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Effect of High Levels of Glucose and Insulin on the Expression of the Human Angiotensinogen Gene in an Opossum Kidney Proximal Tubular Cell Line

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Ce mémoire intitulé

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Sommaire

Dans le but d'étudier l'effet de taux élevés de glucose et d'insuline sur l'expression de l'activité du promoteur du gène humain de l'angiotensinogène (hANG) dans les cellules de tubules proximaux du rein, nous avons construit des gènes de fusion contenant la séquence régulatrice 5' flanquante du gène de l'ANG lié soit au gène du facteur de croissance (hGH) (pOGH(hANG N-1064/+27)) soit au gène d'origine bactérienne, le chloramphénicol acétyl transférase (CAT) (pOCAT(hANG N-1064/+27)) en tant que gènes rapporteurs. Àprès avoir introduit le gène de fusion dans les cellules de tubules proximaux de rein d'opossum (OK), plusieurs transformants stables ont été obtenus.

L'expression des gènes de fusion (pOGH(hANG N-1064/+27)) et (pOCAT(hANG N-1064/+27)) a été évaluée par la présence de l'hGH immuno-réactive sécrétée dans le milieu et par l'activité enzymatique cellulaire de CAT, respectivement. L'addition de taux élevés de D(+)-glucose (25mM) ou de phorbol 12-myristate 13-acétate (PMA) a stimulé de manière dose dépendante l'expression du gène de fusion dans les cellules OK, tandis que l'addition de D-mannitol, de L-glucose et de 2-déoxy-D-glucose (25mM) n'a eu aucun effet. L'effet stimulant du D(+)-glucose (25mM) a été bloqué par l'insuline, la stausporine (un inhibiteur de la protéine Kinase C), le H-7 (un inhibiteur de la protéine Kinase C) et le Tolrestat (un inhibiteur de l'aldolase réductase), mais n'a pas été bloqué par le facteur de croissance-I de l'insuline (IGF-I) et le facteur de croissance-II de l'insuline (IGF-II). L'addition de Tolrestat a inhibé l'augmentation cellulaire de diacylglycérol (DAG) et l'activité enzymatique de la protéine Kinase C stimulés par les hauts taux de glucose, tandis que l'insuline n'a eu aucun effet. L'addition d'insuline a aussi bloqué l'effet stimulant du PMA sur l'expression du gène de fusion dans les cellules OK. Finalement, l'effet inhibiteur de l'insuline sur l'expression du gène de fusion a été bloqué par le PD98059 (un inhibiteur de la MAP Kinase kinase (MEK), mais non par le Wortmannin (un inhibiteur de la phosphatidylinositol-3-Kinase (PI-3-Kinase).

Cette étude démontre que l'effet stimulant de hautes concentrations de D(+)-glucose (25mM) sur l'expression de l'activité promotrice du gène humain de l'angiotensinogène dans les cellules OK est relayée via un mécanisme de signalisation intracellulaire impliquent la protéine Kinase C. De plus, l'addition d'insuline a bloqué l'effet stimulant de hauts taux de D(+)-glucose (25mM) via le mécanisme de signalisation intracellueaire de la MAP Kinase (MAPK). Nos résultats indiquent que l'insuline inhibe l'activation du système rénine-angiotensine (RAS) dans les cellules de tubules proximaux du rein stimulées par de hauts taux de glucose *in vivo*.

Abstract

To investigate the effect of high levels of glucose and insulin on the expression of the human angiotensinogen (hANG) gene promoter activity in kidney proximal tubular cells, we constructed fusion genes containing the 5'-flanking regulatory sequence of the human angiotensinogen gene fused with either the human growth hormone (hGH) gene (pOGH(hANG N-1064/+27)) or with the coding sequence of the bacterial chloramphenicol acetyltransferase (CAT) gene (pOCAT(hANG N-1064/+27)) as a reporter and stably integrated into the opossum kidney proximal tubular cell (OK) genomes.

The level of expression of pOGH(hANG N-1064/+27) and pOCAT(hANG N-1064/+27) was quantified by the amount of immunoreactive human growth hormone (IR-hGH) secreted into the medium and the cellular enzymatic CAT activity, respectively. The addition of a high level of D(+)-glucose (25 mM) or phorbol 12-myristate 13-acetate (PMA) stimulated the expression of the fusion gene in OK cells in a dose-dependent manner, 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 insulin, staurosporine (an inhibitor of protein kinase C), H-7 (an inhibitor of protein kinase C) and Tolrestat (an inhibitor of aldose reductase) but was not blocked by insulin-like growth factor-I (IGF-I) and insulin-like growth factor-II (IGF-II). The addition of Tolrestat inhibited the increase of cellular diacylglycerol (DAG) and protein kinase C enzymatic activity stimulated by high levels of glucose, whereas insulin had no effect. The addition of insulin also blocked the stimulatory effect of PMA on the expression of the fusion genes in OK cells. Finally, the inhibitory effect of insulin on the expression of the fusion gene was blocked by PD98059 (an inhibitor of MAP kinase kinase (MEK)), but not by Wortmannin (an inhibitor of phosphatidylinositol-3-kinase (PI-3 kinase)).

These studies demonstrate that the stimulatory effect of a high D(+)-glucose concentration (25 mM) on the expression of the human angiotensinogen gene promoter activity in OK cells is mediated via the protein kinase C signal transduction pathway. Moreover, the addition of insulin blocked the stimulatory effect of a high level of D(+)-glucose (25 mM) via the MAP kinase (MAPK) signal transduction pathway. Our data indicate that high levels of D(+)-glucose increase, whereas insulin inhibits the activation of the renin-angiotensin-system (RAS) in the renal proximal tubular cells stimulated by a high level of glucose *in vivo*.

List of Abbreviations

ACE	Angiotensin converting enzyme
AGE	Advanced glycosylation end-products
ANG	Angiotensinogen
Ang I	Angiotensin I
Ang II	Angiotensin II
Ang III	Angiotensin III
AR	Aldose reductase
ARI	Aldose reductase inhibitor
AT_1	Angiotensin II type I receptor
AT_2	Angiotensin II type II receptor
CAT	Chloramphenicol acetyltransferase
CRE	cAMP responsive element
DAG	1,2-diacylglycerol
DHAP	Dihydroxyacetone phosphate
DM	Diabetes mellitus
DN	Diabetic nephropathy
ERK	Extracellular regulated protein kinase
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GH	Growth hormone
GIRE	Glucose responsive element
GR	Glucocorticoid receptor
GRE	Glucocorticoid responsive element
hANG	Human angiotensinogen
hGH	Human growth hormone
IDDM	Insulin-dependent diabetic mellitus

IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor-binding protein
IP_3	Inositol 1,4,5-triphosphate
IRE	Insulin responsive element
JG	Juxtaglomerular
mRNA	Messenger RNA
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
MAPKK (MEK)	Mitogen activated protein kinase kinase
NIDDM	Non-insulin-dependent diabetic mellitus
NO	Nitric Oxide
OK	Opossum kidney
PCR	Polymerase chain reaction
PEPCK	Phosphoenolpyruvate carboxykinase
PI	Phosphatidylinositol
PIP	PI-4-phosphate
PIP_2	Phosphatidyl-4,5-biphosphate
PIP3	PI-3,4,5-triphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
rANG	Rat angiotensinogen
RAS	Renin-angiotensin system
RIA	Radioimmunoassay
TGF-β	Transforming growth factor-β
TK	Tyrosine kinase

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I. Introduction

I.1. Diabetic Nephropathy

I.1.1. Characteristics of Diabetic Nephropathy

Diabetic nephropathy (DN) is an important microvascular complication of both insulin-dependent and non-insulin-dependent diabetes mellitus. It is the leading cause of end-stage renal disease (Groggel GC., 1996). It constitutes a major cause of morbidity and mortality among diabetic patients (Andersen AR. et al., 1983). It is estimated that approximately 30-50% of the diabetic patients in North America will eventually develop chronic renal failure (or end stage renal failure) (Parving HH.et al., 1996b).

DN is characterized by arterial hypertension, proteinuria, and progressive loss of renal function. The morphologic feature of DN is glomerular basement membrane thickening and mesangial expansion (Groggel GC., 1996).

The early feature of DN is renal hypertrophy of both glomerular and tubular elements, characterized by thickening of the glomerular and tubular basement membranes, intraglomerular hyperperfusion and hypertension, microalbuminuria, cell enlargement and an accumulation of extracellular matrix (Fine L., 1986; Ziyadeh FN., 1993). In addition, tubulointerstitial fibrosis and renal arteriolosclerosis become apparent during the progression of the disease, and they also correlate with the severity of renal failure (Ziyadeh FN., 1993).

I.1.2. Mechanisms of the Pathogenesis of Diabetic Nephropathy

The pathogenesis of DN is still not fully understood but is likely multifactorial. Intrarenal hemodynamic alterations, metabolic abnormalities and various growth factors and genetic factors

are all thought to be involved in the pathogenesis of DN (Hostetter TH., 1994). A number of factors affect the development of nephropathy, including racial and genetic susceptibility, blood pressure, other intrarenal hemodynamic factors, and metabolic control (Mark EM., 1997). High glucose (reviewed by Wardle FN., 1996), advanced glycation end products (AGEs) (Tsuji H. et al., 1998), as well as growth promoters such as transforming growth factor β (TGF-β) (reviewed by Hoffman BB. et al., 1998), insulin-like growth factor I (IGF-I) (Sugimoto H. et al., 1995), ammonia (Ling H. et al., 1996b) and angiotensin II (Ang II) have been reported to be involved in the development of DN (Ling H. and Heidland A., 1996a).

I.1.2.1. Role of Hyperglycemia

Hyperglycemia is an important etiologic factor in the development of diabetic nephropathy (Raskin P. and Rosenstock J., 1986). The important role of hyperglycemia in the genesis of diabetic renal disease has been strengthened by tissue culture studies, experimental animal models, and clinical trials (Kumar S. and Fuad NZ., 1997). The Joslin Clinic found a strong correlation between the incidence of proteinuria and the degree of hyperglycemia (Krolewski AS. et al., 1985a; 1985b). Recent studies in tissue culture are in support of the belief that elevated ambient glucose levels exert important influences on renal cell growth and extracellular matrix metabolism (Kumar S. and Fuad NZ., 1997). Glucose leads to the expression of TGF-β and cytokine, as well as formation of advanced glycosylation end products (AGEs) which could induce the structural and functional alteration of matrix proteins that have biological effects on various kinds of cells including mesangial cells (Sugiyama S. et al., 1996).

I.1.2.2. Role of Growth Factors

It has been recently recognized that several systemic or intrarenal networks of cytokines, hormones, and growth factors can be modulated as a consequence of abnormal glucose homeostasis and that such effects may play a key role in the induction and progression of DN

(Ziyadeh FN., 1993). Possible mechanisms of these changes could be the direct effects of growth factors such as IGF-I and Ang II (New JP. et al., 1996).

Recent reports indicate that hyperglycemia, increased intraglomerular pressure, and glycated proteins potentially induce overproduction of TGF-β in diabetes. TGF-β stimulates production of extracellular matrix components such as collagen IV, fibronectin, proteoglycans (decorin and biglycan). TGF-β overproduction leads to glomerulosclerosis and TGF-β is a causal factor in myointimal hyperplasia after balloon injury of the carotid artery. It also mediates the modulator effect of Ang II on smooth muscle cell growth. These findings indicate that TGF-β overproduction is a common pathogenic step explaining the well-known association between micro- and macrovascular complications in diabetic patients. TGF-β antagonists, such as decorin, betaglycan, and possibly also heparin, might be potential candidates for future therapy to prevent diabetic vascular disease (Yokoyama H. and Deckert T., 1996).

I.1.2.3. Role of Hemodynamic Alterations

Of the many risk factors identified in the pathogenesis of nephropathy, hemodynamic alterations have been particularly well studied. Increases in glomerular filtration rate, largely driven by increases in plasma flow and glomerular capillary pressure, are apparent in early IDDM and NIDDM. Furthermore, the elevation in capillary pressure may be damaging to glomerular endothelial, epithelial and mesangial cells, thereby initiating and contributing to the progression of DN (O'Bryan GT. and Hostetter TH., 1997).

I.1.2.4. Role of Hypertension

Studies strongly suggest that hypertension is involved in the progression and perhaps the onset of DN (Bretzel RG., 1997). Some antihypertensive agents have demonstrated abilities to reduce urinary albumin excretion either with or without a reduction in systemic arterial pressure

(Weir MR., 1996). Clinical trials have demonstrated that antihypertensive treatment is effective in DN (Parving HH. et al., 1983). Normalization of blood pressure in hypertensive IDDM patients is most important to minimize the loss of kidney function (Baba T. et al., 1997). Experimental evidence suggests that an improvement in intrarenal hemodynamics (reduction in glomerular capillary pressure due to selective efferent arteriolar dilation) and diminished permeability of the glomerular basement membrane to proteins may contribute to the antiproteinuric activity of these drugs (Weir MR., 1996).

I.1.2.5. Role of Renin-Angiotensin System (RAS)

The renin angiotensin system (RAS) has an important role in the physiologic regulation of the renal microcirculation. In conjunction with other vasoactive systems, the RAS may contribute to the imbalance of resistances present at the preglomerular and postglomerular sites which are responsible for glomerular capillary hypertension, a major injurious factor in the diabetic kidney (Correa-Rotter R. et al., 1992). Ang II exerts many hemodynamic as well as nonhemodynamic effects on renal cells that may contribute to the progression of DN. Studies showed that Ang II induces hypertrophy in cultured proximal tubular cells by enhancing protein synthesis and cell size as well as inhibiting collagenolytic and gelatinolytic activities. Moreover, the observations that ACE inhibitors and Ang II receptor antagonists decrease proteinuria and slow the progression of DN, suggesting that glomerular hemodynamics and the local RAS play important roles in the progression of DN (Lewis EJ. et al., 1993; Remuzzi A. et al., 1993). Thus, it has been suggested that the functional and morphologic changes observed in the diabetic kidney may be, in part, secondary to the activation of the intrarenal RAS (Anderson S. et al., 1986a).

I.1.2.6. Role of Genetic Factors

Recent studies suggest that genetic predisposition plays a role in the development of DN which clusters within families, both in IDDM and NIDDM. Epidemiologic and family studies have

demonstrated that only a subset of the patients develop DN, that family clustering of nephropathy is present, and that ethnicity plays an important role in the risk of developing this kidney disease, supporting the hypothesis that genetic predisposition or factors may contribute to the development of DN (Parving HH. et al., 1996a).

The candidate genes for DN have so far been dealing with 1) genes involved in the pathogenesis of hypertension, i.e. genes involved in RAS and cation transport system, and 2) genes coding for components of the glomerular basement membrane and factors regulating mesangial proliferation and extracellular matrix production, i.e. genes coding for collagen IV, N-deacetylase, IL-1 and IL-1 receptor (Ha SK. and Seo JK., 1997).

In experimental and human DN, systemic and glomerular hypertension play roles in the initiation and progression of nephropathy. These hemodynamic changes may be explained in part by alterations in the RAS. Consequently, genes involved in the RAS have been suggested as potential genetic predisposition markers for the development of DN (Ha SK. and Seo JK., 1997).

The most recent results obtained by Rogus JJ. et al. (1998)' family-based study indicated a role of the ANG gene M235T polymorphism, and specifically the T allele is involved in the development of DN in IDDM.

Recent studies have suggested an association between a deletion (D) variant of the ACE gene and DN. ACE (D) genotype was found to have higher urinary albumin levels in a case-control study performed by Dudley CR. et al. (1995). Yoshida H. et al (1996)'s study on ACE polymorphism showed that, in NIDDM, the ACE (D) genotype has a high prognostic value for progressive deterioration of renal function. The deletion polymorphism in the ACE gene reduces the long term beneficial effect of ACE inhibition on the progression of DN in patients with insulin dependent diabetes (Parving HH. et al., 1996c). However, studies by Ringel J. et al. (1997) showed that there was no significant association between the ACE and ANG genotype and

hypertension.

Doria A. et al. (1997) investigated the contribution of polymorphisms in the Ang II type 1 receptor gene (AGTR1). IDDM patients were genotyped for two AGTR1 polymorphisms (T573 to C, and A1166 to C). Their results showed that DNA sequence differences in the AGTR1 gene may modify the noxious effect of hyperglycemia on the kidney. Allele C1166 carriers might especially benefit from nephropathy prevention programs. However, Studies by Chowdhury TA. et al (1997) in the United Kingdom showed that there is no significant association between the hypertension associated AT_1R gene polymorphism and diabetic nephropathy in patients with IDDM.

I.1.3. Therapy for Diabetic Nephropathy

Recently, several major therapeutic interventions have been demonstrated to help prevent the development of, or slow the progression of DN, including blood sugar control, blood pressure control, treatment with ACE inhibitors and dietary protein restriction. The use of aldose reductase inhibitors, the use of drugs that prevent the formation of AGEs, and the use of Ang II receptor antagonists may also be of benefit (Breyer JA. et al., 1995).

Antihypertensive treatment is an effective tool in slowing the progression of early and advanced DN (Arauz-Pacheco C. and Raskin P., 1996). Recent publications support the hypothesis that ACE inhibitors have a unique ability, independent of their antihypertensive effect, to slow the progression of DN (Foote EF., 1995).

Attempts to correct the metabolic abnormalities derived from diabetes are a new topic in the treatment of DN. The effects of HMG CoA reductase inhibitors (antihypercholesterolemic drugs), aldose reductase inhibitors (inhibitors of the polyol pathway) and glycation inhibitors (inhibitors of formation of advanced glycosylation end-products) on DN have been evaluated in animal studies and in some clinical trials (Baba T. et al., 1997). Thus far, results with HMG CoA reductase and

aldose reductase inhibitors have been somewhat conflicting. The potential therapeutic role of glycation inhibition in the treatment of diabetes deserves further study (Baba T. et al., 1997)

Hyperglycemia is one of the most important etiologic factors for DN. Studies on the metabolism and regulation of glucose have made much progress in the past decade.

I.2. Glucose

I.2.1. Glucose Biochemistry

Glucose is widespread in living organisms, in which, with protein and fat, it completes with the triad of the major metabolic fuels. Glucose also constitutes a building block for structural and enzymatic components of cells as well as the extracellular matrix (Ferrannini E. and DeFronzo RA., 1997). In brain, liver, kidney, intestine and placenta, glucose utilization is insulin independent; in adipose tissue, skeletal and heart muscle, glucose uptake depends on insulin (Ferrannini E. and DeFronzo RA., 1997).

As a metabolic substrate, glucose is present in organisms essentially in its simple, monomeric form (α-D-glucopyranose), and as a branched polymer of α-glucose, namely glycogen. Disaccharides of glucose (lactose, maltose, and sucrose) are quantitatively less important (Ferrannini E. and DeFronzo RA., 1997).

Insulin and glucagon represent the two major antagonist hormones involved in the control of glucose metabolic pathways. In the fasting state, glucagon is released to activate glycogenolysis and gluconeogenesis, whereas when insulin secretion falls, it allows the maintenance of stable glycemia despite a lack of exogenous glucose. Such an accurate control mechanism implies that both synthesis and secretion of insulin and glucagon are closely regulated to match fuel production and delivery to meet metabolic demands (Dumonteil E. and Philippe J., 1996).

I.2.2. Glucose Transportation and Metabolism

The transport of glucose to the interior of most of the tissue cells is facilitated by diffusion (Guyton GC and Hall JE., 1996). Glucose transport in mammalian tissues is mediated by a family of structurally related but genetically distinct glucose-transporter proteins (Kahn BB. and Filer JS., 1990). Five different glucose transporters have so far been identified and characterized. They reside in the plasma membrane (and in microsomal membranes), and can specifically and reversibly bind glucose molecules and transfer them across plasma cell membranes in both directions. Present studies indicate that only one type of glucose transporter (termed Glut4) shows clear sensitivity to acute insulin stimulation *in vivo,* and it is this transporter that is abundantly expressed in the classic insulin-sensitive tissues (adipocyte, brown fat, skeletal, heart and smooth muscle) (Kahn BB. and Flier JS., 1990).

Factors that regulate glucose-transporter gene expression in vitro include oncogenes, growth factors, insulin, oral hypoglycemic agents, vanadate, glucocorticoids, ambient glucose levels, and the state of cellular differentiation. In vivo, glucose-transporter gene expression in adipose cells, skeletal muscle, and liver is markedly affected by various altered nutritional and metabolic states (Kahn BB. and Flier JS., 1990).

Immediately on entry into the cells, glucose will be phosphorylated into glucose-6-phosphate. This phosphorylation is promoted mainly by the enzyme glucokinase in the liver or hexokinase in most other cells. Thereafter, it can take one of the two pathways: it can either go through glycolysis and the Krebs cycle to produce ATP, CO_2, and H_2O, or be stored as glycogen (by the process called glycogenesis) (Groop L. et al., 1997).

I.2.3. Effect of Glucose on Insulin Secretion

Glucose has long been recognized as the major physiologic stimulus of insulin secretion in humans (Grodsky GM. et al., 1963). In addition to directly stimulating insulin release from the pancreas, glucose also modulates the β-cell response to non-glucose secretagogues, regulates proinsulin synthesis and processing, and influences β-cell growth (Leathy JL. et al., 1992). The 5'-flanking DNA sequences of the insulin gene were shown to confer glucose responsiveness to a reporter gene when expressed in β-cells (Melloul D., 1993).

The cellular mechanism(s) via which sustained hyperglycemia alters the β-cell response to glucose are no yet fully defined. It was initially proposed that chronic hyperglycemia may cause a generalized down-regulation of glucose transport and metabolism in all cells of the body (DeFronzo RA. et al., 1992). A number of studies also have provided evidence for a primary role of phosphoinositol metabolism and activation of PKC in the final secretory processes that regulate insulin release. The hypothesis is that glucose, after interaction with a specific site on the surface of the β-cell, activates phospholipase C. This enzyme catalyzes the hydrolysis of membrane phosphoinositides, producing DAG and inositol phosphate. The latter, in turn, increases intracellular calcium and activates protein kinase C, both of which have been implicated in the insulin secretory process (Zawalich WS., 1988; Rasmussen H. et al., 1990).

Considerable attention has also been focused on the role of the islet glucose transporter (Glut 2) as a potential site of the defective regulation of glucose-stimulated insulin secretion in diabetes. Several investigators have reported that exposure of rodent islets to hyperglycemia results in decreased levels of Glut 2 protein and mRNA (Orci L. et al., 1990; Milburn JL. et al., 1993).

Hyperglycemia sustains and exacerbates the insulin secretory defects observed in type 2 diabetes, and contribute to insulin resistance in both type 2 and type 1 diabetes. Hyperglycemia not only represents a manifestation of diabetes mellitus, but is a self perpetuating factor that is responsible for sustaining the diabetic state (Simonson DC. et al., 1997).

I.2.4. Hyperglycemia

The normal blood glucose concentration in a person who has not eaten a meal within the past 3 to 4 hours is about 90 mg/ml (5 mM). After a meal containing large amounts of carbohydrates, this level seldom rises above 140 mg/ml (7.8 mM) unless the person has DM (Guyton AC and Hall JE., 1996).

DM affects up to 10% of the general population and up to 20% of people aged 65 years and older. People with diabetes are at high risk of diabetic retinopathy, nephropathy and neuropathy (i.e. microvascular disease) (Kenny SJ. et al., 1995).

Hyperglycemia is an important etiologic factor in the pathogenesis of DM. Although the precise mechanisms are unknown, PKC may play an important role (Kilo C.,1985). There is now considerable evidence that hyperglycemia plays an important role in the pathogenesis of all the major complications of diabetes, including nephropathy, retinopathy, neuropathy and macrovascular disease. In addition to these microvascular and macrovascular complications, hyperglycemia is also closely linked with other physiologic, biochemical and anatomic complications, including basement membrane thickening, protein glycosylation, impaired cellular immunity and abnormalities in cell growth and differentiation (Simonson DC. et al., 1997). Hyperglycemia *per se* plays an important role in exacerbating and sustaining the clinical syndrome of diabetes mellitus, thus leading to a vicious cycle of further hyperglycemia and more profound deficiencies in insulin secretion (Simonson DC. et al., 1997).

I.2.5. Mechanisms of High Glucose Effect

Likely mediators of the effects of high ambient glucose include activation of the polyol pathway, increased PKC activity, nonenzymatic glycation of circulating or matrix proteins, and/or aberrant synthesis or actions of cytokines and vasomodulatory agents. The latter include Ang II,

thromboxane, platelet-derived growth factor, endothelins, IGF-1 and TGF-β (reviewed by Sharma K. and Ziyadeh FN., 1997).

I.2.5.1. Activation of Polyol Pathway (Sorbitol Pathway)

The polyol pathway, which has also been called the sorbitol pathway, has long been suspected to be responsible for some diabetic complications such as cataract, neuropathy, retinopathy and nephropathy. This pathway has been the focus of increased attention (Bleyer AJ. et al., 1994).

As shown in figure I.1, the polyol pathway consists of two enzymes, namely aldose reductase (AR) and sorbitol dehydrogenase. AR, the first and rate-limiting enzyme in this pathway, catalyzes the reduction of glucose to sorbitol, utilizing NADPH as coenzyme. sorbitol dehydrogenase then oxidizes this intermediate sorbitol to fructose, utilizing NAD. The sorbitol pathway has now been found in many tissues, such as lenses, retina, placenta, nerve, erythrocytes, blood vessel, pancreatic duct, kidney and liver etc. (Terashima H., 1988; Fields M. and Lewis CG., 1990) and Enzymes of the sorbitol pathway have been discovered in these tissues (Terashima H., 1988).

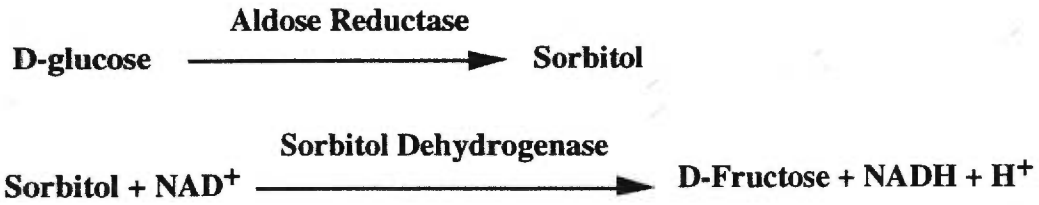


Figure I.1. The Sorbitol Pathway (Polyol Pathway)

In diabetes, the capacity of target tissues to metabolize glucose via the glycolytic pathway is limited (Dvornik D. et al., 1988). A link has thus been indicated between the flux of excess glucose

through the polyol pathway and the development of some diabetic complications (Gabbay KH., 1973), The high levels of glucose in diabetic tissues result in the generation of higher levels of sorbitol by AR, the glucose conversion to sorbitol is eventually further enhanced or promoted (Ziyadeh FN. et al., 1989).

The accumulated intracellular sorbitol can induce a hyperosmotic effect that results in an influx of fluid and subsequent cellular damage (Terashima H., 1988). Increased oxidation of sorbitol to fructose also has been shown to increase the cytosolic ratio of $NADH/NAD^+$ in glomerular mesangial cells and microvasculature (Tilton RG. et al., 1992), which in turn may divert glyceraldehyde-3-phosphate away from the glycolytic pathway toward DAG synthesis and elevation of PKC activity (Wolf BA. et al., 1991; Fumo P. et al., 1994).

Organ dysfunction in diabetes caused by the increased flux of glucose through the polyol pathway has been linked to the hyperglycemia-induced increase in the redox state (high $NADH/NAD^+$ ratio), which appears to be important in the de novo synthesis of DAG and the stimulation of PKC activity (Williamson JR.et al., 1993).

The progression of the changes induced by polyol pathway activation can be arrested by inhibiting AR (Dvornik D. et al., 1988). AR inhibitors are a class of drugs potentially useful in preventing diabetic complications. The major pharmacological action of an AR inhibitor involves competitive binding to aldose reductase and consequent blocking of sorbitol production. Treatment with AR inhibitor, may prevent some of the early features of DN such as glomerular hyperfiltration and the high glucose-induced increase in collagen synthesis *in vitro* (Pedersen MM. et al., 1991; Bleyer AJ. et al., 1994). Recent studies showed that the glucose-induced increase in TGF-ß was prevented by concomitant incubation with epalrestat, an AR inhibitor, in a dose-dependent manner. Morever, the glucose-induced enhancement of PKC activity in the membrane fraction of cultured human mesangial cells was also abolished by epalrestat (Ishii H. et al., 1998). Donnelly SM. et al.'s study (1996) indicated that tolrestat, an AR inhibitor, prevents the functional changes of

glomerular hyperfiltration, mesengial cell hypocontractility, and increased glomerular permeability to albumin. Polyol accumulation may have differential effects on glomerular growth and extracellular matrix accumulation in early DN (Donnelly SM. et al., 1996). Although polyol pathway may not be solely responsible for diabetic complications, studies suggest that therapy with ARIs could be beneficial (reviewed by Zenon GJ. et al., 1990).

I.2.5.2. Increased Formation of DAG and Activity of PKC

PKC is an important enzyme that has been demonstrated to mediate many cellular changes. PKC has also been found to be involved in glucose effects.

I.2.5.2.1. PKC Signal Transduction Pathway

PKC is composed of an ever growing family of lipid dependent serine/threonine protein kinases. Collectively, these isoenzymes are known to mediate critical roles in signal transduction, tumor promotion and cell regulation. PKCs appear to function as switches in acute signal transduction paradigms as well as rheostats in long term cell regulation with multiple mechanisms operating in the regulation of these functions (Blobe GC. et al., 1996).

The PKC family consists of at least 11 isoforms, each isoform shows a distinct tissue distribution, cellular localization, manner of activation, substrate specificity, and sensitivity to phorbol ester treatment, implying differences in function (Nishizuka Y.,1988). These isoforms can be divided into three groups based on their structural features and in vitro activation requirements: the conventional PKC (cPKC) isoforms α, β_1, β_2, and γ (activated by Ca^{2+}, phosphatidylserine, and DAG); the novel PKC (nPKC) isoforms δ, ε, η, and θ (Ca^{2+}-independent enzymes activated by phosphatidylserine and DAG); and the atypical PKC (aPKC) isoforms ζ and λ (insensitive to Ca^{2+} and DAG) (reviewed in Nishizuka Y.,1995). There is convincing evidence that different tissues express different PKC isoenzymes (Nishizuka Y., 1988). Two functional domains are

found in all PKC isoforms, a catalytic carboxy-terminated domain and a regulatory amino-terminal domain (Kazanietz MG. et al., 1993).

PKC is activated in cells by calcium and 1,2-diacylglycerol (DAG), (Berridge MJ., 1987). PKC can also be activated by tumor promoting phorbol esters for which it is the cellular receptor. The phorbol ester binds at the DAG site on the enzyme (Kikkawa U. et al., 1989). PKC activation results in phosphorylation of a number of protein substrates in a wide range of cellular responses (Nishizuka Y.,1988; Qu X. and Donnelly R., 1997), PKC may also be activated by arachidonic acid, an unsaturated fatty acid produced by PLA_2 mediated cleavage of membrane phospholipids (Nishizuka Y., 1988).

Cell stimulation causes most or all of the PKC to translocate from one to another subcellular compartment. PKC has been often observed to travel from the cytosol to the membrane, but recent evidence has revealed PKC translocation to other cell locations such as the nucleus (Whetton AD., 1994), Golgi apparatus (Westermann P. et al., 1996), endoplasmic reticulum (Goodnight J. et al., 1995), perinuclear region (Disatnik MH. et al., 1994), or cytoskeletal elements (Disatnik MH. et al., 1994). The translocation of PKC may, therefore, offer an indication of the activation of PKC in response to specific stimuli (Kraft AS. and Anderson WB., 1983). The most common method for assaying PKC activity involves measuring the transfer of ^{32}P-labeled phosphate to protein or peptide substrate which can be captured on phosphocellulose filters via weak electrostatic interactions (Considine RV. and Caro JF., 1993).

DAG, the hydrophobic product of the phosphodiesterase-mediated cleavage of inositol phospholipids, is thought to have a second-messenger function through the activation of PKC (Nishizuka Y., 1984). DAG can be generated either by de novo synthesis or by stimulated breakdown of phospholipids by PLC (Bell RB., 1986; Berridge MJ., 1983). DAG is generated by hydrolysis of phosphatidyl inositol (PI) (which also generates inositol 1,4,5-triphosphate (IP_3), in turn releasing intracellular Ca^{2+} or phosphatidylcholine (Berridge MJ., 1987; Exton JH., 1990).

Studies by Kreisberg JI. et al (1996) demonstrated cross-talk between the PKC and protein kinase A pathways in that agents which activate the PKC pathway can stimulate phosphorylation of proteins that commonly serve as substrates for protein kinase A.

I.2.5.2.2. Hyperglycemia and PKC Activation

PKC activation leads to increased production of the Jun/Fos (AP-1) transcription factor complex. The observations that the mRNA abundance for both c-fos and c-jun are increased by high glucose in mesangial cell culture (Kreisberg JI. et al., 1994) and in the glomerulus of the STZ-diabetic rat (Sharma K. et al., 1995) support a role for PKC activation in diabetic renal manifestations. High ambient glucose promotes collagen IV gene transcriptional activity in murine mesangial cells, and this likely occurs through PKC activation (Fumo P. et al., 1994). PKC activation may also play a role in the enhanced expression of cytokine-inducible nitric oxide (NO) synthase in murine macrophages and glomerular mesangial cells when cultured in high glucose media in the presence of cytokines or lipopolysaccharide (Sharma K. et al., 1995). Emerging work has implicated activation of specific PKC isozymes (eg. alpha, beta, zeta) in diabetic renal disease (Kikkawa R. et al., 1994).

PKC has been shown to be involved in glucose transport and metabolism, eg. the insulin receptor and glycogen synthase (Shmueli E. et al., 1997). The increase in PKC activity occurred within 2 min of glucose treatment in mesangial cells. High glucose levels stimulate collagen α_1 (IV) transcriptional activity in a reporter mesangial cell line, perhaps through PKC activation (Fumo P. et al., 1994). Haller's study (1995) in vascular smooth muscle cells showed that high extracellular glucose activates PKC, 20 mM glucose caused an increase in total PKC activity at six hours, which was maintained at 24 hours. Glucose induced a translocation of all PKC isoforms except PKC zeta. Confocal microscopy showed that PKC alpha, beta, and epsilon were translocated into the nucleus. PKC delta showed strong association with cytoskeletal structures.

PKC activation has been demonstrated in retina and glomeruli of diabetic rats as well as in retinal capillary endothelial cells exposed to elevated glucose levels in vitro (Lee TS. et al., 1989; Craven PA. and DeRubertis FR., 1989). Modulation of PKC produces glucose-dependent influences on hepatic flow and metabolism (Inaba H. et al., 1995). Elevated glucose concentrations significantly influence vascular Na^+/H^+ antiport activity via glucose-induced PKC dependent mechanisms (Williams B. and Howard RL., 1994). High levels of glucose induce inhibition of insulin receptor tyrosine kinase in vivo, this effect is mediated by a glucose-induced PKC translocation/activation and serine phosphorylation of the insulin receptor (Berti L. et al., 1994). A high glucose level inhibits capacitative Ca^{2+} influx in cultured rat mesangial cells by a PKC-dependent mechanism (Mene P. et al., 1997). A high glucose level has been shown to stimulate fibronectin synthesis by activating PKC (Ayo SH. et al., 1991). Further support for the role of high glucose in this response was the observation that the phorbol ester PMA, which activates PKC directly, mimics the effect of high glucose on extracellular matrix protein mRNA levels (Ayo SH. et al., 1991). The transcription factors c-fos and c-jun (AP-1), which are modulated by PKC activation, are likewise elevated in mesangial cells treated with high glucose (Kreisberg JI. et al., 1994). High glucose was reported to exert its effect on fibronectin synthesis by increasing transcription (Kreisberg JI. et al., 1996).

I.2.5.2.3. High Glucose Levels Increase DAG Synthesis

Activation of PKC by a high glucose level is believed to be due to increased de novo synthesis of DAG from glucose (Figure I.1). Further studies established that glucose exerted its effect on PKC activity by increasing DAG mass through the de novo pathway (Craven PA. et al., 1990). Craven PA. et al. (1990) have demonstrated increased DAG mass in isolated glomeruli by glucose from de novo synthesis of glycerolipids rather than release from inositol phospholipids. Elevation of glucose levels stimulate de novo synthesis of DAG in a variety of tissues, such as heart, retina, and kidney of diabetic rats (Okumura K. et al., 1988; Lee TS. et al., 1989; Craven PA. and DeRubertis FR., 1989) .This DAG molecule disappears quickly. The level of DAG,

however, often increases again with a relatively slow onset, and persists for minutes, occasionally for hours (Nishizuka Y., 1992). The results of Ayo SH. et al (1991) demonstrate that mesangial cells treated with 30 mM glucose display increased levels of DAG within 30 min, DAG remains elevated for up to 1 week in high glucose medium (Ayo SH. et al., 1991). A high glucose level did not stimulate PI hydrolysis, as evidenced by the absence an increase in the water-soluble inositol phosphates, indicating that DAG was not generated though the action of a PI-specific PLC (Ayo SH. et al., 1991). A more reduced cytosolic ratio of NADH/NAD^+ would favor glucose-induced increased de novo synthesis of DAG and associated activation of PKC observed in isolated glomeruli exposed to elevated glucose levels in vitro (Craven PA. et al., 1990).

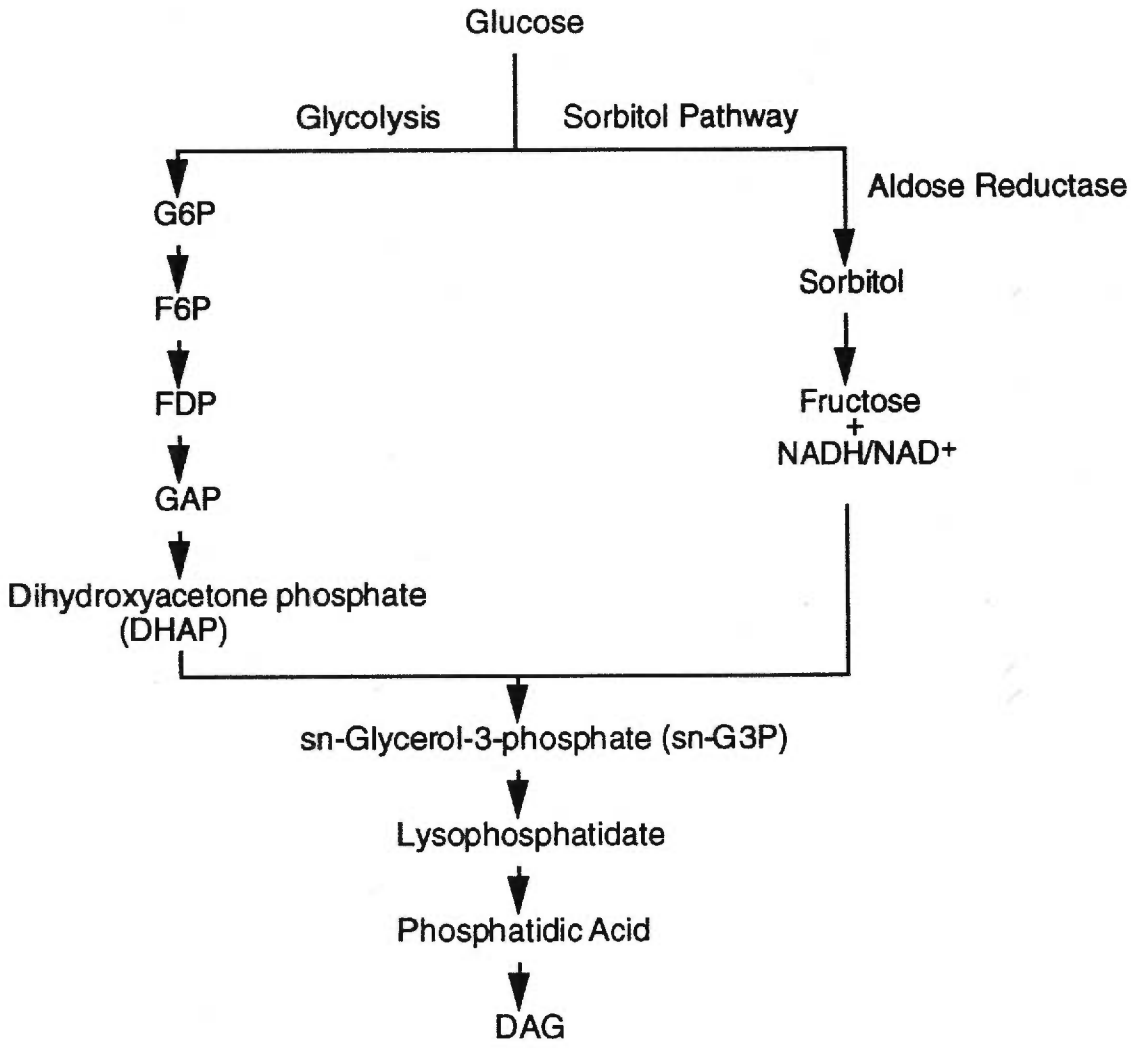


Figure I.2. De novo synthesis of DAG from glucose (redraw from Tilton RG. et al., 1992).

The pathway for the conversion of glucose to DAG involves first the glycolytic conversion of glucose to glyceraldehyde-3-phosphate (accompanied by reduction of NAD^+ to NADH); diversion from the glycolytic pathway then leads to formation of dihydroxyacetone phosphate (accompanied by oxidation of NADH to NAD^+) which is then reduced to glycerol-3-phosphate; the latter is metabolized to phosphatidic acid and then to DAG (Fumo P. et al., 1994). Increased activity of the polyol pathway could increase the cellular NADH/NAD^+ ratio, which favors DAG synthesis (Fumo P. et al., 1994).

De novo synthesis of DAG from dihydroxyacetone phosphate (DHAP) may occur by two pathways. One involves direct acylation of DHAP followed by reduction at carbon-2 (by NADPH). The resulting lysophosphatidic acid is then further acylated to form phosphatidic acid which is then dephosphorylated to yield DAG (Preis JE. et al., 1987). The other involves reduction of DHAP to glycerol-3-phosphate (by NADH) which is then acylated to form lysophosphatidic acid, etc., as in the first pathway. The latter pathway would be favored by increased metabolism of glucose via the sorbitol pathway which has been shown in the lens to increase the NADH:NAD^+ ratio more than the NADPH:$NADP^+$ ratio (Lou MF. et al., 1988; Wolf BA. et al., 1991). DAG mass was measured by an enzymatic assay employing DAG kinase from E. coli to phosphorylate DAG to [^{32}P]phosphatidic acid which was then quantified (Preis JE. et al., 1987).

The glucose-induced increase in DAG levels and its prevention by pyruvate are consistent with increased de novo synthesis of DAG and with the hypothesis that increased DAG levels and associated changes in PKC activity may modulate glucose-induced vascular functional changes. This hypothesis receives further support from the observations that (a) a pharmacological inhibitor of PKC (staurosporine) greatly attenuates the glucose-induced rise in microvascular albumin permeation; (b) pharmacological activation of PKC with the phorbol ester TPA at physiological glucose levels mimics the effects of elevated glucose levels to increase microvascular albumin clearance and blood flow; (c) pharmacological inhibition of the catabolism of endogenous DAG by

the DAG kinase inhibitor MOG at physiological glucose concentrations also mimics glucose-induced increases in albumin clearance and blood flow; and (d) prevention of the glucose-induced rise in DAG accumulation with pharmacological concentrations of pyruvate also prevents glucose-induced rises in microvascular albumin clearance and blood flow (Wolf BA. et al., 1991).

I.2.5.3. Increased Free Radical Activity

Free radical mechanisms are increasingly being implicated in the pathogenesis of tissue damage in diabetes. Various sources of free radicals may modulate oxidative stress in diabetes, including non-enzymatic glycosylation of proteins and monosaccharide autooxidation, polyol pathway activity, indirect production of free radicals through cell damage from other causes, and reduced antioxidant reserve. Ascorbic acid, which may be a principal modulator of free radicals presumably through free radical scavenging, thus preserving levels of other antioxidants such as glutathione (Sinclair AJ. et al., 1992).

I.2.5.4. Nonenzymatic Glycation of Circulating or Matrix Proteins

Hyperglycemia also exerts long-lasting changes in cellular function, which result from non-enzymatic glycosylation of matrix and membrane proteins with subsequent binding of these proteins to specific receptors. These receptors are termed the advanced glycosylation end-products receptors. Their activation leads to an increased release of cytokines and growth factors including PDGF, interleukins, TNF-alpha, and TGF-beta, all of which may act concomitantly in the disease process (reviewed by Haller H. et al., 1996).

Elevated production and/or activity of TGF-β in the kidney is a common final mediator of diabetic renal hypertrophy and extracellular matrix (ECM) expansion in experimental as well as human DN. Overexpression of TGF-β activity can be triggered by PKC activation, either due to hyperglycemia-driven de novo synthesis of DAG or due to growth factor receptor-activated DAG

production resulting from phosphoinositide hydrolysis (Negrete H. et al., 1995). Advanced glycosylation end-products (AGE), endothelins and thromboxane have been shown to stimulate TGF-β production (Kumar S. and Fuad NZ, 1997). Perhaps the most important relevant inducer of TGF-β in renal cells is Ang II, implying that part of the renal protective effect of ACE inhibitors may be by blocking TGF-β production (Wolf G. and Neilson G., 1993; Kagami S. et al., 1994).

I.2.6. Glucose Responsive Element

Glucose induces the transcription of several glycolytic and lipogenic genes in hepatocytes and adipocytes (Goodridge AG., 1987). Glucose catabolism induces the expression of genes such as L-pyruvate kinase (L-PK), S14 (a gene which is directly related to lipogenesis) and fatty acid synthase (FAS) through the glucose responsive element (GIRE) (Doiron B. et al., 1996). These GIREs have all in common the presence of a sequence 5'-CACGTG-3' which binds a transcription factor called USF (upstream stimulatory factor), although the organization of the overall GIRE probably differs from one gene to another (Foufelle F. et al., 1996). Another GIRE which uses a transcription factor named Sp1, has been characterized in the gene for the acetyl-coenzyme A carboxylase (reviewed by Girard J. et al., 1997). The β-cells in the pancreatic islets of Langerhans increase insulin gene transcription in response to increased glucose concentration. German MS.'s study (1994) showed that the full glucose response of the insulin promoter involves the interaction of multiple sequence elements (German MS. and Wang J., 1994). MacFarlane WM.'s study (1994; 1997) showed that glucose modulates the binding activity of the β-cell transcription factor insulin upstream factor 1 (IUF1) in a phosphorylation-dependent manner, and that PKA or PKC are not involved.

I.3. Insulin

I.3.1. Insulin Biochemistry

Insulin, a major hormone of the endocrine pancreas, plays a key role in the control of glucose homeostasis. Insulin is a polypeptide containing 2 chains of amino acids linked by disulfide bridges. Insulin is secreted from the pancreatic β-cells in response to increased levels of plasma glucose and amino acids (i.e. postprandially). Insulin is synthesized as part of a large preprohormone. Preproinsulin has a 23 amino acid signal peptide removed as it enters the endoplasmic reticulum. The remainder of the molecule is then folded, and the disulfide bonds are formed to make proinsulin. Proinsulin is then transferred to the Golgi apparatus to form storage or secretory granules. The C peptide is split off by protease in the secretory granule. The half life of insulin in the circulation is 5 minutes. It is destroyed in the endosomes formed by endocytotic process by insulin protease (Felig P. et al., 1987; Ganong WF. 1995). There are minor differences in the amino acid composition of the molecule from species to species. The differences are generally not sufficient to affect the biologic activity of a particular insulin in heterologous species but are sufficient to make the insulin antigenic (Ganong WF. 1995). The gene for insulin is located on the short arm of chromosome 11 in humans. It has 2 introns and 3 exons.(Ganong WF. 1995).

I.3.2. Insulin Effect

I.3.2.1. General Effects of Insulin

Insulin is among the most potent of anabolic agents and this hormone elicits a variety of metabolic changes in many mammalian tissues, primarily adipose tissue, liver and muscle (Denton RM., 1986). These include increased glucose transport and increased glycogen, fatty acid and protein synthesis along with decreased protein, lipid and glycogen breakdown and reduced glucose biosynthesis (gluconeogenesis). The result is a general increase in anabolism and, in certain circumstances, growth of responsive tissues (Sutherland C. et al., 1997).

The physiological actions of insulin are characterized by a wide variety of cellular effects, including modulation of glucose and amino acid transport; activities of key enzymes in intermediary

metabolism; rates of protein, DNA, and RNA synthesis; transcription of specific genes; and cellular growth and differentiation (Saltiel AR., 1996). Insulin also exerts profound effects on various cellular processes by altering the amount of critical proteins (Jefferson LS., 1980). Insulin changes the rate of protein synthesis in two general ways. First, it affects the rate of protein synthesis in selected tissues (liver, adipose tissue, skeletal and cardiac muscle) at the level of mRNA translation (Granner DK., 1987). Total protein increases in such tissues without a similar change in total mRNA. Secondly, it also has positive and negative effects on the expression of specific genes (Granner DK., 1987). The main effects of insulin are the activation of the transcription of some genes, mRNA translation and glycogen synthase (Denton RM. and Tavaré JM., 1997). Studies have shown that insulin increases phosphorylation of certain proteins, including the insulin receptor, ribosomal protein S6, ATP citrate-lyase, acetyl-CoA carboxylase, and a number of other proteins of unknown function (Avruch J. et al., 1985).

Over the past decade considerable data have been gained suggesting that insulin, normally secreted only by the pancreas, and IGF-1, secreted by cells of the cardiovascular system, regulate normal cardiovascular physiological responses. Furthermore, there is emerging evidence that abnormal actions of these factors may contribute to disease states such as hypertension and atheroslerosis (Sowers JR., 1997)

I.3.2.2. Effect of Insulin on Glucose Homeostasis

One of the most important actions of insulin is to maintain glucose homeostasis (Sutherland C. et al., 1997). Insulin is the dominant glucose lowering hormone, it suppresses endogenous glucose production and stimulates glucose utilization, thereby lowering the plasma glucose concentration. It inhibits hepatic glycogenolysis and gluconeogenesis, it stimulates glycogenesis in liver and muscle, it also stimulates glucose utilization by glycolysis and oxidation. The ability of insulin to increase the rate of glucose transport into muscle and adipose tissue is largely due to the translocation of insulin-regulatable (GLUT-4) glucose transporter (Ganong WF., 1995).

Immediately after a high carbohydrate meal, the glucose that is absorbed into the blood causes rapid secretion of insulin. The insulin in turn causes rapid uptake, storage, and use of glucose by almost all tissues of the body, but especially by the muscles, adipose tissue, and liver (Guyton GC and Hall JE., 1996).

I.3.2.3. Effect of Insulin in Kidney

The kidney is a key site of insulin metabolism. Most of the insulin metabolized is removed from the circulation by glomerular filtration, which is followed by absorption and then degration in the proximal renal tubule. Removal of insulin also proceeds from the postglomerular peritubular circulation (Rabkin R. et al., 1984).

Proximal tubules are a key site of insulin metabolism, which is in part receptor-mediated process (Yagil C. et al., 1988a; 1988b). Insulin is known to regulate both metabolic and transport functions in the renal proximal tubule (Lelongt B. et al., 1993; Kageyama S. et al., 1994). Several in vivo studies in humans and other mammalians have demonstrated the antiphosphaturic effects of insulin (Guntupalli J. et al., 1985). In vitro studies on isolated proximal tubules (Hammerman MR. et al., 1984) have ascertained that insulin has a direct effect on proximal tubular cells resulting in increased Na^+-dependent transport of phosphate (Na^+-Pi cotransport) across the luminal brush-border membrane.

An established renal epithelial cell line has been derived from opossum kidney (OK) (Koyama H. et al., 1978). Yagil C.'s study (1988a; 1988b) demonstrated that the proximal tubular epithelium-like OK cell line possesses insulin-specific receptors on the cell surface. Binding of insulin to these receptors is followed by internalization and then intracellular degradation. Furthermore, these receptors bind insulin in preference to insulin-like growth factor (Hoffman C. et al., 1985; Conti FG. et al., 1987). This OK cell line provides a good model for studying the interaction of insulin with the renal tubular epithelium (Yagil C. et al., 1988a; 1988b).

I.3.4. Insulin Receptor

I.3.4.1. Insulin Receptor Biochemistry

Insulin regulates numerous diverse metabolic processes through binding to high-affinity cell surface receptors (Rosen OM, 1987).

The insulin receptor is a transmembrane glycoprotein complex with a molecular weight of about 460 kDa (Massague J. et al., 1982). It is a tetramer made up of two 135 kDa extracellular α-subunits and two 95 kDa transmembrane β-subunits which are linked together by disufide bonds (Kasuga M. et al., 1982). The α-subunit binds insulin with high affinity and specificity, whereas the β-subunit is a protein and it acts as a transducer though its tyrosine kinase activity (Rosen OM, 1987; Tornqvist HE. et al., 1987). Functionally, the two subunits of the insulin receptor perform the different functions required for transmission of the insulin signal to the cell interior (White MF. and Kanh CR., 1994). 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 (Seino S. et al., 1990). The half-life of the insulin receptor is about 7-12 hours (Ganong WF., 1995). The insulin receptor cDNA has been cloned from human (Ullrich A. et al., 1985) and Drosophila (Petruzzelli L. et al., 1986) tissues.

Insulin receptors are found on many different cells in the body, including cells in which insulin does not increase glucose uptake. Likewise, the postreceptor machinery of insulin signaling, including substrates of the receptor and other signaling intermediates, is present in most cells (Rosen OM, 1987; Cheatham B. and Kahc CR., 1995).

I.3.3.2. Regulation of the Insulin Receptor

Expression of the insulin receptor gene is regulated by both the metabolic status of the cell and the state of differentiation (Mamula PW. et al., 1990). The insulin receptor is in a constant state of turnover (Fehlmann M. et al., 1982; Hedo JA. and Simpson IA., 1984). The number and/or the affinity of insulin receptors are affected by insulin and other hormones, exercise, food, and other factors (Ganong WF., 1995). Exposure to increased amounts of insulin decreases receptor concentration (down regulation), and exposure to decreased insulin levels increases the affinity of the receptors. The number of receptors per cell is increased in starvation and decreased in obesity and acromegaly (Ganong WF., 1995). Studies in many cell types have shown that insulin accelerates receptor degradation by reducing the half-life from 12 hours to about 2-3 hours (Kasuga M. et al., 1981). The number of cell surface insulin receptors correlates inversely with the level of insulin to which the cells have been exposed (Gavin JR. et al., 1974). The phenomenon of the down-regulation plays a central part in the regulation of plasma membrane insulin receptor number and in the pathogenesis of several states of insulin resistance, including obesity and type 2 diabetes (Flier JS., 1983).

I.3.3.3. The Insulin Receptor is a Tyrosine Kinase Receptor

Receptor tyrosine kinases (TKs) are receptor enzymes which respond to ligand binding by catalyzing the transfer of phosphate groups from ATP to tyrosine residues of proteins (Kahn C.R., 1997).

The insulin receptor is a receptor TK. The binding of insulin to the α-subunit activates the intrinsic protein kinase activity of the β-subunit (Denton RM. and Tavaré JM., 1997), which undergoes hormone-dependent autophosphorylation, resulting in increased kinase activity (White MF. and Kahn CR., 1994). Numerous studies have shown that insulin simultaneously produces both the phosphorylation of some proteins and the dephosphorylation of others on serine and threonine residues. ex. insulin generation of a soluble second messenger that directly or indirectly activates a serine/threonine phosphatase; insulin stimulation of a cascade of protein kinases,

resulting in the phosphorylaton of certain cellular proteins (Saltiel AR., 1996). The insulin receptor TK activity appears essential in the mechanism of insulin signal transduction (Rosen OM., 1987).

A number of different intracellular signaling pathways have been shown to be activated by receptor TKs. These activation events include the phosphoinositide 3-kinase (PI3-kinase), 70 kDa S6 kinase, mitogen-activated protein kinase (MAPK), phospholipase C-γ, and the Jak/STAT pathways (Marshall CJ., 1995), they are involved in cell proliferation, differentiation and migration (Schlessinger J. and Ullrich A., 1992; Alroy I. and Yarden Y., 1997). The precise role of each of these pathways in cell signaling remains to be resolved.

I.3.4. Mechanisms of Insulin Actions

I.3.4.1. General Principles

Insulin action involves a network of interrelated and independent pathways with differing levels of divergence regarding mechanisms of regulation (Ellis L. et al., 1986; Chou CD. et al., 1987).

Insulin action at the cellular level can be viewed as existing at three levels (Figure I.3). 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 action 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 transporters required for insulin's effects on glucose, lipid and protein metabolism (Kahn CR., 1997).

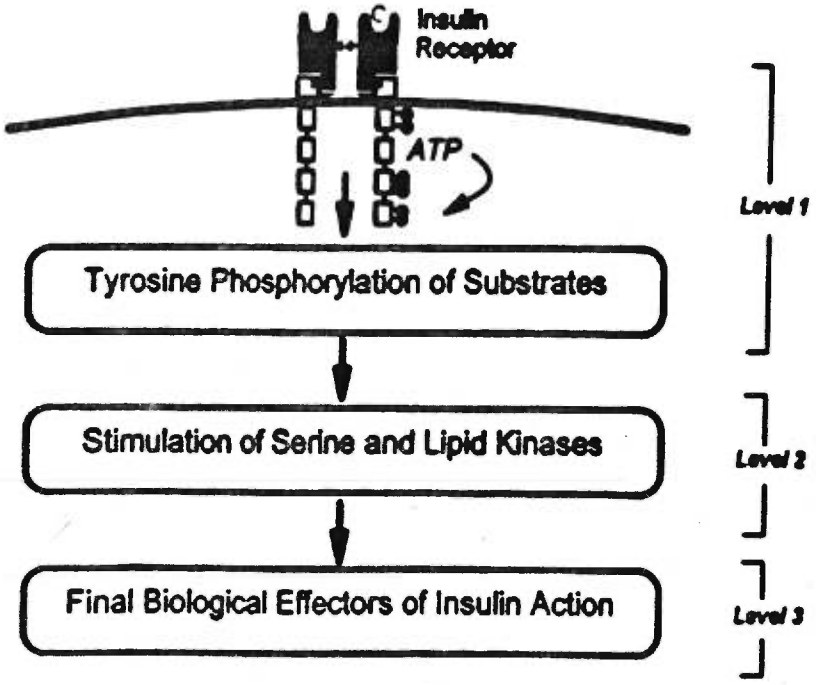


Figure I.3. Three Levels of Insulin Action (Redraw from Denton RM. and Tavaré JM., 1997).

Insulin utilizes distinct signaling pathways for the regulation of transcription of distinct genes (Sutherland C. et al., 1997). One pathway is 'Ras/MAP kinase' pathway, A second insulin signaling pathway involves the enzyme PI-3 kinase. This enzyme generates putative 'second messenger' phosphorylated inositol lipids (Auger KR. et al., 1989), and is required for the insulin regulation of transcription of the genes for PEPCK (Sutherland C. et al., 1997) and hexokinase II (HKII) (Osawa H. et al., 1996). .

Insulin receptor TK activity is required for insulin action on cells, but perhaps not all of insulin signal transduction proceeds via phosphorylation cascades involving endogenous substrate proteins. For example, insulin stimulation of glucose transport has not been associated with any phosphorylation changes of the glucose transporter or in any of the associated proteins in the glucose transport pathway (Gibbs EM. et al., 1986).

Insulin simultaneously produces both the dephosphorylation of certain proteins (i.e. glycogen synthetase, pyruvate dehydrogenase, hormone-sensitive lipase) along with the phosphorylation of other proteins (i.e. ribosomal S6, ATP citrate lyase). The dephosphorylation actions induced by insulin result in the regulation of carbohydrate and lipid metabolism. Processes such as glucose transport and protein synthesis may require insulin-induced kinase reactions (Denton RM. and Tavaré JM., 1997).

The first identified and best characterized substrate of the receptor was named insulin receptor substrate-1 (IRS-1). IRS-1 is a cytoplasmic protein of MW 131 kDa (Sun XJ et al., 1991). Insulin stimulates IRS-1 phosphorylation and the phosphorylated IRS-1 serves as an intracellular ligand and binds to specific domains (termed src homology-2 (SH2)) of intracellular proteins (Kahn CR., 1997). IRS-1 binds to at least six different SH2 domain proteins, and this results in a rapid divergence of signal transduction to many pathways (Koch CA. et al., 1991). Binding of IRS-1 to SH2 proteins such as PI 3-kinase and the phosphotyrosine phosphatase SHPTP2 results in rapid stimulation of their enzymatic activity (Backer JM. et al., 1992; Sugimoto S. et al., 1994). The binding of IRS-1 to SH2 proteins which have no intrinsic enzymatic activity serve as adapter proteins between IRS-1 and other signaling systems (Skolnik EY. et al., 1993; Myers MG. et al., 1994). Alternative insulin substrates may include: (a) Shc, which, like IRS-1 can bind to GRB-2 and through SOS activate Ras (Sasaoka T. et al., 1994); (b), 60 kDa proteins which interact with PtdIns3-kinase (Hosomi Y. et al., 1994); (c), a protein tentatively called 'IRS-2' (Araki E. et al., 1994). All of these proteins are phosphorylated on their tyrosines by the activated insulin receptor. Altogether there appears to be a web of pathways between the receptor on the one hand, and the Ras and/or PI 3-kinase on the other (Denton RM. and Tavaré JM., 1997).

I.3.4.2. Role of PI-3 Kinase in Insulin Action

PI-3 kinase consists of two subunits, a regulatory subunit of molecular weight 85 000

(p85) and a catalytic subunit with a molecular weight of 110 000 (p110). PI-3 kinase is activated following stimulation of receptor-activated tyrosine kinases. It can catalyze the phosphorylation of phosphatidylinositol (PI), PI-4P and PI-4,5P_2 to PI-3P, PI-3,4P_2 and PI-3,4,5P_3 respectively (Folli F. et al., 1992). PI-3P, PI-3,4P_2 and PI-3,4,5P_3 may act as second messengers (Flamigni F. et al., 1997).

PI-3 kinase is one of the key enzymes activated in the signaling pathways of growth factors (Flamigni F. et al., 1997). Involvement of PI-3 kinase in the action of S6 kinase (Chung J. et al., 1994), membrane ruffling (Kotani K. et al., 1994), neurite formation (Kimura K. et al., 1994), activation of PKC (Nakanishi H. et al., 1993), actin polymerization (Eberle M., 1990), and transport of proteins (Schu PV. et al., 1993) has been suggested. Several lines of research, most of them employing specific PI-3 kinase inhibitors such as Wortmannin or LY294002 (Vlahos CJ. et al., 1994), indicate that PI-3 kinase plays an important role in mitogenesis, cytoskeletal rearrangement and vesicle transport (Varticovski L. et al., 1994).

Activation of PI-3 kinase appears to be a critical upstream step for insulin stimulation of Glut4 glucose transporter translocation and glucose uptake, as well as stimulation of certain enzymes involved in protein synthesis, such as p70 S6 kinase (Kahn CR., 1997). The inhibition of PI-3 kinase attenuates insulin-stimulated glucose uptake, suggesting a possible role of the enzyme in vascular movement required for translocation of the insulin-sensitive GLUT-4 transporter (Kahn CR., 1997). These inhibitors can also block other effects of insulin on glucose utilization and gene expression (Saltiel AR., 1996). The interaction of IRS-1 with the SH2 domains of PI-3 kinase results in a stimulation of PI-3 kinase activity (Folli F. et al., 1992). In insulin receptor-transfected Chinese hamster ovary cells, insulin at physiological concentrations increases the amounts of various lipid products of PI-3 kinases. The importance of PI-3 kinase for transmission of growth signals has been suggested by many studies using cell expressing mutant receptors (Whitman M., 1985; Coughlin SR. et al., 1989). PI-3 kinase and $p70^{s6k}$ are necessary for insulin-mediated transcriptional inhibition of the insulin-like growth factor binding protein (IGFBP-1) (Band CJ.

and Posner BI., 1997).

I.3.4.3. Role of the MAP Kinase Signal Transduction Pathway in Insulin Action

The MAPK pathway can be activated by a wide variety of hormones, cytokines, and growth factors including insulin (Begum N. et al., 1996; Xi XP. et al., 1997), epidermal growth factor (Rockow S. et al., 1996), platelet-derived growth factor (Choudhury GG. et al., 1997; Xuereb JM. et al., 1997), nerve growth factor (Kamata H. et al., 1996; Wright JH. et al., 1997), phorbol esters (Seufferlein T. and Rozengurt E., 1996; Schonwasser DC. et al., 1998), nicotine (Cox ME. and Parsons SJ. et al., 1997), okadaic acid (Chen KD. et al., 1998) and hormones that induce oocyte maturation and in T cell activation (Ahn NG. et al., 1992). The targets of MAPK signaling are located within many cellular compartments, The activated MAPK in turn directs the phosphorylation of transcription factors, such as c-Jun and $p62^{TCF}$ (Gille H. et al., 1992), Ets proteins (Whitmarsh AJ. et al., 1995), the protein serine/threonine kinase such as $p90^{rsk}$, cytoskeletal proteins, and cytosolic phospholipase A2 ($cPLA_2$) (Sturgill TW. et al., 1988; Davis RJ. et al., 1994) and intermediate regulators of protein synthesis, such as PHAS-1 (Lin TA. et al., 1994).

MAPK provides a physical link in the signal transduction pathway from the cytoplasm to the nucleus (Davis RJ. 1993). MAPK plays a key role in the transduction of signals through both protein kinases and protein phosphatases (Davis RJ. 1993) Thus, it represents an important intermediate in the network of cellular signal transduction pathways (Davis RJ. 1993). Also, the activation of MAPK is critical for the initiation of immediate-early gene expression and cell cycle progression leading to mitogenesis (Marshall CJ., 1995).

Previous studies have demonstrated that insulin stimulates the insulin receptor TK and the Raf/MEK/ERK pathway (Varies-Smits AMM. et al., 1992; Wood KW. et al., 1992; Thomas SM. et al., 1992). Activation of the MAPK pathway by insulin is initiated by binding to the cell surface

TK receptor, and binding of adapter molecule GRB2 to insulin substrate such as IRS-1, This activates the GDP/GTP exchange factor SOS in the GRB2/SOS complex, which in turn stimulates the GTP loading of Ras. Activation of Ras then results in stimulation of a cascade of serine/threonine phosphorylation involving Raf-1 kinase, MAP kinase kinase (MEK), MAPK (also known as extracellular signal regulated kinase, Erk) and p90 S6 kinase (also called rsk) (Egan SE. et al., 1993). The rsk then phosphorylates and activates several transcription factors which regulate gene expression (Dent P. et al., 1990; Cheatham B. and Kahn CR., 1995). Two isoforms of MAPK, the p44 MAPK (Erk-1), and the p42 MAPK(Erk-2), are expressed in most cell types (Bornfeldt KE. et al., 1997).

Although the Ras-MAP kinase (MAPK) pathway may play a role in the metabolic actions of insulin, this pathway is probably most important in control of transcription factor activity and mitogenesis (Kahn CR., 1997).

I.3.4.4. Role of PKC in Insulin Actions

The ability of insulin to activate PKC has been a much debated topic (Blackshear PJ. et al., 1991). On one hand, PKC has been suggested to be directly activated by insulin and therefore involved in insulin signal transduction. On the other hand, several investigators have hypothesized that PKC may be responsible for attenuation of the insulin signal and, further, that the kinase may be involved in the pathogenesis of insulin resistance in type II diabetes (Considine RV. and Caro JF. et al., 1993). There is disagreement about the ability of insulin to activate PKC; activation of the kinase by insulin has been reported by some investigators, whereas others have failed to show a significant insulin effect (Blackshear PJ. et al., 1991). Insulin induces activation of PKC by means of an increase in DAG (Nishizuka Y., 1995). Whether PKC is a physiologically relevant modulator of insulin receptor kinase activity is currently under investigation (Nishizuka Y., 1995). It has been postulated that this increase in DAG leads to activation of PKC, which in turn mediates the effects of insulin on sugar transport (Farase RV. et al., 1992). PKC might be an important negative

regulator of insulin receptor function (Hüring HU. et al., 1996). In vivo, purified PKC can phosphorylate the insulin receptor, reducing its activity (Bollag GE. et al., 1987). Insulin stimulates the c-fos gene transcription in H4IIE cells through a PKC independent pathway (O'Brien RM. and Granner DK., 1996). The inhibitory effect of insulin on PEPCK gene transcription is not mediated by PKC (O'Brien RM. and Granner DK., 1996).

There is also considerable interest in the proteins that bind to these insulin response sequences, so the isolation and cloning of these proteins is a major goal and the potential involvement of such proteins in the pathogenesis of type II diabetes represent the final step in a signal cascade (O'Brien RM. and Granner DK., 1996).

I.3.5. Insulin Regulated Gene Expression and Insulin Responsive Element

The regulation of specific genes by insulin is clearly a major action of this hormone. Insulin affects the expression of over 100 genes (O'Brien RM. and Granner DK., 1996), and there is no doubt this number will continue to rise. It is possible that the loss of the regulation of one or more of these genes by insulin is responsible for insulin resistance (Sutherland C. et al., 1997). Insulin can regulate gene expression at several levels, but most attention has been given to its role in influencing the transcription of specific genes (O'Brien RM. and Granner DK., 1996).

Although a large number of genes appear to be regulated by insulin, only a subset of these have, to date, been demonstrated to be directly regulated at the transcriptional level by this hormone. Thus, the effects of insulin on gene transcription may be due to an effect on the expression or action of another hormone. In addition, the potential role of insulin-stimulated changes in glucose concentration in the regulation of gene expression must be considered (Sutherland C. et al., 1997).

Insulin responsive elements (IRE) have been identified for several other genes of

carbohydrate metabolism, including phosphoenolpyruvate carboxykinase (PEPCK) (Jitrapakdee S. et al., 1997), liver pyruvate kinase (PK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Alexander-Bridges M. et al., 1991), and glucagon (Philippe J., 1991). Since these insulin-regulated genes have a common function in maintaining glucose homeostasis, it is possible that they are regulated by a common insulin-responsive protein or family of proteins. Isolation and comparison of IRE-binding proteins from different tissues will be required to determine the relationship between insulin regulatory proteins in liver, pancreas, and adipocytes and other insulin-responsive cell types (Johnson TM. et al., 1993).

Insulin stimulates transcription of GAPDH (Leyva F. et al., 1998), α-amylase (Johnson TM. et al., 1993), insulin-like growth factor-binding protein-1 (IGFBP-1) (Suwanichkul A. et al., 1994) and IGFBP-3 (Phillips LS. et al., 1998); it inhibits transcription of phosphoenolpyruvate carboxykinase (PEPCK) (Jitrapakdee S. et al., 1997) and glucagon (philippe J., 1991). Comparison of the cis elements defined as the IREs of these genes reveals they contain one or two AT-rich regions (T(G/A)TTT) (Meisler MH. and Howard G., 1989; O'Brien RM. and Granner DK., 1991). In some genes, such as PEPCK, GAPDH, c-fos and amylase the IRE has been mapped to a short DNA sequence (< 30 bp). The IRE in PEPCK (-416 to -407) is TGGTGTTTTG, The IRE in IGFBP-1 (-284 to -275) is TGTCTTTTTG. The IRE in α-amylase (-165 to -156) is GTTTATTTTG. IRS In other genes only broad insulin response regions have been identified (O'Brien RM. and Granner DK., 1991).

With the continuing characterization of insulin response sequences it becomes evident that no general consensus sequence has been found for an insulin response sequence, and no insulin specific trans-acting transcription factor has yet been identified (Sutherland C. et al., 1997). In addition, no consensus sequences exist for positively or negatively regulated genes, or for genes involved in similar biologic processes (e.g. membrane proteins, enzymes of metabolism, hormones). Interestingly, a relatively short and simple motif (T(G/A)TTT) is found in the promoters of several genes that respond to insulin. However the mechanism by which insulin

signals to this motif appears to be distinct for many of these genes, the exception being the PEPCK, IGFBP-1 and TAT genes. This group of hepatic, insulin-regulated genes shares the ability to bind the glucocorticoid accessory factor, HNF3, at or very close to their T(G/A)TTT-motif. Thus, it would appear that insulin utilizes a number of distinct sequences to regulate gene transcription, and the task of identifying an insulin response sequence in each insulin-regulated gene promoter will therefore be complex (Sutherland C. et al., 1997).

The loss of the appropriate regulation of the expression of specific gene(s) by insulin could cause or contribute to resistance to this hormone (Sutherland C. et al., 1997). Resistance to insulin-stimulated glucose uptake is associated with several cardiovascular disease risk factors, including hypertension, dyslipidemia, and alterations of the blood clotting cascade that accentuate thrombosis (Kotchen TA. et al., 1996).

I.4. Renin-Angiotensin System (RAS)

I.4.1. RAS Cascade

The renin angiotensin system (Figure 1.4), a set of interacting and mutually supportive hormones secreted from the kidney and adrenal cortex, is considered a long-term regulator of (a) sodium balance and extracellular fluid volume, (b) potassium balance, and (c) effective arterial blood pressure. As such, it respond to all influences, both external and internal, that affect any of these three parameters (Sealey JE. and Laragh JH., 1990).

Angiotensinogen (ANG) is synthesized and released predominantly from the liver and subsequently cleaved by an α-glycoprotein enzyme, renin, to form the decapeptide angiotensin I (Ang I). Ang I is then cleaved in vascular beds, particularly in the pulmonary circulation, by angiotensin converting enzyme (ACE) to form angiotensin II (Ang II). This octapeptide is finally inactivated by proteases in the circulation. Ang II is a potent vasoconstrictor and a primary stimulus

for aldosterone secretion from the adrenal gland. Circulating RAS plays an important role in blood pressure and electrolyte as well as fluid homeostasis (Ballermann BJ. et al., 1991).

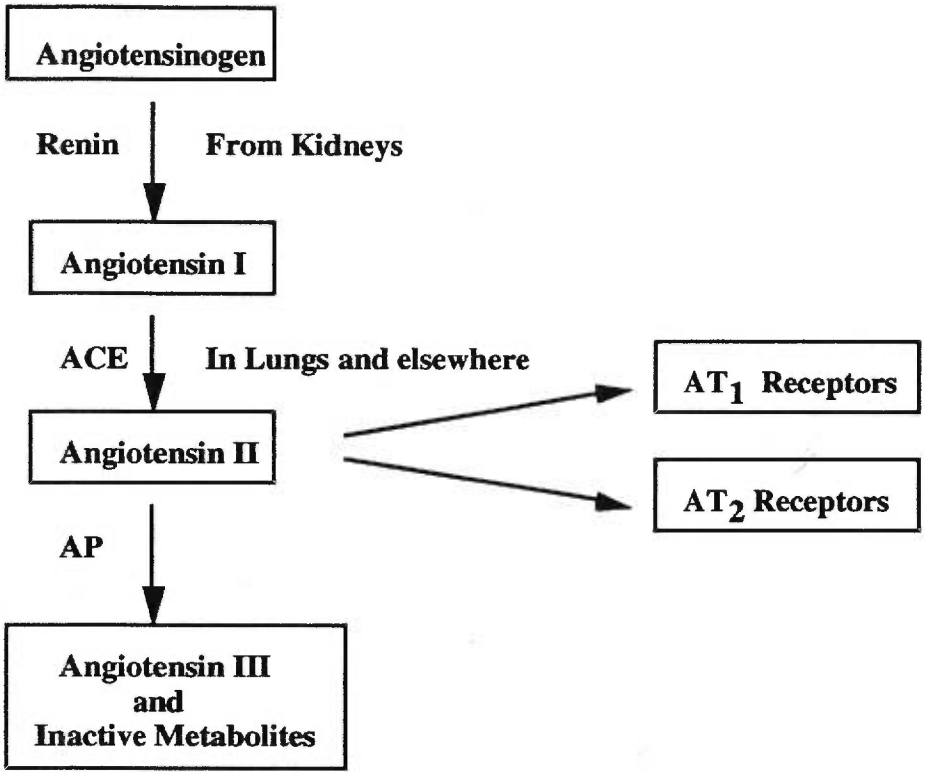


Figure I.4. Renin-Angiotensin System

I.4.2. RAS and Hypertension

I.4.2.1. Genetic Basis of Hypertension

Human essential hypertension, which represents more than 95% of the hypertensive population, is defined by elevated blood pressure without ascertained cause, and is a major risk factor for human cardiovascular morbidity and mortality. Large population based studies, adoption studies, and twin studies have suggested that about 20-40% of human BP variance is genetically determined (Ward R., 1990). It is likely that environmental factors interact with multiple genetic determinants to produce high BP. Identifying the possible genes would not only provide clues

about the mechanisms of the regulation of blood pressure, but also enable identification of subjects at genetically high risk for hypertension for whom preventive measures could then be taken (Hata A., 1995).

Technical advances in defining polygenic diseases in humans over the past few years has promoted numerous studies of the underlying genetic cause of rare Mendelian forms of hypertension, such as glucocorticoid remediable aldosteronism (chimeric duplication of 11 beta-hydroxylase and aldosterone synthase) (Lifton RP. et al., 1992), and Liddle's Syndrome (β and γ subunits of renal-epithelial sodium channel) (Hansson H. et al., 1995). The strongest evidence implicating a gene as the cause of human essential hypertension is for the ANG gene (Jeunemaitre X. et al., 1992).

I.4.2.2. Role of RAS in the Development of Hypertension

The RAS is one of the most important humoral factors underlying the mechanism of hypertension. Among a number of factors regulating blood pressure, the RAS plays an important role in body electrolyte homeostasis, vascular tone and cardiovascular remodeling (Hata A.,1995). Interruption of RAS expression by pharmacological agents attenuates high BP and other pathophysiological aspects of hypertension both in human and animal models (Iyer SN. et al., 1996). It is also well established that inhibition of the RAS (by renin inhibitors, ACE inhibitors, and Ang II antagonists) is highly effective to decrease BP in hypertensive animals and humans (Jeunemaitre X. and Lifton RP., 1993).

The key role of the products of the RAS in the regulation of blood pressure in the human strongly motivates the study of the genes of this system in human hypertension. These genes include those encoding renin, ANG, ACE, and the Ang II receptor (Jeunemaitre X. and Lifton RP., 1993).

The RAS controls blood pressure through the enzymatic production of the vasopressor Ang II from the ANG (Hata A., 1995), as shown in figure 1.3. Inhibitors of Ang II production (ACE inhibitors) or action (Ang II receptor antagonists) are effective antihypertensive agents in many patients (Davisson RL. et al., 1997).

The ANG gene was demonstrated to exhibit genetic linkage and association to essential hypertension. Evidence for genetic linkage between certain variants of the human ANG gene and essential hypertension have been presented. Several studies have demonstrated a link between the ANG gene and hypertension: (a) the ANG gene from the spontaneously hypertensive rat (SHR) cosegregates with pulse pressure in SHR x Wistar Kyoto F_2 intercrosses (Lodwick D. et al., 1995); (b) central administration of ANG antisense oligonucleotides lowers blood pressure in the SHR (Phillips MI., 1997); (c) transgenic mice containing both the human renin and human ANG genes exhibit high plasma Ang II levels, altered baroreflex activity, and chronic hypertension (Merrill DC. et al., 1996); (d) plasma ANG and arterial blood pressure vary proportionally with the number of functional copies of the mouse ANG gene in gene-targeting mice (Kim HS. et al., 1995); (e) the overproduction of Ang II in transgenic mice carrying both human renin and human ANG genes leads to a sustained increase in BP (Fukamizu A. et al., 1993); (f) the inhibition of RAS (by renin inhibitors, ACE inhibitors, and Ang II antagonists) decreases BP in normal state (Schalekamp MADH. et al., 1992); (g) the ANG-deficient mice generated by homologous recombination in mouse embryonic stem cells develop hypotension (Tanimoto K. et al., 1994); (h) The M235T variant is associated with plasma ANG elevation, the M235T variant of ANG is more prevalent among hypertensive than among normotensive subjects in several Caucasian and Japanese populations (Corvol P. et al., 1997; Kunz R. et al., 1997). Several studies have demonstrated a link between the ANG gene and familial hypertension or hypertension of pregnancy (Corvol P. et al., 1997). A variant of the ANG gene, substitution of threonine rather than methionine at codon 235, was specifically associated with hypertension (Williams GH., 1994).

The renin gene is also an attractive candidate in the etiology of essential hypertension. Chronic human renin infusion resulted in severe hypertension with extreme plasma renin activity and plasma renin concentrations in rats transgenic for human ANG (Bohlender J. et al., 1996). Genetic studies have shown that renin is associated with the development of hypertension in several rat strains (Rapp JP. et al., 1989; Kurtz TW. et al., 1990). Studies of Chiang FT. et al. (1997) showed that the renin gene Hind III polymorphism is associated with hypertension. Transgenic animals bearing either a foreign renin gene alone (Peters J. et al., 1996) or in combination with the ANG gene develop precocious and severe hypertension (Ohkubo H. et al., 1990).

Recent studies showed that the association of the ACE gene polymorphism with essential hypertension is still controversial. Insertion/deletion (I/D) polymorphism of the ACE gene has been shown to be a determinant for the serum ACE level and a marker for several cardiovascular diseases (Nakano Y. et al., 1997). However, Maeda Y. et al. (1997)'s study showed that the ACE gene polymorphism is not associated with essential hypertension.

Ang II type 1 (AT_1) receptor has a key role in mediating the vasoconstrictor and growth-promoting effects of Ang II. A A/C transversion located at the 5' end of the 3' untranslated region (position 1166) in the gene for the AT_1 receptor has been reported to be associated with hypertension (Szombathy T. et al., 1998). However, studies by Castellano M. and Agabiti-Rosei E. et al. (1997) did not support a major role of the AT_1 receptor A/C^{1166} polymorphism as a marker of conditions associated with hypertension. Lu D. et al. (1998) explored the use of an adenoviral vector to transfer AT_1 receptor antisense cDNA into neurons and vascular smooth muscle cells and they successfully attenuated the actions of Ang II. The results of Phillips MI (1997) showed the feasibility of adeno-associated virus as a vector for antisense inhibition, which may ultimately be used in gene therapy for hypertension.

I.4.3. Renin

Renin is synthesized mainly in the kidney juxtaglomerular (JG) cells and is secreted into the bloodstream (Jones CA. et al., 1993). It is an aspartyl protease that cleaves the Leu^{10}-Leu^{11} bond of N-terminal ANG (Burton J. and Quinn T., 1988). Renin has species specificity. The human ANG can be cleaved by primate renin, but other mammalian renins can not cleave human ANG (Burton J. and Quinn T., 1988). This glycoprotein hormone has a molecular weight of 37 to 40 kDa (Ganong WF., 1995).

Renin is synthesized as a large preprohormone. Human preprorenin contains 406 amino acid residues. The prorenin that remains after removal of a leader sequence of 23 amino acid residues from the N terminal contains 383 amino acid residues, and after removal of the pro sequence from the N terminal of prorenin, active renin contains 340 amino acid residues. Prorenin has relatively little biologic activity. Some prorenin is converted to renin in the kidneys, and some is secreted. Renin has a half-life in the circulation of 80 minutes or less (Ganong WF., 1995).

Renin is the rate-limiting enzyme in the production of Ang II and control of renin release serves to regulate the activity (Ballermann BJ. et al., 1991). Mechanical, neural, and hormonal stimuli act in an integrated fashion to regulate the rate of renin release in response to the physiologic and pathophysiologic challenges. The release of renin is regulated principally by : i) the sodium chloride load to the macula densa region of the renal tubule; ii) by a baroreceptor sensor in the afferent arteriole; iii) by the renal nerve stimulation via β-adrenoceptors; iv) by the direct negative feedback of Ang II (Churchill PC., 1985).

The mouse and rat renin gene contains nine exons separated by eight intervening sequences, the human renin gene contains ten exons separated by nine introns. The renin gene of the three species spans 12 kb 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 receptor sites, and cAMP induction sequences. The combined action of these elements will result in tissue specific expression and regulation of the gene (Ibarra-Rubio ME. and

Pedraza-Chaverri J., 1993). Transgenic rats overexpressing the mouse renin gene develop hypertension (Bader M. and Ganten D. 1997; Bohlender J. et al., 1997).

Renin genes are also expressed in many tissues other than kidney JG cells. Renin mRNA has been detected in several extrarenal tissues: brain, heart, testes and submaxillary gland (Campbell DJ., 1987; Phillips MI. et al., 1993). The tissue specific expression of these genes has been studied (Tamura K. et al., 1995).

I.4.4. Angiotensinogen

I.4.4.1. Angiotensinogen Biochemistry

ANG is a α-2-glycoprotein with a molecular weight of 55- to 60- kiloDaltons (kDa) (Phillips MI. et al., 1993). ANG consists of 477 amino acids in rodents (Ohkubo H. et al., 1983; Clouston WM. et al., 1988) and 485 amino acids in the human (Kageyama R. et al., 1984). It contains a 24-amino acid leader peptide that is removed to reveal the mature protein (452 amino acids in humans, 453 in rodents) (Lynch KR. and Peach MJ., 1991). Although the ANG gene is expressed in several tissues, including brain, adipose tissue, and kidneys (Lynch KR. and Peach MJ., 1991)., liver is the major sources of circulating ANG (Congiu M. et al., 1992). ANG is the only known precursor of angiotensin peptides and limits the enzymatic reaction of renin by its availability (Phillips MI. et al., 1993). The availability of ANG to be cleaved by renin is the rate-limiting step in the RAS cascade (Takahashi S. et al., 1991). ANG contains several potential sites for N-linked glycosylation. Different glycosylation may be responsible for the isoelectric point size variant of this protein (Tewksbury DA., 1983).

ANG is a ubiquitous, moderately abundant protein with plasma and cerebrospinal fluid concentrations of approximately 10^{-6} M (55-65 μg/ml) and $0.2x10^{-6}$ (11-13 μg/ml), respectively (Germain C. et al., 1984). ANG is constitutively released and is not stored within secretory

granules. (Lynch KR. and Peach MJ., 1991).

Some ANG circulates as a high-molecular-weight form but this is normally only a minor component of plasma ANG (Gordon DB. and Sachin ZN., 1977). However, the latter form of ANG exists at higher plasma levels during the last trimester of pregnancy and is occasionally the predominant form in the plasma of hypertensive pregnant women (Tewksbury DA.,1983). This higher-molecular-weight ANG is an effective renin substrate and consists of ANG that is nonvalently linked to another, unidentified serum protein (Tewksbury DA. and Tyron ES., 1989).

Rat and mouse ANGs are 87% identical. The rodent and human ANG amino acid sequences are 60% and 64% identical overall, while the amino termini (i.e. Ang I) are 100% identical (Lynch KR. and O'Connell DP., 1993).

I.4.4.2. Angiotensinogen Gene

The rat (Tanaki T. et al., 1984), mouse (Clouston WM., 1988), and human (Gaillard-Sanchez I., 1990) ANG genes have been isolated and characterized. All are present as a single copy; the rat gene has been mapped to chromosome 19 (Mori M. et al., 1989), the human gene to chromosome 1 (Gaillard-Sanchez I. et al., 1990), and the mouse gene to chromosome 8 (Clouston WM. et al., 1989).

Several Laboratories have studied the rodent and human ANG gene promoters. These studies have been directed predominantly toward identifying the elements and their corresponding transcription factors that confer hormone responsiveness to the gene (Lynch KR. and Peach MJ., 1991).

The rat gene is the most thoroughly characterized, but the structure and function of the mouse and human genes, so far as is known, are not significantly different. The rat ANG gene is

roughly 12 kilobases (kbp) long and consists of five exons separated by four introns. The first exon encodes only a portion of the 5' untranslated region of the ANG messenger RNA (mRNA) and is separated from remaining exons by a 5 kbp intron (Lynch KR. and Peach MJ., 1991).

The ANG gene contains multiple cis-acting transcriptional elements within a kilobase of the major start site of transcription and that these elements include those ultimately responsive to glucocorticoids, estrogens, and cytokines. This region also contains an enhancer element that confers tissue specificity on ANG gene expression (Lynch KR. and Peach MJ., 1991). The upstream flanking region of the rat ANG gene contains several recognizable promoter elements, including a "TATA" element at position -30 bp (relative to the start site of transcription) and two putative glucocorticoid responsive elements (GREs, 5'-AGAACA-3') at positions -586 bp and -477 bp (Lynch KR. and Peach MJ., 1991).

Previous studies have demonstrated that the 5'-flanking region of the human ANG gene was important for tissue- and cell type-specific expression of the gene in vitro (Fukamizu A. et al., 1990) and in vivo (Takahashi S. et al., 1991). Fukamizu A. et al (1990) analyzed the promoter function of the 5' flanking region (-1,222 to +44) of the human ANG gene by fusing this region to a chloramphenicol acetyl transferase reporter gene and then tested this fusion gene in the human hepatoma cell line HepG-2 and in the human astrocytoma line T98G. They found a number of 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 (Fukamizu A. et al., 1990)

I.4.4.3. Angiotensinogen Messenger RNA

Rat ANG mRNA is roughly 1,800 nucleotides long, consisting of 61, 1,431, and 200-400 nucleotides of 5' untranslated, coding, and 3' untranslated sequence, respectively. The size

heterogeneity of this mRNA is largely because at least four different polyadenylation sites are used. These sites, which were identified by S_1 nuclease mapping, are closely spaced (at 1,650, 1,785, 1,800, and 1,840 nucleotides) (Ben-Ari ET. et al., 1989). Further size heterogeneity of the rat ANG mRNA is a result of an unusual feature of its gene, that is, treatment with glucocorticoids increases liver ANG synthesis and elicits the use of two additional, upstream transcription start sites (at -386 and -328 nucleotides). Although this phenomenon is interesting with regard to mechanisms of glucocorticoid action, these extended ANG mRNA apparently have the same coding potential as the predominant mRNA species and therefore have no obvious bearing on the functioning of the renin-angiotensin system. There is no evidence for alternate splicing of the ANG mRNA transcript (Ohkubo H. et al., 1986; Ben-Ari ET. et al., 1989).

I.4.4.4. Regulation of Angiotensinogen Gene Expression

The regulation of ANG gene expression has been extensively studied. ANG transcription is responsive to diverse hormonal mediators. These agents include circulating steroid hormones (glucocorticoids) (Braiser AR. et al., 1990), estrogens (Krattenmacher R. et al., 1994), and triiodothyronine (Hong-Brown LQ. and Deschepper CF., 1992) and cytokine hormones, such as interleukin-1 and TNF-α or Ang II (Ron D. et al., 1990; Bouhnik J. et al., 1988).

The most studied regulatory phenomenon regarding the ANG gene is it's regulation by glucocorticoids. Earlier studies suggested that glucocorticoid exert their effects at the level of transcription of the ANG gene (Freeman RH. and Rostorfer HH., 1972). Numerous reports have confirmed that glucocorticoids increase the rate of production of ANG protein and mRNA accumulation in liver, primary hepatocytes, and hepatoma cell lines (Deschepper CF. and Hong-Brown LQ., 1993). Two distinct "GREs" have been identified within the 5'-flanking sequence of the angiotensinogen gene (Ben-Ari ET. et al., 1989). It is now known that glucocorticoid hormones induce ANG transcription by binding directly to the GR. The GR is translocated into the nucleus to interact with GREs within the ANG promoter (Brasier AR. and Junyi LI., 1996).

Thyroid hormone and estrogens have been observed to increase ANG mRNA accumulation in a number of tissues. ANG gene expression is also positively affected by sex steroids (Lynch KR. and O'Connell DP., 1993). Cytokines (TNF-α and interleukin-1) also regulate the expression of the ANG gene at the transcriptional level in hepatocytes.(Brasier AR. and Junyi LI., 1996). Infusions of Ang II have been demonstrated to increase plasma angiotensinogen levels in vivo and to increase the rate of angiotensinogen release from liver slices, perfused livers, and isolated hepatocytes (Ben-Ari ET. and Garrison JC., 1988). This response has been argued to constitute a positive-feedback loop that may play a role in renal hypertension (Stuzmann M. et al., 1986).

Angiotensinogen synthesizing tissues respond differentially to glucocorticoid regimens. For example, treatment of rats with dexamethasone resulted in a marked increase in liver angiotensinogen RNA accumulation, a modest increase in whole brain angiotensinogen RNA, and no detectable increase in kidney angiotensinogen RNA (Kalinyak JE. and Perlman AJ., 1987). However, studies in our laboratory have demonstrated clearly that dexamethasone increases the expression of the rat angiotensinogen gene in an immortalized rat proximal tubular cell line (IRPTC) and rat hepatoma cell line (H-4-II-E) in a dose dependent manner (Wang LS. et al., 1998). Likewise, a single dose of estradiol was found to enhance liver angiotensinogen RNA accumulation to increase this RNA in the brain to a lesser extent, but had no effect on the heart angiotensinogen RNA accumulation (Kunapuli SP. et al., 1987). The regulation of angiotensinogen gene expression by insulin also has been reported in studies on cultured cells where this hormone exerts an inhibitory effect (Chang E. and Perlman AJ., 1988).

In conclusion, ANG levels are modulated under different physiological and pathological conditions (Clauser E. et al., 1989), and are also regulated differentially in a tissue-specific manner (Ingelfinger JR. et al., 1986), The cell specific expression of genes is regulated to a large extent at the transcriptional level by the interaction of DNA binding proteins with specific DNA control elements organized normally within the 5'-flanking regions of the transcribed genes (Maniatis T. et

al., 1987).

I.4.5. Angiotensin

I.4.5.1. Angiotensin I

Angiotensin I appears to function solely as the precursor of angiotensin II and does not have any other established action (Ganong WF., 1995).

I.4.5.2. Angiotensin II

I.4.5.2.1. Angiotensin II Biochemistry

Angiotensin II (Ang II) is an octapeptide that is synthesized in the blood from its precursor molecule ANG by successive proteolytic cleavage with renin and ACE. The Ang II concentration is high in the adrenal gland, moderately high in brown fat and ovary, moderate in mesenteric artery, kidney, brain tissue, pituitary, and uterus, and low in the left ventricle, right ventricle, and testes. Ang II is degraded rapidly, its half-life in the human being 1-2 minutes. It is metabolized by aminopeptidase A, the aspartyl-aminopeptidase (Asp AP) which by removal of the amino-terminal Asp^1 produces des-Asp^1-Ang II (Ang III) (Abhold RH. and Harding JW., 1988). Currently the only physiologically relevant processing enzyme recognized is renin; however, several serine proteases such as tonin (Boucher R. et al., 1974) and cathepsin G (Tonnesen MG. et al., 1982) are capable of releasing Ang II directly from angiotensinogen, at least *in vitro.*

I.4.5.2.2. Angiotensin II Receptors

There are at least 2 types of Ang II receptors defined on the basis of their differential pharmacological and biochemical properties, and designated as type 1 (AT_1) and type 2 (AT_2)

receptors (Dzau VJ. et al., 1994).

AT_1 receptors are found in blood vessel walls, the adrenal cortex, the brain, platelets, isolated glomeruli and many other organs (Krieger JE. and Dzau VJ., 1991). The AT_1 receptor comprises two isoforms, AT_{1a} and AT_{1b}, which are strikingly similar in amino acid structure, pharmacological specificity and signal transduction mechanisms (Sasaki K. et al., 1991; Sasamura H. et al., 1992). Both AT_{1a} and AT_{1b} receptor genes encode a protein of 359 amino acids. They differ in 22 amino acids being clustered in the carboxyl terminal putative intracellular tail region (Sasaki K. et al., 1991; Sasamura H. et al., 1992). The AT_1 receptor has been shown to be responsible for all of the physiological effects traditionally associated with angiotensin II, such as smooth muscle contraction, aldosterone release, and the regulation of fluid and electrolyte balance (Dzau VJ. et al., 1994).

The AT_1 receptor appears to be the conventional G protein-linked Ang II receptor (Krieger JE. and Dzau VJ., 1991). Upon binding to AT_1, Ang II stimulates phospholipase C activity, formation of 1,2 DAG, production of inositol-1,4,5 triphosphate (and consequent mobilization of intracellular calcium from intracellular stores), and PKC translocation (Lang U. and Valloton MB., 1987). Recent studies have also shown that AT_1 stimulation is mediated by the activation of the MAP kinase pathway and of immediate early genes. Phosphorylation and dephosphorylation of tyrosine kinases have been associated with AT_1 receptor signal transduction (Reviewed by Regitz-Zagrosek V. et al., 1996).

The functional role for the AT_2 receptor subtype in many fetal tissues suggests that this receptor may be involved in development and/or the regulation of various growth processes. Recent data have demonstrated that the biological effect of the AT_2 receptor is to counteract the effect of the AT_1 receptor (Huang XC. et al., 1996). AT_2 receptors are more plentiful in fetal and neonatal life, but they persist in the brain and other organs in adults. AT_2 receptors have an unknown second messenger that is not IP_3 or cyclic AMP and their functions are unknown. Thus far, the AT_2

receptor does not appear to be G protein-linked, nor has any physiological role been determined (Krieger JE. and Dzau VJ., 1991).

As mentioned above, transgenic animal models, particularly with targeted disruption of AT_1 and AT_2 genes, suggest the contribution of both genes to blood pressure regulation. Genetic polymorphisms have been described in the AT_1 and AT_2 gene or neighbored regions and are used to analyze the association between defects and cardiovascular diseases. AT_1 antagonists are now being introduced into the treatment of hypertension and potential heart failure (Regitz-Zagrosek V. et al., 1996).

I.4.5.2.3. Angiotensin II Effect

Angiotensin II has many different actions, most of which relate, either directly or indirectly, to the regulation of blood pressure, fluid and electrolyte homeostasis (Campbell DJ., 1987). Angiotensin II is one of the most potent vasoconstrictors known, being 4-8 times as active as norepinephrine on a weight basis in normal individuals (Ganong WF. et al., 1995). Ang II also has important effects on the central and peripheral nervous system, the adrenal, kidney, intestine, and heart (Peach MJ., 1977). Angiotensin II acts directly on the adrenal cortex to increase the secretion of aldosterone, and is a major regulator of aldosterone secretion (Fraser R. et al., 1979). Additional actions of angiotensin II include facilitation of the release of norepinephrine by a direct action on postganglionic sympathetic neurons (Hughes J. and Roth RH., 1971), contraction of mesangial cells with a resultant decrease in glomerular filtration rate (Haley DP. et al., 1987), and additional direct effects on the kidneys. Ang II also acts on the brain to increase blood pressure, increase water intake, and increase the secretion of vasopressin and ACTH (Campbell DJ., 1987). Ang II also serves as a myocardial growth factor and controller of sympathetic nervous system output (Campbell DJ., 1987). Other diverse effects include stimulation of uterine smooth muscle contraction, stimulation of erythropoietin, inhibition of the glucagon effects on the liver (Baxter JD. et al., 1987) and modulation of the angiotensin receptor number and the stimulation of angiogenesis

(Hagemann A. et al., 1994).

Ang II increases blood pressure in several different ways. First, it exerts a powerful, direct, and immediate vasoconstrictive effect on the arteriolar bed. At the same time, it acts directly on the renal proximal tubules to increase sodium reabsorption. Additionally, at a somewhat slower pace, it stimulates the adrenal cortex to secrete aldosterone, which acts on the distal nephron to retain sodium in exchange for potassium, which is excreted. The retained sodium is responsible for an accumulation of fluid volume; this, in turn, affects blood pressure. The increased available sodium may also heighten vascular sensitivity to angiotensin and thus induce additional vasoconstrictive influences (Sealey JE. and Laragh JH., 1990).

Intravascular Ang II production stimulates de novo synthesis of its precursor in a positive feedback loop through increased gene expression (Li J. and Brasier AR., 1996).

In the kidney, Ang II plays a key role in renal injury and in the progression of chronic renal disease of diverse causes. In vascular smooth muscle cells, Ang II modulates growth, which may lead to hypertrophy and also may inhibit mitogen-stimulated DNA synthesis (Klahr S. and Morrissey J., 1998). Angiotensin II increases the production of several autocrine factors, including TGF-β, tumor necrosis factor-alpha (TNF-α), and platelet-derived growth factor A chain (PDGF). Angiotensin also increases the release of other growth factors such as endothelin, platelet-activating factor (PAF), and interleukin 6. In addition, it increases the "activity" of nuclear factor-kappaB (NF-kappaB) and the synthesis of angiotensinogen (Klahr S. and Morrissey J., 1998).

I.4.5.2.4. Molecular Mechanisms of Action of Angiotensin II

Ang II elicits its cellular actions by binding to highly specific cell surface receptors and leads to a cascade of events. The cellular mechanisms by which the interaction between Ang II and its receptors evokes a cellular response involve two pathways: the Ca^{2+}-calmodulin branch, which

initiates the contractile response, and the PKC branch, which sustains it. When Ang II binds to its receptor, phosphatidyl-4,5-biphosphate (PIP_2) is sequentially hydrolyzed to liberate "second messengers" or their precursors. This phospholipase C (PLC)-catalyzed process releases water-soluble Inositol 1,4,5-triphosphate (IP_3) from the membrane, which, in turn, stimulates Ca^{2+} release from intracellular organelles (Marsden PA. et al., 1990).

A growing body of evidence suggests that Ang II exerts some of its actions via the activation of proto-oncogenes such as c-fos and c-myc (Li J. and Brasier AR., 1996). The results of studies by Takeuchi K. et al (1990) and Naftilan AJ. et al (1990) suggest that at least some of the effects of Ang II may be associated with the activation of a well-known enhancer element, AP1, found to be important in the control of transcription of several other genes. This activation is dependent on a protein kinase C pathway.

I.4.5.3. Angiotensin III

Ang II is metabolized by aminopeptidase A, which by removal of the amino-terminal Asp^1 produces des-Asp^1-Ang II (Ang III). The Ang III is then metabolized in part by arginyl aminopeptidase (Arg AP) (Abhold RH. et al., 1988). Angiotensin III has about 40% of the pressor activity of angiotensin II but 100% of the aldosterone-stimulating activity (Phillips MI. et al., 1993).

I.4.6. Angiotensin-Converting-Enzyme (ACE)

ACE is a dipeptidyl-carboxypeptidase that splits off histidyl leucine from the physiologically inactive Ang I, forming the octapeptide Ang II. The same enzyme inactives bradykinin. Most of the conversion occurs as the blood passes through the lungs, but there is also conversion in many other parts of the body (Ganong WF., 1995).

endocrine system designed for the general mediation, through the systemic circulation, of the effects of renin on Ang production in plasma (Campbell DJ., 1987).

I.4.7.2. Local RAS (Tissue RAS)

Evidence is accumulating that, in addition to the system that generates circulating Ang II, many different tissues contain independent renin-angiotensin systems that generate Ang II, apparently for local use. The local RAS operates, in whole or in part, independently of the circulating RAS (Campbell DJ., 1987). There are tissue renin-angiotensin systems in the adrenal cortex, renal, testis, ovary, anterior and intermediate lobes of the pituitary, brain etc.(Campbell DJ., 1987).

The detection of mRNAs for angiotensinogen and renin in a tissue is strong evidence for their local synthesis in vivo. ANG is the only known precursor of the Ang peptides; thus, definitive evidence for an independent tissue RAS system requires demonstration of local synthesis of ANG. Recently, ANG mRNA has been detected in 12 different extrahepatic tissues of the rat: kidney, brain, spinal cord, aorta, mesentery, adrenal, atria, lung, stomach, large intestine, spleen, and ovary (Campbell DJ., 1987). Renin mRNA has also been detected in several extrarenal tissues: adrenal. heart, testes, and submaxillary gland. These data for extrarenal renin mRNA were obtained from studies on mice with two renin genes. These two renin genes have different patterns of tissue-specific expression, and for many tissues (adrenal, heart, testes) it is not known to which gene the renin mRNA corresponds; whether these data apply to man and other species with one renin gene remains to be determined (Campbell DJ. and Habener JF., 1986; Campbell DJ., 1987). Recent studies by Wolf G. et al. (1995), Chan JSD. et al. (1992a) and Tang SS. et al. (1994) have demonstrated the presence of renin mRNA in mouse and rat renal proximal tubular cells.

I.4.7.3. Relationship between Circulation RAS and Local RAS

Most of the ACE that cleaves Ang I in the circulation is expressed in endothelial cells and distributed throughout the body (Lieberman J. and Sastre A., 1983). In the kidney, ACE is localized on the vascular endothelial cells of the renal vessels and in the epithelial cells of the brush border of the renal proximal convoluted tubule and juxtaglomerular cells (Danilov SM. et al., 1987).

Serum ACE activity is induced by corticosteroids (Ballermann BJ., 1989) and thyroid hormone (Smallridge RC. et al., 1983). The endogenous kinins and Ang II regulate the developmental expression of renal ACE gene in the rat (Yosipiv IV. and El-Dahr SS., 1995).

The ACE gene of mouse, human, bovine and rabbit has been cloned (Goraya TY. et al., 1994). Serum ACE activity is increased with hyperthyroidism, and a number of diseases, like primary biliary cirrhosis, silicosis, and sarcoidosis (Friedland J. et al., 1978). Studies of Goraya TY. et al (1994) have identified positive (-270 to 0) and negative (-692 to -610) regulatory elements present in the 5'-upstream region of the rabbit somatic ACE transcription unit, which are used both in the vascular endothelial and kidney epithelial cells. There is a TATA-like sequence at -27 and a CRE-like sequence at -52 in the rabbit testis ACE (Kumar RS. et al., 1991). Studies of Zhou Y. et al. (1995) have identified two positive transcriptional elements, a putative cAMP response element (CRE) at -55 and a TATA box at -32, in the promoter for mouse testis ACE. As mentioned before, the association of the ACE gene polymorphism with essential hypertension is still controversial. Further studies are warranted to define the role of ACE gene polymorphism in essential hypertension.

I.4.7. Circulating RAS and Local RAS

I.4.7.1. Circulating RAS

At the present time, the prevailing view holds that the circulating RAS is primarily an

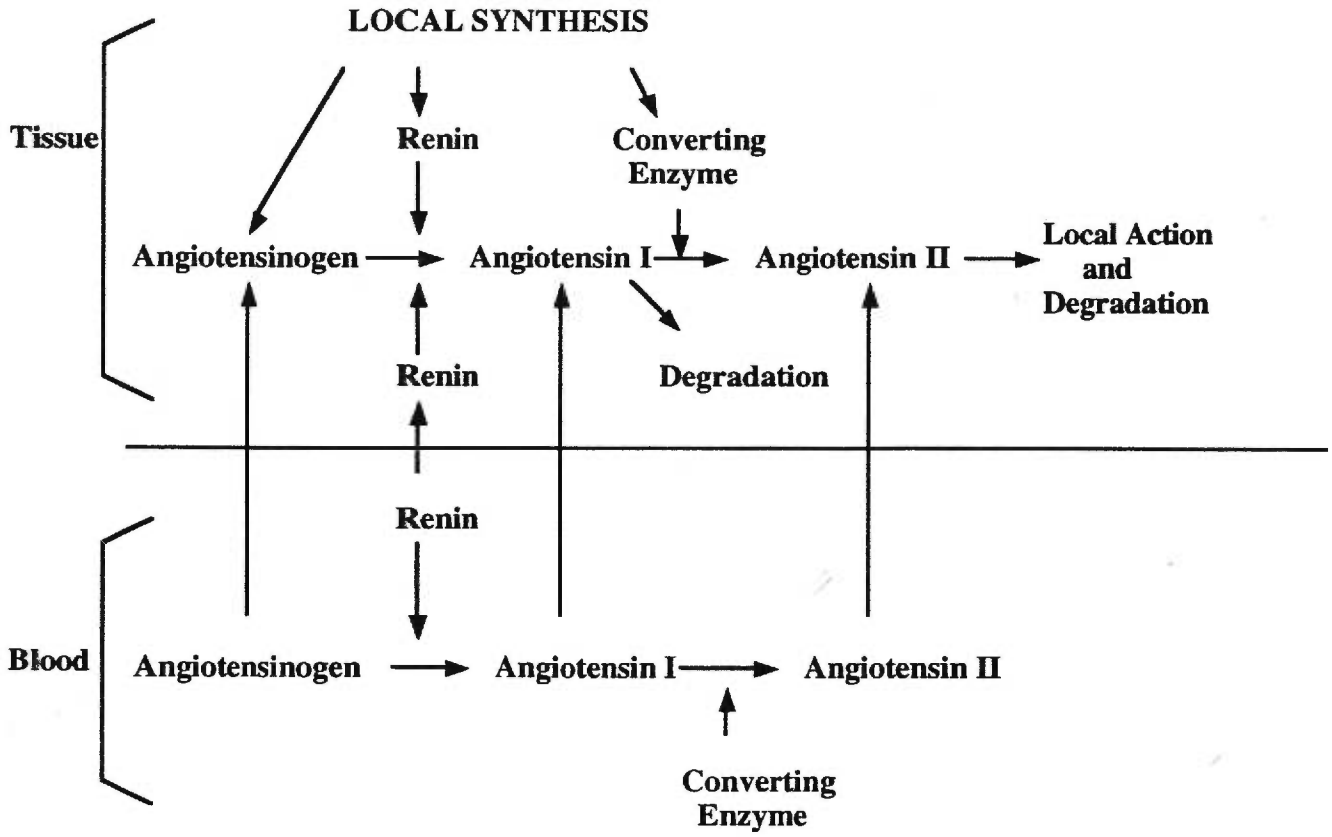


Figure 1.5. Circulation RAS and Local RAS.

The circulating RAS and locally synthesized components may integrate in the generation of Ang (Campbell DJ., 1987). The generation of Ang within a tissue may be either intracellular or extracellular. Intracellular generation of ANG may result from cleavage of either locally synthesized ANG or ANG taken up by the cell; the enzyme responsible for cleavage may be either a cellular enzyme or an enzyme taken up by the cell. The ANG or enzyme taken up by a cell may be plasma derived, or may have been secreted into the interstitial space by neighboring cells. Plasma-derived and locally synthesized ANG may coexist in the interstitial space, where they may be cleaved by either plasma derived renin, or locally synthesized renin or reninlike enzyme, to produce angiotensin (Campbell DJ., 1987).

The circulating RAS provides an extremely rapid and efficient homeostatic response to acute changes in blood pressure and fluid and electrolyte status. In contrast, tissue angiotensin systems may provide a more tonic, and specifically local, influence in those tissues where they exist, for example the regulation of vascular tone, or renal, cardiac, adrenal, or intestinal function (Campbell DJ., 1987).

I.4.8. Intrarenal RAS

The kidney has been traditionally considered to be one of the pivotal organs involved in the systemic actions of the RAS. However, many studies in the last decade have provided convincing evidence that the kidney exhibits a local RAS which may independently function from the systemic actions of the endocrine RAS (Wolf G. et al.,1995). Much of the intrarenal Ang II is formed locally as evidenced by intrarenal Ang II contents that are much greater than can be explained from the circulating Ang II concentration. Intrarenal Ang II is formed from systematically delivered Ang I and from intrarenally formed Ang I derived from systematically delivered ANG as well as locally synthesized ANG (Navar LG. et al., 1997). Both renin and ANG mRNA are detectable in kidney (Phillips MI. et al., 1993). ACE mRNA (based on pulmonary ACE) is strongly expressed in kidney (Whiting P et al., 1991). All the components of the RAS are highly expressed in the developing kidney in a unique spatial and temporal pattern that is associated with nephrogenesis, vascularization, and the proper architectural and functional development of this organ (Hilgers KF. et al., 1997). The kidney RAS is regulated by several factors. Kidney ANG mRNA is increased by dexamethasone and triiodothyronine compared to controls. The kidney RAS is regulated by Na^+ volume reduction and high Ang II (Wolf G.,1995).

Physiological studies in the rat by Seikaly MG. et al. (1990) have shown that the level of luminal Ang II in the renal proximal tubule is as high as 10^{-9} M whereas the level of plasma Ang II is less than 10^{-12} M, a difference of one thousand-fold.

The intrarenal RAS plays a critical role in the paracrine regulation of renal hemodynamics and tubular transport function. Increasing evidence has accumulated that the local intrarenal RAS serves as an important regulator of renal function. In addition to regulating normal renal function, local renal RAS may be triggered in response to renal injury and may contribute to structural and functional abnormalities, including increased glomerulosclerosis and tubulointerstitial fibrosis (Harris RC. and Cheng HF., 1996).

The proximal tubule cells contain all of the components of the RAS necessary for synthesis and secretion of Ang II. Proximal tubular cells express mRNA and protein for ANG (Ingelfinger JR. et al., 1990; Chan JSD. et al., 1992a; Darby IA. and Sernia C., 1995), renin (Yanagawa N. et al., 1991; Moe OW. et al., 1993; Chen M. et al., 1994), ACE (Alhence-Gelas F. et al., 1979; Taugner R. et al., 1982). They bear different types of Ang II receptors, and these cells also exhibit surface proteases such as angiotensinase A which are required for the inactivation of Ang II (Wolf G.,1995). Moreover, studies in the isolated perfused kidney have clearly shown that proximal tubular cells produce considerable amounts of Ang II and these concentrations exceed, by approximately hundred times the systemic concentration of the peptide (Wolf G.,1995). Wang LS's study (1998) show that rat angiotensinogen is expressed in IRPTC cells, the concentration in culture medium and cellular extract are 4.26 ng/ml/24 hours and 93 pg/ml/24 hours, respectively. This local RAS may be important in the regulation of proximal tubule NaCl and $NaHCO_3$ transport. The activation of this RAS may play a pathogenic role in some patients with essential hypertension and in the hypertension and cyst growth seen in autosomal dominant polycystic kidney disease (Moe OW. et al., 1993).

The opossum kidney (OK) proximal tubular cells are an established epithelial cell line which is often studied as a physiological model system of renal proximal tubule function (Nash SR. et al., 1993) . OK cells express a low level of ANG mRNA and ACE (Chan JSD. et al., 1992a; Ingelfinger JR. et al., 1991). Thus, it was conceivable that OK cells could be useful for studing the regulation of the transcription of ANG gene in vitro (Chan JSD. et al., 1992a). Studies have been

done in the last five years to investigate the ANG gene expression in OK cells. L-T_3 is effective at increasing the expression of the rat ANG gene in OK cells via a transcriptional mechanism (Chan JSD. et al., 1992a). Glucocorticoid hormone is effective at stimulating the transcription of the rat ANG gene in OK cells, but stimulation is not observed with testosterone, estradiol, or progesterone. Moreover, glucocorticoid and L-T_3 act synergistically to stimulate the trancription of the ANG gene (Chan JSD.et al., 1992b). β_1-adrenoceptors and dexamethasone act synergistically to stimulate the expression of the rat ANG gene in OK cells via the putative cAMP responsive element (CRE) and glucocorticoid responsive element (GRE) in the 5'-flanking region of the rat ANG gene (Ming M. et al., 1995). Isoproterenol and iodoclonidine stimulate the expression of the rat ANG gene in OK cells in vitro in a dose-dependent manner. The effect of isoproterenol is mediated via the β_1-adrenoceptor and cAMP-dependent PKA pathway (Wang TT. et al., 1994), whereas the effect of iodoclonidine is mediated via the α_2-adrenoceptor and PKC pathway (Wang TT. et al., 1995). A more recent paper of Qian JF et al (1997) showed that the addition of isoproterenol or forskolin enhances the stimulatory effect of CREB on the expression of the rat ANG gene in OK cells. These studies showed that isoproterenol stimulates the expression of the rat ANG gene via the cAMP-dependent PKA and probably via the interaction of the 43-KD cAMP responsive element binding protein (CREB) with the 5'-flanking region of the ANG gene. Furthermore, the above studies indicate the presence of a functional relationship between the renal sympathetic nervous system and the activation of local intrarenal RAS. Terada Y. et al (1995) have investigated the effects of Ang II on serine/threonine cascades in OK cells. They found that Ang II stimulated MAP kinase and S6 kinase activities in OK cells, and this activation is largely dependent on PMA-sensitive PKC (Terada Y. et al., 1995).

Hyperglycemia is an important etiologic factor in the development of diabetic nephropathy (Raskin P. and Rosenstock J., 1986). Clinical studies have shown that ACE inhibitors and Ang II receptor antagonists decrease proteinuria and slow the progression of DN (Lewis EJ. et al., 1993; Remuzzi A. et al., 1993; Gandhi SK. et al., 1996). More recently, studies in our laboratory (Wang TT. et al., 1998) showed that high D(+)-glucose levels directly stimulate the expression of the rat

ANG gene in OK27 cells and that this effect is mediated via the PKC signal transduction pathway. These studies suggest that local renal RAS might play a significant role in the development of diabetic nephropathy.

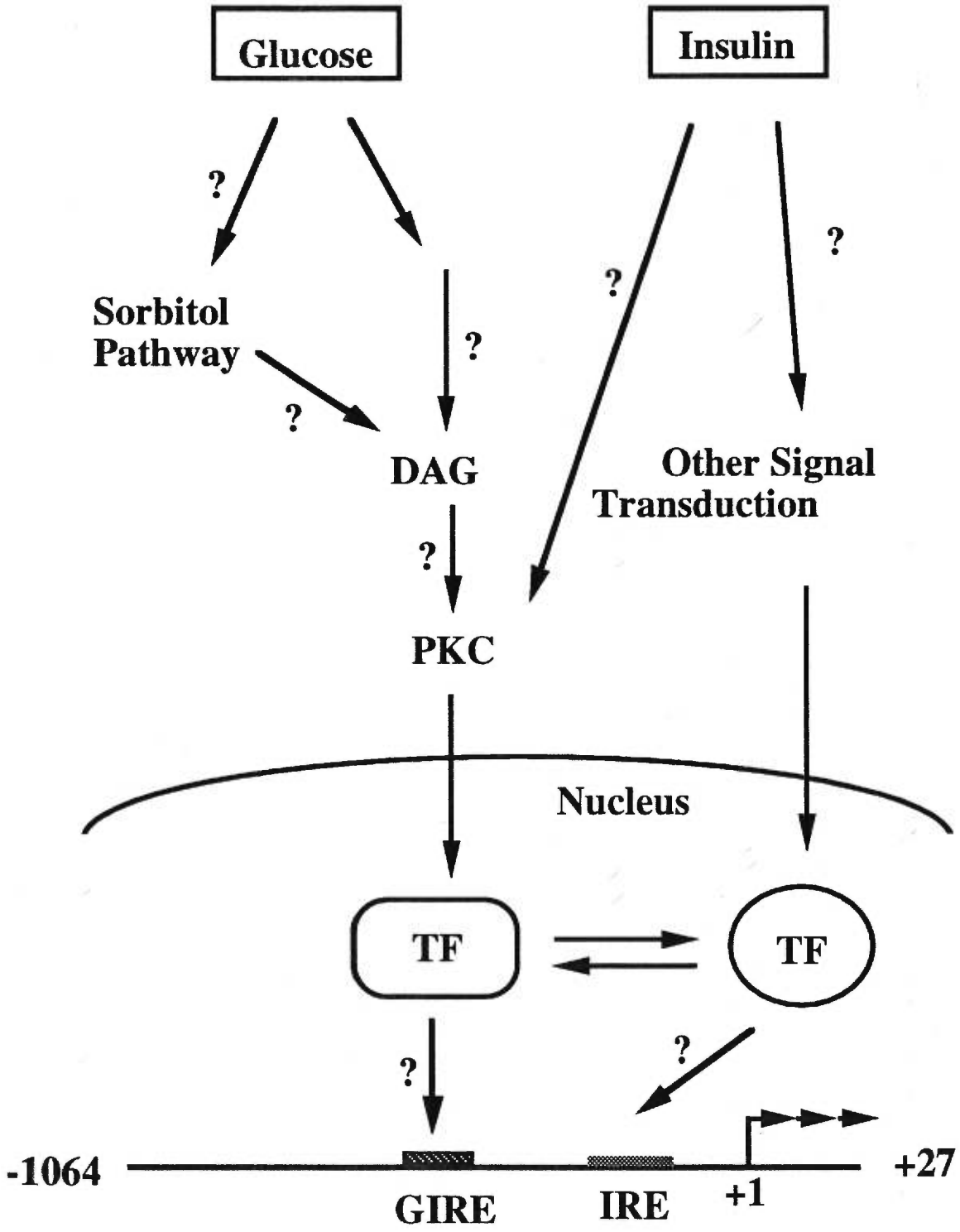
However, no studies have been reported on the effect of hyperglycemia, and/or insulin on the expression of the human ANG gene in kidney.

I.5. Objective of the Present Studies

We hypothesized that there is a reciprocal interaction between diabetes and the expression of RAS genes in the kidney. Abnormal glucose levels and insulin sensitivity in diabetes affect the intrarenal RAS, which in turn contribute to the development of diabetic nephropathy. This is illustrated in the following model(s) (Figure I.6).

The specific objectives of the present studies are 1) To investigate the effects of glucose and insulin on the expression of human angiotensinogen gene promoter activity in opossum kidney proximal tubular (OK) cells. 2) To investigate the molecular mechanisms of action(s) of glucose and insulin on the expression of the human angiotensinogen gene promoter in OK cells.

Figure I.6. Model of High Levels of Glucose and Insulin Effects on the Expression of the Human ANG Gene in OK Cells.



II. Materials & Methods

II.1. Materials

II.1.1. Chemical Reagents and Drugs

	M.W.	Cat. #	Company
Acetyl Coenzyme A	809,600	101907	BMC
Acrylamide	71.08	100-133	BMC
Albumin, Bovine	68,459	A-4503	Sigma
Calcium Chloride, dihydrate	147.0	C-3881	Sigma
2-deoxy-D-Glucose	164.2	D-6134	Sigma
1-(5-Isoquinolinyl sulfonyl)-2-methylpiperazine (H-7)	364.3	I-7016	Sigma
30% Hydrogen Peroxide (H_2O_2)	34.01	21676-3	Aldrich
Geneticin (G418 sulfate)	496.5	11811-031	GIBCO-BRL
D(+)-Glucose	180.2	G-5400	Sigma
Hepes	238.3	H-9136	Sigma
L-Glucose	180.2	G-5500	Sigma
Insulin	5733.5	I-1882	RBI
Insulin-like growth factor I (IGF-I)	7,500	I-3769	Sigma
Insulin-like growth factor II (IGF-II)	7,500	I-2139	Sigma
D-Mannitol	182.2	M-1902	Sigma
L-Dithiothreitol (DTT)	154.2	D-0632	Sigma
N',N'-Methylenebisacrylamide	360.3	15516-024	BRL
PD98059	267.3	513000	Calbiochem
Phorbol 12-myristate 13-acetate (PMA)	616.8	P-8139	Sigma
Penicillin G	1650 u/mg	P-3032	Sigma

Phenylmethylsufonyl Fluoride (PMSF)	174.2	P-7626	Sigma
Potassium Chloride	74.55	P-5405	Sigma
Proteinase K	28,800	745723	BMC
Sodium Bicarbonate	84.01	S-5761	Sigma
Sodium Chloride	58.44	S-5886	Sigma
Sodium Dodecyl Sulfate (SDS)	288.38	1026-693	BMC
Sodium Phosphate, dibasic anhydrous (chemical formula)	142.0	S-5136	Sigma
Sodium Phosphate, monobasic anhydrous (chemical formula)	120.0	S-5011	Sigma
Sodium Sulfate	466.54	S-137	RBI
Streptomycin Sulfate	729 u/mg	860-1860	Calbiochem
N',N',N',N'-Tetra-methylethylenediamine (TEMED)		161-0800	BIO-RAD
Trypan blue stain 0.4%		15250-020	GIBCO-BRL
Trypsin Inhibitor		T-9003	Sigma
Tolrestat	357.3	AY-27773-42	Wyeth-Ayerst
Wortmannin	428.4	681675	Calbiochem

N.B.

BIO-RAD: Bio-Rad Laboratories, Inc. (Richmond, CA, USA)

BMC: Boehringer Mannheim Corp. (Laval, Quebec, Canada)

Calbiochem: Calbiochem-Novabiochem corp. (La Jolla, CA, USA)

GIBCO-BRL: GIBCO-BRL Life Technologies (Burlington, Ontario, Canada)

RBI: Research Biochemicals International (Natick, MA, USA).

Sigma: Sigma Chemical Corporation (St. Louis, MO., USA).

Wyeth-Ayerst: Wyeth-Ayerst Research (Princeton, NJ., USA)

II.1.2. Cell Culture Media and Supplements

All culture media and supplements were purchased from GIBCO-BRL (Quebec, Canada).

	Cat. #
Dulbecco's Modified Eagle's Medium (DMEM), High glucose	12800-082
DMEM, Low glucose	31600-083
DMEM, Base (without glucose)	23800-014
Phenol red solution 0.5%	15100-043
Dulbecco's Phosphate Buffered Saline, 10X	14200-075
MEM sodium pyruvate solution, 100 mM, 10X	11360-070
MEM non-essential amino acids solution, 10 mM, 100X	11140-050
Trypsin-EDTA, 1X	25300-062
Fetal Bovine Serum (FBS)	16140-071

II.1.3. Cell Culture Plasticware

The 6-, 24-, and 96-well plates and 100 x 20 mm Petri dishes were purchased from GIBCO Bethesda Research Laboratories (BRL) (Quebec, Canada).

The 5 ml and 10 ml disposable sterile pipettes were purchased from Costar Corporation (Cambridge, MA, USA).

II.1.4. Isotopes

Na-^{125}I (Cat. #NEZ-033A) and γ-[^{32}P]ATP (3000 Ci/mmol) (Cat. #NEG-502A) were purchased from Dupont, NEN (Boston, MA, USA). D-thero-[dichloroacetyl-1-^{14}C]chloramphenicol (Cat. #CFA747) was purchased Amersham Life Science.

II.1.5. OK Cell Line

The opossum kidney proximal tubular (OK) cell line was obtained from the American Type Tissue Culture Collection (ATCC). It was derived from the kidneys of an adult female American opossum. This cell line has been characterized with respect to morphology, chromosome constitution, tissue-culture requirements, and attainable mitotic arrest. The cells are morphologically epithelial-like (Koyama H. et al, 1978).

OK cells have renal epithelial properties such as the polarized distribution of plasma membrane proteins, the expression of an apical microvilli, and the presence of renal transport systems that are characteristic of the proximal tubule. Parathyroid hormone (PTH), forskolin, and prostaglandin E1 increases cAMP formation in OK cells. PTH inhibits sodium-dependent phosphate transport in OK cells (Koyama H. et al., 1978). Futhermore, these cells have been shown to possess most of the ARs, most importantly, insulin receptor has been localized on the apical and basal membrane of the OK cell (Yagil C. et al., 1988a). Therefore, this cell line is used as the model for proximal tubule cells and these cells have been chosen as a prototypical model for our studies.

II.1.6. Restriction and Modifying Enzymes

All enzymes were purchased either from GIBCO-BRL (Burlington, Ontario, Canada), Boehringer-Mannheim (Dorval, Quebec, Canada), Pharmacia Inc. (Baie d'Urfé, Quebec, Canada) or Sigma Biochemicals (St-Louis Mo. USA).

II.1.7. Vectors

pOGH and pOCAT (Nichols institute of Diagnostics, La Jolla, CA, USA) contain the entire genomic sequence of the human growth hormone (hGH) gene or the chloramphenol acetyltransferase (CAT) gene, respectively.

pRC/RSV (Invitrogen Corporation, San Diego, CA, USA) is a mammalian expression vector, it contains the RSV/LTR promoter/enhancer plus a bovine growth hormone poly-adenylation signal for polyadenylation of transcribed mRNAs.

pTKGH (Nichols institute of Diagnostics, La Jolla, CA, USA) contains tyrosine kinase (TK) promoter/enhancer sequence fused to the 5'-end of the human growth hormone gene

pOGH(hANG N-1064/+27) (Figure II.1) was made by Dr. T.T. Wang in our laboratory. It contains the 5'-flanking region of the human ANG gene fused with the hGH gene (the glucocorticoid responsive element, GRE was deleted) as a reporter gene.

pOCAT(hANG N-1064/+27) (Figure II.1) was made by Dr. X. Chen in our laboratory. It contains the 5'-flanking region of human ANG gene fused with CAT gene as a reporter gene.

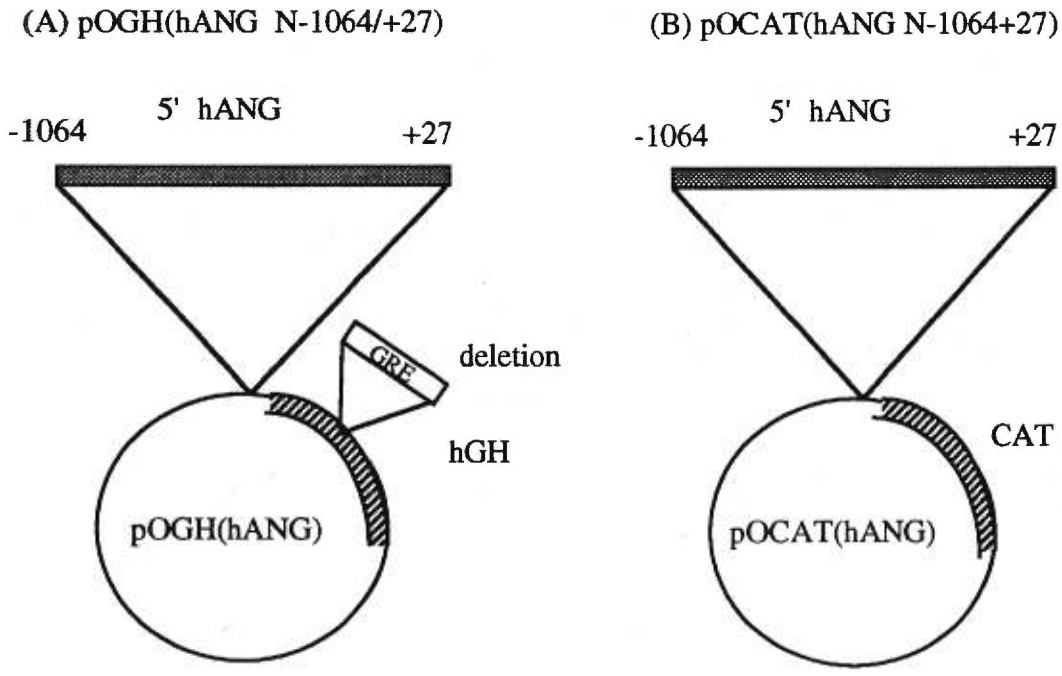


Figure II.1. Construction of Fusion Genes

II.1.8. The Radioimmunoassay Kit for Human Growth Hormone (RIA-hGH)

The RIA-hGH kit was a gift from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases (NIADDK), NIH, USA. It contains hGH antigen and hGH antiserum (rabbit).

II.1.9. Protein Kinase C Enzyme Assay System

Biotrak™ protein kinase C (PKC) assay system was purchased from Amersham™ (code RPN 77) and was used to detect PKC activity.

II.1.10. Diacylglycerol (DAG) Assay Reagents System

DAG assay reagents system (code RPN 200) was purchased from Amersham and used to quantify the cellular levels of DAG.

II.1.11. Protein Assay

The microplate reader (model 3550) was purchased from Bio-RAD. Protein Assay Dye (cat# 500-0006) was purchase from Bio-RAD.

II.1.12. TLC Plates

Thin-layer chromatography (TLC) plates (cat. #05-719-800) were purchased from Fisher Scientific Ltd. (Montreal, Quebec, Canada).

II.1.13. Transfection Buffer

2X Hepes Buffered with Sodium Phosphate (HBSP):

	g	Final Concentration (mM)
Na_2HPO_4	0.21294	1.5
KCl	0.7455	10
NaCl	16.3632	280
Glucose	2.16152	12
Hepes	11.915	50

add ddH_2O to 1000 ml

(adjusted with 1N HCl to pH=7.2)

II.1.14 Radioimmunoassay (RIA) Buffer

The RIA buffer was prepared with:

0.5 M Na_2PO_4 (pH 7.4)	50 ml
0.1 M EDTA (pH 7.0)	50 ml
Triton X-100	1 ml
Bovine Serum Albumin	2.5 (g)

ddH_2O to 500 ml.

II.1.15. Others

All other reagents were of molecular biology grade and purchased either from Sigma Chemicals, Bethesda Research Laboratories (GIBCO-BRL), Boehringer-Mannheim, Pharmacia Inc. or Promega-Fisher Inc.

II.2. Methods

II.2.1. Cloning of the Human Angiotensinogen Gene Promoter

The 5'-flanking region of the hANG gene was cloned by polymerase chain reaction (PCR) from a human liver genomic Library (Clontech, La Jolla, CA, USA) by Dr. X. Chen in our laboratory. The forward primer: 5' GTC AGT GAA TGT ACA GCT TCT GCC 3' and the reversed primer: 5' TAG TAC CCA GAA CAA CGG CAG CTT 3' corresponding to the nucleotide sequences of N-1064 to N-1041 and N-2 to N+27 of the hANG gene (Kageyama R. et al., 1984) were used in PCR, respectively.

The hANG N-1064 to N+27 DNA fragment obtained by PCR was initially cloned in the plasmid, pCRII-1 (In Vitrogen, La Jolla, CA) and subsequently subcloned in the modified expression vectors, pOGH and pOCAT (Wang TT et al., 1994; Ming M et al., 1995).

The sequence and orientation for the fusion genes were confirmed by dideoxy sequencing with S6 primer (Promega-Fisher, Inc.) and restriction enzyme digestion mapping.

II.2.2. Cell Culture

The cells were initially grown in 100 X 20 mm plastic Petri dishes in monolayer in Dulbecco's Modified Eagle's Medium (DMEM), pH 7.45, supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were grown in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. For transfection, cells were trypsinized (0.05% trypsin and EDTA) and plated at 2.5 X 10^5 cells/plate in 6-well plates.

II.2.3. DNA Transfection

The cells were washed with fresh DMEM containing 10% FBS and incubated 2 hours before transfection. The plasmids, pOGH(hANG N-1064/+27) or pOCAT(hANG N-1064/+27) and pRSV-Neo fusion genes were cotransfected (10 μg each well) into OK cells utilizing calcium phosphate-mediated endocytosis. The calcium phosphate precipitate was prepared by adding 2 M $CaCl_2$ (186 μl), DNA (60 μg) and dH_2O (1314 μl) into a 15 ml cultured tube. Then 1500 μl of 2X HBSP was added slowly to the reaction mixture by inserting the pipette near the bottom of the tube. The reaction was carried out at RT without shaking for 5 minutes. After the reaction period, the solution was mixed with 5 strokes (up and down) with pipette and 1 ml of it was added to each well of the cells (5 ml medium) and rocked several times. The cells were incubated in 95% CO_2, 5% O_2 at 37°C and 100% humidity. After overnight incubation, the medium was changed and replaced with fresh DMEM containing 10% FBS.

II.2.4. Selection of Stable Transformants

Geneticin (G418) is an aminoglycoside related to Gentamicin and is used as a selective agent in molecular genetic experiments. It is toxic to both prokaryotic and eukaryotic cells. The resistance gene (neomycin) can be introduced and expressed in eukaryotic cells. Efficient expression is achieved when the Neo DNA sequence is linked to eukaryotic DNA sequences that permit transcription and processing of the Neo coding sequence into mRNA (Jimenez A. et al., 1980). The G418 has no effect on OK cells transfected with Neo gene at 500 μg/ml in the media.

The stable transformants which were able to grow in the presence of G418 (500 μg/ml) and secrete high levels of IR-hGH into the medium were further subcloned using the method of limiting dilution. Cells which have passed through at least three repetitions of limiting dilution and which still secret high levels of IR-hGH after three months in the presence of G418 were considered to be a stable clone.

OKW1 and OKW3 cells are stable transformants with pOGH(hANG N-1064/+27) and

pOCAT(hANG N-1064/+27) integrated into OK cellular genomes, respectively. OK13 cells are stable transformants with pTKGH integrated into the OK cellular genomes. OK13 cells were obtained by Dr. T.T. Wang in our laboratory.

II.2.5. Effect of Different Hormones and Drugs on the Expression of Fusion Genes in OKW1, OKW3, and OK13 cells.

OKW1, OKW3 and OK13 cells were plated at a density of 10^6 cells/plate of 6-well plate and incubated overnight in DMEM containing 5 mM D(+)-glucose and 10% FBS. Then cell growth was arrested by incubation in serum free and a low D(+)-glucose concentration (5 mM) medium for 24 hours. Subsequently, various concentrations of different reagents were added to low concentration (5 mM) or high concentration (25 mM) medium containing 1% depleted fetal bovine serum (dFBS). At the end of the incubation period, media and cells were collected and kept at -20°C until assayed for IR-hGH and cellular CAT activity, respectively.

II.2.6. Depleted Fetal Bovine Serum (dFBS)

The dFBS was prepared by incubation of the FBS with 1% activated charcoal and 1% 1 X 8 ion exchange resin for 16 hours or more at RT with gentle rotation. The serum was then filtered with 0.22 μ bottle top filter and stored at -20°C before use. This procedure removed endogenous steroid and thyroid hormones from the FBS as demonstrated by Samuels HH. et al.(1979).

II.2.7. Iodination of Human Growth Hormone

[^{125}I]-hGH was prepared by a slight modification of the lactoperoxidase method (Thorell JI. and Johnnson BJ., 1971). Sephadex G-100 column (20 X 1.5 cm) was used for separation of iodinated hGH and free iodine. The column was equilibrated with 0.025 M Tris-HCl, pre-ran with 1ml 2.5% BSA and washed with 0.025 M Tris-HCl buffer for 3 bed volumes. To a tube, 10 μl (10

μg) of hGH, 10 μl of 0.5 M phosphate buffer (pH 7.0), 10 μl Na-^{125}I (1 mCi), 10 μl (10 μg) of lactoperoxidase and 10 μl of H_2O_2 (1:7,500 dilution) (25 μl of 33% H_2O_2 + 375 ml dH_2O) were added. The reaction was carried out at room temperature (RT) for 5 minutes. Then, another 10 μl of H_2O_2 was added and left at RT for 5 minutes. At the end of the reaction period, 1 ml of 0.025 M Tris-HCl was added, mixed well, and the reaction solution was applied to the column. The elutates were collected in 0.5 ml aliquots. The radioactivity in each fraction was counted in a LKB gamma counter. The first radioactive peak (i.e. [^{125}I]-hGH) was collected and stored at -20°C for further use. The percentage of total radioactivity incorporated into hGH was 60-70%.

II.2.8. Radioimmunoassay (RIA)

The procedure for RIA was performed according to the method of Chan JSD. et al.(1978) and was carried out at 4°C for four days. Briefly, on day one, 100 μl of RIA buffer, 100 μl of sample or standard hGH and 100 μl of hGH antibody (rabbit, 1:400,000 dilution) were added to the test tubes and incubated at 4°C for overnight. The next day, 100 μl of radioactive hGH (20,000 cpm) was added and further incubated for 24 hours. On the third day, 50 μl of goat anti-rabbit serum (1:10) and 50 μl of normal rabbit serum (1:100) were added and incubated for overnight at 4°C. On the fourth day, the tubes were centrifuged at 780 x g (IEC Centra-8R Centrifuge, USA) for 30 minutes at 4°C. The supernatant was aspirated and the pellet was counted in an LKB gamma counter.

All samples were prepared in triplicate. If the amount of IR-hGH in the sample was too high (out of the detection limit on the standard curve), the samples were diluted with RIA buffer before assay.

The lower limit of sensitivity of the assay was 0.1 ng/ml. The linearity of the standard curve was from 0.2 to 10 ng/ml. The inter- and intra-assay coefficients of variation were 12% (n=10) and 10% (n=10), respectively. The total specific binding was about 15±5%.

A typical standard curve of the RIA-hGH is shown in Figure II.2.

II.2.9. Cell Extraction

The cells were washed twice with 1 x PBS (pH 7.2), digested with 0.5-1.5 ml of Trypsin-EDTA at 37°C for 5 minutes, then the cells were transferred to Eppendorf vials. After centrifugation for 2 minutes at 4°C, the pellets were resuspended in 100 µl of 0.25 M Tris-HCl pH 7.8. Cell extracts were prepared by 3 cycles of freezing and thawing (5 minutes in a dry ice-bath, followed by 5 minutes at 37°C), and centrifugation at 10,000 x g for 10 minutes at 4°C. The supernatants were transferred to new Eppendorf vials and stored at -20 for further use.

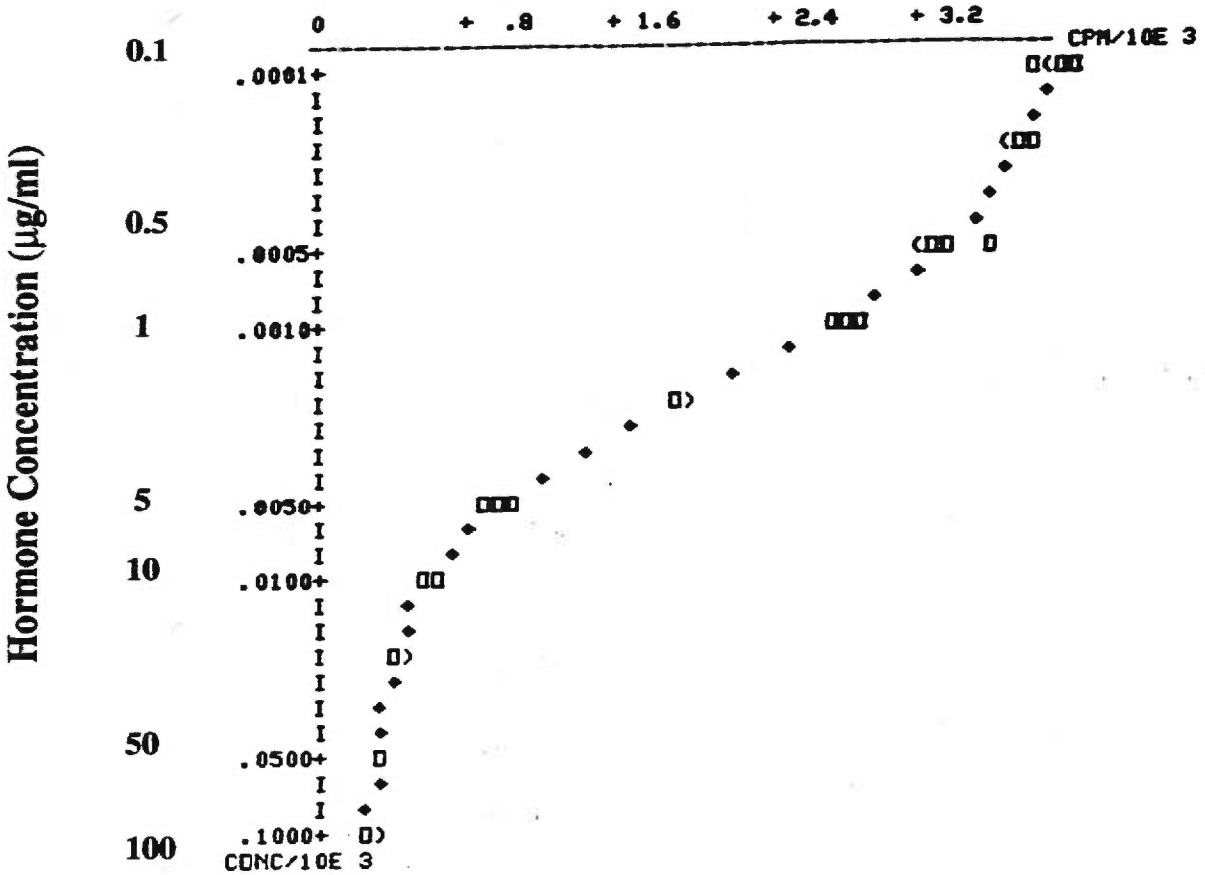


Figure II.2. A Typical Standard Curve of RIA-hGH

II.2.10. Protein Assay

Bovine serum albumin (BSA) was used as a standard. The BSA was diluted to different concentrations (i.e. 0, 1.25, 2.5, 5, 10 μg/ml) in 0.25 M Tris-HCl (pH 7.8). 200 μl of BSA standard or samples were added to the 96-well plate which already contained 50 μl of BIO-RAD protein dye, the optical density was read at 595 nm.

II.2.11. Chloramphenicol Acetyltransferase (CAT) assay

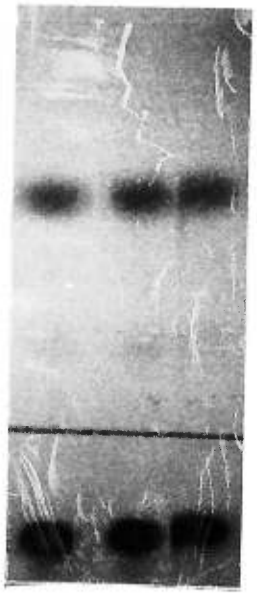


Figure II.3. A Typical Autoradiogram of the CAT Assay.

The reaction mixture contained: cell extract (20-50 μg protein) x μl, 4 mM acetyl coenzyme A (0.53 mM) 20 μl, 71 μl (0.1μCi) of 1M Tris-HCl-[^{14}C]chloramphenicol, and 0.25 M Tris-HCl buffer (pH 7.8) was added to bring the final volume to 146 μl. After incubation at 37°C for 1 hour, the reaction mixture was extracted once with 1 ml of ethyl acetate and the organic phase was lyophilized to dryness. The final samples were resuspended in 10 μl of ethyl acetate and applied to TLC plates, The TLC plates were developed with chloroform:methanol (19:1) and were analyzed by autoradiography. The acetylated and unacetylated forms of [^{14}C]chloramphenicol were scraped from the TLC plates and mixed the scintillation liquid (950 ml toluene + 50 ml Liquifluor), then quantiifited in a β-scintillation counter. A typical autoradiogram of the CAT assay is shown in Figure II.3.

II.2.12. Cell Fractionation and PKC Assay

Prior to PKC assay, OKW1 cells were separated into cytosol and membrane fractions as described below.

II.2.12.1. Separation of Cytosolic and Membrane Fractions

OKW1 cells were incubated with different reagents: 5 mM D(+)-glucose, 25 mM D(+)-glucose, 25 mM D(+)-glucose plus Tolrestat (10^{-4} M), 25 mM D(+)-glucose plus insulin (10^{-5} M) in 100 x 20 mm Petri dishes for one hour. The cells were rinsed twice with ice-cold PBS (approximately 5 ml for each 100 x 20 mm Petri dish plate), and once with 1 ml of buffer A (20 mM Tris-HCL pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulfonylfluoride, 25 ug/ml leupeptin, 330 mM Sucrose, 1 mM DTT, 1 ug/ml Pepstatin A). Cells were scraped from each dish with 1 ml of buffer A and placed directly into high speed centrifuge tube and kept on ice. Three dishes were combined for each tube for a total of 3 ml per tube. Each sample was homogenized with a glass Dounce (type B) homogenizer for 30 strokes while on ice, then centrifuged at 100,00 g for 30 minutes at 4°C. Following centrifugation, the supernatant was

collected for the cytosolic fraction and stored on ice. The pellet was resuspended in 3 ml of buffer B (the same as buffer A except without sucrose and with 0.2% Triton X-100) and homogenized again for 30 strokes on ice, and then incubated at 4°C for 30 minutes. After incubation, the samples were recentrifuged at 100,000 g for 30 minutes and the supernatant was collected as the membrane fraction and stored on ice until assay for PKC. All samples for PKC assay were assayed for protein concentrations (see section 2.2.10). Twenty µg of protein were taken from each sample for the detection of PKC activity.

II.2.12.2. PKC Assay

The Biotrak™ protein kinase C (PKC) assay system from Amersham™ (code RPN 77) was used to detect PKC activity. The protocol for detecting PKC activity was provided by the supplier. This system is based upon the PKC catalyzed transfer of the γ-phosphate group of adenosine-5'-triphosphate to a peptide which is specific for PKC.

Briefly, the following procedures were used in the PKC assay. Samples were prepared as described in II.2.12.1. 25 µl of the component mixture (mixed with equal volumes of calcium buffer, lipid buffer, peptide buffer, and DTT buffer) and 25 µl of each sample were pipetted into appropriately labeled tubes. For blanks, sample buffer was used instead of sample. 5 µl of the magnesium [^{32}P]ATP was carefully pipetted into each tubes. The reactions were simultaneously microcentrifuged for 3 seconds in a microcentrifuge and then incubated in a water bath at 37°C for 30 minutes. The reaction was terminated with 10 µl of stop reagent and microcentrifuged for 3 seconds. 65 µl of terminated reaction mixture was transferred on to the center of each paper-disc and allowed to dry for 5 minutes. Each paper disc was washed with 4 ml of 75 mM orthophosphoric acid in six-well plates. The wash reagent was decanted and disposed as ^{32}P liquid waste. The wash step was repeated three times. Finally, each paper disc was removed from the six-well plate and allowed to dry for 10 minutes. Paper discs were transferred to scintillation vials and 10 ml of scintillation fluid were added to each vial and counted for 2 minutes in a scintillation

counter.

II.2.13. DAG Assay

Amersham's DAG assay reagent system (code RPN 200) was used to quantify the cellular DAG. The protocol for detecting the DAG activity was provided with the assay system. The basis of the assay procedure is a radioenzymatic assay employing the enzyme DAG kinase which quantitatively converts DAG to [^{32}P]phosphatidic acid in the presence of [^{32}P]-γ-ATP and the separation of [^{32}P]phosphatidic acid was achieved by thin-layer-chromatography. The [^{32}P]phosphatidic acid was quantified in a liquid scintillation counter.

Briefly, the following procedures were used in the DAG assay. Cells in each Petri dish were extracted with 3 ml of chloroform:methanol (1:2 v/v). The monophase was mixed and 1.0 ml of chloroform and 1.0 ml of NaCl were added to break the phases. Following centrifugation at 5,000 g for 2 minutes, the lower chloroform phase was taken for DAG assay. Working standards were prepared by using chloroform to dilute standard DAG to 6 concentrations 31.15, 62.5, 125, 250, 500 and 1000 pmol/tube. 100 µl of working standards were pipetted to appropriately labeled polypropylene tubes. 100 µl chloroform was pipetted into the tube marked 0 pmol (zero standard). 100 µl of of each sample was pipetted into labeled tubes. The solutions were evaporated by using a vacuum dryer. 20 µl of detergent solution reagent were added to the dried tubes. The solutions were vortexed and sonicated by using a sonicating water bath for 2 minutes per tube. 70 µl of the reagent mix (contains 10 µl diluted enzyme solution, 50 µl assay buffer, 10 µl 0.02 M dithiothreitol) were then added. The enzyme reaction was initiated by adding 10 µl of tracer solution (prepared by mixing 5 mM ATP with [^{32}P]ATP to give 37kBq, 1.0 µCi per 10 µl aliquot). The contents were mixed and incubated at 25°C for 30 minutes, at the end of the incubation period, 20 µl of 1% (v/v) perchloric acid and 450 µl of chloroform:methanol (1:2, v/v) were added and then mixed. The tubes were left at room temperature for 10 minutes and then centrifuged at 2,000 g for 1 minute. After centrifugation, 150 µl of chloroform and 150 µl of 1%

(v/v) perchloric acid were added to each tube. The tubes were capped and mixed thoroughly by vortexing. After centrifugation at 2,000 g for 1 minute, the upper phase was removed and discarded. This washing procedure was repeated. Working standards which contained predominantly [^{32}P]phosphatidic acid was counted directly. Samples which contained other [^{32}P]-labeled species were separated by TLC on a 10 x 10 cm silica gel thin-layer plate. Separation of different [^{32}P] labeled species was achieved with a solvent consisting of chloroform:methanol:acetic acid (65:15:5, v/v/v). The plate was removed from the tank, air dried and exposed overnight to autoradiography. The band corresponding to [^{32}P]phosphatidic acid was scraped and placed into tubes containing 10 ml of scintillation liquid. Each tube was counted for 2 minutes in a scintillation counter.

II.2.14. Statistical Analysis

The experiments were performed at least two to three times in triplicate. The data were analyzed by Student's "t" test or analysis of variance (Anova). A probability level of $p \leq 0.05$ was regarded as significant.

III. Results

III.1. Effect of Human Growth Hormone (hGH) on the Expression of pOCAT in OKW3 Cells

Figure III.1

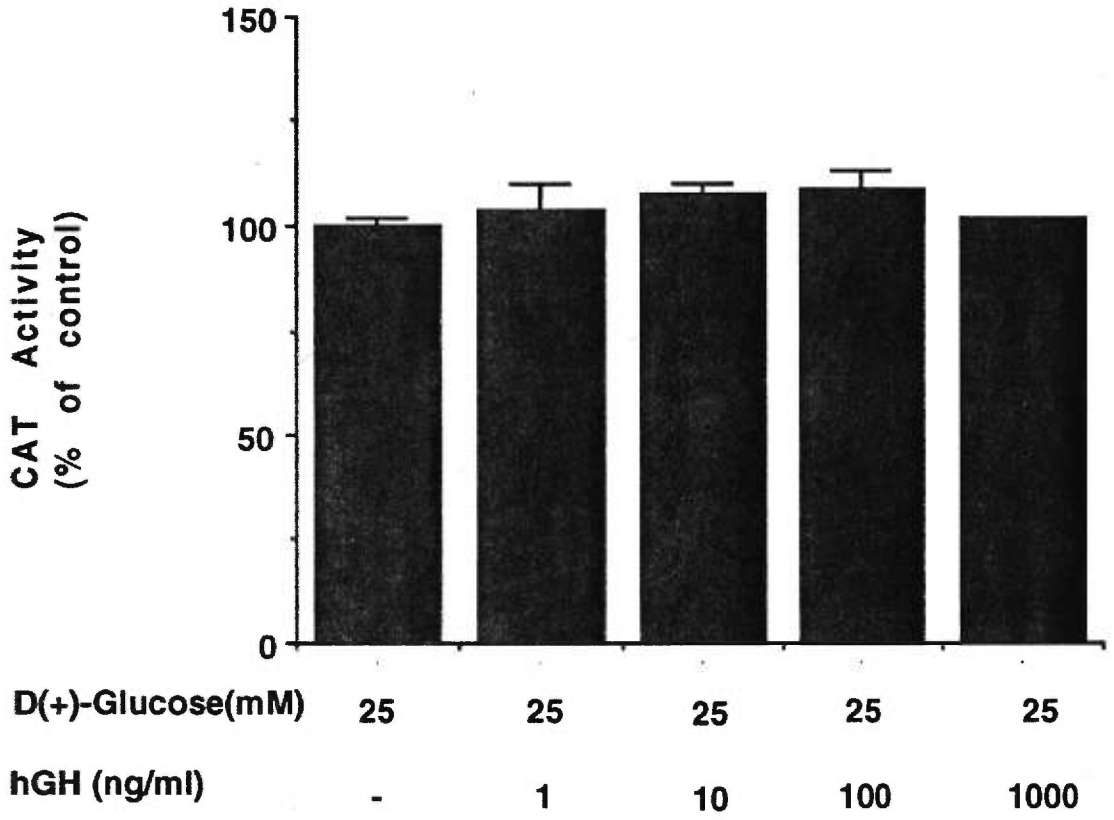


Figure III.1 shows that the addition of hGH had no effect on the expression of pOCAT in OKW3.

Method: Various concentrations of hGH (1 to 1000 ng/ml) were added to OKW3 cells in 25 mM D(+)-glucose medium. Cells were collected after 24 hours of incubation and assayed for cellular enzymatic CAT activity. The cellular enzymatic CAT activity in the medium without hGH (27.7±0.5% convertion, 20 μg protein) was expressed as 100% (control). Each point represents the mean ± S.D. of at least three dishes. Experiments were repeated three times.

III.2. Effect of Different Doses of D(+)-glucose on the Expression of pOGH(hANG N-1064/+27) in OKW1 Cells

Figure III.2

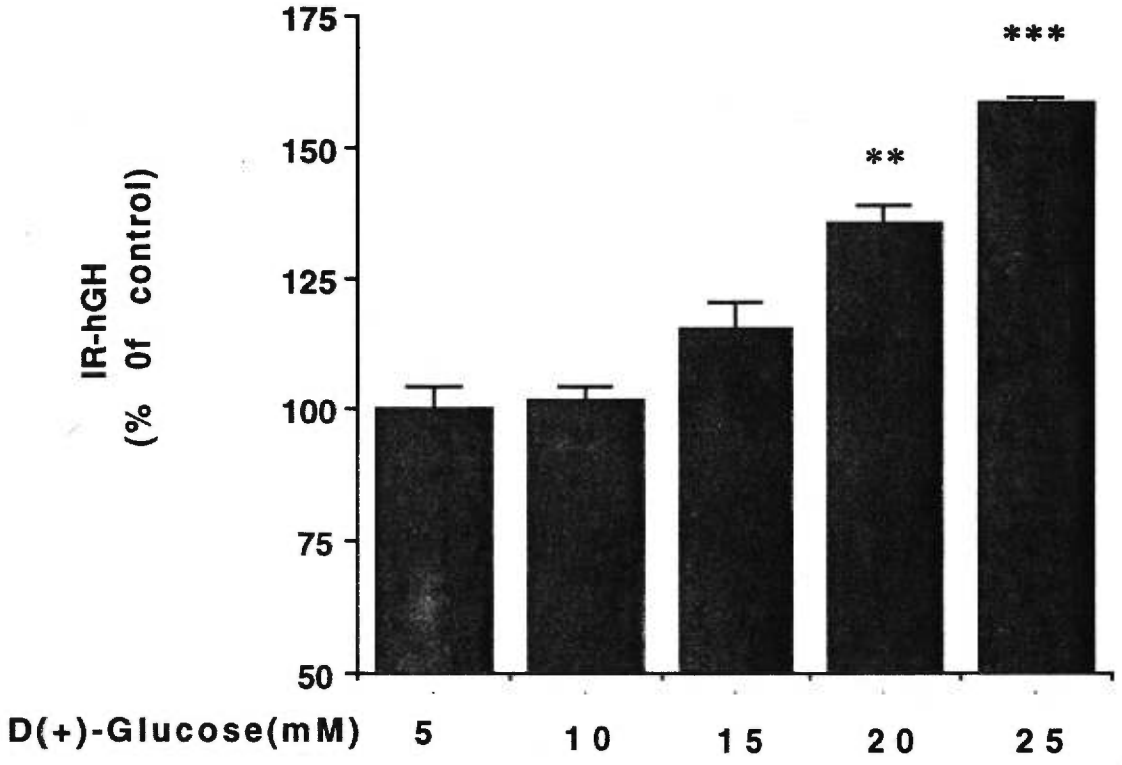


Figure III.2 shows that the expression of pOGH(hANG N-1064/+27) in OKW1 cells in the presence of various concentrations (5 to 25 mM) of D(+)-glucose after 24 hours of incubation. A dose-dependent relationship between D(+)-glucose concentrations and the stimulation of expression of the fusion genes was observed at 5 to 25 mM. The maximum stimulation of expression of the fusion genes was found with 25 mM of D(+)-glucose.

Method: OKW1 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 IR-hGH. The level of IR-hGH in the medium containing low D(+)-glucose (5 mM) (i.e. 0.4±0.02 ng/ml) was expressed as 100% (control). Each point represents the mean ± S.D. of at least three dishes (**$p \leq 0.01$, ***$p \leq 0.005$). Experiments were repeated three times.

III.3. Effect of Different Doses of D(+)-glucose on the Expression of pOCAT(hANG N-1064/+27) in OKW3 Cells

Figure III.3

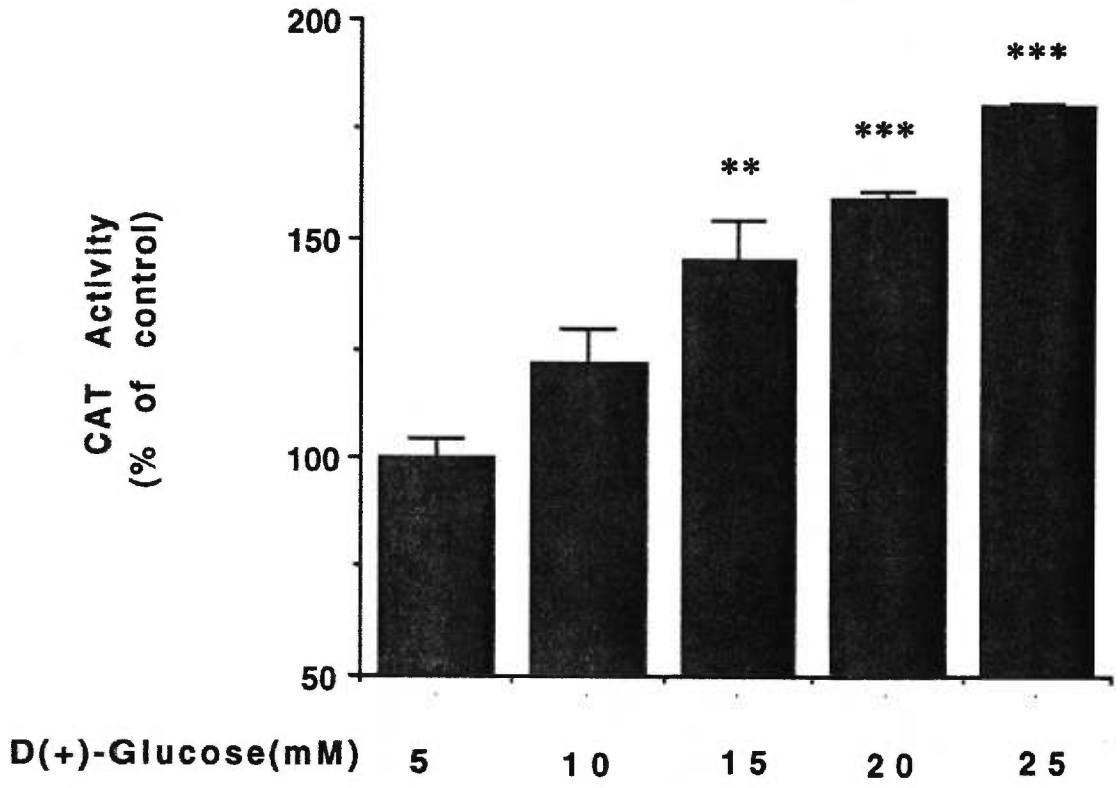


Figure III.3 shows that the expression of pOCAT(hANG N-1064/+27) in OKW3 cells in the presence of various concentrations (5 to 25 mM) of D(+)-glucose after 24 hours of incubation. A dose-dependent relationship between D(+)-glucose concentrations and the stimulation of expression of the fusion genes was observed at 5 to 25 mM. The maximum stimulation of expression of the fusion genes was found with 25 mM of D(+)-glucose.

Method: OKW3 cells were incubated for up to 24 hours in the presence of various concentrations of D(+)-glucose. Cells were collected after 24 hours of incubation and assayed for cellular CAT activity. The cellular CAT activity in the medium containing low D(+)-glucose (5 mM) (i.e. 9.6±0.4% convertion, 20 μg protein) was expressed as 100% (control). Each point represents the mean ± S.D. of at least three dishes (**$p \leq 0.01$ and ***$p \leq 0.005$). Experiments were repeated three times.

III.4. Effects of D(+)-glucose, D-mannitol, L-glucose and 2-deoxy-D-glucose on the Expression of pOGH(hANG N-1064/+27) in OKW1 Cells

Figure III.4

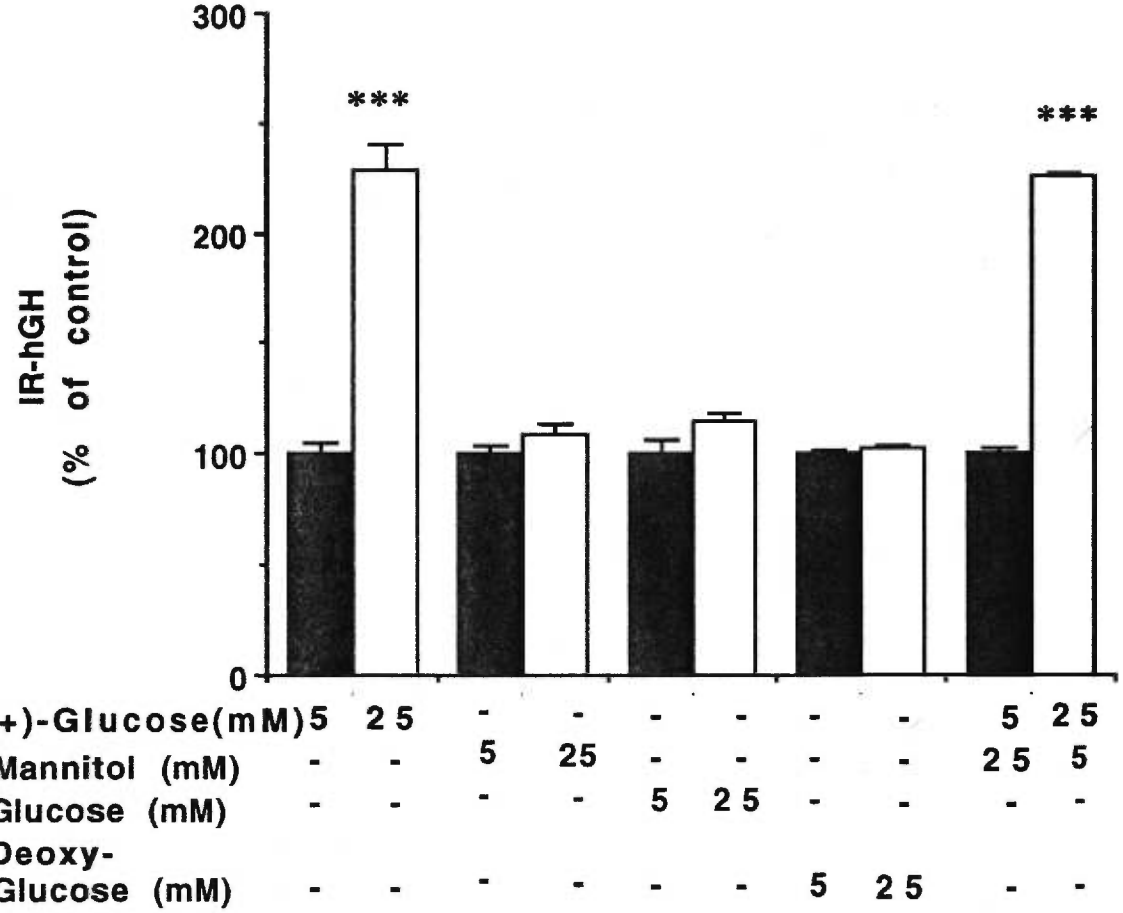


Figure III.4 compares the effects of the addition of 5 or 25 mM of D(+)-glucose, D-mannitol, L-glucose or 2-deoxy-D-glucose on the expression of pOGH(hANG N-1064/+27) in OKW1 cells after 24 hours of incubation. In contrast to D(+)-glucose, high levels (25 mM) of D-mannitol or L-glucose or 2-deoxy-D-glucose had no stimulatory effect on the expression of pOGH(hANG N-1064/+27) in OKW1 cells.

Method: OKW1 cells were incubated up to 24 hours in the presence of low (5 mM) or high (25 mM) levels of D(+)-glucose, D-mannitol, L-glucose and 2-deoxy-D-glucose. Media were harvested after 24 hours of incubation and assayed for IR-hGH. The concentration of IR-hGH in the medium containing 5 mM D(+)-glucose (i.e. 0.4±0.02 ng/ml) was considered as 100% (control). Each point represents the mean ± S.D. of at least three dishes (***$p \leq 0.005$). Experiments were repeated three times.

III.5. Effects of D(+)-glucose, D-mannitol, L-glucose and 2-deoxy-D-glucose on the Expression of pOCAT(hANG N-1064/+27) in OKW3 Cells

Figure III.5.

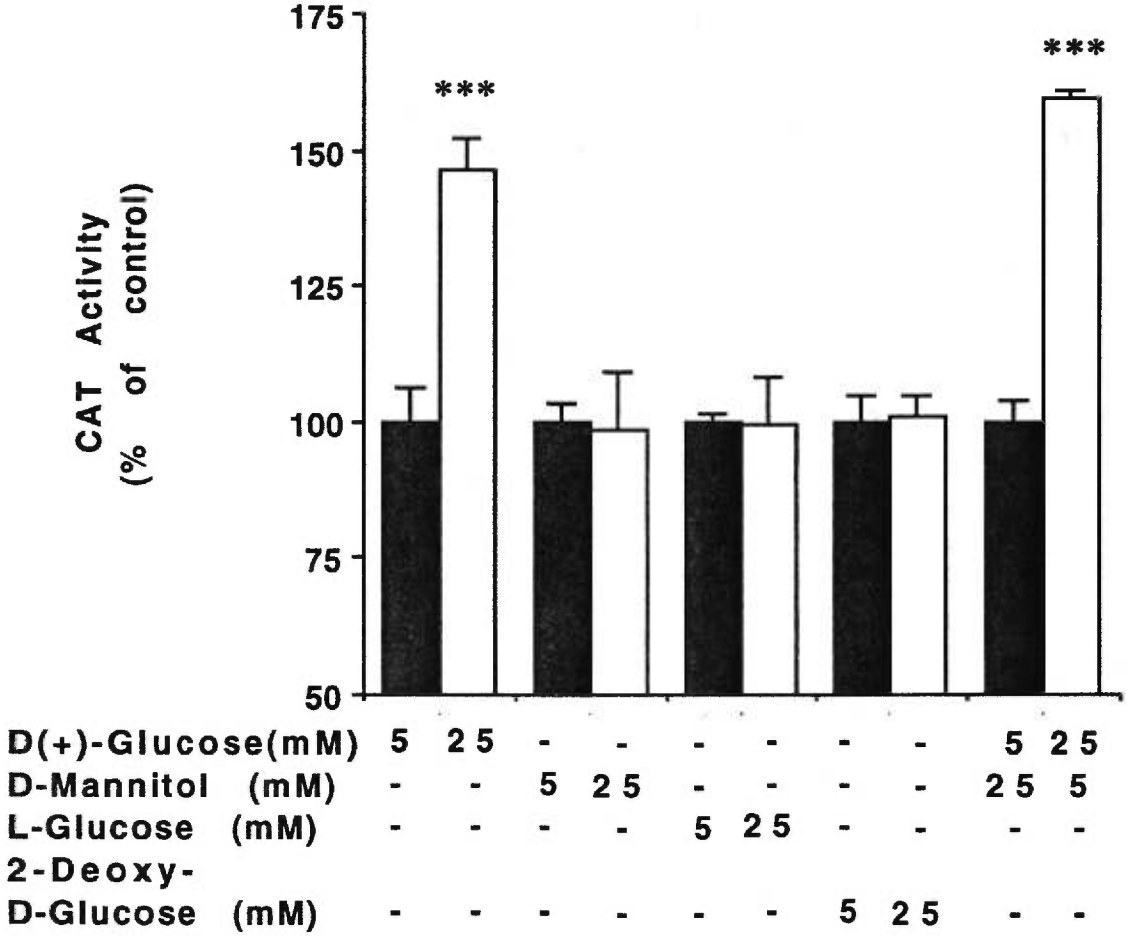


Figure III.5 compares the effects of the addition of 5 or 25 mM of D(+)-glucose, D-mannitol, L-glucose or 2-deoxy-D-glucose on the expression of pOCAT(hANG N-1064/+27) in OKW3 cells after 24 hours of incubation. In contrast to D(+)-glucose, high levels (25 mM) of of D-mannitol or L-glucose or 2-deoxy-D-glucose had no effect on the expression of pOCAT(hANG N-1064/+27) in OKW3 cells.

Method: OKW3 cells were incubated up to 24 hours in the presence of low (5 mM) or high (25 mM) levels of D(+)-glucose, D-mannitol, L-glucose and 2-deoxy-D-glucose. Cells were harvested after 24 hours of incubation and assayed for cellular CAT activity. The cellular CAT activity in the medium containing 5 mM D(+)-glucose (9.6±0.4% conversion, 20 μg protein)was considered as100% (control). Each point represents the mean ± S.D. of at least three dishes (***$p \leq 0.005$). Experiments were repeated three times.

III.6. Effect of Insulin on the Expression of pOGH(hANG N-1064/+27) in OKW1 Cells in Low Glucose Medium.

Figure III.6

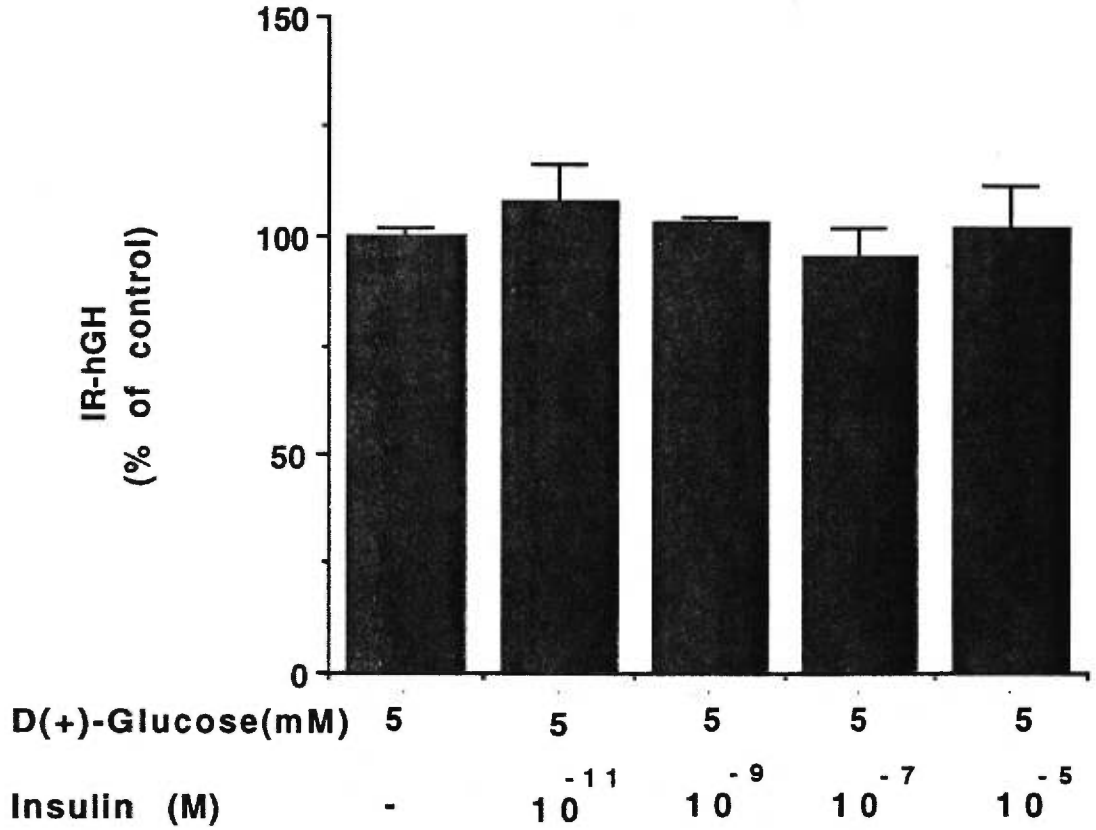


Figure III.6 shows that the addition of insulin (10^{-11} to 10^{-5} M) had no effect on the expression of pOGH(hANG N-1064/+27) in OKW1 cells in low glucose (5 mM) medium.

Method: OKW1 cells were incubated with various concentrations of insulin (10^{-11} to 10^{-5} M) in the presence of 5 mM D(+)-glucose. Media were harvested after 24 hours of incubation and assayed for the level of IR-hGH. The level of IR-hGH in the 5 mM glucose without insulin (i.e. 0.33±0.01 ng/ml)was expressed as 100% (control). Each point represents the mean ± S.D. of at least three dishes. Experiments were repeated three times.

III.7. Effect of Insulin on the Expression of pOCAT(hANG N-1064/+27) in OKW3 Cells in Low Glucose Medium.

Figure III.7

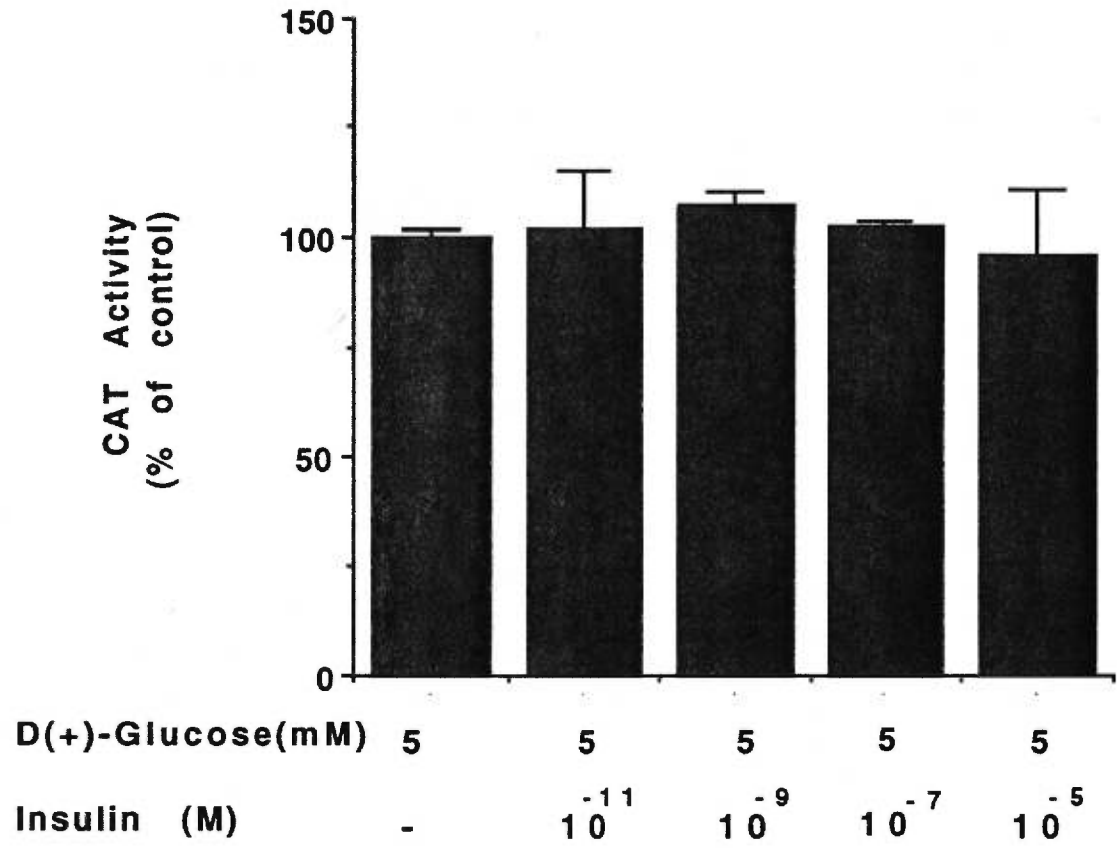


Figure III.7 shows that the addition of insulin (10^{-11} to 10^{-5} M) had no effect on the expression of pOCAT(hANG N-1064/+27) in OKW3 cells in low glucose (5 mM) medium.

Method: OKW3 cells were incubated with various concentrations of insulin (10^{-11} to 10^{-5} M) in the presence of 5 mM D(+)-glucose. Media were harvested after 24 hours of incubation and assayed for the level of CAT activity. The cellular CAT activity in the 5 mM glucose without insulin (i.e. 24±0.4% conversion, 20 μg protein)was expressed as 100% (control). Each point represents the mean ± S.D. of at least three dishes. Experiments were repeated three times.

III.8. Effect of Insulin on the Expression of pOGH(hANG N-1064/+27) in OKW1 Cells in High Glucose Medium.

Figure III.8

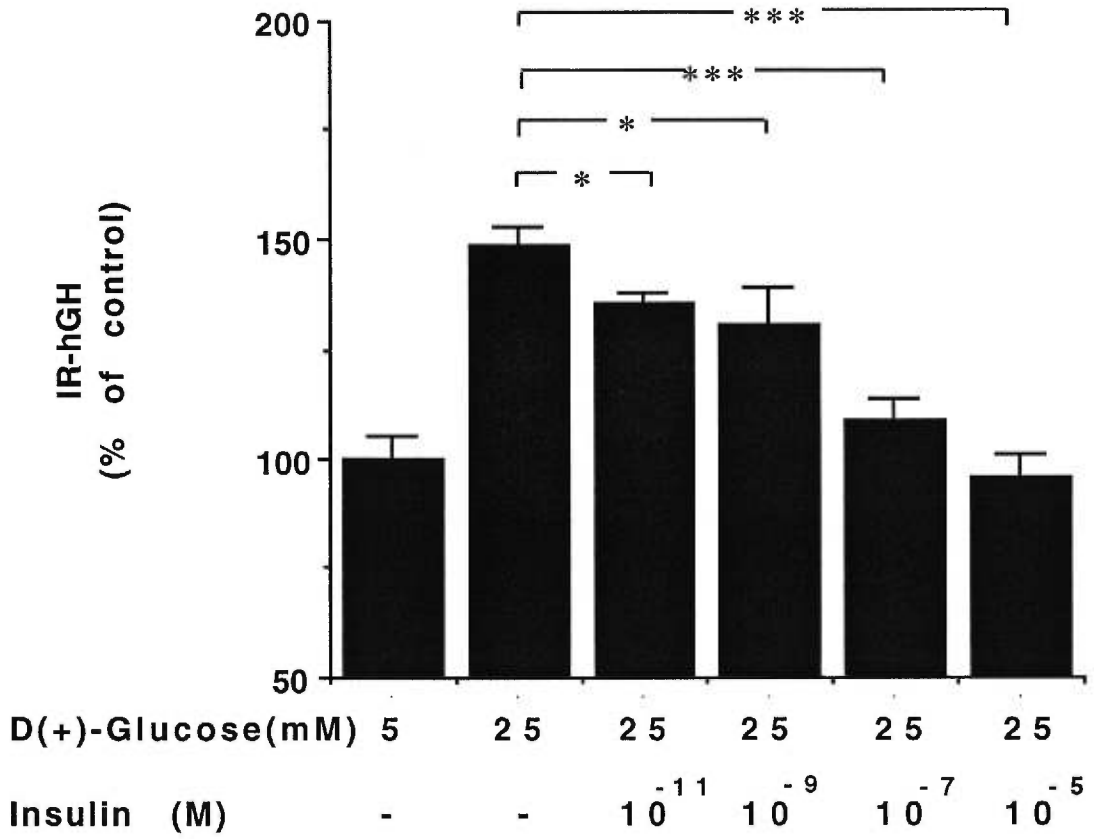


Figure III.8 shows that the addition of insulin (10^{-11} to 10^{-5} M) inhibited the stimulatory effect of D(+)-glucose (25 mM) on the expression of pOGH(hANG N-1064/+27) in OKW1 cells in a dose-dependent manner, respectively. The maximum effective dose for inhibition of the stimulated expression (25 mM D(+)-glucose) of the fusion genes was at greater than 10^{-7} M insulin ($p \leq 0.001$).

Method: OKW1 cells were incubated with various concentrations of insulin (10^{-11} to 10^{-5} M) in the presence of 25 mM D(+)-glucose. Media were harvested after 24 hours of incubation and assayed for the level of IR-hGH. The level of IR-hGH in the 5 mM glucose (i.e. 0.4±0.01 ng/ml) was expressed as 100% (control). Each point represents the mean ± S.D. of at least three dishes (*$p \leq 0.05$ and ***$p \leq 0.005$). Experiments were repeated three times.

III.9. Effect of Insulin on the Expression of pOCAT(hANG N-1064/+27) in OKW3 Cells in High Glucose Medium

Figure III.9

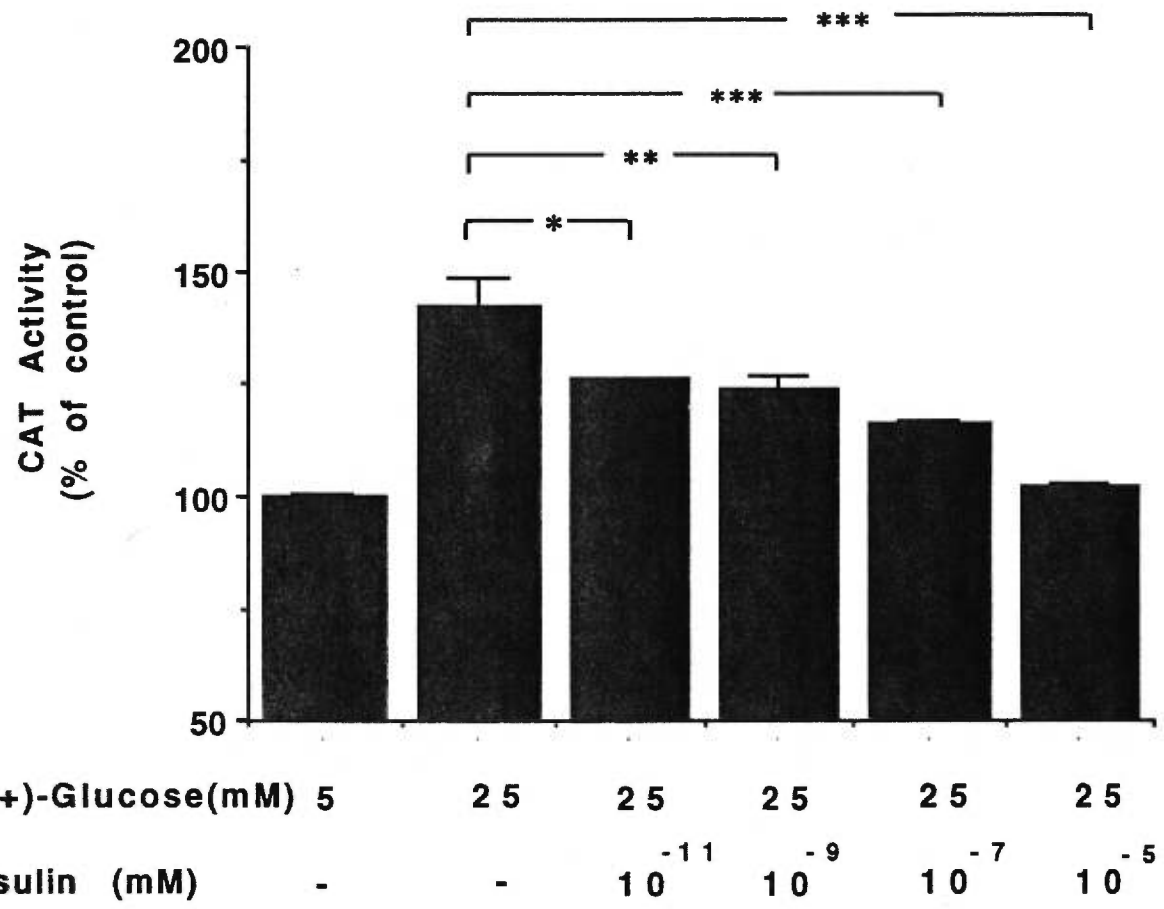


Figure III.9 shows that the addition of insulin (10^{-11} to 10^{-5} M) inhibited the stimulatory effect of D(+)-glucose (25 mM) on the expression of pOCAT(hANG N-1064/+27) in OKW3 cells in a dose-dependent manner, respectively. The maximum effective dose for inhibition of the stimulated expression (25 mM D(+)-glucose) of the fusion genes was at 10^{-5} M insulin ($p \leq 0.001$).

Method: OKW3 cells were incubated with various concentrations of insulin (10^{-11} to 10^{-5} M) in the presence of 25 mM D(+)-glucose. Cells were harvested after 24 hours of incubation and assayed for the level of CAT activity. The cellular CAT activity in the 5 mM glucose (24±0.4% conversion, 20 μg protein) was expressed as 100% (control). Each point represents the mean ± S.D. of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$). Experiments were repeated three times.

III.10. Effects of IGF-I and IGF-II on the Expression of pOGH(hANG N-1064/+27) in OKW1 Cells

Figure III.10

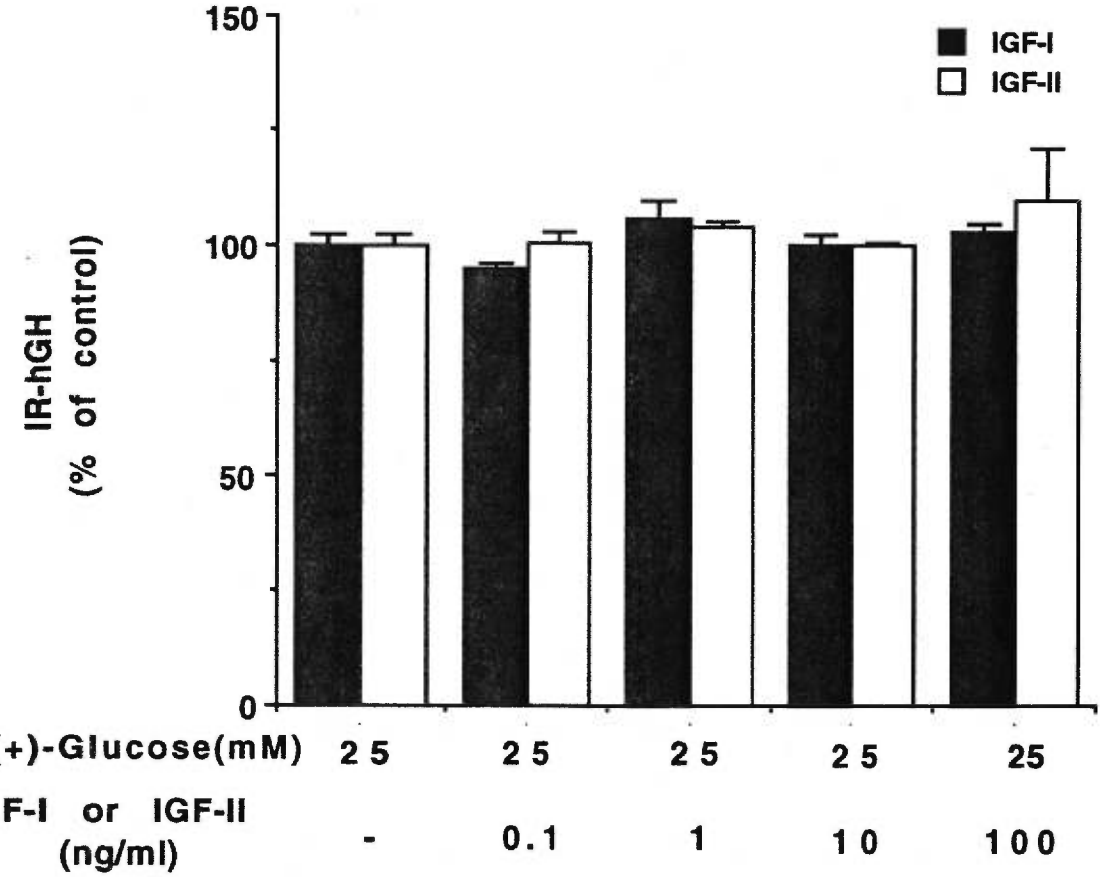


Figure III.10 shows the addition of various concentrations of IGF-I and IGF-II on the expression of pOGH(hANG N-1064/+27) in OKW1 cells, respectively. In contrast to insulin, the addition of IGF-I and IGF-II (0.1 to 100 ng/ml) had no significant effect on the expression of the fusion genes in OKW1 cells as compared to the control (i.e. in the absence of IGF-I or IGF-II).

Method: OKW1 cells were incubated with various concentrations of IGF-I or IGF-II (0.1 to 100 ng/ml) in the presence of 25 mM D(+)-glucose. Media were harvested after 24 hours of incubation and assayed for IR-hGH. The levels of IR-hGH in the medium without the presence of IGF-I or IGF-II (i.e. 0.8±0.01 ng/ml)were expressed as 100% (control). Each point represents the mean ± S.D. of at least three dishes. The solid bars represent the cells incubated in the presence of IGF-1 and the blank bars represent the cells incubated in the presence of IGF-II. Experiments were repeated three times.

III.11. Effects of IGF-I and IGF-II on the Expression of pOCAT(hANG N-1064/+27) in OKW3 Cells

Figure III.11

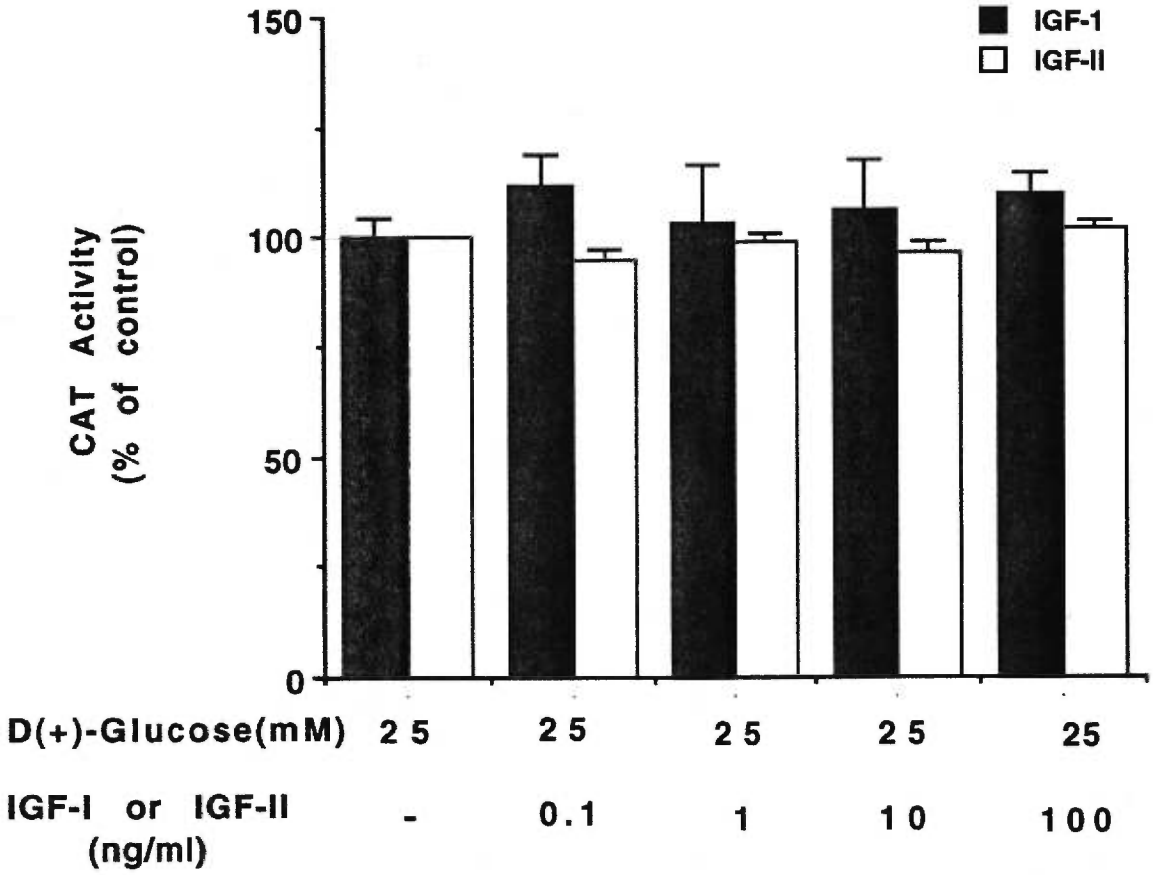


Figure III.11 shows the addition of various concentrations of IGF-I and IGF-II on the expression of pOCAT(hANG -1064/+27) in OKW3 cells, respectively. In contrast to insulin, the addition of IGF-I and IGF-II (0.1 to 100 ng/ml) had no significant effect on the expression of the fusion genes in OKW3 cells as compared to the control (i.e. in the absence of IGF-I or IGF-II).

Method: OKW3 cells were incubated with various concentrations of IGF-I or IGF-II (0.1 to 100 ng/ml) in 25 mM D(+)-glucose medium. Cells were harvested after 24 hours of incubation and assayed for cellular CAT activity. The levels of cellular CAT activity in the medium without IGF-I or IGF-II (8±0.2% conversion, 20 μg protein) were expressed as 100% (control). Each point represents the mean ± S.D. of at least three dishes. The solid bars represent the cells incubated in the presence of IGF-1 and the blank bars represent the cells incubated in the presence of IGF-II. Experiments were repeated three times.

III.12. Effect of Insulin (10^{-7} M) on the Expression of pOGH(hANG N-1064/+27) in OKW1 Cells at Different Time Periods

Figure III.12

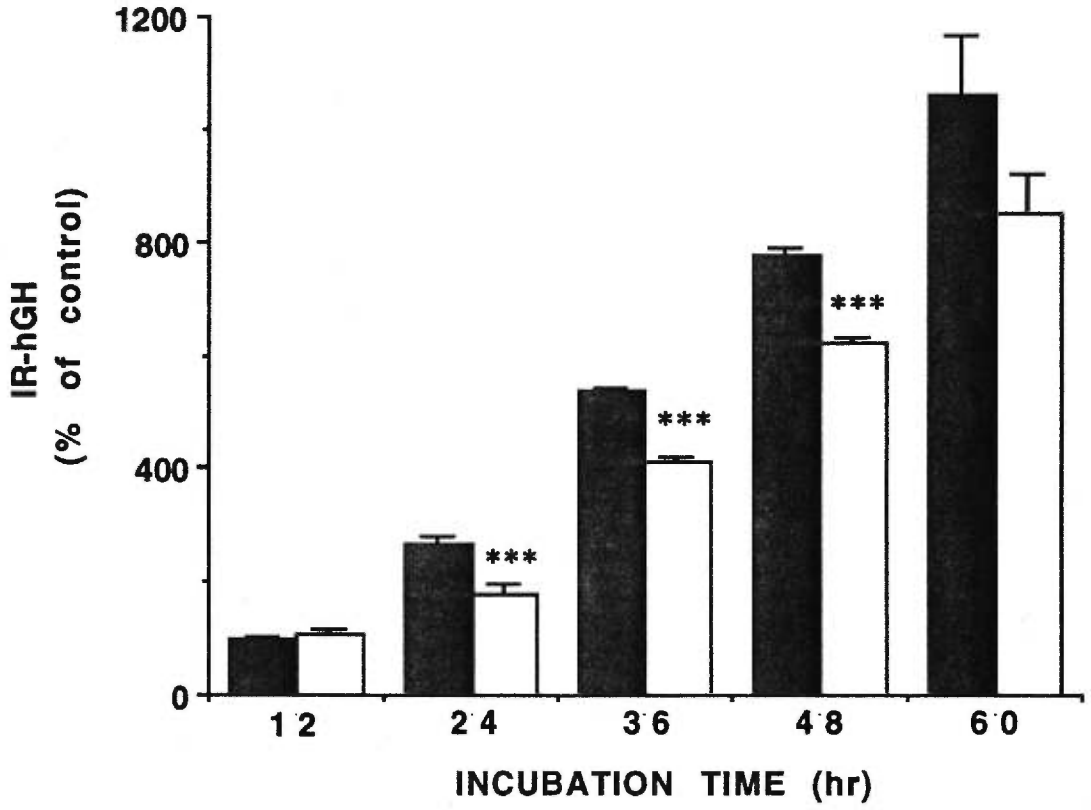


Figure III.12 shows the effect of insulin (10^{-7} M) on the expression of pOGH(hANG N-1064/+27) in OKW1 cells at different time periods. At 12 hours of incubation, there was no significant difference in the expression of the fusion gene in the presence or absence of insulin (10^{-7} M). However, at 24 to 48 hours of incubation, the expression of the fusion gene was consistently lower in the presence of insulin (10^{-7} M) as compared to the control (i.e. in the absence of insulin).

Method: OKW1 cells were incubated for 12 to 60 hours in 25 mM D(+)-glucose media with or without the presence of insulin (10^{-7} M). Cells were harvested after various periods of incubation and assayed for IR-hGH. The level of IR-hGH in the media without the presence of insulin at 12 hours of incubation was considered as 100% (1.22±0.03 ng/ml). The inhibitory effect of insulin was compared with cells that were incubated without insulin. Each point represents the mean ± S.D. of at least three dishes (***$p \leq 0.005$). Experiments were repeated three times.

III.13. Effect of Tolrestat (Aldose Reductase Inhibitor) on the Expression of pOGH(hANG N-1064/+27) in OKW1 Cells

Figure III.13

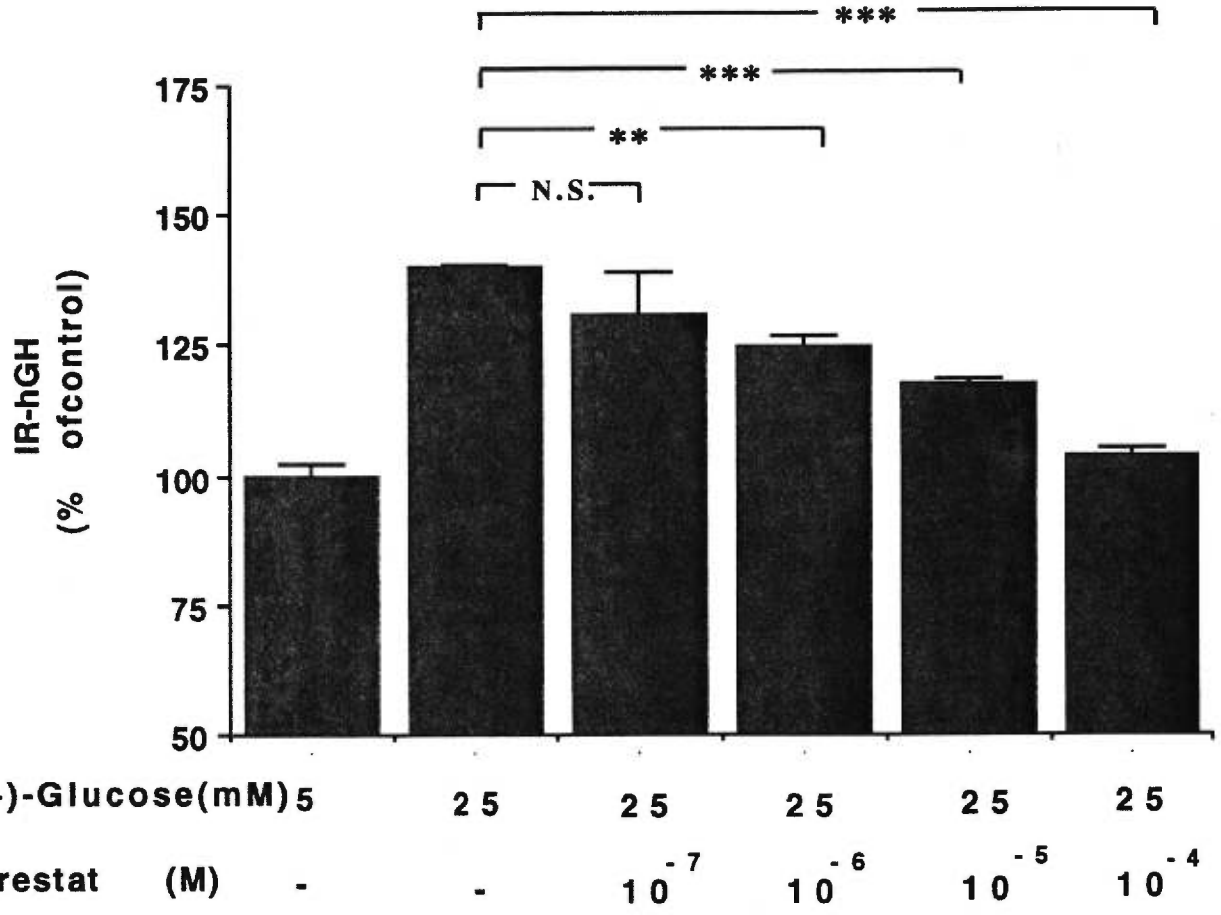


Figure III.13 shows that Tolrestat (10^{-7} to 10^{-4} M) inhibited the stimulatory effect of D(+)-glucose (25 mM) on the expression of pOGH(hANG N-1064/+27) in OKW1 cells in a dose-dependent manner. The effective inhibitory dose for inhibition of the stimulated expression (25 mM D(+)-glucose) of pOGH(hANG N-1064/+27) was at 10^{-6} M Tolrestat ($p \leq 0.01$). At 10^{-4} M Tolrestat, the stimulatory effect of D(+)-glucose (25 mM) on the expression of pOGH(hANG N-1064/+27) was completely abolished.

Method: OKW1 cells were incubated for up to 24 hours in 5 mM or 25 mM D(+)-glucose media with or without Tolrestat . The levels of IR-hGH in the medium containing 5 mM D(+)-glucose (i.e. 0.31±0.01 ng/ml) was expressed as 100% . The inhibitory effect of Tolrestat is compared with cells that were incubated in 25 mM D(+)-glucose (without Tolrestat). Each point represents the mean ± S.D. of at least three dishes (**p≤0.01, ***p≤0.005). Experiments were repeated three times.

Figure III.14 Effect of Tolrestat and Insulin on the Expression of pOGH(hANG N-1064/+27) in OKW1 Cells

Figure III.14.

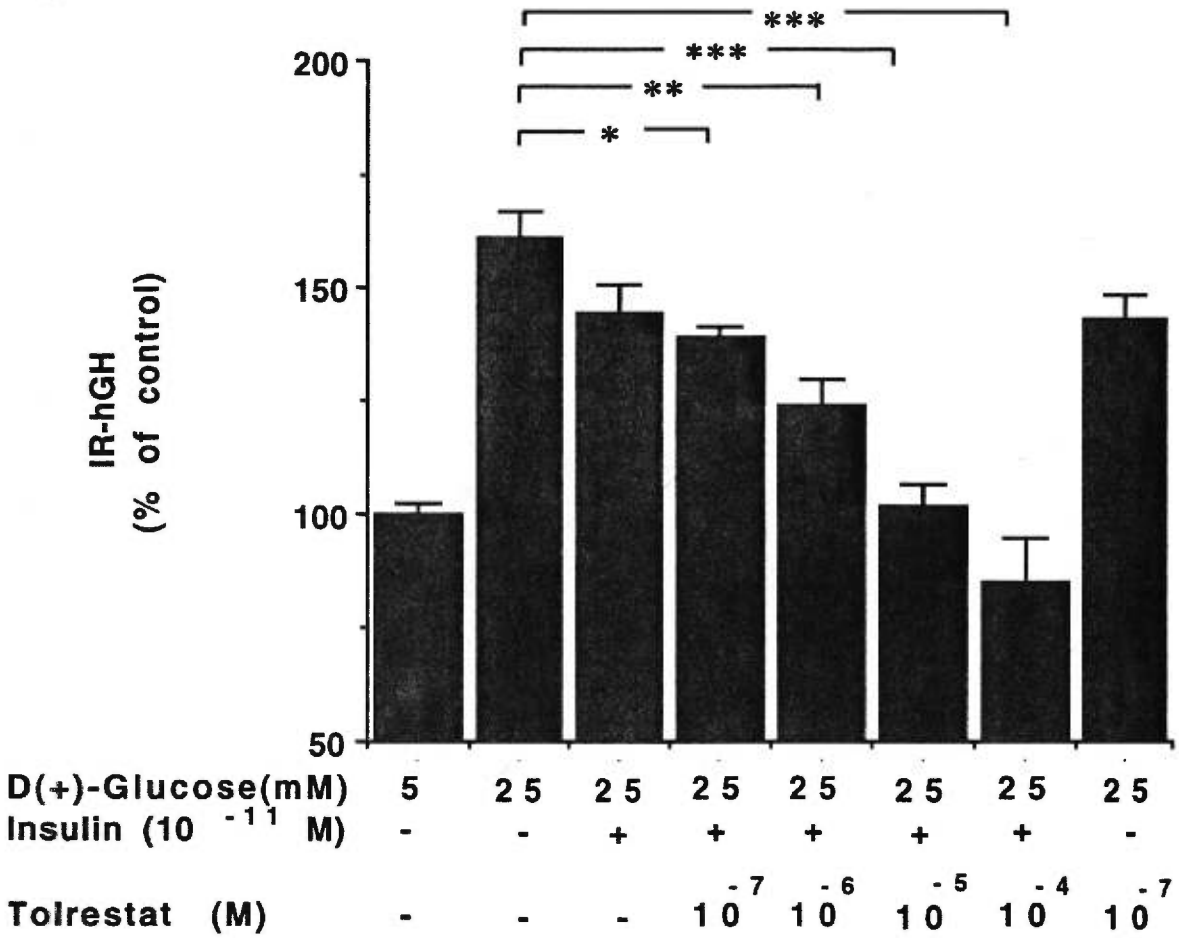


Figure III.14 shows the effect of a combination of both insulin and Tolrestat on the expression of pOGH(hANG N-1064/+27) in OKW1 cells. Insulin (10^{-11} M) and Tolrestat (10^{-7} M) alone were not effective in inhibiting the expression of pOGH(hANG N-1064/+27). However, A combination of insulin (10^{-11} M) and Tolrestat (10^{-7} M to 10^{-4} M) were effective in inhibiting the expression of pOGH(hANG N-1064/+27) in OKW1 cells.

Method: OKW1 cells were incubated for 24 hours in the presence of 5 mM or 25 mM D(+)-glucose plus various concentrations of insulin and / or Tolrestat. Media were harvested after incubation and assayed for IR-hGH. Levels of IR-hGH in the media containing the 5 mM glucose (i.e. 0.4±0.02 ng/ml) in the absence of insulin and Tolrestat were expressed as 100%. The inhibitory effect of insulin and Tolrestat were compared with cells that were stimulated by 25 mM D(+)-glucose. Each point represents the mean ± S.D. of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$). Experiments were repeated three times.

III.15. Effect of D(+)-glucose, Tolrestat and Insulin on Cellular DAG Concentrations

Figure III.15.

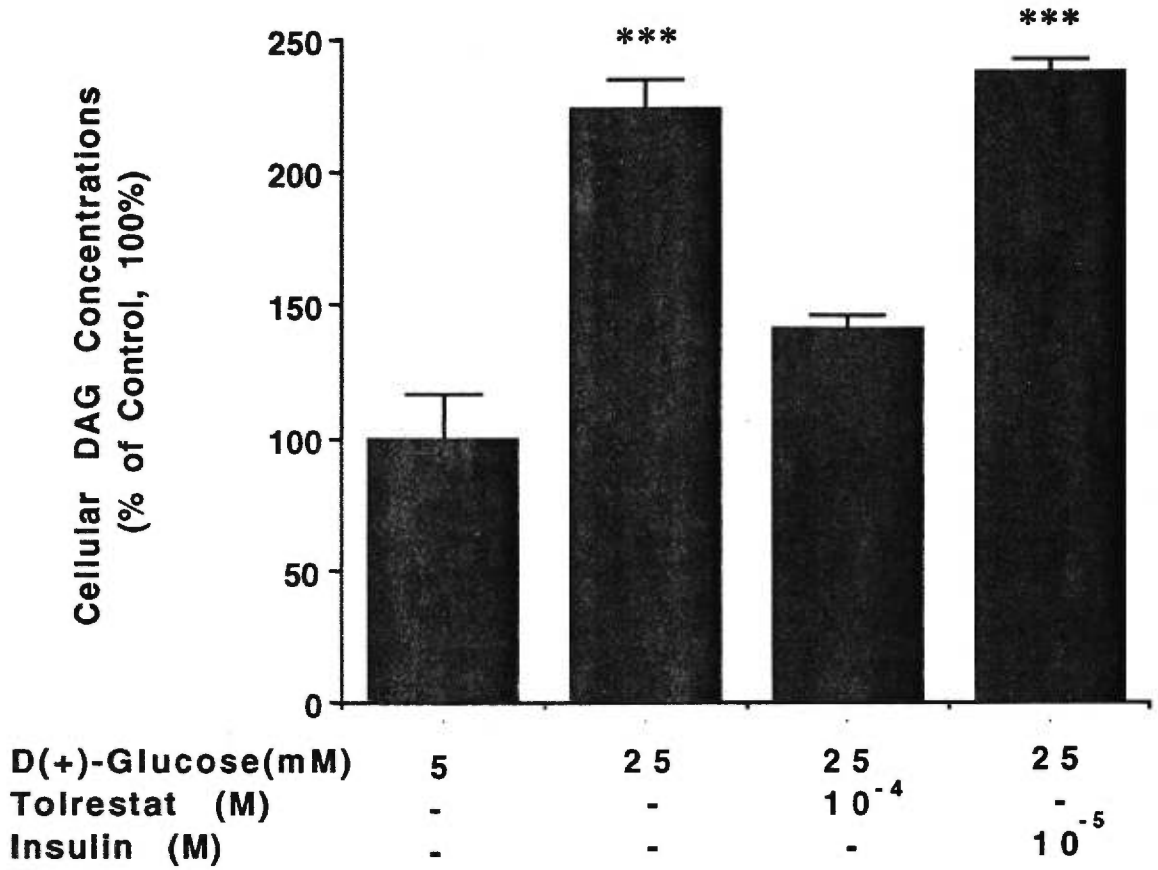


Figure III.15 shows the effect of 5 mM D(+)-glucose, 25 mM D(+)-glucose, 25 mM D(+)-glucose plus Tolrestat or insulin on the cellular DAG level. In high glucose medium (25 mM D(+)-glucose), the cellular DAG level was increased by 220% compared to the control (5 mM D(+)-glucose). The addition of Tolrestat (10^{-4} M) decreased, while insulin (10^{-5} M) did not affect the DAG level.

Method: OKW1 cells were incubated for 30 minutes in the presence of 5 mM, 25 mM D(+)-glucose or 25 mM D(+)-glucose plus Tolrestat (10^{-4} M) or insulin (10^{-5} M). The cells were harvested after incubation and assayed for DAG activity. The DAG concentration in 5 mM D(+)-glucose (0.8 pmol/mg) was expressed as 100%. Each point represents the mean ± S.D. of at least three dishes (***$p \leq 0.005$). Experiments were repeated twice.

III.16. Effects of D(+)-glucose, Tolrestat or Insulin on the Ratio of Membrane/Cytosol PKC Enzymatic Activity in OKW1 Cells

Figure III.16

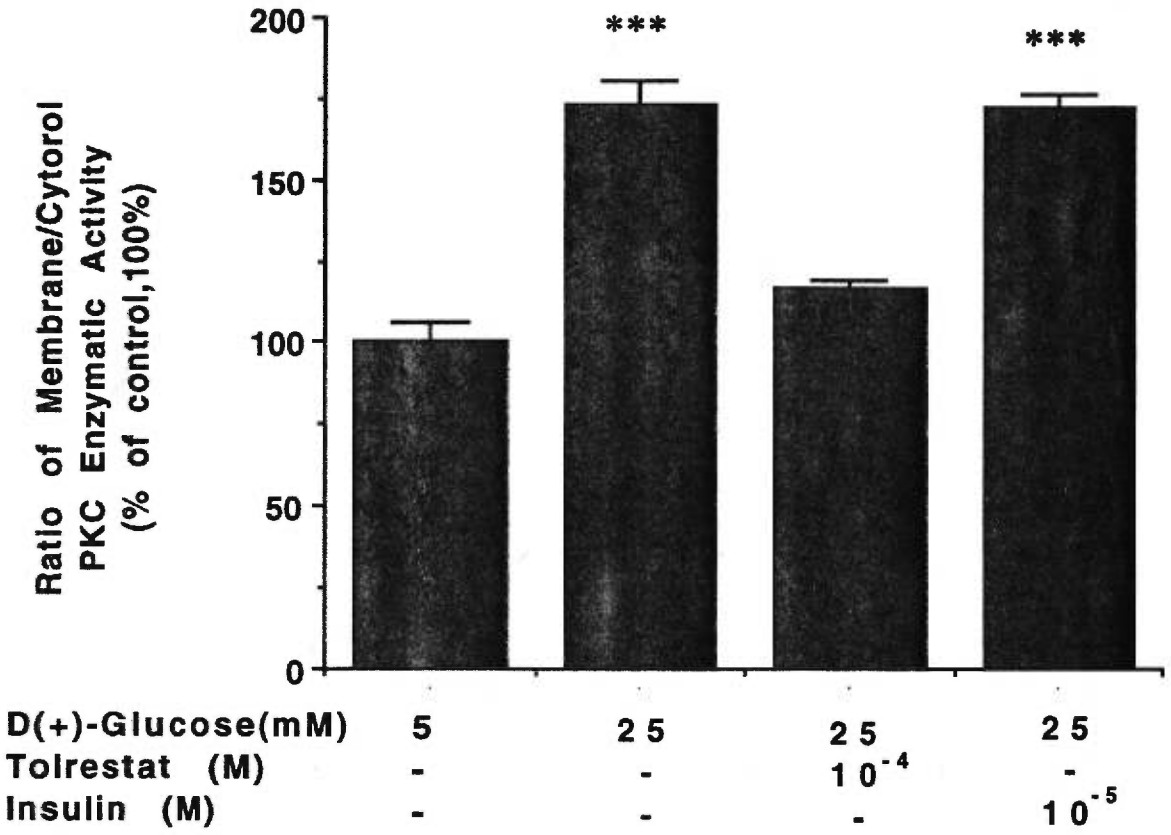


Figure III.16 shows the effect of 5 mM D(+)-glucose, 25 mM D(+)-glucose, 25 mM D(+)-glucose plus Tolrestat or insulin on the ratio of membrane/cytosol PKC enzymatic activity. In 25 mM D(+)-glucose medium, the ratio of membrane/cytosol PKC enzymatic activity was increased by 173% as compared to 5 mM D(+)-glucose medium. The addition of Tolrestat (10^{-4} M) prevented the increase of the ratio of membrane/cytosol PKC enzymatic activity, while insulin (10^{-5} M) did not affect the ratio of membrane/cytosol PKC enzymatic activity.

Method: OKW1 cells were incubated for 1 hour in the presence of 5 mM, 25 mM D(+)-glucose or 25 mM D(+)-glucose plus Tolrestat (10^{-4} M) or insulin (10^{-5} M). Cells were harvested after incubation. The membrane and cytosol fractions were separated. 20 μg of membrane or cytosol fractions were taken for PKC assay. Ratios of membrane to cytosol PKC activity were calculated. The ratio of membrane/cytosol PKC activity in 5 mM D(+)-glucose (i.e. 0.73±0.04) was expressed as 100%. Each point represents the mean ± S.D. of at least three dishes (***$p \leq 0.005$). Experiments were repeated twice.

III.17. Effect of H-7 (PKC Inhibitor) on the Expression of pOGH(hANG N-1064/+27) in OKW1 Cells

Figure III.17

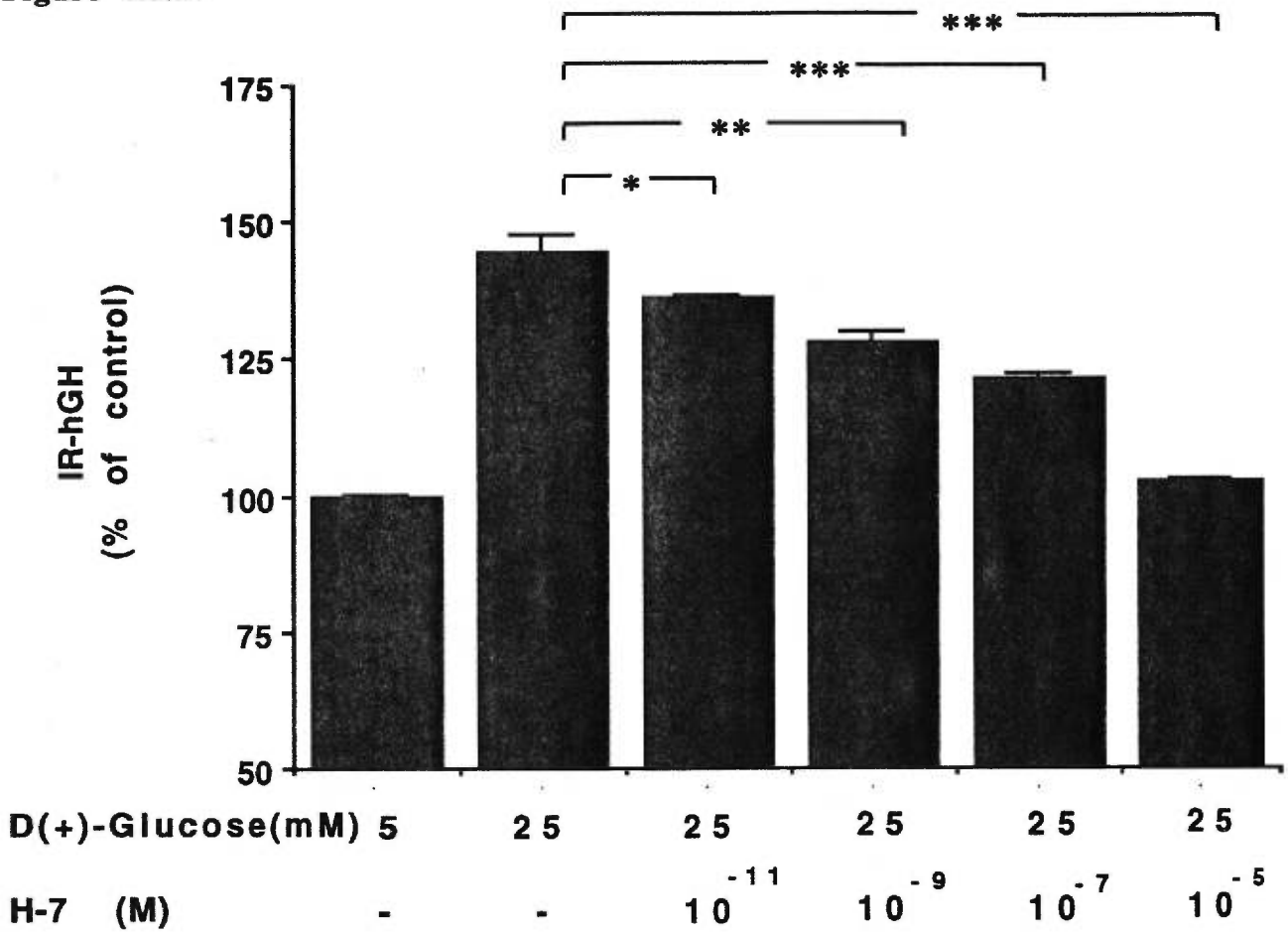


Figure III.17 shows that H-7 (10^{-11} to 10^{-5} M) inhibited the stimulatory effect of D(+)-glucose (25 mM) on the expression of pOGH(hANG N-1064/+27) in OKW1 cells in a dose-dependent manner. The effective inhibitory dose of H-7 was observed at 10^{-11} M. At 10^{-5} M, H-7 completely abolished the stimulatory effect of D(+)-glucose (25 mM) on the expression of pOGH(hANG N-1064/+27)

Method: OKW1 cells were incubated for 24 hours in the presence of 5 mM or 25 mM D(+)-glucose plus various concentrations of H-7. Media were harvested after incubation and assayed for IR-hGH. Levels of IR-hGH in the media containing 5 mM glucose (i.e. 0.36±0.02 ng/ml) in the absence of H-7 were expressed as 100%. The inhibitory effect of H-7 is compared with cells that were stimulated by 25 mM D(+)-glucose. Each point represents the mean ± S.D. of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$). Experiments were repeated three times.

III.18. Effect of Staurosporine (PKC Inhibitor) on the Expression of pOGH(hANG N-1064/+27) in OKW1 Cells

Figure III.18

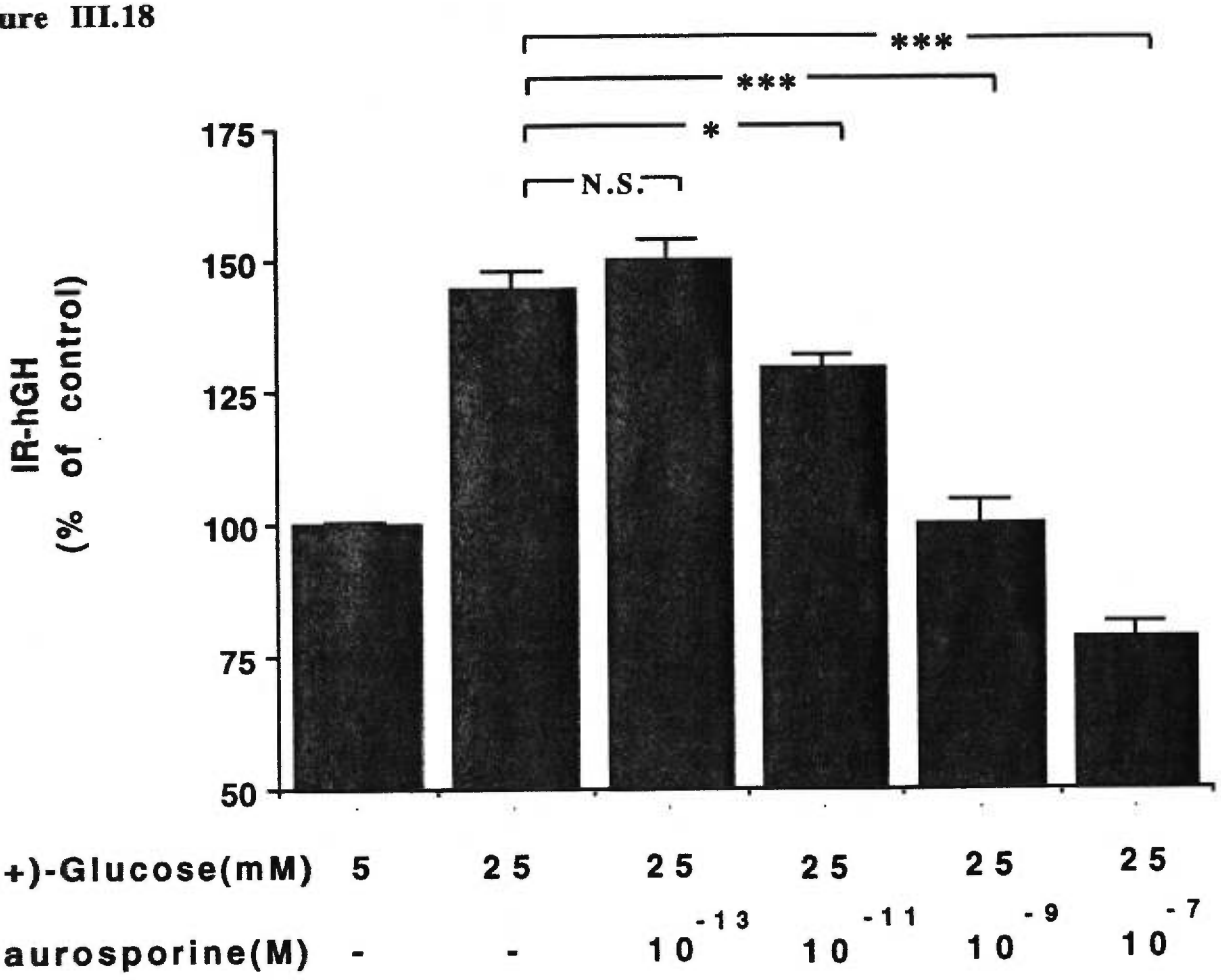


Figure III.18 shows that staurosporine (10^{-13} to 10^{-7} M) inhibited the stimulatory effect of D(+)-glucose (25 mM) on the expression of pOGH(hANG N-1064/+27) in OKW1 cells in a dose-dependent manner. The effective inhibitory dose of staurosporine was at 10^{-11} M. At 10^{-9} M, staurosporine completely abolished the stimulatory effect of D(+)-glucose (25 mM) on the expression of pOGH(hANG N-1064/+27).

Method: OKW1 cells were incubated for 24 hours in the presence of 5 mM or 25 mM D(+)-glucose plus various concentrations of staurosporine (10^{-13} to 10^{-7} M). Media were harvested after incubation and assayed for IR-hGH. Levels of IR-hGH in the media containing the 5 mM glucose (i.e. 0.36±0.02 ng/ml) in the absence of H-7 were expressed as 100%. The inhibitory effect of staurosporine was compared with cells that were stimulated by 25 mM D(+)-glucose. Each point represents the mean ± S.D. of at least three dishes (*$p \leq 0.05$, ***$p \leq 0.005$). Experiments were repeated three times.

III.19. Effect of PMA (PKC Stimulator) on the Expression of pOGH(hANG N-1064/+27) in OKW1 Cells

Figure III.19.

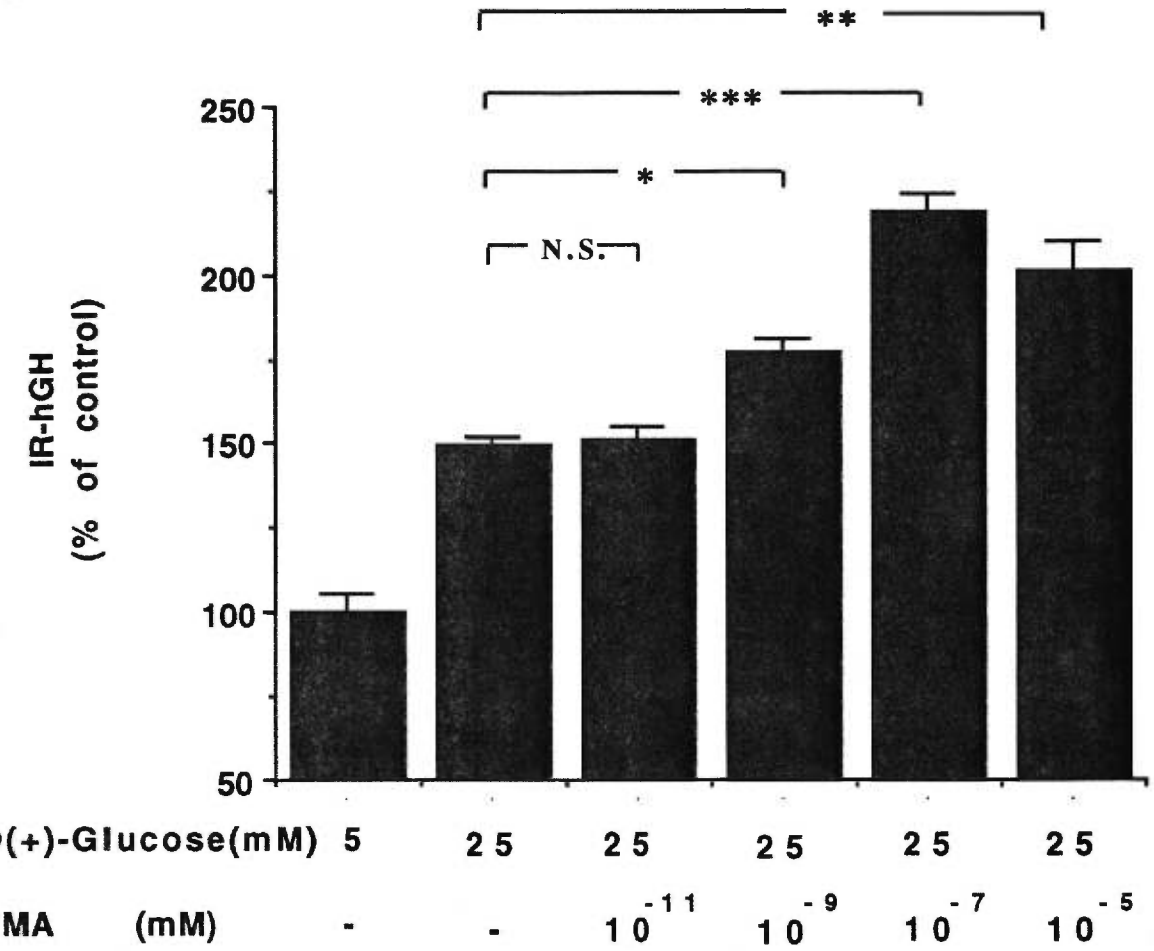


Figure III.19 shows that the addition of PMA (10^{-11} to 10^{-5} M) enhanced the stimulatory effect of D(+)-glucose (25 mM) on the expression of pOGH(hANG N-1064/+27) in OKW1 cells in a dose-dependent manner. The maximal stimulatory effect on the expression of pOGH(hANG N-1064/+27) was found with 10^{-7} to 10^{-5} M PMA.

Method: OKW1 cells were incubated for 24 hours in the presence of 5 mM or 25 mM D(+)-glucose plus various concentrations of PMA (10^{-11} to 10^{-5} M). Media were harvested after incubation and assayed for IR-hGH. Levels of IR-hGH in the medium containing 5 mM glucose (i.e. 0.36±0.02 ng/ml) in the absence of PMA are expressed as 100%. The stimulatory effect of PMA is compared with cells that were stimulated by 25 mM D(+)-glucose. Each point represents the mean ± S.D. of at least three dishes (*$p \leq 0.05$ and ***$p \leq 0.005$). Experiments were repeated three times.

III.20. Antagonist Effect of Insulin and PMA on the Expression of pOGH(hANG N-1064/+27) in OKW1 Cells

Figure III.20 (A)

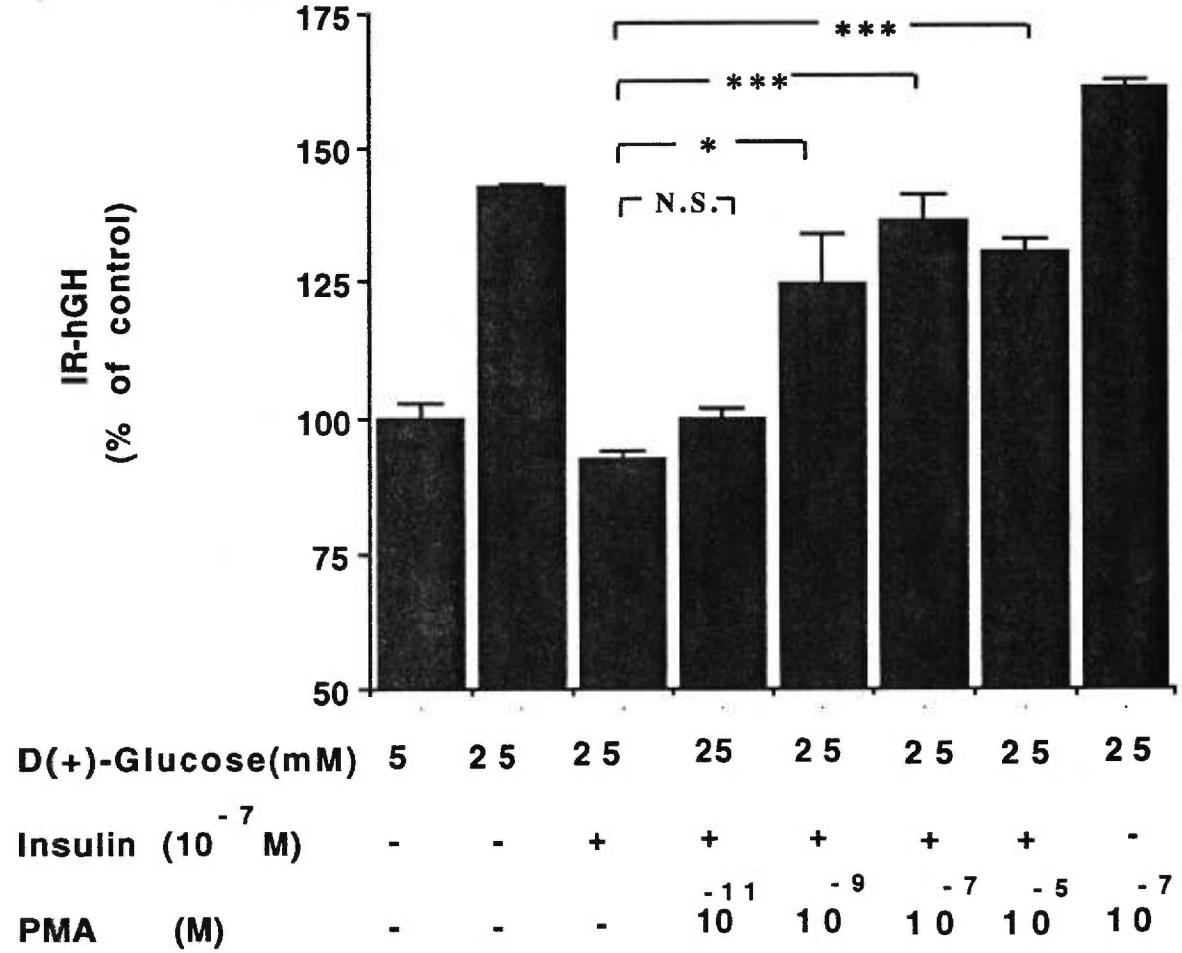


Figure III.20 (A) shows that the addition of PMA (10^{-11} to 10^{-5} M) blocked the inhibitory effect of insulin (10^{-7} M) on the expression of pOGH(hANG N-1064/+27) in OKW1 cells. PMA at concentrations of 10^{-7} to 10^{-5} M completely reversed the inhibitory effect of insulin.

Method: OKW1 cells were incubated for 24 hours in the presence of 5 mM or 25 mM D(+)-glucose plus 10^{-7} M insulin in the absence or presence of various concentrations of PMA (i.e. 10^{-11} to 10^{-5} M). Media were harvested and assayed for IR-hGH. Levels of IR-hGH in the medium containing 5 mM glucose (i.e. 0.36±0.01 ng/ml) in the absence of insulin or PMA were expressed as 100%. The effect of PMA was compared with cells that were incubated with 25 mM D(+)-glucose and in the presence of 10^{-7} M insulin. Each point represents the mean ± S.D. of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$). Experiments were repeated three times.

Figure III.20 (B)

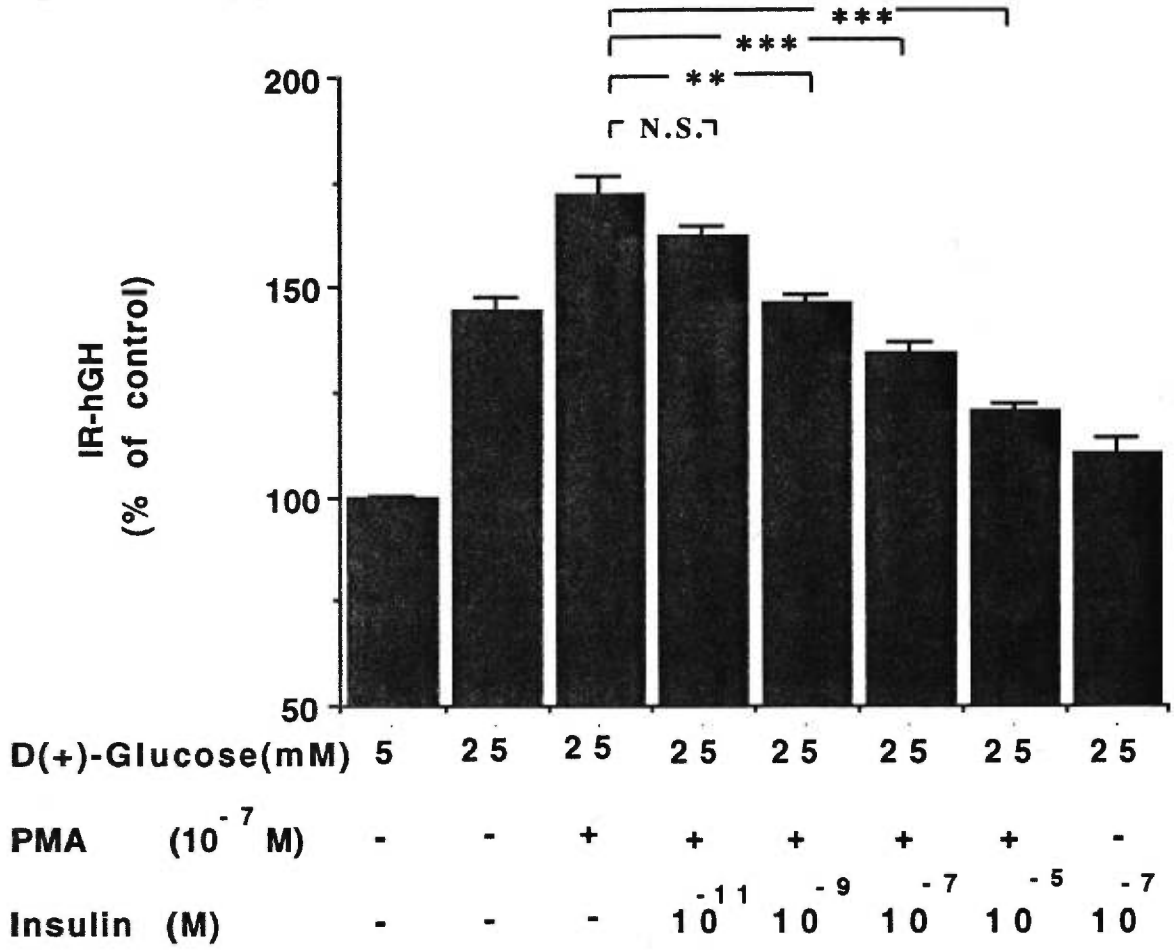


Figure III.20 (B) shows that the stimulatory effect of PMA (10^{-7} M) on the expression of pOGH(hANG N-1064/+27) in OKW1 cells was blocked by insulin in a dose-dependent manner (10^{-11} to 10^{-5} M). Insulin at concentrations of 10^{-7} M completely reversed the stimulatory effect of PMA.

Method: OKW1 cells were incubated for 24 hours in the presence of 5 mM or 25 mM D(+)-glucose plus 10^{-7} M PMA in the absence or presence of various concentrations (i.e. 10^{-11} to 10^{-5} M) of insulin. Media were harvested after incubation and assayed for IR-hGH. Levels of IR-hGH in the medium containing 5 mM D(+)-glucose (i.e. 0.29±0.01 ng/ml) in the absence of PMA and insulin were expressed as 100%. The inhibitory effect of insulin was compared with cells that were stimulated by 25 mM D(+)-glucose plus 10^{-7} M PMA. Each point represents the mean ± S.D. of at least three dishes (**$p \leq 0.01$, ***$p \leq 0.005$). Experiments were repeated three times.

III.21. Effect of Insulin on the Expression of pOGH(hANG N-1064/+27) in OKW1 Cells After Pre-incubation with PMA (10^{-5} M)

Figure III.21

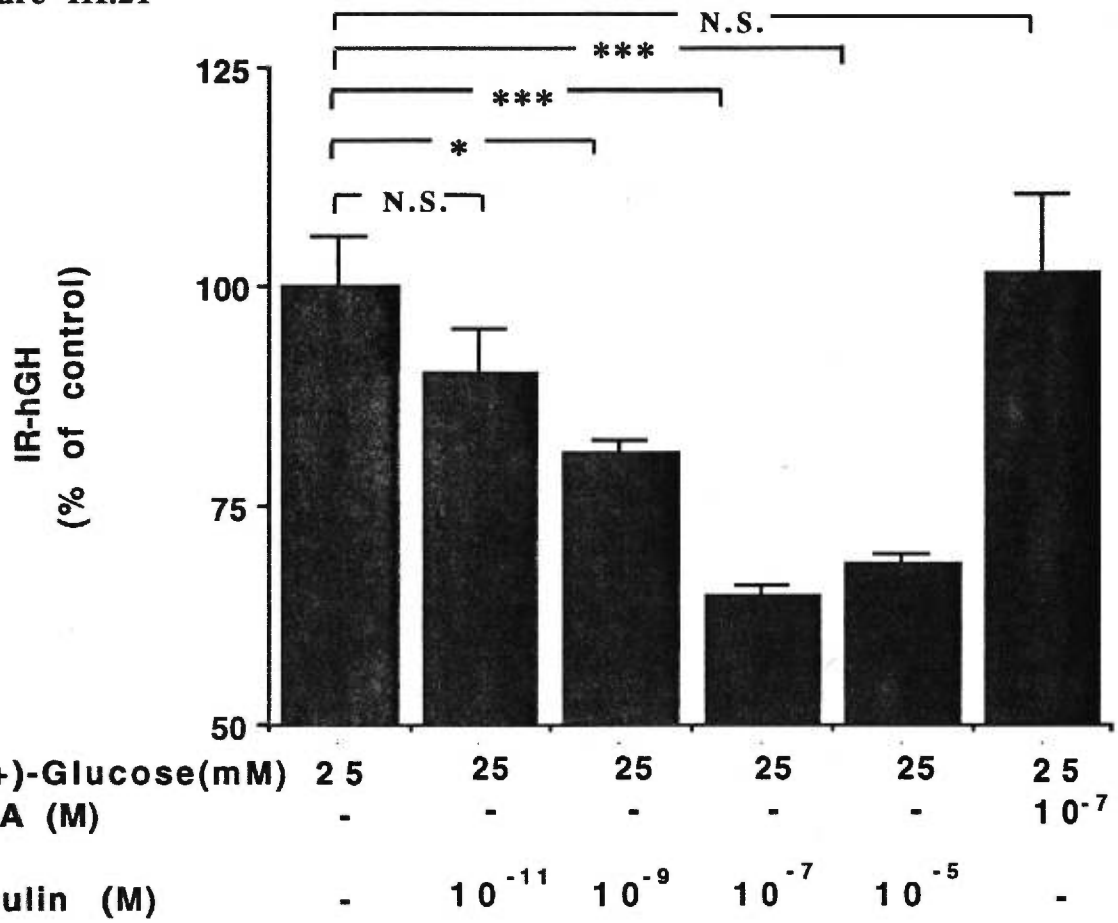


Figure III.21 shows that the overnight incubation of OKW1 cells with a high concentration of PMA (10^{-5} M) abolished the stimulatory effect of PMA (10^{-7} M), but did not block the inhibitory effect of insulin on the expression of pOGH(hANG N-1064/+27).

Method: OKW1 cells were pre-incubated for 24 hours with 10^{-5} M PMA in 25 mM D(+)-glucose medium. Then, cells were incubated for another 24 hours with fresh 25 mM D(+)-glucose medium containing various concentrations of insulin (10^{-11} to 10^{-5} M) or 10^{-7} M PMA. Subsequently, media were harvested and assayed for IR-hGH. Levels of IR-hGH in the media containing 25 mM D(+)-glucose (i.e. 0.30±0.02 ng/ml) in the absence of insulin and PMA in the media were expressed as 100%. The effect of insulin was compared with cells incubated with 25 mM D(+)-glucose alone. Each point represents the mean ± S.D. of at least three dishes (*$p \leq 0.05$ and ***$p \leq 0.005$). Experiments were repeated three times.

III.22. Effect of PD98059 (MEK Inhibitor) on the Expression of pOGH(hANG N-1064/+27) in OKW1 Cells in Low Glucose Medium

Figure III.22

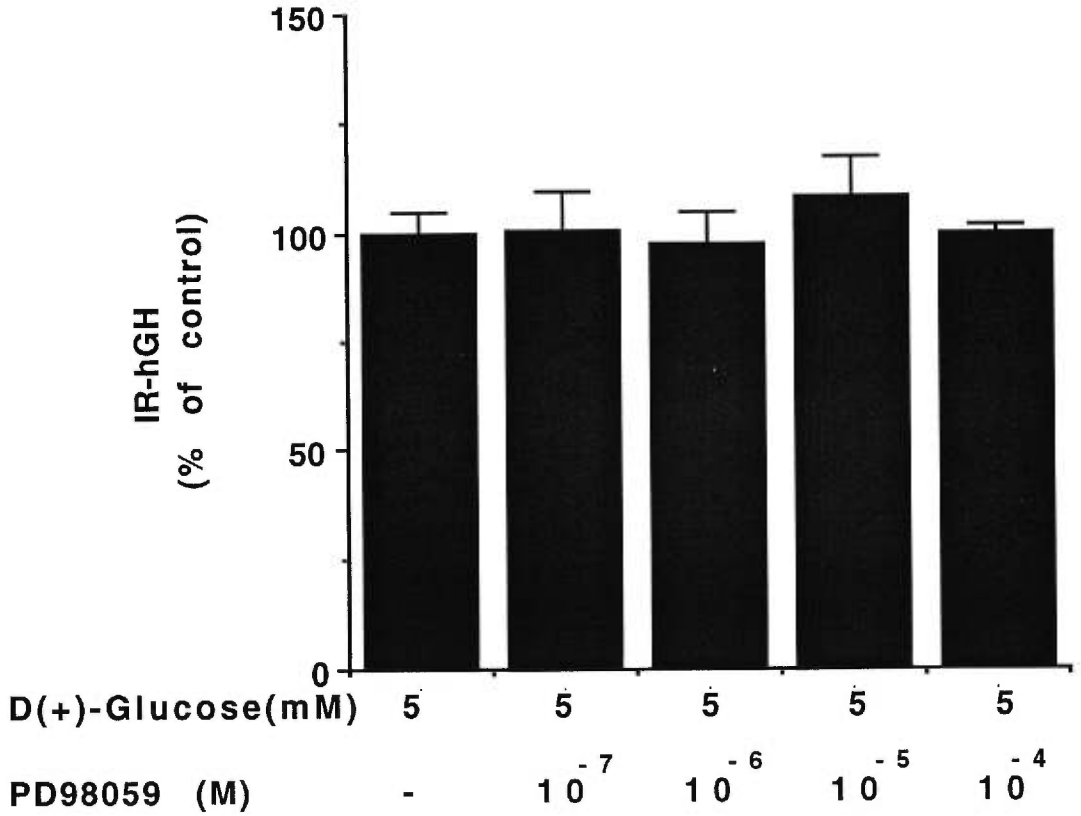


Figure III.22 shows that PD98059 (10^{-7} to 10^{-4} M) have no effect on the expression of pOGH(hANG N-1064/+27) in OKW1 cells when incubated in 5mM D(+)-glucose medium.

Method: OKW1 cells were incubated with various concentrations of PD98059 (10^{-7} to 10^{-4} M) in the presence of 5 mM D(+)-glucose. Media were harvested after 24 hours of incubation and assayed for IR-hGH. The level of IR-hGH in the 5 mM D(+)-glucose without PD98059 was expressed as 100% (control). Each point represents the mean±S.D. of at least three dishes.

III.23. Effect of PD98059 on the Expression of pOGH(hANG N-1064/+27) in OKW1 Cells in High Glucose Medium

Figure III.23

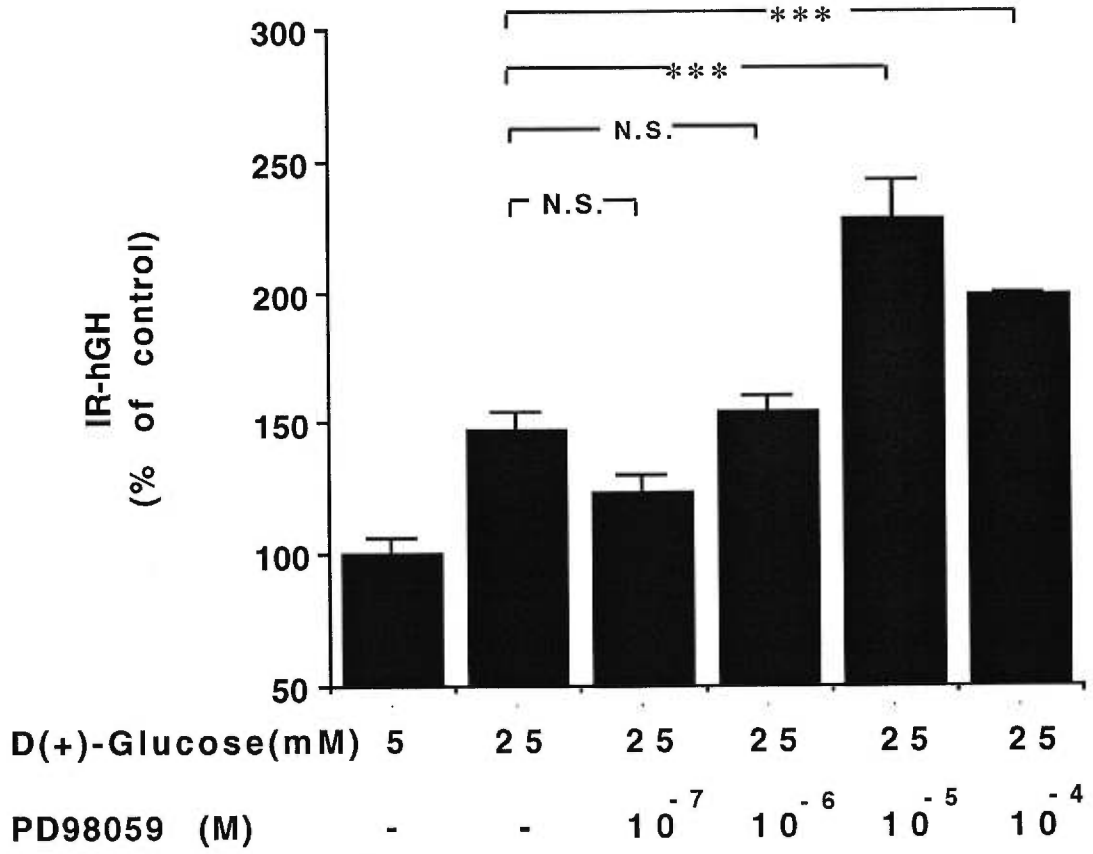


Figure III.23 shows that PD98059 at 10^{-5} to 10^{-4} M significantly enhanced the expression pOGH(hANG N-1064/+27) in OKW1 cells when incubated in 25mM D(+)-glucose medium.

Method: OKW1 cells were incubated with various concentrations of PD98059 (10^{-7} to 10^{-4} M) in the presence of 25 mM D(+)-glucose. Media were harvested after 24 hours of incubation and assayed for the level of IR-hGH. The level of IR-hGH in the 5 mM glucose (i.e. 0.51±0.03 ng/ml) was expressed as 100% (control). Each point represents the mean ± S.D. of at least three dishes (***$p \leq 0.005$).

III.24. Effect of PD98059 on the Expression of pOGH(hANG N-1064/+27) in OKW1 Cells in the Presence of Insulin

Figure III.24

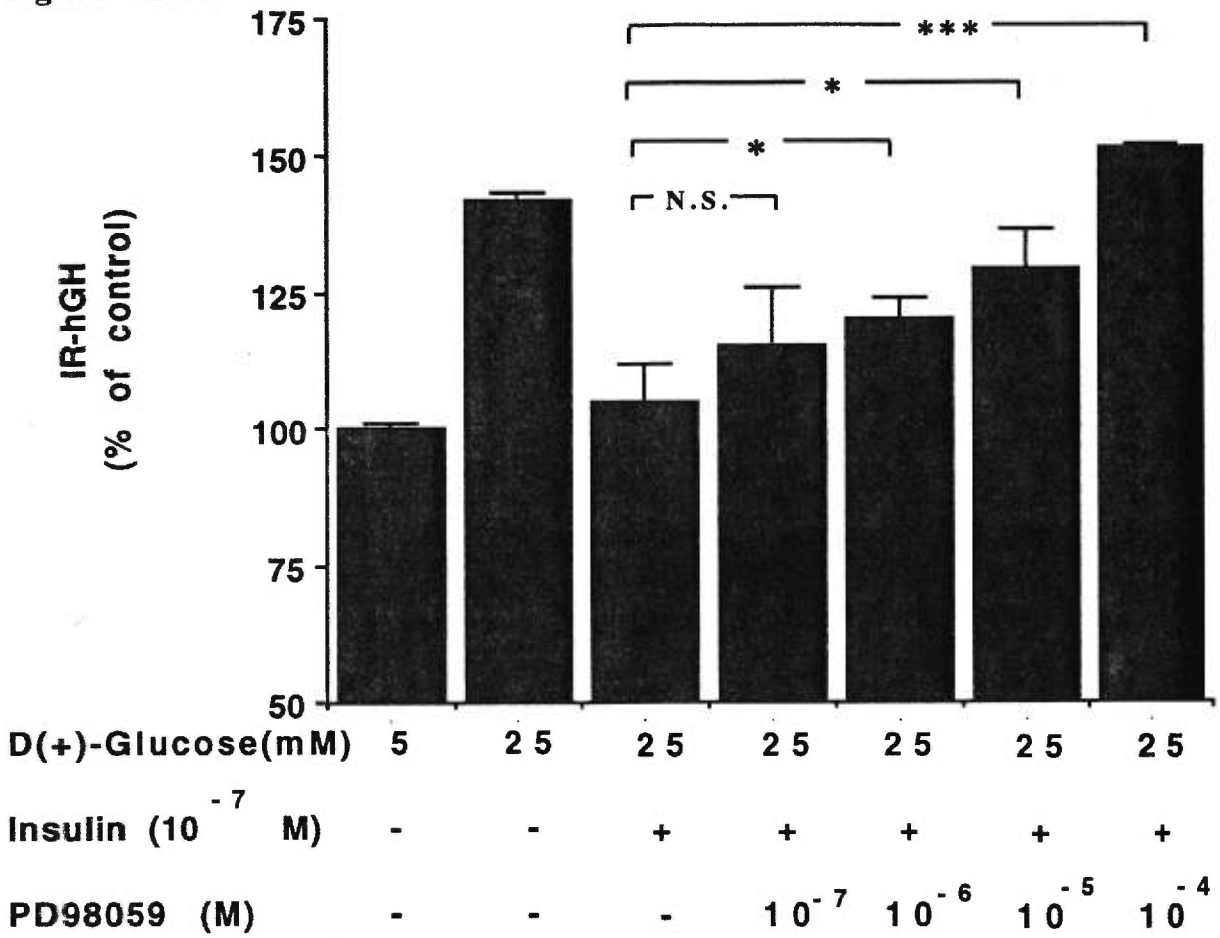


Figure III.24 shows that various concentrations of PD98059 (10^{-7} to 10^{-4} M) blocked the inhibitory effect of insulin (10^{-7} M) on the expression of pOGH(hANG N-1064/+27) in OKW1 cells in a dose-dependent manner. The maximum effective dose for inhibition of the stimulated expression (25 mM D(+)-glucose) of the fusion genes was at 10^{-7} M insulin.

Method: OKW1 cells were incubated for 24 hours in the presence of 5 mM or 25 mM D(+)-glucose plus 10^{-7} M insulin in the absence or presence of various concentrations from 10^{-7} to 10^{-4} M of PD98059. Media were harvested after incubation and assayed for IR-hGH. Levels of IR-hGH in the medium containing 5 mM D(+)-glucose (i.e. 0.44±0.01 ng/ml) in the absence of insulin and PD98059 were expressed as 100%. The effect of PD98059 was compared with cells that were incubated in the presence of 25 mM D(+)-glucose and 10^{-7} M insulin. Each point represents the mean ± S.D. of at least three dishes (*$p \leq 0.05$ and ***$p \leq 0.005$). Experiments were repeated three times.

III.25. Effect of Wortmannin (PI-3 Kinase Inhibitor) on the Expression of pOGH(hANG N-1064/+27) in OKW1 Cells in Low Glucose Medium

Figure III.25

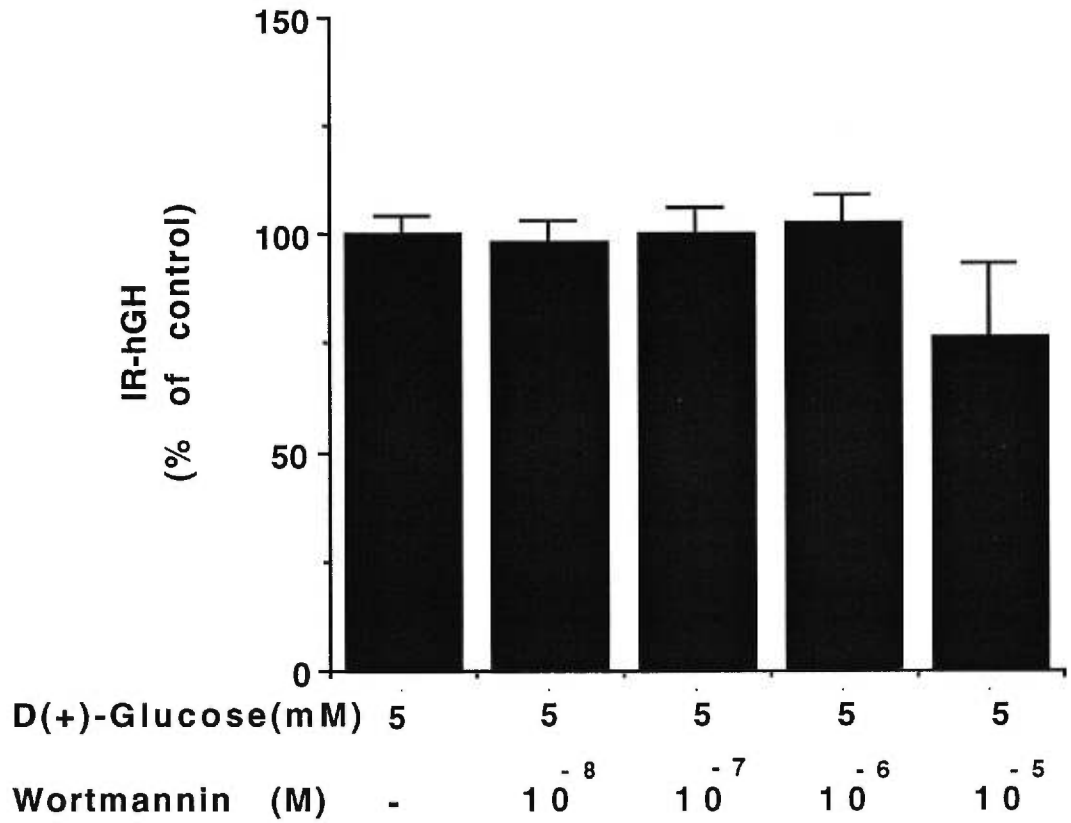


Figure III.25 shows that wortmannin (10^{-8} to 10^{-5} M) have no effect on the expression of pOGH(hANG N-1064/+27) in OKW1 cells in low glucose medium (5mM D(+)-glucose).

Method: OKW1 cells were incubated with various concentrations of wortmannin (10^{-8} to 10^{-5} M) in the presence of 5 mM D(+)-glucose. Media were harvested after 24 hours of incubation and assayed for IR-hGH. The level of IR-hGH in the 5 mM D(+)-glucose without wortmannin was expressed as 100% (control). Each point represents the mean±S.D. of at least three dishes.

III.26. Effect of Wortmannin on the Expression of pOGH(hANG N-1064/+27) in OKW1 Cells in High Glucose Medium

Figure III.26

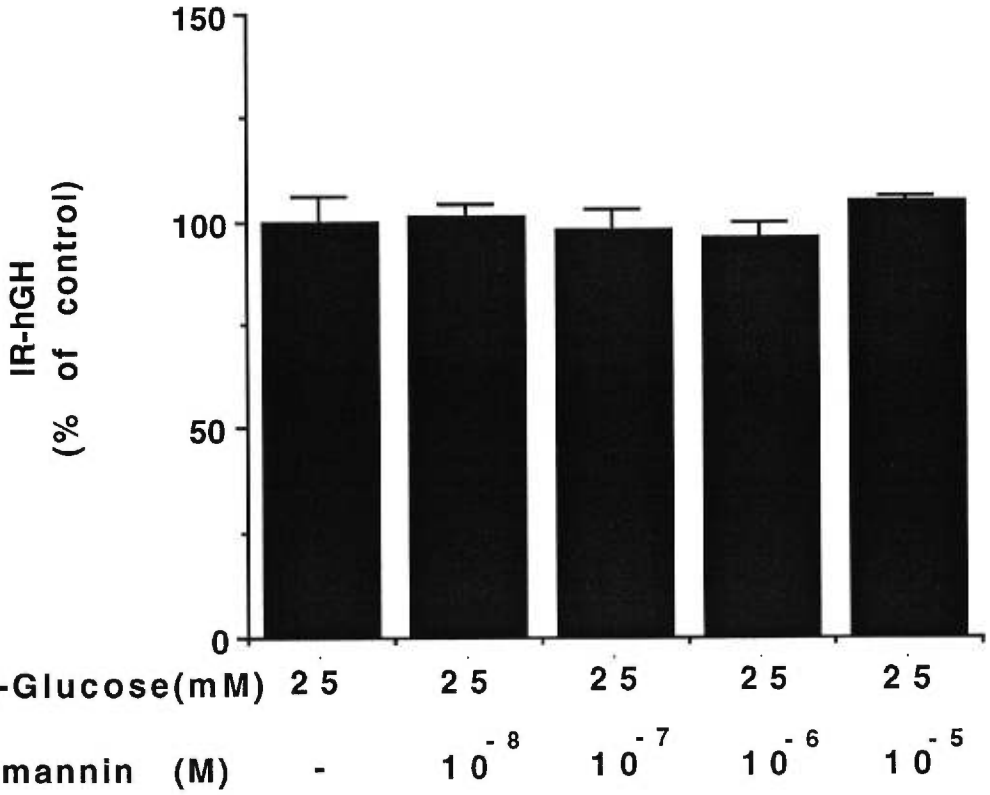


Figure III.26 shows that wortmannin (10^{-8} to 10^{-5} M) have no effect on the expression of pOGH(hANG N-1064/+27) in OKW1 cells in high glucose medium (5mM D(+)-glucose).

Method: OKW1 cells were incubated with various concentrations of wortmannin (10^{-8} to 10^{-5} M) in the presence of 25 mM D(+)-glucose. Media were harvested after 24 hours of incubation and assayed for IR-hGH. The level of IR-hGH in the 25 mM D(+)-glucose without wortmannin was expressed as 100% (control). Each point represents the mean±S.D. of at least three dishes.

III.27. Effect of Wortmannin on the Expression of pOGH(hANG N-1064/+27) in OKW1 Cells in the Presence of Insulin

Figure III.27

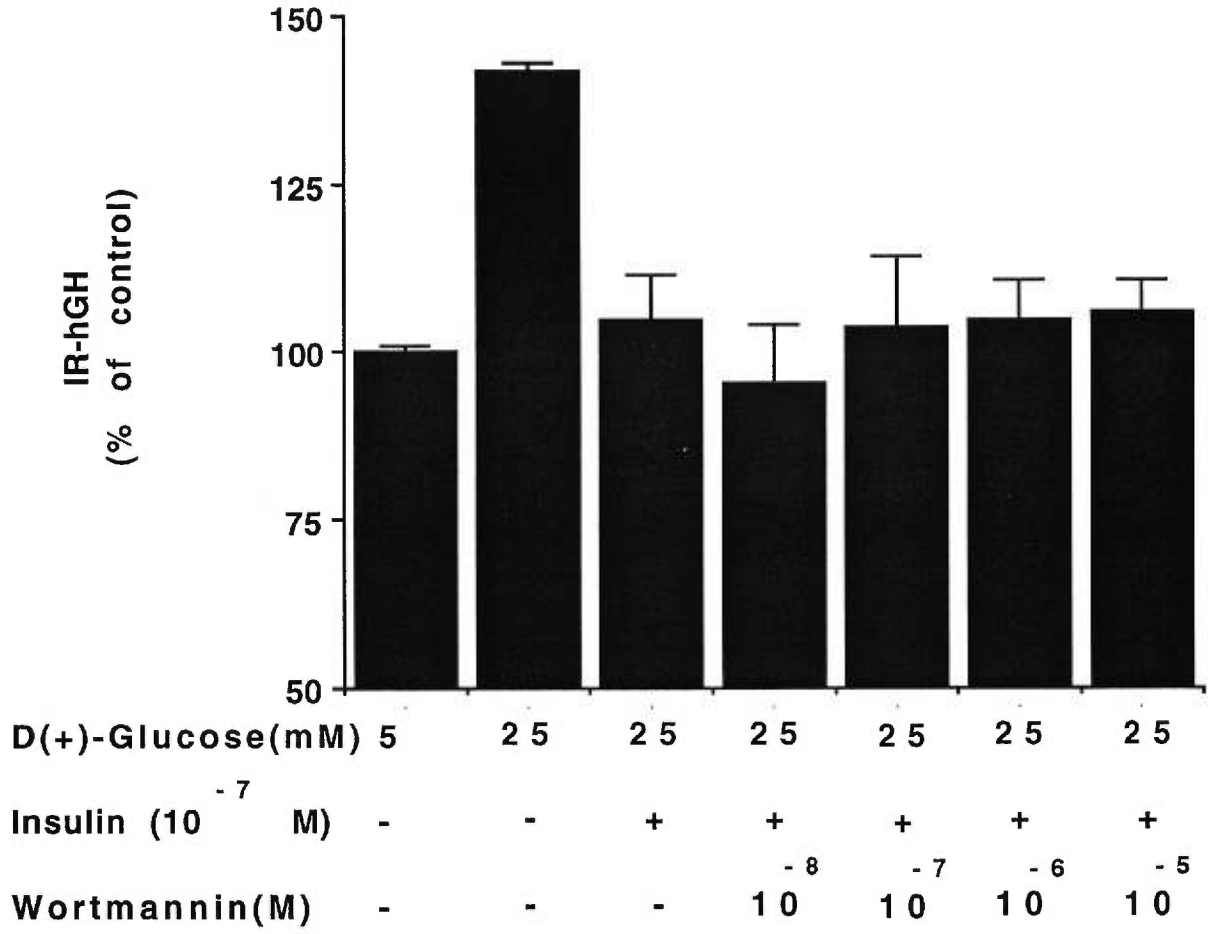


Figure III.27 shows that various concentrations of Wortmannin (10^{-8} to 10^{-5} M) did not block the inhibitory effect of insulin (10^{-7} M) on the expression of pOGH(hANG N-1064/+27) in OKW1 cells.

Method: OKW1 cells were incubated for 24 hours in the absence or presence of various concentrations (10^{-8} to 10^{-5} M) of Wortmannin. Media were harvested after incubation and assayed for IR-hGH. Levels of IR-hGH in the medium containing 5 mM D(+)-glucose (i.e. 0.44±0.01 ng/ml) in the absence of insulin and Wortmannin was expressed as 100%. The effect of Wortmannin was compared with cells that were incubated in the presence of 25 mM D(+)-glucose and 10^{-7} M insulin. Each point represents the mean ± S.D. of at least three dishes. Experiments were repeated three times.

III.28. Effects of D(+)-glucose, Insulin, Tolrestat, Staurosporine, H-7 or PMA on the Expression of pTKGH in OK13 Cells

Figure III.28

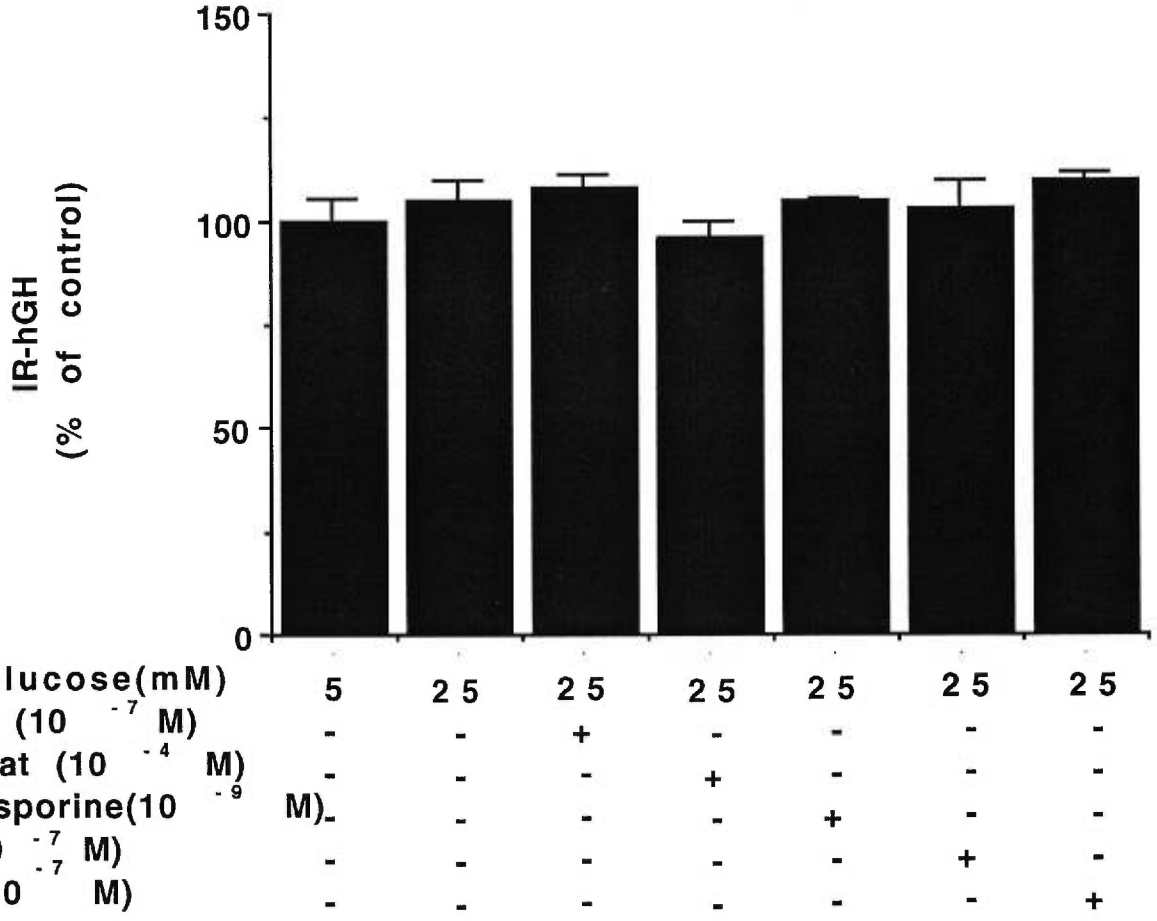


Figure III.28 shows that the addition of insulin (10^{-7} M), Tolrestat (10^{-4} M), staurosporine (10^{-9} M), H-7 (10^{-7} M) or PMA (10^{-7} M) have no effect on the expression of pTKGH in OK13 cells compared to the control (i.e. 5 mM glucose).

Method: OK13 cells were incubated for up to 24 hours in the presence of 5 mM, 25 mM D(+)-glucose, 25 mM D(+)-glucose plus insulin (10^{-7} M), Tolrestat (10^{-4} M), staurosporine (10^{-9} M), H-7 (10^{-7} M) or PMA (10^{-7} M). Media were harvested after incubation and assayed for IR-hGH. The concentration of IR-hGH in the medium containing 5 mM D(+)-glucose (i.e. 0.35±0.02 ng/ml) was expressed as 100%. Each point represents the mean ± S.D. of at least three dishes. Experiments were repeated three times.

III.29. Effect of Angiotensin II on the Expression of pOGH(hANG N-1064/+27) in OKW1 Cells in Low Glucose Medium

Figure III.29

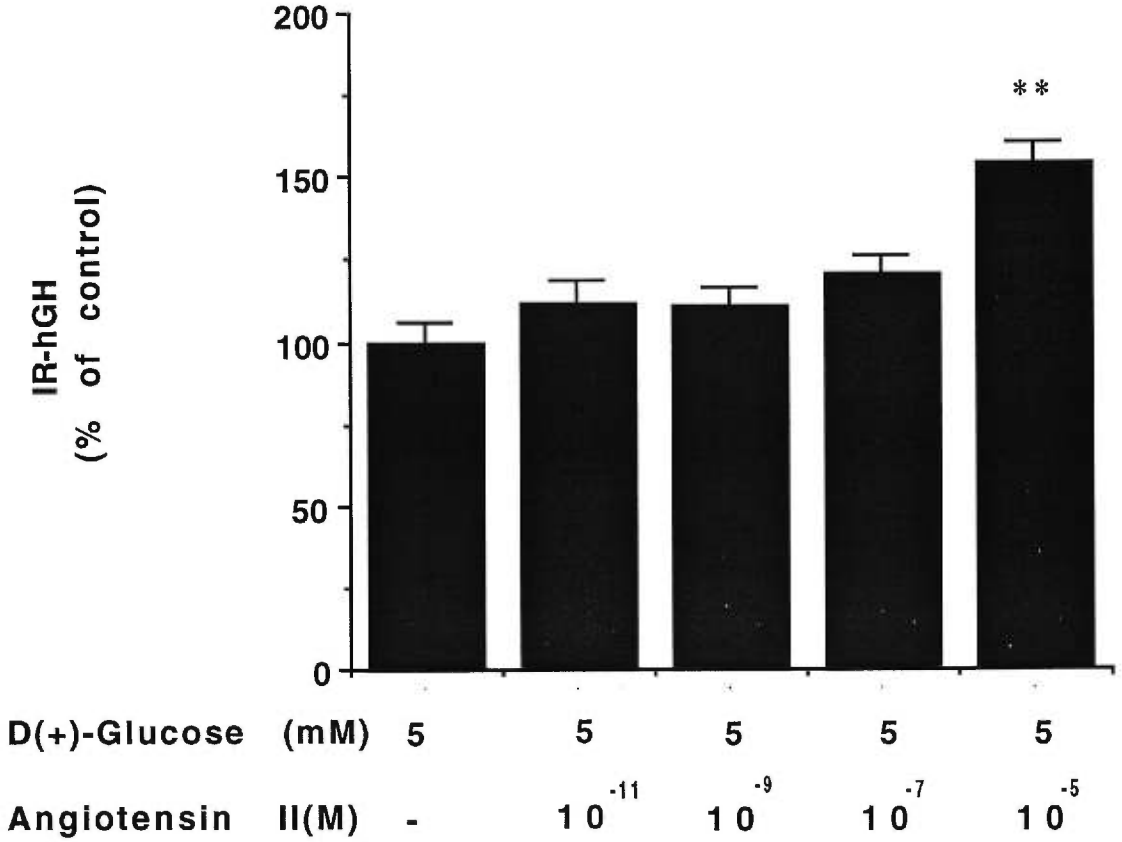


Figure III.29 shows that the addition of Ang II (10^{-11} to 10^{-7} M) have no effect on the expression of pOGH(hANG N-1064/+27) in OKW1 cells in low glucose (5 mM D(+)-glucose). However, Ang II at high concentration (10^{-5} M) stimulates the expression of pOGH(hANG N-1064/+27) in OKW1 cells in low glucose media.

Method: OKW1 cells were incubated with various concentrations of Ang II (10^{-11} to 10^{-5} M) in the presence of 5 mM D(+)-glucose. Media were harvested after 24 hours of incubation and assayed for the level of IR-hGH. The level of IR-hGH in 5 mM glucose without Ang II (i.e. 0.5±0.03 ng/ml) was expressed as 100% (control). Each point represents the mean ± S.D. of at least three dishes (**p≤0.01). Experiments were repeated three times.

III.30. Effect of Angiotensin II on the Expression of pOGH(hANG N-1064/+27) in OKW1 Cells in High Glucose Medium

Figure III.30

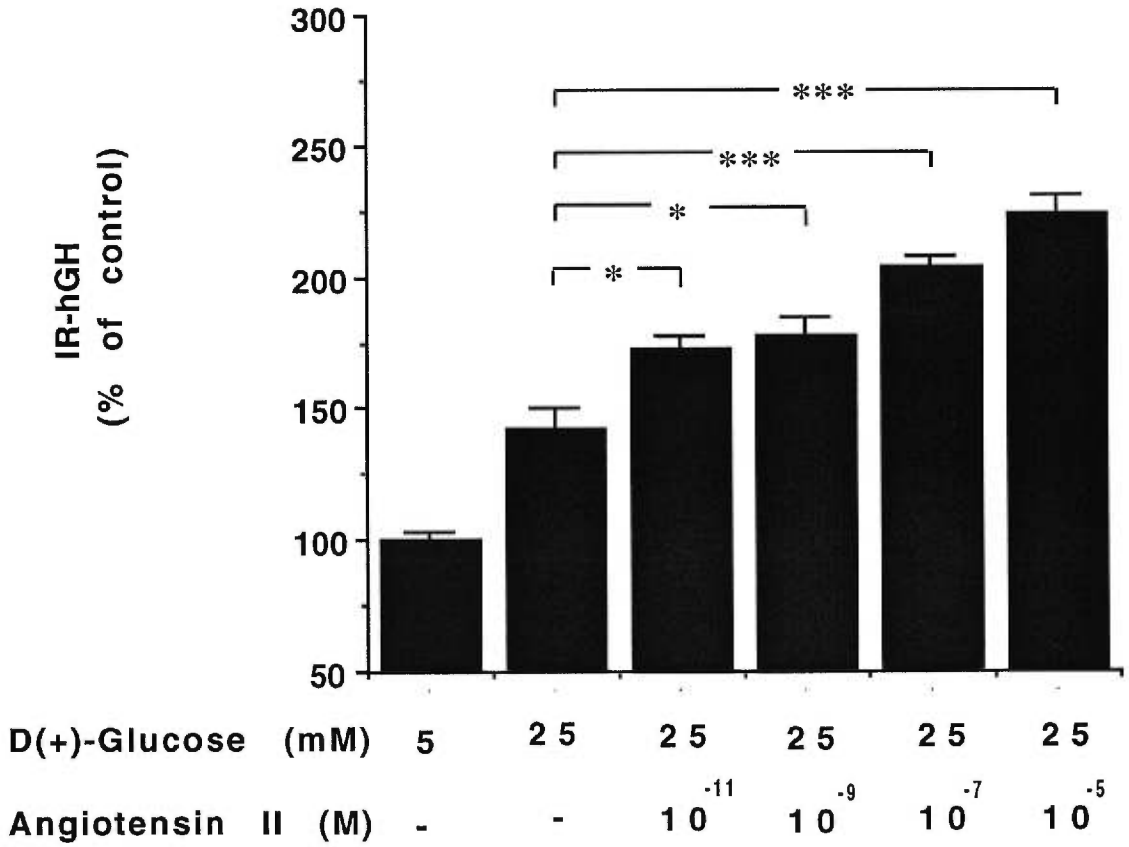


Figure III.30 shows that the addition of Ang II (10^{-11} to 10^{-5} M) stimulated the expression of pOGH(hANG N-1064/+27) in OKW1 cells in high glucose (25 mM D(+)-glucose) medium in a dose-dependent manner.

Method: OKW1 cells were incubated with various concentrations of Ang II (10^{-11} to 10^{-5} M) in the presence of 25 mM D(+)-glucose. Media were harvested after 24 hours of incubation and assayed for the level of IR-hGH. The level of IR-hGH in 5 mM glucose without Ang II (i.e. 0.58±0.02 ng/ml) was expressed as 100%. The effect of Ang II was compared with cells that were incubated in the presence of 25 mM D(+)-glucose without Ang II. Each point represents the mean ± S.D. of at least three dishes (*$p \leq 0.05$ and ***$p \leq 0.005$). Experiments were repeated three times.

III.31. Effect of Insulin and Ang II on the Expression of pOGH(hANG N-1064/+27) in OKW1 Cells.

Figure III.31

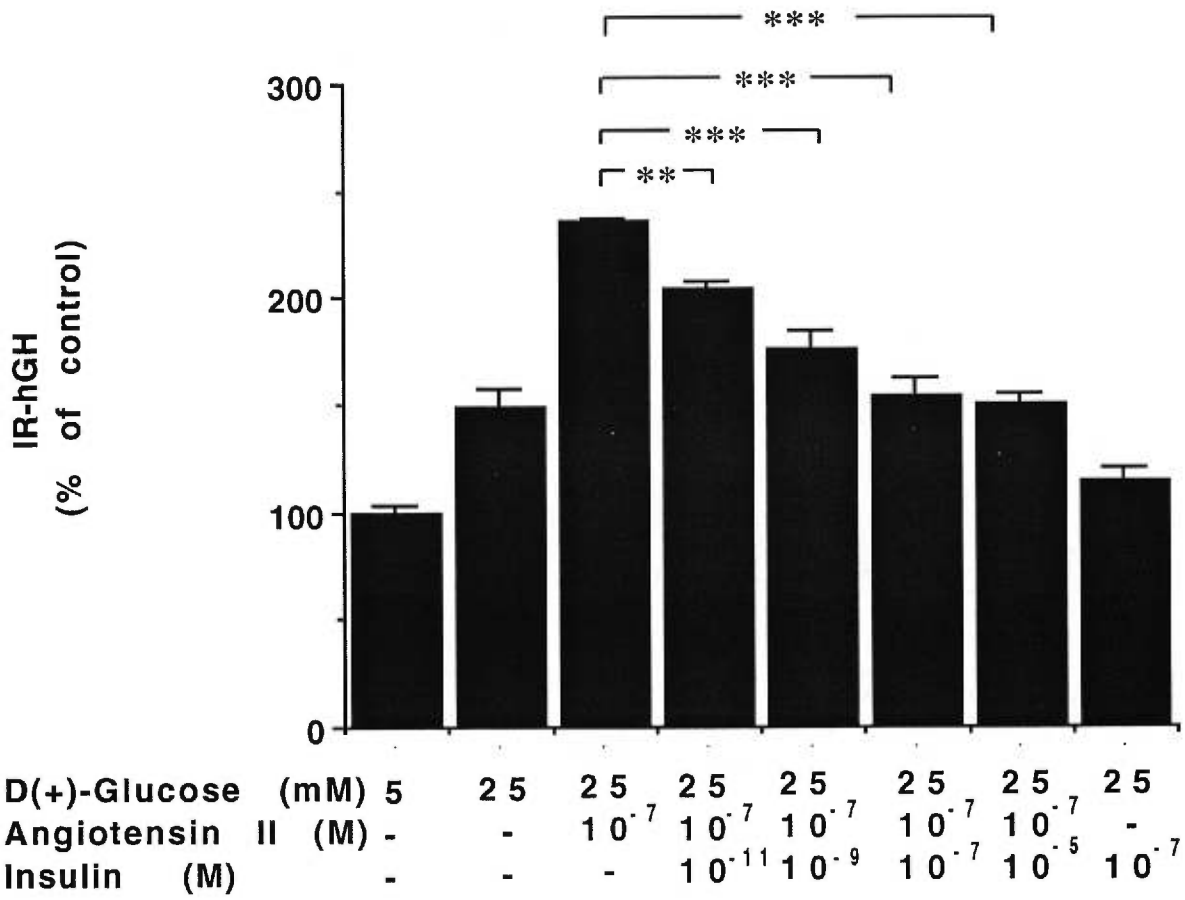


Figure III.31 shows the effect of a combination of both insulin and Ang II on the expression of pOGH(hANG N-1064/+27) in OKW1 cells. The stimulatory effect of Ang II (10^{-7} M) on the expression of pOGH(hANG N-1064/+27) in OKW1 cells was blocked by insulin in a dose-dependent manner (10^{-11} to 10^{-5} M). Insulin at concentrations of 10^{-7} M completely reversed the stimulatory effect of Ang II.

Method: OKW1 cells were incubated in 25 mM D(+)-glucose medium, with 10^{-7} M Ang II in the absence or presence of various concentrations (i.e. 10^{-11} to 10^{-5} M) of insulin. Media were harvested after incubation and assayed for IR-hGH. Levels of IR-hGH in the medium containing 5 mM D(+)-glucose (i.e. 0.58±0.02 ng/ml) in the absence of Ang II and insulin were expressed as 100%. The inhibitory effect of insulin was compared with cells that were incubated with 10^{-7} M Ang II in 25 mM D(+)-glucose. Each point represents the mean ± S.D. of at least three dishes (**$p \leq 0.01$ and ***$p \leq 0.005$). Experiments were repeated three times.

IV. Discussion

IV.1. OK Cell

IV.1.1. Characteristics of the OK Cell Line.

OK is an established renal epithelial cell line derived from opossum kidney . OK cells have many characteristics of renal proximal tubule cells, including specific transport systems for hexoses, amino acids, Pi and Na^+-proton exchange, and receptors for parathyroid hormone, α_2-receptor agonists, and serotonin (Koyama H. et al., 1978; Caverzasio J. et al., 1986). OK cells also have insulin receptors. The binding, internalization, and degradation of insulin by these cells has been studied (Yagil C. et al., 1988a; 1988b). In addition, these cells lack receptors for calcitonin and vasopressin, markers for the distal nephron segments (Yagil C. et al., 1988a; 1988b).

These studies indicate that OK cell line provides a good model for studying the interaction of insulin with the renal tubular epithelium, as well as on the intracellular mechanism of insulin action of Pi transport.

Studies in our laboratories have demonstrated that the OK cell line is a good cellular model to study the expression of the ANG gene in proximal tubular cells (Wang TT. et al., 1994; 1995; 1998).

IV.1.2. Stable Transformed OK Cell Lines

We chose the stable transformed cell lines to study the regulation of the expression of the ANG gene promoter by glucose and insulin instead of transient transfection for the following reasons: 1) In transient transfection, the fusion gene remains within the cell for less than two

weeks. Thus, transient gene transfections must be done repeatedly which sometimes led to results that were not reproducible due to the variation of DNA transfection efficiency. 2) The physical environment of the naked plasmid is not similar to the endogenous gene which should be bound or surrounded by nucleosomes. Thus, the molecular mechanism(s) of regulation of the endogenous ANG gene could be different from the transiently transfected fusion gene.

The cell line with the fusion gene stably integrated into its genome that is able to express the fusion gene following stimulation by various factors would be an ideal in vitro model. Hence, the first objective of our studies was to develop such a cell line.

Clones OKW1, OKW3 and OK 13 are stable transformants with pOGH(hANG N-1064/+27), pOCAT(hANG N-1064/+27) or PTKGH integrated into OK cellular genomes, respectively. These clones have been grown in the medium containing 500 μg/ml of G418 for more than three months and secreted a high amount of IR-hGH or expressed high cellular CAT activity. The levels of hGH or CAT activity reflect the transcriptional activity of the 5' promoter region of human ANG gene or thymidine kinase (TK) gene.

IV.2. Human GH and CAT Gene as Reporter Genes

Both human GH and CAT genes were used as reporter genes in our studies.

Human GH has been used as a reporter gene in the studies of gene regulation (Selder R. et al, 1986; Tomonari A. et al., 1996; Qin W. et al., 1997; Li J. et al., 1998). The advantages of the hGH reporter system including the extreme sensitivity, ease of detection by radioimmunoassay, medium is assayed and the cells do not need to be harvested. The potential disadvantage of this system is the possibility that the secreted hGH itself may have a biological effect on gene expression. To eliminate this possibility, we have studies the effect of hGH on the expression of the hANG gene promoter activity. Our study (Figure III.1) showed that human GH (from 1 to

1000 ng/ml) had no effect on the expression of human ANG gene in the OK cell line. Hence human GH can be used as a reporter gene to study the regulation of the expression of the hANG(N' -1064/+27) gene promoter activity in OK cells.

The CAT gene is also a widely used reporter gene which encodes the enzyme chloramphenicol acetyltransferase (CAT). The advantage of using the CAT reporter gene is that this gene is of prokaryotic origin and is not found in eukaryotic cells. However, in comparison with the RIA-hGH assay, the CAT assay is more labor intensive, expensive and the results have higher variation (i.e. inter- and intra- assay variation for CAT receptor gene are higher).

For the initial experiments, we used both OKW1 and OKW3 cell lines which contained hGH and CAT reporter genes respectively to compare their responses to glucose and insulin. We observed that the responses of OKW1 and OKW3 were consistent. However, we have found that the use of hGH as reporter gene is more reproducible. Hence, we chose the OKW1 cell line which contained the hGH reporter gene to study the regulation of hANG gene in subsequent experiments.

IV.3. The Effect of High Glucose on Renal ANG Gene Expression

While several studies have been described on the expression of the renal RAS genes in experimental DM, conflicting results have been obtained from different groups. Studies of Correa-Rotter R. et al (1992) 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 JE. (1993) reported that there were no significant differences in the expression of renal renin and ANG mRNA in rats two weeks after the induction of diabetes compared to controls. In contrast, Anderson S. et al (1993a) 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 conflicting results, one obvious difference is the duration of diabetes in experimental rats used by these investigators.

Similarly, conflicting results were reported for the effect of high glucose levels on the expression of the RAS gene in renal proximal tubular cells in vitro. The preliminary communications of Ingelfinger JR. et al (1991) reported that an exposure to high glucose level down-regulates the expression of the RAS genes in opossum kidney cells. In contrast, recent studies in our laboratory have shown that the addition of a high level of D(+)-glucose stimulates the expression of the rat ANG gene promoter activity in OK 27 cells in a dose-dependent manner (Wang TT. et al., 1998). The effect of high glucose concentrations (25 mM) on the expression of the rat ANG gene promoter activity was blocked with H-7 or staurosporine but not by Rp-cAMP (an inhibitor of protein kinase I and II) (Wang TT. et al., 1998). These studies demonstrated that the effect of a high level of glucose on the expression of the ANG gene is likely mediated via the PKC pathway. At present, the reasons for the discrepancy between our studies and the studies of Ingelfinger JR. et al (1991) are not known. One possible explanation may be that the OK cells used in the studies of Ingelfinger JR. et al (1991) were heterogeneous in population (i.e. obtained from ATCC) whereas we used subclones of the OK cells. Clearly, more studies are warranted to clarify this discrepancy.

In the present study, we have shown that the hANG gene promoter activity was increased in the presence of high levels of glucose (Figure III.2 and Figure III.3). The addition of D-mannitol, L-glucose and 2-deoxy-D-glucose did not stimulate the expression of pOGH(hANG N-1064/+27) or pOCAT(hANG N-1064/+27) in OKW1 and OKW3 cells, respectively (Figure III.4 and Figure III.5). These results suggest that the effect of high levels of glucose on the expression of hANG gene promoter activity is probably mediated via the metabolic products of D(+)-glucose. Furthermore, these studies also showed that the response of the human ANG gene promoter activity is similar to the rat ANG gene promoter as we previously reported (Wang TT. et al., 1998).

These studies showed that high levels of D(+)-glucose can stimulate the expression of hANG gene expression and that this stimulatory effect is specific.

IV.4. The Molecular Mechanisms of a High Glucose Effect on hANG Gene Expression.

IV.4.1. The Polyol Pathway

The polyol pathway (sorbitol pathway) has long been suspected to be responsible for some diabetic complications including diabetic nephropathy (Bleyer AJ. et al., 1994). Aldose reductase initially catalyzes the reduction of glucose to sorbitol. The progression of the changes induced by polyol pathway activation can be arrested by inhibiting aldose reductase (Dvornik D. et al., 1988). Within 25 years of the discovery of Alrestatin , the first orally active aldose reductase inhibitor, scores of compounds had been tested for their inhibitory effect on aldose reductase (Dvornik D. et al., 1988).

Tolrestat, an carboxylic acid group, is an aldose reductase that was developed by the Wyeth-Ayerst company (Terashima H. et al., 1988). The effect of Tolrestat is through the inhibition of aldose reductase and thus inhibits the increased polyol pathway in diabetics (Fruncillo R. et al., 1996). Tolrestat has been shown to increase sciatic nerve blood flow in diabetic rats (Chang K. et al., 1997); it also corrects the impaired responses of microvessels to histamine and bradykinin in diabetic rats (Fortes ZB. et al., 1996); improves nerve regeneration in diabetic neuropathy (Terada M. et al., 1996); plays a role in the treatment of diabetic peripheral neuropathy (Nicolucci A. et al., 1996) and the primary prevention of diabetic neuropathy (Giugliano D. et al., 1995). Clinical trials in diabetic subjects suggested that Tolrestat may reduce the severity of chronic tissue damage associated with hyperglycemia.

In our study, we used Tolrestat to investigate whether the polyol pathway was involved in the stimulatory effect of high glucose levels on the expression of human ANG in OKW1 cells. Our results showed that Tolrestat blocked the stimulatory effect of 25 mM glucose on the expression of

hANG gene promoter in a dose-dependent manner (Figure. III.13). These observations suggest that the stimulatory effect of high levels of glucose is mediated, at least in part, via the activation of the polyol pathway.

IV.4.2. PKC Signal Transduction Pathway

IV.4.2.1. Activation of PKC

The protein kinase C signal transduction pathway is involved in many intracellular events. We used PKC inhibitor and activator to investigate whether PKC activation is involved in the effect of high levels of glucose on the expression of hANG gene expression in OKW1 cells.

H-7 and staurosporine are PKC inhibitors, with relatively high specificity for PKC. H-7 also can inhibit cAMP-dependent protein kinase, staurosporine at higher concentrations may have actions unrelated to the inhibitory effect of PKC, which include the inhibition of calcium influx through the voltage-dependent calcium channel (Kageyama M. et al., 1991). Hence, we used both H-7 and staurosporine to investigate the effect of PKC inhibitors on the expression of the human ANG gene stimulated by high glucose. Our results show that the addition of H-7 or staurosporine blocked the stimulatory effect of 25 mM glucose on the hANG gene promoter activity in OKW1 cells in a dose-dependent manner (Figure III.17 and Figure III.18).

The addition of PMA (PKC stimulator) enhanced the stimulatory effect of high glucose on the expression of the hANG gene promoter activity in OKW1 cells in a dose-dependent manner (Figure III.19).

These results indicate that a high glucose level exerts its effect on the expression of hANG gene promoter through the activation of PKC enzymatic activity.

It is likely that subcellular localization of individual PKC isoforms is crucial for regulation of PKC activity. Cell stimulation causes most or all of the PKC to translocate from one to another subcellular location. Historically, PKC was assumed to travel from the cytosol to the membrane exclusively (Disatnik MH. et al., 1994; Haller H. et al., 1995), but recent evidence has revealed that the PKC translocation can occur from one location to other cell locations such as the Golgi apparatus (Westermann P. et al., 1996), endoplasmic reticulum (Goodnight J. et al., 1995), nucleus (Haller H. et al., 1995). Subcellular localization of PKC isoforms is cell type- and isoform-specific and is very likely important in regulating PKC access to substrates (Dekker JV. and Parker PJ., 1994).

Our studies showed that the ratio of membrane/cytosol PKC activity is increased in high glucose medium (Figure III.16), although the total PKC activity in the cell did not increase. This data indicates that high glucose levels stimulated the activation of PKC, the activated PKC then translocated from the cytosol to the membrane of the cell.

The above results provide strong evidence that the PKC signal transduction pathway is involved in the high glucose effect on the expression of hANG gene promoter.

IV.4.2.2. Increased Formation of DAG

The PKC family consists of at least 11 isoforms, and PKC can be activated in cells by DAG, calcium, arachidonic acid and/or phosphatidylserine (Berridge NJ., 1986; Exton JH., 1990; Nishizuka Y., 1988). DAG, the hydrophobic product of the phosphodiesterase-mediated cleavage of inositol phospholipids, is thought to have a second-messenger function through the activation of PKC (Nishizuka Y., 1989).

To elucidate whether high glucose activated PKC is DAG-dependent or DAG-independent, we evaluated the DAG level within the cell. The result shows that high glucose also increased the

intracellular DAG level (Figure III.15), indicating that the stimulatory effect of high glucose on the expression of the hANG gene promoter activity is through increased formation of DAG and the activation of PKC enzymatic activity.

IV.4.3. Polyol Pathway and PKC Signal Transduction Pathway

The molecular mechanism of the polyol pathway is not yet clear. The increased polyol pathway first increases the sorbitol production by aldose reductase, and then increases intracellular fructose concentration and subsequently increases the ratio of $NADH^+/NAD$ (Terashima H.,1988). One hypothesis is that increased activity of the polyol pathway could increase the cellular $NADH/NAD^+$ ratio, which favors DAG synthesis (Fumo P. et al., 1994). The elevated DAG levels will in turn increase PKC enzymatic activity (Bell RB., 1986).

To investigate the relation between the polyol pathway and DAG synthesis, we assayed the DAG level after treatment with Tolrestat. Our result shows that the addition of high glucose (25 mM glucose) increased the cellular DAG levels, and the addition of Tolrestat inhibited the increased DAG level stimulated by high glucose (Figure III.15). These data indicate that the high levels of glucose increased the polyol pathway and thus favored DAG synthesis.

These studies support our model (Figure IV.1) that a high glucose level activates the polyol pathway activity, which in turn increases the synthesis of DAG and the subsequent stimulation of PKC activity to increase hANG promoter activity. This is in agreement with the the results of Zhou X. et al (1997) in mesangial cells. Their studies showed that mesangial cell F-actin disassembly in high glucose is likely mediated through DAG-sensitive PKC isoforms and involves the polyol pathway.

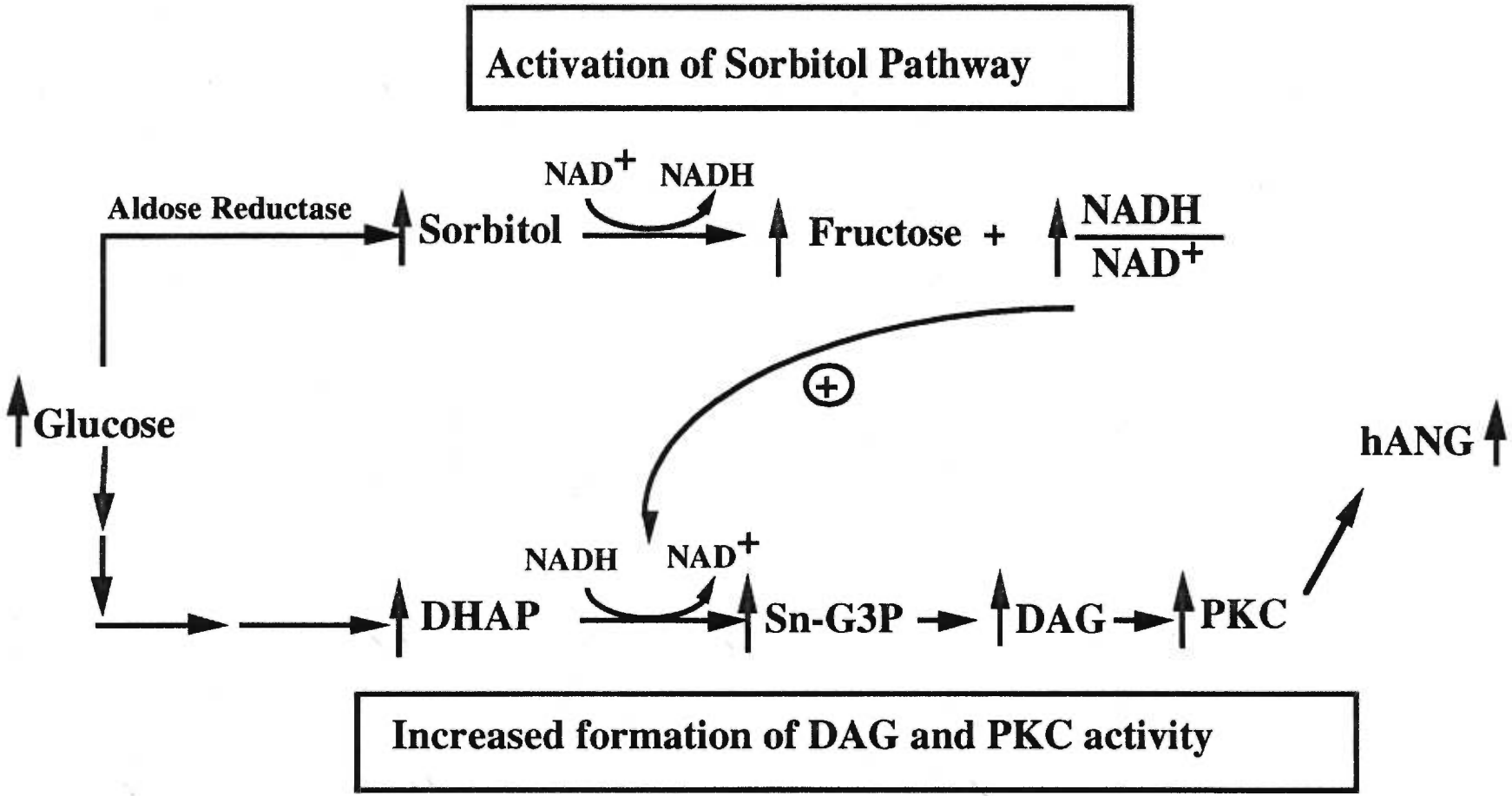


Figure IV.1. Molecular Mechanisms of High Levels of Glucose on the Expression of Human ANG Gene in OK Cells.

IV.5. Effect of Insulin on the Expression of the hANG Gene

Insulin, the most important glucose regulation hormone, has been widely used in the treatment of insulin-dependent diabetes. Thus, the study of the insulin effect on the high glucose stimulated of human ANG gene expression is important.

Studies by Chang E. and Perlman AJ. (1988) have shown that the addition of insulin attenuates the expression of the ANG mRNA in rat hepatoma cells in vitro. Consistent with these results, we have also observed that insulin inhibited the stimulatory effect of a high level of D(+)-glucose on the expression of the human ANG gene activity in a dose-dependent manner (Figure III.8 and Figure III.9). However, insulin has no effect on the expression of the human ANG gene in a low glucose medium (Figure III.6 and Figure III.7). These results, together with those of Kalinyak JE. et al. (1993b), suggest that insulin has no effect on the expression of ANG gene in normal physiological concentration of glucose. However, n the condition of hyperglycemia, insulin may regulate the expression of ANG gene expression.

IV.6. Effect of IGF-I and IGF-II on the Expression of the hANG Gene

Insulin like growth factors (IGFs) are a major class of growth factors, They are peptide mitogens for multiple cell lines (Oh Y. et al., 1993). They share structural similarity with insulin and have their own high affinity receptors on the cell membrane (Czech MP., 1989). The amino acid sequence of the major form of each human IGF was determined in 1978, revealing that IGF-I consists of 70 amino acids (molecular weight 7649 Da) and IGF-II consists of 67 amino acids (molecular weight 7471 Da) with a 65% homology between these two peptides. Many different species produce highly similar IGF-peptides (Rutanen EM. and Pekonen F., 1990). IGF-I has 43% sequence homology with human proinsulin. IGF-I may have a role in the control of glucose homeostasis, facilitated by changes in its binding proteins (Dunger DB. and Acerini CL., 1997). IGFs exert metabolic and mitogenic effects through their own specific receptors which belong to

the tyrosine kinase family (Dunger DB. and Acerini CL., 1997).

To test the specificity of the insulin effect on the expression of the hANG gene in OK cells, we performed experiments with the addition of IGF-I or IGF-II. We did not observe any significant inhibition of the expression of the hANG gene promoter in OKW1 and OKW3 cells in the presence of various concentrations of IGF-I and IGF-II, respectively (Figure III.10 and Figure III.11). Additionally, we have not observed any stimulation or inhibition of expression of pOCAT(hANG N-106/+27) in OKW3 cells treated with human growth hormone preparations at concentrations ranging from 1 to 1000 ng/ml (Figure III.1).

These results suggest that the inhibitory effect of insulin on the expression of the hANG gene promoter activity in OK cells is mediated via the insulin receptor and not the receptor(s) for IGF-I or IGF-II.

IV.7. The Molecular Mechanisms of the Insulin Effect on the Expression of the hANG Gene in a High Glucose Medium

Insulin action involves a network of interrelated and independent pathways with differing levels of divergence regarding the mechanisms of regulation.

IV.7.1. Role of the PKC Signal Transduction Pathway

Our results show that in OKW1 cells, PMA blocked the inhibitory effect of insulin on the hANG gene expression in high glucose media in a dose dependent manner (Figure III.20 (A)). On the other hand, the stimulatory effect of PMA on the expression of hANG gene could be blocked by insulin in a dose-dependent manner (Figure III.20 (B)).

Although we do not completely understand the opposing effect of PMA and insulin on the

activity of the hANG gene promoter, our observations raise the possibility that the antagonistic effect of these two agents may be mediated via a PMA-responsive element and an insulin-responsible element in the 5'-flanking region of the hANG promoter, respectively. We have not identified these responsive elements, however, efforts are underway in this direction.

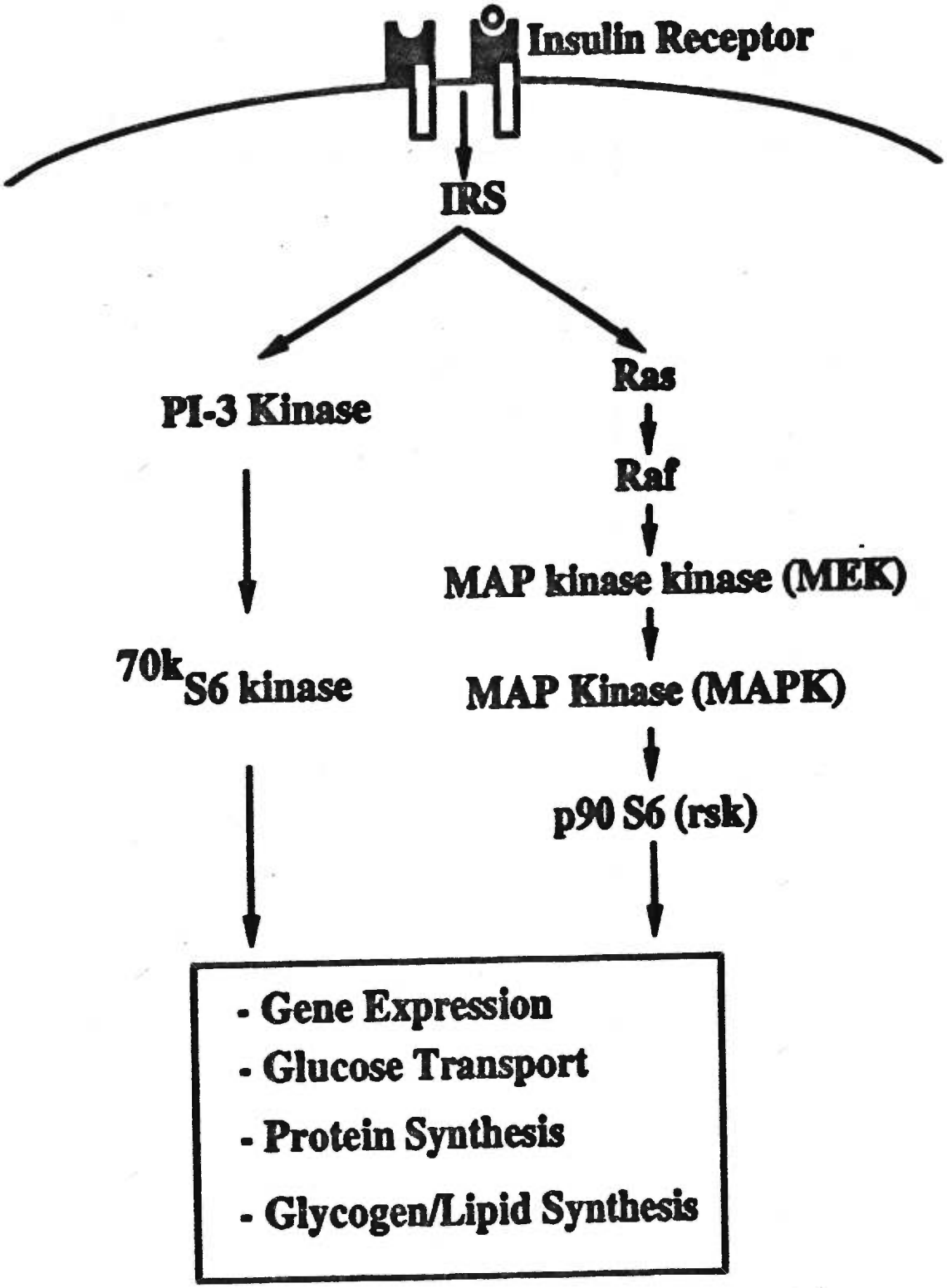
To elucidate the role of PKC in the insulin effect on the expression of the hANG gene promoter, we did a pretreatment study with PMA. Prolonged pretreatment with a high concentration of PMA could down-regulate PKC (Ko KH. et al., 1997). Our studies show that the overnight incubation of OKW1 cells with a high concentration of PMA (10^{-5} M) did not abolish the inhibitory effect of insulin on the expression of pOGH(hANG N-1064/+27) in OKW1 cells, but completely abolished the stimulatory effect of PMA (10^{-7} M) (Figure III.21). Furthermore, we studied PKC enzymatic activity and after 1 hour of insulin treatment, the ratio of membrane/cytosol PKC activity did not change in the presence of high levels of glucose (Figure III.16). These results show that insulin had no effect on the PKC enzymatic activity in the cell.

The above results on the insulin effect indicate that: 1) The inhibitory effect of insulin in the expression of the hANG gene is not through the inhibition of PKC enzymatic activity and it may be mediated downstream from of the PKC signal transduction pathway; 2) another signal transduction pathway is probably involved in the insulin effect on the expression of hANG gene promoter in a high glucose medium.

IV.7.2. Role of the PI-3 Kinase Pathway and MAP Kinase Pathway

Two pathways are well known to be involved in insulin action. One is designated the MAPK pathway . The other is through the activation of PI-3 kinase which is inhibited by the PI-3 kinase inhibitor, Wortmannin (Denton RM. and Tavaré JM., 1997) as shown in Figure IV.2.

Figure IV.2. Molecular Mechanisms of Insulin Effect



IV.7.2.1. PI-3 Kinase Pathway

PI 3-kinase is one of the key enzymes activated in a signaling pathway of growth factors. Activation of PI 3-kinase appears to be a critical upstream step for insulin stimulation of Glut4 glucose transporter translocation and glucose uptake. (Kahn CR., 1997). Insulin stimulates the guanine nucleotide exchange on Rab4 via a PI-3 kinase dependent signaling pathway and that Rab4 is one of the possible targets of insulin action on intracellular vesicle traffic in rat adipocytes (Shibata H. et al., 1997).

Wortmannin, is a direct and specific inhibitor of PI-3 kinase, partially purified from guinea pig neutrophils and rat liver as well as the lipid kinase activity of the anti-p85 immunoprecipitates from these cell lysates (Okada T. et al, 1994). Wortmannin is such a selective inhibitor of PI-3 kinase that it does not inhibit other kinases including cyclic nucleotide-dependent protein kinases, calmodulin-dependent protein kinases II, PKC and MAPK (Okada T. et al., 1994a; 1994b). The concentration required for the half-maximal inhibition was as low as 10 nM (10^{-8} M) or less (Okada T. et al., 1994a; 1994b). The activity of PI-3 kinase was completely inhibited by Wortmannin at 100 nM (10^{-7} M) (Nakamura I. et al., 1995).

Our results show that PI-3 kinase inhibitor, Wortmannin (10^{-8} to 10^{-5} M) has no effect on the expression of hANG gene in low glucose and high glucose medium (Figure III.25, III.26), wortmannin did not affect the inhibitory effect of insulin on the hANG gene expression in a high glucose medium (Figure III.27). This result indicates that PI-3 kinase pathway is not involved in the insulin effect on the hANG gene expression in OKW1 cells.

IV.7.2.2. MAP Kinase (MAPK) Pathway

Studies have demonstrated that insulin stimulation of the insulin receptor tyrosine kinase results in Ras activation and subsequently downstream stimulation of the Raf/MEK/ERK pathway

(Vries-Smits et al. AMM., 1992; Wood KW. et al., 1992; Thomas SM. et al., 1992).

To study the role of the MAP kinase pathway in the insulin effect, PD98059 was used. Studies have shown that PD98059 inhibits the MAP kinase pathway by blocking the activity of MAP kinase kinase (MEK) (Dudley DT. et al., 1995; Pang L. et al., 1995). PD98059 inhibits the phosphorylation and the activation of MAPK substrates both in vitro and in intact cells (Dudley DT. et al., 1995). Half-maximal inhibition was observed at 2 μM PD98059 ($2x10^{-6}$M), with maximal effects at 10-100 μM (i.e. 10^{-5} to 10^{-4} M) (Pang L. et al., 1995). Pretreatment of PC-12 cells with PD98059 completely blocks the 4-fold increase in MAP kinase activity produced by nerve growth factor (Pang L. et al., 1995; Waters SB. et al., 1995).

Our results showed that in low glucose medium, PD98059 alone have no effect on the expression of pOGH(hANG N-1064/+27) in OKW1 cells (Figure III.22). In high glucose, PD98059 at 10^{-5} to 10^{-4} M significantly enhanced the expression of the fusion gene in OKW1 cells (Figure III.23). These studies suggested that the inhibition of the MEK pathway may enhanced the expression of the fusion gene.

Furthermore, the inhibitory effect of insulin on the expression of the fusion gene in OKW1 cells was blocked in a dose-dependent manner by PD98059 (Figure III.24). This result strongly supports the notion that the MAP kinase pathway is involved in the inhibitory effect of insulin on the expression of the ANG gene in OK cells.

The molecular mechanism of the insulin mediated MAPK pathway is not clear. Recent studies of Alblas J. et al (1998) show that in human breast cancer cells, insulin induces transient activation of Erk-2 (one kind of MAPK) which can be blocked by PD98059. Insulin also stimulates prolonged induction of immediate-early gene c-jun, resulting in c-jun expression during the entire G1 period. De Fea K. and Roth RA. (1997) reported that MAPK is capable of phosphorylating and regulating insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation in human embryonic

kidney cells. p38 is a member of the MAP kinase superfamily activated by stress signals and implicated in cellular processes involving inflammation and apoptosis and insulin promotes cell survival by inhibiting the p38-mediated apoptotic pathway (Kummer JL. et al., 1997). Insulin stimulates the serine phosphorylation of the adapter molecule, 66-kDa Shc, which couples receptors to MAPK kinase signaling pathways (Kao AW. et al., 1997).

IV.8. Crosstalk Between the PKC and MAP Kinase Pathways

Intracellular crosstalk between different signaling systems is one of the mechanisms by which cells can integrate multiple hormonal signals for survival and growth. Recent studies have demonstrated that typical G protein-coupled receptors may activate or inhibit the signaling systems classically employed by tyrosine kinase coupled receptors, such as MAPK, PI-3 kinase, Src, FAK, and JAK2 (Schorb W. et al., 1994; Stoyanov B. et al., 1995; Wan Y. et al., 1996).

The integration of the PKC signal into the MAPK cascade is particularly important because it links phospholipid-derived signals to the activation of this kinase pathway (Tolan D. et al., 1997). Many growth factors and G protein-coupled receptor agonists activate phospholipases and phospholipid kinases. Receptors coupled to G protein activate PLC-β, whereas receptor tyrosyl kinases phosphorylate PLC-γ (Cockcroft S. and Thomas GMH., 1992). Subsequent hydrolysis of PIP2 produces DAG, which activates PKC, and IP3 which triggers intracellular calcium mobilization. Growth factors and G-protein-linked receptor agonists can also stimulate the hydrolysis of phosphatidylcholine via both the PLC and PLD routes to provoke the formation of DAG and PtdOH (Morris AJ. et al., 1996).

It is almost certain that stimulation of G-protein-coupled receptors also activates MAPK via the PKC signaling pathway (Lang-Carter CA. et al., 1993; Blumer KJ. and Johnson GL., 1994). The α-isoform has been proposed to phosphorylate and activate Raf-1(Kolch W. et al., 1993), which in turn activates MAPK through a protein kinase cascade. However, the detailed PKC

signaling pathway or network leading to the MAPK activation has not been established unequivocally (MacDonald SG. et al., 1993). It has been proposed that, in T- and B- lymphocytes, PKC links with MAPK at the point of Ras activation. It has also been shown that in COS-7 cells the βγ-dimer of Gi-protein is capable of activating MAP-kinases in a Ras-dependent manner (Crespo P. et al., 1994; Faure M. et al., 1994), but the mechanism of this activation is unknown.

Crosstalk between the PKC and MAPK cascades may occur by different mechanisms, depending on the signaling machinery present in the given cell. Some stimuli show PKC-independent Erk activation, while others show both PKC-dependent and PKC-independent mechanisms in the same cell (Crews CM. et al., 1992). For example in rat fibroblasts, epidermal growth factor (EGF) activates Erk2 by p21ras, whereas in Swiss 3T3 cells, EGF activates Erk2 by both p21ras and PKC. Also, different stimuli can activate MAPK via different pathways within a single cell type. Generally, PKC appears to provide an alternative route of MAPK activation to that utilized by tyrosine kinases, and the two pathways may be subject to independent regulation (Markquardt B. et al., 1994).

Our studies show that a high glucose levels exerts its effect through activation of the PKC and polyol pathway . Insulin can inhibit the stimulatory effect of high glucose on the expression of human ANG gene via the MAPK kinase pathway (Figure IV.3). The exact molecular mechanism of interaction between glucose and insulin is not yet clear, although the crosstalk between PKC and MAPK pathways may be one of the possibilities.

IV.9. OK13

OK13 is a cell line that has been stably transfected with a fusion gene: pTKGH containing the promoter/enhancer DNA sequence of the viral thymidine kinase gene fused with human GH as a reporter.

OK13 cells were used as control cells to examine the effect of glucose, insulin, Tolrestat, staurosporine, H-7 and PMA on the expression of pTKGH. In contrast to the effects of these agents on the promoter activity of the hANG gene, we did not observe any significant stimulation of expression of pTKGH by 25 mM glucose or PMA nor inhibition by insulin, Tolrestat, staurosporine or H-7 (Figure III.28).

These data demonstrate that the promoter/enhancer DNA sequence of the TK gene or the hGH reporter gene is not responsive to the addition of insulin or various drugs. On the other hand, our studies demonstrate that the effect of a high glucose level and insulin on the expression of the human ANG gene promoter activity OKW1 and OKW3 cells is gene specific.

IV.10. Effect of Ang II on the Expression of hANG Gene

In vitro studies on cultured proximal tubular as well as mesangial cells have shown striking similarities between the effects of high glucose and Ang II on the growth properties and the induction of cytokines. High glucose, as well as Ang II stimulate hypertrophy of proximal tubular cells. This hypertrophy is depending on the autocrine induction of TGF-β gene expression (Wolf G. and Neilson G., 1993; Wolf G. et al., 1995). High glucose and Ang II both have been reported to inhibit proximal tubular proteinases (Olbricht CJ. and Geissinger B., 1992; Schaefer L. et al., 1994). High glucose or Ang II induces c-fos and c-jun also have been observed in glomeruli early after induction of diabetes in rats (Kreisberg JI. et al., 1994).

High glucose and Ang II may use similar or parallel signal transduction pathways in cultured renal cells. Activation of PKC in renal cells is a common feature in diabetes, and high glucose alone may be responsible for this induction, presumably because of stimultion of de novo synthesis of DAG (Ayo SH. et al., 1991; DeRubertis FR. and Vraven PA., 1994; Kreisberg JI. and Kreisberg SH., 1995; Cole JA. et al., 1995). Ang II typically activates PKC in mesangial cells and proximal tubular cells through AT_1 receptors (Liu FY. and Cigan MG., 1990).

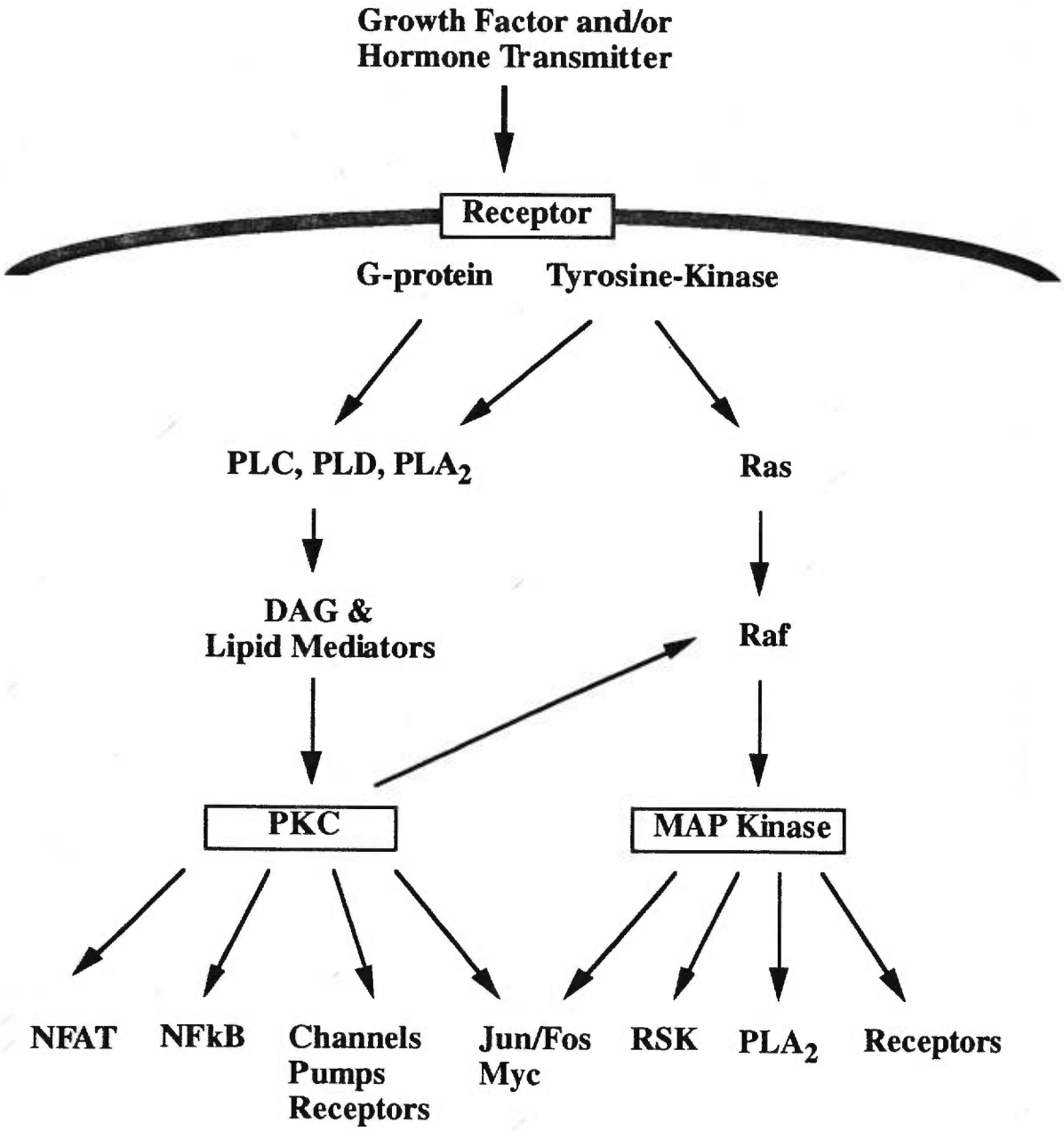


Figure IV.3. Crosstalk Between PKC and MAPK Signal Transduction Pathways.

Our present studies showed that Ang II (10^{-11} to 10^{-5} M) stimulates the expression of the hANG gene in a dose-dependent manner in the presence of high levels (i.e. 25 mM) of glucose (Figure II.30) but not in the presence of 5 mM glucose, except at 10^{-5} M (Figure II.29). The addition of insulin (10^{-11} to 10^{-5} M) blocked the stimulatory effect of Ang II on the expression of pOGH(hANG N-1064/+27) in OKW1 cells (Figure III.31). These results indicate that Ang II may act additively or synergistically with high levels of glucose to stimulate the expression of the ANG gene and the addition of insulin may attenuate this stimulatory effect. The molecular mechanism(s) of action of the insulin may be mediated at least in part, via blocking the PKC signal transduction pathway stimulated by high levels of glucose and Ang II. Hence, insulin may be useful to attenuate or inhibit the development of nephropathy in diabetes by blocking the stimulatory effect of high levels of glucose and Ang II on the expression of renal ANG gene in vivo.

IV.11. Clinical Significance of the Present Studies

The relationship between experimental and clinical diabetes and the RAS is unclear and the available reports vary widely.

RAS plays an important role in DN. In conjunction with other vasoactive systems, the RAS may contribute to the imbalance of resistance present at the preglomerular and postglomerular sites which are responsible for glomerular capillary hypertension, a major injurious factor in the diabetic kidney (Correa-Rotter R. et al., 1992).Clinical studies have shown that ACE inhibitors and Ang II receptor antagonists decrease proteinuria and slow the progression of DN by mechanisms that can not be solely attributed to effective control of systemic hypertension (Lewis EJ. et al., 1993; Remuzzi A. et al., 1993; Gandhi SK. et al., 1996). Studies of Anderson S. and Brenner BM. (1986a) showed that ACE inhibition corrects the glomerular hemodynamic changes observed in diabetes and may arrest the progression of glomerular damage and renal injury. The Ang II receptor antagonist, losartan, decreases proteinuria and slows the progression of DN (Gandhi SK. et al.,

1996). ACE gene polymorphisms may play some role in DN (Marre M. et al., 1994; Schmidt S. et al., 1995). Thus, it has been suggested that the functional and morphologic changes observed in the diabetic kidney may be, in part, secondary to activation of the intrarenal RAS (Anderson S. and Brenner BM., 1986a).

Our studies show that high levels of glucose stimulated the expression of the human ANG gene in opossum kidney proximal tubular cells, and insulin can inhibit this stimulatory effect of high levels of glucose on the human ANG gene expression. The clinical significance of the present study suggests that: Firstly, in diabetes, the elevated glucose levels may stimulate the expression of the ANG gene expression in the proximal tubule via the polyol pathway and the activation of PKC signal transduction pathway; Secondly, insulin is well-known as the glucose lowering hormone that is widely used in the treatment of IDDM. The results of our study indicate that, insulin decreases the expression of ANG gene in the kidney, and thus insulin is an efficient agent in the treatment of DN. Thirdly, the aldose reductase inhibitor might be useful in the treatment of DN involves the inhibition of DAG-sensitive PKC activity. Fourthly, the effect of high levels of glucose on the expression of ANG in proximal tubular cells is through PKC signal transduction pathway, thus a PKC inhibitor may be useful in the treatment of DN. Fifthly, the effect of insulin on the expression of ANG gene in proximal tubular cells is through activation of the MAP kinase pathway and specific activators that might activate the MAP kinase pathway might be useful in the treatment of DN. As mentioned before, PKC and MAPK signal transduction pathways are invoved not only in high glucose effects, but also in many other cellular events. Before put to clinical use, in vivo studies need to be done to clarify the effects of PKC and MAPK related chemicals, and new drugs without side effects need to be developed.

V. Conclusions

We constructed a fusion gene, pOGH(hANG N-10641+27) containing the 5'-flanking regulatory sequence of the human angiotensinogen (ANG) gene as a reporter and stably integrated the fusion gene into the opossum kidney (OK) cell genome.

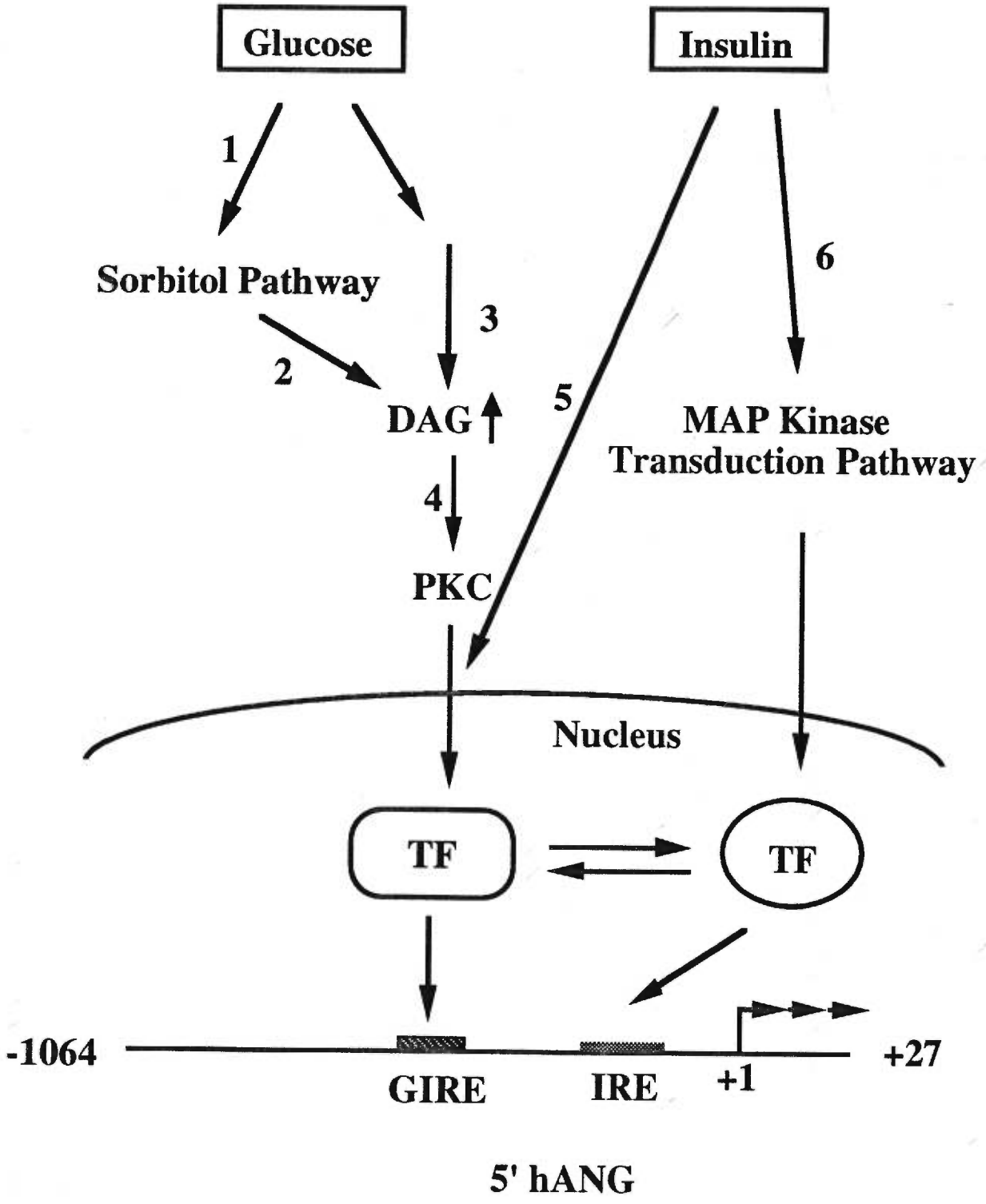
Our studies showed that high levels of D(+)-glucose directly stimulates the expression of the human ANG gene promoter in OK cells. Non-metabolic glucose analogues D-mannitol, L-glucose and 2-deoxy-D-glucose have no such effect. This stimulatory effect of high glucose was blocked by insulin, aldose reductase inhibitor (tolrestat) and PKC inhibitor (staurosporine and H-7). Moreover, high glucose increase the DAG activity and the ratio of membrane/cytosol PKC activity in OK cells. These studies demonstrate that high levels of glucose stimulate the expression of the human ANG gene promoter through the PKC and polyol pathway.

Insulin inhibits the human ANG gene expression in high glucose (25 mM) medium, but not in low glucose (5 mM) medium. Insulin-like growth factor I and II (IGF-I, IGF-II) have no effect in high glucose medium. The inhibitory effect of insulin in high glucose medium was blocked by PKC stimulator (PMA) and MEK inhibitor (PD98059), but not by PI-3 kinase inhibitor (wortmannin). Whereas, pre-incubation with a high concentration of PMA (10^{-5} M) could not abolish the inhibitory effect of insulin on the expression of the human ANG fusion gene in high glucose medium. In addition, insulin has no effect on the high glucose stimulated DAG and PKC enzymatic activity in OK cells. These studies indicate that insulin inhibits the stimulatory effect of high levels of glucose through the MAP kinase pathway and downstream of the PKC signal tranduction pathway.

Up to present, we have demonstrated at least six points in the model of high levels of glucose and insulin effects on the expression of the human ANG gene in OK cells (Figure VI.1).

Our studies suggest that the glucose stimulation of the renal ANG gene may be a very early event in the cascade of biochemical alterations precipitating the loss of renal function in diabetic nephropathy. Our results also provide a biochemical rationale for understanding the beneficial effects of insulin, as well as those of aldose reductase and protein kinase C inhibitors in animal models of diabetic nephropathy.

Figure V.1. Model of High Levels of Glucose and Insulin Effects on the Expression of the Human ANG Gene in OK Cells.



VI. Future Work

1. Investigation of the glucose and insulin effect on ANG mRNA levels by using RT-PCR and northern blot.

1) Rationale: Due to the small amount of ANG mRNA in the OK cells, the ANG mRNA is not detectable by northern blot analysis. Therefore, to study directly the effect of glucose and insulin on the expression of ANG mRNA in OK cells, RT-PCR analysis of the ANG mRNA in OK cells is required.

2) Methods: Multiple oligonucleotides corresponding to the different fragments of the ANG gene cDNA will be synthesized, then RT-PCR could be employed to detect the effect of glucose and insulin on the level of ANG mRNA.

3) Expected Results: These studies will demonstrate whether high levels of glucose might stimulate the ANG mRNA in OK cells. In addition, whether insulin will inhibit the stimulatory effect of high glucose on the expression of ANG mRNA levels in OK cells.

2. Identify the putative glucose-responsive element (GIRE) and insulin-responsive element (IRE) in the human ANG gene promoter region.

1) Rationale: GIRE and IRE have been identified in multiple genes. We speculate that the effect of high glucose and insulin on the expression of the 5' hANG gene promoter might be mediated via the GIRE and/or IRE in the 5' hANG gene promoter region.

2) Methods: Firstly, several new constructs should be made which contain different lengths of the 5'-flanking region of the human ANG gene promoter fused with hGH or CAT gene as reporter gene, and then test the effect of glucose and insulin on the expression of these new fusion genes in OK cells.

3) Expected Results: These studies will demonstrate whether GIRE and IRE are present in the 5' hANG gene promoter region.

3. Identify the PKC isoform that are involved in the glucose effect on the human ANG gene

expression.

1) Rationale: High levels of glucose can activate the DAG-sensitive PKC isoform in OKW1 cells. At least 6 PKC isoforms which are DAG-sensitive have been found.

2) Methods: Antibodies specific toward each isoform of PKC should be employed to detect the presence or absence of these PKC isoform(s) in the cytosol and membrane fractions of OKW1 by immunoblot. Prior to immunoblotting, the OK cells should be incubated in 5 mM glucose, 25 mM glucose, 25 mM glucose plus Tolrestat or insulin.

3) Expected Results: These studies will demonstrate the specific PKC isoform that can be stimulated by high levels of glucose, and whether the addition of Tolrestat and insulin will inhibit the stimulatory effect of glucose.

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Appendix I: Publications

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September 23, 1998

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Saulo Klahr, M.D.
For the Editors

SK/pm

Title: Insulin inhibits the stimulatory effect of glucose on the expression of the angiotensinogen gene in opossum kidney cells

Identification No.: 98-0849

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INSULIN INHIBITS THE STIMULATORY EFFECT OF GLUCOSE ON THE EXPRESSION OF THE ANGIOTENSINOGEN GENE IN OPOSSUM KIDNEY CELLS

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Running Title: Insulin, glucose, angiotensinogen gene and OK cells
Key Words: Renin-angiotensin system, insulin, kidney

ABSTRACT

Background. Angiotensinogen gene expression is stimulated when kidney proximal tubular cells are exposed to high concentrations of glucose, suggesting that high levels of glucose may activate the local intra-renal renin-angiotensin system (RAS) and may play an important rôle in the development of diabetic nephropathy. Intensive insulin therapy has been shown to delay the onset and the progression of the diabetic nephropathy. We hypothesized that insulin could inhibit the stimulatory effect of high levels of glucose on the expression of the ANG gene and subsequently inhibits the activation of local intra-renal RAS in the kidney proximal tubular cells.

Methods. We constructed a fusion gene, pOGH (hANG N-1064/+27) containing the 5'-flanking regulatory sequence of the human ANG gene as a reporter and stably integrated the fusion gene into the opossum kidney (OK) cell genome. The level of expression of the fusion gene was quantified by measuring the amount of immunoreactive human growth hormone (IR-hGH) secreted into the medium.

Results. Exposing the transfected cells to a high level of glucose (25 mM) or phorbol 12-myristate 13-acetate (PMA) stimulated the expression of the fusion gene. The stimulatory effect of glucose (25 mM) was blocked by insulin, H-7 (an inhibitor of protein kinase C) and tolrestat (an inhibitor of aldose reductase) but was unaffected by insulin growth factor-I (IGF-I) or IGF-II. Tolrestat also inhibited the increase of cellular diacylglycerol (DAG) and PKC enzymatic activity stimulated by 25 mM glucose. While insulin did not affect the cellular DAG and PKC activity, it did block the stimulatory effect of high glucose (25 mM) and PMA on the expression of the fusion gene. Finally, PD 98059 (an inhibitor of MAP kinase kinase (MEK)) enhanced the stimulatory effect of

high levels of glucose and blocked the inhibitory effect of insulin on the expression of the fusion gene, whereas Wortmannin (an inhibitor of phosphatidylinositol-3-kinase) had no effect.

Conclusion. These studies demonstrate that the action of insulin, blocking the stimulatory effect of a high level of D(+)-glucose (25 mM) on hANG gene promoter activity, appears to be mediated via the MAP kinase kinase (MEK) signal transduction pathway.

INTRODUCTION

Nephropathy is one of the most serious long-term complications found in diabetic patients. It is estimated that approximatly 30-50% of the diabetic patients will eventually develop chronic renal failure or end stage renal failure (ESRF) (1-3).

While hyperglycemia has been implicated in the etiology of diabetic nephropathy (4), the molecular mechanisms for the development of nephropathy in diabetes, however, are not completely understood. In vivo studies have shown that angiotensin-converting enzyme (ACE) inhibitors and angiotensin-II receptor antagonists decrease proteinuria and slow the progression of nephropathy in diabetic patients, indicating that angiotensin II (Ang II) plays an important role in the development of the nephropathy (5-12).

In animal studies, aldose reductase (AR) and protein kinase C (PKC) activities (13-14) have also been implicated in the etiology of this disease. The relationship between hyperglycemia, Ang II, AR and PKC activities, however, have not yet been defined. To gain insight into this relationship we have characterized the effects of glucose and insulin as well as AR and PKC inhibition on the expression of human ANG gene promoter transfected into immortalized opossum kidney (OK) proximal tubular cells.

Our results show that the expression of the human Ang gene promoter in OK cells was stimulated by a high concentration of glucose (25 mM) and by PMA (phorbol 12-myristate 13-acetate, an activator of PKC). The stimulatory effect of glucose was blocked by insulin, staurosporine (an inhibitor of protein kinase C), H-7 (an inhibitor of protein kinase C) and tolrestat (an inhibitor of aldose reductase (15)). Tolrestat also

blocked the increase of cellular diacylglycerol (DAG) levels and PKC enzymatic activity stimulated by the high level of glucose (25 mM), while insulin had no effect on these parameters. The stimulatory effect of PMA was blocked by insulin. The addition of PD98059 (an inhibitor of MAP kinase kinase (MEK) (16)), enhanced the stimulatory effect of high levels of glucose on the expression of the human ANG gene promoter activity in OK cells and blocked the inhibitory effect of insulin. The addition of Wortmannin (an inhibitor of phosphatidylinositol(PI)-3 kinase (17)) had no effect.

MATERIALS AND METHODS

The plasmid, pRSV-Neo, containing the coding sequence for Neomycin (Neo) with the Rous Sarcoma Virus (RSV) enhancer/promoter sequence fused in the 5'-end of the Neomycin gene was a gift from Dr. Teresa Wang (Dept. of Pathology, Stanford University, Stanford, CA, USA). The plasmid pOGH was purchased from the Nichols Institute of Diagnostics (La Jolla, CA, USA). The pOGH had been modified with the SP6-promoter sequence inserted upstream of the polylinker sites as reported previously (18).

Insulin, staurosporine (an inhibitor of protein kinase C), phorbol 12-myristate 13-acetate (PMA) (an activator of PKC), D(+)-glucose, L-glucose, D-mannitol and 2-deoxy-D-glucose were purchased from Sigma Chemicals (St-Louis, MO, USA). H-7 was purchased from Research Biochemicals Inc. (RBI, Natick, MA, USA). Tolrestat was a gift from Wyeth-Ayerst Research (Princeton, NJ, 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).

Restriction and modifying enzymes were purchased from either Bethesda Research Laboratories (Life Technologies Inc., Burlington, Ontario, Canada), Boehringer-Mannheim (Dorval, Quebec, Canada) or Pharmacia Inc. (Baie d'Urfe, Quebec, Canada). $Na^{125}I$ were purchased from Dupont, New England Nuclear (NEN, Boston, MA, USA).

Radioimmunoassay for human growth hormone (hGH)

The radioimmunoassay kit for hGH (RIA-hGH) was a gift from NIADDK, NIH, USA. The assay procedure has been described previously (19). 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 inter- and intra-assay coefficients of variation were12% (n=10) and 10% (n=10), respectively.

Assays for cellular DAG and PKC enzymatic activity

The DAG levels and PKC activity were quantified with kits (RPN 200 and RPB 77) purchased from Amersham Life Science (Oakville, Ontario, Canada) following the manufacturer's instructions.

Prior to the PKC activity measurement, the OK W1 cells were separated into cytosolic and membrane fractions. Briefly, the cells were harvested in 3 ml of 20 mM Tris HCl, pH 7.5 containing 2 mM EDTA, 0.5 mM EGTA, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 1 mM DTT, 25 µg/ml Leupeptin, 1 µg/ml Pepstatin A and 330 mM

sucrose and kept on ice. Cells were then homogenized with a Dounce-bound glass homogenizer (Type B) and centrifuged at 100,000 X g for 30 minutes at 4°C. The supernatants (cytosolic fraction) were recovered and stored on ice until assayed. The pellets were resuspended in 3 ml of 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, 1 mM DTT, 25 μg/ml Leupeptin, 1 μg/ml Pepstatin A and 0.2% Triton X-100 and incubated on ice for 30 min. Following centrifugation at 100,000 x g for 30 min., the supernatant fractions were collected as the membrane fraction and stored on ice until assayed. The effects of treatments on PKC activity in OK W1 cells were expressed as changes in the ratio of PKC enzymatic activity in the cytosolic and membrane fractions (20).

Cloning of the human angiotensinogen (hANG) gene promoter

The 5'-flanking region of the hANG gene was cloned by the polymerase chain reaction (PCR) from a human liver genomic library (Clontech, La Jolla, CA, USA). The forward primer: 5' GTC AGT GAA TGT ACA GCT TCT GCC 3' and the reversed primer: 5' TAG TAC CCA GAA CAA CGG CAG CTT 3' corresponding to the nucleotide sequences of N-1064 to N-1041 and N-2 to N+27 of the hANG gene (21) were used in PCR, respectively.

The DNA fragment hANG N-1064 to N+27 obtained by PCR was initially cloned in the plasmid, pCRII-1 (In Vitrogen, La Jolla, CA) and subsequently subcloned in the modified expression vector, pOGH (18).

The sequence and orientation of 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 (22,23). We (24), as well as Ingelfinger et al (25) had previously reported that the OK cells express a low level of ANG mRNA. The culture conditions of the OK cells have been described previously (15,24,26,27).

OK cell stable transformants

OK W1 cells are stable transformants with the pOGH (hANG N-1064+27) fusion gene integrated into the OK cellular genome. The method for the selection of OK cell stable transformants with the high expression of the fusion gene was similar to the method described previously for OK 27 cells and OK 13 cells with pOGH (rANG N-1498/+18) and pTKGH stably integrated into the cellular genomes, respectively, (18).

Effect of D(+)-glucose on the expression of the fusion gene

OK W1 cells were plated at a density of 1-2 X 10^5 cells/well in 6-well plates and incubated overnight in DMEM containing 5 mM D(+)-glucose and 10% FBS. To arrest growth, cells were incubated in serum-free and a low glucose concentration (5 mM) medium for 24 hours. Subsequently, cells were incubated for 24 hours in media with

glucose concentrations ranging between 5 to 25 mM and 1% fetal bovine serum (FBS) depleted of endogenous steroid and thyroid hormones (28). Media tonicity was adjusted for the changes in glucose concentration by the addition of D-mannitol. 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 the effect of D(+)-glucose, 5 or 25 mM of L-glucose, D-mannitol or 2-deoxy-D-glucose were added to the culture medium of growth arrested cells and the cells were incubated for 24 hours. The media were then collected and stored at -20°C until assayed for IR-hGH.

Effect of insulin on the expression of the fusion gene

To study the inhibitory effect of insulin on the expression of the fusion gene in OK W1 cells, OK W1 cells were incubated for 24 hours in media with 25 mM glucose and insulin concentrations ranging between 10^{-11} to 10^{-5} M. At the end of the incubation period, media were collected and stored at -20°C until assayed for IR-hGH.

To verify the specificity of the effect of insulin, IGF-I or IGF-II at concentrations ranging between 0.1 to 100 ng/ml or 10^{-11} to 10^{-8} M (final concentration) were added to the culture medium and the cells were incubated for 24 hours. The media were then collected and stored at -20°C until assayed for IR-hGH.

Effect of tolrestat, staurosporine, H-7 and PMA on the expression of the fusion gene

To study the effect of tolrestat, staurosporine, H-7 or PMA on the expression of the fusion gene in OK W1 cells, cell cultures were incubated for 24 hours in media with

25 mM glucose and various concentrations of tolrestat (10^{-7} to 10^{-4} M) , staurosporine (10^{-13} to 10^{-7} M), H-7 (10^{-11} to 10^{-5} M) or PMA (10^{-11} to 10^{-5} M). At the end of the incubation period, media and cells were collected and stored at -20°C until assayed for IR-hGH.

Antagonistic effects of insulin and PMA on the expression of the fusion gene

To investigate the antagonistic effect of insulin and PMA on the expression of the fusion gene in OK W1 cells, PMA at concentrations ranging between 10^{-11} to 10^{-5} M was added along with insulin (10^{-7} M) to media containing 25 mM glucose and the cells were incubated for 24 hours. These experiments were repeated using a fixed concentration of PMA (10^{-7} M) and concentrations of insulin ranging between 10^{-11} to 10^{-5} M. At the end of the incubation period, media were collected and stored at -20°C until assayed for IR-hGH.

Effect of PD98059 and Wortmannin on the expression of the fusion gene

To investigate whether MAP kinase pathway might be involved in mediating the effect of high levels of glucose on the expression of the fusion gene in OK W1 cells, cells were incubated for 24 hours with 5 mM glucose, 25 mM glucose, 25 mM glucose in the absence or presence of PD 98059 (10^{-7} to 10^{-4} M). Similarly, to study the signal transduction pathway of insulin involved in the expression of the fusion gene in OK W1 cells, confluent cultures were incubated for 24 hours in media with 25 mM glucose, 10^{-7} M insulin and concentrations of PD98059 ranging between $10^{-7}$to 10^{-4} M, or

Wortmannin ranging from 10^{-9} to 10^{-5} M. At the end of the incubation period, media and cells were collected and stored at -20°C until assayed for IR-hGH.

Effect of glucose, insulin, tolrestat, H-7 or PMA on the expression of pTKGH in OK 13 cells

The OK 13 cell is a cell line that has been stably transfected with a fusion gene containing the promoter/enhancer DNA sequence of the viral thymidine kinase gene fused with the hGH gene as reporter (18). These cells were plated at a density of 1-2 X 10^5 cells/well in 6-well plates and incubated overnight in DMEM containing 10% FBS. Cell growth was then arrested by incubation in serum-free medium with a low glucose concentration (5 mM) for 24 hours. Subsequently, the cells were incubated in media containing 25 mM glucose and 1% depleted FBS with either insulin (10^{-7} M), Tolrestat (10^{-4} M), H-7 (10^{-7} M) or PMA (10^{-7} M) for 24 hours. At the end of the incubation period, media and cells were collected and kept at -20°C until assayed for IR-hGH.

Statistical analysis

The treatment groups were assayed in triplicate (N=3) and the experiments were repeated at least three times. The data were analyzed with Student's *t* test or an analysis of variance (Anova). A probability level of $p< 0.05$ was regarded as statistically significant.

RESULTS

Effect of D(+)-glucose and insulin on the expression of human Ang gene promoter activity

The expression of the fusion gene, pOGH (hANG N-1064/+18) in OK W1 cells increased in a dose-dependent manner with the concentrations of D(+)glucose in the

culture medium (Figure 1A). In contrast, the addition of 25 mM of D-mannitol, L-glucose or 2-deoxy-D-glucose had no significant effect on the expression of the fusion gene (Figure 1B). The addition of insulin to the culture media abolished the glucose (25 mM) stimulation of promoter expression (Figure 2A). The effective dose of insulin, i.e.10^{-7}M, was routinely used in all of the subsequent experiments. In contrast to insulin, IGF-I or IGF-II at concentrations ranging from 0.1 to 100 ng/ml had no significant effect on the glucose stimulation of promoter expression (Figure 2B).

The glucose stimulation of promoter expression was also inhibited by tolrestat (Figure 3), H-7 (Figure 4A) and staurosporine (Figure 4B). These inhibitors, at the highest concentrations tested, had no effect on cell viability as assessed by dye exclusion (data not shown).

Cellular levels of DAG and PKC enzymatic activity in OK W1 cells

Culture of OK W1 cells in 25 mM gluocse increased DAG levels (Figure 5A) and PKC activity (Figure 5B) by 2- and 1.75-fold, respectively. The addition of tolrestat (10^{-4} M) prevented these increases, while insulin (10^{-5} M) had no inhibitory effect.

Antagonistic effect of insulin and PMA on the expression of the fusion gene

PMA at concentrations of 10^{-11} to 10^{-5} M further stimulated the expression of the fusion gene in OK W1 cells incubated in media with 25 mM glucose (Figure 6). At the maximal stimulatory effective dose, 10^{-7} M, the expression of the fusion gene was increased about 2-fold above the expression level in cells treated with 5 mM glucose.

In experiments where the concentration of insulin was held constant at 10^{-7} M and increasing concentrations of PMA were tested, the inhibitory effect of insulin on the promoter expression was blocked (Figure 7A). On the other hand, the stimulation of promoter expression in OK W1 cells by 25 mM glucose and 10^{-7} M PMA was reversed by insulin treatment (Figure 7B). The loss of this stimulatory effect was proportional to the concentration of insulin. At the highest concentration of insulin tested, 10^{-5} M, promoter expression in OK W1 cells was about 1.2-fold greater than that of cells incubated in media with 5 mM glucose (not statistically significant) (Figure 7B).

Figure 8 shows that while the overnight incubation of OK W1 cells in 25 mM glucose with a high concentration of PMA (10^{-5} M) did not block the inhibitory effect of insulin on the expression of the fusion gene, but the stimulatory effect of PMA (10^{-7} M) was completely abolished.

Effect of PD98059 or Wortmannin on the expression of the fusion gene

Figure 9 shows that the addition of PD 98059 alone at 10^{-5} to 10^{-4} M significantly enhanced the expression of the fusion gene in OK W1 cells when incubated in 25 mM glucose medium. Furthermore, the inhibitory effect of insulin on the expression of the fusion gene in OK W1 cells was blocked in a dose-dependent manner by PD98059 (Figure 10A) but was unaffected by Wortmannin tested at concentrations ranging between 10^{-8} to 10^{-4} M (Figure 10B). These studies demonstrate that the effect of insulin to attenuate the stimulatory effect of a high level of glucose on the expression of fusion gene in OK W1 cells is probably mediated via the activation of MEK activity, but not via phosphatidylinositol-3 kinase activity.

Effect of D(+)-glucose, insulin, tolrestat, staurosporine, H-7 and PMA on the expression of pTKGH in OK 13 cells

Figure 11 shows that the addition of D(+)-glucose (25 mM) in the absence or presence of insulin (10^{-7} M), tolrestat (10^{-4} M), H-7 (10^{-7}M) or PMA (10^{-7} M) had no effect on the expression of pTKGH in OK 13 cells compared to the control (i.e. 5 mM glucose).

DISCUSSION

In vitro studies with renal proximal tubular cells have demonstrated that exposure to high levels of glucose in the medium (i.e. > 25 mM) or Ang II (i.e. > 10^{-8} M) causes cellular hypertrophy and increases the expression of transforming factor-β1 (TGFβ1),collagen type I and type IV (29-35). These studies indicate that the elevated glucose and/or Ang II concentrations may directly or indirectly be responsible for the development of diabetic nephropathy. However, the molecular mechanism(s) of glucose action is not well defined. Recent studies have shown that inhibition of aldose reductase prevents the glucose-induced increase in contractility, TGFβ- and PKC activity in mesangial cells (36-37), suggesting that this glucose effect is mediated, at least in part, via the polyol pathway.

While several laboratories have described the expression of renal RAS genes in experimental diabetes mellitus (38-40) their results are inconsistent. Correra-Rother et al (38) have reported that the renal renin protein and mRNA expression did not differ in diabetic and normal animals, but renal and liver ANG mRNA levels were lower in diabetic animals. Kalinyak et al (39) found no significant differences in the expression of renal renin or ANG mRNA. In contrast, Anderson et al (40) reported small increases

in both renal renin and ANG gene expression. While there is no clear rationalization for these conflicting results, one obvious difference among these studies is the duration of the diabetes.

Conflicting results have also been reported for the effect of high glucose levels on the expression of the RAS gene in renal proximal tubular cells *in vitro*. Ingelfinger et al (25) reported that an exposure to a high level of glucose down-regulates the expression of the RAS genes in opossum kidney cells. In contrast, our recent studies have shown that a high level of glucose (25 mM) stimulates the expression of the rat ANG gene promoter activity in OK 27 cells (38). We have also reported that this effect of high glucose concentrations is blocked by H-7 or staurosporine but not by Rp-cAMP (an inhibitor of protein kinase I and II) (38). The reasons for the discrepancy between our studies (38) and the studies of Ingelfinger et al (25) are not known. One possible explanation might be that the OK cells used in the studies of Ingelfinger et al (25) were heterogeneous in population (i.e. obtained from ATCC) whereas we used subclones of the OK cells. The second possibility is that OK cells express very low levels of ANG mRNA (24,25). Therefore, it may be very difficult to accurately quantify the changes in ANG mRNA levels with Northern blot analysis. In order to increase the sensitivity to detect changes in ANG gene expression, we coupled the ANG gene promoter to the hGH gene, stably transfected this fusion gene into kidney cells and quantified the ANG gene promoter activity by RIA-hGH. The RIA-hGH is very sensitive (i.e. the limit of detection is 0.1 ng/ml or 10 pg/tube) and highly reproducible (i.e. the interassay coefficient of variation is 12%). Due to this assay sensitivity and reproducibility, the growth hormone gene is rapidly becoming a reporter gene of choice. Indeed,

numerous studies have been reported using the hGH gene as reporter to study the regulation of the expression of a particular gene (42-45).

In the present study, we have shown that the human ANG gene promoter activity was increased by high levels of glucose (Figure 1A). D-mannitol, L-glucose and 2-deoxy-D-glucose did not stimulate the expression of the fusion gene in OK W1 cells (Figure 1B), indicating that the effect of a high level of glucose is probably mediated via the metabolic products of D(+)-glucose.

Studies by Chang and Perlman (46) have shown that insulin attenuates the expression of the ANG mRNA in rat hepatoma cells *in vitro*. Consistent with these results, we have also observed that insulin inhibited the stimulatory effect of glucose on the expression of the human ANG gene activity in a dose-dependent manner (Figure 2A). These results together with those of Chang and Perlman (46) suggest that insulin may regulate the ANG gene expression. We did not observe any significant inhibition of the expression of hANG gene promoter in OK W1 cells treated with various concentrations of IGF-I or IGF-II (Figure 2B). These results suggest that the inhibitory effect of insulin on the expression of human ANG gene promoter activity in OK cells is specific for insulin and the insulin receptor.

Tolrestat blocked the stimulatory effect of 25 mM glucose on the expression of human ANG gene promoter in a dose-dependent manner (Figure 3). These observations suggest that the stimulatory effect of high levels of glucose is mediated, at least in part, via the polyol pathway as previously suggested by Tilton et al (47). The mechanisms linking polyol pathway activity to increase hANG promoter activity are not yet clear but may be related to increased synthesis of diacylglycerol (DAG) and

subsequent stimulation of PKC activity, similar to that recently described in mesangial cells by Keogh et al (13) and in proximal tubules by Bleyer et al (48). To further support this hypothesis, our studies showed that tolrestat prevented the increase in DAG levels and PKC activity in OK W1 cells incubated in 25 mM glucose (Figure 5A and 5B). Furthermore, inhibition of PKC activity with H-7 (Figure 4A) or staurosporine (Figure 4B) prevented the glucose stimulation of hANG gene promoter activity, while PMA alone stimulated gene promoter activity (Figure 6).

While we do not completely understand the opposing effects of PMA and insulin on the activity of the hANG gene promoter (Figures 7A and 7B), our observations raise the possibility that the antagonistic effect of these two agents may be mediated via the PKC signal transduction pathway.

Surprisingly, our studies showed that the overnight incubation of OK W1 cells with 10^{-5} M PMA did not abolish the inhibitory effect of insulin on the expression of the fusion gene but did abolish the stimulatory effect of PMA (10^{-7} M) (Figure 8). These studies indicate that the inhibitory effect of insulin on the expression of the ANG gene may be mediated downstream of the PKC signal transduction pathway or other signal transduction pathways.

We were equally surprised that the addition of high levels of PD 98059 (10^{-5} to 10^{-4}) enhanced the stimulatory effect of 25 mM glucose (Figure 9) on the expression of the fusion gene in OK W1 cells. These studies suggest that the inhibition of the MEK pathway may enhance the expression of the fusion gene.

Indeed, our results show that the addition of PD98059 blocked the inhibitory effect of insulin in a dose-dependent manner (Figure 10A), whereas the addition of

Wortmannin had no effect (Figure 10B). These studies support the notion that the activation of the MEK pathway is probably involved in the inhibition of the expression of the ANG gene in OK cells. At present, the molecular mechanism(s) of insulin action on the expression of the fusion gene is not known. However, studies are underway in our laboratory to establish the insulin action.

OK 13 is a cell line that has been stably transfected with a fusion gene: pTKGH containing the promoter/enhancer DNA sequence of the viral thymidine kinase gene fused with human growth hormone as a reporter (18). These cells were used as control cells to examine the effect of glucose, insulin, tolrestat, staurosporine, H-7 and PMA on the expression of pTKGH. In contrast to the effects of these agents on the promoter activity of the hANG gene, we did not observe any significant stimulation of expression of pTKGH by 25 mM glucose or PMA nor inhibition by insulin, tolrestat, staurosporine or H-7 (Figure 11). These data demonstrate that the promoter/enhancer DNA sequence of the TK gene or the hGH reporter gene is not responsive to the addtion of insulin or various drugs, and that the effect of a high glucose level and insulin on the expression of the human ANG gene promoter activity in OK W1 and OK W3 cells is gene specific.

In summary, our studies show that exposure of OK W1 cells to 25 mM glucose directly stimulates the expression of the human ANG gene promoter. This stimulatory effect of high glucose was blocked by insulin, tolrestat, staurosporine and H-7. Our studies suggest that the glucose stimulation of the renal ANG gene may be a very early event in the cascade of biochemical alterations precipitating the loss of renal function in diabetic nephropathy. Our results also provide a biochemical rationale for

understanding the beneficial effects of insulin, as well as those of aldose reductase and protein kinase C inhibitors in animal models of diabetic nephropathy.

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LEGENDS TO FIGURES

Figure 1: (A) Effect of D(+)-glucose on the expression of the fusion gene, pOGH ANG N-1064/+27) in OK W1 cells. Cells were incubated for 24 hours in the presence of various concentrations of D(+)-glucose. Media were collected and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the medium containing 5 mM glucose (i.e. 0.4 ± 0.02 ng/ml/10^6 cells represents the control level. Results are expressed as the percentage of control levels (mean ± S.D. with N=3) (**$p \leq 0.01$ and ***$p \leq 0.005$). (B) Effect of D(+)-glucose, D-mannitol, L-glucose and 2-deoxy-D-glucose on the expression of the fusion gene in OK W1 cells. Cells were incubated for 24 hours in the presence of 5 mM or 25 mM glucose, D-mannitol, L-glucose and 2-deoxy-D-glucose. Media were then harvested and assayed for IR-hGH. The concentration of IR-hGH in the medium containing 5 mM glucose is considered as the control level (i.e. 100%). Results are expressed as the percentage of control levels (mean ± S.D., N=3) (***$p \leq 0.005$). Similar results were obtained from three other experiments.

Figure 2: (A) Inhibitory effect of insulin on the expression of the fusion gene in OK W1 cells in the presence of 25 mM glucose. Cells were incubated for 24 hours in the presence of 5 mM, 25 mM glucose, or 25 mM glucose plus various concentrations of insulin. Media were harvested and assayed for the level of IR-hGH. The levels of IR-hGH in the medium containing 5 mM glucose are

expressed as 100% (control). The inhibitory effect of insulin is compared with those cells that were incubated in 25 mM (D(+)-glucose (without the presence of insulin). Results are expressed as the percentage of controls (mean ± S.D. with N=3) (*$p \leq 0.05$ and ***$p \leq 0.005$). (B) Effect of insulin-like growth factor-I or insulin-like growth factor-II on the expression of the fusion gene in OK W1 cells incubated with 25 mM glucose. Media were harvested and assayed for the level of IR-hGH. The levels of IR-hGH in the medium containing 25 mM D(+)-glucose in the absence of IGF-I or IGF-II are expressed as 100% (control). The effect of IGF-I or IGF-II is compared with those cells that were incubated in 25 mM D(+)-glucose (without the presence of IGF-I or IGF-II). Results are expressed as the percentage of control (mean ± S.D., N=3). The solid bar represents cells incubated in the presence of IGF-I and the blank bar represents the cells incubated in the presence of IGF-II. Similar results were obtained from two other experiments.

Figure 3: Inhibitory effect of tolrestat on the expression of fusion gene in OK W1 cells incubated with 25 mM glucose. Cells were incubated for 24 hours with 5 mM glucose, 25 mM glucose or 25 mM glucose plus various concentrations of tolrestat. The levels of IR-hGH in the medium containing 5 mM glucose (i.e. 0.31 ± 0.01 ng/ml/10^6 cells) are expressed as 100% (control). The inhibitory effect of tolrestat is compared with cells that were incubated in 25 mM D(+)-glucose (without tolrestat). Results are expressed

as the percentage of controls (mean ± S.D., N=3) (**p≤ 0.01 and ***p≤ 0.005). Similar results were obtained from three other experiments.

Figure 4: Inhibitory effect of H-7 or staurosporine on the expression of the fusion gene in OK W1 cells incubated with 25 mM glucose. Cells were incubated for 24 hours in the presence of 5 mM glucose, 25 mM glucose or 25 mM glucose plus various concentrations of H-7 (A) or staurosporine (B). Media were harvested and assayed for the level of IR-hGH. Levels of IR-hGH in the medium containing the 5 mM glucose (i.e. 0.36 ± 0.02 ng/ml/10^6 cells) in the absence of staurosporine or H-7 are expressed as 100% (control). The inhibitory effect of H-7 or staurosporine is compared with cells that were stimulated by 25 mM D(+)-glucose. Results are expressed as the percentage of controls (mean ± S.D., N=3) (*p≤ 0.05, **p≤ 0.01 and ***p≤ 0.005). Similar results were obtained from two other experiments.

Figure 5: Effect of D(+)-glucose, tolrestat and insulin on the cellular levels of DAG and PKC activity in OK W1 cells. (A) After a 30 minute incubation in media with 5 mM glucose, 25 mM glucose or 25 mM glucose plus tolrestat (10^{-4} M) or insulin (10^{-5} M), cells were harvested and assayed for DAG levels. Results are expressed (mean ± S.D., N=6) as a percentage of the levels in cells incubated with 5 mM glucose (i.e. 0.82 nanomol per mg of protein). (B) After a 60 minute incubation in media with 5 mM glucose, 25 mM glucose or 25 mM glucose plus tolrestat (10^{-4} M) or insulin (10^{-5} M), cells were

harvested, cytosol and membrane fractions were separated and assayed for PKC activity. The levels of PKC activity were calculated as picomol of ^{32}P-ATP incorporated per minute per mg protein. The results are expressed (mean ± S.D., N=6) as a percentage of the ratio of PKC activity in the cytosol versus membranous fraction in the cells incubated in 5 mM glucose (i.e. a ratio of 0.73) (***$p \leq 0.005$). Similar results were obtained from two other experiments.

Figure 6: Effect of phorbol 12-myristate 13-acetate (PMA) on the expression of the fusion gene in OK W1 cells in the presence of 25 mM glucose. Cells were incubated for 24 hours in the presence of 5 mM or 25 mM glucose plus various concentrations (i.e. 10^{-11} to 10^{-5} M) of PMA. Media were harvested and assayed for the level of IR-hGH. Results are expressed (mean ± S.D., N=3) as a percentage of the levels found in the medium containing 5 mM glucose (i.e. 0.36 ± 0.02 ng/ml/10^6 cells) (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). Similar results were obtained from two other experiments.

Figure 7: (A) Effect of PMA on the expression of the fusion gene in OK W1 cells incubated with 25 mM glucose and insulin (10^{-7} M). Cells were incubated for 24 hours in the media with 5 mM or 25 mM glucose or 25 mM glucose plus 10^{-7} M insulin in the absence or presence of various concentrations of PMA (i.e. 10^{-11} to 10^{-5} M). Media were harvested and assayed for the level of IR-hGH. Results are expressed as (mean ± S.D., N=3) as a percentage of the

levels found in media from cells incubated with 5 mM glucose (i.e. 0.36 ± 0.01 ng/ml/10^6 cells) (**p≤ 0.01 and ***p≤ 0.005). Similar results were obtained from two other experiments. (B) Effect of insulin on the expression of the fusion gene in OK W1 cells incubated with 25 mM glucose and 10^{-7} M PMA. Cells were incubated for 24 hours in the presence of 5 mM or 25 mM glucose plus 10^{-7} M PMA in the absence or presence of various concentrations (i.e. 10^{-11} to 10^{-5} M) of insulin. Media were harvested and assayed for the level of IR-hGH. Results are expressed (mean ± S.D., N=3) as a percentage of the levels found in the media from cells incubated with 5 mM glucose (i.e. 0.29 ± 0.01 ng/ml/10^6 cells) (*p≤ 0.05 and ***p≤ 0.005).

Figure 8: Effect of a 24 hour pre-incubation with PMA (10^{-5} M) on the expression of the fusion gene in OK W1 cells incubated with 25 mM glucose with or without insulin (10^{-7} M). Cells pre-incubated for 24 hours in the presence of 25 mM glucose and10^{-5} M PMA were transferred into fresh medium containing insulin (10^{-11} to 10^{-5} M) or 10^{-7} M PMA and incubated for 24 hours. The media were then harvested and assayed for IR-hGH. Results are expressed (mean ± S.D., N=3) as a percentage of the levels found in media incubated with 25 mM glucose (i.e. 0.72 ± 0.05 ng/ml/10^6 cells) (*p≤ 0.05, **p≤ 0.01 and ***p≤ 0.005). Similar results were obtained from two other experiments.

Figure 9: Effect of PD 98059 on the expression of fusion gene in OK W1 cells incubated with 25 mM D(+)-glucose. Treatment groups include cells

incubated for 24 hours in the presence of 5 mM glucose, 25 mM glucose, and 25 mM glucose in the absence or presence of PD 98059. Media were harvested and assayed for the level of IR-hGH. Results are expressed (mean ± S.D., N=3) as a percentage of the levels found in media from cells incubated with 5 mM glucose (i.e. 0.53 ± 0.03 ng/ml/10^6 cells). Similar results were obtained from two other experiments.

Figure 10: Effects of PD98059 and Wortmannin on the expression of the fusion gene in OK W1 cells incubated with 25 mM D(+)-glucose and insulin (10^{-7} M). Treatment groups include cells incubated for 24 hours in the presence of 5 mM, 25 mM glucose, and 25 mM glucose with 10^{-7} M insulin with or without PD98059 (A) or Wortmannin (B). Media were harvested and assayed for the level of IR-hGH. Results are expressed (mean ± S.D., N=3) as a percentage of the levels found in media from cells incubated with 5 mM glucose (i.e 0.44 ± 0.01 ng/ml/10^6 cells) (*$p \leq 0.05$ and ***$p \leq 0.005$). Similar results were obtained from two other experiments.

Figure 11: Effect of D(+)-glucose, insulin, tolrestat, staurosporine, H-7 or PMA on the expression of pTKGH in OK 13 cells. Cells were incubated for up to 24 hours in the presence of 5 mM D(+)-glucose, 25 mM D(+)-glucose, or 25 mM D(+)-glucose plus insulin (10^{-7} M), Tolrestat (10^{-4} M), staurosporine (10^{-6}), H-7 (10^{-7} M) or PMA (10^{-7} M). Media were harvested after 24 hours of incubation and assayed for IR-hGH. Results

are expressed (mean ± S.D. with N=3) as a percentage of the levels found in media from cells incubated with 5 mM glucose (i.e. 0.35 ± 0.02 ng/ml/10^6 cells). Similar results were obtained from two other experiments.

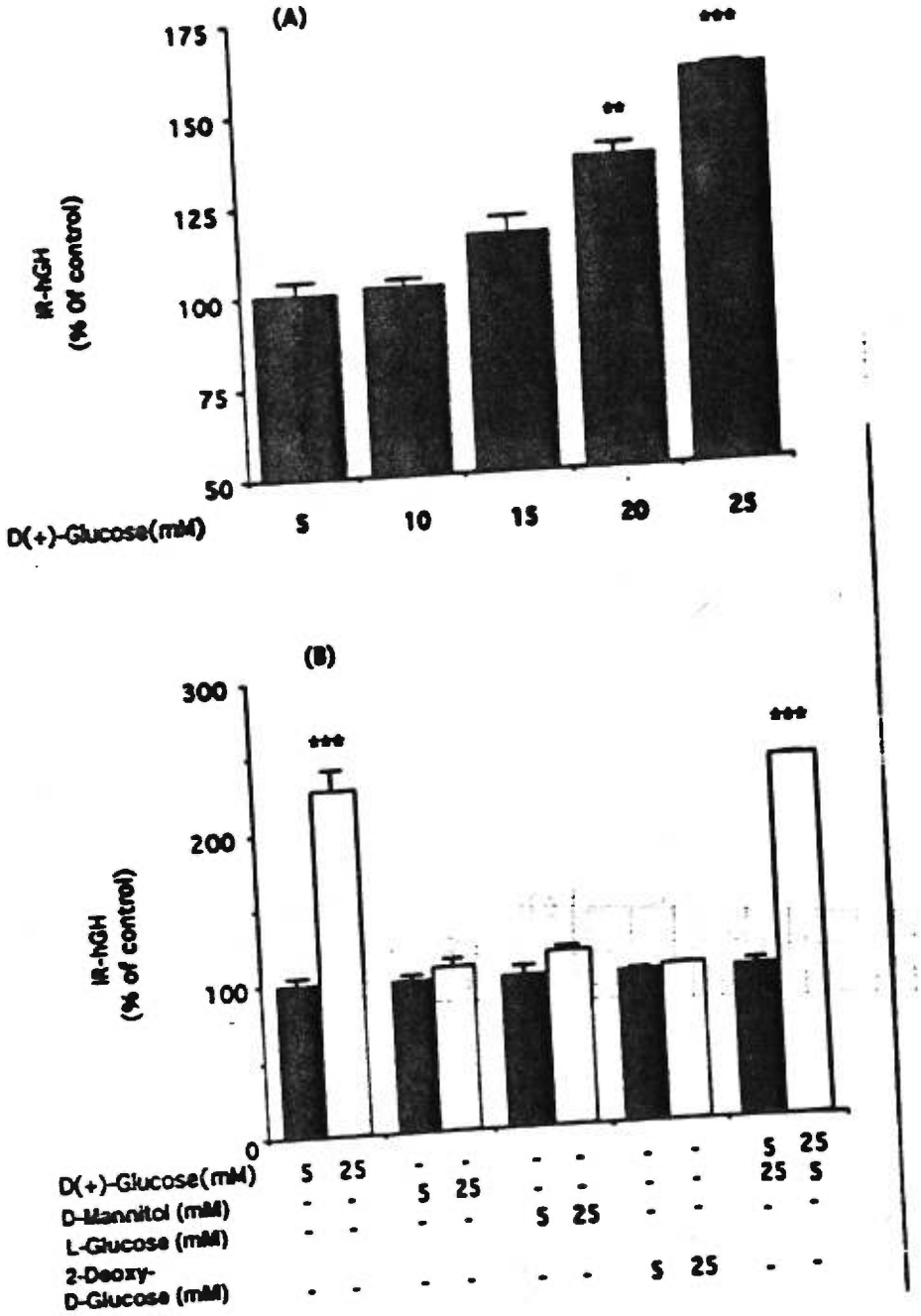
(A)
IR-hGH
(% Of control)
175
150
125
100
75
50
**
***
D(+)-Glucose(mM)
5
10
15
20
25
(B)
IR-hGH
(% of control)
300
200
100
0
***
***
D(+)-Glucose(mM)
D-Mannitol (mM)
L-Glucose (mM)
2-Deoxy-
D-Glucose (mM)

Figure 1

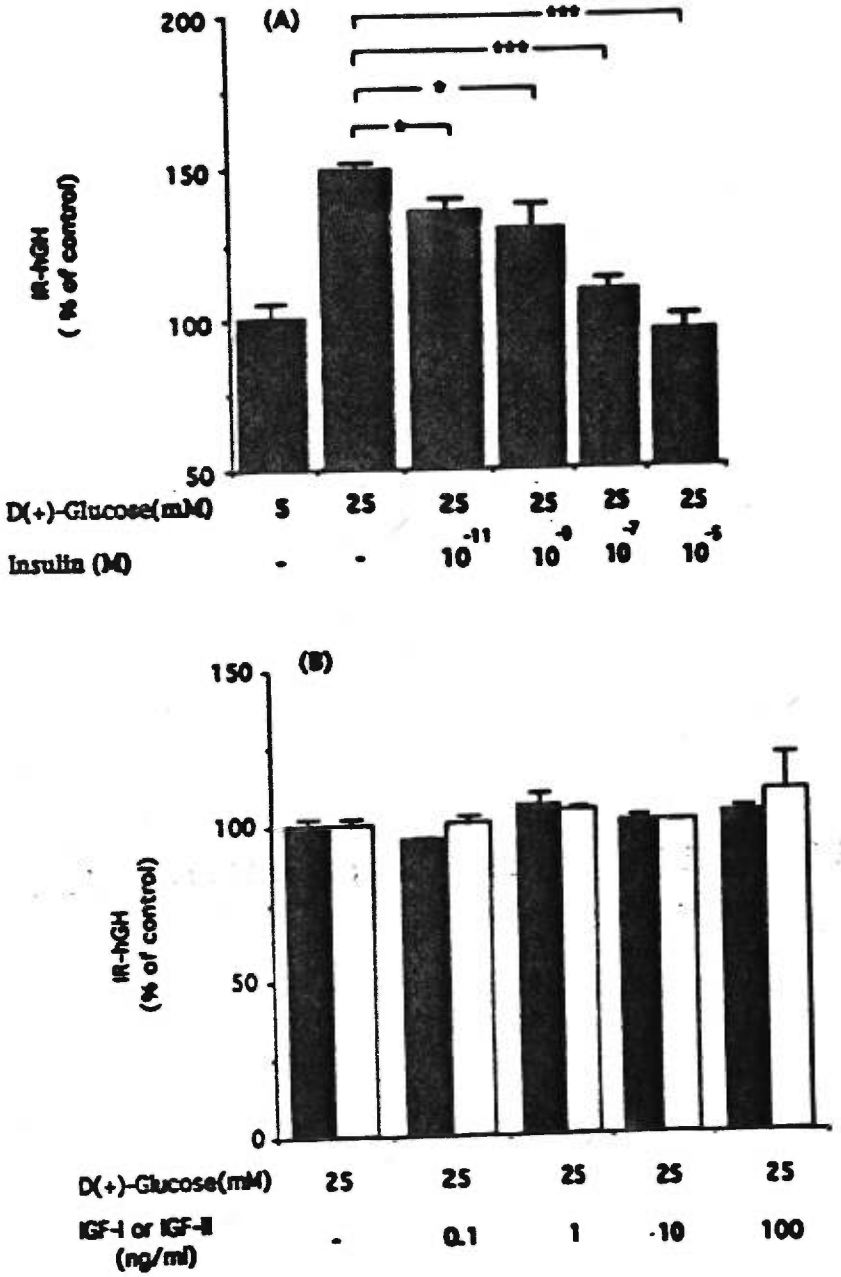
(A)
200
150
100
50
IR-hGH
(% of control)
***
***
*
*
D(+)-Glucose(mM)
5
25
25
25
25
25
Insulin (M)
-
-
10^-11
10^-9
10^-7
10^-5
(B)
150
100
50
0
IR-hGH
(% of control)
D(+)-Glucose(mM)
25
25
25
25
25
IGF-I or IGF-II
(ng/ml)
-
0.1
1
10
100

Figure 2

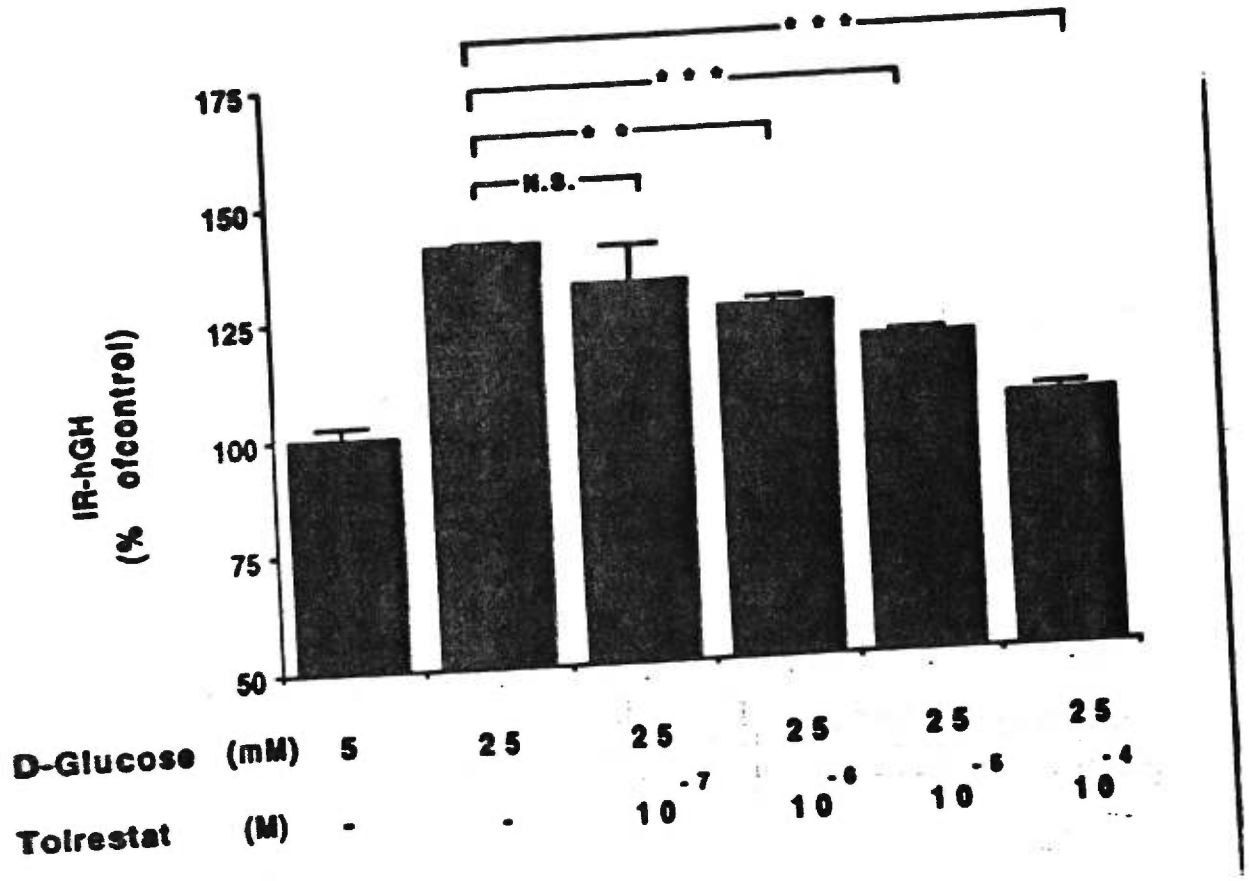
***
***
**
N.S.
175
150
125
100
75
50
IR-hGH
(% of control)
D-Glucose (mM) 5 25 25 25 25 25
Tolrestat (M) - - 10^{-7} 10^{-6} 10^{-5} 10^{-4}

Figure 3

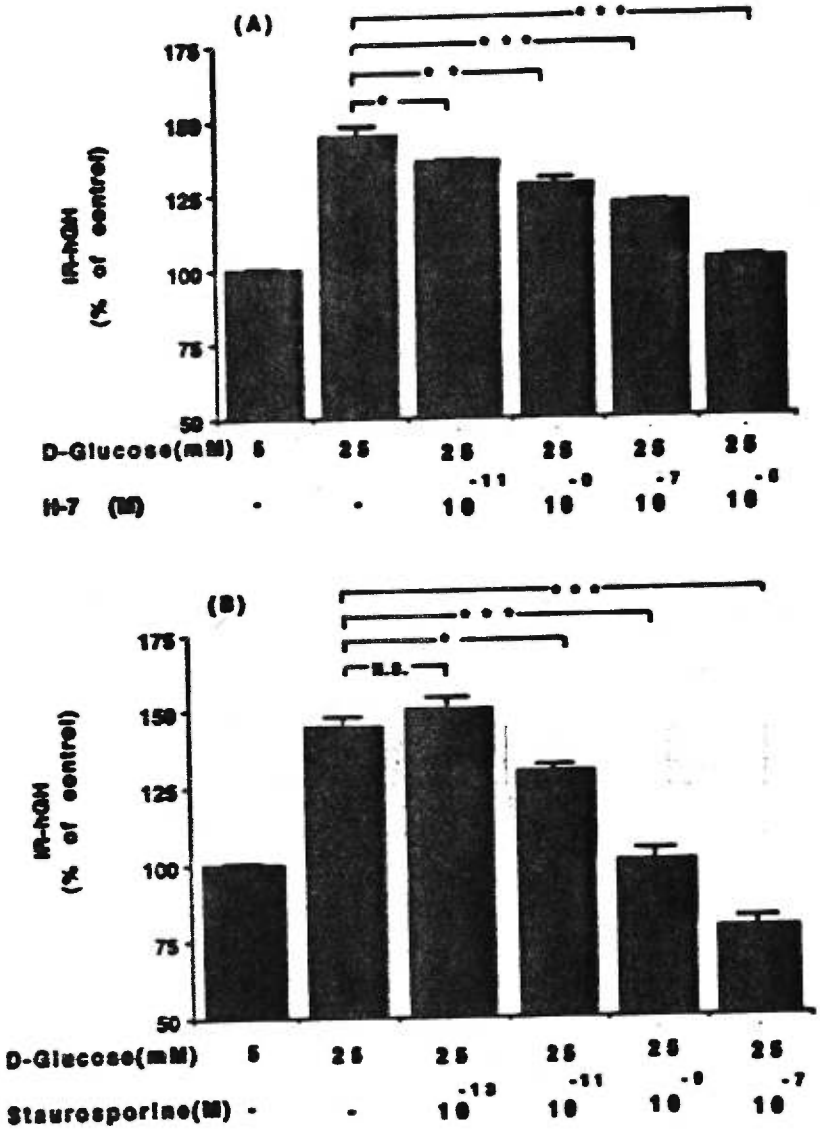
(A)
IR-hGH (% of control)
175
150
125
100
75
50
D-Glucose(mM) 5 25 25 25 25 25
H-7 (M) - - 10^-11 10^-9 10^-7 10^-5
(B)
IR-hGH (% of control)
175
150
125
100
75
50
N.S.
D-Glucose(mM) 5 25 25 25 25 25
Staurosporine(M) - - 10^-13 10^-11 10^-9 10^-7

Figure 4

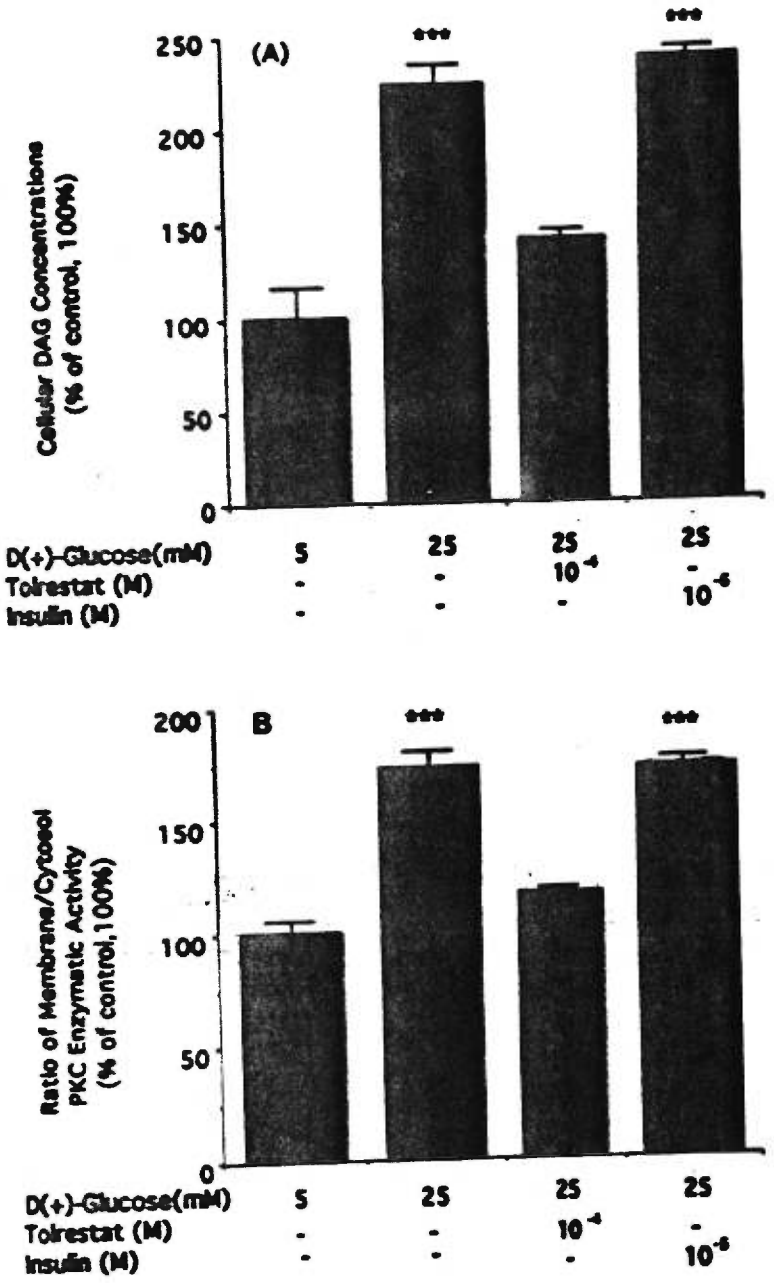
(A)
Cellular DAG Concentrations
(% of control, 100%)
250
200
150
100
50
0
***
***
D(+)-Glucose(mM) 5 25 25 25
Tolrestat (M) - - 10^{-4} -
Insulin (M) - - - 10^{-6}
B
Ratio of Membrane/Cytosol
PKC Enzymatic Activity
(% of control,100%)
200
150
100
50
0
***
***
D(+)-Glucose(mM) 5 25 25 25
Tolrestat (M) - - 10^{-4} -
Insulin (M) - - - 10^{-6}

Figure 5

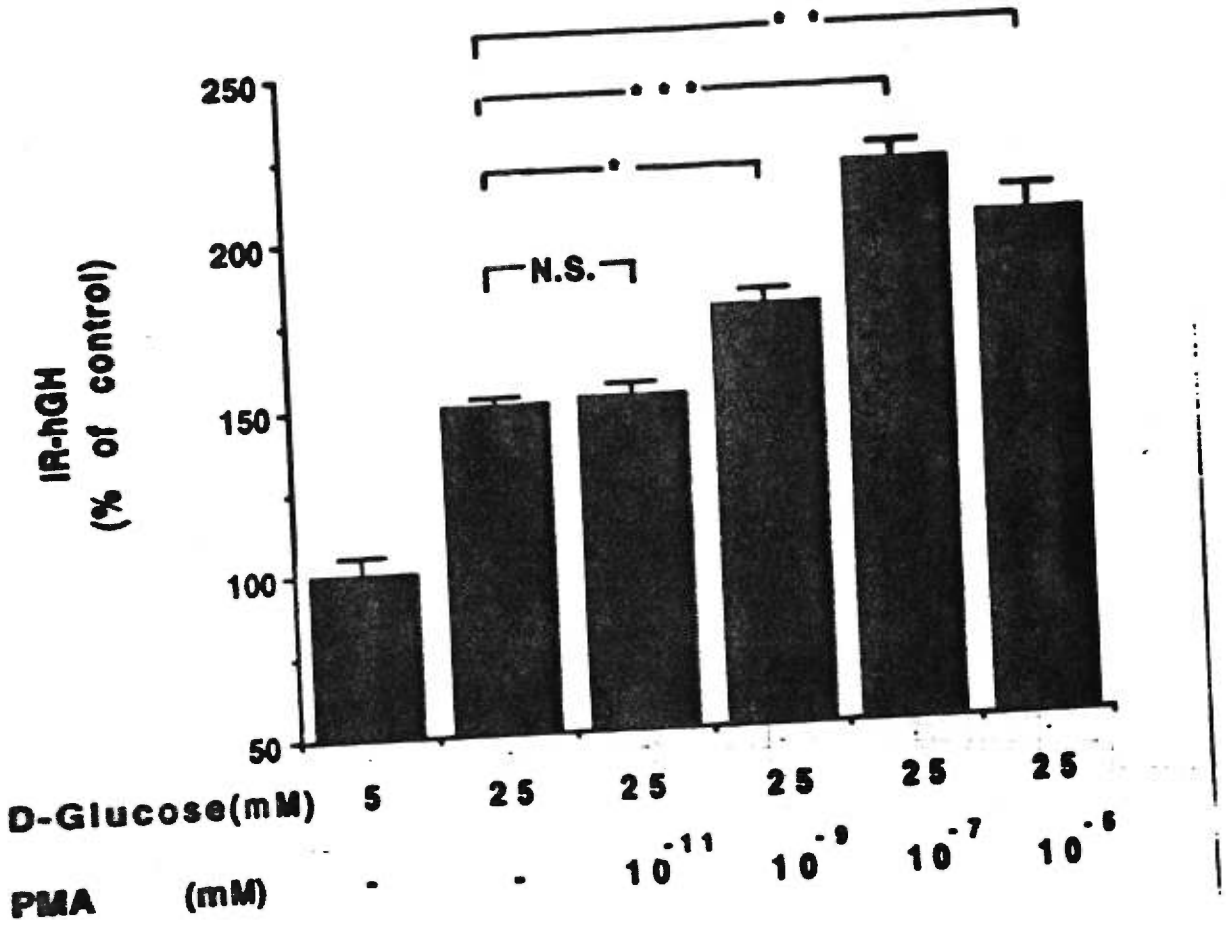
IR-hGH
(% of control)
250
200
150
100
50
N.S.
D-Glucose(mM) 5 25 25 25 25 25
PMA (mM) - - 10^{-11} 10^{-9} 10^{-7} 10^{-5}

Figure 6

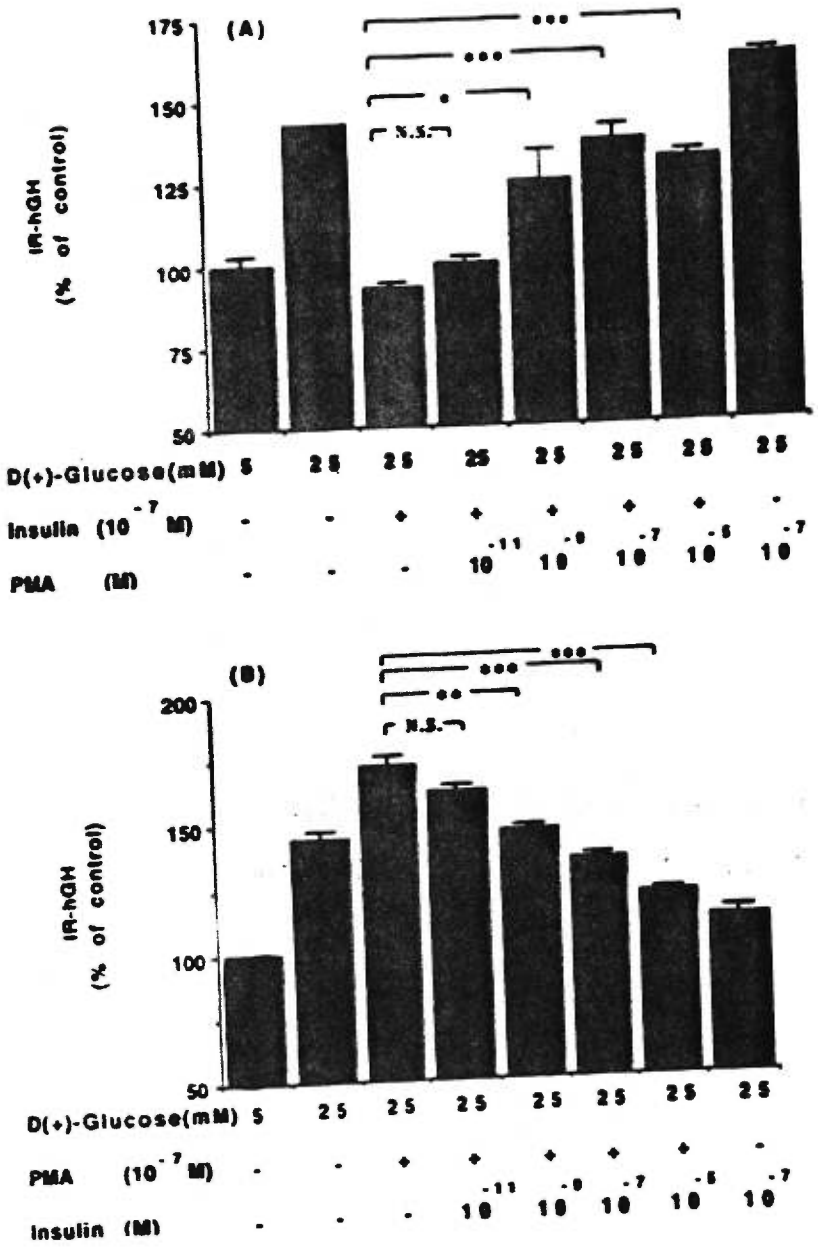
(A)
IR-hGH (% of control)
175
150
125
100
75
50
***
***
*
N.S.
D(+)-Glucose(mM) 5 25 25 25 25 25 25 25
Insulin (10⁻⁷ M) - - + + + + + -
PMA (M) - - - 10⁻¹¹ 10⁻⁹ 10⁻⁷ 10⁻⁵ 10⁻⁷
(B)
IR-hGH (% of control)
200
150
100
50
***
***
**
N.S.
D(+)-Glucose(mM) 5 25 25 25 25 25 25 25
PMA (10⁻⁷ M) - - + + + + + -
Insulin (M) - - - 10⁻¹¹ 10⁻⁹ 10⁻⁷ 10⁻⁵ 10⁻⁷

Figure 7

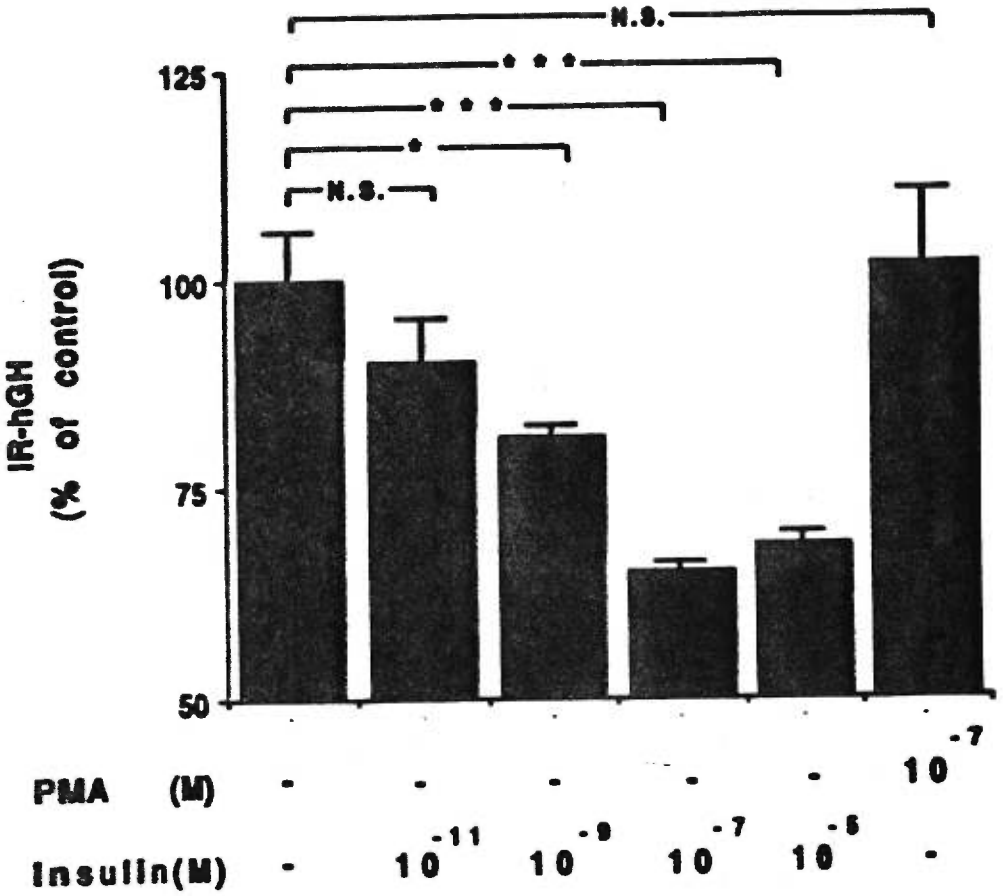
N.S.
* * *
* * *
*
N.S.
125
100
75
50
IR-hGH
(% of control)
PMA (M) - - - - - 10^-7
Insulin(M) - 10^-11 10^-9 10^-7 10^-5 -

Figure 8

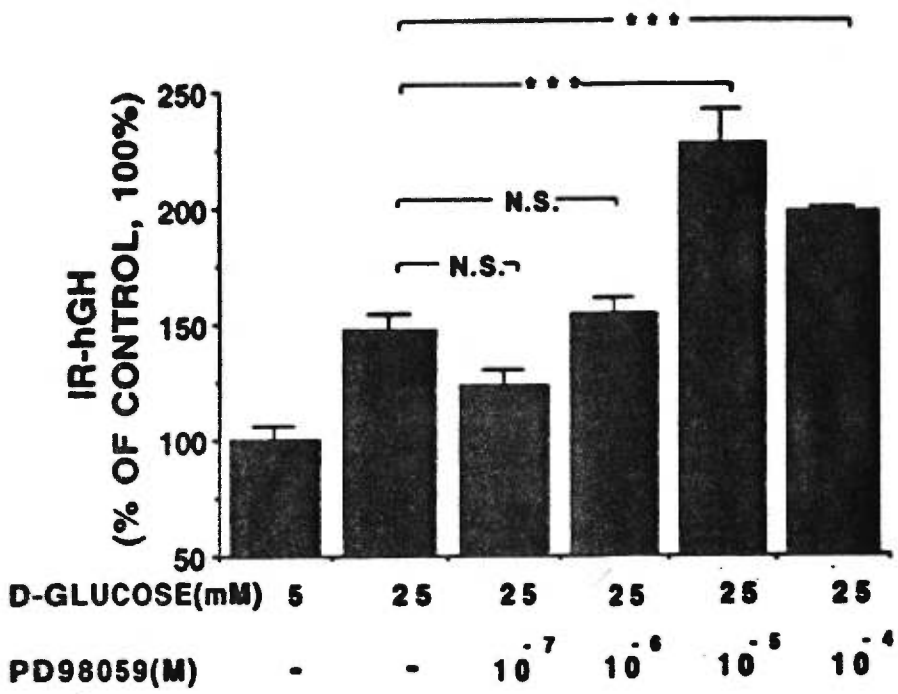
***
**
N.S.
N.S.
IR-hGH
(% OF CONTROL, 100%)
250
200
150
100
50
D-GLUCOSE(mM) 5 25 25 25 25 25
PD98059(M) - - 10^{-7} 10^{-6} 10^{-5} 10^{-4}

Figure 9

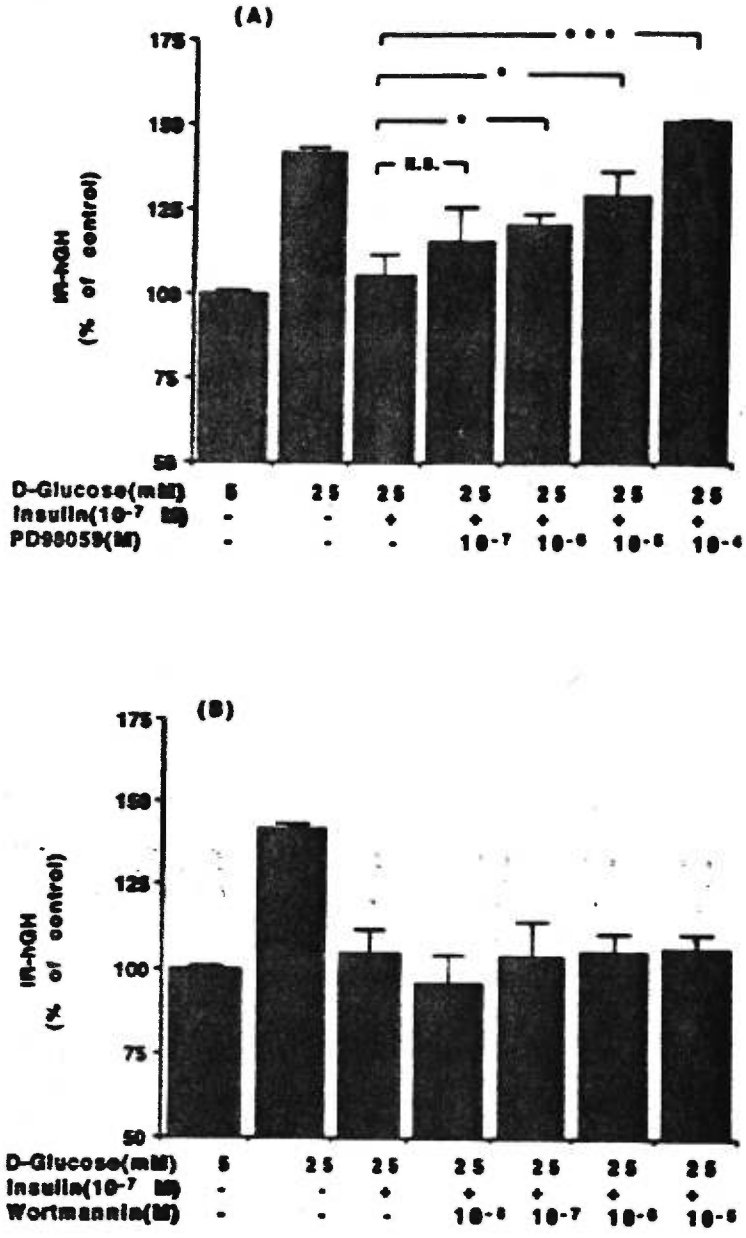
(A)
IR-hGH (% of control)
175
150
125
100
75
50
N.S.
D-Glucose(mM) 5 25 25 25 25 25 25
Insulin(10^{-7} M) - - + + + + +
PD98059(M) - - - 10^{-7} 10^{-6} 10^{-5} 10^{-4}
(B)
IR-hGH (% of control)
175
150
125
100
75
50
D-Glucose(mM) 5 25 25 25 25 25 25
Insulin(10^{-7} M) - - + + + + +
Wortmannin(M) - - - 10^{-8} 10^{-7} 10^{-6} 10^{-5}

Figure 10

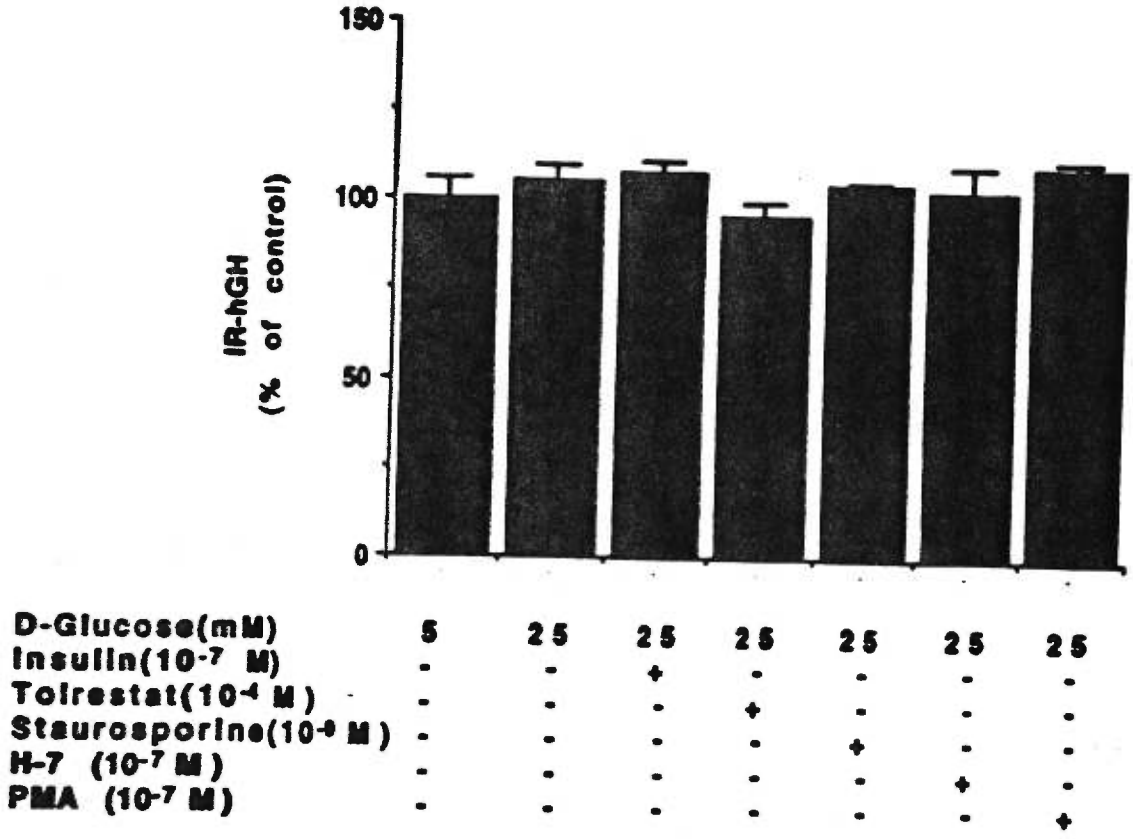
IR-hGH
(% of control)
150
100
50
0
D-Glucose(mM) 5 25 25 25 25 25 25
Insulin(10-7 M) - - + - - - -
Tolrestat(10-4 M) - - - + - - -
Staurosporine(10-8 M) - - - - + - -
H-7 (10-7 M) - - - - - + -
PMA (10-7 M) - - - - - - +

Figure 11

Angiotensinogen Gene Expression Is Stimulated by the cAMP-Responsive Element Binding Protein in Opossum Kidney Cells

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Abstract. It has been reported previously that the addition of isoproterenol or forskolin stimulates the expression of the angiotensinogen (ANG) gene in opossum kidney (OK) 27 cells, an OK cell line with a fusion gene containing the 5′-flanking regulatory sequence of the rat ANG gene fused with a human growth hormone (hGH) gene as a reporter, pOGH (ANG N-1498/+18), permanently integrated into their genomes. To investigate whether the effect of isoproterenol or forskolin on the expression of the ANG gene is mediated via the nuclear 43-kD cAMP-responsive element binding protein (CREB), OK 27 cells were transiently transfected with an expression plasmid containing the cDNA for the 43-kD CREB (pRSV/CREB). The level of expression of the pOGH (ANG N-1498/+18) in OK 27 cells was estimated by the amount of immunoreactive hGH secreted into the culture medium. Transfection of pRSV/CREB alone stimulated the expression of pOGH (ANG N-1498/+18). The addition of isoproterenol or forskolin further enhanced the stimulatory effect of pRSV/CREB on the expression of pOGH (ANG N-1498/+18). The enhancing effect of isoproterenol was inhibited by the presence of propranolol (an inhibitor of β-adrenoceptors) and (*R*)-*p*-adenosine 3′5′-cyclic monophospho-orthioate (Rp)-cAMP (an inhibitor of cAMP-dependent protein kinase A I and II). Transfection of pRSV/CREB had no effect on the expression of thymidine kinase growth hormone in OK 13 cells, an OK cell line with a fusion gene containing the promoter/enhancer DNA sequence of the viral thymidine-kinase gene fused with an hGH gene as a reporter, thymidine kinase growth hormone, permanently integrated into their genomes. These studies demonstrate that isoproterenol stimulates the expression of ANG gene via the cAMP-dependent protein kinase A and probably via the interaction of the 43-kD CREB with the 5′-flanking region of the ANG gene. Our data indicate that the nuclear 43-kD CREB may have a modulatory role on the expression of the ANG gene in OK cells. (J Am Soc Nephrol 8: 1072–1079, 1997)

Angiotensinogen (ANG) mRNA has been localized in rat renal proximal tubules by the techniques of *in situ* hybridization (1) and PCR (2). Furthermore, studies by Wolf and Neilson (3) and Fang *et al.* (4) showed that ANG mRNA is expressed in mouse and rat immortalized proximal tubular cell lines, respectively. We (5), as well as Ingelfinger *et al.* (6), have also demonstrated that the ANG mRNA is expressed in opossum kidney (OK) proximal tubular cells. Thus, these studies demonstrate that the intrarenal angiotensin (Ang) I and II are probably derived from the ANG synthesized in renal proximal tubules.

We have reported previously that thyroid hormone (L-T_3), dexamethasone, 8-bromo-cAMP (8-Br-cAMP), and forskolin stimulate the expression of the ANG gene in OK cells *in vitro* in a dose-dependent manner (5,7,8). We have also demonstrated that isoproterenol stimulates the expression of the ANG gene in OK cells (9). The effect of isoproterenol is mediated via the β_1-adrenoceptor and is blocked by the presence of propranolol (β-adrenoceptor blocker), atenolol (β_1-adrenoceptor blocker), and (*R*)-*p*-adenosine 3′5′-cyclic monophospho-orthioate (Rp)-cAMP (an inhibitor of cAMP-dependent protein kinase A I and II) (9), but not by ICI 118,551 (β_2-adrenoceptor blocker). The specificity of the β_1-adrenoceptor is supported further by our more recent studies that isoproterenol stimulates the expression of the ANG gene when cotransfected with an expression vector containing the cDNA for the β_1-adrenoceptor, but not the cDNA for the β_2-adrenoceptor (10). Our studies confirm the report of Nakamura and Johns (11) that the administration of atenolol blocks the effect of renal nerve stimulation after the increase of the ANG mRNA level in the rat kidney *in vivo*. Furthermore, our studies (9,10) and those of Nakamura and Johns (11) together suggest the presence of a functional relationship between the renal sympathetic nervous system and the activation of local renal renin-angiotensin system. Thus, the local formation of renal Ang II may play an important role in the modulation of the physiology of the renal proximal tubular cells (*i.e.*, sodium and fluid reabsorption) (12–17).

The exact molecular mechanism(s) for the effect of isoproterenol on the expression of the ANG gene in OK cells is unknown. One possibility may be that the addition of isopro-

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Journal of the American Society of Nephrology

/ere harvested 24 h later and assayed for IR-hGH and CAT activity, espectively.

To study the effect of isoproterenol or forskolin on the expression f the fusion gene (pOGH (ANG N-1498/+18)) in OK 27 cells ansfected with pRSV/CREB, cells were incubated in DMEM con- aining 1% depleted FBS, and various concentrations of hormones /ere added on day 1 after DNA transfection. After a 24-h incubation eriod, the media and cells were harvested for hGH assays and CAT nzymatic assays, respectively. The control plasmid pRC/RSV was sed as negative control.

To normalize the efficiency of transfection of the plasmid, 2 μg of TKCAT (a vector with the TK enhancer/promoter fused to the oding sequence of CAT) was cotransfected with pRSV/CREB, pRC/ SV, or pGEM-3 as internal control for normalization. The levels of AT activity in the control groups (*i.e.*, Figure 1, cells transfected ith 2 μg of pTKCAT but without the cotransfection with either RSV/CREB or pRC/RSV) were used as 100% transfection effi- iency. The levels of CAT activity in other groups (*i.e.*, cells cotrans- ected with 2 μg of pTKCAT and pRC/RSV (at various concentra- ons) or pRSV/CREB (at various concentrations) were compared ith the control group as percentage of transfection efficiency. Sub- equently, the IR-hGH levels in groups cotransfected with either RSV/CREB or pRC/RSV were normalized with the percentage of ansfection efficiency as compared with controls (group 1). In each xperiment, an additional group of cells was transfected with 2 μg of RSVCAT (a plasmid with the RSV enhancer/promoter fused to the oding sequence of CAT) as the positive control for the comparison of ansfection efficiency. The transfection efficiency of pTKCAT nged from 25 to 55% compared with pRSVCAT.

The depleted FBS was prepared by incubation with 1% activated harcoal and 1% AG 1 × 8 ion-exchange resin (Bio-Rad Laboratories, ichmond, CA) for 16 h or more at room temperature as described by amuels *et al.* (23). This procedure removed endogenous steroid and hyroid hormones from the FBS as demonstrated by Samuels *et al.* 23).

ffect of pRSV/CREB on the Expression of pOGH ANG N-1498/+18) in OK 27 Cells

OK 27 cells were plated at a density of 1 to 2 × 10^5 cells/well in -well plates and incubated overnight in DMEM containing 10% FBS. hen, 2 μg of pTKCAT with or without 1 to 10 μg of pRSV/CREB r pRC/RSV per well were transiently cotransfected into OK 27 cells. fter an overnight incubation, the media were replaced with fresh edia without FBS and incubated for an additional 24 h. After cubation, cultured media and cells were harvested and kept at 20°C until assayed for IR-hGH and for CAT enzymatic activity, spectively.

ffect of Isoproterenol or Forskolin on the Expression f pOGH (ANG N-1498/+18) in OK 27 Cells ransfected with pRSV/CREB

OK 27 cells were plated at a density of 1 to 2 × 10^5 cells/well in -well plates and incubated overnight in DMEM containing 10% FBS. hen, 2 μg of pTKCAT and 5 μg of pRSV/CREB or pRC/RSV were ansiently cotransfected into OK 27 cells. After an overnight incu- ation, the media were replaced with media containing 1% depleted BS and various concentrations (10^{-13} to 10^{-5} M) of isoproterenol or rskolin and incubated for 24 h. At the end of the incubation period, edia and cells were collected and kept at −20°C until assayed.

Effect of Propranolol or Rp-cAMP on the Expression of pOGH (ANG N-1498/+18) in OK 27 Cells Transfected with pRSV/CREB in the Presence of Isoproterenol

OK 27 cells were plated at a density of 1 to 2 × 10^5 cells/well in 6-well plates and incubated overnight in DMEM containing 10% FBS. Then, 2 μg of pTKCAT and 5 μg of pRSV/CREB or pRC/RSV were transiently cotransfected into OK 27 cells. After an overnight incubation, the media were replaced with media containing 1% depleted FBS, 10^{-9} M isoproterenol, and 10^{-6} M propranolol or 10^{-4} M Rp-cAMP. After 24 h of incubation, media and cells were collected and kept at −20°C until assay.

Effect of pRSV/CREB on the Expression of pTKGH in OK 13 Cells

OK 13 cells were plated at a density of 1 to 2 × 10^5 cells/well in 6-well plates and incubated overnight in DMEM containing 10% FBS. Then, 2 μg of pTKCAT with or without 1 to 10 μg of pRSV/CREB per well were transiently cotransfected into OK 13 cells. After an overnight incubation, the media were replaced with fresh media containing 1% depleted FBS and incubated for an additional 24 h. After incubation, cultured media and cells were collected and kept at −20°C until assayed.

Statistical Analyses

The experiments were performed at least three to four times in triplicate. The data were analyzed with *t* test or ANOVA. A probability level of $P \leq 0.05$ was regarded as significant.

Results

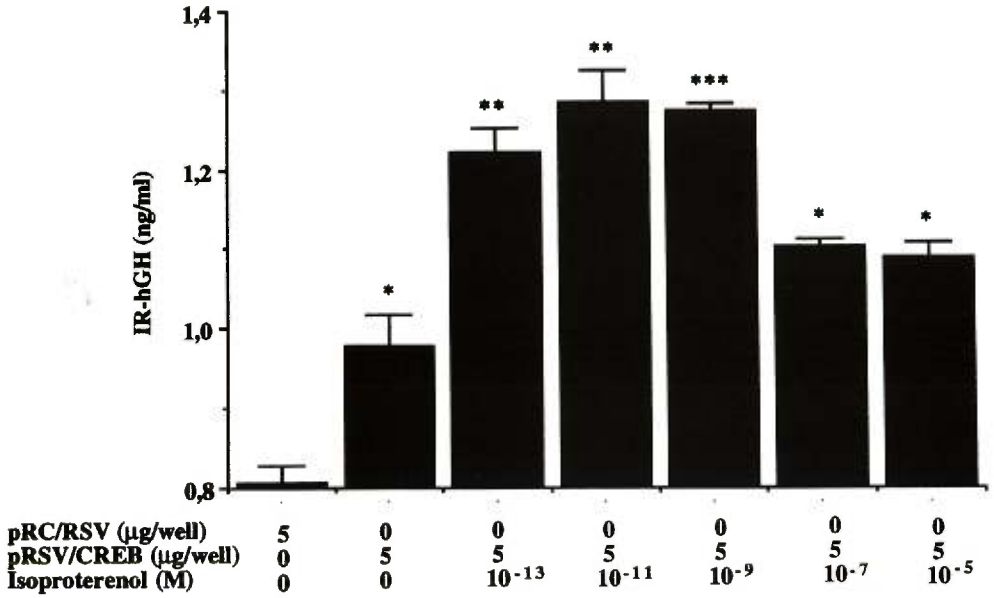
Effect of pRSV/CREB on the Expression of pOGH (ANG N-1498/+18) in OK 27 Cells

Figure 1 shows that the transfection of pRSV/CREB (1 to 10 μg DNA) per well stimulates the expression of pOGH (ANG N-1498/+18) in OK 27 cells. A dose-dependent relationship between pRSV/CREB and the stimulation of expression of the pOGH (ANG N-1498/+18) was observed for pRSV/CREB at 1 to 5 μg of DNA. It appears that the maximal stimulation was found with 5 μg of pRSV/CREB. At doses greater than 5 μg of pRSV/CREB, the expression of pOGH (ANG N-1498/+18) diminished. No significant stimulation of expression of the pOGH (ANG N-1498/+18) was observed with the control plasmid pRC/RSV (1 to 10 μg). These studies indicate that the expression of pOGH (ANG N-1498/+18) could be stimulated directly by CREB alone.

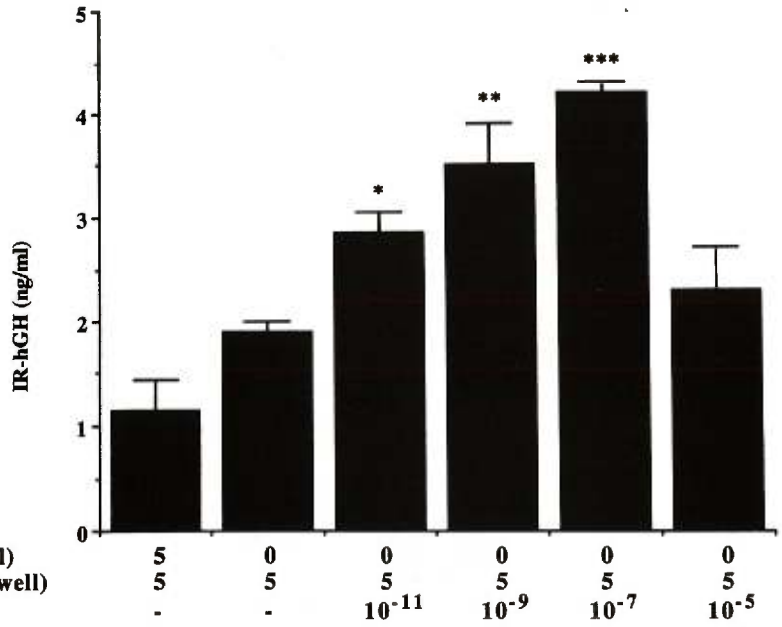
Dose–Response Curve of Isoproterenol or Forskolin on the Expression of pOGH (ANG N-1498/+18) Transfected with pRSV/CREB or pRC/RSV

Figure 2 shows that the addition of isoproterenol (10^{-11} to 10^{-5} M) stimulated the expression of the pOGH (ANG N-1498/+18) when transiently transfected with 5 μg of pRSV/CREB in a dose-dependent manner. It appears that the maximal effect of isoproterenol was found with 10^{-11} to 10^{-9} M isoproterenol. At concentrations greater than 10^{-9} M, the enhancing effect of isoproterenol was diminished.

Similarly, Figure 3 shows that addition of forskolin (10^{-11}



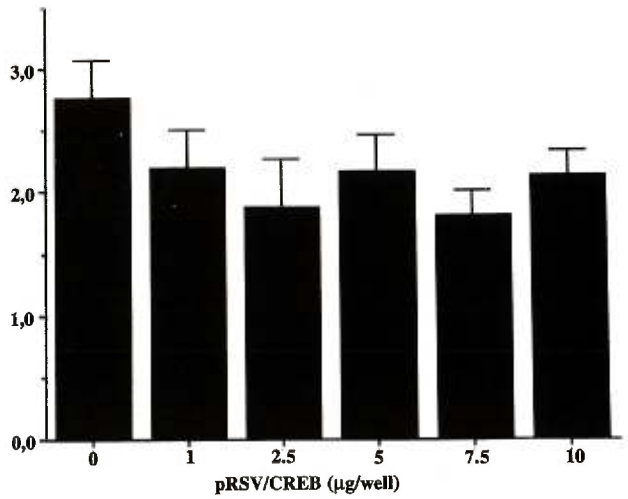
gure 2. Dose–response relationship for the addition of isoproterenol on the expression of pOGH (ANG N-1498/+18) in OK 27 cells ransfected with pRSV/CREB. Five micrograms of pRSV/CREB or pRC/RSV per well (1×10^5 cell) were used in the experiment. The effect isoproterenol is compared with the control cells (transfected with 5 μg of pRSV/CREB but without the addition of isoproterenol). Results : expressed as the mean ± SD of a minimum of three determinations. Similar results were obtained in four independent experiments. The NA transfection efficiency for this experiment was approximately 25% compared with pRSVCAT. $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.005$.



gure 3. Dose–response relationship for the addition of forskolin on the expression of pOGH (ANG N-1498/+18) in OK 27 cells cotransfected th pRSV/CREB. Five micrograms of pRSV/CREB or pRC/RSV per well (2×10^5 cells) were used in the experiment. The effect of forskolin compared with the control cells (transfected with 5 μg of pRSV/CREB but without the addition of forskolin). Results are expressed as the ean ± SD of a minimum of three determinations. Similar results were obtained in three independent experiments. The DNA transfection ficiency for this experiment was approximately 35% compared with pRSV/CAT. $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.005$.

in (27). Thus, the symmetry of the CRE sequence and its 5′- id 3′-flanking sequences determine the relative binding af- nity and specificity of the CREB.

Our previous studies (19) on the DNA structure of the -flanking sequence of the rat ANG gene showed that the DNA sequence of nucleotides N-795 to N-788 (TGACGTAC) is almost identical to the CRE (TGACGTCA) of the somatostatin gene (24), except that the last two nucleotides are in reverse order. With such homology, we raised the question of whether the sequence ANG N-795 to N-788 could be a putative



ure 6. Expression of the pTKGH in OK 13 cells when transiently
ısfected with different concentrations of the plasmid pRSV/CREB.
ɜ levels of transcriptional activity of pTKGH were quantified by
amount of IR-hGH in the medium assayed by RIA-hGH. The
ıcentration of IR-hGH in the medium of cells (1×10^5 cells)
hout transfection with pRSV/CREB is considered as the control
el. Results are expressed as the mean ± SD of a minimum of three
erminations. Similar results were obtained from three additional
ɔeriments. The DNA transfection efficiency in this experiment was
ɔroximately 35% compared with pRSVCAT.

ɛE. Indeed, we have demonstrated that forskolin or 8-Br-
.MP directly stimulated the expression of pTKCAT (ANG
814 to N-761) (a fusion gene containing the 5′-flanking
ɣion [nucleotides N-814 to N-761] of the rat ANG gene
stream of the TK promoter fused to a CAT gene in OK cells)
). Thus, we speculate that CREB might mediate the effect of
ɛskolin or 8-Br-cAMP on the expression of the ANG gene in
ϰ cells via the binding to the putative CRE.

Our present gene transfection experiments showed that
ɛSV-CREB directly stimulated the expression of pOGH
.NG N-1498/+18) in OK 27 cells (Figure 1). The maximal
mulation was at 5 μg of DNA. At higher concentrations of
NA (*i.e.*, >5 μg), the effect of pRSV/CREB was diminished.
: present, we do not know the reasons for this observation.
ıe possible explanation may be that large amounts of DNA
ınsfected into OK cells might exhaust the limited amount of
llular transcriptional factors. Nevertheless, more studies are
ɹrranted to clarify this observation.

Isoproterenol and forskolin are known to increase intracel-
lar cAMP levels. Indeed, our previous studies have shown
at the addition of isoproterenol increased the intracellular
vels of cAMP in OK 27 cells (9). The present studies (Fig-
es 2, 3, and 4) showed that addition of isoproterenol or
ɛskolin further enhanced the stimulatory effect of pRSV/
REB compared with those without the transfection with
ɛSV/CREB. These studies support the hypothesis that iso-
ɔterenol stimulates the synthesis of intracellular cAMP. The
evated intracellular cAMP then activates the cAMP-depen-
ɜnt PKA and phosphorylates the nuclear CREB. Subse-
quently, the CREB binds to the CRE of the rat ANG gene and enhances the gene expression.

At present, we do not understand why forskolin at 10^{-5} M has an inhibitory effect on the expression of the pOGH (ANG N-1498/+18) in OK 27 cells when transfected with pRSV/CREB. One possible explanation may be that the high concentration of forskolin may exert other physiological effects (*i.e.*, Ca^{2+} transport) and subsequently alter the effect of CREB on the expression of the ANG gene in OK cells. Indeed, more experiments are required to clarify this observation.

Our results also showed that the addition of Rp-cAMP or propranolol blocked the enhancing effect of isoproterenol on the stimulatory effect of pRSV/CREB on pOGH (ANG N-1498/+18) gene expression in OK 27 cells (Figure 5). These studies suggest strongly that the effects of cAMP-dependent PKA and β-adrenergic receptor are involved in the expression of the ANG gene and probably are mediated via the CREB. Indeed, experiments are under way in our laboratory to investigate the direct involvement of cAMP-dependent PKA with the 43 kD CREB (*i.e.*, phosphorylation of 43-kD CREB).

Finally, it appears that pRSV/CREB had no effect on the expression of pTKGH in OK 13 cells (Figure 6). Because the expression of pTKGH in OK 13 cells is driven by the promoter/enhancer DNA sequence of the TK gene, these studies demonstrated that the promoter/enhancer DNA sequence of TK gene is not responsive to the addition of 43-kD CREB. The effect of CREB in OK 27 cells may be mediated via the interaction of CREB with the putative CRE (ANG N-795 to N-788) in the 5′-flanking region of the rat ANG gene of the fusion gene. Indeed, preliminary studies in our laboratory (28) have shown that the CREB is able to bind to the CRE of the ANG gene. Nevertheless, more experiments are under way in our laboratory to confirm this observation.

In summary, our present studies demonstrate that CREB directly stimulates the expression of pOGH (ANG N-1498/+18) in OK 27 cells. The stimulatory effect of CREB could be further enhanced by the presence of isoproterenol or forskolin. The addition of Rp-cAMP or propranolol could block the enhancing effect of isoproterenol. Our studies raise the possibility that the molecular mechanism(s) of the effect of renal nerve (that is, via the activation of β-adrenoceptors) on the expression of the renal ANG gene is probably mediated via the PKA and nuclear 43-kD CREB. The local formation of renal Ang II might then modulate the sodium and fluid reabsorption by the renal proximal tubular cells. Hence, the local intrarenal renin-angiotensin system might play a significant role in the modulation of sodium reabsorption.

Acknowledgments

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ntification of a novel mouse hepatic 52 kDa protein that interacts with cAMP reponse element of the rat angiotensinogen gene

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lentify the nuclear protein(s) that interact with the putative IP response element (CRE) of the rat angiotensinogen G) gene (i.e. nt 806–779 upstream of the transcriptional start mouse liver nuclear proteins were prepared for the present es. The DNase 1 footprinting protection analysis revealed nt −799/−788 in the 5′-flanking region of the rat ANG gene rotected by the mouse liver nuclear protein. Gel mobility- assays revealed that the addition of the unlabelled DNA nent, ANG nt −806/−779 competed effectively with the ing of the labelled ANG nt −806/−779 to the mouse liver ear proteins but the addition of unlabelled mutants of ANG 806/−779 were only weakly effective in competing with the led ANG nt −806/−779. The addition of unlabelled CRE ie somatostatin (SOM) gene and the CRE of the tyrosine otransferase (TAT) gene was also ineffective in competing the labelled ANG nt −806/−779. Southwestern blot ysis revealed that the labelled ANG nt −806/−779 interacted with two mouse liver nuclear proteins with apparent molecular masses of 52 and 43 kDa, whereas the labelled SOM-CRE, TAT-CRE and the CRE of the phosphoenolpyruvate carboxykinase (PEPCK) gene interacted with one molecular species of 43 kDa. The binding of the labelled ANG nt −806/−779 to the 52 kDa protein was effectively competed for by the addition of unlabelled ANG nt −806/−779 but not by unlabelled SOM-CRE, TAT-CRE and PEPCK-CRE. Finally, Western blot analysis revealed that polyclonal antibodies against the CRE-binding protein (CREB) interacted with the mouse liver nuclear 43 kDa protein but not with the 52 kDa protein. These studies demonstrate that the CRE of the rat ANG gene (ANG nt −806/−779) interacts with the 43 kDa CREB and a novel 52 kDa protein from mouse liver. The novel 52 kDa protein is immunologically distinct from the 43 kDa CREB. These studies suggest that the 52 kDa protein might have a role in the expression of the hepatic ANG gene.

ODUCTION

have previously reported on the expression of the angiotensinogen (ANG) gene in mouse hepatoma cells (Hepa 1-6) and shown that isoprenaline or 8-Br-cAMP enhances the ulatory effect of dexamethasone on the expression of the G gene in Hepa 1-6 cells [1,2]. The enhancing effect of enaline is blocked by the presence of propranolol (β-nergic receptor blocker), ICI 118,551 (β_2-adrenergic receptor cer) and Rp-cAMP (an inhibitor of cAMP-dependent protein se AI and II), but only minimally by atenolol (β_1-adrenergic otor blocker). These studies demonstrate that the enhancing t of isoprenaline is mediated predominantly via the β_2-nergic receptor and the cAMP-dependent protein kinase A ul transduction pathway.

ie exact molecular mechanism(s) for the enhancing effect of enaline with dexamethasone on the expression of the ANG in Hepa 1-6 cells has not been defined. One possibility might iat the addition of isoprenaline stimulates the synthesis of cellular cAMP, because β-adrenergic receptors are linked igh a guanine nucleotide regulatory protein to adenylate se on the inner part of the plasma membrane of target cells The intracellular cAMP then activates the cAMP-dependent ein kinase AI and II and subsequently phosphorylates the ear 43 kDa cAMP response element-binding protein (CREB) The phosphorylated 43 kDa CREB then interacts with the tive cAMP response element (CRE) (i.e. ANG nt 6/−779 containing the motif of the CRE, TGACGTAC, on 795/−788) in the 5′-flanking region of the rat ANG gene The phosphorylated CREB might also interact with the activated-glucocorticoid receptor complex (GRC), which is bound to the glucocorticoid response elements in the 5′-flanking region of the rat ANG gene when stimulated by dexamethasone. Finally, the bound 43 kDa CREB/GRC unit will act synergistically with the pre-initiation complex to enhance the expression of the ANG gene. This possibility is supported by the studies of Imai et al. [6], who reported that the 43 kDa CREB interacts with the glucocorticoid receptor to stimulate the expression of the phosphoenolpyruvate carboxykinase (PEPCK) gene. Moreover our recent studies showed that the expression of the ANG gene in opossum kidney (OK) cells is stimulated by the transfected plasmid containing the cDNA for the 43 kDa CREB [7]. The addition of isoprenaline further enhanced the stimulatory effect of the 43 kDa CREB on the expression of the ANG gene in OK cells [7].

The objective of our present study was to identify the mouse liver nuclear protein(s) that might interact with the putative CRE (i.e. ANG nt −806/−779) of the rat ANG gene. Our studies demonstrate that the CRE of the rat ANG gene interacts with the 43 kDa CREB and a novel 52 kDa nuclear protein from mouse liver. This novel 52 kDa nuclear protein is immunologically different from the 43 kDa CREB, suggesting that this 52 kDa nuclear protein might have a role in the regulation of expression of the ANG gene in the liver.

MATERIALS AND METHODS

Materials

Rabbit polyclonal antibodies against the C-terminus (residues 295–321) of the 43 kDa CREB were purchased from Santa Cruz

reviations used: ANG, angiotensinogen; ATF, activating transcription factor; CRE, cAMP response element; CREB, CRE-binding protein; DTT, threitol; OK, opossum kidney; PEPCK, phosphoenolpyruvate carboxykinase; SOM, somatostatin; TAT, tyrosine aminotransferase.
o whom correspondence should be addressed.

ınsferred to a nitrocellulose membrane (0.45 μm pore size) :hleicher & Schuell, Keene, NH, U.S.A.). The membrane was :ubated with 10% (w/v) non-fat milk proteins in a binding ffer containing 10 mM Hepes, pH 7.0, 10 mM $MgCl_2$, 50 mM ıCl, 0.25 mM EDTA and 2.5% (v/v) glycerol for 1 h at 4 °C. ıe membrane was then washed at least twice with the binding ffer containing 0.25% non-fat milk proteins. Subsequently the :mbrane was hybridized with ^{32}P-labelled double-stranded gonucleotides (approx. 1.0–2.0 pmol; 10^6 c.p.m./ml) in bind-g buffer containing 0.25% non-fat milk proteins and 300 μg/ml ın-denatured herring sperm DNA at 4 °C overnight. The :mbrane was washed, air-dried and exposed for auto-diography.

In competition assays, a 50–100-fold excess of unlabelled NA fragments was incubated with the membrane overnight fore blotting with the radioactive ANG nt −806/−779.

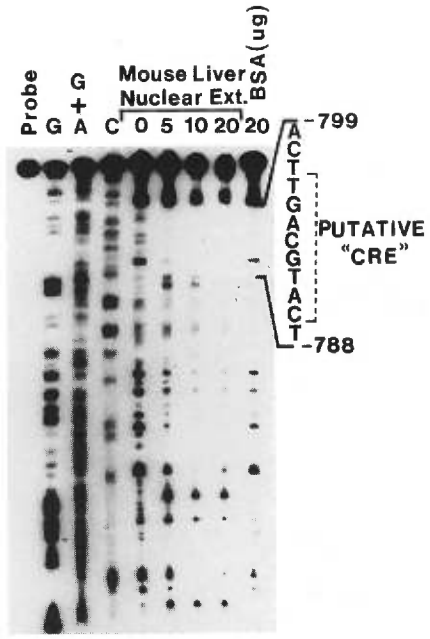
estern blot

estern blot analysis was performed to analyse the mouse liver ıclear proteins by employing rabbit polyclonal antibodies ;ainst the C-terminus (residues 295–321) of the 43 kDa CREB, employing rabbit polyclonal antibodies (Rb#8) against sidues 135–150 of the 43 kDa CREB, Bio-Rad's anti-rabbit ›rseradish peroxidase conjugates and the avidin–horseradish :roxidase conjugates, in accordance with the protocol of the pplier (Bio-Rad, Richmond, CA, U.S.A.).

ESULTS

Nase 1 footprinting protection assay

gure 1 shows that the nucleotides of ANG nt −799/−788 :re protected by the mouse liver nuclear proteins. No protected gion, however, was observed with BSA.



gure 1 DNase 1 footprinting analysis of the DNA fragment, ANG nt -814/−689, of the ANG gene

e DNA was 5′ end-labelled and incubated with BSA (20 μg) or with mouse liver nuclear tract (0–20 μg of protein), as described in the Materials and methods section. The protected NA sequence was ANG nt −799/−788 as indicated. Abbreviation: ug, μg.

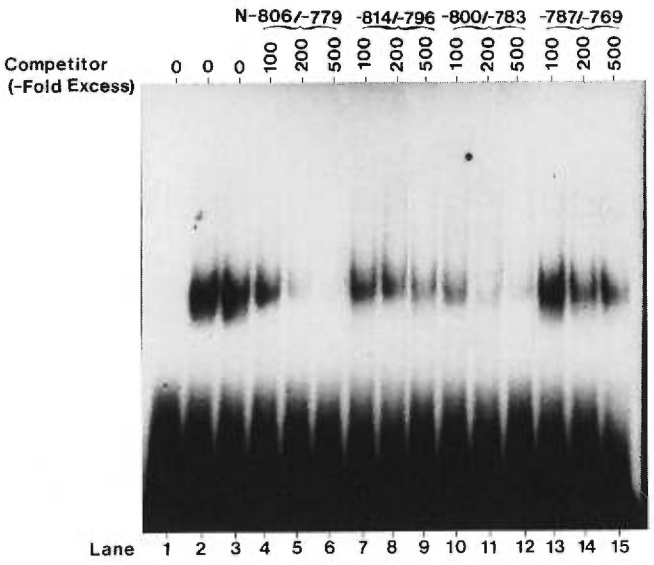


Figure 2 Gel mobility-shift assay of the radioactively labelled DNA fragment ANG nt −806/−779 with the mouse liver nuclear proteins

The labelled DNA probe (0.1 pmol) was incubated with BSA (10 μg) (lane 1) or mouse liver nuclear proteins (10 μg) (lanes 2–15) in the presence of 0.3 i.u. of poly(dI/dC). Competitions with various amounts of unlabelled ANG nt −806/−779, ANG nt −814/−796, ANG nt −800/−783 and ANG nt −787/−769 are shown in lanes 4–6, lanes 7–9, lanes 10–12 and lanes 13–15 respectively. Similar results were obtained in another experiment.

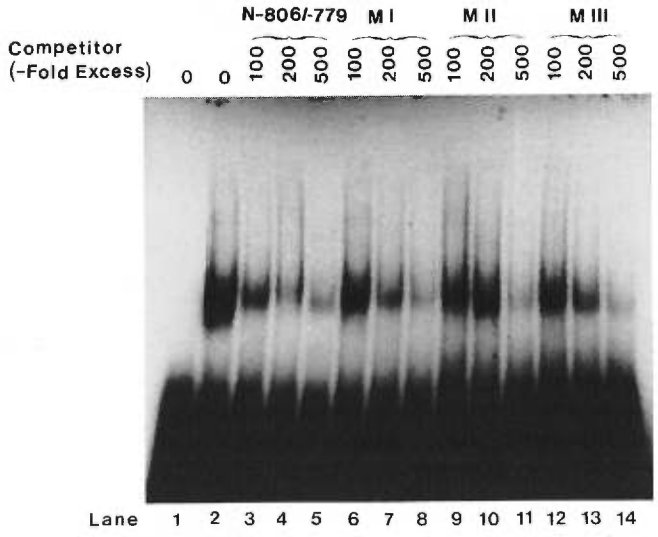
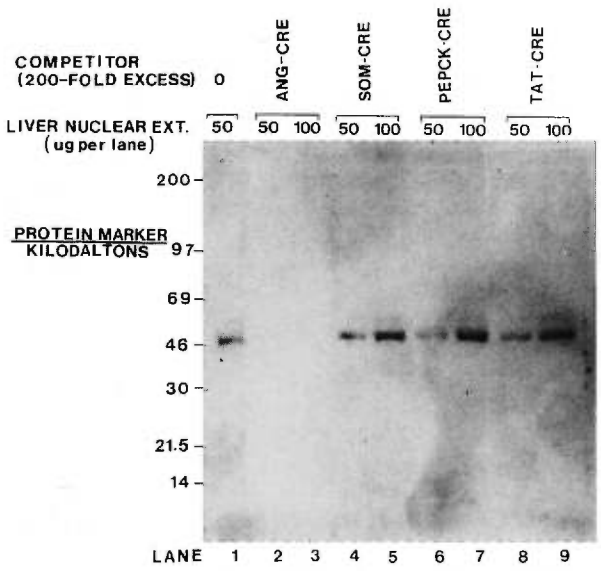


Figure 3 Autoradiography of the gel mobility-shift assay of the radioactively labelled DNA fragment ANG nt −806/−779 with the mouse liver nuclear proteins

The labelled DNA probe (0.1 pmol) was incubated with BSA (10 μg) (lane 1) or mouse liver nuclear proteins (10 μg) (lanes 2–14) in the presence of 0.3 i.u. of poly(dI/dC). Competitions with various amounts of unlabelled ANG nt −806/−779, mutant I, mutant II and mutant III are shown in lanes 3–5, lanes 6–8, lanes 9–11 and lanes 12–14 respectively. Similar results were obtained in two other experiments.

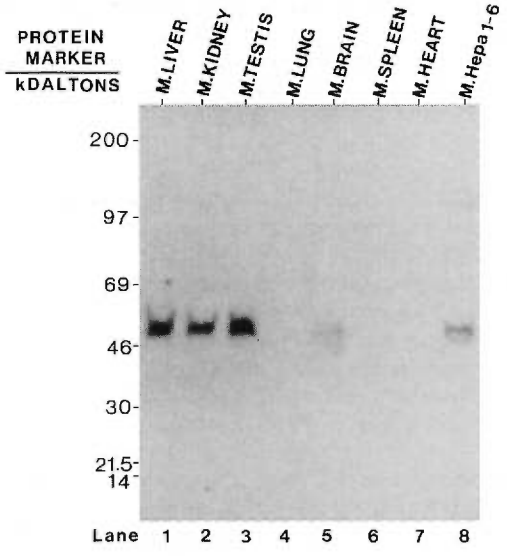
Gel mobility-shift assays

The interaction of the CRE (ANG nt −809/−779) of the rat ANG gene with the mouse liver nuclear proteins was analysed by



ure 6 Effect of addition of the competitor DNA in the Southwestern ılysis with labelled ANG nt −806/−779

ıse liver nuclear extracts (50 or 100 μg per lane) were resolved by SDS/PAGE [4–20% /) gradient gel], transferred to a nitrocellulose membrane, hybridized overnight with 200-fold ıss of unlabelled ANG-CRE (ANG nt −809/−779) (lanes 2 and 3), SOM-CRE (lanes 4 5), PEPCK-CRE (lanes 6 and 7) or TAT-CRE (lanes 8 and 9) at 4 °C. Then the membrane hybridized with radioactively labelled ANG nt −806/−779, washed and subjected to ıradiography.



ure 7 Distribution of the 52 kDa protein in various mouse tissues as ılysed by Southwestern blot analysis

lear extract from various mouse tissues or mouse hepatoma (Hepa 1-6) cells (100 μg per) were resolved by SDS/PAGE [4–20% (w/v) gradient gel], transferred to a nitrocellulose nbrane, hybridized with radioactive ANG nt −806/−779, washed and subjected to ıradiography. Similar results were obtained in another experiment.

Figure 6 shows that the addition of a 200-fold excess of labelled DNA fragment ANG nt −806/−779 competed ectively for the binding of labelled ANG nt −806/−779 to the kDa protein but not of the unlabelled DNA fragment of)M-CRE, PEPCK-CRE or TAT-CRE.

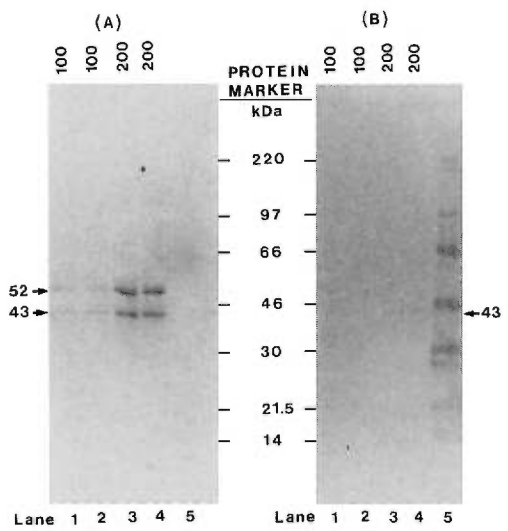


Figure 8 Southwestern and Western blot analysis of immunoreactive 43 kDa CREB from the mouse liver nuclear extract

(**A**) Southwestern blot analysis: different amounts (100 or 200 μg) of mouse liver nuclear extract were resolved by SDS/PAGE [4–20% (w/v) gradient gel], transferred to a nitrocellulose membrane, hybridized with radioactive ANG nt −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 against the C-terminal portion of the the 43 kDa CREB. Rainbow protein markers were used as molecular mass markers. Similar results were obtained in another experiment.

Figure 7 illustrates the tissue distribution of the 52 kDa protein in various mouse tissues. It is apparent that the 52 kDa protein is present in the liver, kidney, testis and brain, as well as in mouse hepatoma (Hepa 1-6) cells. The 52 kDa protein was not detectable in the lung, heart or spleen.

Western blot analysis

Figure 8(A) shows the Southwestern blot analysis of the mouse liver nuclear extract by employing the labelled ANG nt −806/−779. After Southwestern blot analysis, the same membrane was blotted with the polyclonal antibodies against the C-terminus (residues 295–321) of the 43 kDa CREB (Figure 8B). The polyclonal antibodies against the 43 kDa CREB interacted with one species of the 43 kDa CREB-like protein in the mouse liver nuclear extract. The antibodies did not interact with a 52 kDa nuclear protein.

Similarly, Figure 9(A) shows the Southwestern blot analysis of the mouse liver nuclear extract by employing the labelled ANG nt −806/−779. After Southwestern blot analysis, the same membrane was blotted with the polyclonal antibodies (Rb#8) against residues 135–150 of the 43 kDa CREB (Figure 9B). Again, the polyclonal antibodies interacted with the 43 kDa species but not with the 52 kDa species in the mouse nuclear extract. These studies demonstrate that the 52 kDa nuclear protein is immunologically distinct from the 43 kDa CREB.

DISCUSSION

We have previously demonstrated that the transfected pRSV/CREB stimulates the expression of the ANG gene in OK cells in a dose-dependent manner [7]. The addition of isoprenaline further enhances the stimulatory effect of pRSV/CREB [7]. We have also demonstrated that the CREB binds to the CRE (ANG nt −806/−779) of the rat ANG gene [16]. These studies support

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olecular mechanism(s) of action of norepinephrine on the pression of the angiotensinogen gene in opossum kidney cells

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

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

lethods. The fusion genes containing the various lengths of the anking regulatory sequence of the rat ANG gene fused with a an growth hormone (hGH) gene as a reporter were stably sfected into the OK cells. The stimulatory effect of NE on the ression of the fusion genes was evaluated by the amount of unoreactive hGH (IR-hGH) secreted into the culture me- n.

esults. The addition of NE stimulated the expression of the on gene, pOGH (ANG N-1498/+18) in a dose-dependent ner. The stimulatory effect of NE was inhibited in the sence of propranolol, atenolol, Rp-cAMP, yohimbine, staurorine, H-7 and U73122 but not in the presence of ICI 118,551 prazosin. The addition of a combination of isoproterenol and oclonidine synergistically stimulated the expression of pOGH NG N-1498/+18) as compared to the addition of isoproterenol iodoclonidine alone. Furthermore, the addition of NE, forsn, 8-Br-cAMP or phorbol 12-myristate (PMA) stimulated the ression of pOGH (ANG N-806/-779/-53/+18), a fusion gene taining the putative cAMP responsive element (CRE, ANG 06/-779) upstream of the ANG promoter (ANG N-53/+18) in 95 cells, but had no effect on the expression of fusion genes taining the mutant of the CRE.

onclusion. These studies demonstrate that the stimulatory ct of NE on the expression of the ANG gene in OK cells may mediated via both the β_1- and α_2-adrenoceptors and via the E (ANG N-806/-779) in the 5′flanking region of rat ANG e.

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

' **words:** catecholamines, renin-angiotensin system, kidney.

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

To compare the inhibitory effect of propranolol, ateno- , ICI 118,551, yohimbine, prazosin, Rp-cAMP, stauro- rine, H-7 and U73122 on the expression of ANG-GH ion gene in OK cell transformants, various concentra- ns (10^{-13} to 10^{-7} M) of the antagonists or inhibitors were ded in the presence of NE (10^{-9} M) for 24 hours. At the d of the incubation period, media were collected and ot at −20°C until assay for IR-hGH.

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

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

atistical analysis

The experiments were performed at least three to four nes in triplicate. The data were analyzed with Student's est or analysis of variance (ANOVA). A probability level $P \leq 0.05$ was regarded as significant.

ESULTS

fect of norepinephrine on the expression of ANG-GH sion genes in OK cell stable transformants

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

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

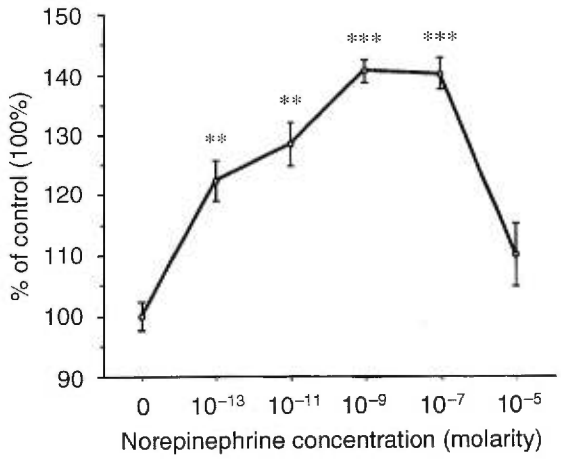


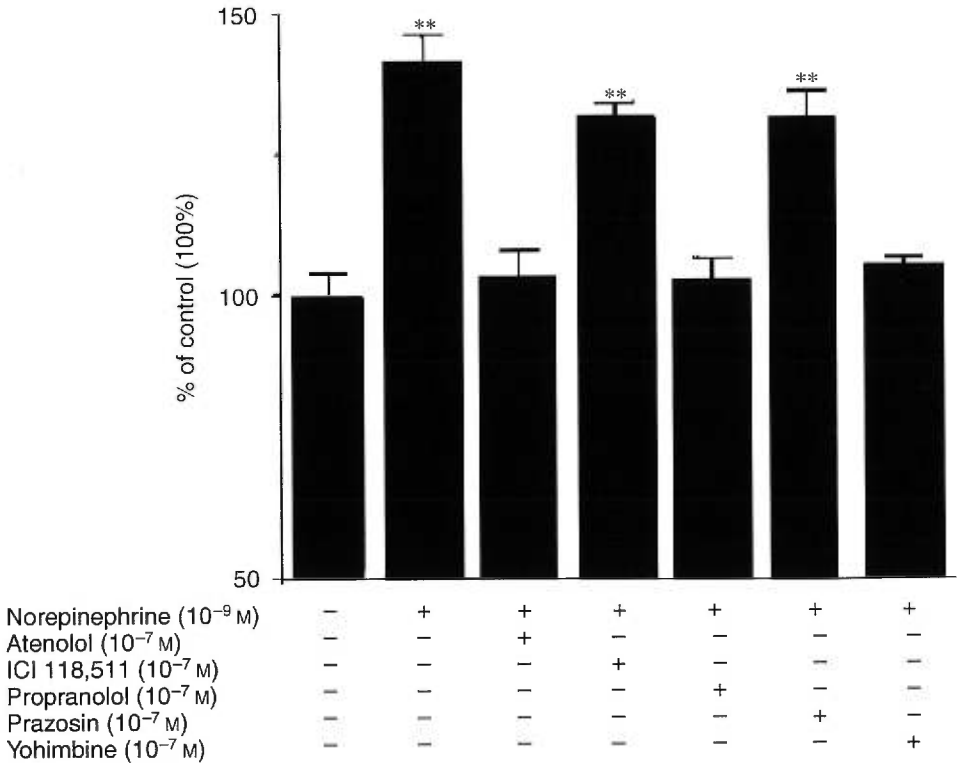
Fig. 1. Effect of norepinephrine on the expression of pOGH (ANG N-1498/+18) in OK 27 cells. Cells were incubated for up to 24 hours in the presence of various concentrations of norepinephrine (10^{-13} to 10^{-5} M). Media were harvested after 24 hours of incubation and assayed for immunoreactive human 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 ± SD of three determinations (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from three other experiements.

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



. 4. Inhibitory effect of adrenoceptor antagonists on the expression of pOGH (ANG N-1498/+18) in OK 27 cells stimulated by norepinephrine $^{-9}$ 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. dia were harvested and assayed for the level of IR-hGH. The concentration of IR-hGH in the absence of norepinephrine or adrenoceptor antagonists he 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 h 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 e obtained from two other experiments.

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

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

Figure 11 shows that the addition of Rp-cAMP (10^{-7} M), 73122 (10^{-7} M) or staurosporine (10^{-7} M) also inhibited e stimulatory effect of NE on the expression of pOGH .NG N-806/-779/-53/+18) in OK 95 cells. These studies monstrate that the stimulatory effect of NE on the pression 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/

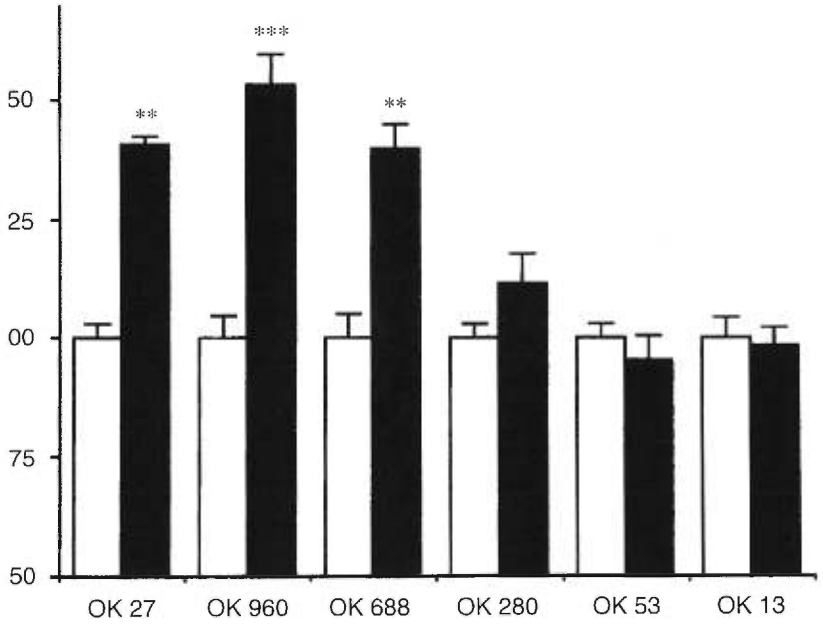
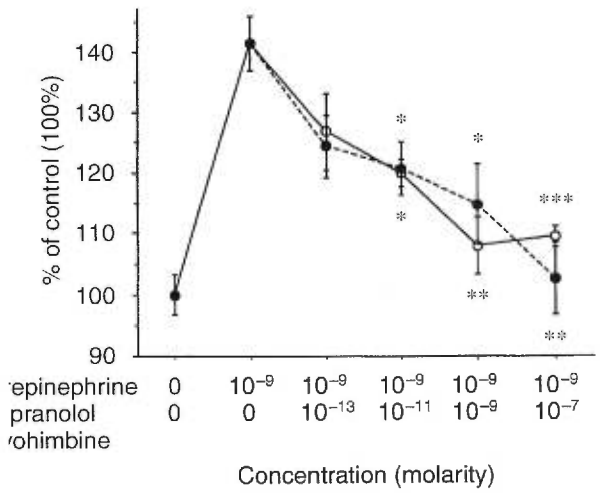


Fig. 8. Effect of norepinephrine on the expression of various ANG-GH fusion genes in OK cells. Cells were incubated for up to 24 hours in the presence of norepinephrine (10^{-9} M). Media were harvested after 24 hours of incubation and assayed for IR-hGH. The concentration of IR-hGH in the medium without 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 ± 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.



). Inhibitory effect of propranolol or yohimbine on the expression of H (ANG N-806/-779/-53/+18) in OK 95 cells stimulated by norepirine (10^{-9} M). OK 95 cells were incubated for up to 24 hours in the nce of norepinephrine (10^{-9} M) and various concentrations (10^{-13} to M) of propranolol or yohimbine. Media were harvested and assayed ie level of IR-hGH. The concentration of IR-hGH in the absence of inephrine or propranolol or yohimbine is the control level (that is, ± 0.06 ng/ml). The inhibitory effect of propranolol or yohimbine was ared to cells which were incubated with 10^{-9} M norepinephrine. lts are expressed as the mean ± SD of three determinations ($^{*}P \leq$ $^{**}P \leq 0.01$ and $^{***}P \leq 0.005$). Symbols are: (○) propranolol; (●) ibine. Similar results were obtained from two other experiments.

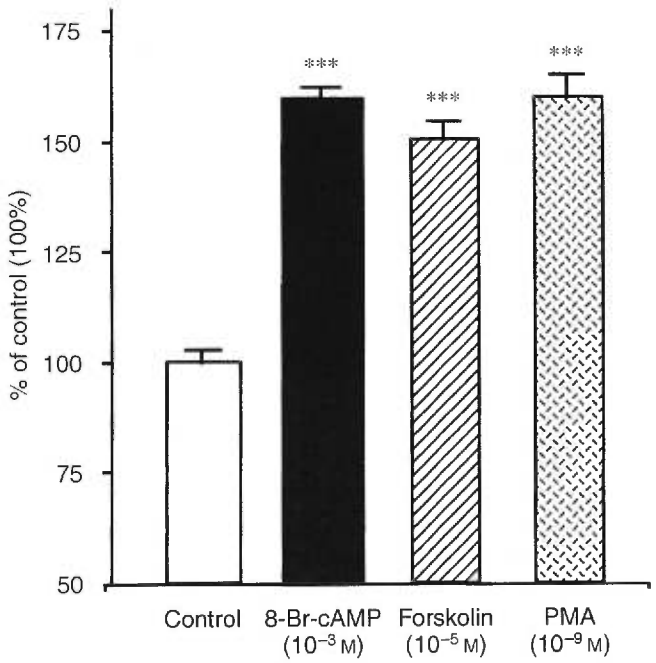


Fig. 10. Effect of 8-bromo-cAMP, forskolin and PMA on the expression of pOGH (ANG N-809/-779/-53/+18) in OK 95 cells. Cells were incubated for 24 hours in the presence of 10^{-3} M 8-Br-cAMP or 10^{-9} M forskolin or 10^{-9} M PMA. The levels of IR-hGH in the media were assayed by RIA-hGH. The concentration 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 ± 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.

signal transduction pathways or via the interaction of pathways in OK 27 cells. The involvement of the PKC way is further supported by the observation that 122 inhibited the stimulatory effect of NE (Fig. 5). e U73122 is an inhibitor of phospholipase C and spholipase A_2, it is conceivable that the addition of 122 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

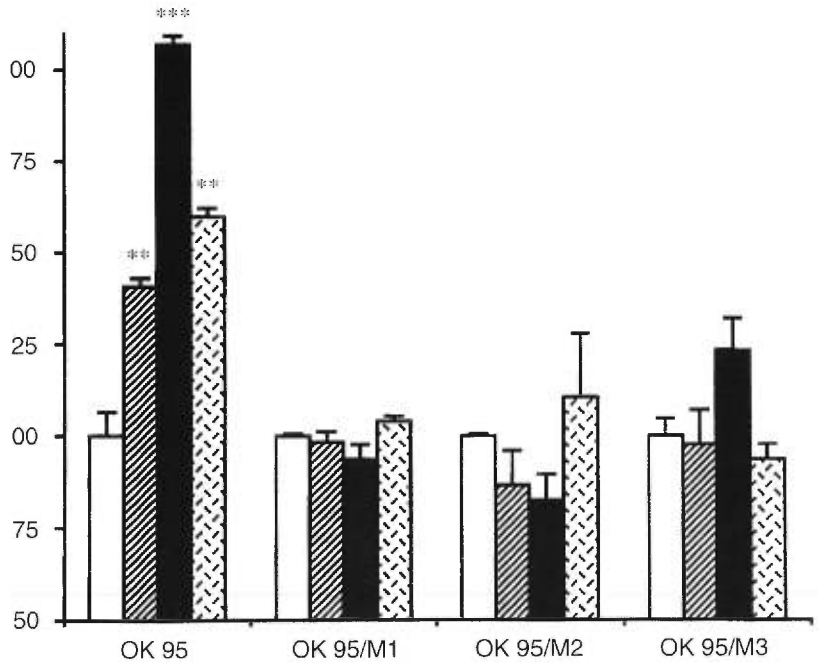


Fig. 12. Effect of norepinephrine, 8-bromo-cAMP and PMA on the expression of pOGH (AnG N-806/-779/-53/+18) and its mutants in OK 95, OK 95/M1, OK 95/M2 and OK 95/M3 cells. Cells were incubated for up to 24 hours in the presence of norepinephrine (10^{-9} M) or PMA (10^{-7} M) or 8-bromo-cAMP (10^{-3} 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 ± SD of three determinations ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.005$). Symbols are: (□) 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.

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

he addition of 8-bromo-cAMP, forskolin or PMA alone stimulated the expression of the pOGH (ANG N-806/- -53/+18) in OK 95 cells compared to the control hout the addition of 8-bromo-cAMP, forskolin or A; Fig. 10). Furthermore, the stimulatory effect of NE the expression of the pOGH (ANG N-806/-779/-53/) in OK 95 cells was inhibited in the presence of cAMP, staurosporine or U73122 (Fig. 11). These stud- provide further support for the notion that the DNA ment, ANG N-806/-779 is the CRE of the rat ANG e.

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

.t present, we do not know the exact molecular mecha- n(s) for the stimulatory effect of NE (that is, the nstream pathway after the activation of PKA and PKC) he expression of pOGH (ANG N-1498/+18) in OK 27 s. One possible explanation may be that NE might ıce the phosphorylation of the nuclear cAMP-respon- 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

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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

Key words: angiotensinogen gene, glucose, opossum kidney cells, growth hormone, renin-angiotensin system.

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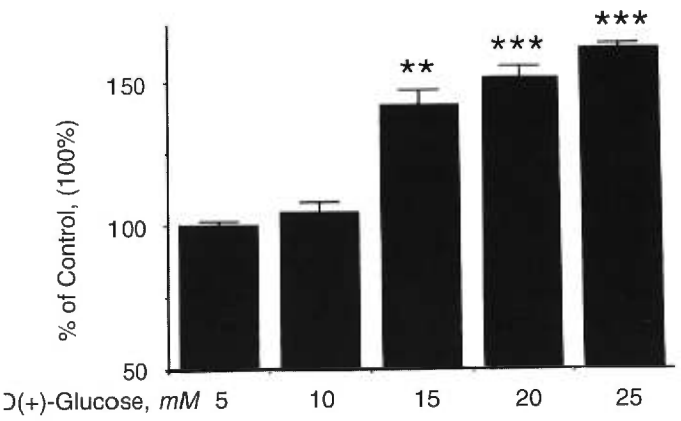


Fig. 1. Effect of D(+)-glucose on the expression of pOGH (Ang N-1498/+18) in opossum kidney (OK) 27 cells. Cells were incubated for up to 24 hours in the presence of various concentrations of D(+)-glucose. Media were collected after 24 hours of incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the medium containing low D(+)-glucose (5 mM) (that is, 1.43 ± 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 ***$P \leq 0.005$). Experiments were repeated three times.

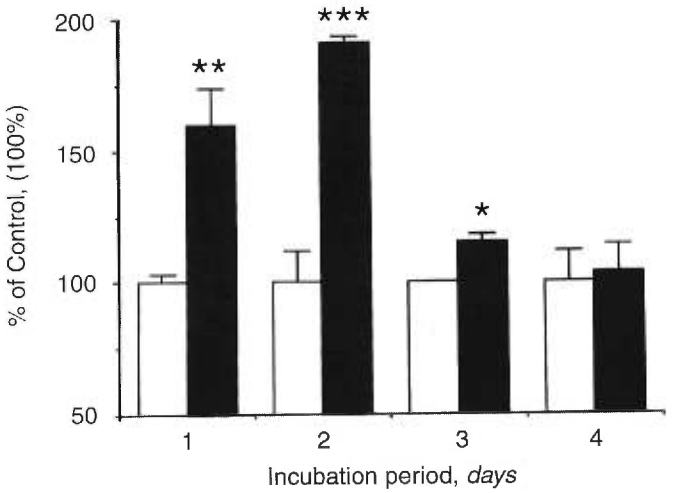


Fig. 2. Effect of incubation period on the expression of pOGH (Ang N-1498/+18) in opossum kidney (OK) 27 cells. Cells were incubated for one to four days in the presence of 5 mM or 25 mM of D(+)-glucose. Media were harvested after various periods of incubation and assayed for IR-hGH. The concentration 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.

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

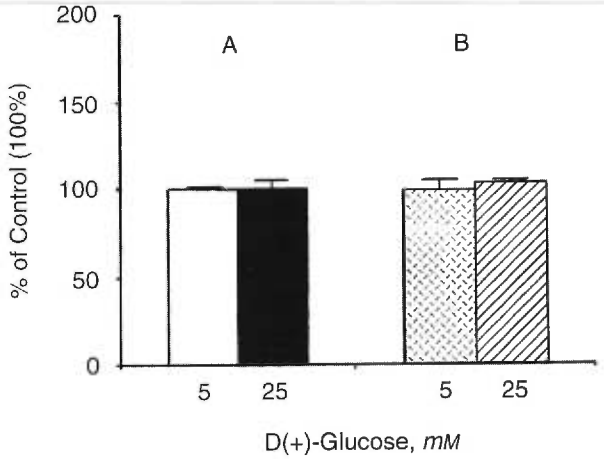
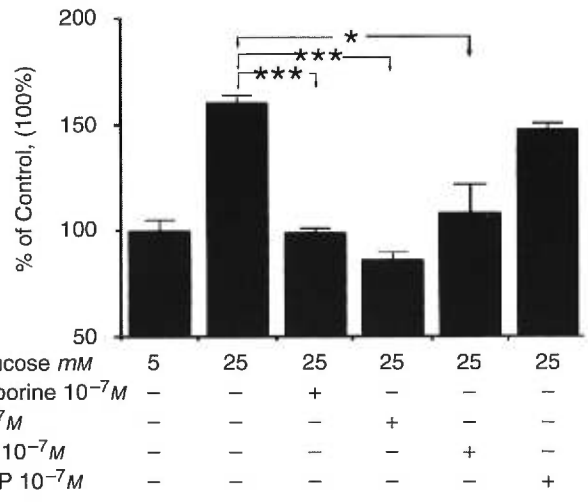
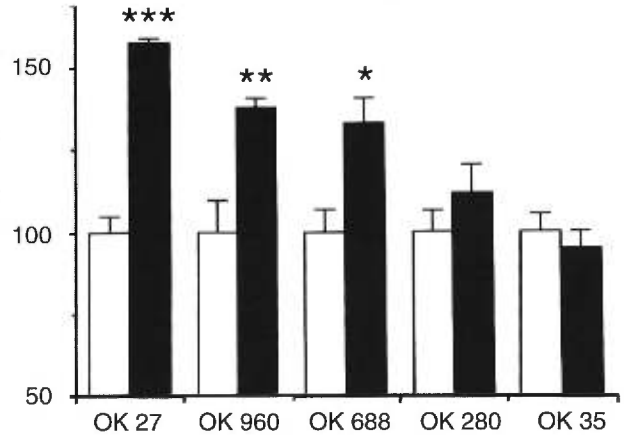


Fig. 3. Effect of the addition of D(+)-glucose on the expression of the pOGH (Ang N-1498/+18) in opossum kidney (OK) 27 cells when the cells were pre-incubated with a high level of glucose or phorbol 12-myristate 13-acetate (PMA). Cells were incubated for 24 hours with 25 mM D(+)-glucose (*A*) or 5 mM 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



6. Inhibitory effect of staurosporine, H-7, U73122 or Rp-cAMP on expression of pOGH (Ang N-1498/+18) in OK 27 cells stimulated by nM D(+)-glucose. Cells were incubated for 24 hours in the presence of M or 25 mM D(+)-glucose plus 10^{-7} M of staurosporine or H-7 or 3122 or Rp-cAMP. Media were harvested and assayed for the level of unoreactive-human growth hormone (IR-hGH). Levels of IR-hGH in medium containing the low D(+)-glucose (5 mM) (that is, 3.72 ± 0.18 nl) in the absence of staurosporine, H-7, U73122 or Rp-cAMP are ressed as 100% (control). The inhibitory effect of staurosporine, H-7, 3122 or Rp-cAMP is compared with cells that were stimulated by 25 D(+)-glucose. Each point represents the mean ± SD of at least three es ($^*P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.005$). Similar results were ained from two other experiments.



7. Effect of D(+)-glucose on the expression of angiotensinogen-wth hormone (Ang-GH) fusion genes in opossum kidney (OK) cells. ls were incubated for up to 24 hours in the presence of 5 mM or 25 mM +)-glucose. Media were harvested after 24 hours of incubation and ayed for IR-hGH. The concentration of IR-hGH in the medium taining low glucose (5 mM) (that is, OK 27 cells, 1.42 ± 0.08 ng/ml; OK , 1.69 ± 0.1 ng/ml; OK 688, 1.43 ± 0.1 ng/ml; OK 280, 1.28 ± 0.1 ng/ml, 35, 1.60 ± 0.12 ng/ml) is considered as the control level. Each point resents 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 eriments.

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

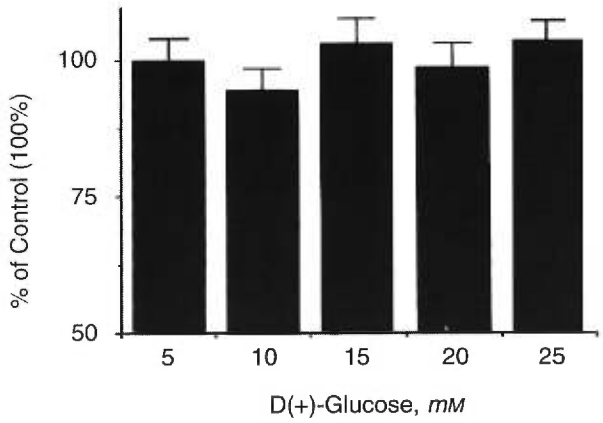


Fig. 8. Effect of D(+)-glucose on the expression of pTKGH in opossum kidney (OK) 13 cells. Cells were incubated for up to 24 hours in the presence of various concentrations of D(+)-glucose. Media were harvested after 24 hours of incubation and assayed for immunoreactive-human growth hormone (IR-hGH). 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.

Studies *in vitro* also showed that the addition of Ang II stimulates the hypertrophy of mouse proximal tubular cells (MCT) and LLC-PK_1 (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

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eprint requests to Dr. John S.D. Chan, University of Montreal, Maison-ve-Rosemont Hospital, Research Center, 5415 Boulevard de l'Assomp-, Montreal, Quebec, Canada H1T 2M4.

PENDIX

bbreviations used in this article are: ACE, angiotensin converting yme; Ang, angiotensinogen; Ang II, angiotensin II; ANOVA, analysis 'ariance; MCT, mouse proximal tubular cells; AT_1-receptor, angioten- II receptor; CRE, cAMP-responsive element; CREB, cAMP-respon- element binding protein; DAG, diacylglycerol; dFBS, depleted fetal ine serum; hGH, human growth hormone; IR-hGH, immunoreactive- nan growth hormone; LLC-PK_1, porcine proximal tubular cells; Neo, omycin; OK, opossum kidney; PKA, protein kinase A; pOGH (Ang 498/+18) and pOGH (Ang N-35/+18), angiotensinogen-growth hor- ne fusion genes; PKC, protein kinase C; PMA, phorbol 12-myristate acetate; RAS, renin-angiotensin system; RIA-hGH, radioimmunoassay human growth hormone; Rp-cAMP, an inhibitor of cAMP-dependent tein kinase A; RSV, Rous Sarcoma Virus; TGF-β, transforming wth factor; TK, thymidine kinase.

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Appendix II: List of Abstracts

1. Wu X.H., Wang T.T. and Chan J.S.D. Effect of glucose, insulin and dexamethasone on the expression of human angiotensinogen gene in opossum kideny cells. The annual meeting of the American Society of Nephrology. Nov.1-5,1997, San Antonio, U.S.A.

2. Wu X.H., Chen X., Wang T.T., Roberts K.D. et Chan J.S.D. Is protein kinase A signal transduction pathway involved in the expression of the angiotensinogen gene in human hepatoma (HepG-2) cells? The annual meeting of Canadian Society of Clinical Investigation, Sept. 25-28, 1997, Vancouver, Canada.

3. Wu X.H., Wang T.T., Chen X., Robert K.D. et Chan J.S.D. Le facteur transcriptionnel CREB (cAMP-responsive element binding protein) et le récepteur glucocorticïdes stimulent synergiquement l'expression du gène de l'angiotensinogen (ANG) dans les cellules hepatoma humaines. XXXVIII Réunion annuelle du Club de Recherches Cliiques du Quebec, Oct. 1996, Quebec, Canada.

4. Wang T.T., Wu X.H., Sajedi N., and Chan J.S.D. Glucose and angiotensinogen gene expression in opossum kidney proximal tubular cells. The annual meeting of Canadian Society of Clinical Investigation, Sept. 25-28, 1997, Vancouver, Canada.

5. Wang T.T., Wu X.H. and Chan J.S.D. Norepinephrine and the cAMP-responsive element binding protein (CREB) on the expression of the rat angiotensinogen (ANG) gene in oppossum kinney (OK) cells. The annual meeting of Canadian Society of Clinical Investigation, Sept. 26-29,1996, Halifax, Canada.

6. Wang T.T., Wu X.H., and Chan J.S.D. Protein kinase A and the cAMP-responsive element binding protein (CREB) stilmulate the expression of the angiotensinogen (ANG) gene in opossum kidney (OK) cells. The annual meeting of the American Society of Nephrology, Nov. 3-6,1996, New Orleans, Louisiana, USA.

7. Wang T.T., Wu X.H., et Chan J.S.D. La norepinephrine (NE) et le facteur transcriptionnel CREB (cAMP responsive-element binding protein) stimulent l'expression du gène de l'angiotensinogène (ANG) dans les cellules rénales du oppossum (OK) XXXVIII Réunion annuelle du Club de Recherches Cliiques du Quebec, Oct. 1996, Quebec, Canada.

8. Chen X., Ming M., Wu X.H., Carrière S., and Chan J.S.D. Molecular mechanism(s) of the synegistical effect of dexamethasome and 8-Bromo-cAMP on the expression of the

angiotensinogen (ANG) gene in opossum kidney cells. The annual meeting of Canadian Society of Clinical Investigation, Sept. 26-29,1996, Halifax, Canada.