that the P-dependent protein kinase AI and II is involved in xpression of the fusion gene stimulated by NE. Simi- the maximal and half-maximal inhibition of the lated expression of the pOGH (ANG N-1498/+18) ound with 10^{-7} M ($P \le 0.05$) and 10^{-11} M ($P \le 0.01$) osporine or U73122, respectively. The addition of H-7 hibitor of PKC) also inhibited the stimulatory effect Ξ on the expression of pOGH (ANG N-1498/+18) in 27 cells in a dose-dependent manner (Fig. 6). The nal and half-maximal inhibition of the stimulated ssion of the pOGH (ANG N-1498/+18) was found 10^{-7} M ($P \le 0.01$) and 10^{-9} M ($P \le 0.05$), respectively. studies indicate that protein kinase C may also ate the effect of NE on the expression of the ANG in OK cells.

t of a combination of both iodoclonidine and oterenol on the expression of pOGH (ANG N-1498/ in OK 27 cells

ure 7 shows that the stimulatory effect of NE on the ssion of pOGH (ANG N-1498/+18) was similar to the ion of isoproterenol or iodoclonidine alone. The ad- of a combination of both isoproterenol and iodo- line, however, significantly enhanced the expression

of the pOGH (ANG N-1498/+18; that is, 230%) compared to the addition of isoproterenol (145%) or iodoclonidine (146%) alone ($P \le 0.05$). These studies indicate that there is probably a synergistic effect of both β_1- and α_2-adreno-ceptors on the expression of pOGH (ANG N-1498/+18) in OK 27 cells.

Effect of NE on the expression of ANG-GH fusion genes and pTKGH in OK cells

Figure 8 shows that the addition of NE (10^{-9} M) stimu-lated the expression of pOGH (ANG N-1498/+18), pOGH (ANG N-960/+18), pOGH (ANG N-688/+18) in OK 27, OK 960 and OK 688 cells compared to the controls (that is, without the addition of NE), respectively. The addition of NE had no stimulatory effect on the expression of pOGH (ANG N-280/+18), pOGH (ANG N-53/+18) and pTKGH in OK 280, OK 53 and OK 13 cells compared to the controls, respectively.

Effect of NE on the expression of pOGH (ANG N-806/-779/-53/+18) in OK 95 cells

Figure 9 shows that the presence of propranolol or yohimbine inhibited the stimulatory effect of NE on the expression of pOGH (ANG N-806/-779/-53/+18) in OK 95 cells in a dose-dependent manner. The maximal and half-maximal inhibition of the stimulated expression of the
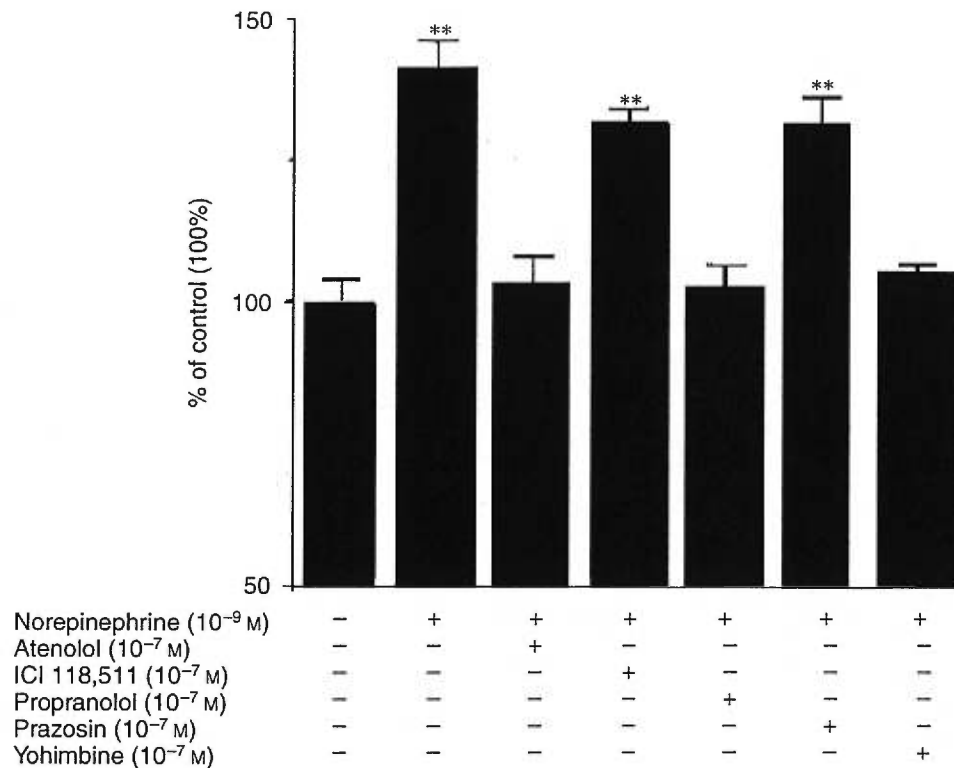
Fig. 4. Inhibitory effect of adrenoceptor antagonists on the expression of pOGH (ANG N-1498/+18) in OK 27 cells stimulated by norepinephrine (10^{-9} M). OK 27 cells were incubated for up to 24 hours in the presence of norepinephrine (10^{-9} M) and 10^{-7} M of various adrenoceptor antagonists. Media were harvested and assayed for the level of IR-hGH. The concentration of IR-hGH in the absence of norepinephrine or adrenoceptor antagonists is the control level (that is, 1.45 ± 0.06 ng/ml of IR-hGH). The inhibitory effect of adrenoceptor antagonists was compared to cells that were incubated with 10^{-9} M norepinephrine. Results were expressed as the mean ± SD of three determinations (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from two other experiments.

pOGH (ANG N-806/-779/-53/+18) was found with 10^{-7} M and 10^{-11} M propranolol or yohimbine, respectively. These studies indicate that the effect of NE on the expression of pOGH (ANG N-806/-779/-53/+18) is mediated via both β_1- and α_2-adrenoceptors, respectively.

Figure 10 shows the result of the addition of 8-bromo-cAMP, forskolin and PMA on the expression of the pOGH (ANG N-806/-779/-53/+18) in OK 95 cells. The addition of 8-bromo-cAMP (10^{-3} M), forskolin (10^{-9} M) or PMA (10^{-9} M) significantly stimulated the expression of pOGH (ANG N-806/-779/-53/+18) in OK 95 cells compared to the controls (that is, absence of 8-bromo-cAMP, forskolin or PMA). Since the DNA fragment, ANG N-806/-779 contains the DNA sequence, TGACGTAC (N-795 to N-788) which is very similar to the consensus CRE (that is, TGACGTCA), these studies indicate that the DNA fragment, ANG N-806/-779, is probably the functional CRE of the rat ANG gene.

Figure 11 shows that the addition of Rp-cAMP (10^{-7} M), U73122 (10^{-7} M) or staurosporine (10^{-7} M) also inhibited the stimulatory effect of NE on the expression of pOGH (ANG N-806/-779/-53/+18) in OK 95 cells. These studies demonstrate that the stimulatory effect of NE on the expression of pOGH (ANG N-806/-779/-53/+18) in OK 95

cells is mediated via both PKA and PKC signal transduction pathway.

Figure 12 shows that the addition of NE (10^{-9} M), 8-Br-cAMP (10^{-3} M) or PMA (10^{-7} M) had no stimulatory effect on the expression of the mutants (that is, M1, M2 and M3) of pOGH (ANG N-806/-779/-53/+18) in OK 95/M1, OK 95/M2 and OK 95/M3 cells compared to the control (without the addition of NE, forskolin or PMA). These studies demonstrate that the DNA sequence, TGACGTAC (N-795/-788) is the motif of the cAMP-responsive element (CRE; that is, ANG N-806/-779) of the rat ANG gene.

Effect of NE or forskolin or 8-Br-cAMP or PMA on the expression of pOGH (ANG N-53/+18) in OK 53 cells

Figure 13 shows that addition of NE (10^{-9} M) or forskolin (10^{-7} M) or 8-Br-cAMP (10^{-3} M) or PMA (10^{-9} M) had no stimulatory effect on the expression of pOGH (ANG N-53/+18) in OK 53 cells. These studies indicate that the minimal promoter (ANG N-53 to N+18) of the rat ANG gene is not sufficient to respond to the addition of NE or PKA or PKC signal transduction pathways.

DISCUSSION

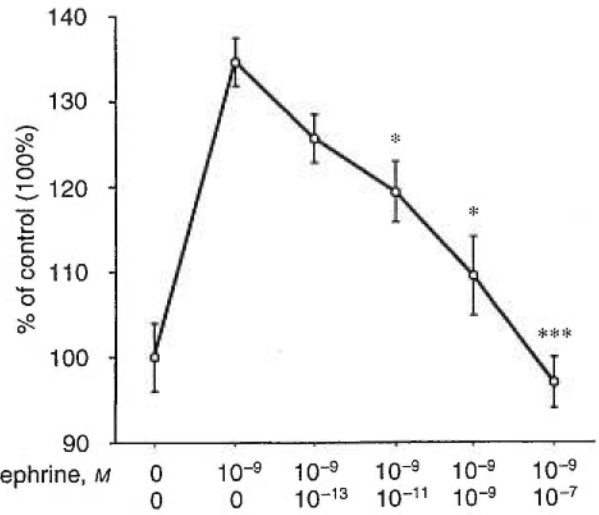Our present studies showed that addition of NE alone directly stimulated the expression of pOGH (ANG N-1498/
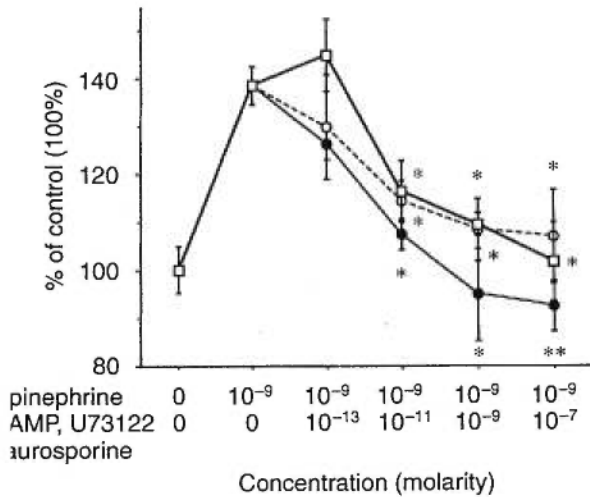
Inhibitory effect of Rp-cAMP or staurosporine or U73122 on the ...sion of pOGH (ANG N-1498/+18) in OK 27 cells stimulated by ...ephrine. Cells were incubated for up to 24 hours in the presence ...epinephrine (10^{-9} M) and various concentrations of Rp-cAMP ...to 10^{-7} M) or staurosporine (10^{-13} to 10^{-7} M) or U73122 (10^{-13} to ...). Media were harvested and assayed for the level of IR-hGH. The ...tration of IR-hGH in the absence of NE or Rp-cAMP or stauro-...: or U73122 is the control level (that is, 2.25 ± 0.06 ng/ml of ...H). The inhibitory effect of Rp-cAMP or staurosporine or U73122 ...mpared to cells that were incubated with 10^{-9} M norepinephrine. ...oint represents the mean $\pm$ SD of three determinations (*$P \leq 0.05$, ... 0.01 and ***$P \leq 0.005$). Symbols are: (●) Rp-cAMP; (○) ...porine; (□) U73122. Similar results were obtained from two other ...nents.
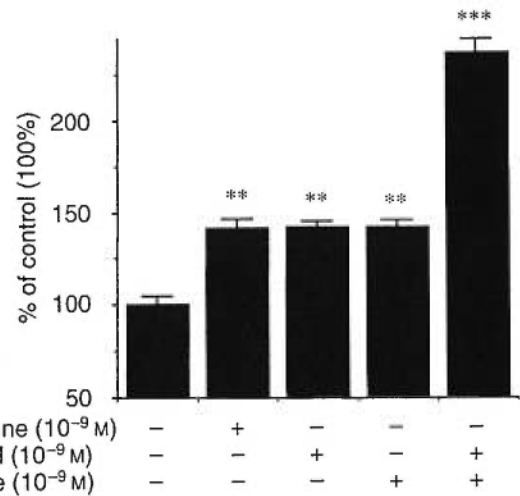

Fig. 6. Inhibitory effect of H-7 on the expression of pOGH (ANG N-1498/+18) in OK 27 cells stimulated by norepinephrine. The cells were incubated for 24 hours in the presence of norepinephrine (10^{-9} M) and various concentrations of H-7 (10^{-13} to 10^{-7} M). Media were harvested and assayed for the level of IR-hGH. The concentration of IR-hGH in the absence of NE represents the control level (that is, 3.14 ± 0.2 ng/ml of IR-hGH). The inhibitory effect of H-7 was compared to cells which were incubated with 10^{-9} M norepinephrine. Results are expressed as the mean $\pm$ SD of three determinations (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from another experiment.

in OK 27 cells in a dose-dependent manner (that is, ... to 10^{-7} M; Fig. 1). At present, we do not understand ...igher concentrations of NE (that is, 10^{-5} M) had no ...latory effects on the expression of the pOGH (Ang ...98/+18) in OK 27 cells. One possible explanation may ...at the exposure of OK cells to high levels of NE may ...sitize or downregulate its own adrenoceptors. Indeed, ...udies (Fig. 2B) showed that the pre-incubation of OK ...lls with 10^{-5} M NE abolished the stimulatory effect of ...soproterenol and iodoclonidine, supporting the notion ...he β- and α-adrenoceptors are subject to desensitiza-...by high levels of NE. Furthermore, these results are ...orted by the observations of Suzuki et al [27] and ...se and Lefkowitz [28] that β- and α-adrenoceptors are ...ct to desensitization by their own agonists. Obviously, ...experiments along these lines are warranted.

...e stimulatory effect of NE was inhibited by the ...nce of propranolol or yohimbine (Fig. 3) as well as by ...resence of atenolol, but not ICI 118,511 and prazosin ...4). These studies demonstrate that the effect of NE ...oe mediated via both β_1-adrenoceptor and α_2-adreno-...or. These studies confirm our previous studies that the ...ation of the β-adrenoceptor or α_2-adrenoceptor alone ...lated the expression of pOGH (ANG N-1498/+18) in ...7 cells [9, 10].

...e addition of Rp-cAMP, staurosporine or H-7 inhib-

Fig. 7. Effect of a combination of both isoproterenol and iodoclonidine on the expression of pOGH (ANG N-1498/+18) in OK 27 cells. Cells were incubated for up to 24 hours in the presence of NE (10^{-9} M) or isoproterenol (10^{-9}) or iodoclonidine (10^{-9} M) or a combination of both isoproterenol (10^{-9} M) and iodoclonidine (10^{-9} M). Media were harvested after 24 hours of incubation and assayed for IR-hGH. The concentration of IR-hGH in the absence of NE or adrenoceptor agonists represents the control level (that is, 3.79 ± 0.05 ng/ml of IR-hGH). Each point represents the mean $\pm$ SD of three determinations (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from two other experiments.

ited the stimulatory effect of NE (10^{-9} M) in a dose-dependent manner (Figs. 5 and 6). These studies indicate that the stimulatory effect of NE may be mediated via either the cAMP-dependent protein kinase A I and II or
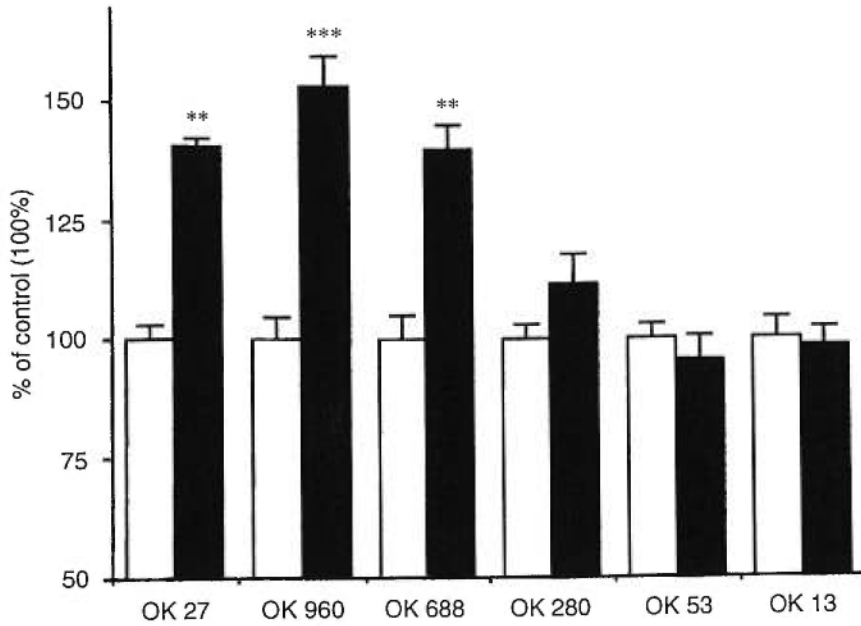
Fig. 8. Effect of norepinephrine on the expression of various ANG-GH fusion genes in OK cells. Cells were incubated for up to 24 hours in the presence of norepinephrine (10^{-9} M). Media were harvested after 24 hours of incubation and assayed for IR-hGH. The concentration of IR-hGH in the medium without norepinephrine (that is, OK 27 cells, 1.45 ± 0.03 ng/ml; OK 960, 1.29 ± 0.04 ng/ml; OK 688, 3.24 ± 0.2 ng/ml; OK 280, 0.37 ± 0.02 ng/ml; OK 53, 0.44 ± 0.02 ng/ml; OK 13, 5.56 ± 0.3 ng/ml) is considered as the control level. Each point represents the mean $\pm$ SD of three dishes ($*P \leq 0.05$, $**P \leq 0.01$ and $***P \leq 0.005$). Similar results were obtained from two other experiments.
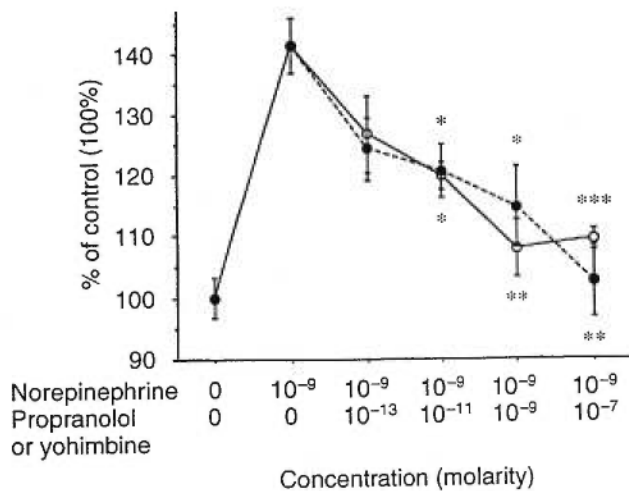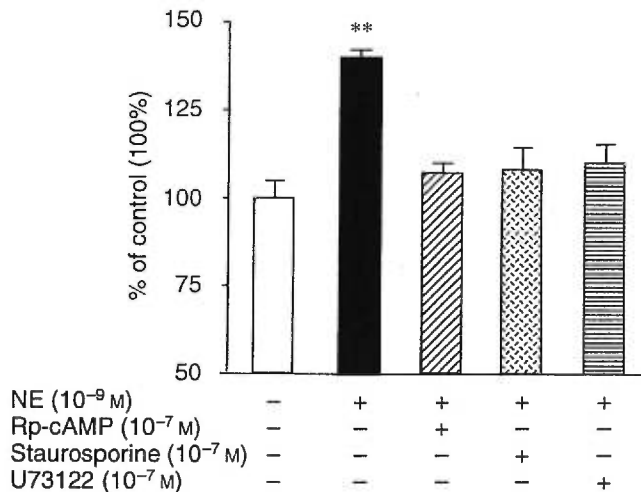

Fig. 9. Inhibitory effect of propranolol or yohimbine on the expression of pOGH (ANG N-806/-779/-53/+18) in OK 95 cells stimulated by norepinephrine (10^{-9} M). OK 95 cells were incubated for up to 24 hours in the presence of norepinephrine (10^{-9} M) and various concentrations (10^{-13} to 10^{-7} M) of propranolol or yohimbine. Media were harvested and assayed for the level of IR-hGH. The concentration of IR-hGH in the absence of norepinephrine or propranolol or yohimbine is the control level (that is, 3.25 ± 0.06 ng/ml). The inhibitory effect of propranolol or yohimbine was compared to cells which were incubated with 10^{-9} M norepinephrine. Results are expressed as the mean $\pm$ SD of three determinations ($*P \leq 0.05$, $**P \leq 0.01$ and $***P \leq 0.005$). Symbols are: (O) propranolol; (●) yohimbine. Similar results were obtained from two other experiments.
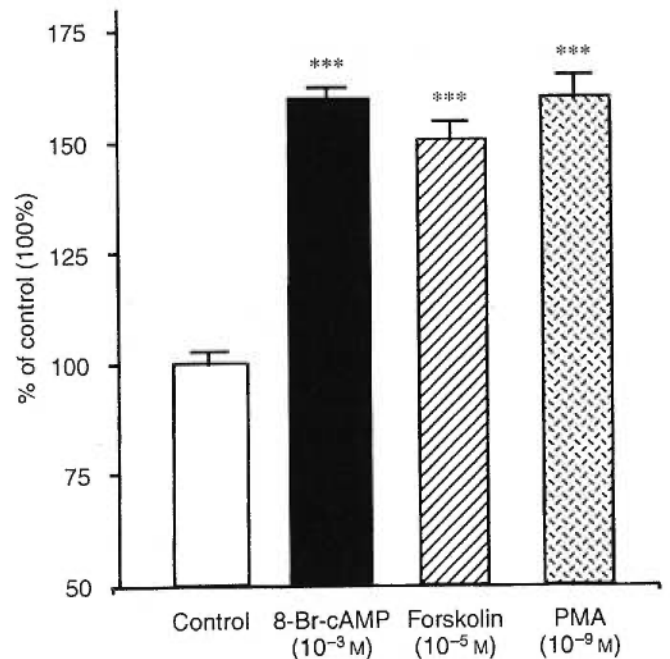
Fig. 10. Effect of 8-bromo-cAMP, forskolin and PMA on the expression of pOGH (ANG N-809/-779/-53/+18) in OK 95 cells. Cells were incubated for 24 hours in the presence of 10^{-3} M 8-Br-cAMP or 10^{-9} M forskolin or 10^{-9} M PMA. The levels of IR-hGH in the media were assayed by RIA-hGH. The concentration of IR-hGH in the absence of 8-bromo-cAMP, forskolin or PMA is the control (that is, 2.82 ± 0.12 ng/ml of IR-hGH). Each point represents the mean $\pm$ SD of three determinations ($*P \leq 0.05$, $**P \leq 0.01$ and $***P \leq 0.005$). Similar results were obtained from two other experiments.

PKC signal transduction pathways or via the interaction of both pathways in OK 27 cells. The involvement of the PKC pathway is further supported by the observation that U73122 inhibited the stimulatory effect of NE (Fig. 5). Since U73122 is an inhibitor of phospholipase C and phospholipase A_2, it is conceivable that the addition of U73122 might prevent the hydrolysis of phosphatidyl-inositol 4,5 biphosphate and would subsequently inhibit the activation of protein kinase C in OK 27 cells. Indeed, this possibility is supported by the studies of Martin et al, who showed that the addition of U73122 abolishes the increase

ig. 11. Inhibitory effect of Rp-cAMP or U73122 or staurosporine on the xpression of pOGH (ANG N-806/-779/-53/+18) in OK 95 cells stimulated y norepinephrine (10^{-9} M). OK 95 cells were incubated for up to 24 ours in the presence of norepinephrine (10^{-9} M) and 10^{-7} M Rp-cAMP r 10^{-7} M U73122 or 10^{-7} M staurosporine. Media were harvested and ssayed for the level of IR-hGH. The concentration of IR-hGH in the bsence of drugs is the control level (that is, 3.6 ± 0.03 ng/ml of IR-hGH). he inhibitory effect of Rp-cAMP or U73122 or staurosporine was ompared to cells, which were incubated with 10^{-9} M norepinephrine. .esults are expressed as the mean $\pm$ SD. of three determinations (*$P \leq$ .05, **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from vo other experiments.

1 particulate PKC activity stimulated by PTH in OK cells ?9]. Our preliminary results also showed that the stimula- ory effect of NE (10^{-9} M) and iodoclonidine (10^{-9} M) was bolished after a 24 hours pre-incubation of OK 27 cells ith 10^{-5} M PMA compared to the control cells without the re-incubation with PMA (unpublished results). These ssults are in agreement that the exposure to PMA will ownregulate the PKC activity [30]. Moreover, our prelim- iary studies showed that NE at 10^{-9} M increased the ellular level of DAG and PKC activity in OK cells but not [E at 10^{-5} M (unpublished results). All these studies ipport the notion that the stimulatory effect of NE is iediated, at least in part, via the PKC signal transduction athway.

At present, we do not have a good explanation for the ssults that the addition of 10^{-7} M atenolol, yohimbine, :aurosporine, U73122 or H-7 alone completely inhibited ie NE (10^{-9} M) stimulation of ANG gene expression (Figs. to 6). One possible explanation may be that the dose 10^{-7} was too high (100-fold excess), since equal molar con- :ntration (10^{-9} M) of atenolol, yohimbine, staurosporine, [73122 or H-7 could only partially block the stimulatory ffect of NE. The second possible explanation may be that iere are multiple isoform(s) of PKC and adenylyl cyclase \C) in OK cells. The effect of NE may be mediated via the iteraction or "cross-talk" of these isoforms of PKC and .C. Indeed, studies have shown that PKC may interact ith specific subtypes of AC but not others in a cell-specific

manner [31]. Thus, it is possible that the addition of high levels (10^{-7} M) of atenolol, yohimbine, staurosporine, U73122 or H-7 might block the "cross-talk" between the PKC and PKA signal transduction pathway. Studies are underway in our laboratory to identify the isoform(s) of PKC and AC in OK cells.

Most interestingly, the stimulatory effect of the addition of a combination of both isoproterenol and iodoclonidine on the expression of the pOGH (ANG N-1498/+18) was significantly higher than the addition of isoproterenol and iodoclonidine alone ($P \leq 0.05$; Fig. 7), suggesting a syner- gistic effect of β- and α_2-adrenoceptors. Since the effect of β- and α_2-adrenoceptors are mediated via protein kinase A (PKA) and protein kinase C (PKC) in OK 27 cells, respectively [9, 10], these studies support the notion that there might be a "cross-talk" between the β- and α_2- adrenoceptors or between the activation of PKA and PKC on the expression of pOGH (ANG N-1498/+18) in OK 27 cells. These results are similar to our recent report [32] that the addition of a combination of SKF-82958 (a D_1-dopa- minergic receptor agonist) and PPHT (a D_2-dopaminergic receptor agonist) significantly enhanced the expression of pOGH (Ang N-1498/+18) in OK 27 cells compared to the addition of SKF-82958 or PPHT alone. The effect of SKF-82958 and PPHT are mediated via the PKA and PKC signal transduction pathway, respectively [32]. At present, we do not understand the molecular mechanism(s) for the synergistic effect of β- and α_2-adrenoceptors in OK cells. Experiments are underway in our laboratory to explore the molecular mechanism(s) of the synergistic effect of β- and α_2-adrenoceptors on the expression of the ANG gene in OK cells.

Our present results showed that the addition of NE (10^{-9} M) stimulates the expression of pOGH (ANG N-1498/+18), pOGH (ANG N-960/+18) and pOGH (ANG N-688/+18) in OK 27, OK 960 and OK 688 cells, respectively. The addition of NE (10^{-9} M), however, had no effect on the expression of pOGH (ANG N-280/+18), pOGH (ANG N-53/+18) and pTKGH in OK 280, OK 53 and OK 13 cells, respectively (Fig. 8). These studies indi- cate that the NE-responsive element is probably localized within nucleotides N-1498 to N-280 in the 5'-flanking region of the rat ANG gene. Indeed, this possibility is supported by those studies (Fig. 9) that the addition of NE (10^{-9} M) stimulated the expression of the pOGH (ANG N-806/-779/-53/+18) in OK 95 cells. The DNA fragment, ANG N-806/-779 contains a CRE-motif (that is, N-795 to N-788, TGACGTAC), which is almost identical to the consensus CRE-motif of the somatostatin gene (that is, TGACGTCA) [33]. Moreover, the stimulatory effect of NE was inhibited in the presence of propranolol or yohimbine (Fig. 9). These studies demonstrate that the NE-responsive element is probably localized in the DNA fragment, ANG N-806/-779 of the rat ANG gene.

On the other hand, we were surprised that the addition
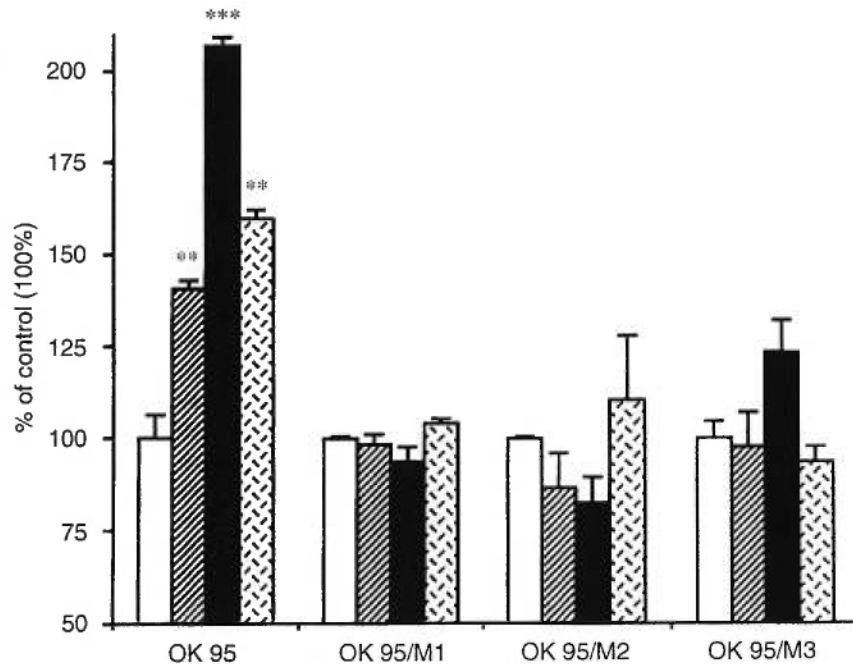
Fig. 12. Effect of norepinephrine, 8-bromo-cAMP and PMA on the expression of pOGH (AnG N-806/-779/-53/+18) and its mutants in OK 95, OK 95/M1, OK 95/M2 and OK 95/M3 cells. Cells were incubated for up to 24 hours in the presence of norepinephrine (10^{-9} M) or PMA (10^{-7} M) or 8-bromo-cAMP (10^{-3} M). Media were harvested after 24 hours of incubation and assayed for IR-hGH. The concentration of IR-hGH in the absence of norepinephrine or 8-bromo-cAMP or PMA represents the control level (that is, OK 95, 3.8 ± 0.2 ng/ml; OK 95/M1, 3.9 ± 0.01 ng/ml; OK 95/M2, 4.2 ± 0.05 ng/ml; OK 95/M3, 3.1 ± 0.14 ng/ml of IR-hGH). Each point represents the mean $\pm$ SD of three determinations ($*P \leq 0.05$, $**P \leq 0.01$ and $***P \leq 0.005$). Symbols are: ($\square$) the control level; (▨) incubation medium in the presence of 10^{-9} M NE; (■) incubation medium in the presence of 10^{-7} M PMA; (▨) medium in the presence of 10^{-3} M 8-bromo-cAMP. Similar results were obtained from four other experiments.

of NE stimulated the expression of pOGH (ANG N-688/+18) in OK 688 cells. We did not find any consensus CRE motif (that is, TGACGTCA) in the promoter region between N-688 to N+281. These observations suggest that there might be an additional functional CRE-like in the region between N-688 to N-281. Studies are underway in our laboratory to identify the putative CRE-like in this region.

The addition of 8-bromo-cAMP, forskolin or PMA alone also stimulated the expression of the pOGH (ANG N-806/-779/-53/+18) in OK 95 cells compared to the control (without the addition of 8-bromo-cAMP, forskolin or PMA; Fig. 10). Furthermore, the stimulatory effect of NE on the expression of the pOGH (ANG N-806/-779/-53/+18) in OK 95 cells was inhibited in the presence of Rp-cAMP, staurosporine or U73122 (Fig. 11). These studies provide further support for the notion that the DNA fragment, ANG N-806/-779 is the CRE of the rat ANG gene.

Most convincingly, the addition of NE, forskolin or PMA had no stimulatory effect on the expression of mutants of the pOGH (ANG N-806/-779/-53/+18) in OK 95/M1, OK 95/M2 and OK 95/M3 cells (Fig. 12), these studies demonstrate that the DNA fragment, ANG N-806/-779 is the CRE of the rat ANG gene.

At present, we do not know the exact molecular mechanism(s) for the stimulatory effect of NE (that is, the downstream pathway after the activation of PKA and PKC) on the expression of pOGH (ANG N-1498/+18) in OK 27 cells. One possible explanation may be that NE might induce the phosphorylation of the nuclear cAMP-responsive element binding protein (CREB) at serine 133 via

either PKA or PKC or a combination of both pathways, since studies have shown that CREB can be phosphorylated at serine 133 by cAMP-dependent protein kinase A [34], or by PKC [35, 36] and phosphorylation increases the binding affinity of 43 kD-CREB to CRE [35, 36] and subsequently enhances the gene expression. This possibility is supported by our most recent studies which showed that the addition of isoproterenol enhances the stimulating effect of 43 kD-CREB on the expression of the pOGH (ANG N-1498/+18) in OK 27 cells [38]. Indeed, more studies are ongoing to explore the molecular mechanism(s) of the stimulatory effect of NE on the expression of the ANG gene in OK cells.

Finally, it is evident that neither NE, forskolin, 8-Br-cAMP nor PMA had any effect on the expression of pOGH (ANG N-53/+18) in OK 53 cells (Fig. 13). Since the expression of the pOGH (ANG N-53/+18) is driven by the minimal promoter (that is, ANG N-53 to +18 contains the putative "CCAT" and "TATA" boxes on nucleotides-50 and -30 upstream of the transcriptional site, respectively [21]) of the rat ANG gene, these studies demonstrate that the minimal promoter of the rat ANG gene was not sufficient to respond to the addition of NE, forskolin, 8-Br-cAMP or PMA. The effect of NE, forskolin, 8-Br-cAMP and PMA in OK 27 is mediated via the putative CRE (ANG N-806 to N-779) in the 5'-flanking region of the rat ANG gene.

In summary, the present studies demonstrate that NE stimulated the expression of pOGH (ANG N-1498/+18) in OK 27 cells. The stimulatory effect of NE was blocked by the presence of propranolol or yohimbine or Rp-cAMP or staurosporine or U73122. Furthermore, we demonstrate
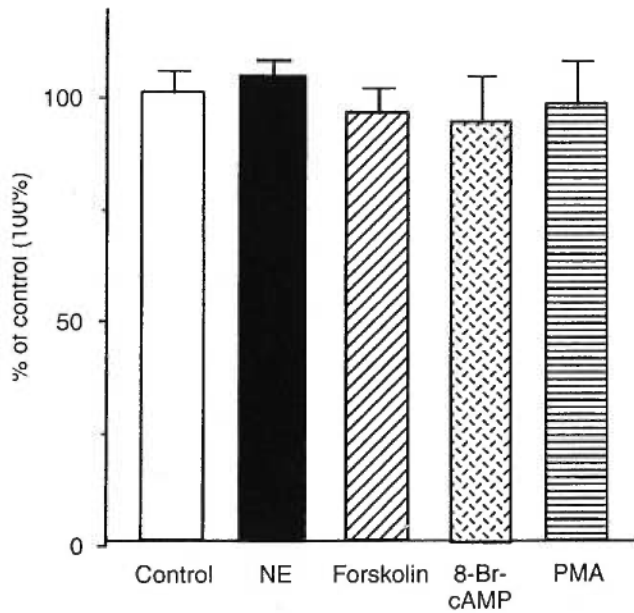
Fig. 13. Effect of norepinephrine or forskolin or 8-bromo-cAMP or PMA [on] the expression of pOGH (ANG N-53/+18) in OK 53 cells. Cells were [inc]ubated for 24 hours in the absence or presence of norepinephrine (10^{-9} M) [or] forskolin (10^{-7} M) or 8-Br-cAMP (10^{-3} M) or PMA (10^{-9} M). Media [we]re harvested 24 hours after the incubation and assayed for IR-hGH. [Ea]ch point represents the mean ± SD of three determinations. The [con]centration of IR-hGH in the absence of norepinephrine, forskolin, [8-B]r-cAMP and PMA is the control level (that is, 2.57 ± 0.09 ng/ml of [IR]-hGH; $*P \leq 0.05$, $**P \leq 0.01$ and $***P \leq 0.005$). Similar experiments [we]re obtained from two other experiments ($*P \leq 0.05$, $**P \leq 0.01$ and [**]$*P \leq 0.005$).

[th]at the effect of a combination of isoproterenol and [io]doclonidine on the expression of the pOGH (ANG [-]1498/+18) in OK 27 cells was significantly higher when [co]mpared to the addition of isoproterenol or iodoclonidine [o]ne. Finally, we demonstrate that the stimulatory effect [of] NE is mediated via the putative cAMP-responsive [ele]ment (CRE) in the 5'-flanking region of the rat ANG [ge]ne. Our studies suggest that the activation of renal nerves [wi]ll release NE, which then stimulates the expression of the [A]NG gene in the renal proximal tubule and increases the [fo]rmation of local renal Ang II. The elevated renal Ang II [su]bsequently modulates the sodium and fluid reabsorption [in] the proximal tubular cells. Hence, the local intrarenal [R]AS plays a significant role in the modulation of sodium [re]absorption.

[A]CKNOWLEDGMENTS

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Appendix IV : Wang TT, Chen X, Wu XH, **Zhang SL**, Chan JSD: Molecular mechanism(s) of action of isoproterenol on the expression of the angiotensinogen gene in opossum kidney proximal tubular cells. *Kidney Int* 55:1713-1723,1999.

Molecular mechanism(s) of action of isoproterenol on the expression of the angiotensinogen gene in opossum kidney proximal tubular cells

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Molecular mechanism(s) of action of isoproterenol on the expression of the angiotensinogen gene in opossum kidney proximal tubular cells.

Background. β-adrenoceptors are present in the renal proximal tubules. We have previously reported that isoproterenol stimulates the accumulation of intracellular cAMP and the expression of the angiotensinogen (ANG) gene in opossum kidney (OK) proximal tubular cells via the β_1-adrenoceptor. We hypothesized that the molecular mechanism(s) of action of isoproterenol on the expression of the ANG gene is mediated via the interaction of the phosphorylated cAMP-responsive element binding protein (CREB) and the cAMP-responsive element (CRE; that is, ANG N-806/-779) in the 5'-flanking region of the rat ANG gene.

Methods. The fusion genes containing the putative ANG-CRE of the rat ANG gene inserted upstream of the rat ANG basal promoter (ANG N-53/+18) fused to a human growth hormone (hGH) gene as reporter were stably cotransfected, with or without the plasmid containing the cDNA for 43 kDa CREB, into the OK cells. The effect of various agonists and antagonists of adrenoceptors on the expression of the fusion genes was evaluated by the amount of immunoreactive hGH secreted into the culture medium. The interactions of OK cellular nuclear protein(s) with the ANG N-806/-779 were determined by gel mobility shift assays and by Southwestern and Western blot analysis.

Results. The addition of isoproterenol, forskolin, or 8-Bromo-cAMP (8-Br-cAMP) stimulated the expression of pOGH (ANG N-806/-779/-53/+18) by 135, 150, and 160%, respectively, but not mutants of the ANG N-806/-779. The stimulatory effect of isoproterenol was blocked in the presence of propranolol, Rp-cAMP, and atenolol, but not by the presence of staurosporine, U73122, and ICI 118,551. Transient transfection of the plasmid containing the cDNA for the catalytic subunit of protein kinase A further enhanced the stimulatory effect of 43 kDa CREB on the expression of the fusion gene. The gel mobility shift assays revealed that the nuclear protein(s) of OK cells binds to the radioactive-labeled ANG N-806/-779.

The binding of the labeled ANG N-806/-779 to the OK cell nuclear protein(s) was displaced by unlabeled ANG N-806/-779, but not by the CRE of the somatostatin gene, the CRE of the tyrosine amino-transferase gene, or the mutants of the ANG N-806/-779. Southwestern blot analysis revealed that the labeled ANG N-806/-779 binds to two nuclear species of 43 and 35 kDa proteins. Western blot analysis, however, revealed that rabbit polyclonal antibodies against the 43 kDa CREB interacted with only the 43 kDa molecular species but not with the 35 kDa species.

Conclusion. These studies demonstrate that the stimulatory effect of isoproterenol on the expression of the ANG gene may be mediated, at least in part, via the interaction of the phosphorylated CREB and the CRE in the 5'-flanking region of the rat ANG gene. The novel 35 kDa nuclear protein that is immunologically different from the 43 kDa CREB may also play a role in the expression of the ANG gene.

Recent studies have shown that the mRNA components of the renin-angiotensin system (RAS), including angiotensinogen (ANG), renin, angiotensin-converting enzyme, and angiotensin II (Ang II) receptor (AT_1 receptor) are expressed in murine (mouse and rat) proximal tubular cell lines [1–4]. We have also demonstrated that the ANG mRNA is expressed in opossum kidney (OK) proximal tubular cells [5]. These studies demonstrate that the intrarenal Ang II is probably derived from the ANG, which is synthesized by the renal proximal tubular cells.

We have previously reported that isoproterenol stimulates the expression of the ANG gene in OK cells [6]. The effect of isoproterenol is mediated via the β_1-adrenoceptor and cAMP-dependent protein kinase A (PKA) pathway [6]. Our studies confirm the reports of Nakamura and Johns that the administration of atenolol (an inhibitor of the β_1-adrenoceptor) blocks the effect of low levels of renal nerve stimulation on the expression of the ANG mRNA levels in the rat kidney *in vivo* [7]. Our studies and those of Nakamura and Johns together indicate the presence of a functional relationship between the renal sympathetic nervous system and the

ation of local intrarenal RAS [6, 7]. Thus, the local
ation of renal Ang II may play an important role
physiology of the renal proximal tubular cells (that
dium and fluid reabsorption) [8–11].

e exact molecular mechanism(s) for the stimulatory
of isoproterenol on the expression of the ANG
in OK cells has not been defined. One possibility
oe that the addition of isoproterenol stimulates the
esis of intracellular cAMP, as β-adrenergic recep-
are linked through guanine nucleotide regulatory
ins to adenylyl cyclase located on the inner part of
lasma membrane of target cells [12]. The intracellu-
AMP then activates the cAMP-dependent protein
e AI and AII, which subsequently phosphorylate
uclear 43 kDa cAMP-responsive element binding
in (43 kDa CREB) [13]. The phosphorylated 43
CREB then interacts with the putative cAMP-
nsive element (CRE; that is, ANG N-806 to N-779
ining the motif of the CRE, TGACGTAC on the
otides N-795 to N-788) in the 5′-flanking region of
t ANG gene [14]. Finally, the bound 43 kDa CREB
ct with the preinitiation complex to enhance the
ssion of the ANG gene. This possibility is sup-
d by our previous studies, which show that the
ion of isoproterenol stimulates the synthesis of in-
llular cAMP in OK cells [6]. Furthermore, our re-
studies show that the addition of isoproterenol fur-
enhances the stimulatory effect of 43 kDa CREB
e expression of the ANG gene in OK cells [15].

e objective of our study was to investigate whether
imulatory effect of isoproterenol on the expression
rat ANG gene is mediated via the interaction of
3 kDa CREB with the putative CRE (that is, ANG
5/-779) of the rat ANG gene. Our studies demon-
that the effect of isoproterenol on the expression
ANG gene is mediated via the CRE of the ANG
The CRE of the rat ANG gene interacts with the
)a CREB and a novel 35 kDa nuclear protein from
K cells. This novel 35 kDa nuclear protein is immu-
ically different from the 43 kDa CREB, suggesting
he 43 kDa CREB and the novel 35 kDa nuclear
in play a regulatory role in the regulation of expres-
of the ANG gene in the kidney.

HODS

rials

e plasmid pRSV-Neo, containing the coding se-
ce for neomycin (Neo) with the Rous sarcoma virus
) enhancer/promoter sequence fused in the 5′-end
Neo gene, was a gift from Dr. Teresa Wang (De-
ient of Pathology, Stanford University, Stanford,
JSA). The plasmid pTKGH, containing the thymi-
kinase (TK) enhancer/promoter sequence fused to
′-end of the hGH gene was purchased from the

Nichols Institute of Diagnostics (La Jolla, CA, USA).
The plasmids containing the β-catalytic subunit of pro-
tein kinase A, pRSV/CATβ, or its mutant, pRSV/
CATβm, were obtained from Dr. Richard A. Maurer
(Oregon Health Sciences University, Portland, OR,
USA) [16].

The radioimmunoassay kit for human growth hor-
mone (RIA-hGH) was a gift from the NIDDK (National
Institutes of Health, Bethesda, MD, USA). The RIA
procedure has been described previously [14]. NIAMDD-
hGH-I-1 (AFP-4793 B) was used for both iodination and
as a hormone standard. The limit of sensitivity of the
assay was 0.1 ng/ml. The interassay and intra-assay coef-
ficients of variation were 12% ($N = 10$) and 10% ($N =$
10), respectively.

R(–)-isoproterenol (+)-bitartrate salt (β-adrenocep-
tor agonist), atenolol (β$_1$-adrenoceptor antagonist), ICI
118,551 (β$_2$-adrenoceptor antagonist), staurosporine [an
inhibitor of protein kinase C (PKC)], U73122 (an inhibi-
tor of phospholipase C and A$_2$), S(–)-propranolol hydro-
chloride (an inhibitor of β$_1$- and β$_2$-adrenoceptors),
8-Bromo-cAMP, forskolin, Rp-cAMP (an inhibitor of
the cAMP-dependent PKA I and PKA II) were all pur-
chased from Research Biochemicals Inc. (Natick, MA,
USA).

Restriction and modifying enzymes were purchased
either from Bethesda Research Laboratories (GIBCO-
BRL, Burlington, Ontario, Canada), Boehringer-Mann-
heim (Dorval, Quebec, Canada), or Pharmacia Inc. (Baie
d'Urfe, Quebec, Canada). Gamma-[^{32}P]-ATP (3000 Ci/
mmol) and Na^{125}I were purchased from Dupont, New
England Nuclear (Boston, MA, USA).

Calcium chloride was purchased from Mallinckrodt,
Inc. (Montreal, Quebec, Canada). Geneticin (G 418)
was purchased from Bethesda Research Laboratories
(GIBCO-BRL). Other molecular biology-grade reagents
were obtained either from Sigma Chemicals (St. Louis,
MO, USA), GIBCO-BRL, Boehringer-Mannheim, Pha-
rmacia Inc., or Promega-Fisher, Inc. (Montreal, Quebec,
Canada).

Oligonucleotides for the putative CRE of the rat ANG
gene (ANG-CRE), N-806 to N-779 (5′ AAG AGA TTA
CT*T GAC GTA C*TG GAT GCA A 3′) [14], mutant 1
(M1; 5′ AAG AGA TTA CT*T GAC TTA C*TG GAT
GCA A 3′), mutant 2 (M2; 5′ AAG AGA TTA CT*T
GAA TTA C*TG GAT GCA A 3′), mutant 3 (M3; 5′
AAG AGA TTA CT*T ATA TTA C*TG GAT GCA A
3′), the CRE of somatostatin gene (SOM-CRE, N-59 to
N-32, 5′GCC TCC TTG GC*T GAC GTC A*GA GAG
AGA 3′) [17], and the CRE of the tyrosine amino trans-
ferase gene (TAT-CRE, N-3660 to N-3634, 5′ CTG CAG
CTT *CTG CGT CAG* CGC CAG TAT 3′) [18] were
synthesized by Biosynthesis Inc. (Lewisville, TX, USA).

Rabbit polyclonal antibodies against the CREB

Rabbit polyclonal antibodies (RB#8) against the amino acid residues 137 to 150 of the 43 kDa CREB were raised in our laboratory. Briefly, the fragments of 43 kDa CREB (amino acid residues 137 to 150) conjugated to keyhole limpet hemocyanin (KLH) were purchased from Biosynthesis Inc. The conjugated peptides were used to immunize New Zealand white rabbits (Charles River Inc., St. Constant, Quebec, Canada) according to the procedure described previously for ovine placental lactogen [19].

Construction of fusion genes

To construct the fusion genes, pOGH (ANG N-806/-779/-53/+18), and its mutants (that is, M1, M2, and M3), the double-strand DNA fragment (ANG N-806/-779) with the Hind III enzyme restriction site on both the 5' and 3' ends was inserted upstream of the minimal promoter of the rat ANG gene (ANG N-53/+18) in the pOGH (ANG N-53/+18) [14], which had been previously digested with the restriction enzyme Hind III and alkaline phosphatase.

The sequence and orientation for the fusion genes were confirmed by dideoxy sequencing with SP6 primer (Promega-Fisher, Inc.) and restriction enzyme digestion mapping.

Cell culture

The opossum kidney (OK) proximal tubular cell line was obtained from the American Tissue Culture Collection (ATCC; Rockville, MD, USA). This cell line is derived from the kidney of a female American opossum and retains several properties of proximal tubular epithelial cells in culture [20, 21] and expresses a low level of ANG mRNA [5]. The culture conditions of the OK cells have been described previously [7, 22–24].

Opossum kidney cell stable transformants

Opossum kidney 95, OK 95/M1, OK 95/M2, OK 95/M3 cells are stable transformants with pOGH (ANG N-806/-779/N-53/+18) or its mutants and pRSV/Neo fusion genes cointegrated into OK cellular genomes. Similarly, OK 96, OK 96/M1, OK 96/M2, OK 96/M3 cells are stable transformants with pOGH (ANG N-806/-779/-53/+18) or its mutants and pRSV-CREB [16] cointegrated into the OK cellular genomes. The method for the selection of OK cell stable transformants with the high expression of the fusion gene was identical to the method described previously for OK 27 cells [6].

Effect of isoproterenol on the expression of the ANG-GH fusion genes in opossum kidney cell stable transformants

Opossum kidney cell stable transformants were plated at a density of 1 to 2×10^5 cells/well in six-well plates and incubated overnight in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS). Then cell growth was arrested by incubation in serum-free medium for 24 hours. Subsequently, various concentrations of isoproterenol (10^{-13} to 10^{-5} M) were added to the culture medium containing 1% depleted fetal bovine serum (dFBS) and incubated for 24 hours. At the end of the incubation period, media were collected and kept at $-20°C$ until assayed for IR-hGH.

To compare the inhibitory effect of propranolol, atenolol, ICI 118,551, Rp-cAMP, staurosporine, and U73122 on the expression of ANG-GH fusion gene in OK cell transformants, 10^{-7} M of the antagonists or inhibitors were cocultured with the isoproterenol (10^{-9} M) for 24 hours. At the end of the incubation period, media were collected and kept at $-20°C$ until assay for IR-hGH.

The depleted FBS was prepared by incubation with 1% activated charcoal and 1% AG 1 × 8 ion-exchange resin (Bio-Rad Laboratories, Richmond, CA, USA) for 16 hours or more at room temperature, as Samuels et al described [25]. This procedure removed endogenous steroid and thyroid hormones from the FBS as Samuels, Stanley and Shapiro demonstrated [25].

Opossum kidney cell nuclear extract preparation

The cellular extract was prepared from 20 plates (150 × 20 mm) of confluent OK cells according to the method of Hennighausen and Lubon with slight modifications [26]. Briefly, cells were harvested by trypsin/ethylenediaminetetraacetic acid (EDTA) digestion and washed once with phosphate-buffered saline (PBS) and once with hypotonic buffer containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM phenylmethylsulfonyl-fluoride (PMSF), and 0.5 mM dithiothreitol (DTT). Then cells were resuspended in the hypotonic buffer (that is, buffer volume to wet weight ratio, vol/wt 3:1) and left on ice for 10 minutes. The cell suspension was transferred to a Dounce (type B) glass homogenizer and was homogenized for 10 strokes. The cell homogenate was centrifuged at 25,000 × g (that is, 14,500 r.p.m. in a SW 24 rotor) for 20 minutes. The nuclear fraction (pellet) was resuspended in 0.2 ml of low-salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 20 mM KCl, 0.2 mM EDTA, 0.2 M PMSF, and 0.5 mM DTT). Subsequently, 0.8 ml of high-salt buffer containing 20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 1.2 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT were added to the cell nuclear suspension and homogenized. The cell nuclear extract was gently stirred at 4°C for 30 minutes and then centrifuged at 25,000 × g for 30 minutes. Finally, the supernatant was dialyzed against a large volume of dialysis buffer (20 mM HEPES, pH 7.0, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT) at 4°C for five hours with several changes of buffer. The dialyzed nuclear extract

is then centrifuged for 20 minutes in an Eppendorf
crocentrifuge at 4°C to remove the precipitate, and
e supernatant (nuclear extract) was stored frozen in
uid nitrogen or at –80°C in aliquots. The protein con-
ntration of the extract was determined by the Bio-
d protein assay using bovine serum albumin (BSA)
standard.

el mobility shift assay

The DNA fragment, ANG N-806 to N-779 was 5′-end
eled with gamma ^{32}P-ATP using T$_4$ polynucleotide
lase. OK cell nuclear proteins (10 μg) or BSA (10 μg)
the presence of 0.3 units of poly (dI-dC) in 20 mM
is-glycine, pH 7.6, and 1 mM EDTA were incubated
: 30 minutes at room temperature. Then the 5′-labeled
obe (0.1 pmol) was added and further incubated for
minutes at room temperature. After chilling on ice,
e mixture was run on a 8% nondenaturing polyacryl-
lide gel and exposed for autoradiography.

In competition assays, a 200-fold excess of unlabeled
VA fragments was added to the reaction mixture and
:ubated for 30 minutes at room temperature prior to
e incubation with the labeled probe.

uthwestern blot

Southwestern blot analysis was performed according
the procedures of Kwast-Welfeld et al, with slight
odifications [27]. Briefly, OK cell nuclear proteins (50
200 μg) ANG N-806/-779/-53/+18) were resolved on a
o 20% gradient polyacrylamide gel containing sodium
decyl sulfate (PAGE-SDS) [28] and were then electro-
insferred onto a nitrocellulose membrane (0.45 mM;
hleicher & Schuell, Keene, NH, USA). The membrane
is incubated with 10% nonfat milk proteins in a binding
ffer containing 10 mM HEPES, pH 7.0, 10 mM MgCl,
mM NaCl, 0.25 mM EDTA, and 2.5% glycerol for one
ur at 4°C for overnight. The membrane was washed,
-dried, and exposed for autoradiography.

estern blot

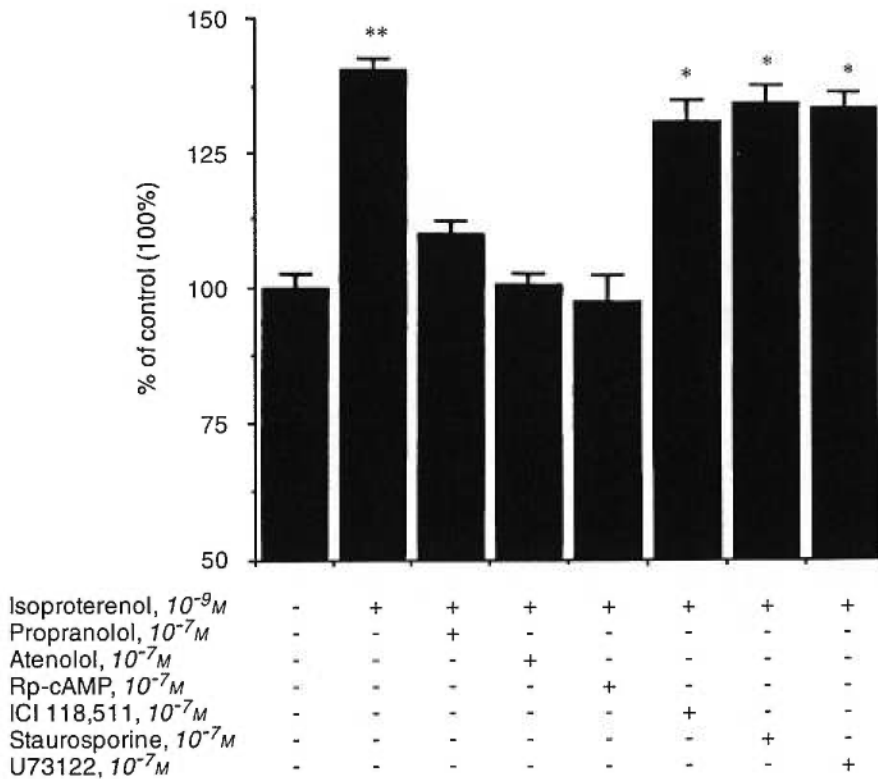Western blot analysis was also performed to analyze
e OK cellular nuclear proteins by employing rabbit
lyclonal antibodies (Rb#8) against the amino acid resi-
es 137 to 150 of the 43 kDa CREB, Bio-Rad's antirab-
horseradish peroxidase (HRP) conjugates, and the
idin HRP conjugates according to the protocol of the
pplier (Bio-Rad Lab).

itistical analysis

The experiments were performed at least three to four
es in triplicate. The data were analyzed with Student's
est or analysis of variance. A probability level of P
s than 0.05 was regarded as significant.
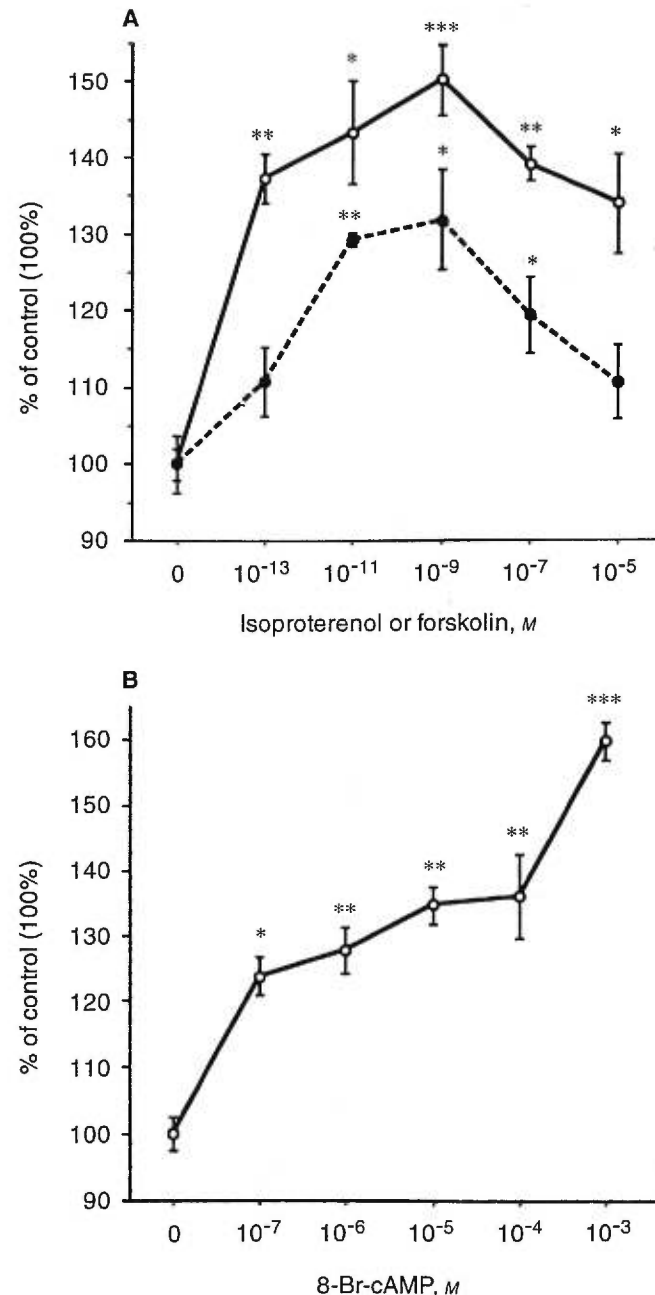
Fig. 1. Effect of isoproterenol, forskolin, or 8-Bromo-cAMP on the expression of pOGH (ANG N-806/-779/-53/+18) in OK 95 cells. Cells were incubated for up to 24 hours in the presence of various concentrations of isoproterenol or forskolin (10^{-13} to 10^{-5} M; *A*) or 8-Bromo-cAMP (10^{-7} M to 10^{-3} M; *B*). Media were harvested after 24 hours of incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the absence of isoproterenol or forskolin or 8-Bromo-cAMP is considered as the control level (that is, 1.65 ± 0.03 ng/ml and 1.79 ± 0.07 ng/ml of IR-hGH in A and B, respectively). Each point represents the mean ± SD of three determinations (*$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.005$). Similar results were obtained from three other experiments.

	−	+	+	+	+	+	+	+
Isoproterenol, 10^{-9} M	−	+	+	+	+	+	+	+
Propranolol, 10^{-7} M	−	−	+	−	−	−	−	−
Atenolol, 10^{-7} M	−	−	−	+	−	−	−	−
Rp-cAMP, 10^{-7} M	−	−	−	−	+	−	−	−
ICI 118,511, 10^{-7} M	−	−	−	−	−	+	−	−
Staurosporine, 10^{-7} M	−	−	−	−	−	−	+	−
U73122, 10^{-7} M	−	−	−	−	−	−	−	+

Fig. 2. **Inhibitory effect of adrenoceptor antagonists, Rp-cAMP, staurosporine, or U73122 on the expression of pOGH (ANG N-809/-779/-53/+18) in OK 95 cells stimulated by isoproterenol (10^{-9} M).** OK 95 cells were incubated for up to 24 hours in the presence of isoproterenol (10^{-9} M) and 10^{-7} M of various adrenoceptor antagonists, Rp-cAMP, staurosporine, or U73122. Media were harvested and assayed for the level of IR-hGH. The concentration of IR-hGH in the absence of isoproterenol or drugs represents the control level (that is, 1.45 ± 0.06 ng/ml of IR-hGH). Results were expressed as the mean $\pm$ SD of three determinations (*$P \leq 0.05$ and **$P \leq 0.01$). Similar results were obtained from two other experiments.

RESULTS

Effect of isoproterenol on the expression of ANG-GH fusion genes and pTKGH in opossum kidney cells

Figure 1 shows the expression of the pOGH (ANG N-806/-779/-53/+18) in OK 95 cells in the presence of various concentrations of isoproterenol (10^{-13} to 10^{-5} M), forskolin (10^{-13} to 10^{-5} M) and 8-Bromo-cAMP (10^{-7} to 10^{-3} M). The maximal stimulation of expression of the pOGH (ANG N-806/-779/-53/+18) was found with 10^{-9} to 10^{-7} M isoproterenol and forskolin (Fig. 1A). The stimulatory effect of isoproterenol was attenuated at concentrations greater than 10^{-7} M (that is, 10^{-5} M). On the other hand, the maximal stimulation of expression of the pOGH (ANG N-806/-779/-53/+18) was found with 10^{-3} M 8-Bromo-cAMP (Fig. 1B).

The inhibitory effect of various adrenoceptor antagonists and inhibitors of PKA or PKC on the expression of the pOGH (ANG N-806/-779/-53/+18) in OK 95 cells stimulated by isoproterenol is shown in Figure 2. Propranolol, Rp-cAMP (an inhibitor of the PKA), and atenolol at 10^{-7} completely inhibited the stimulatory effect of isoproterenol ($P = 0.01$), whereas staurosporine, U73122, and ICI 118,551 had no effect. These studies confirm that the effect of isoproterenol is mediated via the β_1-adrenoceptor and the PKA signal transduction pathway. We have used 10^{-7} M of propranolol, Rp-cAMP, atenolol, and ICI 118,551 to block the effect of isoproter-

enol, because we had reported previously that this dose was the most effective dose to block the stimulatory effect of isoproterenol [6]. Similarly, 10^{-7} M of staurosporine and U73122 was used because this was the most effective dose to block the stimulatory effect of iodoclonidine on the expression of the ANG-GH fusion gene [23].

Figure 3 shows that the addition of isoproterenol (10^{-9} M) stimulated the expression of the pOGH (ANG N-806/-779/-53/+18) in OK 95 cells, but had no stimulatory effect on the expression of the M1, M2, and M3 of pOGH (ANG N-806/-779/-53/+18) in OK 95/M1, OK 95/M2, and OK 95/M3 cells compared with the control (that is, without the addition of isoproterenol), respectively. Similarly, the addition of isoproterenol (10^{-9} M) stimulated the expression of the pOGH (ANG N-806/-779/-53/+18) in OK 96 but had no stimulatory effect on the expression of M1, M2, and M3 of the pOGH (ANG N-806/-779/-53/+18) in OK 96/M1, OK 96/M2, and OK 96/M3 cells, respectively (Fig. 3B).

These studies demonstrate that the mutation of the nucleotides in TGACGTAC completely abolished the response to the addition of isoproterenol, suggesting that the DNA sequence TGACGTAC (N-795 to N-788) is the motif of the CRE of the rat ANG gene.

Figure 4 shows that the addition of propranolol (10^{-7} M), Rp-cAMP (10^{-7} M), atenolol (10^{-7} M) also inhibited the stimulatory effect of isoproterenol (10^{-9} M) on the expression of pOGH (ANG N-806/-779/-53/+18) in
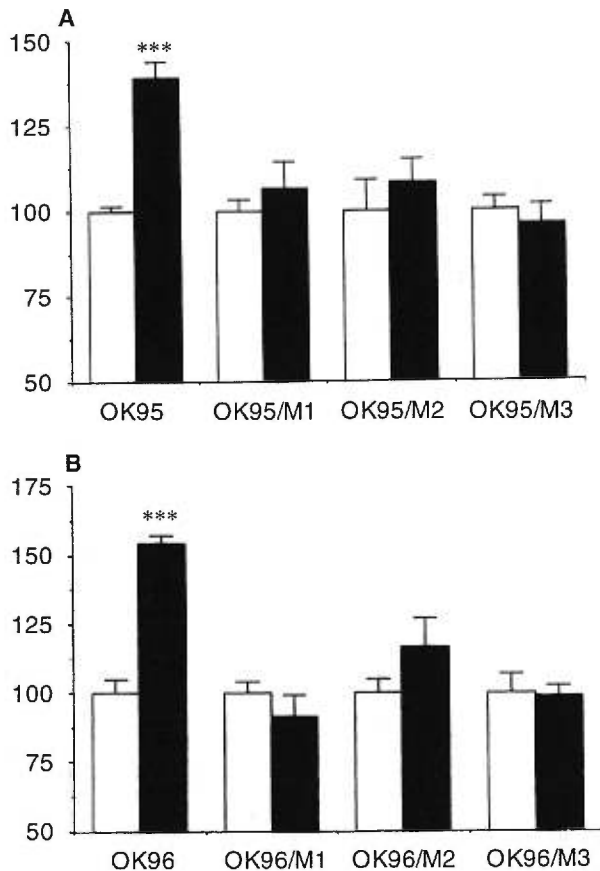
3. Effect of isoproterenol on the expression of pOGH (ANG 06/-779/-53/+18) and its mutants in OK 95, OK 95/M1, OK 95/M2 OK 95/M3 cells (A) or in OK 96, OK 96/M1, OK 96/M2, and OK /13 cells (B). Cells were incubated for up to 24 hours in the presence soproterenol (10^{-9} M). Media were harvested after 24 hours of ibation and were assayed for IR-hGH. The concentration of IR-H in the absence of isoproterenol represents the control level (that)K 95, 3.8 ± 0.2 ng/ml; OK 95/M1, 3.9 ± 0.01 ng/ml; OK 95/M2, ± 0.05 ng/ml; OK 95/M3, 3.1 ± 0.14 ng/ml; OK 96, 7.8 ± 0.1 ng/ OK 96/M1, 4.1 ± 0.2 ng/ml; OK 96/M2, 3.2 ± 0.1 ng/ml; OK 96/ 2.7 ± 0.1 ng/ml of IR-hGH). Each point represents the mean $\pm$)f three determinations (***$P \leq 0.005$). The left blank bar repre-s the control level. The second solid bar represents the incubation lium in the presence of 10^{-9} M isoproterenol. Similar results were ained from four other experiments.

⟨ 96 cells. The addition of staurosporine (10^{-7} M), 3122 (10^{-7} M), and ICI 118,551 (10^{-7} M) had no inhibi-y effect. These studies further demonstrated that the nulatory effect of isoproterenol on the expression of ⟩GH (ANG N-806/-779/-53/+18) in OK 96 cells is me-.ted via the β_1-adrenoceptor and the PKA signal trans-:tion pathway.

fect of pRSV/Catβ and its mutant, pRSV/CATβm, the expression of ANG-GH fusion genes in ossum kidney cells

Figure 5 shows that the transient transfection of the ismid containing the β-catalytic subunit of PKA,

pRSV/CATβ (2 to 10 μg DNA) per well stimulated the expression of pOGH (ANG N-806/-779/-53/+18) in OK 95 cells. It appears that the maximal stimulation was found with 5 μg pRSV/CATβ. At doses greater than 5 μg pRSV/CATβ, the expression of pOGH (ANG N-806/-779/-53/+18) diminished. No significant stimula-tion of expression of the pOGH (ANG N-806/-779/-53/ +18) was observed with the mutant of the β-catalytic subunit of PKA, pRSV/CATβm (2 to 10 μg).

Figure 6 shows that the transient transfection of pRSV/ CATβ (5 μg) also stimulated the expression of pOGH (ANG N-806/-779/-53/+18) in OK 96 cells with the pRSV/CREB cointegrated into the genome. The plasmid pGEM-3 or pRSV/CATβm had no effect on the expres-sion of the M1, M2, and M3 of pOGH ANG N-806/ -779/-53/+18) in OK 96/M1, OK 96/M2, and OK 96/M3 cells, respectively.

These studies demonstrated that the activation of PKA stimulated the expression of the ANG gene and mutation of the nucleotides in the CRE- motif, and ANG N-795/-788 abolished the responsiveness to the PKA sig-nal transduction pathway. These data support the view that the intact CRE motif (that is, ANG N-795/-788) is essential for the PKA and CREB action.
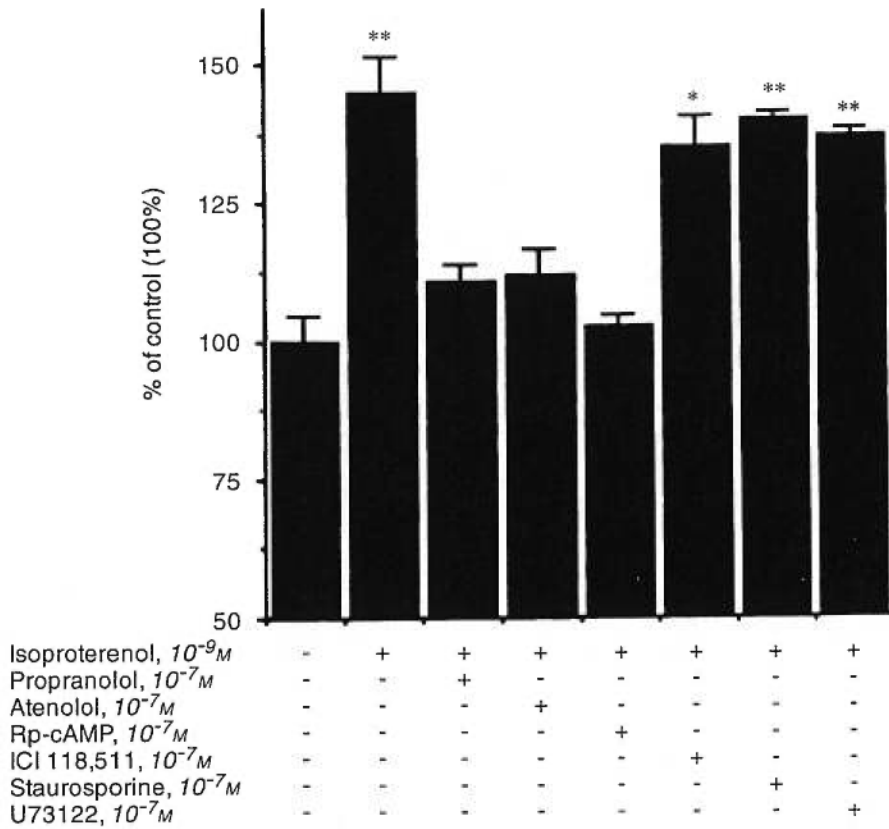
Gel mobility shift assays

The interaction of the putative CRE of the rat ANG gene (ANG-CRE, N-806 to N-779) with OK cellular nuclear proteins was examined by gel mobility shift assays.

Figure 7 shows the binding of the OK cellular extract nuclear proteins with the labeled ANG-CRE. A single specific band appeared with retarded mobility. No slow migrating band was observed when the labeled DNA was incubated with BSA. Furthermore, the binding of labeled ANG N-806/-779 to the nuclear protein(s) was displaced by the unlabeled DNA fragment ANG N-806/ -779, but not by the unlabeled SOM-CRE and the unla-beled mutant 2 and 3 of the ANG N-806/-779 (that is, M2 and M3). The unlabeled TAT-CRE and the M1 of the ANG N-806/-779 were weakly displaced the labeled ANG N-806/-779. These data further confirm that the intact CRE-motif is important for the binding with the OK cellular nuclear protein(s).

Southwestern and Western blot analysis

The interaction of the ANG-CRE (ANG N-806/-779) with nuclear proteins was further examined by South-western blot analysis, as shown in Figure 8A. The labeled ANG N-806/-779 interacts with one major and one minor protein band. The apparent molecular weights of the major and minor protein were 43 and 35 kDa, respec-tively.

After Southwestern blot analysis, the same membrane was blotted with the polyclonal antibodies (Rb #8)

Isoproterenol, 10^{-9}M	-	+	+	+	+	+	+	+
Propranolol, 10^{-7}M	-	-	+	-	-	-	-	-
Atenolol, 10^{-7}M	-	-	-	+	-	-	-	-
Rp-cAMP, 10^{-7}M	-	-	-	-	+	-	-	-
ICI 118,511, 10^{-7}M	-	-	-	-	-	+	-	-
Staurosporine, 10^{-7}M	-	-	-	-	-	-	+	-
U73122, 10^{-7}M	-	-	-	-	-	-	-	+

Fig. 4. Inhibitory effect of adrenoceptor antagonists or Rp-cAMP or U73122 or staurosporine on the expression of pOGH (ANG N-806/-779/-53/+18) in OK 96 cells stimulated by isoproterenol (10^{-9} M). OK 96 cells were incubated for up to 24 hours in the presence of isoproterenol (10^{-9} M) and 10^{-7} M of propranolol or atenolol or ICI 118,551 or Rp-cAMP or U73122 or staurosporine. Media were harvested and assayed for the level of IR-hGH. The concentration of IR-hGH in the absence of drugs represents the control level (that is, 9.6 ± 0.03 ng/ml of IR-hGH). Results are expressed as the mean $\pm$ SD of three determinations (*$P \leq 0.05$ and **$P \leq 0.01$). Similar results were obtained from two other experiments.
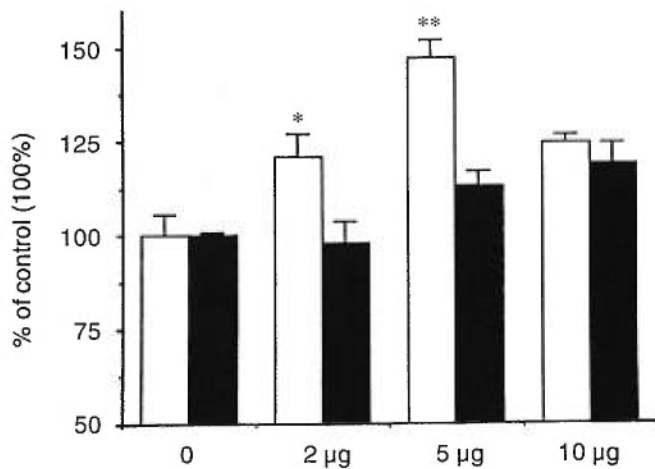
Fig. 5. Basal expression of the pOGH (ANG N-806/-779/-53/+18) in OK 95 cells when transiently transfected with different concentrations of plasmid containing the cDNA for the β-catalytic subunit of protein kinase A (pRSV/CATβ) or with the mutant of the β-catalytic subunit of protein kinase A (pRSV/CATβm) as control. The concentration of IR-hGH in the medium of cells (2×10^5 M cells per well) without transfection with pRSV/CATβ or pRSV/CATβm is considered as the control level (100%). These data were normalized with the DNA transfection efficiency by cotransfection with 2 μg of pTKCAT as internal controls. Results are expressed as the mean $\pm$ SD of three determinations (triplicate). The blank bar represents those cells transfected with pRSV/CATβ and the solid bar those cells transfected with pRSV/CATβm. Similar results were obtained from two independent experiments.
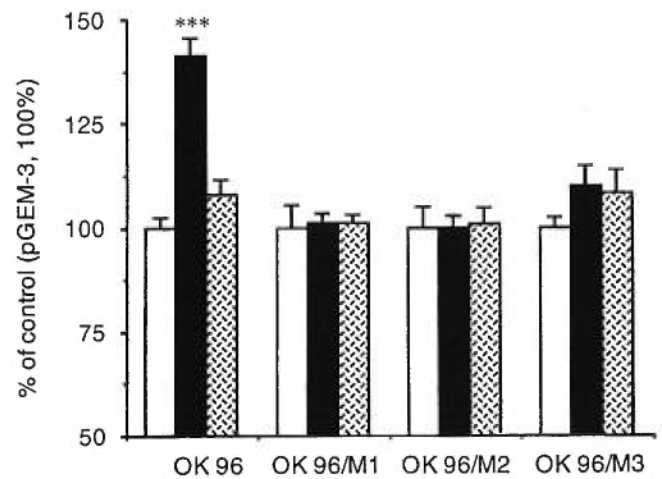
Fig. 6. Effect of transient transfection of the plasmid, pGEM-3 or pRSV/CATβ or pRSV/CATβm on the expression of pOGH (ANG N-806/-779/-53/+ 18) in OK 96 cells with the pRSV/CREB cointegrated in the genome. The levels of transcriptional activity of pOGH (ANG N-806/-779/-53/+18) were quantitated by the amount of IR-hGH in the medium assayed by RIA-hGH. The concentration of IR-hGH in the medium of cells (1×10^5 cells) transfected with pGEM-3 is considered to be the control level (100%). The data were normalized with the DNA transfection efficiency by cotransfection with 2 μg of pTKCAT as internal control. The blank bar represents the cells transfected with pGEM-3. The middle solid bar represents the cells transfected with pRSV/CATβ. The right stippled bar represents the cells transfected with pRSV/CATβm (*$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.005$).
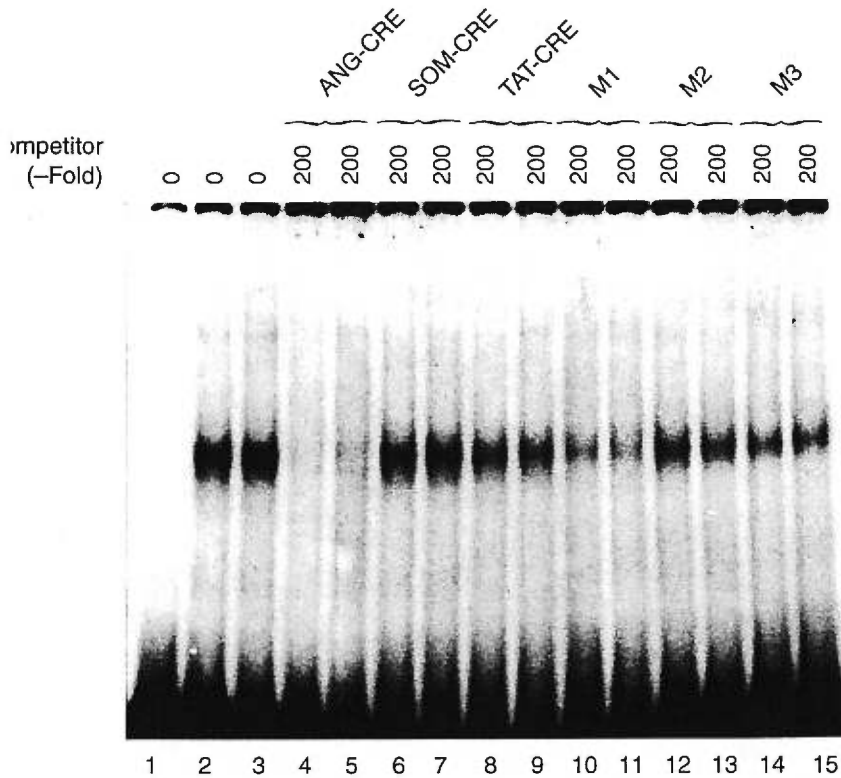
Fig. 7. Gel mobility shift assay of the radioactive-labeled DNA fragment, ANG-CRE (ANG N-806/-779) with the OK cellular nuclear protein(s). The labeled DNA probe (0.1 pmol) was incubated with BSA (10 μg; lane 1) or cellular nuclear protein(s) (10 μg each; lanes 2 through 15) in the presence of 0.03 units of poly dI-dC. Competition with a 200-fold excess of unlabeled ANG-CRE, SOM-CRE, TAT-CRE, M1, M2, and M3 are shown in lanes 4 and 5, lanes 6 and 7, lanes 8 and 9, lanes 10 and 11, lanes 12 and 13, and lanes 14 and 15, respectively. Similar observations were obtained from two other experiments.

.inst the amino acid residues 137 to 150 of the 43 a CREB (Fig. 8B). It is apparent that the polyclonal ibodies interact with the 43 kDa CREB protein in OK cellular nuclear extract. The antibodies did not :ract with the 35 kDa nuclear protein. These studies nonstrate that the 35 kDa nuclear protein is immuno- ically different from the 43 kDa CREB.

;CUSSION

'unctional β-adrenoceptors along the proximal tubule he rat kidney and on the rabbit proximal tubules have :n reported [29–33], suggesting that β-adrenoceptors y contribute a significant role in catecholamine-stimu- :d electrolyte transport. Our previous studies showed t the addition of isoproterenol alone not only stimu- :d the expression of pOGH (ANG N-1498/+18) in : 27 cells in a dose-dependent manner [6], but also lanced the stimulatory effect of 43 kDa CREB on the iression of pOGH (ANG N-1498/+18) in OK 27 cells]. These studies suggest that the molecular mecha- n(s) of action of the isoproterenol, at least in part, y be mediated via the interaction of the phosphory- :d CREB with the putative CRE in the 5′-flanking ion of the rat ANG gene. The nucleotide sequence the putative CRE of the rat ANG gene and other .es was illustrated in Figure 9.
)ur current studies showed that the addition of isopro-

terenol, forskolin, or 8-Bromo-cAMP alone stimulated the expression of pOGH (ANG N-806/-779/-53/+18) in OK 95 cells in a dose-dependent manner (Fig. 1). These studies demonstrate that the DNA fragment, ANG N-806/-779 is the functional CRE of the rat ANG gene.

Currently, we have no explanation for the attenuation of the increase of fusion gene expression in OK 95 stimu- lated by isoproterenol or forskolin at 10^{-7} M or greater. One possible explanation may be that high concentra- tions of isoproterenol or forskolin may desensitize the β-adrenoceptors or adenyl cyclase system, respectively. Indeed, studies by Hausdorff et al have shown that β-adrenoceptors are subject to desensitization by its own agonists [31]. Moreover, studies by Cheng et al demon- strated that isoproterenol at concentrations of 10^{-8} M to 10^{-4} M had no stimulatory effect on cAMP formation in OK cells [32].

The stimulatory effect of isoproterenol on the expres- sion of the pOGH (ANG N-806/-779/-53/+18) in OK 95 cells was inhibited in the presence of propranolol, Rp- cAMP, or atenolol but not in the presence of stauro- sporine, U73122, or ICI 118,551 (Fig. 2). Moreover, the addition of isoproterenol stimulated the expression of the pOGH (ANG N-806/-779/-53/+18) in OK 95 (Fig. 3A) and OK 96 (Fig. 3B) but had no stimulatory effect on the expression of M1, M2, and M3 of the pOGH (ANG N-806/-779/-53/+18) in OK 95/M1, OK 95/M2, and OK 95/M3 cells (Fig. 3A) or in OK 96/M1, OK 96/
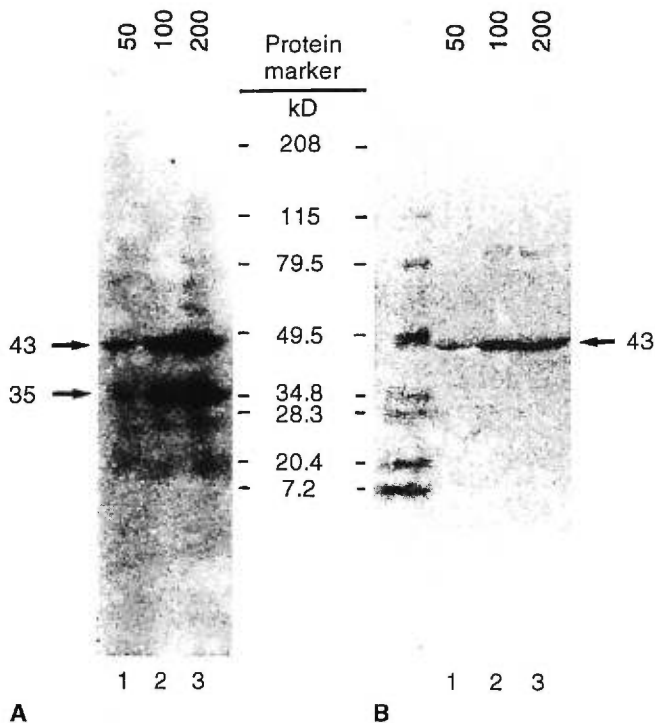
Fig. 8. Southwestern and Western blot analysis of the 43 kDa CREB from the OK cellular nuclear protein(s). (*A*) Southwestern blot analysis: 50, 100, or 200 μg of OK cellular nuclear protein(s) were resolved on a gradient (4 to 20%) PAGE-SDS, transferred onto a nitrocellulose membrane, hybridized with radioactive ANG-CRE (ANG N-806/-779), washed, and subjected to autoradiography. (*B*) Western blot analysis: After Southwestern blot analysis, the same nitrocellulose membrane was blotted with rabbit polyclonal antibodies (Rb #8) against the midregion (amino acid residues 137 to 150) of the 43 kDa-CREB. Bio-Rad's prestained SDS-PAGE protein standard (broad range) was used as molecular weight markers. Similar results were obtained from another experiment.

M2, and OK 96/M3 (Fig. 3B). These studies indicate strongly that the DNA fragment ANG N-806/-779 contains the functional CRE of the rat ANG gene.

We have observed that the level of expression of the fusion gene in OK 96 was twofold greater than in OK 95 cells (that is, 7.8 ± 0.1 ng/ml vs. 3.8 ± 0.2 ng/ml, $P \leq 0.005$). These data indicate that the overexpression of the 43 kDa CREB enhanced the expression of the fusion gene. Mutation of the CRE in OK 96 M1, OK 96 M2, and OK 96 M3, however, attenuated the expression of the fusion gene at levels comparable to those of OK 95. These studies further support the notion that the ANG N-806/-779 contains the functional CRE of the rat ANG gene.

Again, the stimulatory effect of isoproterenol in OK 96 cells was inhibited in the presence of propranolol, Rp-cAMP, or atenolol but not in the presence of staurosporine, U73122, or ICI 118,511 (Fig. 4). These studies further support the notion that the enhancing effect of isoproterenol on the expression of the ANG gene is

mediated via the increase of intracellular cAMP, which then activates the PKA and nuclear CREB to interact with the CRE of the rat ANG gene.

Studies have shown that the CREB could be phosphorylated directly by the catalytic subunit of PKA [33–36]. Our current studies show that the transient transfection of the plasmid containing the β-catalytic subunit of the PKA, pRSV/CATβ, directly stimulated the expression of the pOGH (ANG N-806/-779/-53/+18) in OK 95 cells, whereas the transient transfection of the mutant of the β-catalytic subunit of PKA, pRSV/CATβm, had no effect (Fig. 5). Furthermore, transient transfection of pRSV/CATβ did not stimulate the expression of the M1, M2, and M3 of pOGH (ANG N-806/-779/-53/+18) in OK 96/M1, OK 96/M2, and OK 96/M3 cells, respectively (Fig. 6). These data provide additional evidence that the activation of the PKA pathway stimulated the expression of the ANG gene and that the intact CRE motif is essential for the PKA signal transduction pathway.

At higher concentrations of pRSV/CATβ (that is, more than 5 μg; Fig. 5), the effect of pRSV/CATβ was inhibited. Currently, we do not know the reasons for this observation. One possible explanation may be that large amount of DNA transfected into OK cells might exhaust the limited amount of cellular transcriptional factors. Nevertheless, more studies are warranted to clarify this observation.

To investigate whether ANG N-806/-779 interacts with the nuclear proteins in OK cells, we performed gel mobility shift assays. Our gel mobility shift assays showed that the labeled ANG-CRE interacted with the OK cellular nuclear protein(s) (Fig. 7). The addition of the unlabeled ANG-CRE effectively displaced the labeled ANG-CRE at 200-fold excess of unlabeled DNA, whereas the unlabeled SOM-CRE and mutants (M2 and M3) of the ANG N-806/-779 were not effective in displacing the labeled ANG-CRE. The addition of the unlabeled mutant of the ANG-CRE (that is, M1) and TAT-CRE weakly displaced the labeled DNA. These studies demonstrate that the intact CRE-motif of the ANG-CRE is essential for the binding to the OK cellular nuclear protein(s). Currently, we do not know why M1 (that is, at 200-fold excess) weakly displaced the labeled ANG-CRE. One possible explanation may be that the nucleotide "G" (N-791) is not the "critical" nucleotide for the binding with a nuclear protein(s).

The reason(s) for the lack of displacement by the unlabeled SOM-CRE and TAT-CRE (Fig. 7) is not clear. One possible explanation may be that the ANG-CRE binds with a nuclear protein(s) other than the 43 kDa CREB. Another possible explanation may be that the binding affinity of 43 kDa CREB to the ANG-CRE is lower than another nuclear protein(s) to ANG-CRE. Indeed, our Southwestern blot experiments showed that the labeled ANG N-806/-779 binds to the two OK cellu-

		CRE motif		
3 (N-806)[14]	A A G A G A T T A C T	T G A C G T A C	T G G A T G C A A	(N-779)
S (N-59)[17]	G C C T C C T T G G C	T G A C G T C A	G A G A G A G A G	(N-32)
(N-3660)[18]	C T G C A G C T T	C T G C G T C A	G C G C C A G T A	(N-3634)
3 (Mutant 1)	A A G A G A T T A C T	T G A C **T T** A C	T G G A T G C A A	
3 (Mutant 2)	A A G A G A T T A C T	T G A **A T** T A C	T G G A T G C A A	
3 (Mutant 3)	A A G A G A T T A C T	**T A T A T** T A C	T G G A T G C A A	

9. A comparison of the DNA nucleotide sequence of the cAMP-responsive element (CRE) in the rat angiotensinogen (ANG) gene, ttostatin (SMS) gene, and tyrosine aminotransferase (TAT) gene. The mutated nucleotide(s) in the CRE motif is indicated by a bold letter(s).

nuclear proteins with an apparent molecular weight 3 and 35 kDa (Fig. 8A). Our Western blot analysis he OK cellular nuclear proteins, however, revealed the polyclonal antibodies against the midregion ino acid residues 137 to 150) of the 43 kDa CREB racted with the 43 kDa CREB but not with the 35 ι molecular species (Fig. 8B). These studies demon- te that the 35 kDa nuclear protein is immunologically erent from the 43 kDa CREB.

he exact molecular structure of the 35 kDa nuclear ein is unknown to date. The apparent molecular ght of this nuclear protein is not similar to either Ξ-BP2 or ATF-1 or CREM-related proteins, as er and Habener discussed [37]. Our studies suggest the 35 kDa protein might be a novel CREB-like ein. The physiological role(s) of this 35 kDa nuclear ein is unknown. Experiments such as cloning and :ession of the 35 kDa proteins are definitely war- ed to demonstrate the biological activity of the 35 ι protein.

ι summary, these studies demonstrate that isoproter- ΄ stimulated the expression of the rat ANG gene and iated, at least in part, via the β₁-adrenoceptor, PKA, the interaction of the CREB with the CRE in the anking region of the rat ANG gene. Our studies also onstrate that the CRE ANG N-806/-779 interacts two OK cellular nuclear proteins with an apparent ecular weight of 43 and 35 kDa. It appears that the Da molecular species is immunologically similar to 43 kDa CREB, as reported by Gonzalez and Mont- y [34], whereas the 35 kDa nuclear protein is immu- ιgically different from the 43 kDa CREB. These ob- ations raise the possibility that the novel 35 kDa ear protein may play a role in mediating the effect oproterenol on the expression of the ANG gene in kidney. Finally, our studies suggest that the activation ιe β-adrenoceptor will stimulate the expression of ANG gene in the renal proximal tubule and may ase the formation of local renal Ang II.

KNOWLEDGMENTS

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APPENDIX

Abbreviations used in this article are: ANG, angiotensinogen; AT₁, angiotensin II receptor type 1; CATβ, catalytic subunit β; CRE, cAMP-responsive element; CREB, CRE binding protein; dFBS, depleted fetal bovine serum; G418, geneticin; kDa, kiloDalton; hGH, human growth hormone; OK, opossum kidney; PKA, protein kinase A; RAS, renin-angiotensin system; RSV, Rous sarcoma virus; SOM, somatostatin; TAT, tyrosine amino transferase; TK, thymidine kinase.

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Appendix V : Zhang SL, Filep JG, Hohman TC, Tang SS, Ingelfinger JR, Chan JSD: Molecular mechanism(s) of glucose action on angiotensinogen gene expression in rat proximal tubular cells. *Kidney Int.* 55: 454-464, 1999.

/Iolecular mechanisms of glucose action on angiotensinogen ;ene expression in rat proximal tubular cells

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Iolecular mechanisms of glucose action on angiotensinogen ₁ne expression in rat proximal tubular cells.

Background. Clinical studies have shown that the angioten-n-converting enzyme (ACE) inhibitors or angiotensin II (Ang) receptor antagonists decrease proteinuria and slow the pro-:ession of nephropathy in diabetes, indicating that Ang II lays an important role in the development of nephropathy. /e have previously reported that high levels of glucose stimu-.te the expression of rat angiotensinogen (ANG) gene in opos-ım kidney (OK) proximal tubular cells. We hypothesized that ıe stimulatory effect of D(+)-glucose on the expression of the .NG gene in kidney proximal tubular cells is mediated via *de ovo* synthesis of diacylglycerol (DAG) and the protein kinase : (PKC) signal transduction pathway.

Methods. Immortalized rat proximal tubular cells (IRPTCs) ere cultured in monolayer. The stimulatory effect of glucose n the activation of polyol pathway and PKC signal transduc-on pathway in IRPTCs was determined. The immunoreactive ıt ANG (IR-rANG) in the culture medium and the cellular .NG mRNA were measured with a specific radioimmunoassay nd a reverse transcription-polymerase chain reaction assay, ₂spectively.

Results. D(+)-glucose (25 mM) markedly increased the intra-₂llular levels of sorbitol, fructose, DAG, and PKC activity as 'ell as the expression of IR-rANG and ANG mRNA in RPTCs. These stimulatory effects of D(+)-glucose (25 mM) 'ere blocked by an inhibitor of aldose reductase, Tolrestat. KC inhibitors also inhibited the stimulatory effect of D(+)-lucose (25 mM) on the expression of the IR-rANG in IRPTCs. 'he addition of phorbol 12-myristate 13-acetate further en-anced the stimulatory effect of D(+)-glucose (25 mM) on the xpression of the IR-rANG in IRPTCs and blocked the inhibi-ɔry effect of Tolrestat.

Conclusion. These studies suggest that the stimulatory effect f a high level of D(+)-glucose (25 mM) on the expression of ıe ANG gene in IRPTCs is mediated, at least in part, via ıe *de novo* synthesis of DAG, an activator of PKC signal ransduction pathway.

Hyperglycemia has been implicated in the etiology of diabetic nephropathy [1]. However, the molecular mech-anisms linking hyperglycemia to the development of ne-phropathy are not completely understood. Clinical stud-ies have shown that inhibitors of angiotensin-converting enzyme (ACE) or angiotensin II (Ang II) receptor an-tagonists decrease proteinuria and slow the progression of nephropathy in diabetic patients [2–9], indicating that Ang II plays an important role in this intractable dis-order.

Indeed, *in vitro* studies have shown that high levels of glucose or Ang II stimulate the proliferation of mesan-gial cells and the expression of the mRNA of extracellu-lar matrix proteins [10–13]. Similarly, incubation of mu-rine proximal tubular cells in a high glucose medium or in the presence of Ang II ($\geq 10^{-8}$ M) induces cellular hypertrophy and extracellular matrix protein synthesis [14–21]. Although the results from these studies indicate that elevated glucose and/or Ang II concentrations may be responsible for the development of diabetic nephrop-athy, the mechanisms are poorly understood but may involve increased polyol pathway or protein kinase C (PKC) activity. Indeed, inhibitors of the polyol pathway, as well as protein kinase C antagonists, have prevented glomerular hyperfiltration and have increased urinary albumin excretion in diabetic rats [22, 23].

The mRNA components of the renin-angiotensin sys-tem (RAS), including angiotensinogen (ANG), renin, ACE, and Ang II receptor (AT_1-receptor), are expressed in murine (mouse and rat) immortalized proximal tubu-lar cell lines [24–27]. We have recently reported that the ANG protein is secreted from rat immortalized proximal tubular cells as measured by a specific radioimmunoassay for rat ANG (RIA-rANG) [28], providing evidence that the intrarenal Ang II is derived from the ANG that is synthesized within the renal proximal tubular cells.

In these studies, we have characterized the effect of glucose on the expression of the ANG gene in immortal-

rat proximal tubular cells (IRPTCs). Our results ? that the expression of the ANG in IRPTCs was ılated by high concentrations (25 mM) of D(+)-glu- but not by D-mannitol, L-glucose or 2-deoxy-D-glu- . Furthermore, the addition of Tolrestat (an inhibitor of Idose reductase) or staurosporine (an inhibitor of ?) or H-7 (an inhibitor of PKC) blocked the stimula- effect of glucose. In contrast, the addition of phorbol ıyristate 13-acetate (PMA; a PKC activator) en- :ed the stimulatory effect of glucose and blocked nhibitory effect of Tolrestat. Finally, a high glucose :entration in the culture media increased intracellu- ːvels of sorbitol, fructose, diacylglycerol (DAG), and ː activity, as well as the expression of ANG mRNA ₹PTCs. All of these effects of glucose were blocked ʻolrestat.

ʧHODS

ʻ+)-glucose, L-glucose, D-mannitol, 2-deoxy-D-glu- ʾ, and staurosporine (an inhibitor of PKC) were pur- ːed from Sigma Chemical (St. Louis, MO, USA). H-7 inhibitor of PKC) was purchased from Research ːhemicals Inc. (Natick, MA, USA). Tolrestat (an in- tor of aldose reductase) was a gift from Wyeth- ːrst Research (Princeton, NJ, USA).

ːamma-[^{32}P-ATP] (3000 Ci/mol) and Na^{125}I were pur- ːed from Dupont, New England Nuclear (Boston, ., USA). Restriction and modifying enzymes were ːhased from either Life Technologies Inc. (Burl- ʼon, Ontario, Canada), Boehringer-Mannheim (Dor- Quebec, Canada), or Pharmacia Inc. (Baie d'Urfé, ːbec, Canada).

ʼlioimmunoassay for rat angiotensinogen

ʻhe radioimmunoassay for rat ANG (RIA-rANG) developed in our laboratory (by J.S.D. Chan), and procedure has been previously described [28]. Puri- . rat plasma ANG [that is, greater than 90% pure, as lyzed by polyacrylamide gel electrophoresis con- ɪing sodium dodecyl sulfate (SDS-PAGE)] and the ʲinated rANG were used as the hormone standard l tracer, respectively. This RIA is specific for intact ANG (62 to 65 kDa rANG) and has no cross-reactiv- with pituitary hormone preparations or other rat ʒma proteins [28]. The lower limit of detection for RIA is approximately 2 ng of rANG. The intra-assay l interassay coefficients of variation were 9% (*N* = and 14% (*N* = 10), respectively.

ʻl culture

ʲmmortalized rat proximal tubular cells at passages 11 13 were used in these studies. The characteristics of IRPTCs have been previously described [28]. These ls express the mRNA and protein of ANG, renin, ʑE, and Ang II receptor [27, 28]. ʲmmortalized rat proximal tubular cells were grown

in 100 × 20 mm plastic Petri dishes (Life Technologies Inc.) in low glucose (5 mM) Dulbecco's modified Eagle's medium (DMEM; pH 7.45) supplemented with 10% fe- tal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The cells were grown in a humidi- fied atmosphere in 95% air, 5% CO$_2$ at 37°C. For subcul- turing, cells were trypsinized [0.05% trypsin and ethyl- enediaminetetraacetic acid (EDTA)] and were plated at 2.5 × 10^4 cells/cm^2 in 100 × 20 mm Petri dishes.

Effect of D(+)-glucose on the expression of immunoreactive rat ANG in IRPTCs

Immortalized rat proximal tubular cells were plated at a density of 1 to 2 × 10^5 cells/well in six-well plates and were incubated overnight in low-glucose DMEM containing 10% FBS. Then cell growth was arrested by incubating the cells in serum-free medium with low-glu- cose DMEM for 24 hours. Various concentrations of D(+)-glucose (final concentration 5 to 25 mM) were then added to the culture medium containing 1% depleted FBS, and the cells were incubated for an additional 24 hours. To maintain a constant tonicity, the media were supplemented with D-mannitol, 15 to 25 mM (final con- centration). At the end of the incubation period, media were collected and stored at −20°C until assayed for immunoreactive rat ANG (IR-rANG).

To determine the specificity of D(+)-glucose, 5 or 25 mM of L-glucose or D-mannitol or 2-deoxy-D-glucose were added to the culture medium, and the cells were treated as described earlier here.

The depleted FBS was prepared by incubation with 1% activated charcoal and 1% AG 1 × 8 ion-exchange resin (Bio-Rad Laboratories, Richmond, CA, USA) for 16 to 24 hours at room temperature as Samuels, Standby and Shapiro described [29]. This procedure removes en- dogenous steroid and thyroid hormones from the FBS as Samuels et al demonstrated [29].

Effect of Tolrestat, staurosporine, H-7, or PMA on the expression of IR-rANG in IRPTCs in the presence of high glucose

To study the effects of the PKC signal transduction pathway and the polyol pathway on the expression of IR-rANG in IRPTCs, various concentrations of Tolres- tat (10^{-7} to 10^{-4} M), staurosporine (10^{-11} to 10^{-5} M), H-7 (10^{-11} to 10^{-5} M), or PMA (10^{-11} to 10^{-5} M) were added to the media together with D(+)-glucose (25 mM), and the cells were then incubated for 24 hours. At the end of the incubation period, media were collected and stored at −20°C until assayed for IR-rANG.

To investigate the antagonistic effect of Tolrestat and PMA on the expression of IR-rANG in IRPTCs, various concentrations of PMA (10^{-11} to 10^{-7} M), were added with Tolrestat (10^{-4} M) to the culture media, and the cells were again incubated for 24 hours in the presence of 25 mM D(+)-glucose. At the end of the incubation

)eriod, media were collected and kept at $-20°C$ until issayed for IR-rANG.

Effect of D(+)-glucose and Tolrestat on the ntracellular levels of cellular sorbitol, fructose, DAG, and PKC activity in IRPTCs

To study the effect of Tolrestat on the D(+)-glucose stimulation of cellular sorbitol, fructose, DAG, and PKC activity in IRPTCs, 1 to 2×10^6 cells were plated in 20 × 100 mm Petri dishes and were incubated overnight in DMEM containing 5 mM D(+)-glucose and 10% FBS. Cell growth was then arrested by incubating the cells in serum-free media with low D(+)-glucose (5 mM) for 24 hours. Subsequently, the cells were incubated in media with 5 mM D(+)-glucose, 25 mM D(+)-glucose, or 25 mM D(+)-glucose plus Tolrestat (10^{-4} M) for 30 minutes (that is, for the DAG assay), one hour (PKC assay), or 24 hours (sorbitol and fructose assays). At the end of the incubation period, cells were collected and immediately analyzed for DAG or PKC activity, or were stored at $-80°C$ for subsequent analysis of sorbitol and fructose.

Assays for cellular sorbitol, fructose, DAG, and PKC activity

The methods for the assays of cellular sorbitol, fructose, and myoinositol have been described previously [30]. Briefly, confluent cells were rinsed three times with phosphate-buffered saline and were sonicated for five seconds at approximately 30% of the duty cycle. The cells extracts were snap frozen in liquid nitrogen and were stored at $-20°C$. Deuterium-labeled sorbitol (Isotec, Miamisburg, OH, USA) and myoinositol (Merck Isotopes, Montreal, Canada) were added as internal standards to the thawed samples. The samples were then deproteinized with 5% trichloroacetic acid, extracted with ether, lyophilized, derivatized with hydroxylamine hydrochloride, 4-(dimethylamino) pyridine, and acetic anhydride, and were injected into a gas chromatograph equipped with a SPB-1 fused silica capillary column (Supelco, Bellefonte, PA, USA). Concentrations were calculated on the basis of an eight-point standard curve constructed by the addition of varying amounts glucose, sorbitol, and myoinositol to samples of cells grown in media with 5 mM glucose. Processing and analysis were identical to those used for the unknown samples. This technique gave an instrument response that was linear for polyol concentrations ranging 0.05 µg to 20 µg per sample. Hexose and polyol concentrations in cultured cells were expressed as nmol/mg protein.

The DAG assay kit (RPN 200) was purchased from Amersham Life Science (Oakville, Ontario, Canada). The methods for the assay of DAG in IRPTCs were performed according to the protocol provided by the supplier.

The PKC activity was measured according to the manufacturer's protocol (RPN 77; Amersham Life Science). Prior to the assay, however, the IRPTCs were separated
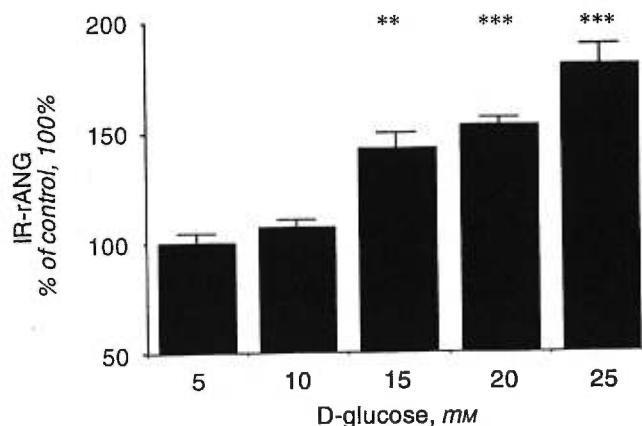
Fig. 1. Effect of D(+)-glucose on the expression of rat angiotensinogen (ANG) in immortalized rat proximal tubule cells (IRPTCs). Cells were incubated to 24 hours in the presence of various concentrations of D(+)-glucose. Media were collected and assayed for immunoreactive rat ANG (IR-rANG). The concentration of IR-rANG in the medium containing low D(+)-glucose (5 mM; that is, 2.75 ± 0.05 ng/ml) was considered as the control level (100%). Each point represents the mean $\pm$ SD of three dishes ($**P \leq 0.01$ and $***P \leq 0.005$). Similar results were obtained from three independent experiments.

into cytosolic and membrane fractions. Briefly, the cells were harvested in 3 ml of buffer A [20 mM Tris-HCl, pH 7.5, containing 2 mM EDTA, 0.5 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N;N′-tetraacetic acid], 1 mM phenyl methyl sulfonyl fluoride, 1 mM dithiothreitol, 25 µg/ml leupeptin, 1 µg/ml pepstatin A, and 330 mM sucrose] and kept on ice. Then, cells were homogenized with a Douce-bound glass homogenizer (type B) and centrifuged at $100,000 \times g$ for 30 minutes at 4°C. Following centrifugation, the supernatants (cytosolic fraction) were stored on ice until assay. The pellets were resuspended in 3 ml of buffer B (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 1 mM phenyl methyl sulfonyl fluoride, 1 mM DTT, 25 µg/ml leupeptin, 1 µg/ml pepstatin A, and 0.2% Triton X-100) and were incubated on ice with agitation for 30 minutes. Finally, the suspensions were centrifuged at $100,000 \times g$ for 30 minutes, and the supernatants were collected as the membrane fraction and were stored on ice until assayed by the PKC assay kit. The PKC activity in the cytosolic and membrane fractions was expressed as pmol of ^{32}P-ATP incorporated per minute per milligram of protein.

Effect of D(+)-glucose and Tolrestat on the expression of ANG mRNA in IRPTCs

To study the effect of D(+)-glucose on the expression of ANG mRNA in the absence or presence of Tolrestat, IRPTCs were incubated in 5 mM D(+)-glucose, 25 mM D(+)-glucose, or 25 mM D(+)-glucose plus Tolrestat (10^{-4} M) for 24 hours. At the end of the incubation period, cells were collected, and total RNA was extracted. One microgram aliquot of total RNA was used in reverse transcrip-
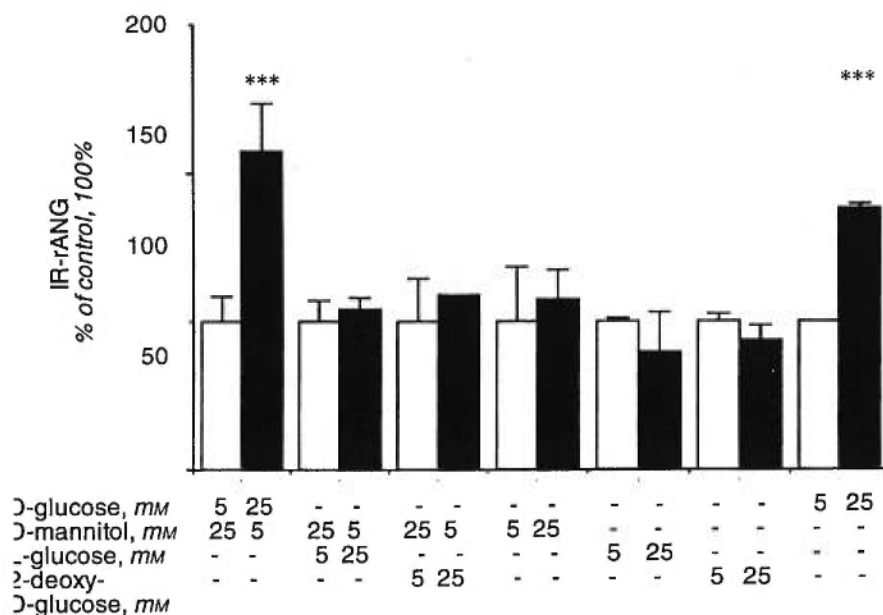
Fig. 2. Effect of D(+)-glucose, D-mannitol, L-glucose, and 2-deoxy D(+)-glucose on the expression of rat ANG in IRPTCs. Cells were incubated for 24 hours in the presence of low (5 mM) or high (25 mM) concentration of D(+)-glucose, D-mannitol, L-glucose, and 2-deoxy D(+)-glucose. Media were harvested after 24 hours of incubation and were assayed for IR-rANG. The concentration of IR-rANG in the medium containing 5 mM D(+)-glucose was considered as the control level (that is, 100%). Each point represents the mean ± SD of three dishes (***$P \leq 0.005$). Similar results were obtained from three other experiments.

tion-polymerase chain reaction (RT-PCR) to quantitate the amount of ANG mRNA expressed in the IRPTCs as described later here.

RT-PCR assay

The RT-PCR assay was performed by using the forward primer, 5' GAG GGG GTC AGC ACG GAC AGC ACC 3', and the reversed primer, 5' GCC CAG AAA GTG CAC CGC ACC TGA 3', corresponding to the nucleotide sequence of N+775 to N+798 and N+1381 to N+1404 of the rat ANG cDNA [31]. Briefly, the total RNA was isolated from the IRPTCs using Trizol reagent (Life Technologies Inc.) according to the protocol of the supplier. The cDNAs were then synthesized using one microgram of total RNA and the Super-Script preamplification system, following the protocol described by the supplier (Life Technologies Inc.). Then, 2 μl of the cDNA reaction mixture was used to amplify the rat ANG cDNA fragment using the PCR-core kit according to the protocol of the supplier (Boehringer-Mannheim Inc.). Furthermore, primers specific for rat glyceraldehyde-phosphate dehydrogenase (G-3-PDH) [32] (forward and reversed primers, 5' TCA CCA CCA TGG AAGA AGG CTG GGG CTC AC3' and 5' GTA GCC CAG GAT GCC CTT TAG 3' corresponding to nucleotides N+329 to N+357 and N+834 to N+852 of rat G-3-PDH) were used in another PCR reaction as an internal control. The RT-PCR reaction mixture was then separated on a 1.5% agarose gel and transferred onto a nitrocellulose membrane. Subsequently, ³²P-labeled oligonucleotides corresponding to the nucleotide N+994 to N+1020 of the rat ANG cDNA [31] and the nucleotide of N+418 to N+448 of rat G-3-PDH [32] were used to hybridize the membrane. Finally,

the membrane was washed and exposed to autoradiography. The relative densities of the PCR bands were determined with a computerized laser densitometer.

Statistical analysis

Three to four separate experiments were performed, and each treatment group was assayed in triplicate. The data were analyzed with the Student's *t*-test or analysis of variance. A probability level of $P \leq 0.05$ was regarded as statistically significant.

RESULTS

Effects of D(+)-glucose on the expression of ANG in IRPTCs

A concentration-dependent relationship between D(+)-glucose levels and the stimulation of expression of the ANG was observed at glucose concentrations ranging between 5 and 25 mM (Fig. 1). Twenty-five mM of D(+)-glucose stimulated the expression of the ANG by 1.75-fold ($P \leq 0.005$).

Figure 2 compares the effect of 5 mM or 25 mM of D(+)-glucose, D-mannitol, L-glucose, or 2-deoxy-D-glucose on the expression of ANG in IRPTCs following a 24-hour incubation. In contrast to D(+)-glucose, D-mannitol, L-glucose, or 2-deoxy-D-glucose at 25 mM had no significant effect on the expression of ANG in IRPTCs compared with those at 5 mM.

Effect of Tolrestat, staurosporine, H-7, or PMA on the expression of ANG in IRPTCs in the presence of high glucose

Figure 3 shows that the addition of Tolrestat (10^{-13} to 10^{-4} M) inhibited the stimulatory effect of 25 mM D(+)-
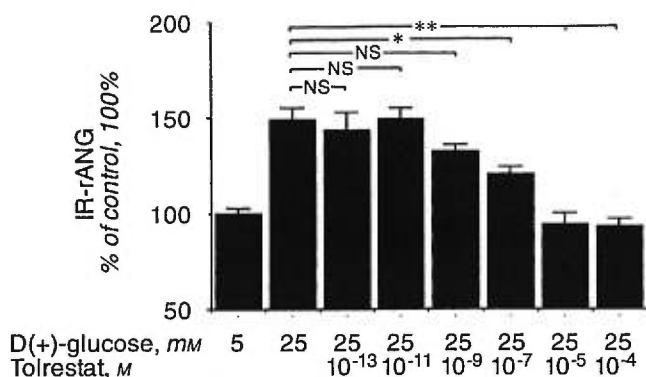
Fig. 3. Inhibitory effect of Tolrestat on the expression of rat ANG in IRPTCs cultured in the presence of 25 mM D(+)-glucose. Cells were incubated for 24 hours in the presence of 5 mM or 25 mM D(+)-glucose in the absence or presence of Tolrestat. Media were harvested and assayed for the level of IR-rANG. The levels of IR-rANG in the medium containing low D(+)-glucose (5 mM; that is, 3.25 ± 0.05 ng/ml) were considered as the controls (100%). The inhibitory effect of Tolrestat is compared with cells that were incubated in 25 mM D(+)-glucose in the absence of Tolrestat. Each point represents the mean ± SD of three dishes (*$P \leq 0.05$, **$P \leq 0.01$, and NS). Similar results were obtained from three other experiments.

Fig. 4. Inhibitory effect of staurosporine or H-7 on the expression of rat ANG in IRPTCs stimulated by 25 mM D(+)-glucose. Cells were incubated for 24 hours in the presence of 5 mM or 25 mM D(+)-glucose in the absence or presence of staurosporine (*A*) or H-7 (*B*). The levels of IR-rANG in the medium containing low D(+)-glucose (5 mM) in A (3.69 ± 0.11 ng/ml) or in B (3.49 ± 0.08 ng/ml) were considered as control (100%). The inhibitory effect of staurosporine or H-7 is compared with cells that were incubated in 25 mM D(+)-glucose (in the absence of staurosporine or H-7). Each point represents the mean ± SD of three dishes (**$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from three other experiments.

glucose on the expression of ANG in IRPTCs in a concentration-dependent manner with a maximal effective inhibitory dose for Tolrestat at 10^{-4} to 10^{-5} M ($P \leq 0.01$).

The addition of staurosporine (10^{-11} to 10^{-5} M; Fig. 4A) or H-7 (10^{-11} to 10^{-5} M; Fig. 4B) also inhibited the stimulatory effect of 25 mM D(+)-glucose on the expression of ANG in IRPTCs in a dose-dependent manner. The half-maximal and maximal inhibitory effects of staurosporine or H-7 were at 10^{-9} and 10^{-5} M, respectively.
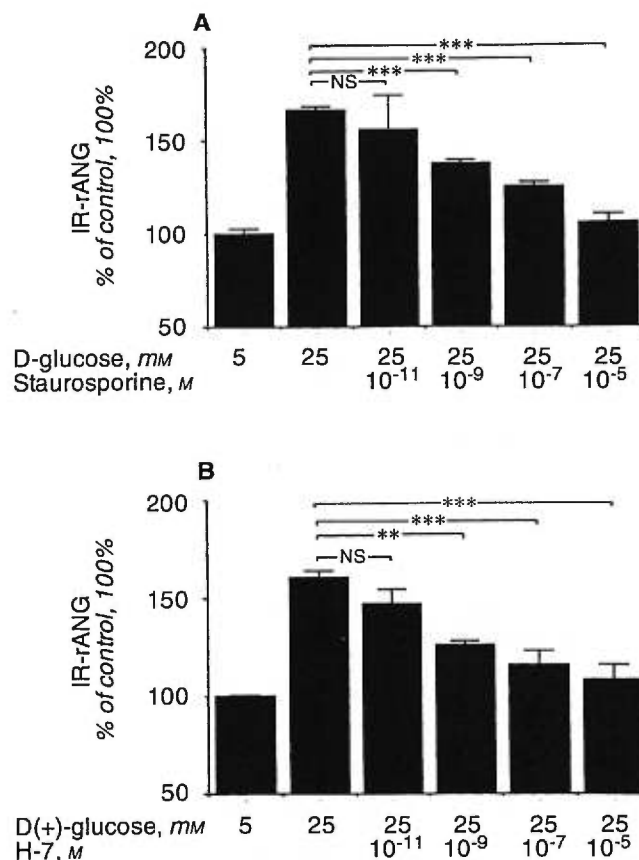
In contrast, the addition of PMA (10^{-11} to 10^{-5} M) enhanced the stimulatory effect of 25 mM D(+)-glucose on the expression of ANG in IRPTCs in a concentration-dependent manner (Fig. 5). The maximal enhancing effect on the expression of the ANG was observed with 10^{-7} M PMA. At higher concentrations, the enhancing effect of PMA appeared to be attenuated.

When cells were preincubated for 24 hours with 10^{-5} M PMA (Fig. 6B) and were then exposed to 25 mM D(+)-glucose or PMA (10^{-7} M), the stimulatory effect of 25 mM of D(+)-glucose or 10^{-7} M PMA on the expression of the ANG was lost (Fig. 6B) compared with the cells without the preincubation with 10^{-5} M PMA (Fig. 6A).

These studies suggest that the stimulatory effect of a high glucose level (25 mM) on the expression of ANG in IRPTCs may be mediated via the PKC signal transduction pathway.

Antagonistic effect of PMA and Tolrestat on the expression of ANG in IRPTCs in the presence of high glucose

Figure 7 shows that the inhibitory effect of Tolrestat (10^{-4} M) on the expression of ANG in IRPTCs treated

with 25 mM D(+)-glucose was reversed in the presence of PMA. PMA, at a concentration of 10^{-9} M, completely blocked the inhibitory effect of Tolrestat (10^{-4} M).
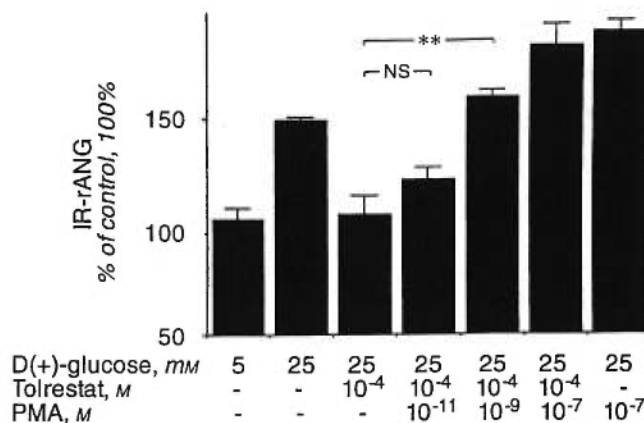
Effect of D(+)-glucose and Tolrestat on the cellular levels of sorbitol, fructose, DAG, and PKC activity in IRPTCs

Incubating IRPTCs for 24 hours in media with 25 mM D(+)-glucose led to increases of 5.63-fold and 33.9-fold in cellular levels of sorbitol and fructose, respectively (Fig. 8). Tolrestat (10^{-4} M) completely prevented the increases in sorbitol but only partially attenuated the increases in fructose (that is, 80% inhibition). Myoinositol levels were unaffected by either high glucose or Tolrestat.

Exposure to high glucose levels (25 mM) also increased the intracellular level of DAG (Fig. 9) and membrane PKC activity (Fig. 10). The addition of Tolrestat (10^{-4} M)
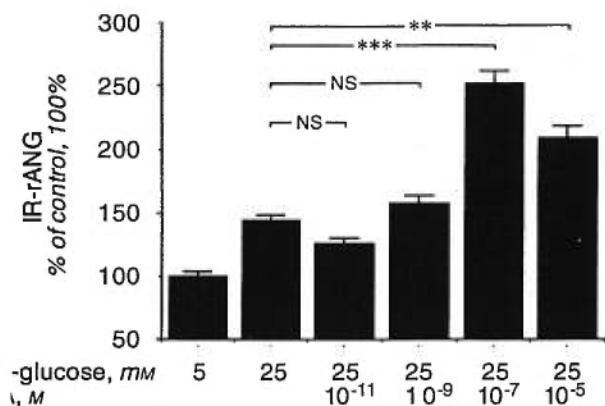
5. Stimulatory effect of phorbol 12-myristate 13-acetate (PMA) ie expression of rat ANG in IRPTCs cultured in the presence of м D(+)-glucose. Cells were incubated for 24 hours in the presence mm or 25 mm D(+)-glucose in the absence or presence of PMA. a were harvested and assayed for the IR-rANG. Levels of IR-Ϡ in the medium containing low D(+)-glucose (5 mm; 3.15 ± 0.05 l) in the absence of PMA were considered as control (100%). The ilatory effect of PMA is compared with cells that were stimulated ; mm D(+)-glucose. Each point represents the mean ± sᴅ of three s (**$P \leq$ 0.01 and ***$P \leq$ 0.005). Similar results were obtained two other experiments.

Fig. 7. Effect of PMA on the expression of rat ANG in IRPTCs in the presence of 25 mᴍ D(+)-glucose and Tolrestat. Cells were incubated for 24 hours in the presence of 5 mᴍ D(+)-glucose, 25 mᴍ D(+)-glucose, or 25 mᴍ D(+)-glucose plus 10^{-4} ᴍ Tolrestat in the absence or presence of various concentrations of PMA (10^{-11} to 10^{-7} ᴍ). Media were harvested and assayed for the level of IR-rANG. Levels of IR-rANG in the medium containing low D(+)-glucose (5 mᴍ; 4.15 ± 0.1 ng/ml) in the absence of Tolrestat or PMA were considered as the controls (100%). The effect of PMA is compared with cells that were incubated in the presence of 25 mᴍ D(+)-glucose and 10^{-4} Tolrestat. Each point represents the mean ± sᴅ of three dishes (**$P \leq$ 0.01). Similar results were obtained from another experiment.
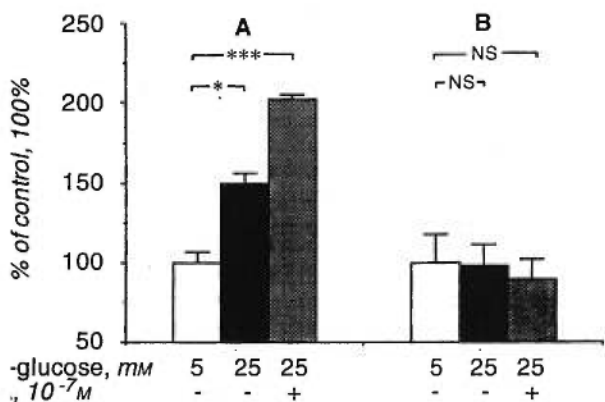
6. Effect of D(+)-glucose on the expression of rANG in IRPTCs cubated with a high level of PMA. Cells were preincubated for urs with 5 mm D(+)-glucose (*A*) or 5 mm D(+)-glucose and 10^{-5} м (*B*). Then the media were replaced with fresh media containing D(+)-glucose, 25 mm D(+)-glucose, or 25 mm D(+)-glucose plus PMA and were incubated for an additional 24 hours. Then the a were harvested and assayed for immunoreactive rANG (IR-Ϡ). The concentration of IR-rANG in the medium containing 5 (+)-glucose in panels A and B (1.86 ± 0.12 ng/ml and 4.16 ± 0.72 ., respectively) were considered as the controls (100%). Each point sents the mean ± sᴅ of three dishes (*$P \leq$ 0.05 and ***$P \leq$ 0.005). ar results were obtained from another experiment.

oited the increases in DAG and PKC activity stimu- l by high glucose levels (25 mм).

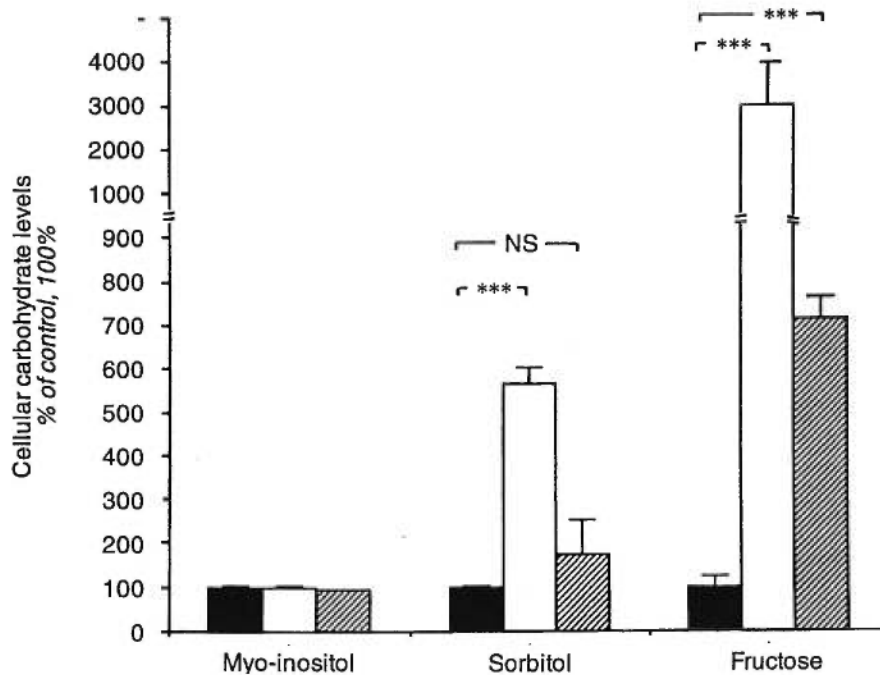**ct of D(+)-glucose and Tolrestat on the cellular Ϡ mRNA level in IRPTCs**

gure 11 shows that a high glucose concentration nм) stimulated the accumulation of the ANG mRNA

to levels that were twofold higher than those in control cells cultured in media containing 5 mᴍ glucose ($P \leq$ 0.05). Tolrestat (10^{-4} ᴍ) inhibited the stimulatory effect of high glucose (25 mᴍ) on the expression of ANG mRNA.

DISCUSSION

Although several studies have investigated the relationship between diabetes and the expression of RAS genes (that is, ANG, renin, ACE, and AT₁-receptors) in the kidney of rats, the results have been inconsistent [33–35]. Correa-Rotter, Hostetter and Rosenberg [33] and Kalinyak et al [34] reported that renal renin and ANG mRNA levels were not different between control and diabetic rats. Anderson, Jung and Ingelfinger, however, have found a small but significant increase in renal renin and ANG gene expression in diabetic rats [35]. The reason for this apparent discrepancy is not yet known, but may be related to the use of different strains of rats or the collection of tissue samples at different time periods after the induction of diabetes with streptozotocin.

Very few studies have described the effect of high glucose levels on the expression of RAS genes in renal proximal tubular cells *in vitro*. In preliminary studies of heterologous opossum kidney (OK) cells, we reported [36] that high glucose down-regulates the expression of RAS genes in OK cells. In more recent studies of a subclone (OK 27) of the OK cells stably transfected with the plasmid, pOGH (ANG N-1498/+18), we found that

Fig. 8. Effect of D(+)-glucose and Tolrestat on the cellular levels of myoinositol, sorbitol, and fructose in IRPTCs. Cells were incubated for 24 hours in the presence of 5 mM D(+)-glucose, 25 mM D(+)-glucose, or 25 mM D(+)-glucose plus Tolrestat (10^{-4} M). Cells were harvested after 24 hours of incubation and assayed for sorbitol, fructose, and myoinositol. The levels of myoinositol (93.74 ± 2.32 nmol/mg), sorbitol (0.08 ± 0.003 nmol/mg), and fructose (0.248 ± 0.06 nmol/mg) in the cells incubated in low D(+)-glucose (5 mM) in the absence of Tolrestat were considered as the controls (100%). Each point represents the mean ± SD of five dishes of cells (***$P \leq 0.005$). Symbols are: (■) control, 5 mM glucose; (□) cells incubated in 25 mM D(+)-glucose; (▨) cells incubated in 25 mM D(+)-glucose plus 10^{-4} Tolrestat. Similar results were obtained from another experiment.
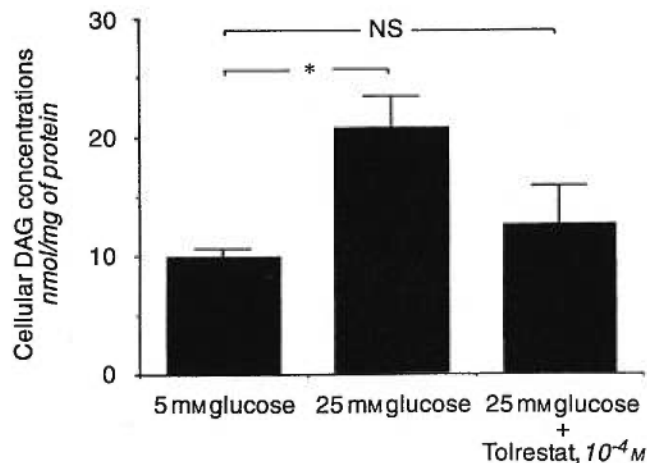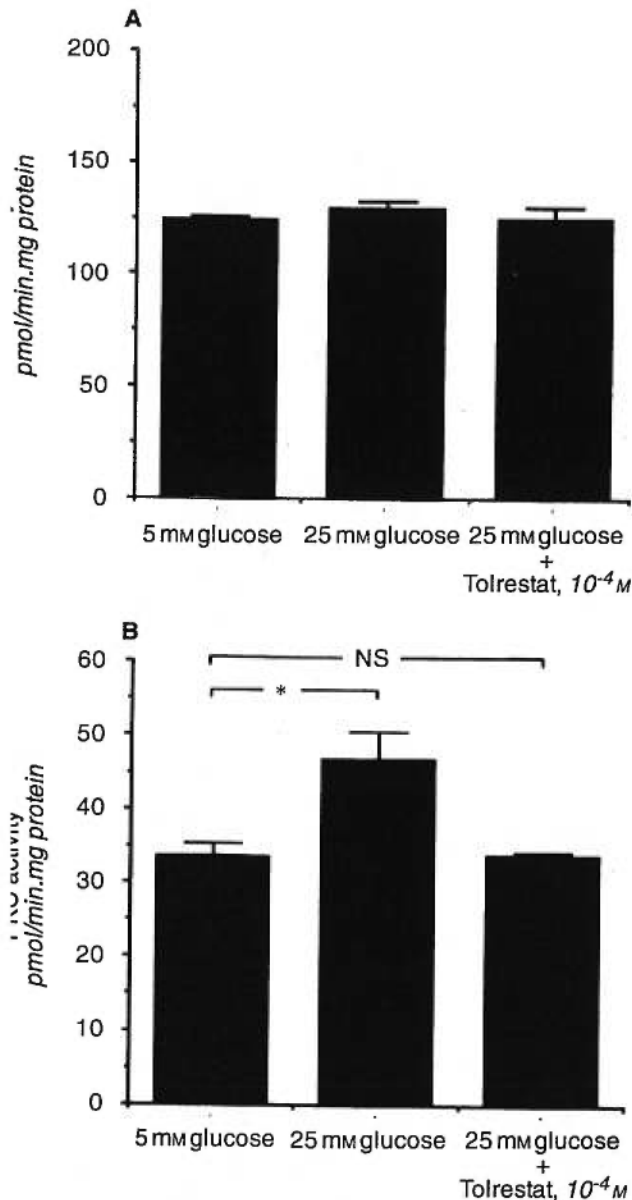

Fig. 9. Effect of D(+)-glucose and Tolrestat on the cellular levels of diacyglycerol (DAG) in IRPTCs. After a 30-minute incubation in media with 5 mM D(+)-glucose, 25 mM D(+)-glucose, or 25 mM D(+)-glucose plus Tolrestat (10^{-4} M). Cells were harvested and assayed for DAG levels. The levels of DAG activity in the cells are expressed as nanomol per mg of protein. Each point represents the mean ± SD of six determinations (*$P \leq 0.05$). Similar results were obtained from another experiment.

25 mM D(+)-glucose stimulated the expression of the rat ANG gene promoter activity in OK cells [37]. We also reported that this increased promoter activity was blocked by H-7 and staurosporine but not by Rp-cAMP (an inhibitor of protein kinase A I and II) [37], suggesting that the effect of glucose on the expression of the ANG gene was mediated via the PKC signal transduction pathway. Currently, the reasons for the discrepancy between the previous [36] and our recent studies [37] in OK cells

are not known. One possible explanation might be that a heterogenous population of OK cells was used in the previous studies (obtained from the American Tissue Culture Collection) [36], whereas our recent studies [37] used a subclone (OK 27) of the OK cells obtained from the American Tissue Culture Collection. Clearly, more studies are warranted to clarify this discrepancy.

A novel finding of the study is that exposure of IRPTCs to high concentrations of D(+)-glucose stimulates the expression of the ANG in a concentration-dependent manner (Fig. 1). We did not observe any stimulation of the expression of ANG by L-glucose, D-mannitol, or 2-deoxy-D-glucose (Fig. 2), indicating that the effect of high D(+)-glucose levels on the expression of ANG in IRPTCs is independent of changes in osmolarity and is probably mediated via the metabolism of D(+)-glucose.

Our study also demonstrated that inhibition of aldose reductase with Tolrestat blocked the stimulatory effect of 25 mM D(+)-glucose on the expression of ANG in IRPTCs (Fig. 3). Furthermore, the addition of staurosporine (Fig. 4A) and H-7 (Fig. 4B) also blocked the stimulatory effect of high D(+)-glucose on the expression of ANG in IRPTCs, suggesting that the effect of high levels of glucose is mediated, at least in part, via the polyol pathway as Tilton et al suggested [38]. Activation of the polyol pathway results in stimulation of *de novo* synthesis of DAG, which subsequently could stimulate the PKC activity in IRPTCs. The involvement of PKC in the expression of ANG in IRPTCs is further supported by the observation that the addition of PMA stimulated the expression of ANG in IRPTCs (Fig. 5). Consistent

A

B

. 10. Effect of D(+)-glucose and Tolrestat on the protein kinase C (C) activity in IRPTCs. After 60 minutes of incubation in media h 5 mM D(+)-glucose, 25 mM D(+)-glucose, or 25 mM D(+)-glucose s Tolrestat (10^{-4} M), cells were harvested, and cytosol and membrane :tions were separated and assayed for PKC activity. The levels of C activity were expressed as picomol of ^{32}P-ATP incorporated per ute per mg protein. The effect of 25 mM glucose, 25 mM glucose s Tolrestat (10^{-4} M) is compared to the 5 mM glucose (control). Each nt represents the mean ± SD of six determinations (*$P \leq 0.05$). iilar results were obtained from another experiment.

h these findings, the stimulatory effect of PMA was olished after a 24-hour preincubation of IRPTCs with ɔM D(+)-glucose in the presence of 10^{-5} M PMA (Fig.) compared with the control cells without the preincu-:ion with PMA (Fig. 6A). These results are consistent h the notion that the prolonged exposure to PMA will wn-regulate the PKC activity and protein expression

levels [39]. The observation that the addition of PMA abolished the inhibitory effect of Tolrestat on the expression of ANG in IRPTCs (Fig. 7) suggests that direct activation of PKC activity by PMA could stimulate the ANG expression independently of the polyol pathway. These studies also suggest that the PKC activation occurs downstream of the polyol pathway stimulated by high levels of glucose.

To examine whether the effect of glucose could be mediated via the *de novo* synthesis of DAG and subsequent activation of PKC activity, we assayed the cellular levels of sorbitol, fructose, DAG, and PKC activity in IRPTCs exposed to high glucose in the presence or absence of Tolrestat. Indeed, culture of IRPTCs in 25 mM D(+)-glucose dramatically increased the intracellular level of sorbitol and fructose (Fig. 8) and markedly increased the levels of DAG (Fig. 9) and membrane PKC activity (Fig. 10) compared with IRPTCs cultured in medium containing 5 mM glucose. The addition of Tolrestat (10^{-4} M) blocked the increase in sorbitol, fructose, and DAG levels and PKC activity stimulated by 25 mM D(+)-glucose (Figs. 8, 9, and 10). These data provide additional support to the notion that the effect of a high level of glucose (25 mM) on the expression of the ANG in IRPTCs is mediated, at least in part, via the *de novo* synthesis of DAG and the activation of PKC activity. Apparently, more experiments are warranted to determine the isoform(s) of PKC involved. Cellular myoinositol levels were also quantitated in IRPTCs exposed to 25 mM glucose in the presence or absence of Tolrestat (Fig. 8). Myoinositol levels are considerably decreased in some tissues from diabetic animals, and some have interpreted this change as evidence for decreased phosphoinositide turnover and decreased DAG production, possibly leading to decreased PKC activity [40]. Our studies were in agreement with Ziyadeh et al [41], however, that cellular myoinositol levels in IRPTCs were unaffected by glucose or Tolrestat.

Finally, the effects of glucose on ANG appear to occur at the mRNA level. Indeed, exposure of IRPTCs to a high glucose concentration (25 mM) stimulated the expression of the ANG mRNA by twofold ($P \leq 0.05$) compared with levels in control cells (that is, 5 mM glucose; Fig. 11). The addition of Tolrestat (10^{-4} M) completely blocked the stimulatory effect of 25 mM D(+)-glucose. Currently, it is uncertain whether glucose could increase the transcriptional level or might affect the stability of the ANG mRNA in IRPTCs. Studies are underway in our laboratory to investigate these possibilities.

In these studies, we have not quantitated the levels of Ang II in the medium to demonstrate a parallel increase of ANG and Ang II in the medium stimulated by high glucose levels. Studies by Tang et al [27], however, have shown that the Ang II is presented in the incubation medium of IRPTCs. Clearly, more experiments are war-
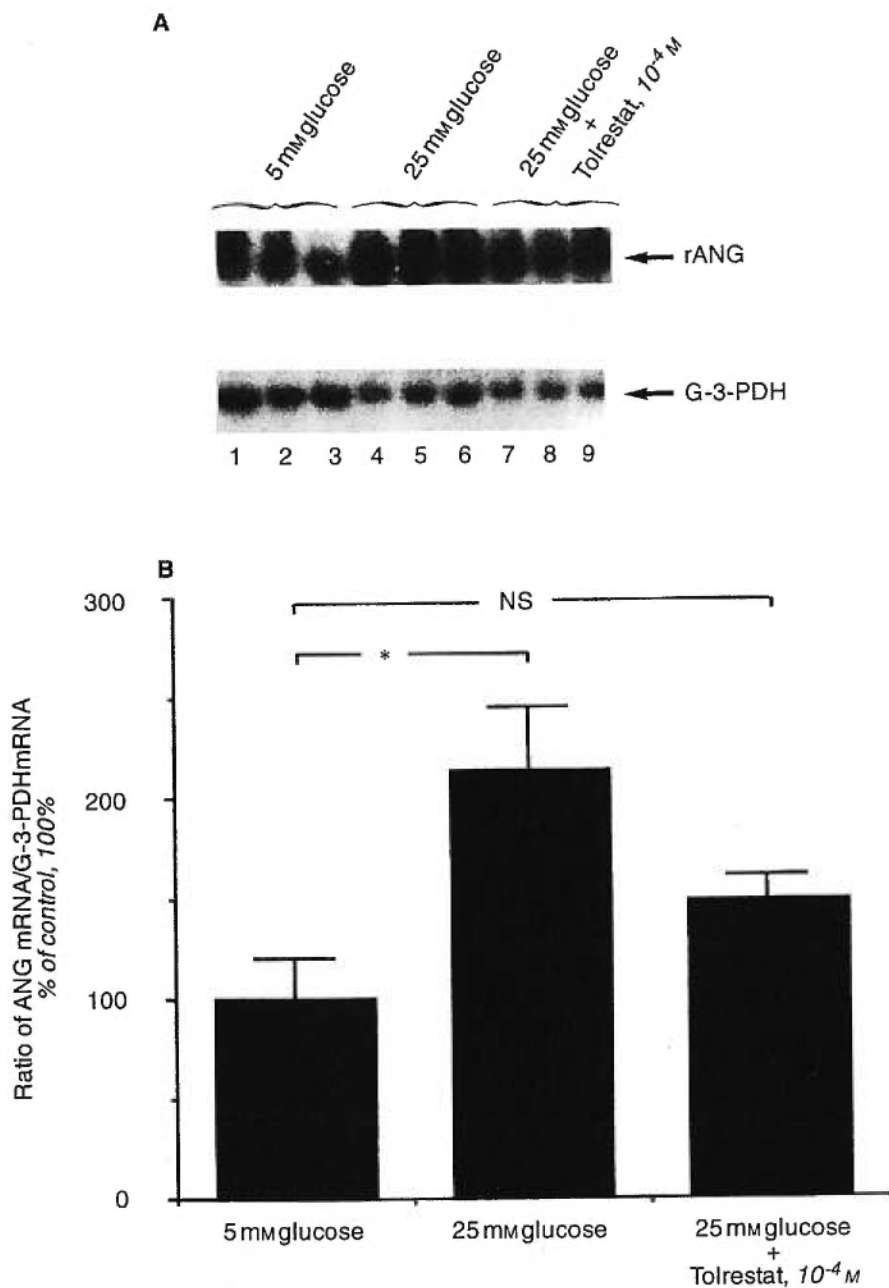
Fig. 11. Effect of D(+)-glucose and Tolrestat on the expression of rat ANG mRNA in IRPTCs. After a 24-hour incubation in media with 5 mM D(+)-glucose, 25 mM D(+)-glucose, or 25 mM plus Tolrestat (10^{-4} M), cells were harvested and assayed for ANG mRNA levels with a RT-PCR assay. The DNA fragments of the RT-PCR reaction mixture were separated on a 1.5% agarose gel and were then transferred onto a nitrocellulose membrane. Subsequently, the membrane was blotted with a ^{32}P-labeled oligonucleotide corresponding to the nucleotides N+944 to N+1020 of rat ANG and N+418 to N+448 of rat G-3-PDH, respectively. The relative densities of the PCR band of ANG (629 bp DNA fragment) were compared with the G-3-PDH control (523 bp DNA fragment; A). The level of ANG mRNA in the cells incubated in low D(+)-glucose (5 mM; that is, a ratio of 0.83 ± 0.17 unit: ANG/G-3-PDH), in the absence of Tolrestat was considered as the control (100%; B). Each point represents the mean $\pm$ SD of three dishes (*$P \leq 0.05$). Similar results were obtained from another experiment.

ranted to correlate the levels of expression of the ANG and Ang II in the medium, and studies are underway in our laboratory.

In summary, our studies showed that high D(+)-glucose levels directly stimulate the expression of the ANG gene in IRPTCs. We have further demonstrated that the stimulatory effect of high glucose was blocked by Tolrestat, staurosporine, and H-7, implicating the involvement of both the polyol pathway and the PKC signal transduction pathway in the overexpression of renal ANG gene under hyperglycemic conditions. These results suggest that the aldose reductase inhibitors and PKC might be useful agents for the prevention and/or attenuation of glucose-induced ANG gene expression and consequently development of diabetic nephropathy. However, it remains to be investigated whether aldose reductase inhibitors may be beneficial in the long term in the treatment of diabetic nephropathy.

ACKNOWLEDGMENTS

APPENDIX

Abbreviations used in this article are: ACE, angiotensin-converting enzyme; ANG, angiotensinogen; Ang II, angiotensin II; DAG, diacylglycerol; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; G-3-PDH, glyceraldehyde-phosphate dehydrogenase; IRPTCs, immortalized rat proximal tubular cells; IR-rANG, immunoreactive-rat angiotensinogen; OK, opossum kidney; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RAS, renin-angiotensin system; RIA, radioimmunoassay; RT-PCR, reverse transcription-polymerase chain reaction.

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Appendix VI : Zhang SL, Chen X, Filep JG, Tang SS, Ingelfinger JR, Chan JSD: Insulin inhibits angiotensinogen gene expression via the mitogen-activated protein kinase (MAPK) pathway in rat proximal tubular cells. *Endocrinology* (Acceptance pending), 1999.

July 9, 1999

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INSULIN INHIBITS ANGIOTENSINOGEN GENE EXPRESSION VIA THE MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) PATHWAY IN RAT KIDNEY PROXIMAL TUBULAR CELLS

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Running title: Insulin, glucose, angiotensinogen gene, rat proximal tubular cells

ABSTRACT

The present study aimed to investigate the molecular mechanism(s) of insulin action on angiotensinogen (ANG) secretion and gene expression in kidney proximal tubular cells exposed to high levels of glucose. Immortalized rat proximal tubular cells (IRPTC) were cultured in monolayer. The levels of rat ANG and ANG mRNA in the IRPTC were quantified by a specific radioimmunoassay for rat ANG (RIA-rANG) and by a reverse transcription-polymerase chain reaction (RT-PCR) assay. Insulin inhibited the stimulatory effect of a high level of glucose (25 mM) and phorbol 12-myristate 13-acetate (PMA, an activator of protein kinase C) on the secretion of ANG and the expression of the ANG mRNA in IRPTC. This inhibitory action of insulin on the ANG secretion and gene expression was blocked by PD98059 (an inhibitor of mitogen-activated protein kinase kinase (MEK)) but not by Wortmannin (an inhibitor of phosphatidylinositol-3-kinase). PD98059 was effective in inhibiting the phosphorylation of MEK 1/2 and p44/42 MAP kinase in IRPTC stimulated by insulin. These studies demonstrate that insulin prevents the stimulatory effect of high levels of glucose on the expression of the renal ANG gene in IRPTC, at least in part, via the MAPK kinase (MEK) signal transduction pathway, subsequently inhibiting the activation of the local renal renin-angiotensin system (RAS).

Key words: Insulin, glucose, renin-angiotensin system, mitogen-activated protein kinase.

INTRODUCTION

Messenger RNAs (mRNA) for renin-angiotensin system (RAS) components, including angiotensinogen (ANG), renin, angiotensin-converting enzyme (ACE) and angiotensin II receptor (AT_1-receptor) are expressed in murine (mouse and rat) kidney proximal tubular cell lines (1-5). We have recently reported that the ANG protein is secreted from rat immortalized renal proximal tubular cells (IRPTC) as measured by a specific radioimmunoassay for rat ANG (RIA-rANG) (6), providing evidence that the intrarenal Ang II is probably derived from the ANG that is synthesized within renal proximal tubular cells.

Several studies have shown that incubation of murine proximal tubular cells in a high glucose (i.e 25 mM) medium or in the presence of high levels of Ang II (i.e. 10^{-8} M) induces cellular hypertrophy and extracellular matrix protein synthesis (7-12). We have previously reported that high levels of glucose stimulate the expression of the rat ANG gene in opossum kidney (OK) proximal tubular cells (13) and in immortalized rat proximal tubular cells (IRPTC) (14). This stimulatory effect of glucose is blocked in the presence of inhibitors of protein kinase C (PKC) and aldose reductase. These studies indicate that high levels of glucose may activate the local renal RAS via the stimulation of ANG gene expression. The local formation of Ang II may contribute to the induction of hypertrophy observed in proximal tubular cells in early diabetes.

Insulin therapy of patients with insulin-dependent diabetic mellitus (IDDM) delays the onset and slows the progression of nephropathy (15-16). Studies in diabetic rats have shown that normalization of blood glucose by insulin reverses established glomerular hyperfiltration, renal hypertrophy and extracellular matrix protein synthesis

(17-18). The molecular mechanism(s) of the beneficial effects of insulin treatment, however, is not completely understood.

In the present studies, we investigated whether insulin might attenuate or inhibit the stimulatory effect of glucose on ANG gene expression *in vitro* and to study the possible underlying molecular mechanism(s) of action. Our studies showed that insulin inhibits the stimulatory effect of high levels of glucose (i.e. 25 mM) and PMA on the expression of the rat ANG gene in IRPTC. PD98059 (an inhibitor of MAP-kinase kinase (MEK)) blocked the inhibitory effect of insulin on the ANG gene expression as well as on the phosphorylation of the MEK 1/2 and p44/42 MAP kinase. The addition of Wortmannin (an inhibitor of phosphatidylinositol-3-kinase (PI-3 kinase)), however, did not reverse the inhibitory effect of insulin.

MATERIALS AND METHODS

D(+)-glucose, insulin and phorbol 12-myristate 13-acetate (PMA, an activator of PKC) were purchased from Sigma Chemicals (St-Louis, MO, USA). Insulin growth factor I and II (IGF-I and IGF-II) were purchased from Life Technologies Inc.(Burlington, Ontario, Canada). PD98059 and Wortmannin were purchased from Calbiochem Inc (La Jolla, CA, USA).

Gamma-[^{32}P-ATP] (3000 Ci/mol) and Na^{125}I were purchased from Dupont, New England Nuclear (NEN, Boston, MA, USA). Oligonucleotides were synthesized by Life Technologies Inc (Burlington, Ontario, Canada). Restriction and modifying enzymes were purchased from either Life Technologies Inc, Boehringer-Mannheim (Dorval, Quebec, Canada) or Pharmacia Inc. (Baie d'Urfé, Quebec, Canada).

Phospho Plus MEK 1/2 antibody and Phospho Plus p44/42 MAP kinase kits were purchased from New England Biolabs Ltd. (Mississauga, Ont, Canada). The Phospho Plus MEK 1/2 and Phospho Plus p44/42 MAP kinase antibody kits are assays for rapid analysis of MEK 1/2 (Ser 217/221) and p44/42 MAP kinase (Thr 202/Tyr 204) phosphorylation status, respectively, that function in a mitogen-activated protein kinase cascade.

Radioimmunoassay for rat angiotensinogen

The radioimmunoassay for rat ANG (RIA-rANG) was developed in our laboratory (JSDC), and the procedure has been previously described in detail (6). Purified rat plasma ANG (i.e. greater than 90% pure, as analyzed by polyacrylamide gel electrophoresis containing sodium dodecyl sulfate (SDS-PAGE)) and iodinated rANG were used as the hormone and tracer, respectively. This RIA is specific for intact rat ANG (i.e. 62-65 kilodalton rANG) and has no cross-reactivity with pituitary hormone preparations or other rat plasma proteins (6). The lower limit of detection for the RIA is approximately 2 ng of rANG. The intra- and inter-assay coefficients of variation were 9% (n=10) and 14% (n=10), respectively.

Cell culture

IRPTC at passages 11 to 13 were used in the present studies. The characteristics of IRPTC have been previously described (4). These cells express the mRNA and protein of ANG, renin, angiotensin-converting enzyme and angiotensin-II receptor (4).

IRPTC were grown in 100 X 20 mm plastic Petri dishes (Life Technologies Inc) in normal glucose (i.e. 5 mM) Dulbecco's Modified Eagle Medium (DMEM) (pH 7.45), supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 µg/ml of streptomycin. The cells were grown in a humidified atmosphere in 95% air, 5% CO_2 at 37°C. For subculturing, cells were trypsinized (0.05% trypsin and EDTA) and plated at 2.5 X 10^4 cells/cm^2 in 100 X 20 mm Petri dishes.

Effect of glucose and insulin on the secretion of IR-rANG in IRPTC

IRPTC were plated at a density of 1-2 X 10^5 cells/well in 6-well plates and incubated overnight in normal glucose (i.e. 5 mM) DMEM containing 10% FBS. Cell growth was arrested by incubating the cells in serum-free medium with 5 mM glucose DMEM for 24 hours. Various concentrations of insulin (10^{-13} to 10^{-5} M) were then added to a high (25 mM) glucose culture medium containing 1% depleted fetal bovine serum (dFBS) in the presence or absence of PMA (10^{-7} M) and the cells were incubated for an additional 24 hours. At the end of the incubation period, media were collected and stored at-20°C until assayed for IR-rANG. The depleted FBS (i.e. depletion of endogenous steroid and thyroid hormones) was prepared by incubation with 1% activated charcoal and 1% AG 1X 8 ion-exchange resin (Bio-Rad Laboratories, Richmond, CA, USA) for 16 to 24 hours at room temperature as described by Samuels et al (19).

To asses the specificity of the insulin effect, IGF-I or IGF-II at concentrations ranging between 1.3 X 10^{-11} to 1.3 X 10^{-8} M (final concentration) were added to the culture medium, and the cells were incubated for 24 hours. To study whether the

inhibitory effect of insulin is mediated via the PKC signal transduction pathway, IRPTC were pre-incubated for 24 hours with 10^{-5} M PMA in a 25 mM glucose medium. Then, the cells were incubated with fresh 25 mM glucose medium containing 10^{-7} M PMA or various concentrations of insulin (10^{-11} to 10^{-5} M) for 24 hours. To explore the involvement of the MAP kinase and PI-3 kinase pathways in mediating the effect of high levels of glucose or insulin on the secretion of the IR-rANG from IRPTC confluent cells were incubated for 24 hours in media with 5 mM glucose, 25 mM glucose or 25 mM glucose and PMA (10^{-7} M) with or without insulin (10^{-7} M) plus various concentrations of PD98059 or Wortmannin. At the end of the incubation period, media were collected and stored at -20°C until assayed for IR-rANG.

Expression of ANG mRNA in IRPTC

To study the effect of glucose and insulin on the expression of ANG mRNA in IRPTC, the cells were incubated in 5 mM glucose, 25 mM glucose medium, 25 mM glucose medium and insulin (10^{-7} M) in the absence or presence of PD 98059 (10^{-5} M) for 24 hours. At the end of the incubation period, cells were collected and total RNA was isolated using Trizol reagent (Life Technologies Inc. Burlington, Ontario, Canada) according to the protocol of the supplier. The total RNA was used in a reverse-transcription polymerase chain reaction (RT-PCR) to quantitate the amount of ANG mRNA expressed in IRPTC as described previously (14). Briefly, 1µg of total RNA was used to synthesize the cDNAs by employing the Super Script pre-amplification system, following the protocol described by the supplier (Life Technologies Inc). Then, 2 µl of the cDNA reaction mixture was used to amplify the rat ANG cDNA fragment using the

PCR-core kit according to the protocol of the supplier (Boehringer-Mannheim Inc). The forward primer, 5' CCT CGC TCT CTG GAC TTA TC 3', and the reversed primer, 5' CAG ACA CTG AGG TGC TGT TG3', corresponding to the nucleotide sequence of N+676 to N+695 and N+882 to N+901 of the rat cDNA (20) were used in PCR. Furthermore, primers specific for rat β-actin (21) (forward and reversed primers, 5' ATG CCA TCC TGC GTC TGG ACC TGG C3' AND 5' AGC ATT TGC GGT GCA CGA TGG AGG G3' corresponding to nucleotide N+155 to N+179 of exon 3 and nucleotide N+115 to N+139 of exon 5 of rat β-actin) were used in another PCR reaction as an internal control. The RT-PCR reaction mixture was then separated on a 1.5% agarose gel and transfered onto a Gene-Screen Plus nylon membrane (Dupont, NEN). Subsequently, ^{32}P-labeled oligonucleotides corresponding to the nucleotide N+775 to N+798 of the rat ANG cDNA (20) and nucleotide of N+9 to N+35 of exon 4 of rat β-actin (21) were used to hybridize the membrane. Finally, the membrane was washed and exposed to autoradiography. The relative densities of the PCR bands were determined with a computerized laser densitometer.

Phosphorylation of MEK 1/2 and p44/42 MAP kinase in IRPTC

The effect of glucose and insulin on the activation of MAPK signal transduction pathways in IRPTC was evaluated by the phosphorylation of MEK 1/2 and p44/42 MAP kinase by employing Phospho Plus MEK 1/2 and Phospho Plus p44/42 MAP kinase antibody kits, respectively. Briefly, cells were plated at 5×10^4 cells/well in 6-well plates in 5 mM glucose DMEM containing 10% FBS, and were synchronized in 5 mM glucose-medium for 24 hours. Subsequently, the cells were incubated in a 5 mM

glucose, 25 mM glucose, 25 mM glucose and PD98059 (10^{-5} M) for 15 minutes. Then insulin (10^{-7} M) was added and the cells were incubated for another 10 minutes. Cells were lysed in 100 µl of lysis buffer (62.5 mM Tris-HCl, pH 6.8 containing 2% sodium dodecyl sulfate (SDS) (w/v), 10% glycerol, 50 mM DTT and 0.1% bromophenol blue (w/v)) and harvested in Eppendorf tubes. The cell lysates were sonicated for 2 seconds, heated at 95°C for 5 minutes and then centrifuged at 12000 X g for 2 minutes at 4°C. Small aliquots (20 µl) of the supernatants were subjected to polyacrylamide gel (10%) electrophoresis containing sodium dodecyl sulfate (SDS-PAGE) and then transferred onto a nitrocellulose membrane (Hybond C Extra, Amersham Life Science, Oakville, Ontario, Canada). The membrane was then blotted for the phosphorylated MEK 1/2 by employing the Phospho Plus MEK 1/2 or Phospho Plus p44/42 MAP kinase antibody kit.

Statistical analysis

Three to four separate experiments per protocol were performed and each treatment group was assayed in triplicate. The data were analysed with the Student's t test or Anova analysis. A probability level of $p \le 0.05$ was regarded as statistically significant.

RESULTS

Effects of glucose and insulin on the secretion of IR-rANG in IRPTC

The secretion of the IR-rANG was increased (i.e. 150%) in IRPTC with a high level of glucose (i.e. 25 mM) in the culture medium compared to normal glucose (i.e. 5 mM)

(Figure 1A) (p≤ 0.005). The addition of insulin to the culture medium abolished the high glucose (25 mM)-stimulated secretion of the IR-rANG in IRPTC in a dose-dependent manner (Figure 1A) with a maximal effect observed at 10^{-7} to 10^{-5} M. This effective dose of insulin, i.e. 10^{-7} M, was therefore routinely used in all subsequent experiments. Unlike insulin, IGF-I or IGF-II, at concentrations ranging from 1.3×10^{-11} to 1.3×10^{-8} M had no significant effect on glucose stimulated secretion of IR-rANG in IRPTC (Figure 1B).

Antagonistic effect of insulin and PMA on the secretion of the IR-rANG in IRPTC

PMA at 10^{-7} M stimulated the secretion of the IR-rANG by approximately 150% (p≤ 0.005) in IRPTC cultured in a normal (5 mM) glucose medium (Figure 2A). This increase was similar to that observed in IRPTC incubated in 25 mM glucose medium. Addition of insulin to the culture medium inhibited the PMA stimulation of secretion of the IR-rANG in IRPTC incubated in 5 mM glucose medium (Figure 2A). PMA (10^{-7} M) augmented the stimulatory effect of a high level (25 mM) of glucose to stimulate the secretion of the IR-rANG from IRPTC compared to control cells cultured in 5 mM glucose (p≤ 0.005) (Figure 2B). Insulin at 10^{-7} to 10^{-5} M completely inhibited the combined effect of PMA and high glucose (25 mM) medium on the secretion of the IR-rANG from IRPTC (Figure 2B).

Figure 3 shows that the overnight incubation of IRPTC in 25 mM glucose with a high concentration of PMA (10^{-5} M) did not block the inhibitory effect of insulin on the secretion of the IR-rANG. The stimulatory effect of PMA (10^{-7} M), however, was abolished. These studies demonstrate that the insulin inhibition of the stimulatory

effect of PMA on the secretion of the IR-rANG is independent of PKC activation and may be mediated downstream in the PKC signal transduction pathway or via another signal transduction pathway.

Effect of PD98059 or Wortmannin on the secretion of the IR-rANG

Figure 4 shows that PD 98059 (10^{-11} to 10^{-7} M) had no effect on the secretion of the IR-rANG from IRPTC stimulated by 25 mM glucose, wheras PD98059 at 10^{-5} M significantly (p≤ 0.01) enhanced the stimulatory effect of 25 mM glucose. Similarly, PD98059 at 10^{-7} to 10^{-6} M had no effect on the secretion of the IR-rANG in IRPTC stimulated by 25 mM glucose plus 10^{-7} M PMA (Figure 4B). Again, PD98059 at 10^{-5} and 10^{-4} M significantly (p≤ 0.005) enhanced the stimulatory effect of 25 mM glucose and PMA (10^{-7} M). These studies indicate that the inhibition of MAP kinase enhances the stimulatory effect of high levels of glucose on the secretion of the ANG independent of the PKC pathway. On the other hand, the inhibitory action of insulin on the secretion of the IR-rANG from IRPTC cells was blocked by PD98059 in a dose-dependent manner (Figure 5A) but was unaffected by Wortmannin (10^{-8} to 10^{-4} M) (Figure 5B). These studies suggest that the inhibitory action of insulin is probably mediated via the activation of MEK activity, but not via activation of phosphatidylinositol-3-kinase.

Effect of high glucose and insulin on the cellular ANG mRNA level in IRPTC

Figure 6 shows that a high glucose concentration (25 mM) stimulated the accumulation of the ANG mRNA. ANG mRNA levels were 2.0-fold higher than those found in control cells cultured in a medium containing 5 mM glucose (p≤ 0.05). Insulin

(10^{-7} M) completely inhibited the stimulatory effect of a high glucose (25 mM) on ANG mRNA in IRPTC. The addition of PD98059 blocked the inhibitory effect of insulin.

Effect of high glucose and insulin on the phosphorylation of MEK 1/2 and p44/42 MAP kinase in IRPTC

Figure 7 shows that insulin activated the phosphorylation of MEK 1/2 in IRPTC. The addition of PD98059 inhibited the phosphorylation of MEK 1/2. Similarly, PD98059 also inhibited the phosphorylation of p44/42 MAP kinase stimulated by insulin (Figure 8). These studies demonstrate that insulin is effective in activating the MAPK signal transduction pathways in IRPTC.

DISCUSSION

The present studies demonstrate that a novel action of insulin is to prevent the stimulatory effect of high levels of glucose on the expression of ANG gene in rat renal proximal tubular cells. This inhibitory action is mediated, at least in part, through activation of the MAPK signal transduction pathway.

While several previous reports have described the expression of renal RAS genes in experimental diabetes mellitus (22-24), conflicting results have been obtained from different groups. Studies of Correra-Rother et al (22) found that the renal renin protein and mRNA expression were not different between the diabetic and normal animals, but that renal and liver ANG mRNA levels were lower in the diabetic group. Kalinyak et al (23) reported that there were no significant differences in the expression of renal renal and ANG mRNA in rats two week after the induction of diabetes

compared to controls. In contrast, Anderson et al (24) reported a small increase in renal renin and ANG gene expression in rats 6 to 8 weeks after induction of diabetes. While there is no clear rationalization for these, one obvious difference is the duration of diabetes in experimental rats used by these investigators.

The secretion of IR-rANG from IRPTC was increased by 1.5-fold in the presence of a high glucose (25 mM) medium compared to a normal glucose (5 mM) medium (Figure 1A). This level of stimulation is similar to our previous studies which showed that a high level of glucose (25 mM) stimulated the expression of the rat ANG gene by 1.5-fold in OK cells (13) and in IRPTC (14). Studies by Chang and Perlman (25) have shown that insulin attenuated the expression of the ANG mRNA in rat hepatoma cells *in vitro*. More recently, Aubert et al (26) also demonstrated that insulin down-regulated ANG gene expression and secretion in cultured adipose tissue. Consistent with these findings, we have also observed that insulin inhibited the stimulatory effect of glucose on the secretion of IR-rANG in a dose-dependent manner (Figure 1A). These results, together with those of Chang and Perlman (25) and Aubert et al (26), suggest that insulin may down-regulate ANG gene expression. We did not observe any significant inhibition of the secretion of IR-rANG in IRPTC treated with various concentrations of IGF-I or IGF-II (Figure 1B), suggesting that the inhibitory action of insulin on the secretion of IR-rANG in IRPTC is specific for insulin and the insulin receptor.

The present studies show that PMA (10^{-7} M) stimulated the secretion of IR-rANG in IRPTC incubated either in a normal (5 mM) glucose medium (Figure 2A) or in a high (25 mM) glucose medium (Figure 2B), supporting the hypothesis that the effect of high glucose levels on the expression of the ANG gene is mediated via the protein kinase C

pathway (13,14). Indeed, the involvement of PKC in modulating the expression of ANG in IRPTC is confirmed by our recent studies where it was reported that the stimulatory effect of a high level (25 mM) of glucose on the expression of rat ANG gene in OK cells (13) and IRPTC (14) is blocked in the presence of inhibitors of PKC (i.e. staurosporine and H-7) and that PMA (10^{-7} M) increased the ANG mRNA level in IRPTC when incubated in 5 mM glucose medium (unpublished results). It is interesting that insulin blocked the stimulatory effect of PMA on the secretion of IR-rANG in IRPTC in a dose-depedent manner (Figure 2A and 2B). Whereas overnight incubation of IRPTC with a high dose of PMA (10^{-5} M) did not abolish the inhibitory effect of insulin on the secretion of IR-rANG but it did abolish the stimulatory effect of a lower dose of PMA (10^{-7} M) (Figure 3). These results are consistent with the notion that the prolonged exposure to PMA will downregulate the PKC activity and protein expression levels (27). While we do not understand the molecular mechanism(s) of the opposing effect of PMA and insulin on the secretion of IR-rANG from IRPTC, our observations raise the possibility that the inhibitory effect of insulin on the secretion of the ANG may be mediated downstream of the PKC signal transduction pathway or mediated via other signal transduction pathways.

It is also interesting to note that PD 98059 (an inhibitor of MEK (28)) at concentrations of 10^{-5} M or greater enhanced the stimulatory effect of 25 mM glucose (Figure 4A) and 25 mM glucose plus PMA (Figure 4B) on the secretion of the rANG. These results indicate that the stimulatory effect of high glucose (25 mM) and PMA on the secretion of ANG may be enhanced by inhibition of the MEK signal transduction pathway. Indeed, our results show that PD 98059 blocked the inhibitory effect of

insulin in a dose-dependent manner (Figure 5A), whereas the addition of Wortmannin (an inhibitor of phosphatidylinositol-3-kinase activity (29)) had no effect (Figure 5B). Furthermore, our preliminary studies (Zhang et al, unpublished results) showed that PMA (10^{-7} M) did not stimulate the phosphorylation of p44/42 MAP kinase in IRPTC. These data are consistent with the notion that the inhibitory effect of insulin is mediated, at least in part, via the MEK pathway and independent of PKC signal transduction pathway.

The effects of glucose and insulin on ANG gene expression appears to occur at the mRNA level. Exposure of IRPTC to a high glucose concentration (25 mM) significantly ($p \leq 0.05$) stimulated the expression of ANG mRNA (i.e. an increase of 2-fold) compared to expression in control cells (cultured in 5 mM glucose medium) (Figure 6). Insulin (10^{-7} M) completely blocked the stimulatory effect of 25 mM glucose. PD98059 reversed the inhibitory effect of insulin. At present, it is uncertain whether insulin decreases ANG mRNA levels at the transcriptional level or affects the stability of the ANG mRNA in IRPTC. Studies are ongoing in our laboratory to investigate these possibilities.

At present, we do not understand the exact molecular mechanism(s) of high glucose levels on the expression of the ANG gene in IRPTC. One possibility may be that high glucose levels may stimulate *de novo* synthesis of diacylglycerol (DAG) from metabolized glucose via the polyol pathway as suggested by Tilton et al (30) leading to an increase protein kinase C activity. Indeed, our recent studies showed that high levels of glucose increase the cellular levels of DAG and PKC activity in IRPTC (14). Once PKC is activated, it may phosphorylate the 43 kilodalton (kDa) cAMP-responsive

element binding protein (CREB) or CREB-like nuclear protein(s), since 43 kDa CREB contains the site of phosphorylation by protein kinase C (31). Moreover, recent studies by Kreisberg et al (32) have shown that PMA and high glucose levels stimulate the phosphorylation of 43 kDa CREB. Phosphorylated CREB then binds to the cAMP-responsive element (CRE) of the rat ANG gene (TGACGTAC, nucleotides N-795 to N-788) (33). Subsequently, the bound CREB stimulates the expression of the rat ANG gene. This possibility is supported by our recent studies which demonstrated that the transient transfection of 43 kDa CREB into OK cells stimulates the expression of rat ANG gene promoter activity (34) and that ANG-CRE binds with the 43 kDa-CREB (35).

Similarly, we do not understand the precise molecular mechanism(s) of action of the inhibitory effect of insulin on the expression of ANG gene in IRPTC. One possibility might be that insulin activates the MAP kinase signal transduction pathway as shown in Figures 7 and 8 and induces the phosphorylation or expression of certain protein(s). The insulin-induced protein(s) then suppress(es) the expression of the ANG gene via a yet undefined pathway. Indeed, recent studies have shown that insulin induces c-Fos expression via MAP kinase but not PI-3 kinase in 32 D mouse myeloid progenitor cells (36) and in vascular smooth muscle cells (37), suggesting that MAP kinase signal transduction pathway is important for insulin action. Whether insulin will also induce the expression of c-Fos gene in IRPTC, remains, however, to be studied. Alternatively, the inhibitory effect of insulin may be mediated, at least in part, via an insulin-responsive element (IRE) in the 5'-flanking region of the ANG gene. Several studies have reported that insulin stimulates and/or inhibits the gene expression via the putative IRE in the 5'-flanking region of various genes (38-40). To our best knowledge,

the IRE in the rat ANG gene has not yet been identified. Studies along these lines, however, are underway in our laboratory.

It is unlikely that the inhibitory action of insulin on the expression of the ANG gene is mediated via the PI-3 kinase signal transduction pathway, because Wortmannin failed to block the inhibitory effect of insulin on the secretion of the ANG from IRPTC (Figure 5).

In summary, our studies demonstrate that the exposure of IRPTC to 25 mM glucose directly stimulates the expression of the rat ANG gene in IRPTC. This stimulatory effect of high glucose was blocked by insulin via the MAPK kinase signal transduction pathway. These findings raise the possibility that the expression of the renal ANG gene may be stimulated by hyperglycemic states *in vivo*. Consequently, the increased local formation of renal Ang II may contribute to renal remodeling (i.e. renal hypertrophy observed in early diabetes). Insulin therapy may therefore attenuate this event by inhibiting the activation of local renal RAS. Furthermore, chronic treatment with inhibitors of angiotensin-converting enzyme (ACE) and AT_1-angiotensin II (Ang II) receptor have also been shown to delay the onset and the development of nephropathy in patients with insulin-dependent diabetic mellitus (41-45). These studies raise the possibility that a combination of both insulin and ACE-inhibitor(s) or AT_1-receptor antagonists may be a more effective therapy than the treatment with either insulin or ACE-inhibitor alone. Therfore, this therapeutic approach should be explored in experimental animals and in patients with diabetic mellitus.

ACKNOWLEDGEMENTS

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LEGENDS TO FIGURES

Figure 1: (A) Inhibitory effect of insulin on the secretion of immunoreactive rat angiotensinogen (IR-rANG) from immortalized renal proximal tubular cells (IRPTC). Cells were incubated for 24 hours in the presence of 5 mM glucose, 25 mM glucose, or 25 mM glucose plus various concentrations of insulin. Media were harvested and assayed for the level of IR-rANG. The levels of IR-rANG in the medium containing 5 mM glucose are expressed as 100% (control, i.e. 3.05 ± 0.21 ng/ml/10^6 cells). The inhibitory effect of insulin is compared to those cells that were incubated in 25 mM glucose (without the presence of insulin). Results are expressed as the percentage of controls (mean $\pm$ S.D., N=3) (**$p \leq 0.01$ and ***$p \leq 0.005$). (B) Lack of effect of insulin-like growth factor-I or insulin-like growth factor-II on the secretion of IR-rANG in IRPTC incubated with 25 mM glucose. Media were harvested and assayed for the level of IR-rANG. The levels of IR-rANG in the medium containing 25 mM glucose in the absence of IGF-I or IGF-II are expressed as 100% (control, i.e. 4.75 ± 0.25 ng/ml/10^6 cells). The effect of IGF-I or IGF-II is compared with those cells that were incubated in 25 mM glucose (without IGF-I or IGF-II). Results are expressed as the percentage of control (mean $\pm$ S.D., N=3). The blank bar represents cells incubated in the presence of IGF-I and the solid bar represents the cells incubated in the presence of IGF-II. Similar results were obtained in two other experiments.

Figure 2: Inhibitory effect of insulin on the secretion of immunoreactive rat angiotensinogen (IR-rANG) from immortalized renal proximal tubular cells (IRPTC) challenged with phorbol 12-myristate 13-acetate (PMA). Cells were incubated for 24 hours in the presence of 5 mM glucose and PMA (10^{-7} M) (A) or 25 mM glucose and PMA (10^{-7} M) (B) plus various concentrations of insulin. The levels of IR-rANG in the medium containing 5 mM glucose (i.e. 2.75 ± 0.01 ng/ml/10^6 cells in A and 3.17 ± 0.03 ng/ml/10^6 cells in B) are expressed as 100% (control). The inhibitory effect of insulin is compared with cells that were incubated in the presence of PMA (10^{-7} M) (without insulin). Results are expressed as the percentage of controls (mean ± S.D., N=3) (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). Similar results were obtained in three other experiments.

Figure 3: Effect of a 24 hour preincubation with PMA (10^{-5} M) on the secretion of immunoreactive rat angiotensinogen (IR-rANG) from immortalized renal proximal tubular cells (IRPTC). After the preincubation of 24 hours in the presence of 25 mM glucose and 10^{-5} M PMA, the cells were incubated in fresh medium containing insulin (10^{-11} to 10^{-5} M) or 10^{-7}M PMA for another 24 hours. The media were then harvested and assayed for IR-rANG. Results are expressed (mean ± S.D., N=3) as a percentage of the levels found in media incubated with 25 mM glucose without insulin and PMA (i.e. 7.2 ± 0.72 ng/ml/10^6 cells) (*$p \leq 0.05$ and **$p \leq 0.01$). Similar results were obtained in two other experiments.

Figure 4:   Effect of PD98059 on the secretion of IR-rANG from IRPTC. IRPTC were incubated for 24 hours in the presence of 5 mM glucose, 25 mM glucose or 25 mM glucose plus PD98059 (10^{-11} to 10^{-5} M) (A) or 5 mM glucose, 25 mM glucose, 25 mM glucose and PMA 10^{-7} M plus various concentrations of PD98059 (10^{-7} to 10^{-4} M) (B). The levels of IR-rANG in the medium containing 5 mM glucose (i.e. 2.87 ± 0.34 ng/ml/10^{6} cells in A and 2.36 ± 0.02 ng/ml/10^{6} cells in B) are expressed as 100% (control). The effect of PD98059 is compared with cells that were incubated in 25 mM glucose (A) or 25 mM glucose plus PMA (10^{-7} M) (B). Results are expressed as the percentage of controls (mean $\pm$ S.D., N=3) (**$p \leq 0.01$ and ***$p \leq 0.005$). Similar results were obtained in two other experiments.

Figure 5:   Effect of PD98059 and Wortmannin on the secretion of IR-rANG from IRPTC in the presence of insulin. Treatment groups include cells incubated for 24 hours in the presence of 5 mM glucose, 25 mM glucose and 25 mM glucose plus 10^{-7} M insulin with or without PD98059 (A) or Wortmannin (B). Media were harvested and assayed for the level of IR-rANG. The effect of PD98059 or Wortmannin is compared with cells that were incubated in 25 mM glucose medium and in the presence of insulin (10^{-7} M). Results are expressed (mean $\pm$ S.D. with N=3) as a percentage of the levels found in media from cells incubated with 5 mM glucose (i.e. 4.4 ± 0.1 ng/ml/10^{6} cells) (**$p \leq 0.01$ and ***$p \leq 0.005$). Similar results were obtained in two other experiments.

Figure 6: Effect of glucose and insulin on the expression of rat angiotensinogen mRNA in immortalized renal proximal tubular cells. After a 24 hour incubation period in media with 5 mM glucose, 25 mM glucose, 25 mM glucose plus insulin (10^{-7} M) in the absence or presence of PD98059 (10^{-5} M), cells were harvested and assayed for ANG mRNA levels with a RT-PCR assay. The DNA fragments of the RT-PCR reaction mixture were separated on a 1.5% agarose gel and then transfered onto a nylon membrane. Subsequently, the membrane was blotted with a ^{32}P-labeled oligonucleotide corresponding to the nucleotide N+775 to N+798 of rat ANG and N+9 to N+35 of exon 4 of rat β-actin, respectively. The relative densities of the PCR band of ANG were compared with the β-actin control (A). The level of rANG mRNA in the cells normalized in 5 mM glucose (i.e. ratio of 0.94 ± 0.03: ANG/β-actin) was considered as control (100%) (B). Each point represents the mean ± S.D. of three dishes (*p≤ 0.05). Similar results were obtained from another experiment.

Figure 7: Effect of glucose and insulin on the phosphorylation of MEK 1/2 in immortalized renal proximal tubular cells (IRPTC). After a 24 hour incubation period in 5 mM glucose, cells were incubated in 5 mM glucose, 25 mM glucose, 25 mM glucose and PD98059 for 10 minutes. Then, insulin (10^{-7} M) was added and further incubated for 15 minutes. Cells were harvested and assayed for the phosphorylated MEK 1/2 by employing the Phospho Plus MEK 1/2 antibody kit. The relative densities fo the

phosphorylated MEK 1/2 in the cells incubated in 5 mM glucose media was considered as control (100%). C, control phosphorylated MEK 1/2 supplied by the supplier. Each point represents the mean ± SD of three dishes (*p≤ 0.05, **p≤ 0.01 and ***p≤ 0.005). Similar results were obtained in three other experiments.

Figure 8: Effect of glucose and insulin on the phosphorylation of p44/42 MAP kinase in immortalized renal proximal tubular cells (IRPTC). After a 24 hour incubation period in 5 mM glucose, cells were incubated in 5 mM glucose, 25 mM glucose, 25 mM glucose and PD 98059 for 10 minutes. Then, insulin (10^{-7} M) was added and further incubated for 15 minutes. Cells were harvested and assayed for the phosphorylated p44/42 MAP kinase by employing the Phospho Plus p44/42 MAP kinase antibody kit. The relative densities for the phosphorylated p44/42 MAP kinase in the cells incubated in 5 mM glucose media was considered as control (100 %). C, control phosphorylated p44/42 AP kinase supplied by the supplier. Each point represent the mean ± SD of three dishes (*p≤ 0.05, **p≤ 0.01 and ***p≤ 0.005). Similar results were obtained in another experiment.
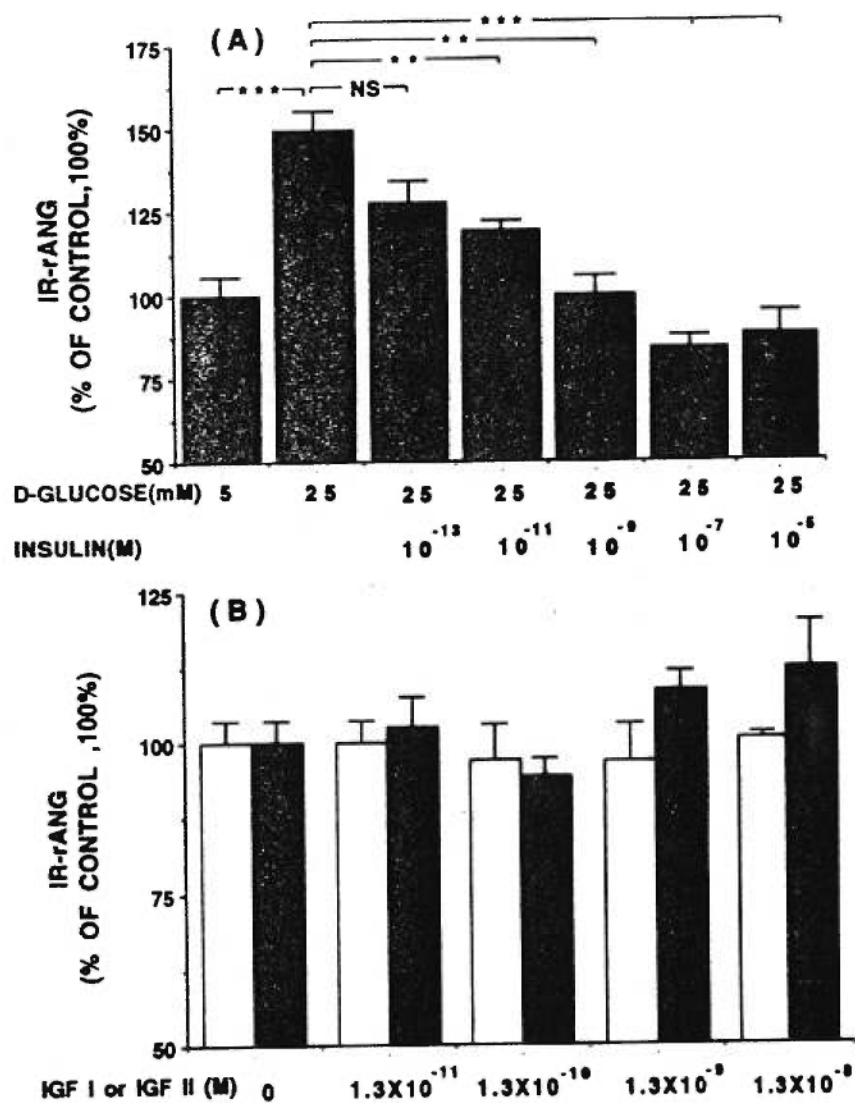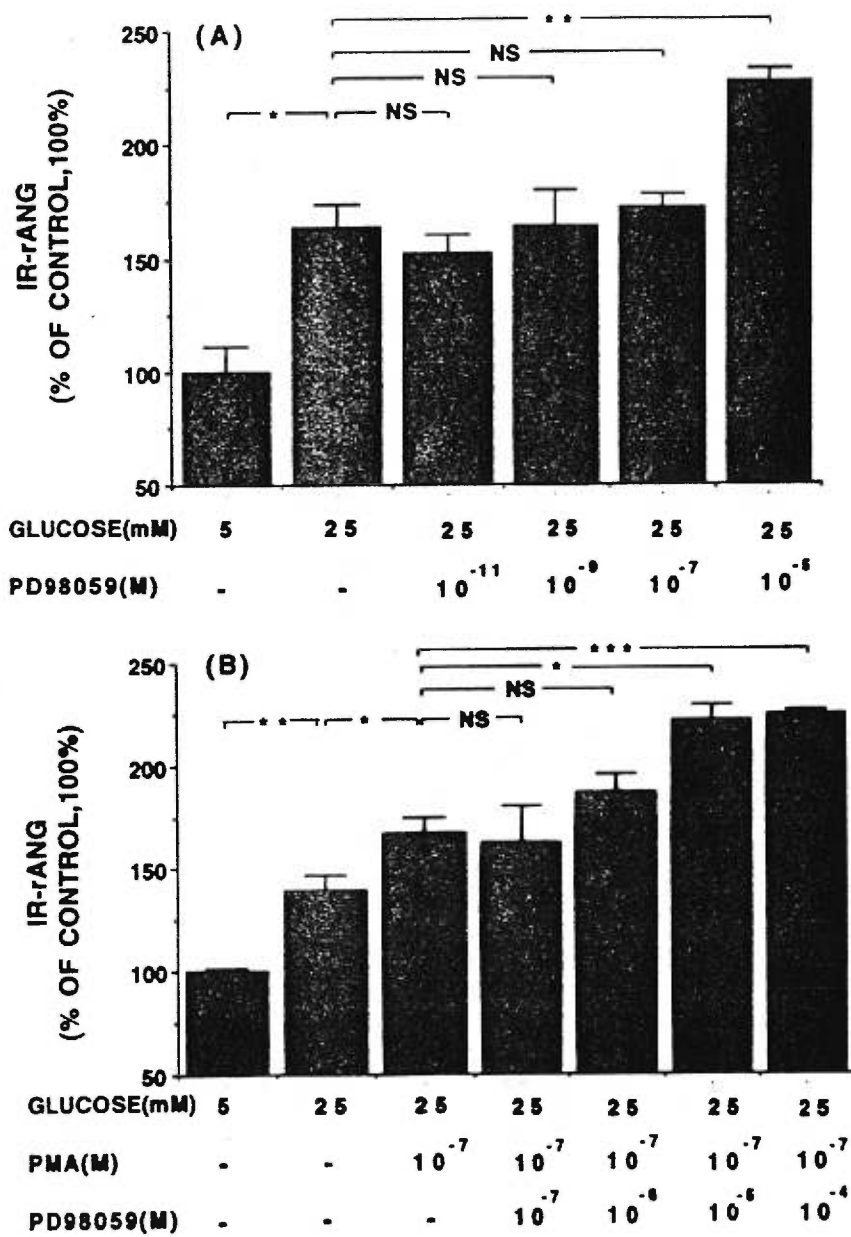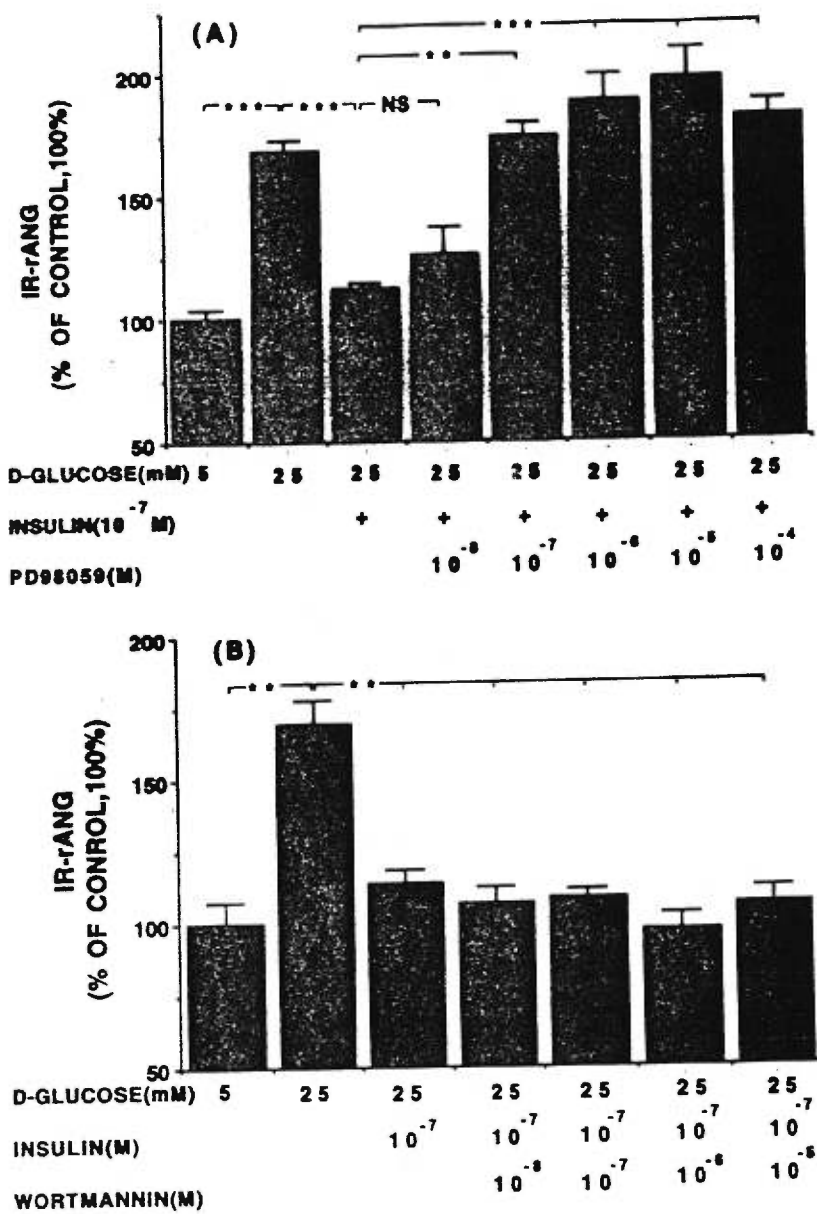
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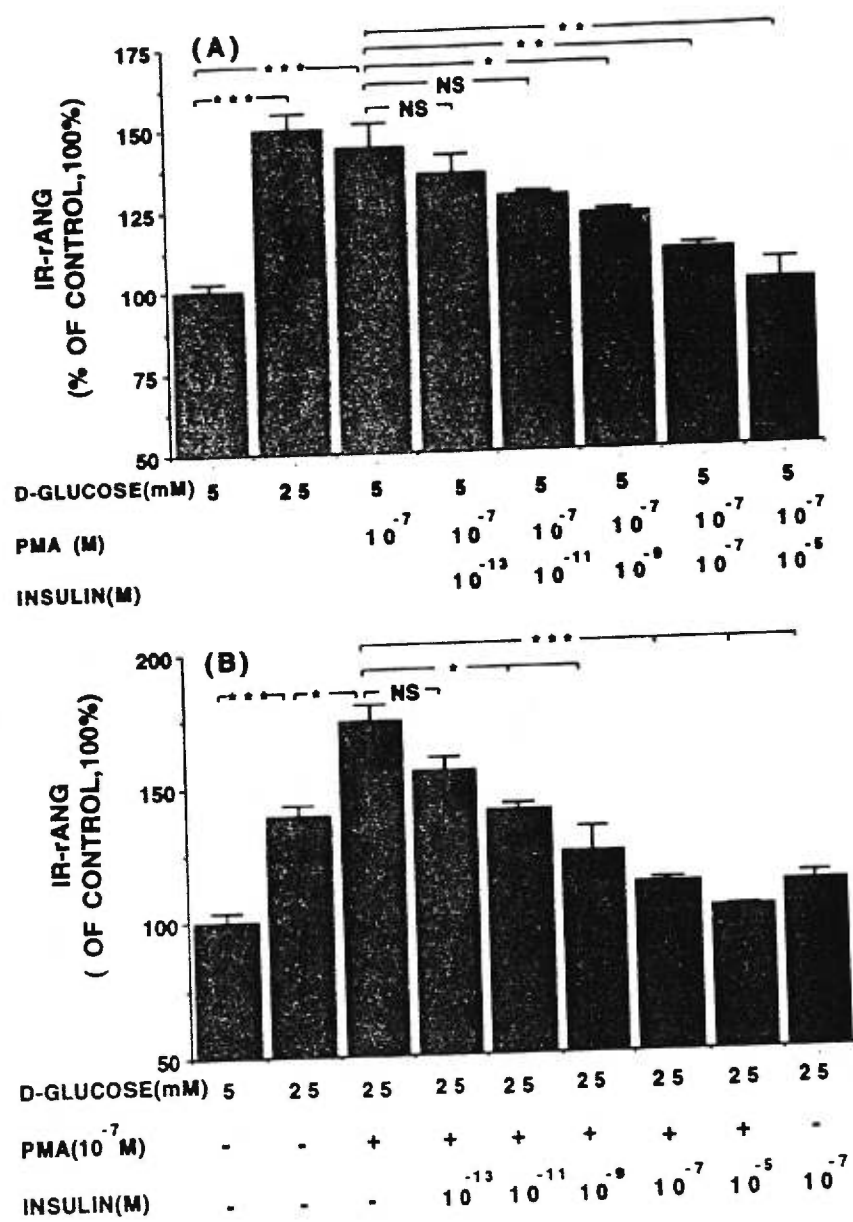
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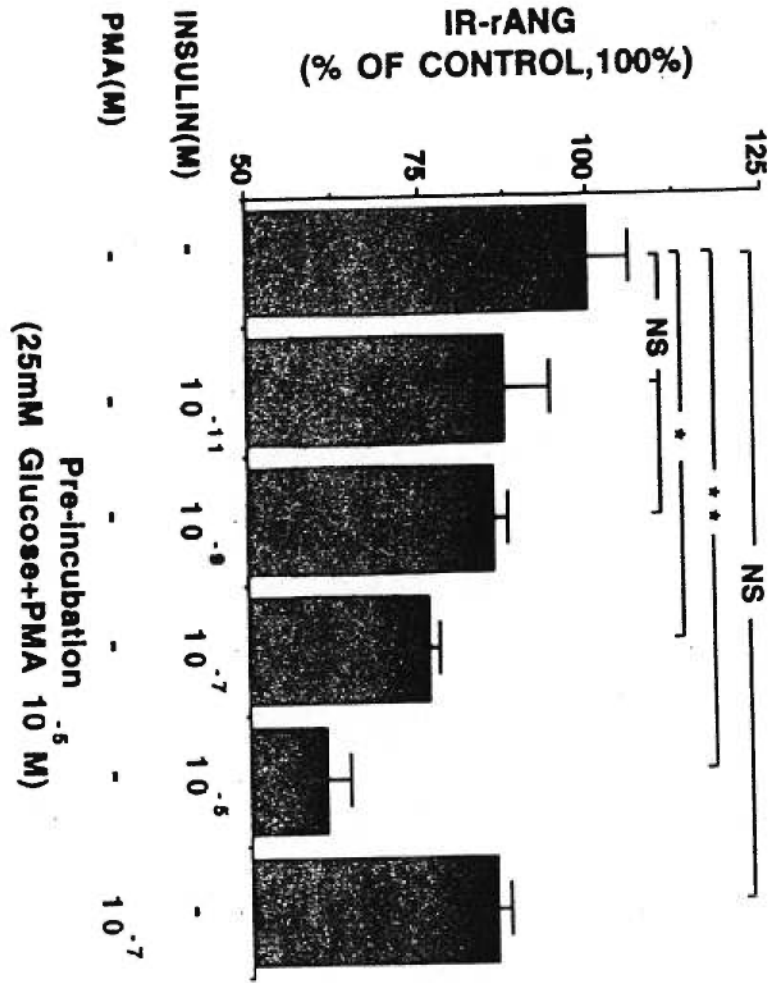
Figure 3

Figure 4

Figure 5

Figure 6

(A)
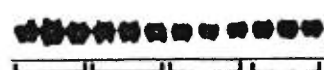
				P-MEK 1/2
				C
D-GLUCOSE (mM)	5	25	25	25
INSULIN (10⁻⁷M)	-	-	+	+
PD98059 (10⁻⁵M)	-	-	-	+

(B)

D-GLUCOSE(mM)	5	2 5	2 5	2 5
INSULIN(M)	-	-	10^{-7}	10^{-7}
PD98059(M)	-	-	-	10^{-8}

Figure 7

Figure 8