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Cardiovascular and sensory abnormalities in a rat model of insulin resistance: beneficial effect of an antioxidant and an angiotensin-1 converting enzyme inhibitor

by

Mahmoud Ali Ismael

Department of Physiology
Faculty of Medicine

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Presented by:

Mahmoud Ali Ismael

The thesis has been evaluated by the following persons:

Madhu Anand-Srivastava
President

Réjean Couture
Research director

Pierre Beaulieu
Member

Abstract

Glucose-fed rat is a model of insulin resistance that displays hypertension and sensory polyneuropathy. This study aimed at comparing the beneficial effects of an antioxidant (N-acetyl-L-cysteine, NAC) and an angiotensin-1 converting enzyme inhibitor (ramipril) in glucose-induced tactile and cold allodynia, hypertension, plasma levels of glucose, insulin, malondialdehyde (MA) and 4-hydroxynonenal (4-HNE), liver/aortic superoxide anion, changes of skeletal muscle insulin receptor substrate-1 (IRS-1) protein expression and of tissue kinin B_1 receptor mRNA. **Methods:** Male Wistar rats (50-75 g) were given 10% D-glucose in their drinking water during 11 and 20 weeks. NAC (1-2 g/kg/day orally) and ramipril (1 mg/kg/day in drinking water) were administered for the last 4-5 weeks. **Results:** Systolic blood pressure, plasma levels of insulin and glucose as well as insulin resistance (HOMA index) were significantly higher in rats treated with glucose for 20 weeks. This was associated with a higher production of superoxide anion and NADPH oxidase activity in aorta and liver and with a marked reduction of IRS-1 protein expression in the gastrocnemius muscle. Tactile and cold allodynia occurred after six weeks of glucose treatment and B_1 receptor mRNA was increased in the spinal cord and renal cortex at 11 weeks. NAC restored all these alterations in glucose-fed rats and decreased plasma MA and 4-HNE levels. Although ramipril provided the same therapeutic effect as that of NAC on blood pressure and allodynia, it was less effective in reducing insulin resistance and failed to reduce liver/aortic NADPH oxidase activity and plasma levels of MA and 4-HNE. Ramipril normalized superoxide anion only in the aorta.

Conclusion: The beneficial effects of NAC and ramipril on insulin resistance, hypertension and allodynia were linked to the reduction of the oxidative stress and kinin B_1 receptor expression. The antioxidant effect of NAC involved the inhibition of NADPH oxidase and lipid peroxidation while that of ramipril was exerted most strongly in vascular tissue independently of NADPH oxidase and lipid peroxidation.

Key words: allodynia, diabetes, hypertension, insulin resistance, kinin B_1 receptor, N-acetyl-L-cysteine, oxidative stress, polyneuropathy

Résumé

L'hypertension et les polyneuropathies apparaissent chez le rat recevant du glucose, un modèle de résistance à l'insuline. Cette étude a pour but de comparer, chez le rat recevant du glucose, les effets bénéfiques d'un antioxydant (N-acétyl-L-cystéine, NAC) et d'un inhibiteur de l'enzyme de conversion de l'angiotensine-1 (ramipril) sur l'allodynie tactile et au froid, l'hypertension, la glycémie, l'insulinémie, les taux plasmatiques de malondialdéhyde (MA) et de 4-hydroxynonenal (4-HNE), l'anion superoxyde dans l'aorte et le foie, les changements d'expression protéique de «insulin receptor substrate-1» (IRS1) dans le muscle gastrocnemius ainsi que sur l'expression tissulaire (ARNm) du récepteur B_1 des kinines. **Méthodes :** Des rats mâles de 50-75 g ont reçu 10% de glucose dans l'eau de boisson pendant 11 et 20 semaines. NAC (1-2 g /kg/jour oralement) et ramipril (1 mg/kg/jour dans l'eau de boisson) ont été administrés pendant les 4 et 5 dernières semaines. **Résultats :** La pression systolique, la glycémie, l'insulinémie ainsi que la résistance à l'insuline (indice HOMA) étaient significativement augmentées chez le rat recevant du glucose pendant 20 semaines. Ceci était associé avec une plus grande production de l'anion superoxyde et de l'activité de la NADPH oxydase dans l'aorte et le foie et avec une réduction marquée de l'expression de l'IRS-1 dans le gastrocnemius. L'allodynie tactile et au froid apparaissaient après six semaines de traitement au glucose et l'ARNm du récepteur B_1 était augmenté dans la moelle épinière et le cortex rénal à 11 semaines. Le NAC a corrigé toutes ces anomalies chez le rat recevant du glucose et a diminué les taux de MA et 4-HNE. Bien que le ramipril ait produit les mêmes effets thérapeutiques que le NAC sur la pression artérielle et l'allodynie, il s'est avéré moins efficace à réduire la résistance à l'insuline et n'a pas

réduit l'activité de la NADPH oxidase dans le foie ou l'aorte ou encore les taux de MA et de 4-HNE. Le ramipril a toutefois normalisé la production d'anion superoxide dans l'aorte.

Conclusion : Les effets bénéfiques du NAC et du ramipril sur la résistance à l'insuline, l'hypertension et l'allodynie sont liés à la réduction du stress oxydatif et à l'expression du récepteur B_1 des kinines. L'effet antioxydant du NAC implique l'inhibition de la NADPH oxydase et de la peroxydation des lipides, tandis que celui du ramipril est exercé principalement sur les vaisseaux indépendamment de la NADPH oxidase et de la peroxydation des lipides.

Mots clés : allodynie, diabète, hypertension, résistance à l'insuline, récepteur B_1, stress oxydatif, polyneuropathie et N-acétyl-L-cystéine

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CHAPTER FOUR

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List of abbreviations

AGEs	Advanced glycation end products
ACEI	Angiotensin-converting enzyme inhibitor
Ang II	Angiotensin II
ANOVA	Analysis of variance
B_1R	Kinin B_1 receptor
B_2R	Kinin B_2 receptor
BH_4	Tetrahydrobiopterin
CAP	Cbl-associated protein
CoQ_{10}	Coenzyme Q_{10}
CNS	Central nervous system
Cu/Zn-SOD	Cu/Zn superoxide dismutase
CO_2	Carbon dioxide
DNP	Diabetic neuropathic pain
DPNP	Diabetic peripheral neuropathic pain
Gab-1	Growth factor receptor-binding protein 2-associated binder-1
GLUT	Glucose transporter
Grb-2	Growth factor receptor-binding protein 2
GPX	Glutathione peroxidase
GSH	Reduced glutathione
GSSH	Oxidized glutathione
$^{\bullet}HRO_2^{-}$	Hydroperoxyl
H_2O_2	Hydrogen peroxide

HOMA	Homeostasis Model assessment
HOCl	Hydrochlorous acid
iNOS	Inductible Nitric Oxide Synthase.
IP3	Inositol 1, 4, 5-triphosphate
IRS-I	Insulin receptor substrate-1
LA	Lipoic acid
LDL	Low-density lipoprotein
MAPKs	Mitogen-activated protein kinase
MDA	Malondialdehyde
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NAC	N-acetyl-L-cysteine
NO	Nitric oxide
$^{\bullet}O_2^-$	Superoxide anion
OH	Hydroxyl group
PARP	Poly ADP-ribose polymerase
PBN	Phenyl-N-tert-butylnitrone
PI 3-kinase	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RO_2	Peroxyl group
SOD	Superoxide dismutase
SBP	Systolic Blood Pressure

SH	Sulfhydryl group
SH2	Src homology 2
SHR	Spontaneous Hypertensive Rats
SREBPs	Sterol Regulatory Element Binding Proteins
ZDF	Zucker Diabetic Fatty rats

Dedication

To my wife, Salma

To my kids, Firdus, Lemia, Mohamed, Ali, Yusef

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I wish to express my sincere appreciation to my supervisor Professor Réjean Couture for his assistance in the preparation of this thesis and who read my numerous revisions and helped to make some sense out of the confusion. Thanks to the El-Mergeb University, Elkhoms-Libya which provided me with the financial means to complete this project. Finally, thanks to my wife, children, and numerous friends who endured this long process with me, always offering support and love.

CHAPTER ONE

INTRODUCTION

1. Introduction

1.1 Diabetes Mellitus

Definition: Diabetes mellitus is a chronic disease caused by inherited and/or acquired deficiency in production of insulin by islets of pancreas, or by the ineffectiveness of the insulin produced. Deficiencies lead to increased concentrations of glucose in the blood, which in turn damage many of the body's systems, in particular the blood vessels and nerves. It is probably the most important metabolic disease and is widely spread all over the world. Diabetes mellitus is a chronic disease that requires long-term medical consideration both to limit the development of its harmful complications and to manage them when they do occur. Because of the huge premature morbidity and mortality associated with the disease, prevention of complications is the key issue.

1.2 Type 1 Diabetes Mellitus

It was formerly known as insulin-dependent diabetes, in which the pancreas fails to produce the insulin which is essential for survival. Most cases of type 1 diabetes are immune-mediated characterized by autoimmune destruction of insulin-producing ß cells in the islets of langerhans of the pancreas by CD4+ and CD8+ T cells and macrophages infiltrating the islets (Foulis et al., 1991). This form develops most frequently in children and adolescents, but is being increasingly noted in adult people. The disease accounts for about 10% of all cases of diabetes, occurs most commonly in people of European descent and affects 2 million people in Europe and North America.

There is a marked geographic variation in incidence, with a child in Finland being about 400 times more likely than a child in Venezuela to acquire the disease (Gillespie, 2006). (Figure 1)

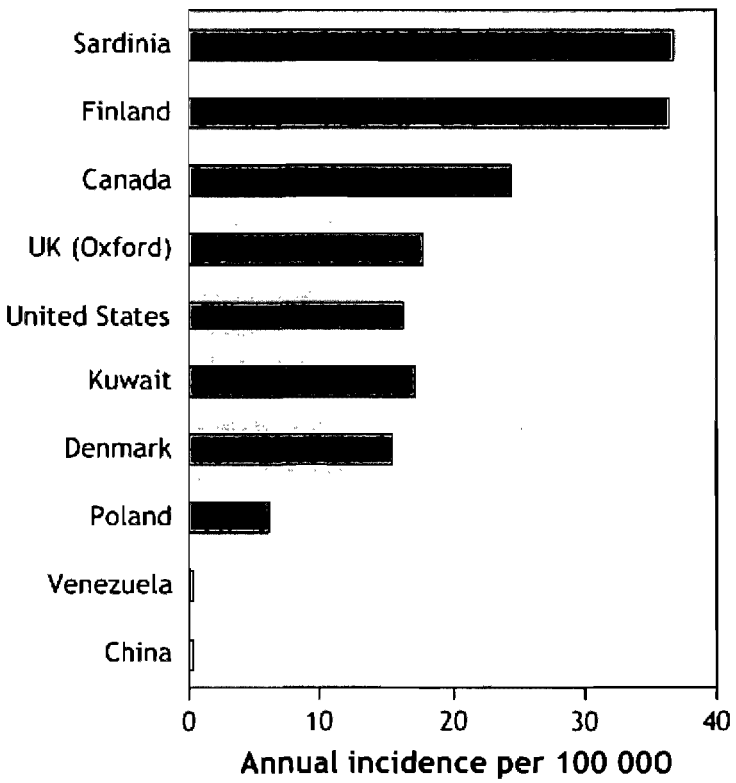

Figure 1 Geographic variation in annual incidence of type 1 diabetes.

The current global increase in incidence of 3% per year is well reported. Furthermore, the incidence of type 1 diabetes will be 40% higher in 2010 than in 1998 (Onkamo et al., 1999). This rapid rise strongly suggests that the action of the environment on susceptibility genes contributes to the evolving epidemiology of type 1 diabetes (Gillespie, 2006). People with type 1 diabetes depend on regular insulin injections with multiple self care tasks to achieve best blood glucose control.

1.3 Type 2 Diabetes Mellitus

It was formerly named non-insulin-dependent diabetes, which results from the body's inability to respond properly to the action of insulin produced by the pancreas. Type 2 diabetes is much more common than type 1 and approximately accounts for 90% of all diabetes cases worldwide. It occurs most frequently in adults, but is being noted increasingly in adolescents as well (Likitmaskul et al., 2003; Hotu et al., 2004). Most of type 2 diabetic patients are not diagnosed until the individual has had the disease for many years, and the microvascular complications of diabetes (retinopathy, nephropathy and neuropathy) are already present (Spijkerman et al., 2003). Lifestyle modification is the cornerstone of both treatment and attempts to prevent type 2 diabetes (Mann et al., 2004).

1.4 Global prevalence of diabetes

The prevalence of diabetes for all age-groups worldwide was estimated to be 2.8% in 2000 and 4.4% in 2030. The number of people with diabetes is expected to increase from 171 million in 2000 to 366 million in 2030. The proportion of diabetic patients is increasing due to growth of population, aging, change in life style, and increasing prevalence of obesity and physical inactivity (Woods et al., 2004). The morbidity and mortality associated with diabetes are related to the short- and long-term complications. These complications include hypoglycemia and hyperglycemia, increased risk of infections, microvascular and macrovascular complications.

1.5 Complications associated with Diabetes Mellitus

1.5.1 Microangiopathy

The microangiopathy is arising from small blood vessels disease which includes:

(a) Diabetic retinopathy

Diabetic retinopathy is one of the most common microvascular complications, the most frequent cause of new cases of blindness in Europe and North America in age group of 30 to 70–74 years (Villar et al., 1999; Stitt et al., 2002). Recent clinical studies have revealed that the presence of diabetic retinopathy is predictable of diabetic nephropathy (Villar et al., 1999; El-Asrar et al., 2001; Rossing et al., 2002). Due to its high content of unsaturated lipids and high oxygen demand, the retina represents a site which is partially prone to hyperglycemia induced free radical generation and lipid oxidation. Furthermore, recent studies have shown that NF-kB activation in retinal pericytes is responsible for hyperglycemia-induced loss of pericytes observed in diabetic retinopathy. Loss of vision due to certain types of glaucoma and cataract occurs primarily due to age, but is more common in diabetes. Moreover, cataract is common in diabetic person where superoxide anion in the mitochondria is elevated as a result of hyperglycemia (Vinson, 2006). Clinical trials suggest that blood glucose control and control of hypertension can delay the onset and progression of diabetic retinopathy and loss of vision associated with diabetes (Grassi, 2003; Fong et al., 2004a; Fong et al., 2004b). Morover, loss of vision due to diabetic retinopathy can be prevented by early detection and treatment of vision-threatening retinopathy by regular eye examinations and timely intervention with laser treatment, or through surgery in cases of advanced retinopathy.

(b) Peripheral neuropathy

Neuropathy is an early clinical sign of diabetes affecting sensory and autonomic peripheral functions. Studies suggest that about 30-50% of people with type 2 diabetes are affected to some degree of neuropathy (Dickinson et al., 2002; Feldman, 2003). Moreover, diabetic neuropathies are the most common chronic disturbing complications of diabetes mellitus because of the pain, discomfort, and disability. Pain or numbness in the legs or feet may be the most common complaint from diabetic neuropathic patients (Park et al., 2004), who also display autonomic dysfunction (especially erectile dysfunction and altered cardiac vagal response). The pathogenesis of diabetic neuropathic pain is still unknown, yet several mechanisms were proposed. These include: (1) axonal degeneration/regeneration, (2) neuroma properties, which cause ectopic impulse generation and ephaptic transmission, (3) small-fiber diseases, which involve the Aδ and C-fibers, (4) dorsal root ganglion involvement, and (5) central sensitization and neural plasticity. The early detection and diagnosis of diabetic neuropathies are important to reverse and prevent their progression (Park et al., 2004). Diabetic patients who are inadequately treated have higher morbidity and complication rates related to neuropathy than patients whose blood glucose is closely controlled. Peripheral neuropathy represents the main etiologic factor involved in the development of diabetic foot ulceration and lower limb amputation.

c) Erectile dysfunction

Erectile dysfunction is commonly associated with diabetes and occurs at an earlier age in such patients than in the general population. Hyperglycemia has pathologic effect on peripheral tissue innervation and vascularization, both of which are critical for erectile function. Oxidative stress to cavernous tissue may be an important causative factor to erectile dysfunction in diabetic patients (Ryu et al., 2005).

(d) Diabetic nephropathy

Nephropathy is an irreversible complication of diabetes representing 30% of all new cases of end-stage renal failure and the most distressing and money-consuming complication in patients with diabetes throughout the world. The principal lesion of diabetic nephropathy occurs in renal glomeruli and is called diabetic glomerulosclerosis. Hyperglycemia is responsible for the development and progression of diabetic nephropathy through different metabolic pathways, including increased oxidative stress, renal polyol formation, activation of protein kinase C (PKC)-mitogen-activated protein kinases (MAPKs), and accumulation of advanced glycation end products, as well as hemodynamic factors such as systemic hypertension and increased intraglomerular pressure (Kikkawa, 2000).

(e) Diabetic cardiomyopathy

This prominent cardiovascular complication has been recognized as a microvascular disease that may lead to heart failure. Pathogenesis of diabetic cardiomyopathy involves vascular endothelial cell dysfunction, and cardiomyocyte necrosis (Devereux et al., 2003). Chronic hyperglycemia induces several biochemical

changes including increased non-enzymatic glycation, sorbitol-myoinositol-mediated changes, redox potential alterations, and protein kinase C (PKC) activation, all of which have been implicated in diabetic cardiomyopathy (Farhangkhoee et al., 2006)

1.5.2 Macrovascular complications

The development of macrovascular complications, including cardiac, cerebrovascular, and peripheral vascular complications, is an important concern considering that a substantial proportion of premature deaths in patients with type 1 diabetes mellitus (Deckert et al., 1978), and most deaths in type 2 diabetes mellitus are related to macrovascular disease (Morrish et al., 1990; Morrish et al., 1991).

(a) Hypertension

Patients with diabetes have a much higher rate of hypertension than would be expected in the general population. There is an increased prevalence of hypertension among diabetic patients (Sowers et al., 2000). Population studies suggest that blood pressure in excess of 140/90 mmHg is found in nearly 30% of adults having type 2 diabetes. Moreover, both conditions are strongly age-dependent, and exhibit large geographical variations. These two medical conditions tend to occur together in the same patients; approximately 2/3 of diabetics will have hypertension while hypertensive persons, have a substantially increased risk of diabetes. Elevated blood pressure is known to contribute to diabetic microvascular and macrovascular complications. Hypertension in patients with diabetes increases the risk of coronary artery disease. In the general population, the prevalence of coronary artery disease lies at around 1% to 4 %, but this may increase by as much as fourfold in older adult diabetic patients, compared with non diabetic individuals of the same age. Also, the

risk of heart failure has been shown to increase twofold for diabetic men and fivefold for diabetic women, relative to nondiabetic individuals (Krum, 2003). In addition, more than one third of patients with myocardial infarction also suffer from clinically diagnosed type 2 diabetes (Norhammar et al., 2002). Hypertension increases the incidence of stroke in patients with diabetes. Survival rates and recovery from stroke are reduced in patients with diabetes compared with patients without diabetes; also hypertension increases the risk of peripheral vascular disease and subsequent foot ulcers and amputations in patients with diabetes. Hypertensive diabetic patients are also at increased risk for diabetes-specific complications including retinopathy and nephropathy. Heart disease accounts for approximately 50% of all deaths among people with diabetes in industrialized countries. Diabetes mellitus induces abnormal changes in the structure of different components of the heart including the plasma membrane and other cytoplasmic organelles of cardiomyocyte. Pathological findings include cell hypertrophy, neuropathy, interstitial fibrosis, myocytolysis, apoptosis and lipid deposits in the heart of diabetic patients (Adeghate, 2004).

(b) Peripheral vascular disease

The diabetic foot disease, due to changes in blood vessels and nerves, often leads to ulceration and subsequent limb amputation. It is one of the most costly complications of diabetes, especially in communities with inadequate footwear. It results from both vascular and neurological disease processes. The impairment of microcirculation of diabetic patients leads to secondary complications in lower limbs, as foot infections and ulcerations. These microcirculatory changes, which are mainly functional rather than structural, are responsible for the impaired ability of the microvasculature to vasodilate in response to injury, and nerve reflex related

microvascular vasodilatation is also impaired in the diabetic population (Schramm et al., 2006). Diabetes is the most common cause of non-traumatic amputation of the lower limb, which may be prevented by regular inspection and good care of the foot.

1.6 Diabetes Mellitus and oxidative stress

1.6.1 Source of oxidative stress in diabetes

Oxidative stress has been considered to be a common pathological factor of diabetes complications and appears a target for therapeutic treatments (Shih et al., 2002). Tissue exposure to hyperglycemia results in increased production of reactive oxygen species (ROS). Furthermore, ROS and reactive nitrogen species (RNS) are products of normal cellular metabolism, and recognized for playing a double role as both harmful and beneficial to living systems (Valko et al., 2006). Beneficial effects of ROS occur at low/moderate concentrations and involve physiological roles in cellular responses to anoxia, in defense against infectious agents, in a number of cellular signaling systems, and induction of a mitogenic response. Oxidative stress is defined in general as excess formation and/or inadequate removal of highly reactive molecules such as ROS and RNS (Turko et al., 2001; Maritim et al., 2003). The sources for the overproduction of ROS in diabetes are widespread and include enzymatic pathways, auto-oxidation of glucose, and mitochondrial superoxide production. ROS include free radicals such as superoxide anion ($^{\bullet}O_2^-$), hydroxyl (OH), peroxyl (RO_2), hydroperoxyl ($^{\bullet}HRO_2^-$) and nonradical species such as hydrogen peroxide (H_2O_2) and hydrochlorous acid (HOCl) (Turko et al., 2001; (Evans et al., 2002). RNS include free radicals like nitric oxide ($^{\bullet}NO$) and nitrogen dioxide ($^{\bullet}NO_2^-$) as well as nonradicals such as peroxynitrite ($ONOO^-$), nitrous oxide (N_2O) and alkyl peroxynitrate (RONOO) (Turko et al., 2001; Evans et al., 2002). Reactive molecules,

superoxide anion, $^{\bullet}NO$ and $ONOO^{-}$ are the main species which play important roles in diabetic complications. In diabetes, the antioxidant defense is blunted whereas the generating system is stimulated (Dickinson et al., 2002). Induction of ROS formation can result from different additive mechanisms. These mechanisms include direct intracellular effect of glucose in cells subjected to increase glucose uptake during hyperglycemia (renal, retinal, and some nerves cells) and indirect via the extracellular formation of advanced glycation end products (AGEs). Excess generation of oxidative stress has pathological consequences including damage to proteins, lipids and DNA. $^{\bullet}O_2^{-}$ can activate several damaging pathways in diabetes including accelerated formation of AGE, polyol pathway, hexosamine pathway and PKC, nicotinamide adenine dinucleotide phosphate (NAD(P)H) (Kitada et al., 2003), mitochondrial electron-transport chain (Brownlee, 2001), all of which were proven to be involved in micro- and macrovascular complications. In addition, $\bullet O_2^{-}$ and H_2O_2 stimulate stress-related signalling mechanisms such as NF-κB, p38-MAPK and STAT-JAK resulting in vascular smooth muscle cells migration and proliferation. In endothelial cells, H_2O_2 mediates apoptosis and pathological angiogenesis (Taniyama and Griendling, 2003).

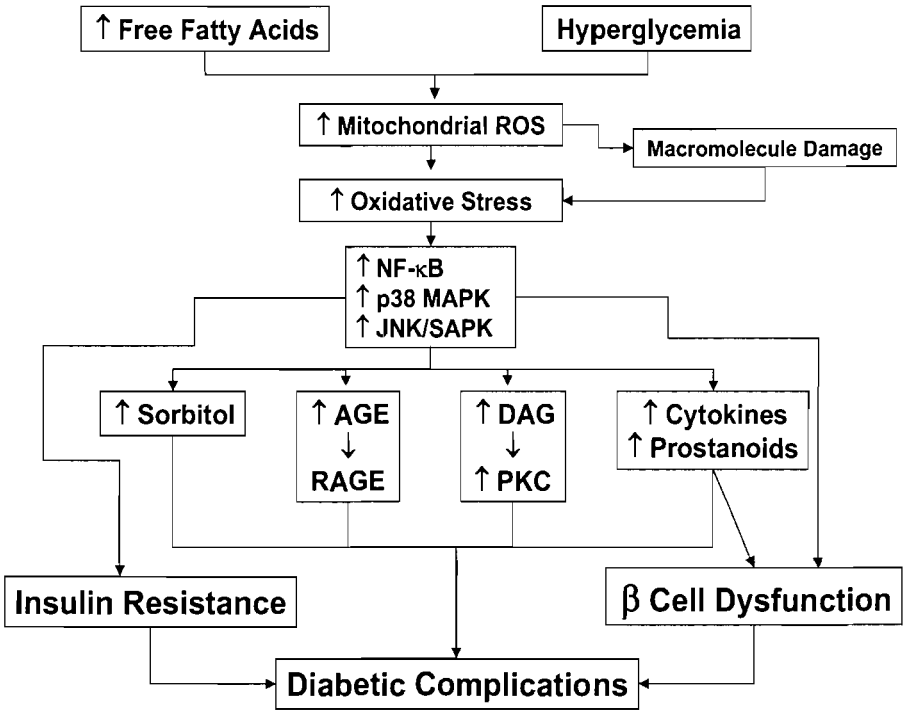

Figure 2 Relationship between oxidative stress and the development of type 2 diabetes (Evans et al., 2002).

Figure 2, shows the proposed causative link between hyperglycemia, elevated free fatty acid (FFA), mitochondrial ROS generation, oxidative stress, activation of stress-sensitive pathways (NF-kB, p38 MAPK, JNK/SAPK, and others), insulin resistance, ß-cell dysfunction, and diabetic complications (Nishikawa et al., 2000). The activation of NAD(P)H oxidase by protein kinase C produces the predominant source of reactive oxygen species in vasculature that directly lead to diabetic complications and cardiovascular disease (Kitada et al.,2003; Feldman, 2003). ROS can stimulate oxidation of low-density lipoprotein (LDL), and oxidized-(ox)-LDL, which is not recognized by the LDL receptors. The ox-LDL which formed can be taken up by scavenger receptors in macrophages leading to foam cell formation and atherosclerotic plaques as shown in Figure 3 (Taniyama et al., 2001).

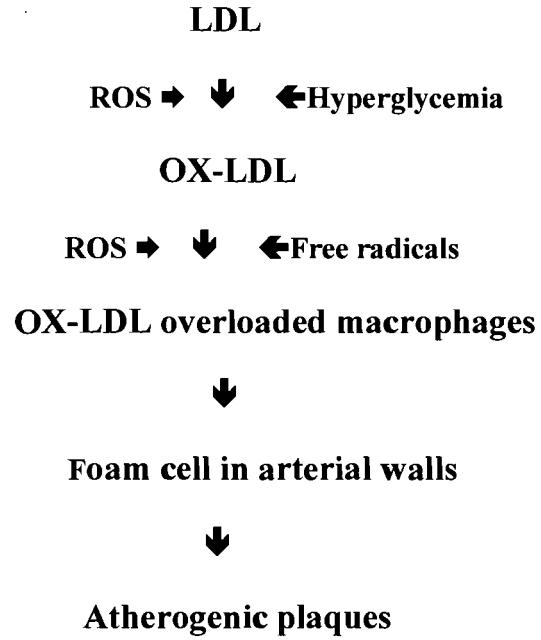

Figure 3 Pathogenic cascade of hyperglycemia, oxidative stress, LDL oxidation, arteriogenic plaque formation and myocardial infarction rate.

Under normal conditions, $^{\bullet}O_2^-$ is immediately eliminated by natural defense mechanisms, but in excess $^{\bullet}O_2^-$ reacts with $^{\bullet}NO$ immediately and generates cytotoxic $ONOO^-$, which is a strong oxidant. This reaction has several consequences. First, $ONOO^-$ alters functions of biomolecules by protein nitration and lipid peroxidation. Increase levels of nitrotyrosine are associated with apoptosis of myocytes, endothelial cell and fibroblasts in diabetes (Turko et al., 2001). Second, $ONOO^-$ causes single-strand DNA breakage which in turn activates nuclear enzyme poly (ADP-ribose) polymerase (PARP) (Soriano et al., 2001). Third, it decreases $^{\bullet}NO$ bioavailability causing impaired relaxation and inhibition of the antiproliferative effects of $^{\bullet}NO$ (Maritim et al., 2003). Furthermore, $ONOO^-$ oxidizes tetrahydrobiopterin (BH_4), an important cofactor for NOS, and causes uncoupling of NOS, which produces $^{\bullet}O_2^-$

instead of 'NO (Maritim et al., 2003). All these pathological modifications contribute to the pathogenesis of vascular complications of diabetes.

1.7 Antioxidants and diabetes

1.7.1 Defense system against oxidative stress

Exposure to free radicals from different sources has led organism to develop a series of defense mechanisms (Cadenas, 1997). The defense mechanisms against free radical-induced oxidative stress involve: (1) preventive mechanisms, (2) repair mechanisms, (3) physical defenses, and (4) antioxidant defenses (Valko et al., 2006). Under normal physiological conditions, our body constantly produces ROS and RNS, which are eliminated by antioxidant enzymes as primary antioxidants. But when the production of ROS and RNS is significantly increased, the enzyme systems are rapidly overloaded. The oxidation may be slowed down by secondary antioxidants provided in the diet. Under normal conditions, there is a balance between the activities and the intracellular levels of these antioxidants. This balance is essential for the survival of organisms and their health.

(a) Primary antioxidants: active detoxification

The cell has antioxidant enzymes, which are very effective defense systems because enzymes have the property to eliminate free radicals in a constant manner. This line of defense is composed of three major antioxidant enzymes, superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase, which differ from each other in structure, tissue distribution, and cofactor requirement. Their substrates are reactive species; they change superoxide anions and hydrogen peroxide into non-harmful products.

SOD: $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

GPX: $ROOH + 2GSH \rightarrow ROH + H_2O +$ glutathione

Catalase: $2\ H_2O_2 \rightarrow O_2 + H_2O$

SOD is the first line of defense against ROS by preventing changes in NF-kB, polyol pathway, AGE formation and PKC activity. There are 3 different forms of SOD: Mn-SOD, a tetramer molecule present in mitochondria, Cu/Zn-SOD, a dimer molecule present in the plasma and a tetramer form in the cytosol. SOD works immediately to convert the superoxide radical O_2^- to hydrogen peroxide (H_2O_2), which is toxic for the cell as it is involved in the formation of hydroxyl radical. H_2O_2 is then detoxified to water either by catalase in the lysosomes or by glutathione peroxidase in the mitochondria.

Catalase is a tetramer containing NAPH molecule which stabilizes the active site and heme molecule necessary for enzymatic activity. Catalase reacts very rapidly, without requiring any energy. Contrary to catalase, peroxidases need energy from the cell and cofactors as ascorbate for ascorbate peroxidase and both glutathione and selenium for selenium-dependent glutathione peroxidase (GPX). GPX is a tetramer present in plasma and cytosol; it mediates the transformation of GSH to GSSH (Bharath et al., 2002). GSH plays a critical role as a cellular antioxidant, reacting with free radicals nonenzymatically, and also in the reduction of peroxides, catalyzed by GPX.

Prolonged exposure to hyperglycemia increases the generation of free radicals and reduces capacities of the antioxidant defense system. The pathogenesis of diabetic complications is strongly related to cellular injury caused by intracellular alterations in the metabolism of natural defense system against oxidative stress. Simple inactivation of enzymes by glycating proteins, for example, glycation of SOD, also

lead to DNA cleavage (Kaneto et al., 1994). The most common antioxidant deficiencies reported in diabetes are lower levels of ascorbate, glutathione and superoxide dismutase, also the concentration of reduced glutathione has been seen in diabetic neutrophils and monocytes (Venugopal et al., 2002). Also the activity and expression of SOD and glutathione peroxidase are decreased in diabetic models (Maritime et al., 2003).

b) Secondary antioxidants: passive detoxification

The passive detoxification is a second complementary line of defense including compounds able to significantly slow down the effects of free radicals that have not been eliminated by the enzymatic defense systems. Non enzymatic systems include vitamins A, C and E; glutathione; α-lipoic acid; carotenoids; trace elements like copper, zinc and selenium; coenzyme Q_{10} (CoQ_{10}); and cofactors like folic acid, uric acid, albumin, and vitamins B_1, B_2, B_6 and B_{12}. These systems are located in cell membrane, cystol, and plasma where they play specific functions. Numerous studies demonstrated that antioxidant vitamins and supplements can help lower the markers indicative of oxidative stress and lipid peroxidation in diabetic patients and animal models (Mayne, 2003). A number of studies have reported vitamins C and E and beta-carotene deficiency in diabetic patients and experimental diabetic animals (Penckofer et al., 2002; Naziroglu and Butterworth, 2005). Vitamin E (tocopherols and tocotrienols) is a lipophilic vitamin that prevents lipid peroxidation. It exists in 8 different forms, of which alpha-tocopherol is the most active form in humans. Alpha-tocopherol mainly eliminates lipid peroxyl radicals while gama-tocopherol is able to scavenge peroxinitrites. Hydroxyl radical reacts with tocopherol forming a stabilized phenolic radical which is reduced back to the phenol by ascorbate and NAD(P)H

dependent reductase enzymes (Hensley et al., 2000; Hensley et al., 2004). Vitamin C is a hydrophilic molecule and is the strongest physiological antioxidant acting in the organism's aqueous environment. It has been shown to be an important antioxidant, to regenerate vitamin E through redox cycling, and to raise intracellular glutathione levels (Zaidi and Banu, 2004). Thus vitamin C plays an important role in protein thiol group protection against oxidation (Rahimi et al., 2005). In contrast to vitamin A, the combination of vitamins C and E can also be safely used in high doses to prevent diabetes and cardiovascular disease (Hatzigeorgiou et al., 2006).

CoQ_{10} is a lipid soluble antioxidant, endogenously synthesized compound that acts as an electron carrier in the Complex II of the mitochondrial electron transport chain. At higher concentrations, it scavenges $^{\cdot}O_2^{\cdot}$ and improves endothelial dysfunction in diabetes. Furthermore, it inhibits lipid peroxidation by either scavenging free radicals directly or reducing alpha-tocopheroxyl radical to alpha-tocopherol (Kagan et al., 1990; Ernster and Dallner, 1995; Forsmark-Andree et al., 1995; Lass and Sohal, 1998; Abusheikha et al., 1998). The concentration of CoQ homologues in plasma, tissue homogenates and mitochondria can be increased by dietary CoQ_{10} supplementation. CoQ supplementation can modulate the plasma aminothiol redox status towards antioxidants and lower protein oxidative damage in skeletal muscle mitochondria (Vahle et al., 2002). Furthermore, CoQ intake enhances the antioxidative potential of tissues by elevating the endogenous amounts of alpha - tocopherol (Kamzalov et al., 2003).

Glutathione, a water-soluble tripeptide (γ-L-Glu-L-Cys-Gly) is the most abundant intracellular nonprotein thiol compound in mammalian cells (Sies, 1999). It plays a crucial role in antioxidant defense, nutrient metabolism and in regulation of

pathways essential for the whole body homeostasis (Deneke and Fanburg, 1989; Kretzschmar, 1996). It occurs in reduced thiol (GSH) and oxidized disulfide forms (GSSG). GSH is linked to many physiologic processes including detoxification of xenobiotics, modulation of signal transduction, prostaglandin metabolism, regulation of immune response, and enzyme activities. Synthesis of glutathione depends on the intake of cysteine. Cysteine availability is known as the rate-limiting factor in GSH synthesis. Glutathione deficiency leads to increase oxidative stress and may therefore play a key role in the pathogenesis of many diseases. Low levels of glutathione are found in persons with arthritis, diabetes, and cardiac injuries (Julius et al., 1994) or in neurodegenerative pathologies including dementia (Jenner, 1994).

Alpha-Lipoic acid (LA), a dithiol compound derived from octanoic acid, is used as a potent antioxidant, and has special criteria making it a powerful antioxidant. These criteria include radical quenching, metal chelation (Packer et al., 1995), amphiphilic character, bioavailability and safety, interaction with other antioxidants, and metabolic regeneration (Packer et al., 1995). LA scavenges hydroxyl radicals, hypochlorous acid, peroxynitrite, and singlet oxygen. Dihydrolipoic acid also scavenges superoxide and peroxyl radicals and can regenerate thioredoxin, vitamin C, and glutathione, which in turn can recycle vitamin E. In addition to its antioxidant properties, LA increases glucose uptake through recruitment of the glucose transporter-4 to plasma membranes, a mechanism that is shared with insulin-stimulated glucose uptake by activating elements of the insulin-signaling pathway. Furthermore, recent trials have demonstrated that treatment of insulin-resistant fatty Zucker rats with LA increased both oxidative and nonoxidative glucose metabolism and enhanced insulin sensitivity (Jacob et al., 1996). In experimental and clinical studies, LA markedly reduced the symptoms of diabetic pathologies, including

cataract formation (Maitra et al., 1995), vascular damage (Hofmann et al., 1999), and improved neural blood flow, endoneural glucose uptake and metabolism and nerve conduction (Ruhnau et al., 1999; Smith et al., 2004). Treatment with α-lipoic acid has also been shown to prevent diabetic nephropathy (Siu et al., 2006).

1.8 N-acetyl-L-cysteine (NAC)

N-acetyl-L-cysteine (NAC) developed in the 1960s, is a sulfhydryl-containing compound, which is a stable derivative of the amino acid cysteine, has antioxidant properties and makes up part of the tripeptide glutathione. NAC is rapidly absorbed into various tissues following an oral dose, is deacetylated and metabolized in the intestine and liver, and incorporated into disulfide protein peptides. A peak plasma level of NAC occurs approximately one hour after an oral dose and at 12 hours post-dose it is undetectable in plasma (De et al., 1989).The biological activity of NAC is attributed to its sulfhydryl group while its acetyl substituted amino group affords its protection against oxidative and metabolic processes. NAC can be administered orally, intravenously and via respiratory nebulizer.

1.8.1 Mechanism of action of NAC as antioxidant and anti-inflammatory agent

NAC is rapidly metabolized to cysteine, which is a direct precursor in the synthesis of intracellular GSH. In this way, it acts as an antioxidant by restoring the pool of intracellular reduced GSH (Santangelo, 2003). NAC can also have reducing and antioxidant properties by acting as a direct scavenger of free radicals such as $OH^{\bullet}$ and H_2O_2 and $O_2^{-\bullet}$ (Aruoma et al., 1989; Benrahmoune et al., 2000). Moreover, as a direct consequence of its antioxidant and SH-donating properties, NAC restores cellular redox-status and can in this way modulate the activity of redox-sensitive cell-

signaling and transcription pathways such as NF-κB which regulates a variety of proinflammatory genes (Desaki et al., 2000), and the p38, ERK1/2, SAPK/JNK, c-Jun and c-Fos pathways (Zafarullah et al., 2003; Wuyts et al., 2003). The possible sites of action of NAC as antioxidant and anti-inflammatory agent on chronic obstructive pulmonary disease are shown in Figure 4 (Sadowska et al., 2007).

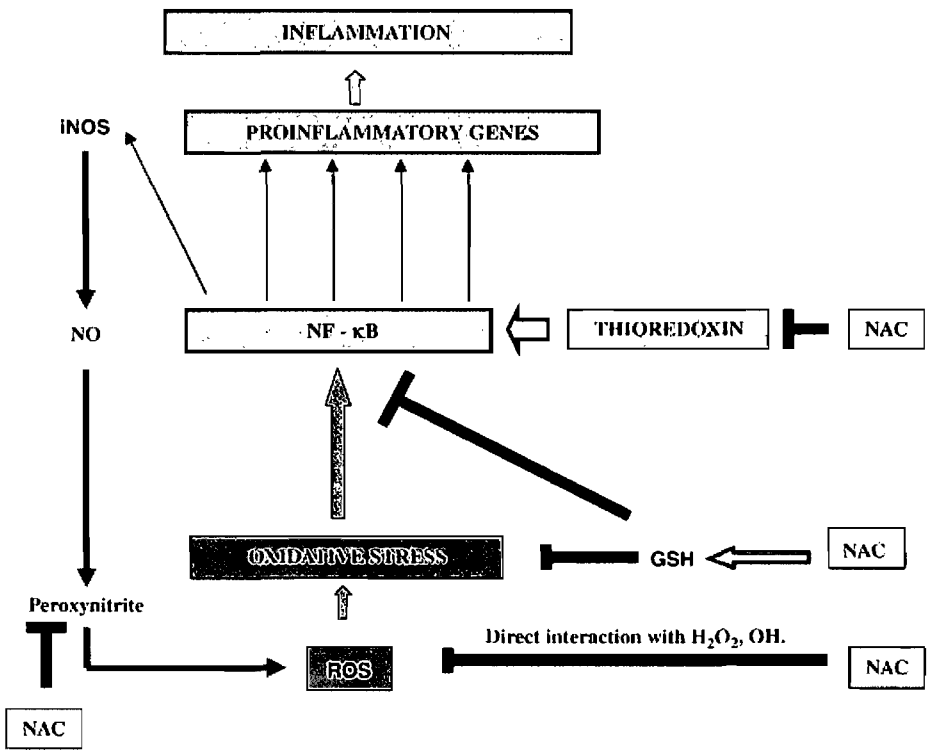

Figure 4 Possible sites of action of N-acetyl-L-cysteine (NAC). NAC inhibits the oxidative stress by acting as direct ROS scavenger and by changing the cellular redox status. This, in turn, may influence NF-κB activation and modulate the inflammatory response (Sadowska et al., 2007).

1.8.2 Therapeutic uses of NAC

NAC has been in clinical practice since 1960s (Ziment, 1986; Flanagan, 1987; de and De, 1993; van, 1995). Initially, NAC was introduced as a mucolytic agent for

the treatment of respiratory diseases such as chronic bronchitis and cystic fibrosis (Webb, 1962; Richardson and Phipps, 1978). It acts as an expectorant by stimulating both ciliary action and the gastro-pulmonary vagal reflex, thereby clearing the mucus from the airway (Sheffner, 1966). For this reason, NAC is used clinically in bronchopulmonary diseases to reduce both the viscosity and the tenacity of mucus, as well as to facilitate its removal. In the late 1970s, NAC was recognized as an antidote for the therapy of acute acetaminophen intoxication (Prescott et al., 1977; Prescott et al., 1979). Recent studies have shown an effect of NAC in the prevention of atheromatous plaque formation, NAC inhibits the oxidation of LDL which accumulates in the vascular wall and promotes a local inflammatory process contributing to the progression of atheromatous plaque (Van et al., 2005). More recently NAC was found to prevent fructose-induced insulin resistance and hypertension in rats (Song et al., 2005). NAC also increased fat degradation and decreased body weight gain in conditions of excess sucrose intake (Diniz et al., 2006). Chronic treatment with NAC in Spontaneously Hypertensive Rats (SHR) decreased blood pressure by improving sympathetic functions and β-adrenergic pathway (Girouard et al., 2003). Furthermore, NAC exerts protective effect against glucose toxicity on pancreatic β-cells in various models of diabetes, reduces blood glucose and increases glucose-induced insulin secretion (Ho et al., 1999; Kaneto et al., 1999; Tanaka et al., 1999). NAC has been shown to be a strong antioxidant, to exert antigenotoxic and anticarcinogenic properties, and to detoxify free radicals that cause DNA changes in diseases (e.g., cancer). These effects of NAC have been attributed to its ability to act as an analogue of cysteine and precursor of reduced glutathione (GSH), to improve the activities of glutathione S-transferases, glutathione peroxidase, glutathione reductase, NADH- and NAD(P)H-quinone reductase, and probably, to

promote DNA repair by protecting ADP-ribosyltransferase activity (Albano et al., 1984; De et al., 1984; De et al., 1984; De et al., 1985; De et al., 1985; De et al., 1986; Perchellet et al., 1987; Dorsch et al., 1987; De and Ramel, 1988; Cesarone et al., 1988).

1.9 Angiotensin-converting enzyme (ACE) inhibitor

Angiotensin II (Ang II), is a potent vasoconstrictor that is involved in the regulation of vascular functions such as cell growth, apoptosis, migration, inflammation, and fibrosis (Touyz and Schiffrin, 2000; Wolf and Wenzel, 2004). Ang II is an important growth modulator of blood vessels and renal organogenesis during development. It plays a critical role in regulating blood pressure and fluid homeostasis in physiological conditions. Ang II contributes to altered vascular tone, endothelial function, structural remodeling and to vascular inflammation, characteristic features of vascular damage in hypertension, atherosclerosis, vasculitis, and diabetes (Griendling and Ushio-Fukai, 2000; Touyz and Schiffrin, 2000). Angiotensin-1 converting enzyme (ACE) inhibitors are widely accepted as vascular protective agents. They are frequently used as the first line of treatment of hypertension in type 2 diabetic patients, and have been shown to improve insulin sensitivity and to reduce cardiovascular complications in diabetes (Henriksen et al., 1996; McFarlane et al., 2003). The mechanism by which ACE inhibition improves insulin sensitivity and reduces the development of type 2 diabetes in patients with essential hypertension is not yet completely elucidated and goes beyond the blockade of the renin-angiotensin system (Couture and Girolami, 2004). Treatment with ACEI attenuated ROS formation and prevented phenotypic changes in the heart (cardiomyocyte hypertrophy, perivascular fibrosis) and in the aorta of diabetic rats (Fiordaliso et al.,

2006). ACE is a major link between the renin-angiotensin system and the kinin system, because it not only converts Ang I to Ang II but also degrades kinins.

1.9.1 Ramipril

Ramipril is an ACE inhibitor. As inactive prodrug, ramipril is converted to ramiprilat in the liver by esterase enzymes, and is used to treat hypertension and heart failure, to reduce proteinuria and renal disease in patients with nephropathies, and to prevent stroke, myocardial infarction, and cardiac death in high-risk patients. Ramiprilat is mostly excreted by the kidneys. The half-life of ramiprilat is variable (3 - 16 hours), and is prolonged by heart and liver failure, as well as kidney failure. Ramiprilat, the active metabolite, competes with angiotensin I for binding at the angiotensin-converting enzyme, blocking the conversion of angiotensin I to angiotensin II. As angiotensin II is a vasoconstrictor and a negative-feedback mediator for renin activity, lower concentrations result in a decrease in blood pressure and an increase in plasma renin. Ramiprilat may also act on kininase II, an enzyme identical to ACE that degrades the vasodilator bradykinin. Recent studies have shown that ramipril, reduced the accumulation of advanced glycation end products in experimental diabetic nephropathy (Forbes et al., 2002).

1.10 Diabetic neuropathic pain

Functional and structural impairments of peripheral nervous system in diabetic individual are generally defined as diabetic neuropathy, which is a common complication of both type 1 and type 2 diabetes (Boulton et al., 2005). Peripheral neuropathy affects about 30% of people with diabetes mellitus. Between 16% and 26% of diabetes patients experience chronic pain. This may be referred to as diabetic

neuropathic pain (DNP) or diabetic peripheral neuropathic pain (DPNP) (Jensen et al., 2006). Most commonly, it manifests as sensory loss, which predisposes to foot abnormalities and high risk of ulceration. It has been reported that between 4 and 33% of patient with diabetes mellitus suffer from the painful type of neuropathy, which is recognized as one of the most difficult type of pain to treat (Ziegler et al., 1992; Daousi et al., 2004). Diabetic neuropathic pain can occur either spontaneously or as a result of exposure to mild painful stimuli (hyperalgesia) or to abnormal non painful stimuli (allodynia) (Brown and Asbury, 1984; Clark, Jr. and Lee, 1995). Diabetic neuropathy, during its natural course progresses from initial functional changes to late poorly reversible structural changes. Various interconnected pathogenetic concepts of diabetic neuropathy have been proposed based on metabolic and vascular factors, mostly derived from long-term hyperglycemia. The etiology of diabetic neuropathy is multifactorial, long term hyperglycemia, increased oxidative stress and altered protein kinase C activity and poly ADP-ribose polymerase (PARP) activation, all these lead to development of neuropathy (Yagihashi, 1995; Sima and Sugimoto, 1999; Yagihashi, 2006; Yagihashi et al., 2007) (Fig 5).

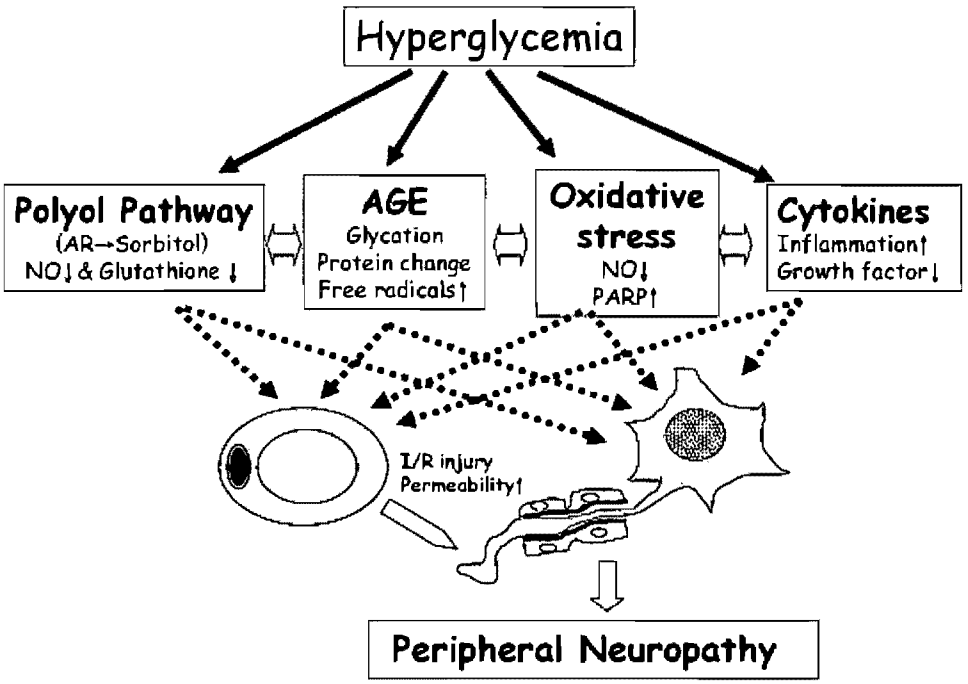

Figure 5 Multifactorial etiology of diabetic neuropathy. Hyperglycemia increases polyol pathway, AGE formation, oxidative stress and cytokine release. These factors interact or operate independently in diabetic neuropathy. They can affect nerve tissues either directly or indirectly through nutrient vascular tissues (Yagihashi et al., 2007).

These pathogenic mechanisms have been targeted in several experimental and clinical trials (Yagihashi et al., 2007). The experimental rat model of chronic glucose feeding presents all the hallmarks of type 2 diabetes such as the increases of plasma levels of glucose and insulin, arterial hypertension, insulin resistance and increases in the production of superoxide anion (marker of the oxidative stress) in the heart and aorta (Midaoui and de Champlain, 2002; Midaoui and de Champlain, 2005). Further studies have shown sensory abnormalities, namely tactile and cold allodynia, after 4 weeks of treatment with glucose. However, glucose-fed rats did not exhibit thermal hyperalgesia up to 20 weeks of treatment. Thermal hyperalgesia is mediated by myelinated Aδ and unmyelinated primary afferent neurons, which include both peptidergic and non peptidergic C-fibers. Foot withdrawal responses evoked by low rates of skin heating are primarily mediated by C-fiber nociceptors (Handwerker and

Kobal, 1993; Yeomans and Proudfit, 1996). Whereas C-fibers are thought to be involved in the transmission of warm sensations, Aδ-fibers are stimulated by cold stimulus (Pierau and Wurster, 1981). On the contrary, tactile allodynia is a central phenomenon mediated by large myelinated Aβ-fibers (Woolf et al., 1992; Shortland et al., 1997; Pitcher and Henry, 2000). Thus, sensory neuropathy measured in glucose-fed rats may affect primarily sensory Aβ and Aδ fibers and less likely polymodal C-fibers.

A recent study showed an impairment of cutaneous endothelium-related vasodilation and C-fiber-mediated vasoconstriction in peripheral diabetic neuropathy. Peripheral nerve perfusion is reduced by diabetes and this makes an important contribution to neuropathy in patient and animal models (Tuck et al., 1984; Cameron et al., 2001; Quattrini et al., 2007). Treatment of diabetic neuropathic pain is based on four cornerstones: (a) causal treatment aimed at (near)-normoglycemia, (b) treatment based on pathogenetic mechanisms, (c) symptomatic treatment, and (d) avoidance of risk factors and complications (Ziegler, 2006). A clinical study in diabetic polyneuropathic patients has shown that the oral treatment with alpha-lipolic acid for five weeks improves the neuropathic symptoms and deficits (Ziegler et al., 2006). Inhibition of xanthine oxidase (an important source of ROS that contributes to neurovascular dysfunction in experimental diabetes) could be a potential therapeutic approach to diabetic neuropathy and vasculopathy (Inkster et al., 2007). Systemic injection of the ROS scavenger phenyl-N-tert-butylnitrone (PBN) relieved mechanical allodynia in a model of neuropathic pain (Kim et al., 2004). Intraperitoneally administration of NAC resulted in significant reduction of hyperalgesia after chronic constriction of the sciatic nerve in rats (Naik et al., 2006).

1.11 Insulin signaling and action

Insulin is the most potent anabolic hormone and is essential for normal tissue development, growth, and maintenance of body glucose homeostasis. It is secreted by the pancreatic ß cells of islets of Langerhans in response to increased circulating levels of glucose and amino acids after a meal. Insulin regulates glucose homeostasis at different organ sites, reducing hepatic glucose output (via decreased gluconeogenesis and glucogenolysis) and increasing the rate of glucose uptake, primarily into striated muscle and adipose tissue (Goalstone and Draznin, 1997). Gluconeogenesis represents the generation of glucose from non-sugar carbon substrates and occurs during periods of fasting, starvation or intense exercise. Glycogenolysis refers to enzymatic breakdown or catabolism of the polysaccharide glycogen into molecules of glucose and molecules of glucose 1-phosphate.

Insulin signaling at the target tissue results in a large array of biological functions. It is essential for normal growth and development and for normal metabolism of glucose, fat and protein. The insulin pathway is critical for the regulation of intracellular and blood glucose levels and the avoidance of diabetes. Moreover, studying the signaling pathways involved in insulin action increases our understanding of the pathophysiology of insulin resistance related to type 2 diabetes and can help to identify the key molecules for the development of more effective therapeutic.

1.11.1 Insulin receptor

The insulin receptor (IR) belongs to a family of transmembrane receptors with intrinsic tyrosine kinase activity (Schlessinger, 1993). It is an heterotetrameric protein made of two extracellular α subunits and two transmembrane β subunits (Fig 6).

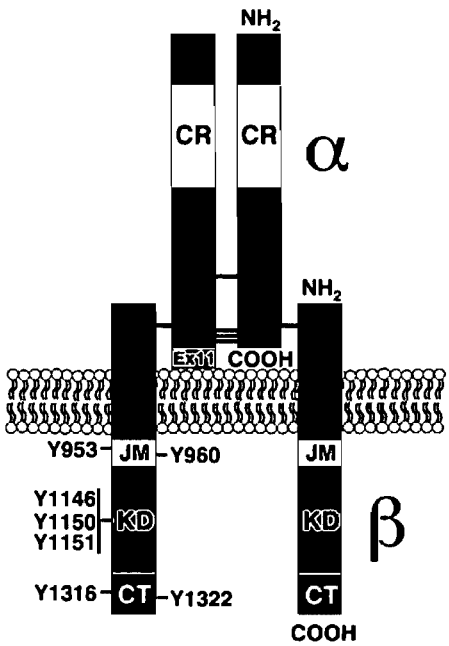

Figure 6 Structure of the insulin receptor. CR, Cysteine-rich domain; JM, juxtamembrane domain; KD, kinase domain; CT, carboxyl-terminal domain. The positions of the tyrosine autophosphorylation sites are indicated. The left side depicts IR-B, which includes 12-amino acids alternatively spliced exon 11 (Ex11) at the carboxyl terminus of the α -subunit. The right side depicts IR-A. The extracellular - and intracellular ß-subunits are indicated. The horizontal black bars represent disulfide linkages (De Meyts and Whittaker, 2002).

Binding of insulin to α subunits induces a conformational change resulting in the autophosphorylation of a number of tyrosine residues present in β subunits (Van, Baron et al., 2001). These residues are recognized by phosphotyrosine-binding (PTB) domains of adaptor proteins such as members of the insulin receptor substrate family (IRS) (Lizcano and Alessi, 2002). Receptor activation leads to the phosphorylation of key tyrosine residues on IRS proteins, some of which are recognized by the Src homology 2 (SH2) domain of the p85 regulatory subunit of PI 3-kinase (a lipid

kinase). The binding of insulin to the α subunit of IR not only concentrates insulin at its site of action, but also induces conformational changes in the receptor, which in turn stimulates the tyrosine kinase activity intrinsic to the β subunit of the IR and triggers the signaling cascades (Fig 7). Insulin receptors trans phosphorylate several immediate substrates (on Tyr residues) including IRS1 – 4, Shc, and Gab 1, Cbl, APS, and $P60^{dok}$. Each of these provides specific docking sites for other signaling proteins containing Src homology 2 (SH2) domains (White and Yenush, 1998). These events lead to the activation of downstream signaling molecules including PI-3 kinase.

The four IRS proteins are highly homologous with overlapping and differential tissue distribution. Studies with genetic deletion in mouse models and cell lines indicate that the IRS proteins serve complimentary functions in different tissues as immediate substrates for insulin and IGF-I receptors. Combined heterozygous deletions of insulin receptor, IRS-1, and IRS-2 in different tissues develop severe insulin resistance in skeletal muscle and liver and marked β-cell hyperplasia. A recent study suggested tissue-specific differences in the roles of IRS proteins to mediate insulin action, with IRS-1 playing a prominent role in skeletal muscle and IRS-2 in liver (Kido et al., 2000). Also IRS-2 promotes β cell replication, function, and survival, especially during metabolic stress (Park et al., 2006). Furthermore, recent studies showed that IRS-3 and IRS-4 impair IGF-1-mediated IRS-1 and IRS-2 signaling in cells (Tsuruzoe et al., 2001).

Activation of the insulin receptor evokes increased transcription of SREBP and the phosphorylation of members of the IRS family, SHC and Cbl. Upon tyrosine phosphorylation, these proteins interact with signaling molecules through their SH2

domains, which results in the activation of a variety of signaling pathways, including PI 3-kinase signaling, MAPK activation and the activation of the Cbl/CAP complex. These pathways act in a coordinated manner to regulate glucose, lipid and protein metabolism.

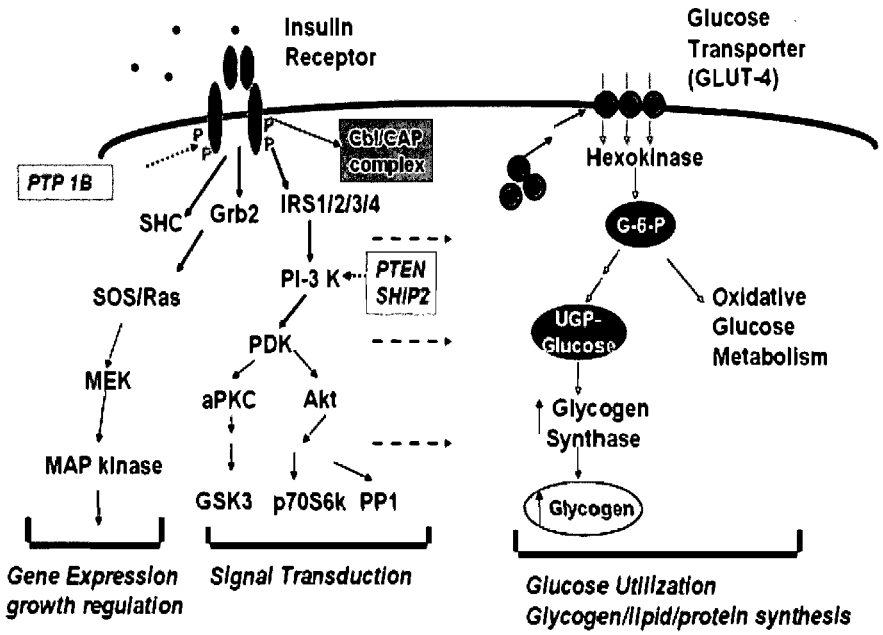

Figure 7. Activation of the insulin receptor evokes increased transcription of SREBP and the phosphorylation of members of the IRS family, SHC and Cbl, Gab1, SIRPS, and adaptor protein containing PH and SH2 domains APS. This results in the activation of a variety of signaling pathways, including PI 3-kinase signaling, MAPK activation and the activation of the Cbl/CAP. Taken from www.endotext.org/Diabetes/diabetes4/diabetesframe4.htm (Li and Zhang 2007).

1.11.2 Glucose transporters

Glucose is cleared from the bloodstream by transporters (GLUTs) which include a family of highly related 12 transmembrane domain-containing proteins (Joost and Thorens, 2001). The GLUT family contains 13 known members and can be classified into three classes based upon their structural characteristics (Joost et al., 2002). Class1 includes GLUTs 1–4 which are, by far, the best characterized transporters of the family. Class II includes GLUT5 (a fructose-specific transporter), and GLUTs 7, 9, and 11 (Joost and Thorens, 2001). Class III includes GLUTs 8, 10, 12, and the proton-myoinositol symporter H^+-*myo*-inositol cotransporter (HMIT1) (Joost and Thorens, 2001). GLUT4 is expressed primarily in striated muscle and adipose tissue and, unlike most other GLUT isoforms, is sequestered in specialized intracellular membrane compartments under basal conditions (Bryant et al., 2002). GLUT4 is the only known insulin-responsive GLUT which is highly and specifically expressed in muscle and adipose tissue, the major sites of postprandial glucose disposal. Interestingly, overexpression of the human GLUT4 gene in muscle and fat tissue of the diabetic *db/db* mouse, which lacks the leptin receptor, protects these animals from insulin resistance and diabetes (Brozinick et al., 2001)

1.12 Hypothesis (article #1)

Long term treatments with N-acetyl-L-cysteine (NAC), a potent antioxidant, and ramipril (an angiotensin-1 converting enzyme inhibitor) can reverse arterial hypertension and insulin resistance in a rat model of diabetes induced by glucose feeding. It is expected that these beneficial effects are partly due to the inhibition of the oxidative stress (associated to overproduction of aortic/hepatic superoxide anion and plasma lipid peroxidation) and to the normalization of skeletal muscle insulin receptor substrate-1 (IRS-1) protein expression. Decreases in the expression of IRS-1 may account for insulin resistance as these proteins play an important role in insulin signaling.

Experimental approach: Male Wistar rats were given tap water only (control) or water supplemented with 10% D-glucose for 20 weeks. Treatments with NAC (2 g/kg/day) and ramipril (1 mg/kg/day) were initiated at 16 weeks in the drinking fluid. Body weight and blood pressure were measured weekly during the last four weeks. Systolic blood pressure was measured by tail-cuff photoplethysmography, Plasma glucose concentrations were measured with a glucometer. The Homeostasis Model Assessment (HOMA) was used as an index of insulin resistance and calculated by the following formula: insulin (μU / ml) x glucose (mmol/L) ÷ 22.5. Superoxide anion production was measured in isolated aortic and hepatic small slices using the lucigenin-enhanced chemiluminescence method. Total IRS-1 protein levels in skeletal muscle (gastrocnemius muscle) were measured by Western blot. Plasma samples were analyzed for malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) which are specific markers of lipid peroxidation.

1.13 Hypothesis (article #2)

Long term treatments with N-acetyl-L-cysteine (NAC), a potent antioxidant, and ramipril (an angiotensin-1 converting enzyme inhibitor) can improve sensory polyneuropathy such as tactile and cold allodynia in addition to their anti-hypertensive effects in glucose-fed rats. It is suggested that these sensory abnormalities are associated with the oxidative stress and the induction of kinin B_1 receptor expression. Inhibition of oxidative stress may provide a protective effect by preventing the induction of this pro-nociceptive receptor. This should be consistent with the inhibition of tactile and cold allodynia recently reported with B_1 receptor antagonists. Blockade of this receptor in the central nervous system also reversed high blood pressure in glucose-fed rats.

Experimental approach: Male Wistar rats (50-75 g) were given 10% D-glucose in their drinking water for 11 weeks or tap water only (controls). Treatment with NAC (1 g/kg/day, gavage) or ramipril (1 mg/kg/day in drinking water) was initiated after six weeks, for a period of five weeks. Blood glucose was measured weekly with the glucose oxidase-impregnated test strip. Systolic arterial blood pressure was measured weekly before and after the administration of NAC and ramipril by tail-cuff photoplethysmography. Tactile allodynia was assessed by measuring the hindpaw withdrawal threshold to a calibrated series of six von Frey filaments (2, 4, 6, 8, 10 and 15 g). Cold allodynia was assessed using the acetone drop method. At the term of the protocol of 11 weeks, rats were anaesthetised with CO_2 inhalation and then decapitated. Approximately 10 mg of rat tissues (thoracic spinal cord and renal cortex) were isolated and put in RNA later stabilisation reagent, and the relative quantification of B_1 receptor gene expression was analyzed by the $2^{-\Delta Ct}$ method

CHAPTER TWO

Article #1: Comparative effects of N-acetyl-L-cysteine and ramipril on arterial hypertension, insulin resistance and oxidative stress in chronically glucose-fed rats

COMPARATIVE EFFECTS OF N-ACETYL-L-CYSTEINE AND RAMIPRIL ON ARTERIAL HYPERTENSION, INSULIN RESISTANCE AND OXIDATIVE STRESS IN CHRONICALLY GLUCOSE-FED RATS

Short running title: N-acetyl-L-cysteine and ramipril in diabetes

Adil El Midaoui[a], Mahmoud Ali Ismael[b], Huogen Lu [a], I. George Fantus[a], Jacques de Champlain[b] and Réjean Couture[b]

Banting and Best Diabetes Center[a], University of Toronto, Ontario, Canada and Department of Physiology[b], Université de Montréal, Montréal, Quebec, Canada

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Correspondence and requests for reprints to Réjean Couture, Ph.D., Department of Physiology, Faculty of Medicine, Université de Montréal, CP 6128, Succursale Centre-ville, Montreal, Quebec, Canada H3C 3J7
Tel: [information retirée / information withdrawn] Fax: [information retirée / information withdrawn]
E-mail: [information retirée / information withdrawn]

Sumitted to J. Hypertension

2.0 Abstract

Objectives of the study : To compare the ability of N-acetyl-L-cysteine (NAC) and ramipril (ACEI) to reverse arterial hypertension, insulin resistance, the oxidative stress, lipid peroxidation and changes of skeletal muscle insulin receptor substrate-1 (IRS-1) protein expression in a rat model of diabetes.

Methods: Rats were given tap water only (control) or water supplemented with 10% D-glucose for 20 weeks. Treatments with NAC (2 g/kg/day) and ramipril (1 mg/kg/day) were initiated at 16 weeks in the drinking fluid.

Results: Systolic blood pressure, plasma levels of insulin and glucose as well as insulin resistance (HOMA index) were significantly higher in rats treated with glucose for 20 weeks. This was associated with a higher production of superoxide anion and NADPH oxidase activity in aorta and liver and with a marked reduction of IRS-1 protein expression in the gastrocnemius muscle. NAC prevented all these alterations. Although ramipril provided the same therapeutic effect as NAC, on blood pressure and IRS-1 protein, it had a lesser effect on insulin resistance. At the same time superoxide anion production was lowered only in aorta but not in liver by ramipril. Furthermore, it did not reduce NADPH oxidase activity in aorta or liver and failed to reduce plasma levels of 4-hydroxynonenal and malondialdehyde in contrast to NAC.

Conclusions: The data indicate a pathologic role for oxidative stress in both hypertension and insulin resistance and that ramipril antioxidant activity is exerted most strongly in vascular tissue. In contrast to ramipril, the antioxidant effect of NAC is dependent of NADPH oxidase and affects lipid peroxidation.

Key words: hypertension, insulin resistance, oxidative stress, N-acetyl-L-cysteine, ramipril

2.1 Introduction

The "metabolic syndrome" is an emerging epidemic worldwide and consists of an association of multiple cardiovascular risk factors (1). These factors, including hypertension, insulin resistance, and obesity, directly contribute to the higher incidence of cardiovascular disease as well as to the development of type 2 diabetes. An increase in oxidative stress is one of the main hypotheses suggested to explain the enhanced risks associated with metabolic syndrome [1, 2, 3]. Oxidative stress may result from either excessive production of reactive oxygen species (ROS), especially the superoxide anion ($O_2^{\bullet -}$), or from reduced antioxidant reserve. Previous studies have suggested that increased $O_2^{\bullet -}$ production may be involved in the pathogenesis and complications of both diabetes and hypertension [3, 4]. In animal studies, arterial tissue $O_2^{\bullet -}$ levels were reported to be increased in spontaneously hypertensive rats (SHR) [5, 6], insulin resistant rats [3] and obese rats [4]. Treatment with the thiol compound, alpha-lipoic acid was reported to lower blood pressure in SHR [7], to enhance insulin-stimulated glucose metabolism and to reduce insulin resistance in fatty Zucker rats [8], and to increase tissue levels of gluthatione in mice [9]. We previously reported that the treatment with alpha-lipoic acid prevented the sustained elevation of blood pressure, the basal overproduction of $O_2^{\bullet -}$ in cardiovascular tissues and attenuated the development of insulin resistance in 4 and 12 week glucose-fed rats [10-13]. Moreover, N-acetyl-L-cysteine (NAC), another potent antioxidant acting as a free radical scavenger and glutathione donor [14], was found to prevent fructose-induced insulin resistance and hypertension in rats [15]. NAC also decreased body weight gain in conditions of excess sucrose intake [16]. Chronic treatment of SHR with NAC decreased blood pressure and heart rate by inhibiting sympathetic activity

and restoring cardiac β-adrenoceptor function [17]. The improvement of the cardiovascular function in SHR was also associated with the normalization of tissue lipid peroxidation and of the balance between oxidized and reduced glutathione [18, 19]. Furthermore, NAC exerts protective effects against glucose toxicity on pancreatic β-cells in various models of diabetes, reduces blood glucose and increases glucose-induced insulin secretion [20, 21, 22].

Angiotensin-I converting enzyme (ACE) inhibitors, frequently used as first line treatment of hypertension in type 2 diabetic patients, have been shown to improve insulin sensitivity and to reduce cardiovascular complications in diabetes [23, 24]. The mechanism by which ACE inhibition improves insulin sensitivity and reduces the development of type 2 diabetes in patients with essential hypertension is not yet completely elucidated [25]. Yavuz et al. [26] have reported that ACE inhibitors improve insulin sensitivity and endothelial dysfunction and decrease lipid peroxidation in patients with essential hypertension. Moreover, studies have shown that ramipril, an ACE inhibitor reduced the accumulation of advanced glycation end products in experimental diabetic nephropathy [27]. It is therefore tempting to propose that ACE inhibitors exert their beneficial effects on glucose metabolism and blood pressure through their antioxidant properties in hypertensive and insulin resistant states.

We thus postulated that an increase in oxidative stress could contribute not only to the development of hypertension and insulin resistance but also to their maintenance. In this study, we investigated whether a chronic treatment of 4 weeks with the antioxidant, NAC, or the ACE inhibitor ramipril could reverse the established blood pressure elevation, insulin resistance, plasma lipid peroxidation and the rise in $O_2^{\bullet -}$ and NADPH oxidase activity in aorta and liver tissues in the experimental model

of chronic glucose feeding. Moreover, we evaluated the effect of 20 weeks of glucose feeding on skeletal muscle insulin receptor substrate-1 (IRS-1) protein levels in the presence and absence of NAC or ramipril.

2.2 Materials and Methods

2.2.1 Animals and protocols

Male Wistar rats weighing 70-80 g were purchased from Charles River, St-Constant, Que., Canada, a few days prior to experiments and were housed two per cage, under controlled conditions of temperature (23 °C) and humidity (50 %), on a 12 h light-dark cycle and allowed free access to normal chow diet (Charles River Rodent # 5075) and drinking water. All research procedures and the care of the animals were in compliance with the guiding principles for animal experimentation as enunciated by the Canadian Council on Animal Care and were approved by the Animal Care Committee of our University. Thirty six rats were given 10 % D-glucose in their drinking water during 16 weeks. For the subsequent four weeks, they were randomly administered 10% D-glucose (n = 18), D-glucose plus NAC (2 g/kg/day, n = 7) or D-glucose plus ramipril (1 mg/ kg/day, n = 11) in their drinking water. Control rats (n=14) received tap water for the entire 20 weeks. Fluid consumption of each rat was monitored every two days to adjust the dosage of NAC and ramipril throughout the study.

Body weight and blood pressure were measured weekly during the last four weeks. Systolic blood pressure was measured by tail-cuff photoplethysmography (Harvard Apparatus Ltd) and registered on a MacLab/8 system. The average of five blood pressure readings was recorded for each measurement. At the end of treatment, rats were killed by decapitation after light anesthesia with CO_2. Blood was rapidly collected from sectioned carotids and transferred into a chilled vacutainer tube containing 0.63 mg/ml heparin for plasma biochemistry. All blood samples were drawn early in the morning after fasting overnight (16 h). Slices of liver and aorta

were sampled and placed in liquid nitrogen and stored at -80 °C until the measurement of $O_2^{\bullet-}$ and NADPH activity.

2.2.2 Laboratory analysis

Plasma glucose concentrations were measured with a glucometer (Elite, Bayer Inc, Toronto, Canada). Insulin levels were determined by radioimmunoassay (Rat insulin RIA kit, Linco Research, St. Charles, MO) using 100 µl plasma. The Homeostasis Model Assessment (HOMA) was used as an index of insulin resistance and calculated by the following formula: insulin (µU / ml) x glucose (mmol/L) ÷ 22.5 [28].

2.2.3 Aortic and hepatic superoxide anion measurement

Superoxide anion production was measured in isolated aortic and hepatic small slices using the lucigenin-enhanced chemiluminescence method as described previously [29, 30]. Briefly, small slices from aorta and liver tissues were preincubated in Krebs-Hepes buffer (saturated with 95 % O_2 and 5 % CO_2, at room temperature) for 30 min and then transferred to a glass scintillation vial containing 5 µmol/L lucigenin for the determination of basal $O_2^{\bullet-}$ levels. The chemiluminescence was recorded every minute for 15 min at room temperature in a liquid scintillation counter (Wallac 1409, Tuku, Finland). Lucigenin counts were expressed as cpm/mg of dry weight tissue. Moreover, the activation of NAD(P)H oxidase activity in the samples was assessed by adding 0.1 mM NADPH to the vials before counting. Basal superoxide induced luminescence was then subtracted from the luminescence value induced by NADPH.

2.2.4 Skeletal muscle IRS-1 protein levels

Total IRS-1 protein levels in skeletal muscle were measured by Western

blotting as follows: gastrocnemius muscle was homogenized in lysis buffer: 50 mM Tris pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5 mM sodium orthovanadate, 0.1% beta-mercaptoethanol, 1% Triton X-100, 5 mM sodium pyrophosphate, 10 mM sodium-beta-glycerol phosphate, 0.1 mM PMSF, 1 μg/ml Aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin and 1 μg/ml microcystin. Equal amounts of total cell lysates (40 μg) were separated on 10% sodium dodecyl sulphate (SDS) polyacrylamide gels by electrophoresis and transferred onto nitrocellulose membranes. Nonspecific binding was blocked with 5 % BSA and membranes were then probed with anti-IRS-1 antibody (1/1000, Upstate Biotechnology, Lake city, New York). The blots were visualized using HRP-conjugated secondary antibody (1/2500 goat anti-Rabbit IgG-HRP, Santa Cruz Biotechnology) followed by chemiluminescence. The protein band intensities were quantified by densitometry.

2.2.5 Malondialdehyde and 4-hydroxynonenal analysis

To assess lipid peroxidation, plasma samples were analyzed for malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) by a modification of a previously described method [31]. Briefly, plasma aldehydes and 100 pmol of benzaldehyde-*ring*-D5 (internal standard) were derivatized by pentafluorobenzyl hydroxylamine hydrochloride (PFBHA·HCl) to form the aldehydes-PFBHA derivative. Plasma protein was precipitated by methanol, and aldehydes were separated by hexane extraction. The hydroxyl group of 4-HNE was further derivatized by 50 μl of *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) at 60°C for 15 min. Finally, 50 μl of hexane were added to each sample, and 1 μl was analyzed by gas chromatography-mass spectrometry (GC-MS). The Hewlett-Packard 5973N MSD GC-MS was equipped

with an HP-5 ms capillary column (30 cm length, 0.25 mm ID, 0.25 μm film thickness), and the mass spectrometry was performed in negative-ion chemical ionization with methane as the reagent gas.

2.3 Drugs

Except where otherwise stated, all chemical components of solutions and drugs were purchased from Sigma-Aldrich Canada.

2.4 Statistical analysis of data

Data are expressed as mean ± SEM of values obtained from (n) rats. Statistical analysis of data was performed with Graph-Pad Prism software. Data were subjected to a one-way analysis of variance (ANOVA), followed by the Bonferroni/Dunn multiple comparison test to estimate the significance of differences between groups. Significance was considered when $P < 0.05$.

2.5 Results

2.5.1 Systolic blood pressure and body weight

As shown in Table 1, chronic administration of glucose for 16, 18 and 20 weeks resulted in a significant increase in systolic arterial blood pressure when compared to control rats. The increase in systolic blood pressure in glucose-fed rats was not significantly reduced by NAC after two weeks but was significantly reduced after 4 weeks of treatment. On the other hand, the increase in systolic blood pressure was reversed to control values after two and four weeks treatment with ramipril. Twenty weeks glucose feeding had no significant effect on final body weight in comparison to control animals (Table 2). A treatment of four weeks with NAC significantly reduced body weight in chronically glucose-fed rats. In contrast, four weeks treatment with ramipril had no significant effect on body weight in 20 week glucose-fed rats (Table 2).

2.5.2 Metabolic parameters

Twenty weeks of glucose feeding resulted in a significant increase in plasma levels of glucose in comparison to controls (Table 2). Four weeks of treatment with either NAC or ramipril normalized the hyperglycemia in glucose-fed rats to control values. As shown in Table 2, plasma insulin levels were significantly increased (by 81 %) in glucose-treated rats in comparison to control animals. Four weeks of treatment with NAC normalized plasma insulin levels while ramipril reduced these by 42 % compared to untreated glucose-fed rats. Insulin resistance as expressed by the HOMA index was increased by 166 % in glucose-fed rats in comparison to control animals (Table 2). A treatment of four weeks with NAC reversed insulin resistance, while ramipril reduced it by 21 % ($p > 0.05$) in glucose-fed rats.

2.5.3 Oxidative stress parameters

As shown in Figure 1 panels A and B, basal $O_2^{\bullet-}$ production was significantly ($P<0.05$) increased in 20 weeks glucose-fed rats in aorta and liver tissues in comparison with control. Four weeks of treatment with NAC normalized the rise in both aortic ($P<0.01$) and hepatic ($P<0.05$) basal $O_2^{\bullet-}$ production in glucose-fed rats. A treatment of four weeks with ramipril also normalized the increase in basal $O_2^{\bullet-}$ production in aortic tissue ($P<0.01$). Ramipril failed to significantly reduce basal $O_2^{\bullet-}$ production in hepatic tissue in 20 weeks glucose-fed rats. However, the residual value of basal $O_2^{\bullet-}$ production in this group was no longer significantly different from control values. As shown in Figure 2, panels A and B, NADPH oxidase activity was significantly increased in 20 weeks glucose-fed rats in aorta ($P<0.05$) and liver ($P<0.05$) tissues in comparison with control. Four weeks of treatment with NAC normalized ($P<0.05$) the increase in NADPH oxidase activity in both tissues in glucose-fed rats. In contrast, four weeks of treatment with ramipril had no effect on NADPH oxidase activity in aorta or liver tissues in glucose-fed rats. As illustrated in Figure 3, panels A and B, plasma levels of 4-hydroxynonenal and malondialdehyde were not altered by 20 weeks of glucose feeding. Four weeks treatment with NAC, however, decreased significantly ($P<0.01$) plasma levels of 4-hydroxynonenal and malondialdehyde in glucose-fed rats. A treatment of four weeks with ramipril had no significant effect on these markers in 20 weeks glucose-fed rats (Figure 3A, 3B). As shown in Figure 4, 20 weeks glucose feeding resulted in a significant ($P<0.05$) decrease of IRS-1 protein levels in the gastrocnemius muscle in comparison to control animals. A treatment of four weeks with either NAC or ramipril normalized ($P<0.05$) the decrease of IRS-1 protein levels in gastrocnemius muscle in glucose-fed rats.

2.6 Discussion

In this study, it is reported for the first time that chronic high glucose (10% in drinking water) feeding for 20 weeks increased the production of vascular and hepatic $O_2^{\bullet-}$ along with NADPH oxidase activity. Rats treated chronically with glucose displayed a sustained increase in systolic blood pressure, insulin resistance and a decrease in IRS-1 protein levels in the gastrocnemius muscle. All these abnormalities were restored by a four week treatment with NAC which was associated with the inhibition of vascular and hepatic oxidative stress and a decreased plasma lipid peroxidation. Contrary to NAC, the therapeutic effect of ramipril in chronically glucose-fed rats was not associated with the inhibition of NADPH oxidase activity or lowering of plasma lipid peroxidation markers.

The increase in oxidative stress in glucose-fed rats is in agreement with a previous study performed in the same strain of rats with a regimen of high sucrose [16]. NAC reduced body weight and plasma oxidative stress and normalized metabolic functions in hepatic tissue and thus enhanced fat degradation in chronically sucrose-fed rats [16]. In the present study, NAC also reduced body weight and decreased the overproduction of $O_2^{\bullet-}$ and NADPH oxidase activity in aorta and liver of 20 week glucose-fed rats. Whereas lipid peroxidation was not increased in glucose-fed rats, NAC reduced plasma levels of 4-hydroxynonenal and malondialdehyde, suggesting a protective effect of NAC which is not related to the state of insulin resistance.

The increased blood pressure in 20 week glucose-fed rats is in agreement with our previous short term studies performed in four week glucose-fed rats [10, 11, 12] and with the rat model of fructose feeding which exhibited arterial hypertension [32]. Similar to our study, increases in basal $O_2^{\bullet-}$ production and NADPH oxidase activity

were reported in aorta from insulin-resistant fructose-fed rats [3] and NAC prevented the increase in blood pressure elevation in association with a decrease in oxidative stress in 10 week fructose-fed rats [15]. Thus, our study in 20 week glucose-fed rats supports the involvement of vascular oxidative stress not only in the development of hypertension but also in its maintenance. Likewise hepatic oxidative stress may contribute to the development of insulin resistance induced by glucose feeding. The decrease of IRS-1 protein level in the gastrocnemius muscle of glucose-fed rats is in agreement with the investigation of Giorgino et al. [33] who have reported that total IRS-1 protein level is decreased in skeletal muscle of type 1 diabetic hyperglycemic rats. The present study with NAC and ramipril suggests an involvement of oxidative stress downstream to the inhibition of the renin-angiotensin system in the decrease of IRS-1 protein in skeletal muscle in a type 2 model of diabetes. The recovery of IRS-1 expression by these drugs is believed to contribute to the retablishment of insulin signaling in skeletal muscle and to the inhibition of insulin resistance.

It is well known that ACE inhibitors exert beneficial effects on blood pressure and insulin sensitivity through the inhibition of the renin-angiotensin system and of the kinin metabolism [23-26, 34]. In this study, we tested the hypothesis that ramipril could normalize the vascular and/or metabolic abnormalities through its antioxidant properties in the model of glucose-induced insulin resistance. Interestingly, we found that ramipril normalized the rise in blood pressure in parallel with a reduction in aortic basal superoxide anion production in 20 week glucose-fed rats. However, it had a weaker, only partial, effect to improve insulin resistance. This was associated with a lack of improvement in basal hepatic superoxide production. Furthermore, ramipril failed to decrease plasma levels of 4-hydroxynonenal and malondialdehyde as well as NADPH oxidase activity in aorta and liver tissues in chronically glucose-fed rats. This

finding suggests that ACE inhibitors may exert their beneficial effects on blood pressure and oxidative stress, at least in part, through an NADPH oxidase-independent pathway involving additional mechanisms such as the kinin-NO pathway [25]. In addition, the normalization of skeletal muscle IRS-1 protein by ramipril, while insulin resistance was not reduced to normal in contrast to the effect of NAC treatment, indicates that additional defects in insulin signaling exist, likely mediated by oxidative stress, in liver and/or muscle. These data suggest that the "antioxidant" effects of ramipril may be limited to vascular tissue, a more efficient target of ACE inhibitors.

In conclusion, this study suggests that the reversal of hypertension and insulin resistance in chronically glucose-fed rats is linked to the inhibition of vascular and hepatic oxidative stress mediated, at least in part, by an NADPH oxidase dependant pathway. The results also indicate that while NAC exerts effective antioxidant activity in multiple tissues, ramipril appears to preferentially target the vasculature.

2.7 Acknowledgment

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Table 1

Effect of four weeks treatment (from 16 to 20 weeks) with NAC or ramipril on systolic blood pressure (SBP) in glucose-fed rats.

	SBP (mmHg)		
	Week 16	**Week 18**	**Week 20**
Control (14)	123.4 ± 3.6	125.8 ± 1.5	124.9 ± 1.4
Glucose (18)	149.0 ± 2.2 ***	158.2 ± 1.8 ***	154.9 ± 2.5***
Glucose + NAC (7)	149.6 ± 5.2 ***	150.5 ± 8.7 ***	127.7 ± 3.6 †††
Glucose + ramipril (11)	152.9 ± 6.4 ***	134.0 ± 5.5 †††	127.5 ± 3.4 †††

Data are means ± SEM of values obtained from (n) rats. Statistical comparison to control (*) or to glucose-treated rats (†) is indicated by ***, †††$P < 0.001$, respectively.

Table 2

Effects of chronic glucose feeding (20 weeks) combined or not with NAC or ramipril treatments (final four weeks, week 16-20) on final body weight, plasma levels of glucose and insulin as well as insulin resistance (HOMA).

	Control n = 14	**Glucose n = 18**	**Glucose+NAC n = 7**	**Glucose+Ramipril n = 11**
Body weight (g)	615.5 ± 18.7	604.6 ± 19.5	458.9 ± 8.8 ††	611.0 ± 21.9
Plasma glucose (mmol/L)	5.5 ± 0.2	6.9 ± 0.2**	5.1 ± 0.1 ††	5.9 ± 0.2 ††
Plasma insulin (ng/ml)	3.1 ± 0.3	5.6 ± 0.4**	2.0 ± 0.8 ††	4.4 ± 0.5
HOMA	13.6 ± 1.8	36.2 ± 5.1**	13.0 ± 4.6 ††	28.6 ± 4.5

Data are means ± SEM of values obtained from (n) rats. Statistical comparison to control (*) or to glucose-treated rats (†) is indicated by **, ††$P < 0.01$, respectively.

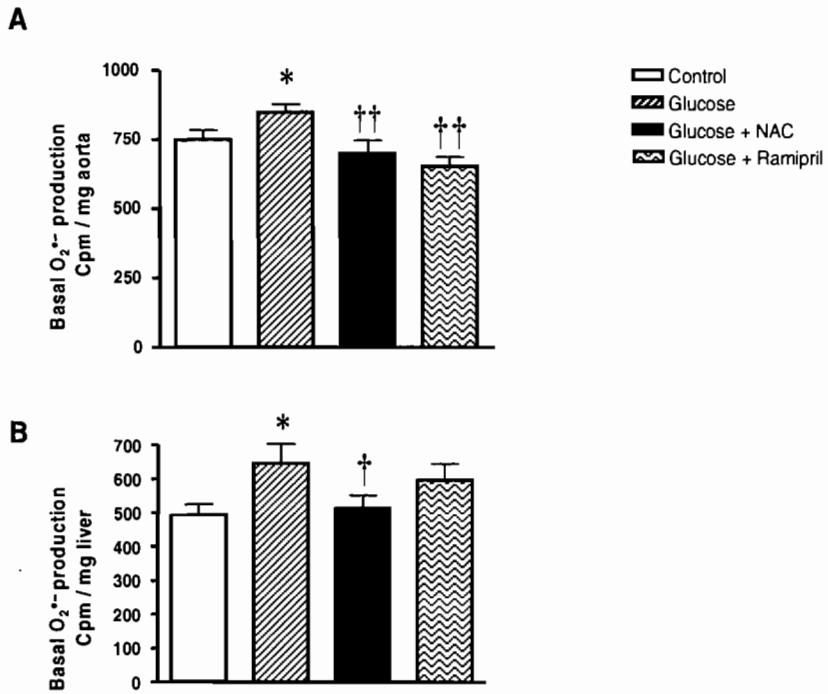

Figure 1

Effects of 20 weeks of glucose feeding combined or not with four weeks (week 16-20) of NAC or ramipril treatment on superoxide anion production in the aorta (A) and liver (B). *P<0.05 vs control; † P<0.05 vs glucose, †† P<0.01 vs glucose.

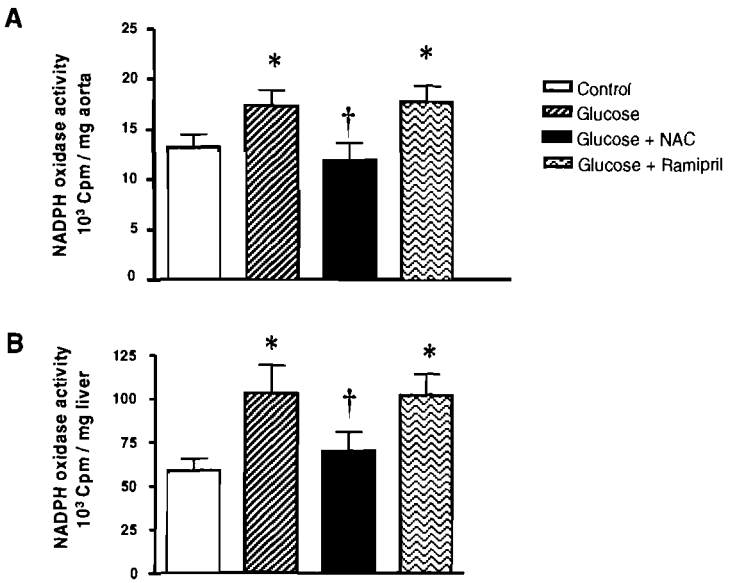

Figure 2

Effects of 20 weeks of glucose feeding combined or not with four weeks (week 16-20) of NAC or ramipril treatment on NADPH oxidase activity in the aorta (A) and liver (B). *P<0.05 vs control; † P<0.05 vs glucose.

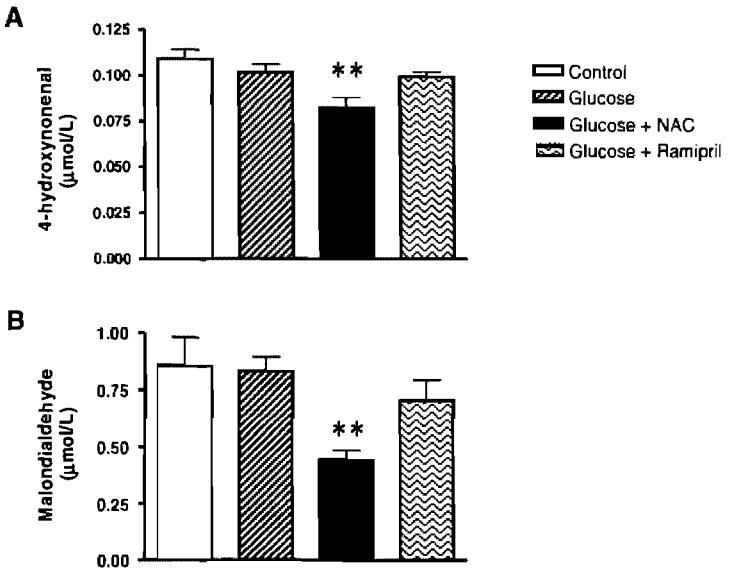

Figure 3

Effects of 20 weeks glucose feeding combined or not with four weeks (week 16-20) of NAC or ramipril treatment on plasma levels of 4-hydroxynonenal (A) and malondialdehyde (B). $^{**}P<0.01$ vs control.

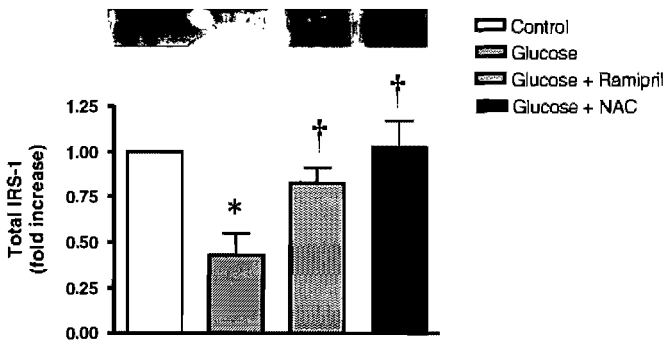

Figure 4

Effects of 20 weeks of glucose feeding combined or not with four weeks (week 16-20) of NAC or ramipril treatment on IRS-1 protein levels in the gastrocnemius muscle (expressed in arbitrary unit). *P<0.05 vs control; † P<0.05 vs glucose.

CHAPTER THREE

Article # 2: Blockade of sensory abnormalities and kinin B_1 receptor expression by N-acetyl-L-cysteine and ramipril in a rat model of insulin resistance

BLOCKADE OF SENSORY ABNORMALITIES AND KININ B_1 RECEPTOR EXPRESSION BY N-ACETYL-L-CYSTEINE AND RAMIPRIL IN A RAT MODEL OF INSULIN RESISTANCE

Mahmoud Ali Ismael[1], Sébastien Talbot[1], Cynthia L. Carbonneau[2], Christian M. Beausejour[2], Réjean Couture[1*]

[1]Department of Physiology, Faculty of Medicine, Université de Montréal, C.P. 6128, Succursale Centre-ville, Montréal, Québec, Canada H3C 3J7

[2]Centre Hospitalier Universitaire Ste-Justine and Department of Pharmacology, Université de Montréal, Quebec, Canada H3T 1C5

Short running title: Allodynia and kinin B_1 receptor in diabetes

*Corresponding author

Tel.: [information retirée / information withdrawn] fax. [information retirée / information withdrawn]

E-Mail address: [information retirée / information withdrawn]

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3.0 Abstract

Glucose-fed rats is a model of insulin resistance that displays sensory polyneuropathy and hypertension. This study aimed at comparing the beneficial effects of N-Acetyl-L-cysteine (NAC, antioxidant) and ramipril (angiotensin-1 converting enzyme inhibitor) on tactile and cold allodynia induced by chronic glucose feeding. Impact of these treatments was also assessed on hypertension, hyperglycaemia and kinin B_1 receptor expression.

Male Wistar rats (50-75 g) were given 10% D-glucose in their drinking water for 11 weeks or tap water only (controls). Treatment with NAC (1 g/kg/day, gavage) or ramipril (1 mg/kg/day in drinking water) was initiated after six weeks, for a period of five weeks. Glucose feeding led to significant increases in systolic blood pressure (tail-cuff photoplethysmography) and kinin B_1 receptor mRNA in the spinal cord and renal cortex (real-time QRT-PCR) along with tactile and cold allodynia (application of von Frey filaments and acetone to the plantar surface of the hindpaws). NAC and ramipril reversed gradually tactile and cold allodynia from 6 to 11 weeks. Hypertension, hyperglycaemia and kinin B_1 receptor expression were also normalized at 10-11 weeks. Data suggest an association between the expression of B_1 receptor, the oxidative stress (documented in a previous study) and the sensory abnormalities and hypertension encountered in this model of insulin resistance.

Key words: ACE inhibitor; allodynia; arterial hypertension; diabetes; insulin resistance; N-Acetyl-L-cysteine; oxidative stress; pain neuropathy

3.1 Introduction

Diabetes Mellitus is a serious illness with multiple complications and premature mortality, accounting for at least 10% of total health care expenditure in several countries (King et al., 1998). The World Health Organization has estimated that there will be around 300 millions of clinically diagnosed type 2 diabetes worldwide by the year 2025 (Gorus et al., 2004). The number of diabetic patients is increasing due to growth of population, aging, change in life style, and increasing prevalence of obesity and physical inactivity (Woods et al., 2004). Diabetes is associated with depletion of cellular antioxidant defence system and increased level of reactive oxygen species, which are known as important factors in the onset and progression of diabetes and its complications. Proteins that are damaged by oxidative stress have decreased biological activity leading to loss of energy metabolism, cell signaling, transport, and ultimately, to cell death (Vincent et al., 2004).

Antioxidants or nutrients with high antioxidant capacity may offer a unique therapeutic option for the treatment of diabetes and its complications. Treatment with scavengers of reactive oxygen species improves nerve blood flow, oxygenation and function in experimental diabetes (Cameron et al., 1994; Nagamatsu et al., 1995). N-Acetyl-L-cysteine (NAC) is an endogenous antioxidant acting as a free radical scavenger and precursor of glutathione. It was found to prevent fructose-induced insulin resistance and hypertension in rats (Song et al., 2005), to decrease blood pressure in spontaneously hypertensive rats by decreasing sympathetic activity and restoring cardiac β-adrenergic function (Girouard et al., 2003), and to inhibit the development of diabetic neuropathies (Sagara et al., 1996; Love et al., 1996). Diabetic neuropathies, among the most common complications of type 1 and type 2 diabetes

(Boulton et al., 2005), affect about 33% of patients with diabetes mellitus and are recognized as one of the most difficult type of pain to treat (Daousi et al., 2004). Pain associated with diabetic neuropathy can occur either spontaneously or as a result of exposure to mildly painful stimuli (hyperalgesia) or to stimuli not normally perceived as painful (allodynia) (Brown and Asbury, 1984; Clark and Lee, 1995).

Angiotensin-1 converting enzyme inhibitors (ACEI) have been shown to improve insulin sensitivity and to reduce cardiovascular complications in diabetes {Henriksen et al., 1996; McFarlane et al., 2003). ACEI improve insulin sensitivity and endothelial dysfunction and decrease lipid peroxidation in essential hypertensive patients (Yavuz, et al., 2003). The ACEI ramipril reduced the accumulation of advanced glycation end products in experimental diabetic nephropathy (Forbes et al., 2002). The mechanisms of the therapeutic effects of ACEI include inhibition of the oxidative stress, angiotensin II formation and of kinin catabolism (Couture and Girolami, 2004).

Recently, we reported that a treatment of four weeks with NAC or ramipril normalized the elevation of blood pressure and attenuated insulin resistance along with a reduction in aortic basal superoxide anion production in 20 weeks glucose-fed rats (El Midaoui et al., 2007). B_1 receptor was induced in several tissus in glucose-fed rats and the inhibition of the oxidative stress with a diet enriched with alpha-lipoic acid prevented allodynia and the up-regulation of B_1 receptor. Finally, blockade of the B_1 receptor reversed allodynia and hypertension in this model of insulin resistance (Dias et al., 2007; Lungu et al., 2007).

Thus, the present study was undertaken to determine whether a chronic treatment of five weeks with NAC or ramipril can also reverse tactile and cold allodynia induced by chronic glucose feeding. Furthermore, we tested the possibility

that either treatment could affect the induction and overexpression of kinin B_1 receptor in two selected tissues, the renal cortex and spinal cord.

3.2 Materials and methods

3.2.1 Experimental animals and treatments

Male Wistar rats (50-75 g Charles River, St-Constant, Que., Canada) were housed one per cage, under controlled conditions of temperature (23°C) and humidity (50 %), on a 12 h light-dark cycle and allowed free access to normal chow diet (Charles River Rodent) and tap water (control rats, n = 8) or 10 % D-glucose (Sigma-Aldrich Canada) in the drinking water (diabetic rats, n = 23) during 11weeks. From 6 to 11 weeks glucose-fed rats were randomly divided into three groups; Group 1: vehicle (n = 8), Group 2: NAC (1 g/kg/day, n = 7) and Group 3: ramipril (1 mg/kg/day, n = 8). Ramipril was added to the drinking water, while NAC was given by gavage once daily. Fluid consumption for each rat was monitored every two days to adjust the dose of ramipril throughout the study. All research procedures and the care of the animals were in compliance with the guiding principles for animal experimentation as enunciated by the Canadian Council on Animal Care and were approved by the Animal Care Committee of our University. Effects of NAC and ramipril were determined weekly on body weight, water and food intake, blood glucose, systolic blood pressure, and sensory abnormalities (tactile and cold allodynia).

3.2.2 Measurement of blood glucose and blood pressure

Blood glucose was measured weekly with the glucose oxidase-impregnated test strip and a reflectance meter (Accu-Check III, Boehringer Mannheim, Germany) from a drop of blood obtained by tail pinprick. Systolic arterial blood pressure was measured weekly before and after the administration of NAC and ramipril by tail-cuff photoplethysmography (Harvard Apparatus Ltd.) with the use of a cuff placed around

the tail and recorded on a MacLab/8 system. For each measurement, three individual readings were averaged.

3.2.3 Behavioural testing

Behavioural signs representing tactile and cold allodynia were assessed with the rats placed on a wire mesh floor beneath an inverted plastic cage (20 x 10 x 10 cm). The rats were allowed to adapt for about 15 min or until explorative behaviour ceased.

3.2.4 Tactile allodynia

Tactile allodynia was assessed by measuring the hindpaw withdrawal threshold to a calibrated series of six von Frey filaments (2, 4, 6, 8, 10 and 15 g) (Stoelting, Wood Dale, IL) using the up-down method of Chaplan et al., (1994). Starting with the filament that has the lowest force (2 g), the filament was applied perpendicularly to the mid-plantar surface with sufficient force to cause the filament to buckle slightly. Brisk withdrawal or paw flinching was considered as positive response. Each filament was applied five times to each paw (for 6-8 s per stimulation, with an inter stimulus interval of 1-2 min). Minimum recording of five positive responses (50 %) out of 10 stimulations for both paws was considered to be the threshold (in grams). Absence of a response (less than five withdrawals) prompted use of the next graded filament of increasing weight. Maximal withdrawal threshold in control rats was fixed at 15 g.

3.2.5 Cold allodynia

Cold allodynia was assessed using the acetone drop method described by Choi *et al.* (1994). With the rats in the same conditions of testing, an acetone bubble formed at the end of a standard plastic syringe was placed to the plantar surface of the

hindpaws. Acetone was applied five times to each paw at intervals of 3-5 min. Normal rats either ignore the stimulus or occasionally respond with a small and brief withdrawal. Allodynic rats respond with a prompt and intense paw withdrawal or escape behaviour to acetone application. The frequency of paw withdrawal was expressed as a percentage (the number of paw withdrawals ÷ number of trials × 100).

3.2.6 SYBR green-based quantitative RT-PCR

At the term of the protocol of 11 weeks, rats were anaesthetised with CO_2 inhalation and then decapitated. Approximately 10 mg of rat tissues (thoracic spinal cord and renal cortex) were isolated and put in RNA later stabilisation reagent (QIAGEN, Valencia, CA, USA). Total RNAs were extracted from tissue according to the manufacturer's protocol. First-strand cDNA synthesized from 400 ng total RNA with random hexamer primers was used as template for each reaction with the QuantiTect Rev Transcription Kit (QIAGEN). SYBR Green-based real-time quantitative PCR was performed as described (Aoki et al., 2002). Mx3000p (stratagene) was used for the signal detection and the PCR was performed in 1 × SYBR Green Master mix (QIAGEN) with 300 nM of each primer. For standardization and quantification, rat 18s was amplified simultaneously. The following primer pairs were designed by Vector NTI and used: 5'- GCAGCGCTTAACCATAGCGGAAAT -3' (upper, 367–391) and 5'- CCAGTTGAAACGGTTCCCGATGTT -3' (lower, 478-454) for amplification of rat B_1 receptor (GenBank accession No. NM_030851); 5'- TCAACTTTCGATGGTAGTCGC CGT -3' (upper, 363-386) and 5'- TCCTTGGATGTGGTAGCCGTTTCT -3' (lower, 470-447) for amplification of rat 18s (GenBank accession No. X01117). PCR conditions were: 95°C for 15 min, followed by 46 cycles at 94°C for 15 s, 60°C for 30 s and 72°C for 30 s. The cycle threshold (Ct) value represents the cycle number at which a fluorescent signal rises

statistically above background (Wada et al., 2000). The relative quantification of gene expression was analyzed by the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001).

3.3 Drugs

Ramipril, N-Acetyl-L-cysteine and all other reagents were purchased from Sigma-Aldrich Canada, Ltd (Oakville, ON, Canada).

3.4 Statistical analysis of data

Data are expressed as mean ± SEM of values obtained from (n) rats. Statistical analysis of data was performed with Graph-Pad Prism software. Data were subjected to a one-way analysis of variance (ANOVA), followed by the Bonferroni/Dunn multiple comparison test to estimate the significance of differences between groups. Significance was considered when $P < 0.05$.

3.5 Results

3.5.1 Effect of NAC and ramipril on body weight, blood glucose and blood pressure in glucose-fed rats

As shown in Table 1, rats fed with 10% glucose had their body weight slightly but not significantly reduced when compared to control rats during the protocol of 11 weeks. However the body weight of rats retreated with NAC or ramipril was significantly reduced at 11 and 10 weeks of treatment, respectively. Plasma levels of glucose were significantly increased in glucose-fed rat at 6, 8, 9, 10 and 11 weeks when compared with age-matched control rats. The hyperglycaemia was significantly reduced in the rats treated with NAC from 8 to 11 weeks and with ramipril from 9 to 11 weeks when compared with age-matched glucose-fed rats. Systolic blood pressure was significantly higher in glucose-fed rats at 6 weeks and increased further at 10 and 11 weeks. Treatment with NAC prevented the increase in systolic blood pressure in glucose-fed rats to values which were not significantly different from control values. Ramipril reduced the increase in systolic blood pressure in glucose-fed rats to values which were slightly lower than control values at 10 and 11 weeks.

3.5.2 Effect of NAC and ramipril on food and water intake in glucose-fed rats

As shown in Table 2, food intake was significantly decreased while water intake was significantly increased in glucose-fed rats when compared to control rats receiving tap water during the protocol of 11 weeks. Whereas ramipril had no influence on food intake, NAC showed a significant inhibitory effect at 8 and 11 weeks in glucose-fed rats. Water intake in the ramipril group was significantly increased at 8 and 10 weeks, but there was no significantly change with NAC.

3.5.3 Effects of NAC and ramipril on tactile and cold allodynia in glucose-fed rats

Glucose-fed rats displayed significant and sustained tactile allodynia (decrease in paw withdrawal threshold) from 6 to 11 weeks when compared to age-matched controls (Fig.1). NAC and ramipril caused a progressive and significant reduction of tactile allodynia in glucose-fed rats after 3, 4 and 5 weeks of treatment; the anti-allodynic effect of NAC was quite similar to that produced by ramipril. Tactile allodynia was completely abolished by either treatment after 5 weeks.

Response to acetone stimulation was significantly higher (cold allodynia) at 6 weeks in glucose-fed rats and remained stable up to 11 weeks. The reduction of cold allodynia by NAC in glucose-fed rats reached significance after 3-4 weeks. The inhibition of cold allodynia by ramipril was similar to that achieved with NAC and reached significance at 3, 4 and 5 weeks post-treatment (Fig. 2). Cold allodynia in glucose-fed rats was completely abolished by NAC and ramipril after 5 weeks of treatment.

3.5.4 Effects of NAC and ramipril on kinin B_1 receptor expression in glucose-fed rats

Kinin B_1 receptor mRNA was under expressed in the spinal cord and renal cortex of control rats (Fig. 3). Glucose feeding for a period of 11 weeks increased by 13.6-fold B_1 receptor mRNA in the same tissues. This marked increased of B_1 receptor expression in glucose-fed rats was significantly reversed by 5 weeks treatment with NAC or ramipril. Complete inhibition of receptor expression was achieved in the renal cortex.

3.6 Discussion

In a recent study, we found that four weeks treatment with NAC was associated with the inhibition of vascular and hepatic oxidative stress and NADPH activity along with the reduction of plasma lipid peroxidation, hyperglycaemia, hyperinsulinemia, insulin resistance and hypertension in 20 weeks glucose-fed rats (El Midaoui et al., 2007). Thus, the present study in 11 weeks glucose-fed rats confirms the anti-hypertensive and anti-hyperglycaemic effects of NAC and reveals for the first time a potent anti-allodynic effect under insulin resistance. It is worth noting that NAC acts as a cysteine donor to maintain intracellular glutathione level (endogenous antioxidant enzyme) and mitochondrial oxidative metabolism by protecting the cytochrome oxidase complex I from NO-mediated damage (Moncada; 2000). NAC has been shown to exert neuroprotective effect for a range of neuronal cell type against a variety of stimuli {Cooper and Kristal, 1997).

Ramipril also exerted anti-hypertensive and anti-allodynic effects with a temporal profile similar to that of NAC. The beneficial effect of ramipril on sensory abnormalities and hypertension is also thought to be associated with the inhibition of the oxidative stress as a similar treatment with ramipril normalized the basal over production of aortic superoxide anion in 20 weeks glucose-fed rats (El Midaoui et al., 2007). Because NADPH oxidase has been suggested to be the major superoxide anion-generating enzyme in the vascular wall following the activation of AT_1 receptors by angiotensin II (Cai et al., 2003), ramipril can interfere directly at the source of the oxidative stress by preventing the activation of NADPH oxidase by angiotensin II.

One should consider, however, that in addition to preventing the conversion of angiotensin I into angiotensin II, ramipril can block the degradation of kinins into

inactive metabolites. The protection of endogenous bradykinin by ramipril is expected to preserve endothelial function by reducing the oxidative stress following the release of endothelial nitric oxide (NO) upon activation of B_2 receptors in blood vessels (Couture and Girolami, 2004). Activation of kinin B_2 receptor has also been shown to facilitate glucose uptake by triggering the translocation of GLUT-4 transporter via insulin-dependent and -independent pathways in skeletal muscles (for review see Couture and Girolami, 2004). However, it is unlikely that the endogenous kinins-NO system is involved in the anti-allodynic effect of ramipril because blockade of kinin B_1 and B_2 receptors with selective antagonists reversed tactile and cold allodynia in glucose-fed rats (Dias et al., 2007; Lungu et al., 2007). The latter studies plead in favour of a role for kinin receptors in glucose-induced sensory abnormalities. This statement is further supported by the present results showing the inhibition by NAC or ramipril of the up-regulation of B_1 receptor mRNA in the spinal cord and renal cortex in glucose-fed rats. Earlier studies provided evidence that the oxidative stress induced by chronic glucose feeding is the trigger mechanism of B_1 receptor induction and overexpression both in the spinal cord and peripheral tissues (El Midaoui et al., 2005; Lungu et al., 2007). Hence, it is tempting to propose that the anti-allodynic effect of NAC and ramipril in glucose-fed rats is associated to the inhibition of oxidative stress that leads to the subsequent inhibition of B_1 receptor overexpression.

Impact of NAC and ramipril was also assessed on water and food intake in glucose-fed rats. As usually seen in diabetic states, water intake was increased (dipsogenia) in these rats. Because glucose is given in the drinking water, these rats increased their consumption of glucose that was compensated by a decrease of food intake. The increase of water intake induced by ramipril in glucose-fed rats is believed to be a consequence of the diuretic effect generally observed under treatment with

ACEI. Interestingly, the higher glucose intake resulting from the higher water intake seen in ramipril treated rats did no affect food intake. Thus the lost of body weight induced by ramipril can hardly be explained by the diet. The inhibition of angiotensin II as trophic factor and/or inhibitor of apoptotic mechanism may better explain the reduction of the body mass. Whereas NAC did not significantly affect water intake in glucose-fed rats, it did decrease significantly food intake at some end points. Because NAC contains sulphydryl (SH) groups which repels and provokes nausea, it is likely that this may cause inhibition of appetite and may account for the slight loss of body weight in these rats at the end of the five weeks treatment.

Conclusion

The inhibition of the oxidative stress by NAC (an antioxidant) and ramipril (by preventing the pro-oxidative effect of Ang II) and the subsequent inhibition of B_1 receptor up-regulation can explain the anti-allodynic effect of these drugs in glucose-fed rats. Thus antioxidants and ACEI may have therapeutic value in the treatment of diabetic sensory polyneuropathy.

3.7 Acknowledgements

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Table 1. Effect of NAC and ramipril on body weight, blood glucose and blood pressure in glucose-fed rats

	6 weeks	7 weeks	8 weeks	9 weeks	10 weeks	11 weeks
Control (n=8)						
Body weight (g)	369± 8	429±10	470±13	491±13	509±18	523±16
Plasma glucose (mM)	5.9± 0.1	6.2± 0.2	5.7± 0.2	5.3± 0.1	5.7± 0.2	4.9± 0.1
Blood pressure (mmHg)	120±2	n.d.	n.d.	n.d.	134± 3	139± 1
Glucose (n=8)						
Body weight (g)	355± 9	410±12	465±13	469±14	493±16	517±16
Plasma glucose (mM)	7.8± 0.3***	5.8± 0.3	7.0± 0.6***	6.6± 0.2***	8.9± 0.9***	6.9± 0.2***
Blood pressure (mmHg)	146± 4***	n.d.	n.d.	n.d.	161± 3***	161± 3***
Glucose+NAC (n=7)						
Body weight (g)	364± 9	385±14	421±18	435±19	450±19	463±15*
Plasma glucose (mM)	8.4± 0.4***	5.8± 0.3	5.8± 0.3†††	5.0± 0.2†††	5.4± 0.2†††	5.4± 0.2†††
Blood pressure (mmHg)	148± 4***	n.d.	n.d.	n.d.	129± 3†††	137± 3†††
Glucose+ramipril (n=8)						
Body weight (g)	369± 7	373±18	464±27	434±16	439±20*	469±20
Plasma glucose (mM)	8.0± 0.3***	5.5± 0.2	6.7± 0.3	5.3± 0.2†††	5.0± 0.2†††	5.3± 0.1†++
Blood pressure (mmHg)	149± 3***	n.d.	n.d.	n.d.	123± 3†††*	121± 2†††***

Values represent the mean ± S.E.M obtained from (n) rats. Statistical comparison to control (*) and glucose (†) is indicated by * P< 0.05, ***†††P< 0.001.Treatment with NAC (1 g /kg/day) and ramipril (1 mg/kg/day) were given from 6 to 11 weeks in glucose-treated rats. Not determined (n.d.).

Table 2.Effect of NAC and ramipril on food intake and water intake in glucose-fed rats

	6weeks	7weeks	8weeks	9weeks	10weeks	11weeks
Control (8)						
Food intake(g)	23.6±1.2	29.9±1.9	30.6±1.1	31.7±0.9	31.9±0.8	30.7±1.3
Water intake(ml)	89.4±5.2	98.7±6.4	92.5±11.5	76.9±3.4	96.2±4.9	95.6±5.9
Glucose (8)						
Food intake(g)	16.4±0.5***	18.5±0.9***	19.2±1.2***	17±1.3***	16.4±1.1***	21.4±1.6***
Water intake(ml)	142.5±12.2***	155±8.2***	140.0±5.9***	161.3±4.4***	161.9±4.6***	170.0±4.6***
Glucose+NAC (7)						
Food intake(g)	14.9±1.0***	18.4±1.3***	12.8±0.8***††	14.9±0.9***	14.1±1.2***	13.7±0.9***††
Water intake(ml)	142.9±7.5***	141.4±7.5***	147.1±8.6***	155.7±7.5***	144.3±5.7***	155.7±6.6***
Glucose+Ramipril (8)						
Food intake(g)	19.6±1.1	22.0±1.2***	16.4±1.2***	16.0±1.0***	16.2±1.1***	17.1±1.0***
Water intake(ml)	137.5±7.6***	171.3±5.5***	173.8±12.1***††	156.3±3.7***	195.0±3.3***††	195.0±4.2***

Values represent the mean ±S.E.M obtained from (n) rats. Statistical comparison to control (*) and glucose (†) is indicated by †† $P < 0.01$, †††*** $P< 0.001$. Treatment with NAC (1 g /kg/day) and ramipril (1 mg/kg/day) were given from 6 to11 weeks in glucose-treated rats.

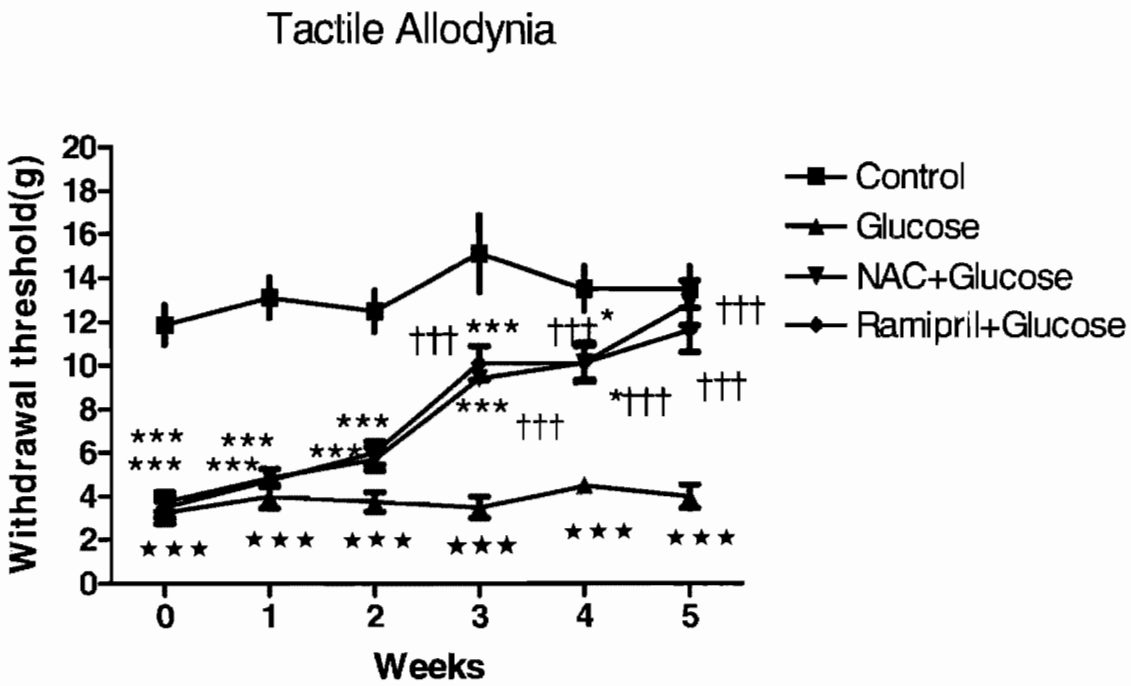

Figure 1

Effect of a treatment of five weeks with N-Acetyl-L-cysteine (1 g/kg/day, gavage) or ramipril (1 mg/kg/day, in drinking water) on tactile allodynia induced by glucose feeding (10 % in drinking water). Control rats received tap water only. Glucose was given for a period of 11 weeks and treatment with NAC or ramipril was initiated after six weeks. Data are means ± SEM of (n) rats as indicated in Table 1. Statistical comparison with controls (*) or glucose-fed rats (†) is indicated by *P< 0.05, ***†††P< 0.001.

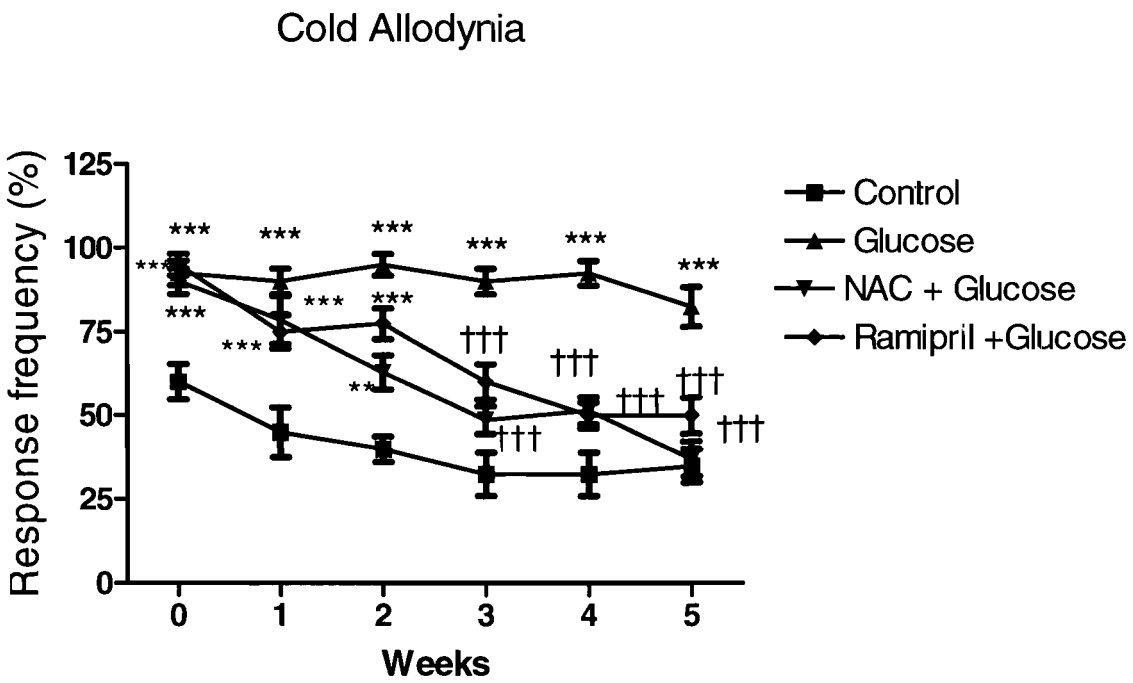

Figure 2

Effect of a treatment of five weeks with N-Acetyl-L-cysteine (1 g/kg/day, gavage) or ramipril (1 mg/kg/day, in drinking water) on cold allodynia induced by glucose feeding (10 % in drinking water). Control rats received tap water only. Glucose was given for a period of 11 weeks and treatment with NAC or ramipril was initiated after six weeks. Data are means ± SEM of (n) rats as indicated in Table 1. Statistical comparison with controls (*) or glucose-fed rats (†) is indicated by **P< 0.01, ***†††P<0.001.

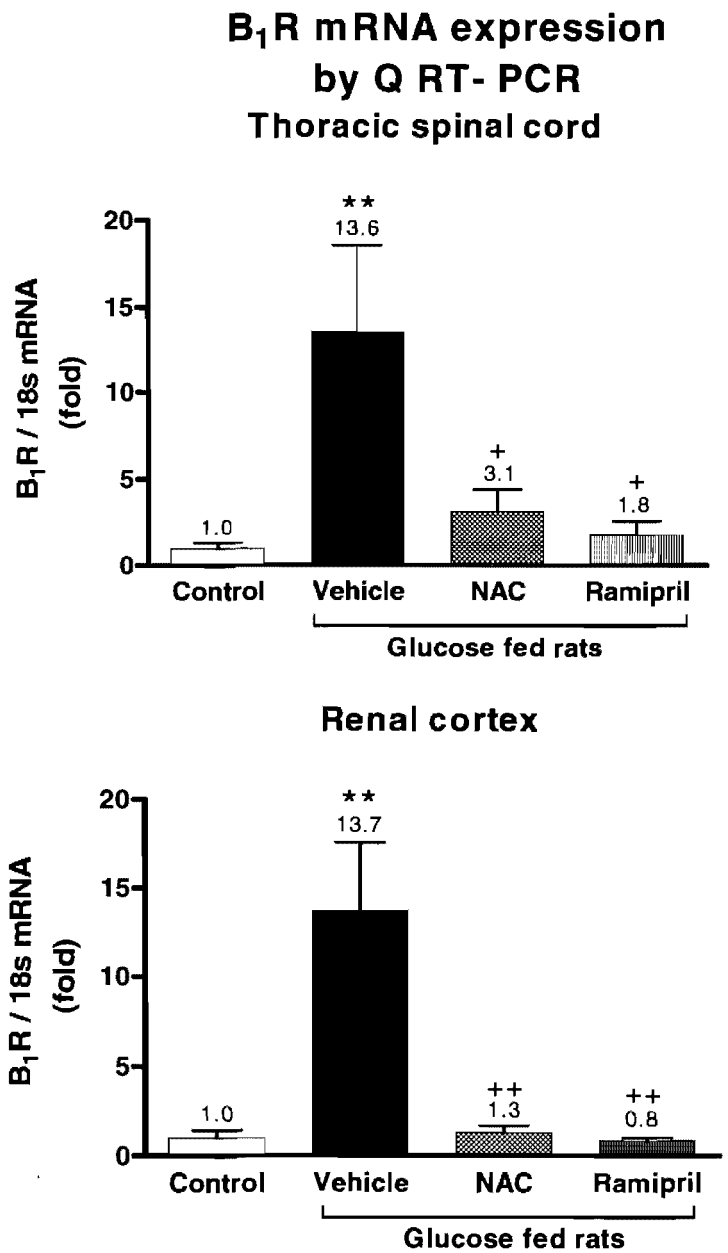

Figure 3

Effect of a treatment of five weeks with N-Acetyl-L-cysteine (1 g/kg/day, gavage) or ramipril (1 mg/kg/day, in drinking water) on B_1R mRNA over expression induced by glucose feeding (10 % in drinking water) in the thoracic spinal cord and renal cortex. Control rats received tap water only. Glucose was given for a period of 11 weeks and treatment with NAC or ramipril was initiated after 6 weeks. Data are means ± SEM of (5 to 7) rats per groups. Statistical comparison with controls (*) or glucose-fed rats ($^+$) is indicated by $^+ P<0.05$, ** $^{++} P < 0.01$.

CHAPTER FOUR

GENERAL DISCUSSION

4.0 Diabetes and oxidative stress

The relationship between hyperglycaemia, oxidative stress, cellular and endothelial dysfunction has been documented in recent studies. Increased formation of superoxide radicals and inactivation of nitric oxide in condition of hyperglycaemia is one of the probable causes of the evolution of vascular complications in diabetes mellitus. Moreover, data from animal and cell culture models of diabetes as well as clinical trials with antioxidants strongly implicate hyperglycemia-induced oxidative stress in diabetic neuropathy (Vincent et al., 2004). In animal studies, arterial tissue $O_2^{\bullet -}$ levels were reported to be increased in insulin resistant rats (Kashiwagi et al., 1999), and in spontaneously hypertensive rats (Suzuki et al., 1995; Kerr et al., 1999). This gives rationale to look for a relationship between the oxidative stress, diabetes and its complications. Thus inhibition of the oxidative stress with different antioxidants should prevent diabetes and its complications.

Antioxidants are compounds that have a chemical affinity for free radicals and bond with free radicals before they can cause harmful effect and damage. Antioxidants are: 1) enzymes such as catalase, peroxidase, and superoxide dismustase (SOD); 2) peptides such as glutathione, phenolic compounds like vitamin E and plant flavonoids; 3) nitrogen compounds which include various amino acids and carotenoids. Other agents may have antioxidant effects through replenishing mechanisms. Vitamin C, for instance, helps to recycle vitamin E, and N-acetyl-L-cysteine (NAC) provides an important component of glutathione.

The effects of antioxidants on oxidative stress are measured by specific observable biomarkers. These markers include the enzymatic activities of catalase, SOD, GSH-Px, and GSH-reductase, as well as thiobarbituric acid reactants levels, an indirect measurement of free-radical production that has been shown to be consistently elevated in diabetes. In our study, superoxide anion production was measured in isolated aortic and hepatic small slices using the lucigenin-enhanced chemiluminescence method, and by this method we measured the effect of NAC and ACEI (ramipril) on oxidative stress in glucose-fed rats.

NAC acts as a free radical scavenger and a cysteine donor to maintain intracellular glutathione level (endogenous antioxidant enzyme) and mitochondrial oxidative metabolism by protecting the cytochrome oxidase complex I from NO-mediated damage (Moncada, 2000, De Vries et al., 1993). It did prevent fructose-induced insulin resistance and hypertension in rats (Song et al,.2005), and exerted protective effect against glucose toxicity on pancreatic β-cells in various models of diabetes in addition to reducing blood glucose and increasing glucose-induced insulin secretion (Ho et al.,1999; Kaneto et al.,1999; Tanaka et al., 1999). NAC was found to inhibit the development of functional and structural abnormalities of the peripheral nerve in streptozotocin-induced diabetic rats (Sagara et al., 1996), to inhibit diabetic neuropathy and to protect against neuropathies caused by chemotherapy drugs (Love et al., 1996). Thus NAC is neuroprotective for a range of neuronal cell type against a variety of stimuli (Cooper et al., 1997).

Moreover, studies using alpha-lipoic acid, another antioxidant, were reported to lower blood pressure in SHR (Vasdev et al., 2000), to increase insulin-stimulated glucose metabolism and to reduce insulin resistance in fatty Zucker rats (Jacob et al.,1996) in addition to increasing tissue levels of gluthatione in mice (Busse et al.,1992). Treatment with alpha-lipoic acid also prevented the sustained elevation of blood pressure, the basal over production of $O_2^{\bullet -}$ in cardiovascular tissues and attenuated the development of insulin resistance in 4 and 12 weeks glucose-fed rats (Midaoui and de Champlain, 2002; Midaoui et al., 2003; 2005).

4.1 Advantages and disadvantages of the model of glucose-fed rats

We focused on the effects of NAC and ACEI (ramipril) in glucose-fed rats. This model of insulin resistance has several advantages over the commonly used genetic models of type 2 diabetes. First, the diabetes induced by glucose is controllable and much less costly than the commercially available genetic models. Second, this experimental model does mimic the North American diet enriched of carbohydrates (soft drinks, fruit juices). Third, the model of glucose-fed rats is not linked to obesity, and allows studying diabetes and its complications without association with hyperlipidemia; for instance, the body weight is not affected in glucose-fed rats. On the other hand, the model of glucose-fed rats is not perfect and one possible disadvantage to mention is the fact that it is not a real model of type 2 diabetes as it does present the characteristics of a pre-diabetic state with insulin resistance. This provides the possibility, however, to study the mechanisms involved in the development of the early phase of diabetes. Hyperglycaemia is indeed borderline and not very striking. The reason why glucose levels in glucose-fed rats is not as high as in diabetic subjects is likely due to the presence of hyperinsulinemia. Indeed,

hyperinsulinemia increases glucose uptake and would prevent hyperglycaemia to occur. However, pancreas insufficiency and insulin deficit are expected to occur in a later stage and may lead to higher hyperglycaemia. Importantly, it is worth noting that glucose-fed rats present other hallmarks generally described in type 2 diabetes such as arterial hypertension, hyperinsulinemia, insulin resistance and polyneuropathy.

The model of glucose has other advantages over the commonly used model of streptozotocin diabetic rats (STZ). Rats treated with STZ develop type 1 and not type 2 diabetes. In our study, we focused on the effects of NAC and ramipril on the complications (hyperglycaemia, hypertension, hyperinsulinemia, and sensory abnormalities) associated with a model of insulin resistance (pre type 2 diabetic phase). A recent study was performed in STZ-induced diabetes treated with an ACEI (lisinopril, 5 mg/kg/d), an antioxidant (NAC, 0.5 g/kg/d) or with their combination. Authors showed that the treatment with the ACEI or NAC attenuated ROS formation and prevented phenotypic changes in the heart (cardiomyocyte hypertrophy, perivascular fibrosis) and in the aorta of seven weeks STZ rats. Also, systolic blood pressure was significantly lower in the two groups receiving ACEI, alone or in combination with NAC. Blood glucose level remained elevated in all groups of STZ- diabetic rats, suggesting a dissociation with hyperglycaemia (Fiordaliso et al., 2006). In that case, the increased oxidative stress is most likely induced by angiotensin II and its inhibition with lisinopril can account for the reduction of ROS and the cardiac complications.

We reported that the increased oxidative stress plays an important role in the development of complications in glucose-fed rats and contributes to the up-regulation of B_1 receptor (Dias et al., 2007; Lungu et al., 2007). It was reported that B_1 receptor is

induced in several peripheral organs, the spinal cord and brain in STZ-diabetic rats (Couture et al., 2001; Abdouh et al., 2003; Vianna et al., 2003; Ongali et al., 2004; Campos et al., 2005). The STZ model of type 1 diabetes was used to study the involvement of B_1 receptor in thermal hyperalgesia (Gabra et al., 2005).

A final concern that deserves to be addressed is the reduction of food intake caused by glucose feeding. The consumption of glucose decreased the appetite for food as documented in the second study. One can raise the possibility that this model can mask a malnutrition effect as rats would eat less proteins and other essential nutrients from the diet. Although the real impact of this issue on our data is difficult to address, an unpublished study performed in our laboratory showed that the calories balance (total calories ingested daily from the chow diet and 10 % glucose solution) was maintained throughout the treatment with glucose. This means that rats, contrary to obese human beings, show a strict regulation of their daily calories intake. Thus greater glucose ingestion is finely compensated by a reduction of eating by the central mechanisms of satiety.

Although ramipril failed to interfere with the mechanism of food intake in glucose-fed rats, NAC reduced further food intake induced by glucose. This may account for the loss of body weight measured in rats receiving NAC. The reduction of food appetite by NAC appears independent of the water intake because glucose-fed rats receiving ramipril drank more without changing their food intake. Because NAC contains sulfhydryl (SH) groups which repels and provokes nausea, it is likely that this may cause inhibition of appetite. Although the impact of this side effect on insulin resistance and the

reduction of oxidative stress is not known at the present time, it should be mentioned that a diet enriched with alpha-lipoic acid, another antioxidant, had the same beneficial effects as that of NAC on insulin resistance, oxidative stress and arterial hypertension in glucose-fed rats (Midaoui et al., 2003; 2005; Lungu et al., 2007)

NAC and Ramipril were not given to control groups for the following reasons:

1- B_1R is not expressed in control rats. We do not expect that an antioxidant or an ACEI would induce this receptor.

2- Oxidative stress is not abnormal in controls and therefore antioxidants should be ineffective.

3- In control rats, blood pressure and metabolic parameters are normal and the rats do not display sign of allodynia.

NAC and ACEI are expected to normalize alterations and to exert minimal effect on control values. Nevertheless, one cannot exclude an effect of these drugs on lipid peroxidation in control rats. It is also advisable to provide the same treatments to controls to assess any unexpected effects exerted by pharmacological treatments. This should be considered in the design of future experiments.

4.2 Observations made in the two articles

In the first study, we investigated the ability of NAC and ramipril (ACEI) to reverse arterial hypertension, insulin resistance, the oxidative stress, lipid peroxidation and changes of skeletal muscle insulin receptor substrate-1 (IRS-1) protein expression in glucose-fed rats. For that purpose we used young male Wistar rats (50-75 g) as rats were treated with 10 % D-glucose for a period of 20 weeks. Large rats are more difficult to manipulate, to assess blood pressure and require greater amounts of drugs. We found that

chronic administration of glucose for 16, 18 and 20 weeks resulted in a significant increase in systolic arterial blood pressure when compared to control rats. The increase in systolic blood pressure in glucose-fed rats was not significantly reduced by NAC after two weeks but was significantly reduced after four weeks treatment with NAC. On the other hand, the increase in systolic blood pressure was reversed to control values after two and four weeks of treatment with ramipril. Whereas 20 weeks (first study) and 11 weeks (second study) glucose feeding had no significant effect on final body weight, a treatment of four or five weeks with NAC reduced significantly body weight in chronically glucose-fed rats. Whereas four weeks treatment with ramipril had no significant effect on body weight in 20 weeks glucose-fed rats, ramipril reduced body weight in the second study. This discrepancy cannot easily be explained as in both studies the same dose of ramipril was provided; the age of rats when treatment with ramipril was initiated was however different (from 16 to 20 weeks versus 6 to 11 weeks in the first and second study, respectively). One should notice that the effect of ramipril was not sustained and was seen only after four weeks of treatment; values at three and five weeks were not significantly different from non treated glucose-fed rats. Chronic glucose feeding for 20 weeks increased the production of oxidative stress as assessed by the measurement of vascular and hepatic $O_2^{\bullet -}$, and NADPH oxidase activity. This finding is in agreement with a previous study performed in the same strain of rats with a regimen of high sucrose (Diniz et al., 2006). The treatment of glucose-fed rats with NAC for four weeks inhibited vascular and hepatic oxidative stress and plasma lipid peroxidation, suggesting the involvement of oxidative stress in the cardiovascular and metabolic abnormalities occurring in this model of insulin resistance. The restoration of IRS-1

protein in skeletal muscle of chronically glucose-fed rats by NAC also suggests that part of the beneficial effect related to the inhibition of the oxidative stress is mediated by the improvement of insulin receptor function.

The first study also revealed that treatment with ramipril for four weeks normalized high blood pressure and reduced insulin resistance and hyperinsulinemia chiefly through its antioxidant properties at the level of vascular tissues because aortic but not hepatic basal superoxide anion production was inhibited by ramipril in 20 weeks glucose-fed rats. Also the treatment with ramipril had no effect on NADPH oxidase activity in aorta and liver tissues in glucose-fed rats and failed to decrease plasma levels of 4-hydroxynonenal and malondialdehyde. These findings suggest that ramipril may exert its beneficial effects on blood pressure, insulin resistance and the oxidative stress through an NADPH oxidase independent pathway.

The second study was undertaken to determine whether a chronic treatment of five weeks with NAC or ramipril can also reverse sensory abnormalities (tactile and cold allodynia) in the experimental rat model of insulin resistance induced by chronic glucose feeding. This study, initiated in young male Wistar rats (50-75 g) for the reasons given above, confirms the anti-hypertensive and anti-hyperglycemic effects of NAC and reveals for the first time a potent anti-allodynic effect in glucose-fed rats. Ramipril also exerted anti-hypertensive and anti-allodynic effects with a temporal profile similar to that obtained with NAC.

Part of the beneficial effect of ACEI (ramipril) might be attributed to inhibition of kinin B_1 receptor activity in addition to the blockade of the renin-angiotension system.

Treatment with ramipril reversed the up-regulation of B_1 receptor expression in tissues (spinal cord and renal cortex) of glucose-fed rats. In a previous study, we provided evidence that B_1 receptors are involved in hypertension and allodynia in the model of glucose. For instance, a treatment with SSR240612, a non-peptide B_1 receptor antagonist, prevented high blood pressure in glucose-fed rats (Dias, M.Sc. Thesis, 2007), as well as tactile and cold allodynia (Dias et al., 2007). Moreover, i.c.v. injection of R-715, another B_1 receptor antagonist, inhibited arterial hypertension in glucose-fed rats (Estevão et al., 2006). R-715 was used to dissociate the contribution of central and peripheral B_1 receptors because this antagonist does not cross the blood brain barrier. When injected intraperitoneally, R-715 failed to affect high blood pressure but blocked tactile and cold allodynia in glucose-fed rats (Lungu et al., 2007). These results allowed us to conclude that arterial hypertension induced by glucose is mediated by central B_1 receptors while allodynia is mediated by peripheral B_1 receptors.

ACEI can also prevent the degradation of kinins and by this way can exert vasodilatation and decrease blood pressure elevation through B_2 receptor activation. This concept was recognized as a beneficial affect of ACEI in the treatment of hypertension (Couture and Girolami, 2004). Thus ACEI appear to exert paradoxical effect on the kinin system which can be explained by the opposite effects of kinins in the periphery (B_2 receptor mediated vasodilatation and decrease blood pressure via the endothelial-NO pathway) and the CNS (B_1 receptor mediated vasoconstriction and increase blood pressure through activation of the sympathetic nervous system).

The beneficial effect of NAC and ramipril with regard to sensory abnormalities also appear to be due to inhibition of the oxidative stress and B_1 receptor expression. Both treatments were found to inhibit the expression of B_1 receptors in the spinal cord and peripheral tissue (renal cortex) and B_1 receptor antagonists blocked allodynia (Dias et al., 2007; Lungu et al., 2007). The use of a diet supplemented with alpha-lipoic acid, another antioxidant, blocked allodynia and prevented the induction and overexpression of B_1 receptors in the spinal cord and several peripheral tissues of glucose-fed rats (Lungu et al., 2007). Hypertension, insulin resistance, hyperinsulinemia and the overproduction of aortic superoxide anion were also normalized in glucose-fed rats by the diet enriched of alpha-lipoic acid.

ACEI are currently among the best medications, and their therapeutic effects in cardiovascular disease appear to be exerted through several mechanisms: 1- inhibition of the renin-angiotensin system by preventing the conversion of angiotensin I to angiotensin II; 2- inhibition of the oxidative stress by preventing the activation of NADPH oxidase by angiotensin II on the AT_1 receptor; 3- inhibition of the metabolic degradation of kinins into inactive peptides, thus allowing the release of NO from endothelial cells upon activation of B_2 receptors by endogenous kinins; 4- enhancement of B_2 receptor expression (Ongali et al., 2003); 5- inhibition of B_1 receptor expression in the CNS and peripheral tissues, most likely through their antioxidant properties. The latter mechanism regarding the inhibition of the deleterious effect of B_1 receptor on the cardiovascular system and sensory abnormalities was discovered in studies conducted in the frame of this thesis.

4.3 Perspectives

Additional studies using conventional models of type 2 diabetes (Obese diabetic Zucker rats, obese db/db diabetic mice) are needed to consolidate the contribution of B_1 receptors in the cardiovascular and sensory abnormalities occurring in diabetes. Also, it would be relevant to demonstrate whether B_1 receptor antagonists can block the oxidative stress and metabolic changes (hyperglycaemia, hyperinsulinemia, hyperlipidemia, insulin resistance) in diabetes. The role of kinin receptors in other complications, including retinopathy, nephropathy and vasculopathy (vascular remodelling, fibrosis, apoptosis) are subjects which warranty further investigations.

Summary and Conclusion

The salient findings of this thesis are the following:

1- Chronic treatment with 10 % glucose in drinking water for 11 or 20 weeks induced hyperglycaemia, hyperinsulinemia, insulin resistance, arterial hypertension, aortic and hepatic oxidative stress (over production of superoxide anion and increased NADPH oxidase activity), reduction of skeletal muscle insulin receptor substrate-1 (IRS-1) and sensory abnormalities (tactile and cold allodynia) in rats. These alterations were accompanied by an induction and up-regulation of kinin B_1 receptor mRNA in the spinal cord and renal cortex.

2- A treatment with the antioxidant N-acetyl -L-cysteine (1-2 g/kg/day) or the ACEI ramipril (1 mg/kg/day) initiated 4-5 weeks prior to the end of the treatment with glucose (11 and 20 weeks) restored all these abnormalities occurring in glucose-fed rats. Although NAC and ramipril provided the same beneficial therapeutic effects, dissociation was seen on the oxidative stress. Whereas NAC prevented the

increased production of superoxide anion and NADPH oxidase activity in aorta and liver, ramipril targeted the oxidative stress on vascular tissue by a mechanism independent of NADPH oxidase activity. Also NAC but not ramipril reduced plasma levels of 4-hydroxynonenal and malondialdehyde, two markers of lipid peroxidation.

It is concluded that arterial hypertension and sensory abnormalities encountered in the model of insulin resistance induced by glucose may be due to the induction and overexpression of central and peripheral kinin B_1 receptors. The therapeutic effect afforded by NAC and ramipril involves the inhibition of kinin B_1 receptor overexpression which is triggered by the oxidative stress. Thus any strategies aimed at blocking the oxidative stress and/or the B_1 receptors would prevent the development of diabetes and its complications.

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