SUBRATA KUMAR BISWAS

INFLAMAÇÃO RENAL E ESTRESSE OXIDATIVO EM RATOS ESPONTANEAMENTE HIPERTENSOS ANTES DO DESENVOLVIMENTO DA HIPERTENSÃO ARTERIAL E NO DIABETES MELLITUS PRECOCE

CAMPINAS

2007

Este exemplar corresponde à versão final da **Tese de Doutorado** apresentada ao Programa de Pós-Graduação Clínica Médica da Faculdade de Ciências Médicas da UNICAMP, para obtenção do título de Mestre em Clínica Médica, área de Clínica Médica, do (a) aluno (a) **SUBRATA KUMAR BISWAS.**

Campinas, 01 de fevereiro de 2007.

Prof (a). Dr (a). José Butori Lopes de Faria
Orientador (a)

SUBRATA KUMAR BISWAS

RENAL INFLAMMATION AND OXIDATIVE STRESS IN SPONTANEOUSLY HYPERTENSIVE RATS BEFORE DEVELOPMENT OF HYPERTENSION AND IN EARLY DIABETES

Tese de Doutorado apresentada à Pós-graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção de título de Doutor em Clínica Médica, área de concentração em Clínica Médica.

Orientador: José Butori Lopes de Faria

CAMPINAS

2007

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DA FACULDADE DE CIÊNCIAS MÉDICAS DA UNICAMP

Bibliotecário: Sandra Lúcia Pereira – CRB-8ª / 6044

B545r

Biswas, Subrata Kumar
Inflamação renal e estresse oxidativo em ratos espontaneamente hipertensos antes do desenvolvimento da hipertensão e no diabetes precoce. / Subrata Kumar Biswas. Campinas, SP : [s.n.], 2007.

Orientador : José Butori Lopes de Faria
Tese (Doutorado) Universidade Estadual de Campinas. Faculdade de Ciências Médicas.

1. Diabetes mellitus. 2. Nefropatia diabética. 3. Hipertensão. 4. Inflamação. 5. Estresse oxidativo. I. Faria, José Boturi Lopes de. II. Universidade Estadual de Campinas. Faculdade de Ciências Médicas. III. Título.

Título em ingles: Renal inflammation and oxidative stress in spontaneously hypertensive rats before development of hypertension and in early diabetes

Keywords:

- Diabetes mellitus
- Diabetic nephropathy
- Hypertension
- Inflammation
- Oxidative stress

Área de concentração: Clínica Médica
Titulação: Doutorado em Clínica Médica

Banca examinadora:
Profº.Drº. José Butori Lopes de Faria
Profº Drº Kleber Gomes Franchini
Profº Drº Roger Frigério Castilho
Profª Drª Dulce Elena Casarini
ProfªDrª Zuleica Bruno Fortes

Data da defesa: 01 / 02 / 2007

Banca examinadora da tese de Doutorado

Orientador(a): Prof(a). Dr(a). José Butori Lopes de Faria

Membros:

Professora Doutora Dulce Elena Casarini _____

Professora Doutora Zuleica Bruno Fortes _____

Professor Doutor Roger Frigério Castilho _____

Professor Doutor Kleber Gomes Franchini _____

Professor Doutor José Butori Lopes de Faria _____

Curso de pós-graduação em Clínica Médica da Faculdade de Ciências Médicas da Universidade Estadual de Campinas.

Data: 01/02/2007

To my parents

Rekha Biswas and

Santosh Kumar Biswas

ACKNOWLEDGEMENTS

I am extremely pleased to have the opportunity to thank all the people whose support and encouragement has made this thesis possible. I have done the experiments, data analysis and interpretation, manuscript preparation, and sometimes I have taken independent decision for further experiments. However, direct and indirect participation of many persons was involved in the whole process.

I would like to express my gratitude to my advisor, Dr. Jose Butori Lopes de Faria, for accepting me in his laboratory. His guidance helped me to be confident and to develop myself as an independent researcher. He kindly allowed me not only to take independent decisions, but also to argue on scientific issues. Due to his constant cooperation and encouragement, I was able to participate in international conferences in Athens and Copenhagen, to review manuscripts for high impact journals, to publish several letters of criticism, and to write and submit grant applications.

I would like to thank Dr. Jacqueline M. Lopes de Faria, Dr. Roger F. Castilho, Dr. Roberto B. Amazonas, Dr. Wilson Nadruz and the professors of nephrology discipline for their helpful suggestions in different stages of my work. I would like to thank all of my colleagues in the laboratory, in particular Lilia, Kamila, Camila, Denise, Elisa, Vanessa, Daniela, Leonardo, Bruno, and many others with whom I have done the experiments and who were always ready to help me. I am grateful to Denise for helping me in the laboratory and in preparation of this thesis. I am also grateful to Sergio Magalhaes for technical assistance.

I would like to thank my teachers in Bangladesh, in particular Dr. Suhrab Ali, Dr. Iqbal Arslan, Dr. Liaquat Ali, Dr. Khorshed Alam and Dr. Forhadul Hoque Mollah, for their encouragement in doing science. I would also like to thank all of my friends, especially Dr. Achintya Ghosh, Dr Zahid Hassan and Dr. Ashesh Chowdhury, for their suggestion and support in my academic and personal life.

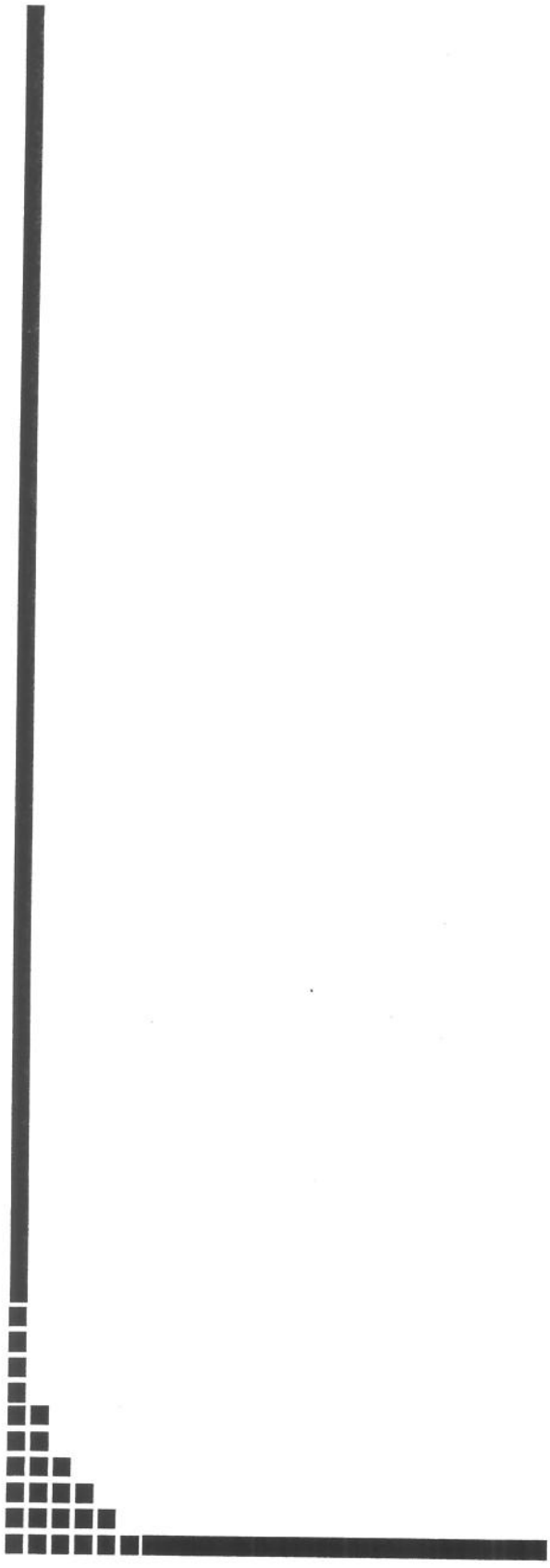
Finally, I am emotional to express my feelings about my dearest family members, my parents, my dada-boudy, my sister Keka, my wife Sonchita and her parents and sisters (Nondita and Ratna), and the new members of our family Palak, Kanak, Progga and Anabil for their constant support and encouragement, and most of all for loving me enough to let me follow my dreams.

I acknowledge the support that I received from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) in the form of a scholarship.

I would like to add here my non-institutional e-mail addresses to receive mails from anybody interested in my thesis and related topics: su.biswas@yahoo.com, subrata.kb@gmail.com

AGE	advanced glycation end-products
BP	blood pressure
DM	diabetes mellitus
ESRD	end-stage renal disease
HAS	hipertensão arterial sistêmica
H_2O_2	hydrogen peroxide
HTN	hypertension
ICAM-1	intercellular adhesion molecule-1
IGF	insulin like growth factor
IL	interleukin
IκB	inhibitor of nuclear factor-κB
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
NADPH	nicotinamide adenine dinucletide phosphate (reduced form)
NF-κB	nuclear factor-κB
$NO^{\bullet}$	nitric oxide
8-OHdG	8-hydroxy-2'-deoxyguanosine
$O_2^{\bullet-}$	superoxide
$OH^{\bullet}$	hydroxyl radical
$ONOO^-$	peroxynitrite
PKC	protein kinase C

ROS	reactive oxygen species
SHR	spontaneously hypertensive rat
SOD	superoxide dismutase
TGF-β	transforming growth factor-β
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
WKY	Wistar-Kyoto



RESUMO

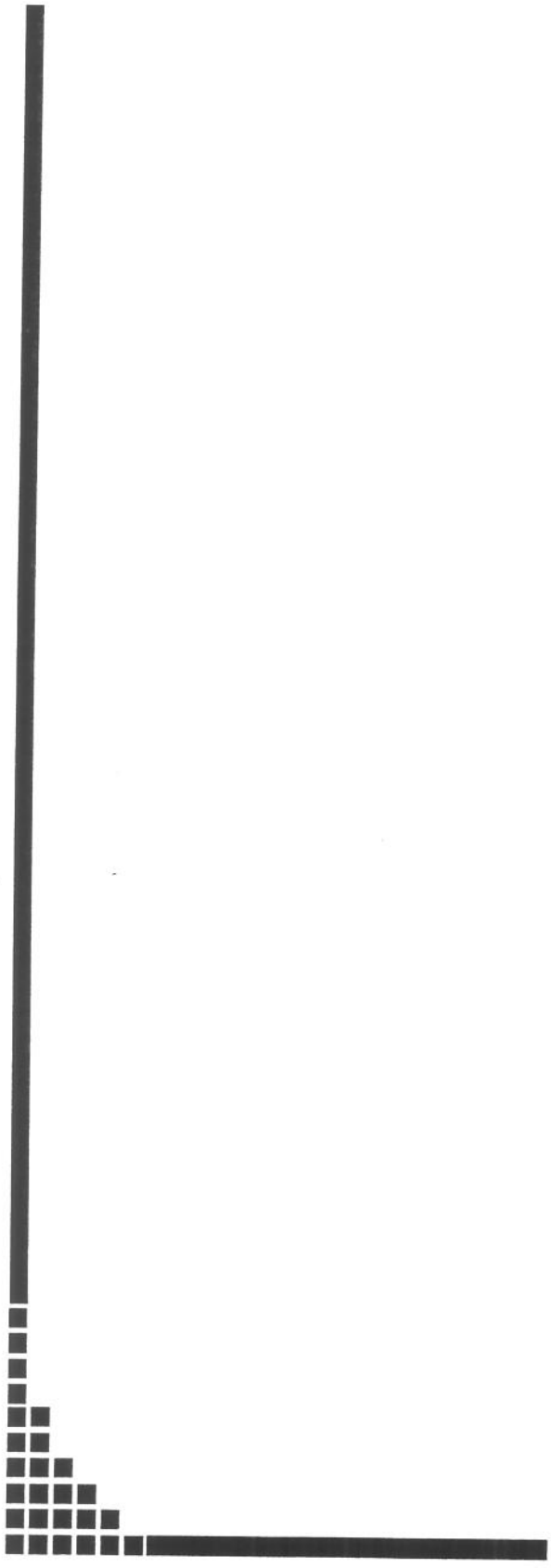
Hipertensão arterial sistêmica (HAS) e diabetes mellitus (DM) freqüentemente coexistem em humanos e constituem uma importante causa de nefropatia e de doença renal terminal. Tanto a HAS quanto o DM podem induzir a inflamação renal e o estresse oxidativo, os quais estão implicados na patogênese da nefropatia. HAS, inflamação renal e estresse oxidativo são eventos altamente interdependentes; e na presença do DM a complexidade deste relacionamento aumenta. Na presente série de estudos nos propusemos investigar a relação entre HAS, inflamação renal e estresse oxidativo na ausência ou presença de DM em ratos espontaneamente hipertensos (SHR), um modelo aceito como representativo da HA primária ou essencial. Os estudos foram descritos nos seguintes artigos publicados (ou aceitos):

Artigo I: Neste artigo, identificamos a anormalidade primária entre inflamação e estresse oxidativo nos rins de ratos SHR. Inflamação renal e estresse oxidativo foram quantificados em ratos SHR pré-hipertensos de 2 a 3 semanas de idade e em ratos geneticamente normotensos, Wistar-Kyoto (WKY), usados como controle. A inflamação renal e o estresse oxidativo estavam nitidamente elevados em ratos SHR de 3 semanas se comparados aos ratos WKY controles. Além disso, observamos evidência de aumento do estresse oxidativo, mas não da inflamação, em ratos SHR de 2 semanas de idade comparados com ratos WKY da mesma idade. Além disso, o emprego de antioxidantes foi capaz de reduzir a inflamação renal em ratos SHR pré-hipertensos. Assim, concluimos que o estresse oxidativo ocorre antes da inflamação como uma alteração primária nos rins de ratos SHR pré-hipertensos.

Artigo II: Neste segundo artigo, investigamos se a presença da HAS modifica a infiltração renal de macrófagos ou o estresse oxidativo em fase precoce de um modelo de DM experimental. Ratos SHR e WKY de 12 semanas de idade foram tornados diabéticos e estudados após 10 dias. Após a indução do DM, o estresse oxidativo renal estava mais elevado em ratos com HAS (SHR), mas não nos ratos controles (WKY) normotensos. Em contrapartida, a infiltração renal de macrófagos estava elevada de forma semelhante em ambos os grupos, WKY e SHR diabéticos. Neste artigo, concluimos que a combinação do DM e da HAS afeta adversamente o estresse oxidativo renal, mas esta combinação não tem nenhum efeito aditivo na infiltração renal de macrófagos, pelo menos em fase inicial do DM.

Artigo III: No terceiro artigo investigamos o efeito da idade da indução do DM na infiltração renal de macrófagos em ratos normotensos (WKY) e hipertensos (SHR). A indução do diabetes em ratos adultos, mas não em ratos pré-púbere, levou ao aumento da infiltração de macrófagos nos rins dos ratos WKY e SHR. Concluímos que a indução do DM em ratos pré-púbere, aparentemente protege da infiltração renal precoce de macrófagos, enquanto que nos ratos adultos a indução do DM leva a exacerbação da infiltração renal de macrófagos.

Em resumo, a presente tese fornece evidência de que o estresse oxidativo aparece como uma conseqüência primária nos rins de ratos SHR antes do desenvolvimento da inflamação renal ou da HAS. A presença da HAS exacerba o estresse oxidativo renal em estágio inicial do DM experimental. Em contrapartida, a infiltração renal de macrófagos encontra-se aumentada em fases iniciais do DM experimental, não dependendo da presença ou da ausência da HAS mas sim da idade em que foi feita a indução do DM.



ABSTRACT

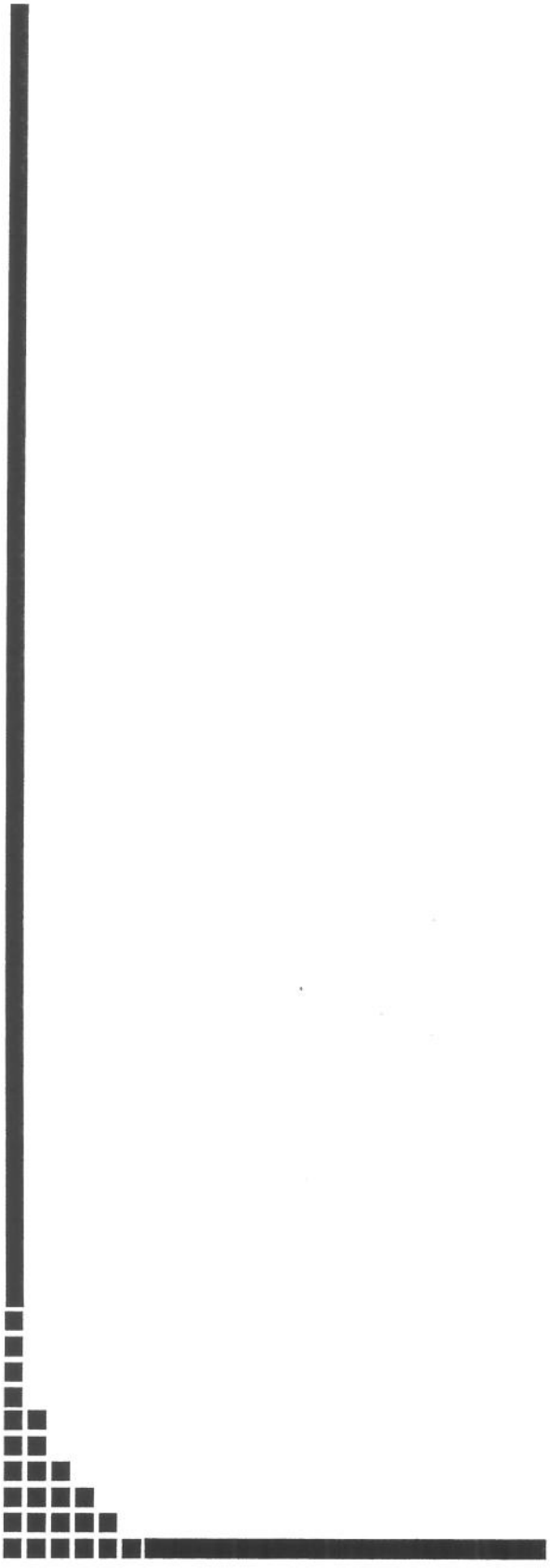
Hypertension (HTN) and diabetes mellitus (DM) frequently coexist in human and constitute the most important causes of nephropathy and end-stage renal disease. Both HTN and DM can induce renal inflammation and oxidative stress, which have been implicated in the pathogenesis of nephropathy. Systemic HTN, renal inflammation and oxidative stress are highly interdependent events; presence of diabetes adds further complexity to this relationship. The present series of studies were therefore undertaken to investigate the relationship among HTN, renal inflammation and oxidative stress in the absence or presence of experimental diabetes in the spontaneously hypertensive rats (SHR), an established animal model of essential/primary HTN. The studies were described in detail in the following published (or accepted) papers:

Paper I: The paper I identified the primary abnormality between inflammation and oxidative stress in the kidney in SHR. Renal inflammation and oxidative stress were evaluated in 2- and 3-week-old prehypertensive SHR and age-matched genetically normotensive control Wistar-Kyoto (WKY) rats. Renal inflammation and oxidative stress were found clearly elevated in SHR at 3-week of age compared with age-matched WKY rats. Additionally, there was an evidence of increased renal oxidative stress, but not inflammation, in 2-week-old SHR compared with age-matched WKY rats. Moreover, antioxidant treatment reduced renal inflammation in prehypertensive SHR. The paper I therefore concluded that the oxidative stress appears before inflammation as a primary abnormality in the kidney in prehypertensive SHR.

Paper II: The paper II investigated whether the presence of HTN modifies renal macrophage infiltration and oxidative stress at the early stage of experimental diabetes. Diabetes was induced in SHR and WKY rats at 12 weeks of age for 10 days. Renal oxidative stress was found elevated in hypertensive SHR, but not in normotensive WKY rats, after induction of diabetes. On the other hand, renal macrophage infiltration was higher in both WKY and SHR groups after induction of diabetes. The paper II concluded that the combination of diabetes and HTN adversely affects oxidative stress in the kidney, but the combination has no additive effect on renal macrophage infiltration, at least, in early diabetes.

Paper III: The paper III identified the effect of age of induction of experimental diabetes on renal macrophage infiltration in normotensive (WKY) and hypertensive (SHR) rats. Induction of diabetes in adult rats, but not in prepubertal rats, markedly increased macrophage infiltration in the kidney both in WKY and SHR strains. The paper III concluded that the prepubertal induction of diabetes apparently protects against early renal macrophage infiltration while the induction of diabetes in the adults induces exaggerated macrophage infiltration in the kidney.

In summary, the present thesis provides evidence that the oxidative stress appears as a primary abnormality in the kidney before development of renal inflammation or systemic HTN in SHR, and that the presence of HTN adversely affects renal oxidative stress in the early stage of experimental diabetes. Renal macrophage infiltration, on the other hand, increases markedly in the early stage of experimental diabetes, regardless of the presence or absence of HTN but depending on the age of induction of diabetes.



CHAPTER 1

Introdução

Hipertensão arterial sistêmica (HAS) é um problema médico e de saúde pública importante e crescente. Tem sido relatado que aproximadamente 50 milhões de indivíduos nos Estados Unidos e um bilhão em todo o mundo são acometidos pela HAS (Chobanian et al., 2003). A prevalência da HAS ajustada para idade na população geral adulta na América Latina varia de 26 a 42% (Burlando et al., 2004). A HAS é a causa primária da insuficiência renal crônica terminal (IRCT) em um terço dos casos, sendo a segunda causa de IRC (US Renal Data System, 2006). Entretanto, a causa da HAS não é clara na HAS essencial, a qual constitue 95% de todos os casos de HAS. Como a causa específica da HAS é desconhecida na grande maioria dos casos, as orientações para o tratamento atual desta doença segue uma conduta geral de simplesmente reduzir a pressão arterial. A identificação das causas específicas da HAS pode propiciar a escolha de tratamentos mais efetivos ou mesmo previnir a HAS.

Diabetes Mellitus (DM) é um grupo de doenças metabólicas caracterizadas por hiperglicemia por defeito na secreção de insulina, na sua ação, ou em ambas. Do ponto de vista etiológico o DM tem sido classificado em tipo 1 ou 2, gestacional e outros tipos específicos (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003). A longo prazo a hiperglicemia crônica do DM desempenha papel primordial na lesão, disfunção e falência de vários órgãos especialmente: os olhos, rins, nervos, coração e vasos sangüíneos (The Diabetes Control and Complications Trial Research Group, 1993; United Kingdom Prospective Diabetes Study Group, 1998; Stratton et al., 2000). Entre as complicações de longo prazo, a nefropatia diabética é uma das principais complicações do DM. Cerca de 20-30% dos pacientes com DM tipo 1 ou tipo 2 desenvolvem evidências de nefropatia e a maioria deles eventualmente progridem para IRCT (American Diabetes Association, position statement, 2003). A nefropatia diabética é causa freqüente de IRCT no Brasil (Lopes de Faria et al., 1995) e nos países desenvolvidos é a primeira causa de IRCT. Conseqüentemente, DM e HAS são os principais responsáveis pela maioria dos casos de nefropatia e IRCT. Infelizmente, DM e HAS com freqüência coexistem em humanos (Tarnow et al., 1994) e essa combinação aumenta a incidência e a

1. INTRODUCTION

1.1. General consideration

1.1.1. Hypertension: Hypertension (HTN), or elevated arterial blood pressure (BP), is an increasingly important medical and public health problem. It has been reported that approximately 50 million individuals in the United States and 1 billion individuals worldwide are affected by HTN (Chobanian et al., 2003). The age-adjusted prevalence of HTN in adult general population in different countries of Latin America ranges from 26 to 42% (Burlando et al., 2004). The prevalence of HTN increases with increasing age of the population. Recent data suggest that individuals who are normotensive at 55 years of age have a 90% lifetime risk for developing HTN (Vasan et al., 2002). The relationship between BP and risk of cardiovascular disease events is continuous, consistent and independent of other risk factors. It has been shown that the higher the BP, the greater the chance of myocardial infarction, heart failure, stroke, and kidney disease (Chobanian et al., 2003). The World Health Organization reports that about 62% of cerebrovascular disease and 49% of ischemic heart disease are attributable to suboptimal BP control (World Health Report, 2002). Furthermore, HTN is the primary diagnosis in one-third of new cases of end-stage renal disease (ESRD) and thereby HTN is the second commonest cause for ESRD (US Renal Data System, 2006). However, the cause of HTN is not clear in case of primary or essential HTN, which constitutes about 95% of all cases of HTN. Primary HTN clusters in family, and it results from a complex interaction of genetic and environmental factors. Many pathophysiological factors have been implicated in the pathogenesis of primary HTN including activation of renin-angiotensin-aldosterone system and sympathetic nervous system, overproduction of sodium retaining hormones and vasoconstrictors, deficiency of vasodilators, long-term high sodium intake, inadequate dietary intake of potassium and calcium, vascular alterations, diabetes mellitus, insulin resistance, obesity, and altered cellular ion transport (Oparil, 2003). On the other hand, the secondary HTN constitutes only 5% of all cases of HTN and it results from a known cause like renovascular disease, renal failure, pheochromocytoma, aldosteronism or other monogenic involvement (Carretero and Oparil, 2000). As the specific cause of HTN is

unknown for most of the cases of HTN, current treatment guidelines follow a generic approach to lowering BP. Identification of specific causes could help to select more effective approaches to treating and even preventing HTN.

1.1.2. Diabetes mellitus: Diabetes Mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. DM has been etiologically classified into: type 1 and type 2 DM, gestational DM, and other specific types (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003). The prevalence of diabetes is steadily increasing worldwide and several reports predict that DM is going to take an epidemic nature during the first quarter of the 21st century (King et al., 1998; Zimmet et al., 2001; Wild et al., 2004). In fact, the worldwide prevalence of DM for all age-groups was estimated to be 2.8% in 2000 and 4.4% in 2030, and the total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 (Wild et al., 2004). In Latin American consensus on DM and HTN, the prevalence of diabetes in adult population has been reported to be close to 7% in Latin American countries (Burlando et al., 2004). In another study, the prevalence rate of diabetes was found 7.6% in Brazilian population (Malerbi and Franco, 1992).

The chronic hyperglycemia of DM plays a major role for the long-term damage, dysfunction and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels (The Diabetes Control and Complications Trial Research Group, 1993; United Kingdom Prospective Diabetes Study Group, 1998; Stratton et al., 2000). Among the long-term complications, nephropathy resulting from diabetes, the diabetic nephropathy, is one of the major complications of DM. About 20-30% of patients with type 1 or type 2 diabetes develop evidence of nephropathy and a majority of them eventually progresses to ESRD (American Diabetes Association, position statement, 2003). Diabetic nephropathy is a frequent cause of chronic kidney disease in Brazil (Lopes de Faria et al., 1995), and diabetic nephropathy is the leading cause of ESRD in the Western world. Recent statistics showing that diabetic nephropathy is the cause for more than half of new cases of ESRD, and the adjusted incidence and prevalence rate of ESRD reached to 339 and 1542 per million population, respectively, in the United States in 2004 (US Renal Data System,

2006). Due to the rising trend of ESRD, the enormous suffering of the patients with ESRD, and the huge cost of health care related with dialysis and renal replacement therapy required by every patient, ESRD is considered as a medical catastrophe of worldwide dimension (Ritz et al., 1999).

Clinical hallmarks of diabetic nephropathy include a progressive increase in urinary albumin excretion and a decline in glomerular filtration rate, which occur in association with an increase in BP (Cooper, 1998). These renal functional changes develop as a consequence of structural abnormalities, including glomerular basement membrane thickening, mesangial expansion with extracellular matrix accumulation, changes in glomerular epithelial cells, and tubulointerstitial fibrosis (Giunti, 2006). Pathogenesis of diabetic nephropathy is complex and is an area of active investigation. Diabetic nephropathy results from the interaction between genetic susceptibility and environmental insults, primarily metabolic and hemodynamic in origin (Giunti, 2006). Although a number of candidate genes have been studied with controversial results, the fact that diabetic nephropathy occurs only in a subset of diabetic patients and tends to cluster in families is consistent with the hypothesis of a role of genetic susceptibility in the development of this complication (American Diabetes Association, position statement, 2003; Canani et al., 1999). Relevant metabolic factors include hyperglycemia-induced increased formation of reactive oxygen species, advanced glycation end-products (AGE), polyols, and activation of protein kinase C (PKC) isoforms; and hemodynamic factors include systemic HTN, intraglomerular HTN, and the role of vasoactive hormones, such as angiotensin II. Metabolic and hemodynamic pathways may converge to produce various factors like transforming growth factor-β (TGF-β), insulin like growth factor (IGF), nuclear factor (NF)-κB and vascular endothelial growth factor (VEGF) leading to diabetic nephropathy and ultimately to ESRD (as reviewed in Cooper, 1998; and Wolf, 2004).

Taken together, DM and HTN are 2 major diseases with profound impact on the life of the patients as well as on the family and the society. Once DM or HTN affects a person, he or she has to live with the disease for the remaining years of life. Furthermore, DM and HTN are responsible for most of the cases of nephropathy and ESRD.

Unfortunately, DM and HTN frequently coexist in human (Tarnow et al., 1994), and the combination increases the incidence and severity of nephropathy sometimes in an additive manner (Cooper et al., 1988; Stratton et al., 2006). Previous studies identified that the combination of DM and HTN induces early renal fibronectin accumulation (Righetti et al., 2001), and additively affects the renal cortical expression of specific isoforms of PKC and tubulin (Osicka et al., 2003). However, the underlying mechanism by which HTN exaggerates renal lesions in diabetes is largely unknown.

1.2. Oxidative stress and inflammation: basic aspects

1.2.1. Pro-oxidant, antioxidant and oxidative stress: Chemically, **oxidation** is defined as the removal of electrons and ***reduction*** as the gain of electrons (Mayes and Botham, 2003). The general meaning of the term ***oxidant*** is 'oxidizing agent'. In reactions, a free radical may act as an oxidizing agent by taking a single electron from other species, or as a reducing agent by donating a single electron to other species (Halliwell, 2006). The term ***pro-oxidant*** is not well defined; it is generally considered that a pro-oxidant is any substance that can generate reactive species or capable of inducing oxidative stress. However, an ***antioxidant*** is defined as any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell and Whiteman, 2004). ***Oxidative stress*** is conventionally defined as an imbalance between pro-oxidant stress and antioxidant defense. However, recent evidence indicates that the disruption of redox signaling is an important aspect of oxidative stress, sometimes more important than the pro-oxidant—antioxidant imbalance or the tissue damage induced by such imbalance (Jones, 2006). Therefore a new definition of oxidative stress has been proposed as "an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage" (Jones, 2006). Consequences of oxidative stress can be very subtle to very serious (including oxidative damage to biomolecules, disruption of signal transduction, mutation and cell death) depending upon the balance between reactive species generation and the antioxidant defense (Halliwell and Whiteman, 2004).

1.2.2. Free radical: A **free radical** is any species that contains one or more unpaired electrons, that is, electrons singly occupying an atomic or molecular orbital (Halliwell and Whiteman, 2004). Because electrons are more stable when paired together in orbitals, free radicals are generally reactive with other species (Halliwell, 1989). Unpaired electrons have a strong tendency to form pair to be stable. Therefore, a radical might donate its unpaired electron to another molecule, or it might steal an electron from another molecule in order to pair. However, if a radical gives one electron to, or takes one electron from, another molecule, that other molecule itself becomes a radical. Thus an important feature of free radical mediated reactions is that they tend to proceed as chain reaction (Halliwell, 1989).

1.2.3. Reactive species: The three different classes of **reactive species** relevant in biology and medicine are: a. reactive oxygen species (ROS), b. reactive nitrogen species, and c. reactive chlorine species. A reactive species may be a free radical or a nonradical in structure (Halliwell and Whiteman, 2004). ROS is a collective term that includes both oxygen radicals and certain nonradicals that are oxidizing agents and/or are easily converted into radicals. Superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are examples of radical and nonradical ROS, respectively. Similarly, reactive nitrogen species is a collective term including radicals (nitric oxide, $NO^{\bullet}$) and nonradicals (peroxynitrite, $ONOO^-$); and reactive chlorine species is also a collective term including radicals (atomic chlorine, $Cl^{\bullet}$) and nonradicals (hypochlorus acid, HOCl) (Halliwell, 2006). A list of important reactive species in biological system is shown in Table 1.

Among the reactive species, the free radical superoxide anion ($O_2^{\bullet-}$) is of critical importance. Because $O_2^{\bullet-}$ is the primary species produced in the cells, and many other reactive species of physiological significance, including H_2O_2, hydroxyl radical ($OH^{\bullet}$), and $ONOO^-$, are derived from $O_2^{\bullet-}$ as products of the downstream reaction cascade (Munzel et al., 2002). The $O_2^{\bullet-}$ is produced by the mitochondrial electron-transport chain, and by NADPH oxidase, xanthine oxidase, cyclooxygenase and lipoxygenase, nitric oxide synthase and cytochrome P450 (as reviewed in Schnachenberg, 2002).

Table 1. Important reactive species in biological system (adapted from Halliwell, 2006)

Free radicals	**Nonradicals**
Reactive oxygen species	
Superoxide, $O_2^{\bullet-}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $OH^{\bullet}$	Singlet oxygen, $O^1{}_2\Delta g$
Peroxyl, $RO_2^{\bullet}$	Organic peroxides, ROOH
Alkoxyl, $RO^{\bullet}$	Peroxynitrite, $ONOO^-$
Carbonate, $CO_3^{\bullet-}$	Peroxynitrous acid, ONOOH
Reactive chlorine species	
Atomic chlorine, $Cl^{\bullet}$	Hypochlorous acid, HOCl
	Chlorine gas, Cl_2
	Nitryl (nitronium) chloride, NO_2Cl
Reactive nitrogen species	
Nitric oxide, $NO^{\bullet}$	Nitrous acid, HNO_2
Nitrogen dioxide, $NO_2^{\bullet}$	Nitrosyl cation, NO^+
	Nitroxyl anion, NO^-
	Dinitrogen tetroxide, N_2O_4
	Dinitrogen trioxide, N_2O_3
	Peroxynitrite, $ONOO^-$
	Peroxynitrous acid, ONOOH
	Alkyl peroxynitrites, ROONO

1.2.4. Oxidative tissue injury: There are many pathways for inducing ROS-mediated oxidative damage to biomolecules. One such pathway starts from the interaction between two commonly found free radicals in vivo, $O_2^{\bullet-}$ and $NO^{\bullet}$.

$$O_2^{\bullet-} + NO^{\bullet} \rightarrow ONOO^{-} \text{ (Peroxynitrite)}$$

The reaction product is peroxynitrite, which at physiological pH, rapidly protonates to peroxynitrous acid (ONOOH). This powerful oxidizing and nitrating agent can directly damage proteins, lipids, and DNA (Halliwell, 2006). Nitration of tyrosine residues of proteins generates nitrotyrosine which is widely used as a biomarker for oxidative and nitrosative stress. Although nitrotyrosine is not a specific biomarker for peroxynitrite formation, as there are several other nitrating agents in vivo (Halliwell, 1997). However, nitration of proteins is very dangerous for the cell or the organism. Nitration of structural proteins, including neurofilaments and actin, can disrupt filament assembly with major pathological consequences (Beckman and Koppenol, 1996). On the other hand, nitration of signaling molecules or transcription factors can greatly modify the physiological function of the affected proteins (as discussed in a letter by Biswas and Lopes de Faria, 2005). Furthermore, peroxynitrite mediates calcium-dependent mitochondrial dysfunction and cell death via activation of calpains (Whiteman et al., 2006).

Hydroxyl free radical ($OH^{\bullet}$) induced lipid peroxidation and DNA hydroxylation are also major pathways for oxidative damage. The $OH^{\bullet}$ radical is the most reactive species known to chemistry as it can attack and damage almost every molecule found in living cells (Halliwell, 1989). The $OH^{\bullet}$ radical can react with the ring structure of guanine in DNA forming the adduct 8-hydroxy-2'-deoxyguanosine (8-OHdG) radical which can propagate a chain reaction through the DNA and cause chemical alteration of the bases as well as DNA strand breakage. Imperfect repair of such DNA damage can lead to mutations, arrest of cell growth or apoptosis (Evans et al., 2004). The $OH^{\bullet}$ radical can also initiate chain reaction by reacting with membrane lipids leading to lipid peroxidation. The overall effects of lipid peroxidation are to decrease membrane fluidity, increase the

leakiness of the membrane, and damage membrane proteins, thereby inactivating receptors, enzymes, and ion channels (Halliwell, 2006).

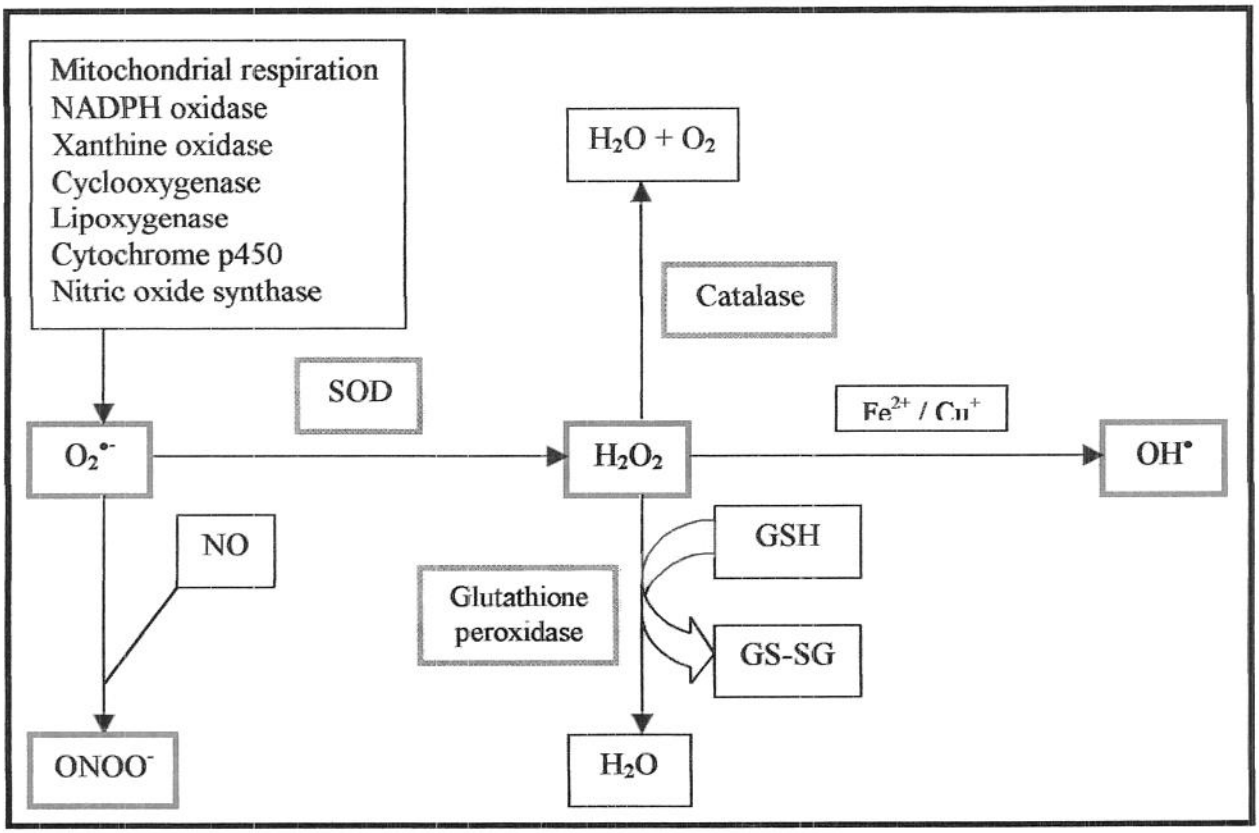


Figure 1. Major pro-oxidant—antioxidant reactions relevant in biological system (adapted from Schnachenberg, 2002). Superoxide ($O_2^{\bullet-}$) produced from a number of sources acts as a primary reactive species. $O_2^{\bullet-}$ rapidly reacts with nitric oxide (NO) to produce peroxynitrite ($ONOO^-$), or catalyzed by superoxide dismutase (SOD) to produce hydrogen peroxide (H_2O_2). H_2O_2 can be neutralized by catalase or glutathione peroxidase. However, in presence of transition metal ions, like iron (Fe^{2+}) and cupper (Cu^{+}), highly toxic hydroxyl free radicals ($OH^{\bullet}$) can be produced from H_2O_2 via the Fenton reaction. Reactive species are shown in red and antioxidant enzymes are shown in green boxes. GSH, reduced glutathione; GS-SG, oxidized glutathione.

1.2.5. Antioxidant system: To minimize the oxidative damage, antioxidant systems have been evolved. Enzymatic antioxidants like superoxide dismutase (SOD), catalase and ghluathione peroxidase, and non-enzymatic antioxidants like vitamins C and E, glutathione (reduced form, GSH) and beta-carotene provide major protection against oxidative stress by neutralizing or scavenging reactive species or by breaking the chain reactions (as reviewed in Scandalios, 2005). In addition, transferrin, ceruloplasmin and albumin also play antioxidant role by sequestering transition metal ions, like iron and copper, as the metal ions rapidly react with H_2O_2 to yield highly toxic hydroxyl radical ($OH^{\bullet}$) by Fenton reaction (Halliwell, 1989). Major pro-oxidant-antioxidant reactions are summarized in Figure 1.

However, reactive species are not always harmful. They help phagocytes to kill microorganisms and modulate signaling events by redox (reduction and oxidation) regulation and thereby affect the phosphorylation and dephosphorylation of enzymes and transcription factors (Halliwell, 2006).

1.2.6. Inflammation: Inflammation is a complex reaction in the vascularized connective tissue in response to exogenous and endogenous stimuli. It is a protective response the ultimate goal of which is to rid the organism of both the initial cause of cell injury and the consequences of such injury. However, exaggerated or unregulated prolong inflammatory process can induce tissue damage and is the cause for many chronic diseases (Collins, 1999). A critical component of inflammation is the infiltration of inflammatory cells, like neutrophils, monocytes and lymphocytes, to the site of stimulus. Leukocyte infiltration to the site of inflammation is a highly coordinated process involving margination, rolling and adhesion of leukocytes to the vascular endothelium, transmigration across the endothelium and migration toward a chemotactic stimulus. Participation of a number of adhesion molecules, including selectins, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), and their respective leukocyte receptors, and chemokines like monocyte chemoattractant protein 1 (MCP-1) or interleukin 8 (IL-8) is crucial for the inflammatory cellular infiltration (Collins, 1999). At the site of inflammation, activated inflammatory cells release many

enzymes (neutral proteases, elastase, collagenase, acid hydrolases, phosphatases, lipases etc.), reactive species (superoxide, hydrogen peroxide, hydroxyl radical, hypochlorus acid etc.) and chemical mediators (eicosanoids, complement components, cytokines, chemokines, nitric oxide etc.) and thereby induce tissue damage and oxidative stress (Collins, 1999).

1.2.7. Nuclear factor-κB: The nuclear factor-κB (NF-κB), a transcription factor of major importance in inflammation, regulates the gene expression of proinflammatory cytokines, chemokines, inflammatory enzymes, adhesion molecules, and receptors (Barnes and Karin, 1997). The NF-κB/Rel family includes NF-κB1 (p50/p105), NF-κB2 (p52/p100), p65, RelB, and c-Rel. Most members of this family form dimers with each other, the heterodimer consisting of p50 and p65 subunits being the most prevalent activated form of NF-κB (Tak and Firestein, 2001). In resting cells, NF-κB dimers remain in the cytoplasm as an inactive form bound to the inhibitory protein IκB. Upon cellular activation by extracellular stimuli, IκB is phosphorylated, ubiquitinylated, and ultimately degraded by the proteasome system. As a result, NF-κB dimers are translocated into the nucleus and activate the transcription of target genes (Chen et al., 1999). The transcription factor NF-κB can be activated by a number of different stimuli, including bacterial lipopolysaccharides, viral agents, phytohemagglutinin, cytokines (tumor necrosis factor-α and IL), and PKC activators (phorbol esters) (Barnes and Karin, 1997). Importantly, oxidative stress or intracellular redox status, is also involved in the activation of NF-κB; particularly, H_2O_2 has been found to activate NF-κB and antioxidants have been demonstrated to block NF-κB activation (Anderson et al., 1994; Flohe et al., 1997).

1.2.8. Inflammation and oxidative stress: Above discussion indicates that the generation of reactive species by the inflammatory cells can induce oxidative stress. On the other hand, oxidative stress may induce inflammation through NF-κB mediated pro-inflammatory gene expression. Figure 2 depicts this close and interdependent relationship between inflammation and oxidative stress, although the sequence of events is not so simple. Many other redox-sensitive signal transduction pathways like c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK), and transcription factor

activator protein-1 (AP-1) also participate to set up a vicious cycle between inflammation and oxidative stress (Vaziri and Rodriguez-Iturbe, 2006). If oxidative stress appears as the primary abnormality in an organ, inflammation will eventually develop and will further accentuate oxidative stress. Conversely, if inflammation is the primary event, oxidative stress will develop as a consequence which will further exaggerate inflammation (Vaziri and Rodriguez-Iturbe, 2006). Therefore, identification of primary abnormality could be of great clinical importance, as the treatment of the primary disorder could ensure a sustain relief from the problem.

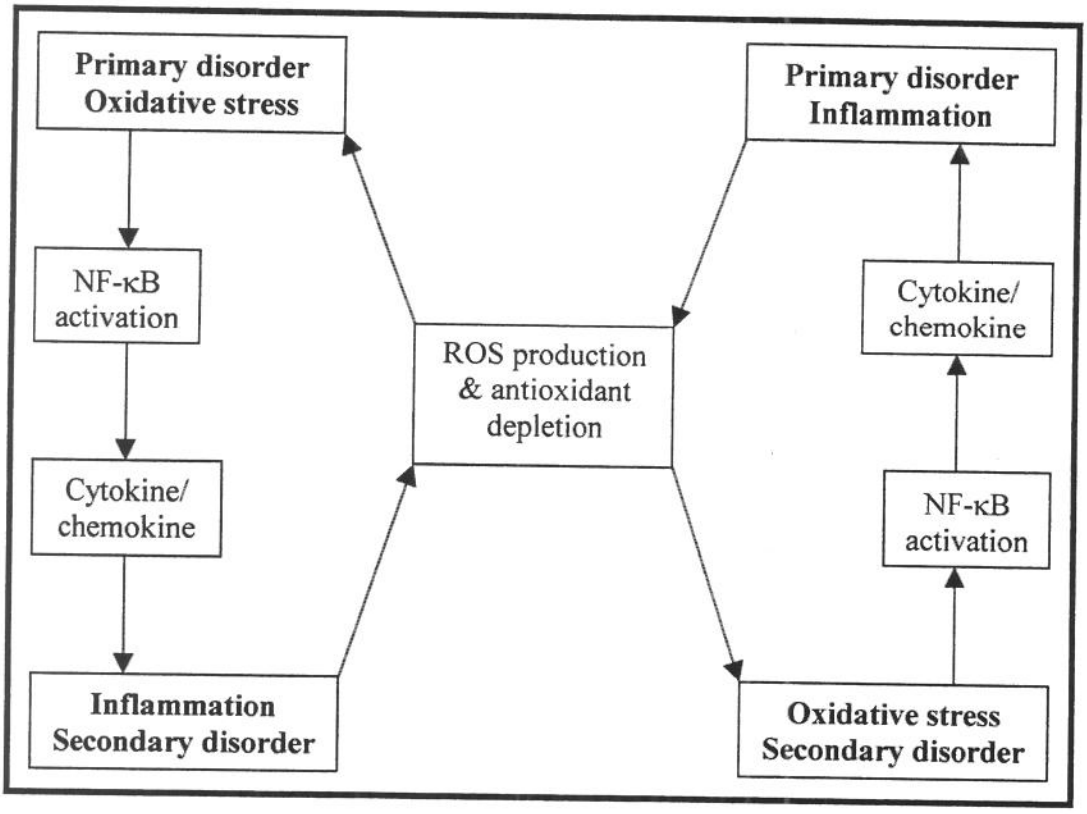


Figure 2. Overview of the relationship between inflammation and oxidative stress. When oxidative stress appears as a primary disorder inflammation develops as a secondary disorder and further enhances oxidative stress. On the other hand, inflammation as a primary disorder can induce oxidative stress as a secondary disorder which can further enhance inflammation. NF-κB, nuclear factor-κB; ROS, reactive oxygen species.

1.3. Oxidative stress and inflammation in the kidney in HTN

The intricate relationship between inflammation and oxidative stress is not just a theoretical linkage rather numerous data demonstrated simultaneous presence of inflammation and oxidative stress in an inseparable manner in different organs, particularly in the kidney (Vaziri, 2004; Rodriguez-Iturbe et al., 2004a; Johnson et al., 2005; Vaziri and Rodriguez-Iturbe, 2006). Renal inflammation and oxidative stress is a common feature in spontaneously hypertensive rats (SHR) and in different other models of hereditary and acquired HTN (as reviewed in Vaziri, 2004; Vaziri and Rodriguez-Iturbe, 2006). SHR are frequently used as a model of essential/primary HTN in humans. As in humans with primary HTN, the SHR born normotensive and remain normotensive until around 4 weeks of age. Then the BP gradually increases in SHR and by around 10 to 12 weeks of age they become fully hypertensive (Okamoto and Aorki, 1963). On the other hand, Wistar-Kyoto (WKY) rats, genetically normotensive counter-part of SHR, remain normotensive throughout the life.

Several studies demonstrated increased levels of renal inflammation and oxidative stress in adult hypertensive SHR compared to age-matched WKY rats (Nava et al., 2003; Rodriguez-Iturbe et al., 2003; Zhan et al., 2004; Rodriguez-Iturbe et al., 2005). It has been claimed that such renal abnormalities in SHR could be a cause of HTN as the kidney plays a major role in the regulation of BP (Guyton, 1991). Inflammation and oxidative stress-induced enhanced superoxide production in the kidney can reduce the bioavailability of nitric oxide. Altered superoxide, nitric oxide, and superoxide-nitric oxide balance can alter renal vascular tone, sodium reabsorption and signaling events, and thereby may elevate BP (as reviewed in Rodriguez-Iturbe et al., 2004a; Johnson et al., 2005). Moreover, tubulointerstitial inflammatory cells in SHR express angiotensin II that may further enhance sodium retention due to local elevation of this vaso-active peptide (Rodriguez-Iturbe et al., 2002). Indeed, supporting this theoretical basis of HTN in SHR, antioxidants have been shown to reduce BP and renal inflammation (Nava et al., 2003; Rodriguez-Iturbe et al., 2003; Cuzzocrea et al., 2004), and anti-inflammatory agents have been shown to reduce BP and renal oxidative stress in SHR (Rodriguez-Iturbe et al., 2002;

Rodriguez-Iturbe et al., 2005). Furthermore, induction of oxidative stress in otherwise normal healthy rats has been found to induce HTN (Vaziri et al., 2000; Makino et al., 2003). However, all these findings failed to establish renal inflammation or oxidative stress as a cause of HTN, since HTN, per se, has been found to induce oxidative stress (Vaziri and Ni, 2005), raising the possibility that renal inflammation and oxidative stress could be a mere consequence of HTN in SHR. More or less similar relationship among HTN and renal inflammation and oxidative stress has been obtained in other models of HTN (as reviewed in Vaziri, 2004; Vaziri and Rodriguez-Iturbe, 2006). Taken together, it has been concluded that systemic HTN and renal inflammation and oxidative stress, three tightly associated and closely interdependent events, are apparently inseparable.

Few studies have attempted to separate HTN, and renal inflammation and oxidative stress to identify the primary abnormality among them. Chabrashvili et al. (2001) showed that the renal expression of NADPH oxidase subunit, p47phox, is elevated in 4-week-old SHR. They speculated from this finding that the renal oxidative stress precedes the development of HTN in SHR (Wilcox, 2005). In fact, NADPH oxidase is the major source of pro-oxidant superoxide in the vascular tissue (Griendling et al., 2000), and this enzyme system has been found upregulated in different animal models of HTN associated with oxidative stress (Chabrashvili et al., 2001; Zhan et al., 2004). The NADPH oxidase enzyme complex is composed of membrane-bound subunits (gp91phox and p22phox) and cytosolic subunits (p47phox, p67phox and p40phox). Upon activation, some subunits, including p47phox, are phosphorylated and translocated to the membrane and participate in superoxide generation (Babior, 1999). However, only upregulation of a subunit of NADPH oxidase does not necessarily mean an elevation of oxidative stress. Therefore the speculation of Chabrashvili et al. (2001) was not convincing. Moreover, it was uncertain whether the over-expression of p47phox in the kidney of 4-week-old SHR was an effect of elevated BP in the study by Chabrashvili *et al.*, as several studies have shown that the BP is already elevated in the SHR at 4 weeks of age compared with age-matched WKY rats (Lopes de Faria et al., 1997; Imig et al., 1993). However, apart from oxidative stress, it has recently been shown that the renal interstitial inflammation and pro-inflammatory

transcription factor NF-κB are already elevated in 3-week-old prehypertensive SHR compared to age-matched WKY rats (Rodriguez-Iturbe et al., 2004b).

The findings of above studies, with some limitations, suggest that the inflammation and oxidative stress in the kidney may precede the development of HTN in SHR raising the possibility that those renal abnormalities could be causally linked to HTN. However, it is unknown whether inflammation or oxidative stress is the primary abnormality in the kidney in SHR. Although inflammation and oxidative stress are closely interdependent events, it is reasonable to think that one of them may appear first as a primary abnormality and, by inducing another, may ultimately lead to HTN in SHR.

1.4. Oxidative stress and inflammation in the kidney in DM

As in the case of HTN, renal inflammation and oxidative stress is also a common finding in DM. Mitochondrial respiratory chain, NADPH oxidase enzyme system and Nox4 (homologue of gp91phox subunit of NADPH oxidase) have been found to generate superoxide in high glucose conditions (Nishikawa et al., 2000; Susztak et al., 2006; Gorin et al., 2005). AGE-RAGE (Receptor for AGE) interaction, which is exaggerated in DM, has also been shown to induce oxidative stress (Bierhaus et al., 2005). However, superoxide generated by mitochondrial electron transport chain is considered as the primary source of reactive species in diabetes and is the major contributor of microvascular complications (Brownlee, 2005). Partial reduction of oxygen in the electron transport chain due to high levels of intracellular glucose (cells in which glucose uptake is relatively insulin-independent) generates superoxide, which inhibits glyceraldehyde-3 phosphate dehydrogenase, a key enzyme of glycolytic pathway (Brownlee, 2001). Upstream glycolytic intermediates, as a consequence, accumulate and activate several pathways of hyperglycemic damage, including PKC, polyol, hexosamine, and AGE pathways (Brownlee, 2001). PKC activation, for example, induces a number a pathogenic consequences by affecting the expression of endothelial nitric oxide synthase (eNOS), endothelin-1, VEGF, TGF-β, collagen and fibronectin. Furthermore, activation of PKC activates NADPH oxidase enzyme system and thereby further enhances oxidative stress

(Brownlee, 2001). Several signal transduction pathways, including NF-κB, are activated as a consequence of hyperglycemia-induced oxidative stress (Nishikawa et al., 2000), resulting in expression of cell adhesion molecules and chemokines and thereby inflammatory cellular infiltration (Barnes and Karin, 1997). AGE-RAGE interaction also constitutes an important mechanism of sustained inflammation through NF-κB activation in DM (Bierhaus et al., 2001).

Numerous studies have demonstrated elevated inflammation and oxidative stress in the kidney in diabetes, and such renal abnormalities have been found to induce features of diabetic nephropathy (as reviewed in Wolf, 2004). In cultured human mesangial cells, experimental evidence of Kiritoshi et al. (2003) suggested that high glucose increases mitochondrial ROS production, resulting in NF-κB activation, cyclooxygenase-2 gene and protein expression, and prostaglandin E_2 synthesis. Kiritoshi et al. (2003) therefore concluded that this chain of events might contribute to the pathogenesis of diabetic nephropathy through renal hemodynamic alterations. In cultured podocytes, high extracellular glucose induces ROS production through NADPH oxidase and mitochondrial pathways, and thereby induces activation of proapoptotic p38 MAPK and caspase 3, and apoptosis of podocytes (Susztak et al, 2006). It has also been shown that NADPH oxidase Nox4-mediated generation of ROS induces glomerular hypertrophy and fibronectin expression in early diabetes. Administration of antisense oligonucleotides for Nox4 reduces diabetes-induced ROS generation, renal hypertrophy, fibronectin expression, and Akt/PKB and ERK1/2 activation (Gorin et al., 2005). Moreover, NADPH oxidase inhibitor, apocynin, reduces or prevents diabetes-induced oxidative stress, podocyte depletion, albuminuria, and mesangial matrix expansion (Asaba et al., 2005; Susztak et al, 2006). Participation of superoxide in the pathogenesis diabetic nephropathy has been demonstrated in SOD overexpressing mice. In transgenic *db/db* mice overexpressing SOD, diabetes-induced albuminuria, glomerular accumulation of TGF-β, collagen IV, nitrotyrosine, and mesangial matrix were significantly reduced, and diabetes-induced decline in renal function was largely prevented (DeRubertis FR et al., 2004). Antioxidant DL-alpha-lipoic acid has also been shown to prevent enhanced renal cortical oxidative stress in early diabetes (Obrosova et al., 2003).

Inflammatory cellular infiltration in the glomerulus and tubulointerstitial area has been demonstrated in the very early stage of DM. A prominent glomerular macrophage infiltration has been observed just after 3 days of streptozotocin induced DM in rats (Young et al., 1995). Sassy-Prigent et al. (2000) have found elevated glomerular macrophage infiltration starting from 8 days after the induction of diabetes, which was preceded or associated with an increased glomerular expression of ICAM-1, VCAM-1, and MCP-1. This diabetes-induced renal inflammatory process was associated with glomerular hypertrophy, glomerular accumulation of type IV collagen and mesangial expansion (Sassy-Prigent et al., 2000). In *db/db* mice, an animal model of type 2 DM, Chow et al. (2004) found an exaggerated macrophage infiltration in the kidney which was correlated with albuminuria, elevated plasma creatinine, glomerular and tubular damage, and renal fibrosis. Glomerular and tubulointerstitial macrophage infiltration has also been demonstrated in human progressive diabetic nephropathy, and the interstitial macrophage infiltration has been found strongly correlated with serum creatinine, proteinuria and interstitial fibrosis (Furuta et al., 1993; Nguyen et al., 2006). In addition to the association between renal inflammation and features of diabetic nephropathy several studies evaluated the effects of anti-inflammatory agents in the prevention of diabetic kidney disease. Diabetes-induced glomerular macrophage infiltration, ICAM-1 expression and NF-κB activation associated with albuminuria, mesangial expansion, glomerular hypertrophy and glomerular expression of collagen and TGF-β in the kidney have been partly or completely prevented by anti-inflammatory agents like mycophenolate mofetil, erythromycin or methotrexate (Utimura et al., 2003; Tone et al., 2005; Yozai et al., 2005). Prevention of diabetes-induced renal inflammation and associated renal damage in ICAM-1 or MCP-1 deficient animals provides further evidence of the participation of inflammatory process in the pathogenesis of diabetic nephropathy (Okada et al., 2003; Chow et al., 2005; Chow et al., 2006).

HIPÓTESES E OBJETIVOS

HAS e DM são capazes de induzir inflamação e estresse oxidativo renal. Entretanto, tem sido sugerido que a inflamação e o estresse oxidativo podem estar presente no rim de ratos SHR antes do desenvolvimento da HAS e sem DM. Além disso, estudos recentes indicam proteção relativa a nefropatia diabética em pacientes com DM tipo 1 com início pré-púbere comparado aos pacientes com DM tipo 1 de início após a puberdade. O mecanismo para tal comportamento é amplamente desconhecido (Finne et al., 2005; Svensson et al., 2006). Conseqüentemente, o propósito da presente tese foi identificar o evento primário renal em SHR antes de se tornarem hipertensos, se inflamação ou estresse oxidativo, e investigar se a presença da HAS ou da idade de indução do DM afeta a inflamação e o estresse oxidativo renal em ratos diabéticos.

Hipótese: Estresse oxidativo renal pode preceder a inflamação e a HAS em ratos geneticamente hipertensos, e a HAS/idade de indução do DM podem modificar a inflamação renal e o estresse oxidativo em ratos diabéticos.

Os objetivos específicos da presente tese foram:

1. identificar a anormalidade primária entre HAS, inflamação e estresse oxidativo renal,
2. investigar o efeito da combinação de HAS e DM na infiltração renal de macrófagos e no estresse oxidativo, e
3. determinar o efeito da idade de indução do DM na infiltração renal de macrófagos.

2. HYPOTHESIS AND OBJECTIVES

HTN and DM both are known to induce inflammation and oxidative stress in the kidney. However, inflammation and oxidative stress have been claimed to be present in the kidney even in the absence of HTN or DM in pre-hypertensive SHR. Furthermore, recent studies indicate a relative protection against diabetic nephropathy and ESRD in prepubertal onset of type 1 diabetes compared to pubertal or postpubertal onset, the mechanism of which is largely unknown (Finne et al., 2005; Svensson et al., 2006). Therefore, the purpose of the present thesis were to identify the primary renal abnormality between inflammation and oxidative stress before development of HTN in SHR, and to investigate whether the presence of HTN or the age of induction of experimental diabetes affects renal inflammation and oxidative stress in diabetic animals.

Hypothesis: Renal oxidative stress may appear before renal inflammation and systemic HTN in genetically hypertensive animals, and systemic HTN/age of induction of diabetes may modify renal inflammation and oxidative stress in diabetic animals.

Specific objectives of the present thesis were

1. to identify the primary abnormality among systemic HTN, renal inflammation and oxidative stress,
2. to investigate the effect of combination of HTN and DM on macrophage infiltration and oxidative stress in the kidney, and
3. to find out the effect of age of induction of experimental diabetes on renal macrophage infiltration.

CHAPTER 2

Paper I

Which comes first: renal inflammation or oxidative stress in spontaneously hypertensive rats?

Biswas SK, Lopes de Faria JB. Which comes first: renal inflammation or oxidative stress in spontaneously hypertensive rats? Free Radic Res; in press 2007.

ABSTRACT

The present study was undertaken to identify whether inflammation or oxidative stress is the primary abnormality in the kidney in spontaneously hypertensive rats (SHR). Renal inflammation and oxidative stress were evaluated in 2- and 3-week-old prehypertensive SHR and age-matched genetically normotensive control Wistar-Kyoto (WKY) rats. Blood pressure was similar in WKY and SHR rats at 2- and 3-week of age. Renal inflammation (macrophage and nuclear factor-κB) was elevated in SHR at 3-week, but not at 2-week, of age compared with age-matched WKY rats. Renal oxidative stress (nitrotyrosine, 8-hydroxy-2'-deoxyguanosine and p47phox) was also clearly elevated in 3-week-old SHR compared with age-matched WKY rats. Additionally, NADPH oxidase subunit p47phox was found elevated in 2-week-old SHR compared to age-matched WKY rats. Moreover, antioxidant (N-acetyl-L-cysteine and Tempol) treatment reduced renal inflammation in prehypertensive SHR. We therefore conclude that the oxidative stress appears before inflammation as a primary abnormality in the kidney in prehypertensive SHR.

Keywords: Oxidative stress, inflammation, SHR, p47phox, nitrotyrosine, NF-κBp65

1. INTRODUCTION

Spontaneously hypertensive rats (SHR) are commonly used as a model of human essential hypertension (HTN). SHR remain normotensive until 4 to 5 weeks of age, and then the blood pressure (BP) gradually increases in these animals and they become fully hypertensive by around 10 to 12 weeks of age.[1] Recent studies have convincingly shown that the HTN in different animal models, including SHR, is tightly associated with oxidative stress and inflammation in the kidney.[2-8] Antioxidants have been found to reduce renal inflammation and BP as well as renal oxidative stress in adult SHR.[2,7] On the other hand, anti-inflammatory agents have been shown to reduce oxidative stress and BP along with reduction of renal inflammation in SHR.[3,6] Thus, inflammation and oxidative stress in the kidney and systemic HTN are interdependent in adult hypertensive SHR.

Recently it has been demonstrated that the inflammation and oxidative stress develop in the kidney of SHR at or before the age of 4 weeks, the age at which the rats are still normotensive.[5,9] This evidence, therefore, strongly supports the hypothesis that the renal inflammation and/or oxidative stress may be causally linked to the development of systemic HTN in SHR. However, it is still unknown whether renal inflammation is the cause or the effect of renal oxidative stress in SHR. Considering the clinical relevance of identification of primary cause in the pathogenesis of HTN, the present study was designed to identify the primary abnormality between renal inflammation and oxidative stress in SHR. We studied oxidative stress and inflammation in the kidney of 2 and 3 weeks old prehypertensive SHR and age-matched genetic control Wistar Kyoto (WKY) rats. On the basis of the observational findings, we further proceeded with an interventional approach in which we treated 2-week-old SHR with antioxidants, N-acetyl-L-cysteine (NAC) and Tempol, for 1 week. Our findings indicate that the oxidative stress appears before inflammation as a primary abnormality in the kidney in prehypertensive SHR.

2. MATERIALS AND METHODS

2.1. Animals and Experimental Protocol

The protocol for this study complied with the guidelines established by the Brazilian College of Animal Experimentation (COBEA) and was approved by the Institutional Ethical Committee. All reagents were purchased from Sigma, St Louis, Mo., USA, unless stated otherwise. The SHR (n=26) and their genetically normotensive control WKY (n=24) rats derived from animals supplied by Taconic (Germantown, NY, USA) and bred in our animal facility were used in this study. We studied BP, and renal inflammation and oxidative stress in male SHR and WKY rats at 2 different age points: (i) 2-wk group, consisting of SHR (n=12) and WKY (n=11) rats that were 2 weeks of age (14-15 days), and (ii) 3-wk group, consisting of SHR (n=14) and WKY (n=13) rats that were 3 weeks of age (20-22 days). Rats were sacrificed by overdose of pentobarbital (Hypnol®), and both of the kidneys were removed immediately, carefully wiped on cotton gauze, decapsulated and cut longitudinally into 2 halves. One half was fixed immersing in a solution of Methacarn[10] and subsequently embedded in paraffin. A small part of the cortical tissue was homogenized in homogenization buffer. The remaining cortical tissue was frozen in liquid nitrogen for subsequent collection of nuclear extract and determination of reduced form of glutathione (GSH).

A separate group of 2-week-old 10 SHR rats were used for antioxidant intervention study. Five of them were treated with thiol-based antioxidant NAC (Calbiochem, 200 mg/kg/day, intraperitoneal, in the morning) and superoxide dismutase mimetic Tempol (4-hydroxy-2,2,6,6,-tetramethylpiperidine-1-oxyl) (Calbiochem, 250 mg/kg/day, intraperitoneal, in the evening). Another 5 rats were treated with the vehicle normal saline as control. After 1 week of treatment, that is, at the age of 3-week, the rats were sacrificed and the kidney tissues were processed as described above.

2.2. Blood Pressure Determinations

Systolic blood pressure was obtained by tail-cuff plethysmography (3 to 5 determinations per rat) in unanaesthetized rats using an MK III physiograph (Narco Bio-System, Houston, TX, USA). A small cuff suitable for measuring blood pressure in 2 to 3 weeks old rats was validated in our laboratory and used in the present study. The rats were habituated to the procedure before taking the blood pressure readings.

2.3. Preparation of Renal Cortical Extract

Renal cortical extract was prepared in tissue homogenization buffer (30 mM Tris-HCl, pH 7.5, 10 mM EGTA, 5 mM EDTA, 1 mM DTT and 250 mM sucrose) supplemented with a cocktail of protease inhibitors as described previously.[11]

2.4. Preparation of Nuclear Extract

Nuclear extract of the kidney cortex was prepared as described previously[12] with few modifications. Snap-frozen kidney cortices were pulverized in liquid nitrogen and homogenized in homogenization buffer A (250 mM sucrose, 10 mM Hepes, pH 7.6, 25 mM KCl, 1 mM EDTA, 10% glycerol, 0.1 mM PMSF and 2.5 µg/ml aprotinin) with a Dounce homogenizer. The homogenate was layered over buffer B (1 M sucrose, 10 mM Hepes, pH 7.6, 25 mM KCl, 1 mM EDTA, 10% glycerol, 0.1 mM PMSF and 2.5 µg/ml aprotinin) and centrifuged at 3900×*g* for 10 min at 4^0C. The pellet was resuspended in buffer A/glycerol (9:1,w/w) and layered over buffer B/glycerol (9:1,w/w). The gradient was centrifuged at 48,000×*g* for 30 min at 4^0C. The semi-purified nuclear pellet was resuspended in nuclear extraction buffer (10 mM Hepes, pH 7.6, 400 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.1 mM PMSF). Nuclear proteins were then extracted on ice for 30 min, and the particulate material was removed by centrifugation at 13,000×*g* for 10 min at 4^0C. An aliquot of 10 µL supernatant containing nuclear extract was collected for measuring total protein concentration (Bradford),[13] and the remaining supernatant was mixed in Laemmli sample buffer,[14] heated at 100^0C for 5 minutes, and stored at -80^0C.

2.5. Immunohistochemistry

To detect macrophage infiltration, methacarn-fixed paraffin-embedded renal tissue sections (4 μm) were dewaxed and rehydrated. After microwave exposure and blocking with nonfat milk, slides were incubated with a 1:50 dilution of monoclonal mouse anti-rat ED1 antibody (Serotec, Oxford, UK) followed by alkaline phosphatase labeled polymer (Dako EnVision system, DAKO corporation, Carpinteria, CA, USA) conjugated with anti-mouse antibody, developed with fast red (Dako EnVision system, DAKO) and counterstained with hematoxylin. To detect oxidative stress-induced DNA base modification, immunohistochemistry was done for 8-hydroxy-2'-deoxyguanosine (8-OHdG, a DNA base-modified product) in methacarn-fixed paraffin-embedded renal tissue sections. After microwave exposure and blocking of endogenous peroxidase with 3% H_2O_2, slides were incubated with a 1:50 dilution of a mouse monoclonal anti-8-OHdG antibody (N45.1; Japan Institute for the Control of Aging, Japan), and subsequently a 1:200 dilution of a biotinylated secondary anti-mouse IgG antibody (Vector, Burlingame, CA). After incubation with avidin-biotin complex (ABC) reagent (Dako, Glostrup, Denmark), slides were developed in diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. For negative controls, staining was performed omitting the primary antibody or by using an irrelevant immunoglobulin. Tubulointerstitial cells containing 8-OHdG positive nuclei were counted in 50 sequential high power microscopic fields (x400), and 3 to 5 sections were evaluated for each animal.

2.6. Determination of Reduced Glutathione (GSH) Concentration

Renal cortical GSH level was measured by the method of Beutler *et al.*[15] with few modifications. A small piece of frozen kidney cortex was weighed and was directly homogenized in cold 10% trichloroacetic acid on ice. Homogenate was centrifuged at 3000 rpm for 15 min at 4^0C. The supernatant was reacted with 0.3 M phosphate buffer and 0.04% 5,5'dithio-bis 2-nitrobenzoic acid (DTNB). Absorbance was read at 412 nm and the GSH concentration was expressed as micromole GSH per gram frozen tissue. GSH (reduced form) was used as an external standard for preparation of a standard curve.

2.7. Western Blotting

Renal cortical homogenate was used for quantification of nitrotyrosine, p47phox and Nox4 (homologue of NADPH oxidase subunit gp91phox), and renal cortical nuclear extract was used for quantification of nuclear factor-κB (NF-κB) p65 subunit. Fifty μg of cortical or nuclear protein was separated on 10% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose membrane using Mini-Protean II Dual Slab Cell apparatus (Bio-Rad Laboratories, Hercules, CA, USA). After blocking nonspecific binding, the membranes were incubated with a mouse monoclonal anti-nitrotyrosine antibody (1:2000; clone 1A6, Upstate, Lake Placid, NY), a mouse monoclonal anti-p47phox antibody (1:500, BD Transduction Laboratories, BD Biosciences Pharmingen, NJ, USA), a rabbit polyclonal anti-Nox4 antibody (1:2500; a gift of Dr. Karen Block, University of Texas Health Science Center at San Antonio) or a rabbit polyclonal anti-NF-κB-p65 antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were subsequently incubated with horseradish peroxidase–conjugated appropriate secondary antibodies (Santa Cruz). Immunoreactive bands were made visible using the enhanced chemiluminescence method (Super Signal CL-HRP Substrate System; Pierce, Rockford, IL, USA). To verify the uniformity of protein load and transfer efficiency across the test samples, membranes were reprobed for actin (goat polyclonal anti-actin antibody, 1:1000, Santa Cruz), or stained with Ponceau S stain in case of nuclear proteins. Exposed films were scanned with a laser densitometer (Bio-Rad) and were analyzed quantitatively with Multi-Analyst Macintosh Software for Image Analysis Systems (Bio-Rad).

2.8. Statistical Analysis

Statistical significance was assessed by unpaired comparisons using Student's *t* test. The number of 8-OHdG positive cells in immunohistochemistry was not normally distributed; nonparametric Mann-Whitney U test was used in this case. All data are expressed as means±SD except the number of 8-OHdG, which is expressed as median (range). Statistical significance was set at $p<0.05$. All analyses were performed using statistical software StatView (SAS Institute Inc., Cary, NC, USA).

3. RESULTS

In the present study, we did age-matched comparison between WKY and SHR rats, and abnormality of any parameter of SHR has been considered when it significantly differed from that of age-matched WKY rats. As we did not perfuse the rats/kidneys, we could not completely exclude the possibility of blood contamination of our samples. However, we processed tissues from WKY and SHR groups exactly the same way, and therefore the effect of possible blood contamination should be equal in both groups resulting in no/negligible effect on the overall result.

3.1. Body Weight and Systolic Blood Pressure

As we observed in previous studies,[11,16] the SHR used in this study were also smaller than the age-matched WKY rats (body weight: 2-wk group, 32±6 g vs. 23 ± 4 g; 3-wk group, 67±8 vs. 36±6; WKY vs. SHR respectively; $p<0.001$ in both age groups). However, systolic BP (in mm Hg) was not different between WKY and SHR rats at 2 weeks (WKY: 81±11 vs. SHR: 80±13) and at 3 weeks (WKY: 97±10 vs. SHR: 103±14) of age (Figure 1).

[Insert Figure 1 about here]

3.2. Inflammatory Events

3.2.1. Macrophage infiltration

Inflammatory process in the kidney cortex was evaluated by counting macrophage (ED1-positive cell) infiltration in the tubulointerstitial area. Tubulointerstitial macrophage infiltration was similar in WKY (21.3±4.7/HPF) and SHR (24.5±4.3/HPF) rats at 2 weeks of age (n=5 in each group, Figure 2). However, macrophage infiltration in the tubulointerstitial area was found elevated in 3-week-old SHR compared to age-matched WKY rats (WKY: 18.9±1.2/HPF vs. SHR: 24.3±1.6/HPF, p=0.0002, n=5 in each group, Figure 2).

[Insert Figure 2 about here]

3.2.2. NF-κB activation

To identify the underlying mechanism of the inflammatory process, we studied activation status of the pro-inflammatory transcription factor NF-κB by quantifying the intra-nuclear fraction of the p65 subunit. Activated NF-κB regulates the expression of adhesion molecules and chemokines, and thereby regulates the infiltration of inflammatory cells in the tissue.[17] Western blot analysis of the renal cortical nuclear extract showed that the intra-nuclear p65 (expressed in densitometric unit) was lower in SHR than in WKY rats at 2 weeks of age (WKY: 1.0±0.10 vs. SHR: 0.43±0.11, p=0.003) (Figure 3). However, the expression and/or nuclear translocation of the p65 subunit was found elevated by 2.8 fold in 3-week-old SHR compared to age-matched WKY rats (WKY: 1.0±0.39 vs. SHR: 2.81±0.48, p=0.001) (Figure 3). Probably, the base-line inflammatory gene expression pattern was not affected by the lower levels of intra-nuclear NF-κBp65 in SHR at 2 weeks of age, for which we observed similar levels of tubulointerstitial macrophage infiltration between WKY and SHR rats at 2-week time point.

[Insert Figure 3 about here]

3.3. Oxidative Stress

3.3.1. Renal cortical nitrotyrosine

Oxidative and nitrosative stress-induced protein modification (nitration of tyrosine residues leading to formation of nitrotyrosine) was assessed in the renal cortical tissue by Western blot analysis. Although nitrotyrosine is not a specific marker for the *in vivo* generation of peroxynitrite, it has widely been used as a marker of oxidative and nitrosative stress.[18] We detected a major band of nitrated protein of unknown identity at around 55 kD position as it was previously demonstrated in the kidney tissue using the same antibody.[19] Considering this 55 kD protein, nitrotyrosine levels (expressed as a ratio of nitrotyrosine/actin in densitometric units) were found similar between WKY (1.0±0.26) and SHR (1.32±0.44) rats at 2 weeks of age (Figure 4). However, nitrotyrosine level was found elevated by 1.6 fold in 3-week-old SHR compared to age-matched WKY rats (WKY: 1.0±0.26 vs. SHR: 1.62±0.29, p=0.019) (Figure 4).

[Insert Figure 4 about here]

3.3.2. Oxidative stress-induced DNA damage

The DNA base guanine-containing nucleoside 2'-deoxyguanosine can be modified to 8-hydroxy-2'-deoxyguanosine (8-OHdG) by excessive oxidative stress.[20] We detected renal cortical cells containing the modified base 8-OHdG in the nucleus by immunohistochemistry using monoclonal antibody against 8-OHdG. The staining pattern was heterogeneous, involving tubular cells mainly in the inner cortex and medulla. There were different grades of positive staining, some nuclei were intensely stained and some others were faintly stained or stained with intermediate intensity (Figure 5). However, the median (range) number of cortical cells containing positively stained nuclei was not different between WKY {158(22-294)} and SHR {59(21-236)} rats at 2 weeks of age (n=5). But the median number of cells containing positively stained nuclei were elevated in 3-week-old SHR compared to age-matched WKY rats {WKY: 27(25-119) vs. SHR: 191(64-492), p=0.049, n=5 in each group}.

[Insert Figure 5 about here]

3.3.3. NADPH oxidase

NADPH oxidase is the major source of pro-oxidant superoxide in the vascular tissue.[21] This enzyme system has been found upregulated in different animal models of HTN associated with oxidative stress.[9,19] Recently, it has been shown that high blood pressure, *per se*, can promote the expression of NADPH oxidase subunits in the arterial wall.[22] To study the participation of this enzyme system in the pathogenesis of renal oxidative stress and associated inflammatory events in prehypertensive SHR, we investigated the expression of p47phox, a regulatory subunit of NADPH oxidase, and Nox4, a homologue of gp91phox subunit of NADPH oxidase system.

The expression of p47phox in the renal cortex was found significantly elevated in 2-week-old SHR compared to age-matched WKY rats (WKY: 1.0±0.29 vs. SHR: 1.75±0.27, p=0.01, expressed as a ratio of p47phox/actin in densitometric unit) (Figure 6). The expression of p47phox was found further elevated by more than 6 folds in 3-week-old SHR compared to age-matched WKY rats (WKY: 1.0±0.47 vs. SHR: 6.59±1.0, p<0.001) (Figure 6). However, the expression of Nox4 was not different between SHR and WKY rats at 2- or 3-week time points (data not shown).

[Insert Figure 6 about here]

3.3.4. Reduced glutathione (GSH)

Renal cortical GSH concentration was assessed as a measure of non-enzymatic antioxidant. GSH concentration (in micromole/g frozen tissue) was found elevated in SHR at 2 weeks (WKY: 3.08±0.59 vs. SHR: 4.14±1.03, p=0.013, n=10 in each group) and at 3 weeks (WKY: 4.49±0.55 vs. SHR: 5.0±0.47, p=0.038, n=10 in each group) of age compared to age-matched WKY rats. The increased GSH levels at 2 and 3 weeks of age in SHR may be an adaptive response to protect the kidney from underlying pro-oxidant stress.

[Insert Figure 7 about here]

3.4. Antioxidant Treatment

Antioxidant treatment with NAC and Tempol in 2-week-old SHR for 1 week did not alter systolic BP (control: 106±9 vs. treated group: 103±11 mm Hg, n=5 in each group). However, renal cortical oxidative stress, as assessed by nitrotyrosine levels, was significantly reduced by antioxidant treatment (control: 1±0.32 vs. treated group: 0.49±0.21, nitrotyrosine/actin ratio, p=0.022, n=5 in each group, Figure 7). We evaluated renal cortical inflammatory process in the same group of rats to answer whether renal inflammation could be a consequence of renal oxidative stress in prehypertensive SHR. We found that the renal cortical macrophage infiltration in the tubulointerstitial area was significantly reduced in the treated group compared with control group (control: 22.45±3.10/HPF vs. treated group: 18.17±2.53/HPF, p=0.044, n=5 in each group, Figure 8). In addition, NF-κBp65 level in the renal cortical homogenate was also found significantly reduced in treated group compared with control group (control: 1±0.31 vs. treated group: 0.63±0.14, NF-κBp65/actin ratio, p=0.039, n=5 in each group, Figure 7). However, intra-nuclear fraction of p65 failed to show a significant decrement in the treated group (control: 1±0.14 vs. treated group: 0.80±0.39 densitometric unit).

[Insert Figure 8 about here]

4. DISCUSSION

It is well accepted that the systemic HTN, renal inflammation and oxidative stress are tightly associated events in different models of HTN.[4,8,23] The association of these 3 events is not just a simple coexistence, rather they are closely interdependent in a manner that by modulating any of them the status of other 2 could be modulated, at least partly.[2,3,6,7] This fact has been highlighted in several recent reviews and the importance of identification of the primary event among HTN, renal inflammation and oxidative stress has been emphasized.[8,23] Since the events are interdependent, identification and targeting of the primary culprit may be of great clinical importance. Although a great deal of work has already been done on this issue, the novelty of the present study is the simultaneous investigation of renal inflammation and oxidative stress at 2 different time points in very young prehypertensive SHR. Findings of our baseline study and antioxidant intervention suggest that the oxidative stress appears as a primary abnormality in the kidney that leads to renal inflammation before development of hypertension in SHR.

In the present work, we decided to study very young prehypertensive SHR of 2 and 3 weeks of age and age-matched WKY rats, because previous studies had confirmed that the BP does not differ between WKY and SHR rats at 2 and 3 weeks of age.[5,24] Although the accuracy of the tail-cuff plethysmography has been questioned, the BP data obtained in the present study are similar to those obtained by Rodriguez-Iturbe *et al.*[5] and Dickhout and Lee[24]. The SHR rats studied were normotensive and the systolic BP of the SHR was similar to that of WKY rats both at 2 and 3 weeks of age. Therefore, we consider that the BP did not modify renal inflammation or oxidative stress in the animals we studied. According to the objective of the study, we did all experiments and comparisons in an age-matched fashion to explore the differences in renal inflammation and oxidative stress between SHR and age-matched WKY rats. The inflammatory/oxidative stress parameters we investigated are likely to be different between 2-week and 3-week of age within a rat strain, however we did not study this age-effect in the present work.

The markers of renal inflammation and oxidative stress showed clear elevation in SHR at 3 weeks of age compared with age-matched WKY rats. However, at 2-week, there

was no evidence of elevated renal inflammation in the SHR, as the macrophage infiltration was similar and intra-nuclear NF-κBp65 level was even lower in 2-week-old SHR compared to age-matched WKY rats. On the other hand, among the 5 oxidative stress markers studied, the expression of NADPH oxidase subunit p47phox showed significant elevation in the kidney of 2-week-old SHR compared to age-matched WKY rats.

We consider that the findings obtained at 2-week time point need careful interpretation. Our data clearly indicate that the inflammatory process does not start in the kidney of 2-week old SHR. The question is whether oxidative stress starts at 2-week time point. Since oxidative stress is typically defined as a disturbance in the pro-oxidant/antioxidant balance in favor of the former, leading to potential damage,[25] our data do not show a direct evidence of oxidative stress at 2-week time point. However, present data indicate that the upregulation of p47phox subunit of NADPH oxidase starts at (or before) the age of 2 weeks in the kidney, which shows marked elevation at the age of 3 weeks with associated oxidative damage. The elevation of the antioxidant GSH levels at 2- and 3-week time points compared with age-matched WKY rats also provide a functional evidence of enhanced pro-oxidant production in SHR.

To further elucidate whether inflammation or oxidative stress is the primary abnormality in the kidney in prehypertensive SHR, we treated 2-week-old SHR rats with antioxidants, NAC and Tempol. Although antioxidants are known to reduce BP in SHR,[2,7,19,26] we did not observe an alteration of BP with NAC and Tempol therapy for 1 week. This finding is not unlikely as we treated prehypertensive rather than hypertensive rats. Welch *et al.*[26] observed BP reduction in hypertensive SHR, but not in normotensive WKY rats, with oral Tempol therapy for 2 weeks. However, in the present study, antioxidant therapy reduced renal oxidative stress that was associated with a significant reduction of tubulointerstitial macrophage infiltration in the renal cortex. Total cortical NF-κBp65 level was also significantly reduced with antioxidant therapy, but the reduction of intra-nuclear fraction of p65 failed to reach the level of significance. Although we are unable to provide a definitive explanation of this last finding, the regulation of inflammatory gene expression by the NF-κB system is a complex issue and it does not

solely depend on the p65 level or it's transmigration into the nucleus. However, results of the antioxidant therapy suggest that oxidative stress, but not inflammation, is the primary abnormality in the kidney in prehypertensive SHR.

The NADPH oxidase enzyme complex is composed of membrane-bound subunits (gp91phox and p22phox) and cytosolic subunits (p47phox, p67phox and p40phox). Upon activation, some subunits, including p47phox, are phosphorylated and translocated to the membrane and participate in superoxide generation.[27] Chabrashvili *et al.*[9] previously showed that the p47phox expression is elevated in the kidney of 4-week-old SHR. They suspected from this finding that the oxidative stress in the kidney precedes the development of HTN in SHR.[28] However, several studies including our previous study have shown that the BP is already elevated in the SHR at 4 weeks of age compared with age-matched WKY rats.[16,29] Therefore, it was uncertain whether the over-expression of p47phox in the kidney of 4-week-old SHR was an effect of elevated BP in the study by Chabrashvili *et al.*[9] The findings reported in the present study confirmed that the p47phox overexpression occur in the kidney of SHR at 2 weeks of age, long before the development of HTN in this rat strain. In contrast to the findings of Chabrashvili *et al.*[9] and present study, Zhan *et al.*[19] did not observe an overexpression of p47phox, instead they found overexpression of gp91phox and p22phox subunits in SHR. Although this discrepancy is difficult to explain, Zhan *et al.*[19] studied 24-week-old SHR that were hypertensive for long duration.

Regarding the renal inflammatory process, similar to the findings of the present study, it has recently been shown that the renal interstitial inflammation and pro-inflammatory transcription factor NF-κB are already elevated in the prehypertensive SHR of 3 weeks of age.[5] However, this last study did not investigate the relation of oxidative stress with inflammation in the kidney of prehypertensive SHR. In the present study, to the best of our knowledge, for the first time we investigated the relationship between renal inflammation and oxidative stress in prehypertensive SHR, and we have identified primary abnormality among HTN, renal inflammation and oxidative stress in young SHR.

conclusion, the present study apparently separates 2 closely interdependent and associated events, renal inflammation and oxidative stress, in SHR; and indicates that the oxidative stress appears before inflammation as a primary abnormality in the kidney in prehypertensive SHR. The identification of primary abnormality may have major implications in the prevention of HTN, because the renal oxidative stress and associated inflammation that appears before the development of HTN in SHR may be involved in the subsequent development of HTN and nephropathy.

Acknowledgments

This work was supported by grants from Fundacao de Amparo a Pesquisa do Estado de Sao Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq). Dr. Subrata Biswas was on receipt of a scholarship from CAPES. We thank Dr. Jacqueline M. Lopes de Faria, Dr. Roger Castilho and Dr. Roberto Amazonas for helpful suggestions and Dr. Wilson Nadruz for helping in isolation of nuclear extract. We also thank Ms. Denise S Souza, Ms. Dulcineia M Albuquerque, Ms. Kamila C Silva, Ms. Camila C Pinto, Ms. Vanessa C Pansiera, Ms. Daniela R Melo, Mr. Leonardo D Lobo and Mr. Bruno S Pessoa for assistance in laboratory procedures. We are grateful to Sergio Magalhaes for technical assistance.

REFERENCES

[1] Okamoto K, Tabei R, Fukushima M, Nosaka S, Yamori Y, Ichijima K, Haebara H, Matsumoto M, Maruyama T, Suziki Y, Tamegai IM. Further observations of the development of a strain of spontaneously hypertensive rats. *Jpn Circ J* 1966;30:703-716.

[2] Nava M, Quiroz Y, Vaziri ND, Rodriguez-Iturbe B. Melatonin reduces renal interstitial inflammation and improves hypertension in spontaneously hypertensive rats. *Am J Physiol Renal Physiol* 2003;284:F447-F454.

[3] Rodriguez-Iturbe B, Ferrebuz A, Vanegas V, Quiroz Y, Mezzano S, Vaziri ND. Early and sustained inhibition of nuclear factor-κB prevents hypertension in spontaneously hypertensive rats. *J Pharmacol Exp Ther* 2005;315:51-57.

[4] Rodriguez-Iturbe B, Pons H, Herrera-Acosta J, Johnson RJ. Role of immunocompetent cells in nonimmune renal diseases. *Kidney Int* 2001;59:1626-1640.

[5] Rodriguez-Iturbe B, Quiroz Y, Ferrebuz A, Parra G, Vaziri ND. Evolution of renal interstitial inflammation and NF-κB activation in spontaneously hypertensive rats. *Am J Nephrol* 2004;24:587-594.

[6] Rodriguez-Iturbe B, Quiroz Y, Nava M, Bonet L, Chavez M, Herrera-Acosta J, Johnson RJ, Pons HA. Reduction of renal immune cell infiltration results in blood pressure control in genetically hypertensive rats. *Am J Physiol Renal Physiol* 2002;282:F191-F201.

[7] Rodriguez-Iturbe B, Zhan C, Quiroz Y, Sindhu RK, Vaziri ND. Antioxidant-rich diet relieves hypertension and reduces renal immune infiltration in spontaneously hypertensive rats. *Hypertension* 2003;41:341-346.

[8] Vaziri ND. Roles of oxidative stress and antioxidant therapy in chronic kidney disease and hypertension. *Curr Opin Nephrol Hypertens* 2004;13:93-99.

[9] Chabrashvili T, Tojo A, Onozato ML, Kitiyakara C, Quinn MT, Fujita T, Welch WJ, Wilcox CS. Expression and cellular localization of classic NADPH oxidase subunits in the spontaneously hypertensive rat kidney. *Hypertension* 2002;39:269-274.

[10] Righetti AE, Boer-Lima PA, Lopes de Faria JB. The presence of genetic hypertension stimulates early renal accumulation of fibronectin in experimental diabetes mellitus. *Diabetologia* 2001;44:2088-2091.

[11] Silveira LA, Bacchi CE, Pinto GA, Lopes de Faria JB. The genetics of hypertension modifies the renal cell replication response induced by experimental diabetes. *Diabetes* 2002;51:1529-1534.

[12] Thai MV, Guruswamy S, Cao KT, Pessin JE, Olson AL. Myocyte enhancer factor 2 (MEF2)-binding site is required for GLUT4 gene expression in transgenic mice. Regulation of MEF2 DNA binding activity in insulin-deficient diabetes. *J Biol Chem* 1998;273:14285-14292.

[13] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye binding. *Anal Biochem* 1976;72:248-254.

[14] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-685.

[15] Beutler E, Duron O, Mikus B. Improved method for the determination of blood glutathione. *J Lab Clin Med* 1963;16:882-888.

[16] Lopes de Faria JB, Zoukhri D, Lorenzi M. Mesangial cell abnormalities in spontaneously hypertensive rats before the onset of hypertension. *Kidney Int* 1997;52:387-392.

[17] Tak PP, Firestein GS. NF-κB: A key role in inflammatory disease. *J Clin Invest* 2001;107:7-11.

[18] Halliwell B. What nitrates tyrosine? Is nitrotyrosine specific as a biomarker of peroxynitrite formation *in vivo*? *FEBS Lett* 1997;411:157-160.

[19] Zhan DC, Sindhu RK, Vaziri ND. Up-regulation of kidney NADPH oxidase and calcineurin in SHR: reversal by lifelong antioxidant supplementation. *Kidney Int* 2004;65:219-227.

[20] Kasai H, Nishimura S. Hydroxylation of deoxyguanosine at the C-8 position by ascorbic acid and other reducing agents. *Nucleic Acid Res* 1984;12:2137-2145.

[21] Griendling KK, Sorescu D, Ushio-Fukai M. NADPH oxidase: role in cardiovascular biology and disease. *Circ Res* 2000;86:494-501.

[22] Vaziri ND, Ni Z. Expression of Nox-1, gp91phox, p47phox and p67phox in the aorta segments above and below coarctation. *Biochim Biophys Acta* 2005;1723:321-327.

[23] Touyz RM. Molecular and cellular mechanisms in vascular injury in hypertension: role of angiotensin II. *Curr Opin Nephrol Hypertens* 2005;14:125-131.

[24] Dickhout JG, Lee RM. Blood pressure and heart rate development in young spontaneously hypertensive rats. *Am J Physiol* 1998;274:H794-H800.

[25] Sies H. *Oxidative Stress: Oxidants and Antioxidants.* New York: Academic Press; 1991.

[26] Welch WJ, Mendonca M, Blau J, Karber A, Dennehy K, Patel K, Lao YS, Jose PA, Wilcox CS. Antihypertensive response to prolonged tempol in the spontaneously hypertensive rat. *Kidney Int* 2005;68:179-187.

[27] Babior BM. NADPH oxidase: an update. *Blood* 1999;93:1464-1476.

[28] Wilcox CS. Oxidative stress and nitric oxide deficiency in the kidney: a critical link to hypertension? *Am J Physiol Regul Integr Comp Physiol* 2005;289:913-935.

[29] Imig JD, Falck JR, Gebremedhin D, Harder DR, Roman RJ. Elevated renovascular tone in young spontaneously hypertensive rats. Role of cytochrome p-450. *Hypertension* 1993;22:357-364.

LEGENDS TO FIGURES

Figure 1. Systolic blood pressure in WKY and SHR rats during early development. Absolute values of blood pressure (means±SD) in mm Hg are given in the text. Number of rats: 2-wk group-12 SHR and 11 WKY rats; and 3-wk group-14 SHR and 13 WKY rats.

Figure 2. Immunohistochemical detection of macrophage (ED1-positive cell) infiltration in the kidney of WKY and SHR at 2 weeks (A, B) and 3 weeks (C, D) of age. Positive staining was observed as bright-red granular staining. Tubulointerstitial macrophage infiltration was evaluated by counting ED1-positive cells in 20 cortical high power fields and expressed as positive cells per high power field (E). Three sections from each animal were evaluated. Bars are representing means±SD; n=5 in each group. HPF, high power field; *p=0.0002 vs. age-matched WKY rats. Sections were counterstained with hematoxylin (original magnification x400). Scale bar=50 μm.

Figure 3. NF-κB activation as measured by intra-nuclear p65 protein levels during early development in SHR and age-matched WKY rats. Representative Western blots (upper panel) for SHR and WKY rats at 2 and 3 weeks of age. Densitometric analysis (lower panel) of intra-nuclear p65 in WKY and SHR rats. Equal loading was confirmed by Ponceau S stain. Values obtained in SHR groups are expressed relative to those of WKY groups, which are arbitrarily assigned a value of 1.0. Bars are representing means±SD of at least 3 independent experiments (n=4 in each group); *p=0.003 and #p=0.001 vs. age-matched WKY group.

Figure 4. Renal cortical nitrotyrosine levels in SHR and age-matched WKY rats. Representative Western blots (upper panel) for SHR and WKY rats at 2 and 3 weeks of age. Actin was used as control for protein loading. Densitometric analysis of nitrotyrosine/actin ratio (lower panel) in WKY and SHR rats. Values obtained in SHR groups are expressed relative to those of WKY groups, which are arbitrarily assigned a value of 1.0. Bars are representing means±SD of at least 3 independent experiments; n=5 in each group; *p=0.019 vs. age-matched WKY group.

Figure 5. Immunohistochemical detection of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in the kidney of WKY and SHR at 2 weeks (A, B) and 3 weeks (C, D) of age. Positive staining was observed as brown-stained nuclei. Number of 8-OHdG positive cells was found elevated in 3-week old SHR, but not in 2-week old SHR, compared with age-matched WKY rats. Sections were counterstained with hematoxylin (original magnification x400). Scale bar=50 μm.

Figure 6. Expression of p47phox in the kidney cortex in SHR and age-matched WKY rats. Representative Western blots (upper panel) for SHR and WKY rats at 2 and 3 weeks of age. Actin was used as control for protein loading. Densitometric analysis of p47phox/actin ratio (lower panel) in WKY and SHR rats. Values obtained in SHR groups are expressed relative to those of WKY groups, which are arbitrarily assigned a value of 1.0. Bars are representing means±SD of at least 3 independent experiments; n=5 in each group; *p=0.01, and #p<0.001 vs. age-matched WKY group.

Figure 7. Renal cortical nitrotyrosine and NF-κB p65 in control and antioxidant treated (NAC+T) SHR rats. Representative Western blots for nitrotyrosine (A) and NF-κBp65 (B). Actin was used as control for protein loading. Densitometric analysis of nitrotyrosine/actin ratio (C) and p65/actin ratio (D). Value obtained in treated group is expressed relative to that of control group, which is arbitrarily assigned a value of 1.0. Bars are representing means±SD of at least 3 independent experiments; *p=0.022 and #p=0.039 vs. control group; n=5 in each group; NAC+T, N-acetyl-L-cysteine and Tempol.

Figure 8. Immunohistochemical detection of macrophage (ED1-positive cell) infiltration in the kidney of control (A) and antioxidant treated (B) rat. Tubulointerstitial macrophage infiltration was evaluated by counting ED1-positive cells in 20 cortical high power fields and expressed as positive cells per high power field (C). Four sections from each animal were evaluated. Bars are representing means±SD; *p=0.044 vs. control group, n=5 in each group. NAC+T, N-acetyl-L-cysteine and Tempol; HPF, high power field. Sections were counterstained with hematoxylin (original magnification x400). Scale bar=50 μm.

FIGURES

Figure 1

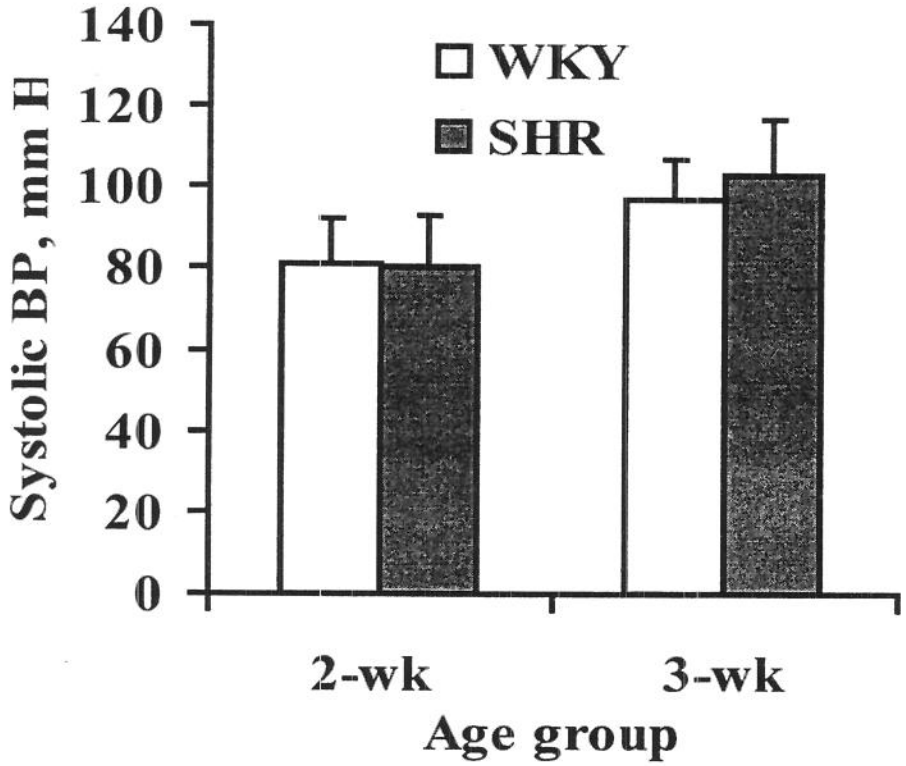
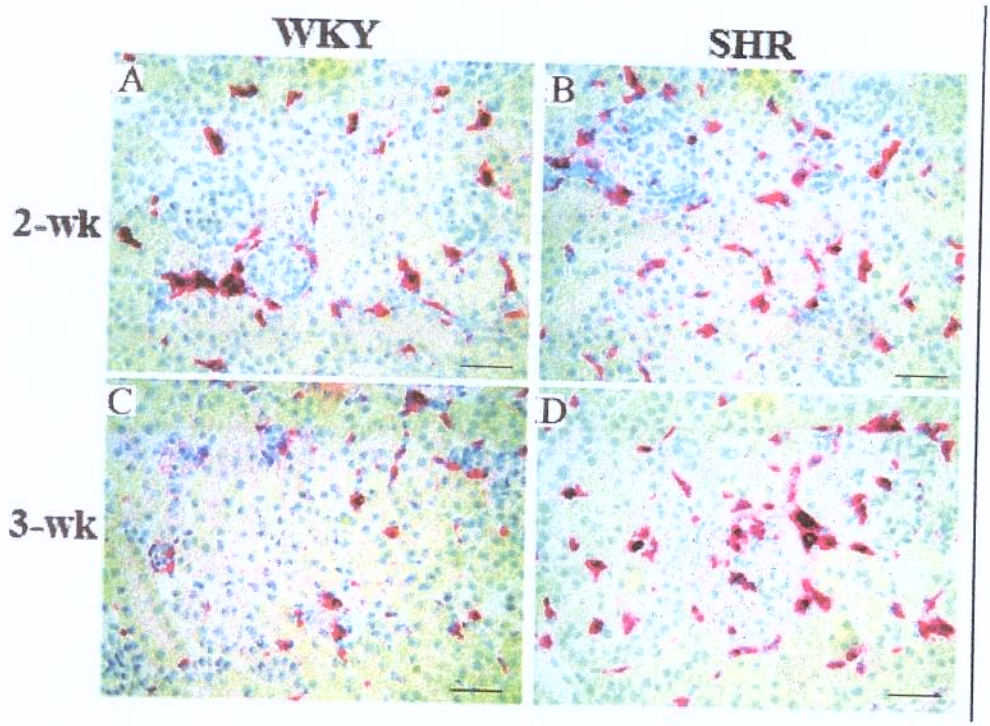


Figure 2


WKY
SHR
A
B
2-wk
C
D
3-wk

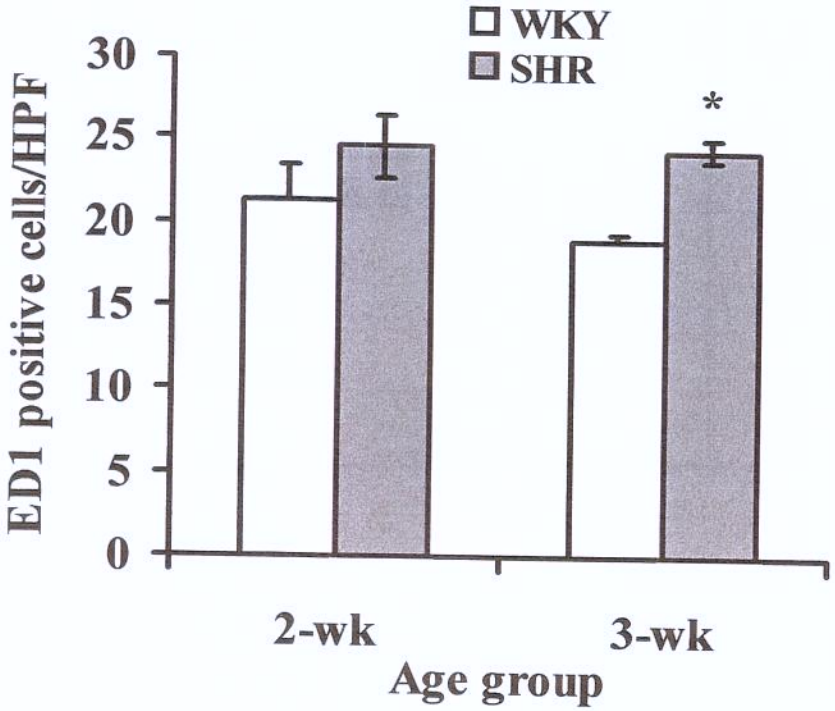
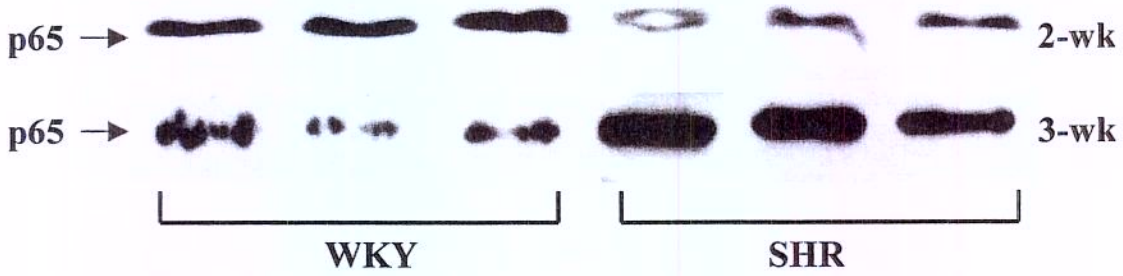

WKY
SHR
30
25
20
15
10
5
0
ED1 positive cells/HPF
*
2-wk
3-wk
Age group

Figure 3


p65
p65
2-wk
3-wk
WKY
SHR

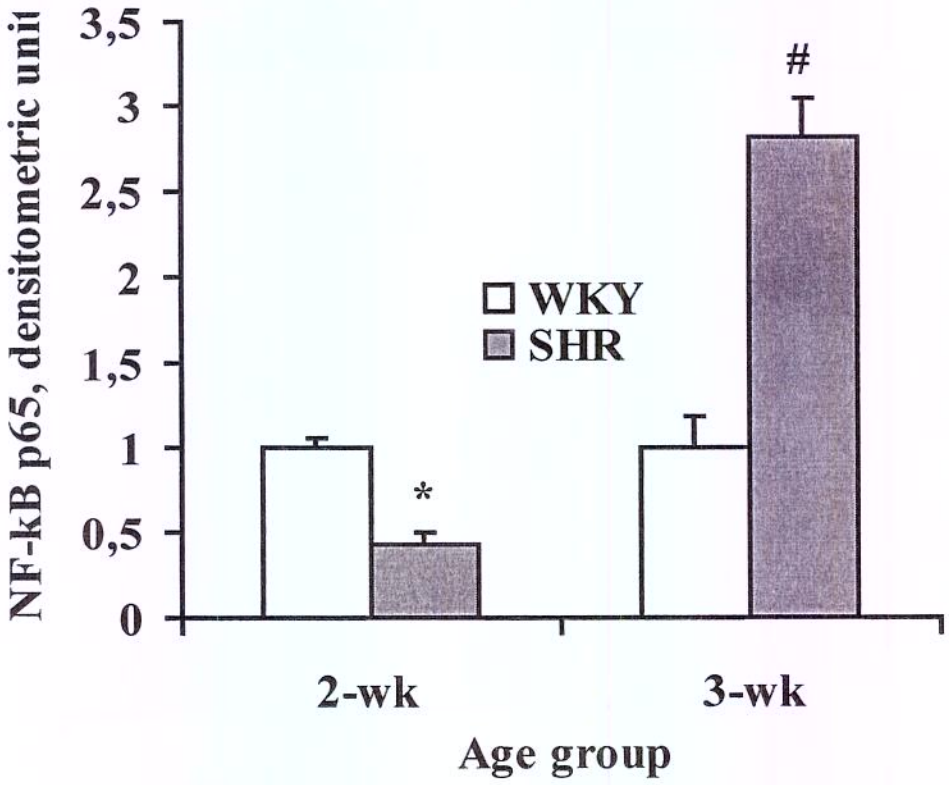

NF-kB p65, densitometric unit
3,5
3
2,5
2
1,5
1
0,5
0
WKY
SHR
*
#
2-wk
3-wk
Age group

Figure 4

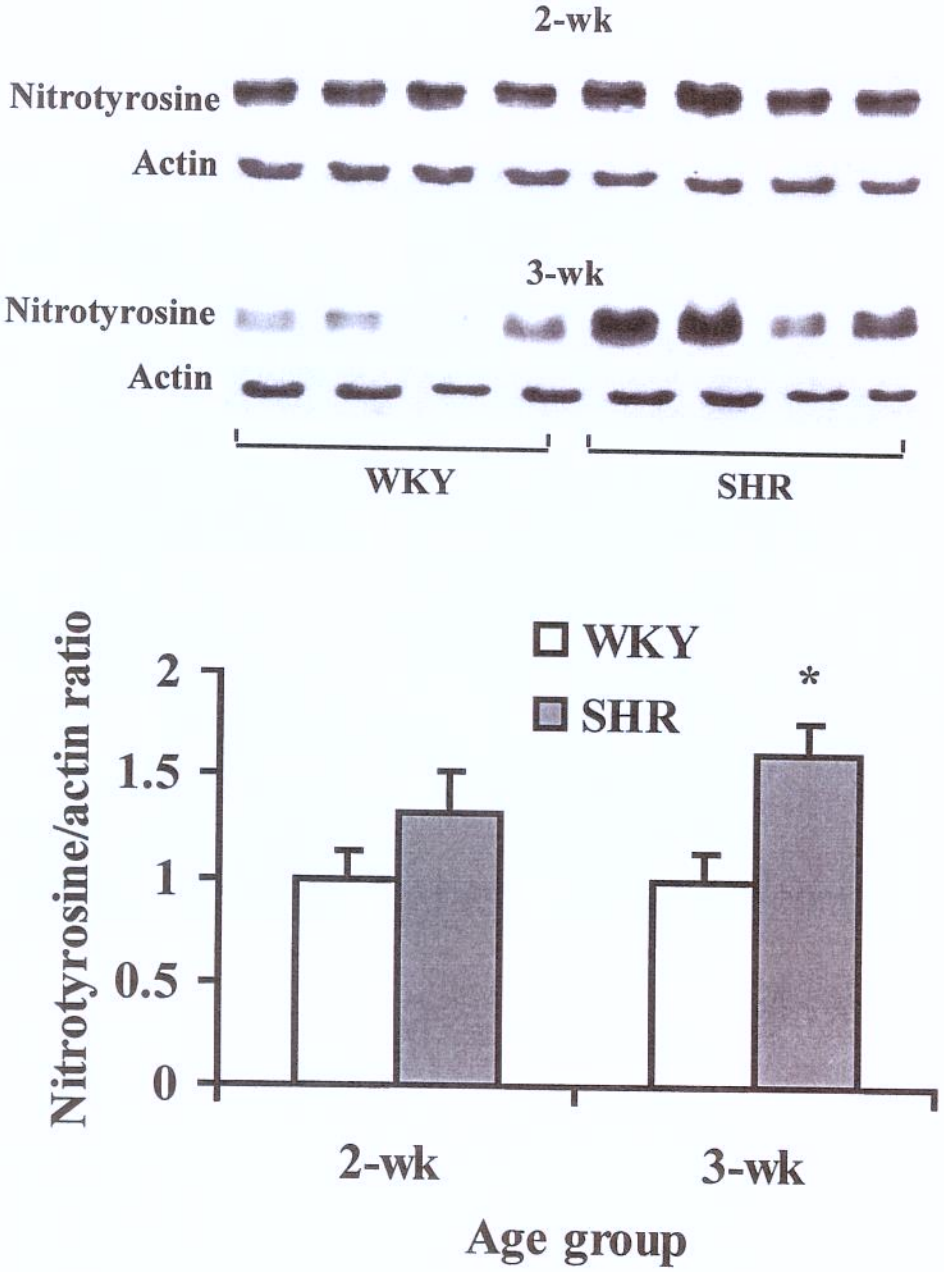

2-wk
Nitrotyrosine
Actin
3-wk
Nitrotyrosine
Actin
WKY
SHR
Nitrotyrosine/actin ratio
2
1.5
1
0.5
0
WKY
SHR
*
2-wk
3-wk
Age group

Figure 5

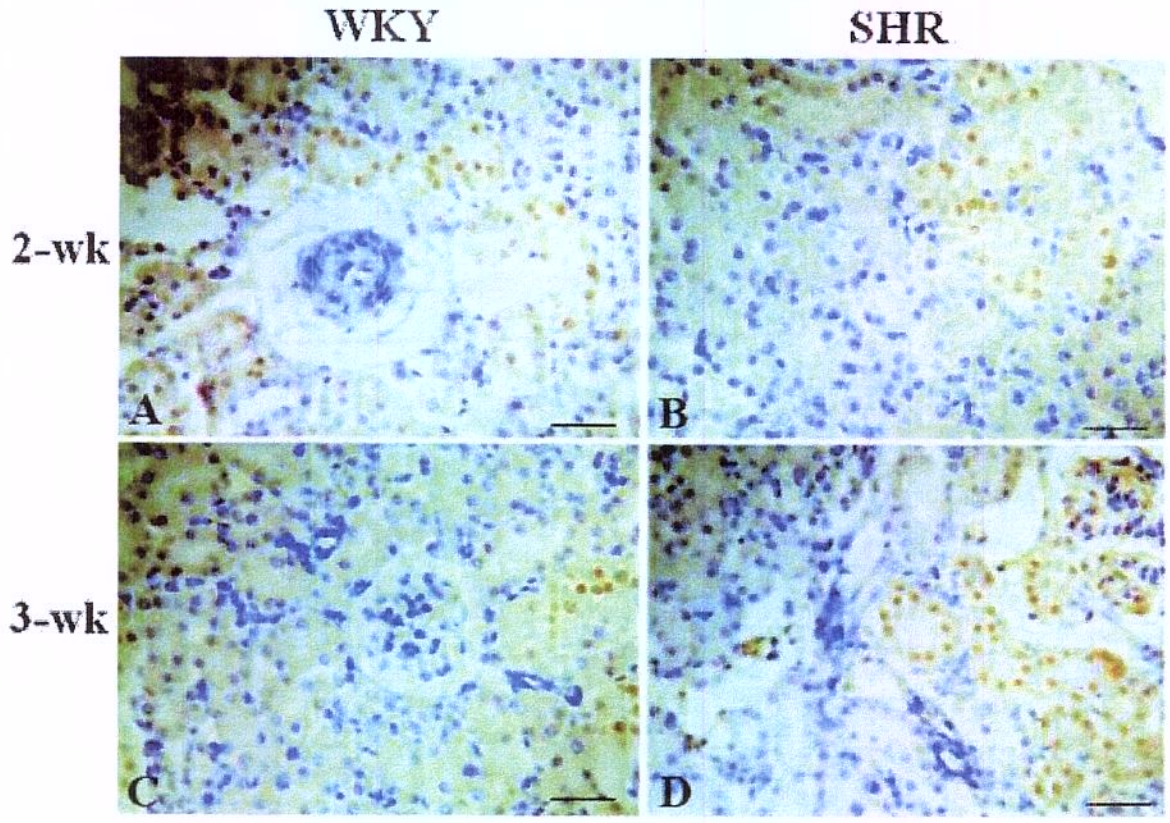


Figure 6

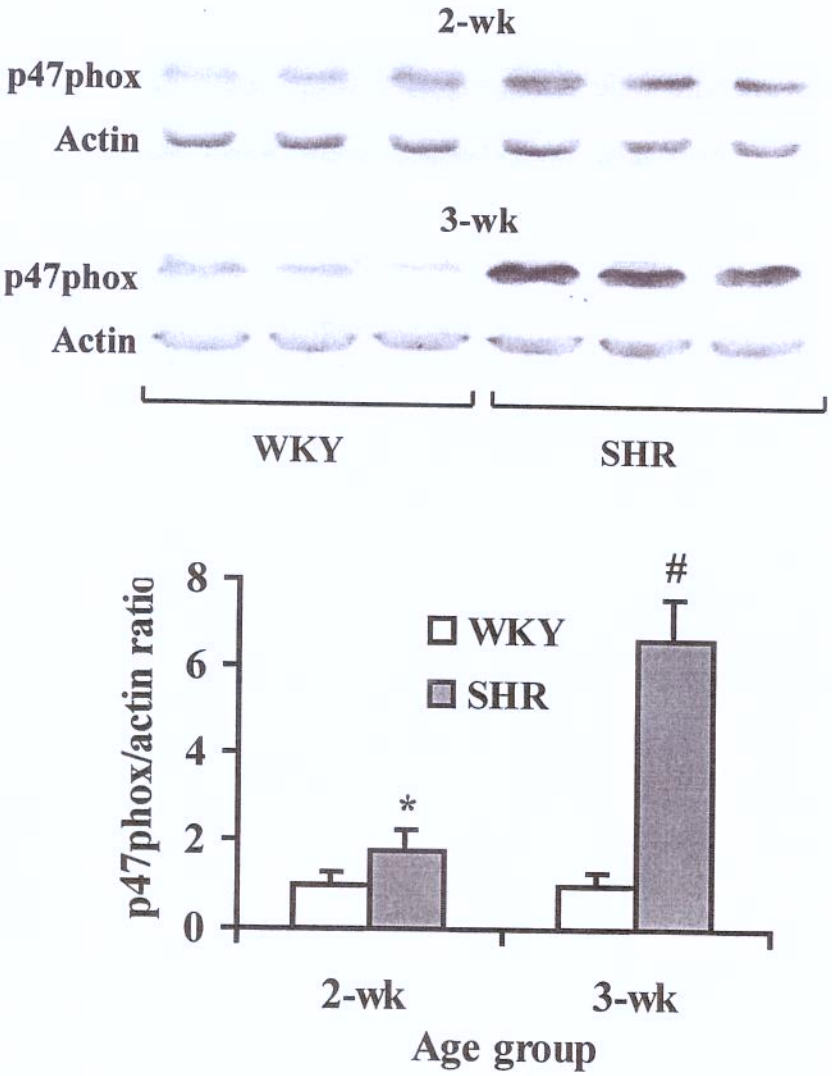

2-wk
p47phox
Actin
3-wk
p47phox
Actin
WKY
SHR
p47phox/actin ratio
8
6
4
2
0
WKY
SHR
*
#
2-wk
3-wk
Age group

Figure 7

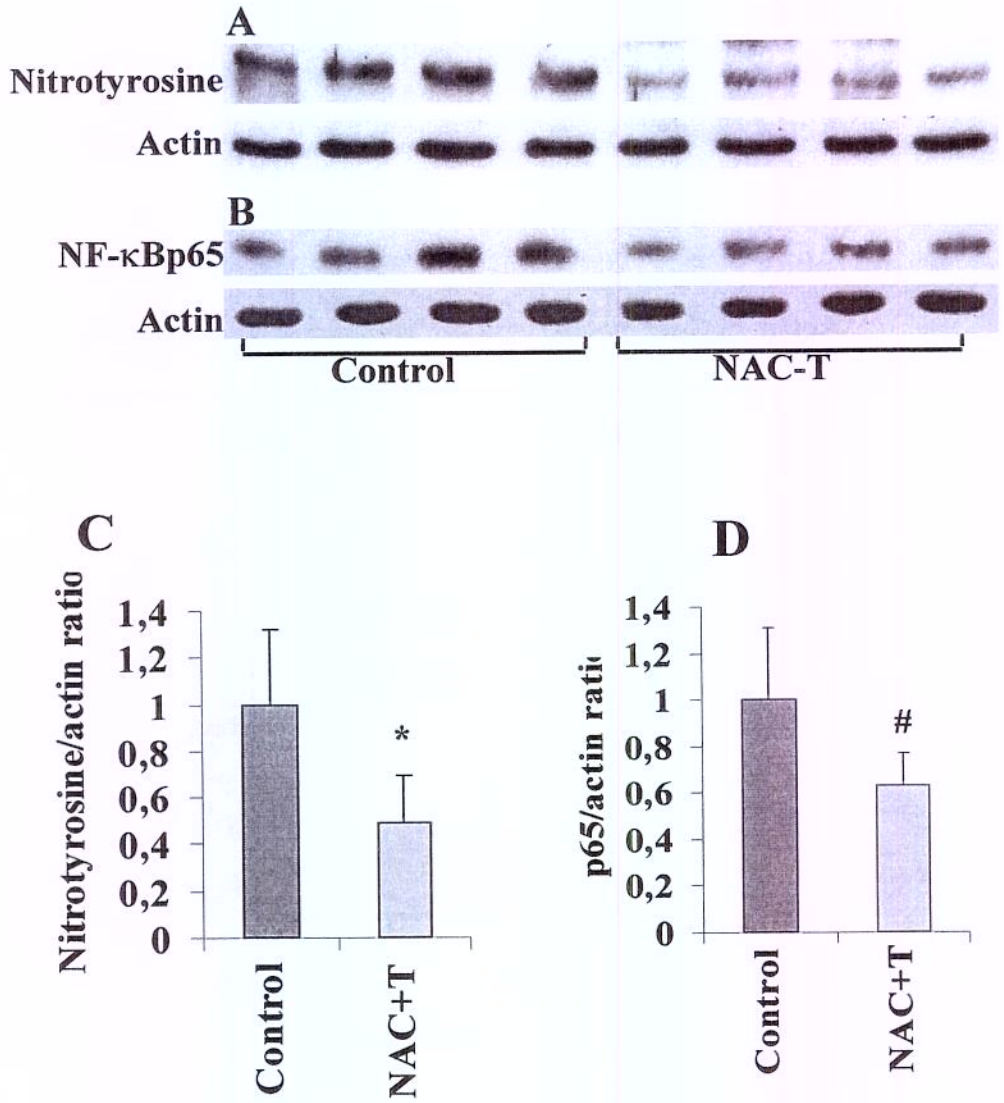
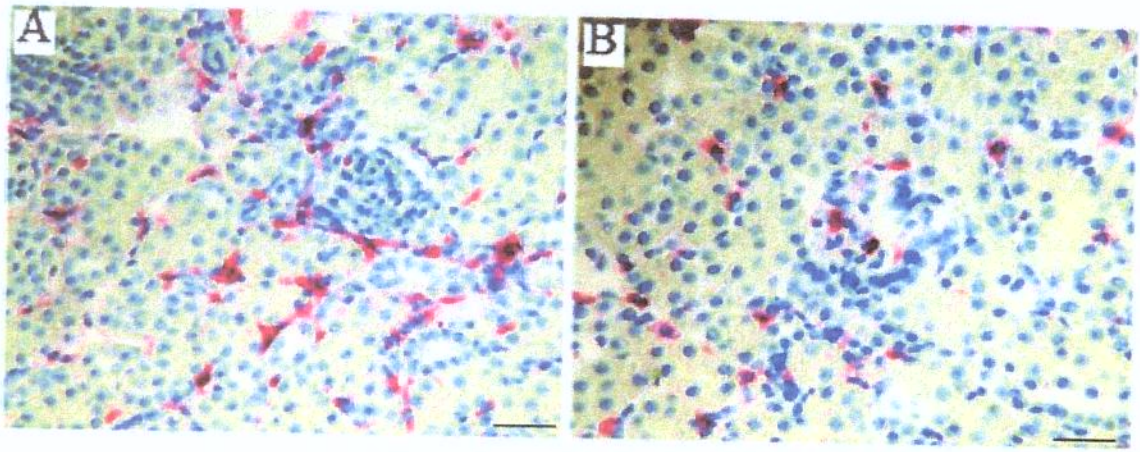
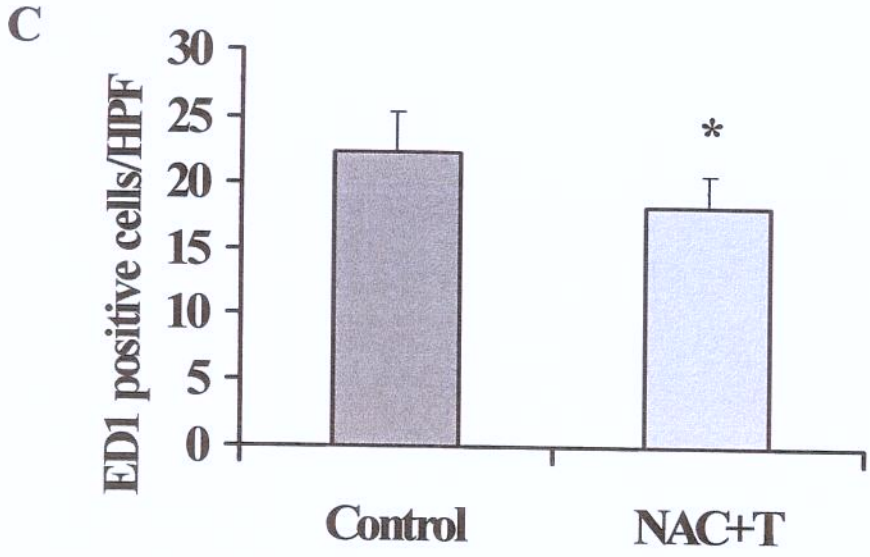
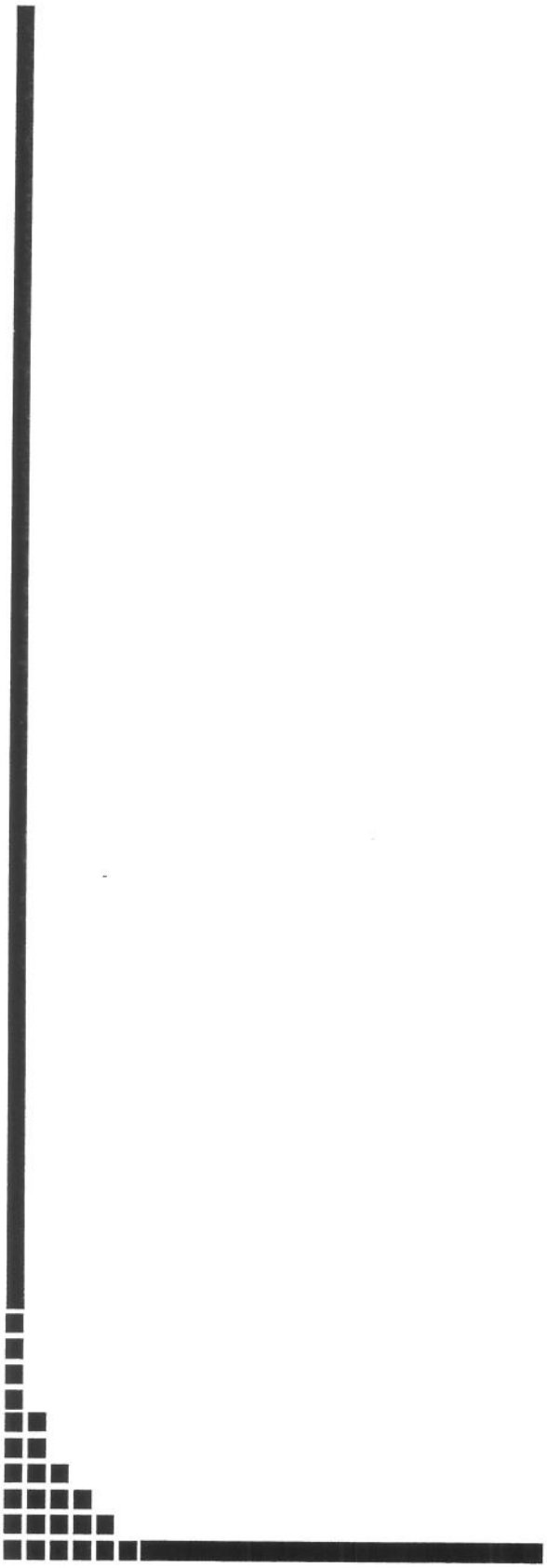

A
Nitrotyrosine
Actin
B
NF-κBp65
Actin
Control
NAC-T
C
Nitrotyrosine/actin ratio
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
Control
NAC+T
D
p65/actin ratio
1,4
1,2
1
0,8
0,6
0,4
0,2
0
#
Control
NAC+T

Figure 8







CHAPTER 3

Paper II

Hypertension induces oxidative stress but not macrophage infiltration in the kidney in the early stage of experimental diabetes mellitus

Biswas SK, Lopes de Faria JB. Hypertension induces oxidative stress but not macrophage infiltration in the kidney in the early stage of experimental diabetes mellitus. Am J Nephrol 2006; 26: 415-422.

Original Report: Laboratory Investigation

American Journal of Nephrology

Am J Nephrol 2006;26:415–422
DOI: 10.1159/000095707

Received: June 7, 2006
Accepted: July 30, 2006
Published online: September 7, 2006

Hypertension Induces Oxidative Stress but Not Macrophage Infiltration in the Kidney in the Early Stage of Experimental Diabetes Mellitus

Subrata K. Biswas Jose B. Lopes de Faria

Renal Pathophysiology Laboratory, Division of Nephrology, Department of Internal Medicine, Faculty of Medical Sciences, State University of Campinas (UNICAMP), Campinas, São Paulo, Brazil

Key Words

Diabetic nephropathy · Hypertension · Inflammation · Macrophage · Oxidative stress · Spontaneously hypertensive rats

Abstract

Background: The combination of diabetes and hypertension increases the incidence and severity of kidney disease in an additive manner. Inflammatory and oxidative stress mechanisms contribute to renal damage in both diabetes and hypertension. Therefore, we investigated whether renal macrophage infiltration and oxidative stress events are additive from the beginning in diabetic animals with coexisting hypertension. ***Methods:*** Diabetes was induced in spontaneously hypertensive rats (SHRs) and their genetically normotensive control Wistar Kyoto (WKY) rats by streptozotocin injection at 12 weeks of age for 10 days, and the effects of hyperglycemia on renal macrophage infiltration and oxidative stress were evaluated. ***Results:*** Blood pressure was higher in SHR than in WKY groups. Markers of oxidative stress-induced DNA and protein modification, 8-hydroxy-2′-deoxyguanosine (8-OHdG) and nitrotyrosine, respectively, and the antioxidant glutathione levels were found to be similar in WKY-control and WKY-diabetic groups. However, 8-OHdG was significantly elevated ($p = 0.014$), the nitrotyrosine level tended to be elevated ($p = 0.068$) and the glutathione level was significantly reduced ($p = 0.034$) in the SHR-diabetic group compared to the SHR-control group. On the other hand, glomerular and tubulointerstitial macrophage infiltration was significantly higher in both WKY-diabetic and SHR-diabetic groups than the respective control groups. ***Conclusions:*** A short duration of diabetes mellitus induces renal oxidative stress in the presence of hypertension; however, renal macrophage infiltration becomes evident in early diabetes regardless of the presence or absence of hypertension. We conclude that the combination of diabetes and hypertension adversely affects oxidative stress in the kidney, but the combination has no additive effect on renal macrophage infiltration, at least in early diabetes.

Introduction

Diabetes mellitus and hypertension are two major independent risk factors for chronic kidney disease leading to end-stage renal disease [1, 2]. Unfortunately, these two risk factors frequently coexist, and their combination leads to the increased incidence and severity of renal complications [3, 4]. Multiple mechanisms, including inflammation and oxidative stress in the kidney, have been implicated in the pathogenesis of diabetic nephropathy [5].

Hyperglycemia is known to enhance the production of reactive oxygen species from the mitochondrial electron transport chain [6] and from the membrane-associated NADPH oxidase enzyme [7]. Mitochondrial superoxide

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0250–8095/06/0265–0415$23.50/0

Accessible online at:
www.karger.com/ajn

Dr. Jose B. Lopes de Faria, MD, PhD, Renal Pathophysiology Laboratory
Division of Nephrology, Department of Internal Medicine
Faculty of Medical Sciences, State University of Campinas (UNICAMP)
13084-971 Campinas, São Paulo (Brazil)
Tel. +55 19 3788 7499, Fax +55 19 3788 7366, E-Mail jblfaria@fcm.unicamp.br

activates several pathways of hyperglycemic damage including polyol, protein kinase C and advanced glycation end-product pathways [6]. Moreover, the hyperglycemia-induced generation of mitochondrial superoxide can activate pro-inflammatory transcription factor nuclear factor (NF)-κB [6]. Activation of NF-κB induces expression of cell adhesion molecules and chemokines leading to inflammatory cellular infiltration [8]. Therefore, enhanced oxidative stress and increased macrophage infiltration in the kidney are commonly observed both in human diabetes and in animal models of diabetes mellitus [9–17]. However, exaggerated oxidative stress and enhanced inflammatory cellular infiltration in the kidney are also well-known features of systemic hypertension. In fact, hypertension has been shown to induce renal inflammation and oxidative stress; on the other hand, renal inflammation and oxidative stress have also been proposed to be the cause of hypertension [18, 19].

Several studies have shown that anti-inflammatory and antioxidant agents are able to reduce renal functional and structural abnormalities in both diabetes and hypertension [10, 11, 18], indicating that the renal inflammatory and oxidative stress events play a prominent role in the pathogenesis of diabetic and hypertensive nephropathy. However, the exact sequence of events by which inflammation and oxidative stress interact to exacerbate diabetic renal disease with concomitant hypertension is largely unknown. In the present study, we investigated whether renal inflammatory and oxidative stress events are additive from the beginning in diabetic animals with coexisting hypertension. Our findings indicate that the presence of hypertension clearly induces renal oxidative stress without inducing an additional influence on renal macrophage infiltration in the early stage of experimental diabetes.

Subjects and Methods

Animals and Experimental Protocol

The protocol for this study was complied with the guidelines established by the Brazilian College of Animal Experimentation (COBEA) and was approved by the Institutional Ethical Committee. All reagents were purchased from Sigma (St Louis, Mo., USA), unless stated otherwise. Spontaneously hypertensive rats (SHR) and their genetically normotensive control Wistar-Kyoto (WKY) rats, derived from animals supplied by Taconic (Germantown, N.Y., USA) and bred in our animal facility, were used in this study. Rats were housed in a room maintained at around 22°C, exposed to a 12-hour dark/light cycle, and allowed free access to food and tap water. Diabetes was induced in 12-week-old hypertensive male SHR and their normotensive counterpart male WKY rats by a single injection of streptozotocin (STZ, 50 mg/kg) dissolved in sodium citrate buffer (pH 4.5) via the tail vein after an overnight fast. Control groups received only vehicle (citrate buffer). Plasma glucose levels were measured using an enzymatic colorimetric GOD-PAP assay (Merck, Darmstadt, Germany) 72 h after the injection of STZ or citrate buffer. A plasma glucose concentration of >15 mM was considered diabetic for these experiments.

For different types of experiments 27 WKY (14 control and 13 diabetic) and 24 SHR (13 control and 11 diabetic) rats were used in the present study. Five to seven rats from each group were sacrificed 10 days after induction of diabetes by CO_2 gas. The abdomen was opened via a midline incision and the right kidney was removed immediately, decapsulated, weighed and further processed for homogenization of the cortical tissue. The left kidney was similarly removed and cut longitudinally into two halves. Part of the cortical tissue of one half was frozen in liquid nitrogen and preserved at –80°C, and the other half was fixed by immersing in a solution of methacarn (60% methanol, 30% chloroform and 10% glacial acetic acid). One day before sacrifice, urine samples were collected by placing each rat in individual metabolic cages for 24 h. Urine samples were analyzed by single radial immunodiffusion to determine the albumin excretion rate (AER) as described previously [20].

Blood Pressure Determinations

Systolic blood pressure was obtained by tail-cuff plethysmography (3–5 determinations per rat) in unanesthetized rats using an MK III physiograph (Narco Bio-System, Houston, Tex., USA) the day before the induction of diabetes and on the 9th day after induction. Rats were habituated to the procedure before taking the blood pressure readings.

Preparation of Renal Cortical Extract

Renal cortical extract was prepared in tissue homogenization buffer (30 mM Tris-HCl, pH 7.5, 10 mM EGTA, 5 mM EDTA, 1 mM DTT and 250 mM sucrose) supplemented with a cocktail of protease inhibitors as described previously [21].

Immunohistochemistry

To detect macrophage infiltration, methacarn-fixed paraffin-embedded renal tissue sections (4 μm) were dewaxed and rehydrated. After microwave exposure and blocking with nonfat milk, slides were incubated with a 1:50 dilution of monoclonal mouse anti-rat ED1 antibody (Serotec, Oxford, UK) followed by alkaline phosphatase-labeled polymer (Dako EnVision System, DAKO Corporation, Carpinteria, Calif., USA) conjugated with anti-mouse antibody, developed with fast red (Dako EnVision System) and counterstained with hematoxylin. To detect oxidative stress-induced DNA base modification, immunohistochemistry was done for 8-hydroxy-2′-deoxyguanosine (8-OHdG, a DNA base-modified product) in methacarn-fixed paraffin-embedded renal tissue sections. After microwave exposure and blocking of endogenous peroxidase with 3% H_2O_2, slides were incubated with a 1:50 dilution of a mouse monoclonal anti-8-OHdG antibody (N45.1; Japan Institute for the Control of Aging, Japan), and subsequently with a 1:200 dilution of a biotinylated secondary anti-mouse IgG antibody (Vector, Burlingame, Calif., USA). After incubation with avidin-biotin complex reagent (Dako, Glostrup, Denmark), slides were developed in diaminobenzidine tetrahydrochloride and counterstained with hematoxylin. For negative

in hypertensive SHR rats during early diabetes. Our finding of a nonsignificant elevation in antioxidant GSH in normotensive rats, but a significant reduction in GSH in hypertensive rats after the induction of diabetes, supports this explanation. However, to obtain a definitive explanation, a complete evaluation of the pro-oxidant/antioxidant system might be necessary.

In the present study, we also studied albuminuria and observed that urinary AER was similarly elevated in both normotensive and hypertensive rats after 10 days of diabetes induction. This finding suggests that the hypertension or hypertension-induced oxidative stress does not affect urinary albumin excretion in the very early stage of experimental diabetes. However, in our previous communication we showed that long-term diabetes along with hypertension augmented albuminuria and renal fibronectin expression, which was completely prevented by blood pressure normalization, independent of the class of antihypertensive agents used [20]. Taken together, our data support the concept that the normalization of blood pressure may improve diabetic nephropathy through a reduction of oxidative stress.

Over the last several years, some published studies have evaluated renal oxidative stress and some other studies have focused on renal inflammation in the early stage of experimental diabetes. However, the novelty of the present study is the simultaneous evaluation of both oxidative stress and inflammation in the kidney, in a model that allowed us to identify distinct effects of hypertension on renal oxidative stress and inflammation in early diabetes. In fact, we are not aware of any other study comparing the effects of diabetes and the effects of a combination of diabetes and hypertension on both renal oxidative stress and inflammation in early diabetes using an appropriate normotensive and hypertensive animal model. While this paper was being prepared, Hartner et al. [26] published data on the effects of diabetes and hypertension on macrophage infiltration and matrix expansion in the kidney. Similar to our present finding, they demonstrated that the combination of diabetes and hypertension does not exert an additive effect on renal inflammation at 5 weeks of experimental diabetes [26]. However, Hartner et al. did not evaluate renal oxidative stress, and they used the mRen-2 transgenic rat as a model of hypertension in which renal macrophage infiltration was markedly elevated compared to the normotensive control Sprague-Dawley rat. Therefore, the control groups were not comparable in the study by Hartner et al., which might have affected their finding. However, a similar finding obtained in the present study confirms the finding of Hartner et al. [26].

Diabetes-induced oxidative stress may lead to inflammatory cellular infiltration through NF-κB-mediated pro-inflammatory gene expression [6, 8]. However, several other pathways exist for the renal inflammation in diabetes, and the precise mechanisms that induce leukocyte infiltration in the kidney in early diabetes have not yet been fully identified [24]. But the contribution of renal inflammation and oxidative stress to diabetic kidney disease has repeatedly been demonstrated in experimental diabetes. Hyperglycemia-induced renal oxidative stress and macrophage infiltration have been found to be associated with albuminuria, mesangial matrix expansion and expression of fibronectin and collagen I in experimental diabetes, and these functional and structural renal abnormalities have largely been eliminated by anti-inflammatory or antioxidant agents [10, 11, 16]. Therefore, the present trend is to consider diabetic nephropathy as an inflammatory disease regulated by redox balance [24].

In conclusion, we provide experimental evidence that the combination of diabetes and hypertension adversely affects oxidative stress in the kidney, but the combination has no additive effect on renal macrophage infiltration, at least in early diabetes. Our data support the notion that oxidative stress contributes to the acceleration of kidney damage caused by diabetes mellitus in combination with hypertension. The renoprotective effect of early correction of hypertension in diabetes may therefore come through modulation of redox status.

Acknowledgments

We thank Dr. Jacqueline M. Lopes de Faria, Dr. Roberto Amazonas and Dr. Roger F. Castilho for helpful suggestions. We are grateful to Sergio Magalhaes for technical assistance. We also thank Ms Kamila C. Silva, Ms Camila C. Pinto, Ms Vanessa C. Pansiera, Ms Daniela R. Melo, Mr. Leonardo D. Lobo and Mr. Bruno S. Pessoa for assistance in laboratory procedures.

Part of this work was presented at the 41st Annual Meeting of the European Association for the Study of Diabetes, Athens, Greece, 2005.

This work was supported by grants from Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq). Dr. Subrata Biswas was on receipt of a scholarship from CAPES.

References

1 US Renal Data System: USRDS 2001 Annual Data Report. Bethesda, National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, 2001.
2 Klag MJ, Whelton PK, Randall BL, Neaton JD, Brancati FL, Ford CE: Blood pressure and end-stage renal disease in men. N Engl J Med 1996;334:13–18.
3 Hypertension in Diabetic Study (HDS): I. Prevalence of hypertension in newly presenting type 2 diabetic patients and the association with risk factors for cardiovascular and diabetic complications. J Hypertens 1993;11:309–317.
4 Cooper ME, Allen TJ, O'Brien RC, Macmillan PA, Clarke B, Jerums G: Effects of genetic hypertension on diabetic nephropathy in the rat – functional and structural characteristics. J Hypertens 1988;6:1009–1016.
5 Wolf G: New insights into the pathophysiology of diabetic nephropathy: from haemodynamics to molecular pathology. Eur J Clin Invest 2004;34:785–796.
6 Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y: Normalizing mitochondrial superoxide production blocks three pathways of hyperglycemic damage. Nature 2000;404:787–790.
7 Guzik TJ, Mussa S, Gastaldi D, Sadowski J, Ratnatunga C, Pillai R, Channon KM: Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase. Circulation 2002;105:1656–1662.
8 Barnes PJ, Karin M: Mechanisms of disease: nuclear factor-κB – a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 1997;336:1066–1071.
9 Furuta T, Saito T, Ootaka T: The role of macrophages in diabetic glomerulosclerosis. Am J Kidney Dis 1993;21:480–485.
10 Asaba K, Tojo A, Onozato ML, Goto A, Quinn MT, Fujita T: Effects of NADPH oxidase inhibitor in diabetic nephropathy. Kidney Int 2005;67:1890–1898.
11 Utimura R, Fujihara CK, Mattar AL, Malheiros DM, De Lourdes Noronha I, Zatz R: Mycophenolate mofetil prevents the development of glomerular injury in experimental diabetes. Kidney Int 2003;63:209–216.
12 Obrosova IG, Fathallah L, Liu E, Nourooz-Zadeh J: Early oxidative stress in the diabetic kidney: effect of DL-alpha-lipoic acid. Free Radic Biol Med 2003;34:186–195.
13 Ishii N, Patel KP, Lane PH, Taylor T, Bian K, Murad F: Nitric oxide synthesis and oxidative stress in the renal cortex of rats with diabetes mellitus. J Am Soc Nephrol 2001;12:1630–1639.
14 Kakkar R, Mantha SV, Radhi J, Prasad K, Kalra J: Antioxidant defense system in diabetic kidney: a time course study. Life Sci 1997;60:667–679.
15 Young BA, Johnson RJ, Alpers CE, Eng E, Gordon K, Floege J: Cellular events in the evolution of experimental diabetic nephropathy. Kidney Int 1995;47:935–944.
16 Sassy-Prigent C, Heudes D, Mandet C, Belair M, Michel O, Perdereau B: Early glomerular macrophage recruitment in streptozotocin-induced diabetic rats. Diabetes 2000;49:466–475.
17 Kato S, Luyckx VA, Ots M, Lee KW, Ziai F, Troy JL: Renin-angiotensin blockade lowers MCP-1 expression in diabetic rats. Kidney Int 1999;56:1037–1048.
18 Rodriguez-Iturbe B, Quiroz Y, Nava M, Bonet L, Chavez M, Herrera-Acosta J: Reduction of renal immune cell infiltration results in blood pressure control in genetically hypertensive rats. Am J Physiol Renal Physiol 2002;282:F191–F201.
19 Vaziri ND: Roles of oxidative stress and antioxidant therapy in chronic kidney disease and hypertension. Curr Opin Nephrol Hypertens 2004;13:93–99.
20 Pavan MV, Ghini B, Castro M, Lopes De Faria JB: Prevention of hypertension attenuates albuminuria and renal expression of fibronectin in diabetic spontaneously hypertensive rats. Am J Nephrol 2003;23:422–428.
21 Silveira LA, Bacchi CE, Pinto GA, Lopes de Faria JB: The genetics of hypertension modifies the renal cell replication response induced by experimental diabetes. Diabetes 2002;51:1529–1534.
22 Beutler E, Duron O, Mikus B: Improved method for the determination of blood glutathione. J Lab Clin Med 1963;16:882–888.
23 Halliwell B: What nitrates tyrosine? Is nitrotyrosine specific as a biomarker of peroxynitrite formation in vivo? FEBS Lett 1997;411:157–160.
24 Galkina E, Ley K: Leukocyte recruitment and vascular injury in diabetic nephropathy. J Am Soc Nephrol 2006;17:368–377.
25 Sies H: Oxidative Stress: Oxidants and Antioxidants. New York, Academic Press, 1991.
26 Hartner A, Veelken R, Wittmann M, Cordasic N, Hilgers KF: Effects of diabetes and hypertension on macrophage infiltration and matrix expansion in the rat kidney. BMC Nephrol 2005;6:6.

CHAPTER 4

Paper III

Prepubertal induction of experimental diabetes protects against early renal macrophage infiltration

Biswas SK, Lopes de Faria JB. Prepubertal induction of experimental diabetes protects against early renal macrophage infiltration. Pediatric Nephrology; in press 2007

Abstract

The present study was undertaken to identify whether the age of induction of experimental diabetes modifies macrophage infiltration in the kidney. Renal macrophage infiltration was studied 10 days after the induction of experimental diabetes in 4-week-old prepubertal and 12-week-old adult male rats of normotensive (Wistar-Kyoto rats, WKY) and hypertensive (spontaneously hypertensive rats, SHR) background. Renal macrophage infiltration was evaluated by immunohistochemistry for ED1. Plasma glucose levels were similar in all diabetic groups. Adult SHR rats were hypertensive and induction of diabetes did not alter BP in any group. Induction of diabetes in prepubertal rats did not induce macrophage infiltration in the kidney. But in adult rats, tubulointerstitial macrophage infiltration was increased in both WKY (22.86±3.93 vs. 7.86±2.16 per high power field, $p<0.001$) and SHR (26.41±5.91 vs. 11.48±1.23, $p<0.001$) groups after induction of diabetes. Glomerular macrophage infiltration was also increased after induction of diabetes in adult WKY group (1.83±0.50 vs. 1.16±0.26 per glomerular cross section, $p=0.029$), which was not significant in adult SHR (2.52±0.34 vs. 1.95±0.35). We conclude that the prepubertal induction of diabetes apparently protects against early renal macrophage infiltration while the induction of diabetes in adults induces exaggerated macrophage infiltration in the kidney.

Keywords: Diabetic nephropathy, Macrophage, Prepubertal diabetes, Hypertension, SHR, ESRD

Introduction

Diabetic nephropathy is the leading cause of end-stage renal disease (ESRD) all over the world. Glycemic control, duration of diabetes and the presence of hypertension contribute to the development of diabetic nephropathy. Interestingly, some studies have identified that the age at onset also modifies the development of nephropathy in patients with childhood-onset type 1 diabetes [1, 2]. Recently, two large nationwide population-based studies demonstrated that a prepubertal, compared to pubertal or post-pubertal, onset of diabetes significantly reduces the risk of developing diabetic nephropathy and ESRD [3, 4]. So far, the mechanism behind this effect of age at onset of diabetes on diabetic kidney disease is not clear.

Inflammatory process is known to contribute to the pathogenesis/progression of nephropathy. Renal interstitial inflammation, particularly macrophage infiltration, has shown to be well correlated with functional and structural renal damage in almost all types of chronic kidney disease, including diabetic nephropathy [5]. Moreover, both animal and human studies suggest a pathogenic role of renal macrophage infiltration in diabetic nephropathy [6, 7]. However, it is not known whether the age at onset of type 1 diabetes, or the age at induction of experimental diabetes, modifies macrophage infiltration in the kidney. Therefore, we investigated renal macrophage infiltration after induction of experimental diabetes in prepubertal (weanling) and adult rats of normotensive and hypertensive background.

Methods

Animal handling and experiments performed in the present study complied with the guidelines established by the Brazilian College of Animal Experimentation (COBEA). As hypertension and diabetes frequently coexist in human, we tested our hypothesis in clinically relevant models of diabetes and hypertension, created by inducing diabetes in spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats. Prepubertal (4-week-old) and adult (12-week-old) male WKY and SHR rats were rendered

diabetic by tail-vain injection of streptozotocin (STZ, 50mg/kg). Age-matched control rats received the vehicle citrate buffer. Rats were housed in a room maintained at around 22^0C, exposed to a 12-hour light/dark cycle, and allowed free access to food and tap water. Plasma glucose levels were measured using a glucose oxidase-based colorimetric method 72 hours after the injection of STZ or citrate buffer. Plasma glucose concentration of >15 mM was considered diabetic for these experiments. Systolic blood pressure was measured by tail-cuff plethysmography using an MK III physiograph (Narco Bio-System, Houston, TX, USA) before the day of induction of diabetes and on the day before sacrifice. Rats were sacrificed by CO_2 gas after 10 days of induction of diabetes. Kidneys were removed, decapsulated, cut longitudinally into 2 halves and fixed immersing in a solution of methacarn (60% methanol, 30% chloroform and 10% glacial acetic acid). Subsequently, methacarn-fixed kidney tissues were embedded in paraffin and 4 μm sections were stained immunohistochemically using ED1 antibody to detect macrophage infiltration as described previously [8]. Briefly, the slides were incubated with a 1:50 dilution of monoclonal mouse anti-rat ED1 antibody (Serotec, Oxford, UK) followed by alkaline phosphatase labelled polymer (Dako EnVision system, DAKO corporation, Carpinteria, CA, USA) conjugated with anti-mouse antibody. The slides were developed with fast red (Dako EnVision system, DAKO) and counterstained with hematoxylin. ED1 positive cells were counted in 50 sequential cortical glomeruli and 20 high power microscopic fields (400x) for the evaluation of glomerular and tubulointerstitial macrophage infiltration, respectively. Three to 5 sections were stained and evaluated from each rat.

Statistical analysis

Effects of diabetes on different parameters were statistically compared among different groups of same strain with ANOVA followed by Bonferroni corrected *t*-test. The results were expressed as means±SD, and the statistical significance was set at $p<0.05$.

Results

Body weight was decreased in all diabetic groups compared to age-matched controls, but the decrement was not significant in prepubertal WKY groups (Table 1). WKY rats were

normotensive although the systolic BP was higher (p=0.012) in adult controls than the prepubertal controls. Prepubertal SHR rats were normotensive and the adult SHR rats were clearly hypertensive. Experimental diabetes of 10 days duration did not alter BP in any group (Table 1). Plasma glucose levels were significantly elevated (p<0.001) in all diabetic groups compared with control groups, and the glucose levels did not show any difference among the diabetic groups (Table 1). Induction of diabetes in prepubertal rats of WKY and SHR strains for the duration of 10 days did not induce macrophage infiltration in the kidney, neither in the glomerulus nor in the tubulointerstitial area. Interestingly, induction of diabetes for the same duration in adult rats increased glomerular and tubulointerstitial macrophage infiltration in both WKY and SHR rats, although the glomerular macrophage infiltration failed to reach the level of significance in SHR (Table 1, Figure 1). Since the duration of diabetes and the levels of hyperglycemia were similar between prepubertal and adult rats, age at induction of diabetes and/or related events are likely to be involved in the mechanism of renal macrophage infiltration, at least in the rat strains that we studied here. We repeated parts of the experiments that were done earlier for a different study [8], and a similar finding was obtained.

Discussion

Macrophage infiltration in the kidney has been demonstrated in different stages of diabetic nephropathy and anti-inflammatory agents have been found beneficial in preventing albuminuria, glomerular injury and mesangial matrix deposition [6, 7, 9]. Inflammatory mechanisms therefore appear to play a crucial role in the pathogenesis of diabetic nephropathy. In the present study, for the first time, we observed a distinct effect of age of induction of diabetes on renal macrophage infiltration in 2 different rat models of normotensive and hypertensive background. Induction of diabetes in prepubertal weanling rats of 4 weeks of age appears to be relatively protected against renal macrophage infiltration, as the induction of diabetes for 10 days elevated macrophage infiltration in adult rats but not in prepubertal rats. We consider that the elevated renal macrophage infiltration in adult diabetic rats was not mediated by the direct toxic effect of STZ, as the

prepubertal rats did not show this feature in spite of induction of diabetes with the same dose of STZ.

In the present study, adult control rats showed a tendency to have lower number of resident macrophage in the kidney compared to prepubertal controls. This tendency was non-significant, but stronger in SHR than WKY rats. Due to this age-related variation in the number of resident macrophage we performed age-matched comparison considering that the number of macrophage in control rat is normal or physiological for that specific age. The effect of age of induction of diabetes on renal macrophage infiltration was found more or less similar in WKY and SHR strains, although the finding in SHR may partly be influenced by the age-related variation in the number of resident macrophage in control rats.

In normotensive WKY rats, the systolic BP was slightly elevated in adult rats compared to prepubertal rats and the elevation was significant between control groups. This small but significant rise in blood pressure of adult WKY rats may participate in exaggerated macrophage infiltration in the kidney after induction of diabetes. However, it seems unlikely, as the macrophage infiltration did not increase in prepubertal SHR after induction of diabetes in spite of similar level of BP between prepubertal SHR and adult WKY. Moreover, high BP itself did not induce renal macrophage infiltration, which is evident by comparing macrophage infiltration between prepubertal and adult control groups of SHR.

The notion that the prepubertal duration of diabetes contributes less than the postpubertal duration to the risk of diabetic complications is controversial. However, two large clinical studies recently revealed a lower risk of developing diabetic kidney disease in patients with prepubertal (0-9 years) onset of type 1 diabetes compared to those with pubertal (10-14 years) onset [3, 4]. Moreover, among the patients with prepubertal onset of diabetes, the risk of nephropathy was found considerably lower in those patients whose diabetes occurred before age 5 years [3, 4]. Although the reasons are not known, puberty-associated complex hormonal, metabolic and physiological changes may be involved in pubertal or postpubertal susceptibility to diabetic nephropathy [10]. Several studies have investigated

the mechanism behind the effect of age at onset of type 1 diabetes on the susceptibility to nephropathy. Lane et al. previously demonstrated an elevated renal expression of transforming growth factor-β1 (TGF-β1) after induction of experimental diabetes in postpubertal Sprague-Dawley rats but not in prepubertal rats [11]. As the TGF-β1 is the key factor in the pathogenesis of diabetic nephropathy [12], the finding of Lane et al. [11] is potentially important in explaining the effect of age at onset of type 1 diabetes on the susceptibility to nephropathy. However, it is not clear how the age at induction of diabetes regulates renal TGF-β1 expression, or macrophage infiltration in the kidney as found in the present study.

We conclude that the age of induction of experimental diabetes influences macrophage infiltration in the kidney. Prepubertal induction of diabetes apparently protects against early renal macrophage infiltration while the induction of diabetes in adults induces exaggerated macrophage infiltration in the kidney. This experimental evidence of animal models may partly explain the mechanism of relative lower risk of diabetic nephropathy in prepubertal onset of type 1 diabetes in human.

Acknowledgments

We thank Dr. Jacqueline M. Lopes de Faria and Dr. Roberto Amazonas for helpful suggestions. We are grateful to Sergio Magalhaes for technical assistance. We also thank Ms Denise S. Souza, Ms Kamila C. Silva, Ms Camila C. Pinto, Ms Vanessa C. Pansiera, Ms Daniela R. Melo, Mr. Leonardo D. Lobo and Mr. Bruno S. Pessoa for assistance in laboratory procedures. This work was supported by grants from Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq). Dr. Subrata Biswas was on receipt of a scholarship from CAPES.

References

1. Krolewski AS, Warram JH, Christlieb AR, Busick EJ, Kahn CR (1985) The changing natural history of nephropathy in type I diabetes. Am J Med 78:785-794
2. Donaghue KC, Fairchild JM, Craig ME, Chan AK, Hing S, Cutler LR, Howard NJ, Silink M (2003) Do all prepubertal years of diabetes duration contribute equally to diabetes complications? Diabetes Care 26:1224-1229
3. Finne P, Reunanen A, Stenman S, Groop PH, Gronhagen-Riska C (2005) Incidence of end-stage renal disease in patients with type 1 diabetes. JAMA 294:1782-1787
4. Svensson M, Nystrom L, Schon S, Dahlquist G (2006) Age at onset of childhood-onset type 1 diabetes and the development of end-stage renal disease: a nationwide population-based study. Diabetes Care 29:538-542
5. Eardley KS, Zehnder D, Quinkler M, Lepenies J, Bates RL, Savage CO, Howie AJ, Adu D, Cockwell P (2006) The relationship between albuminuria, MCP-1/CCL2, and interstitial macrophages in chronic kidney disease. Kidney Int 69:1189-1197
6. Chow F, Ozols E, Nikolic-Paterson DJ, Atkins RC, Tesch GH (2004) Macrophages in mouse type 2 diabetic nephropathy: correlation with diabetic state and progressive renal injury. Kidney Int 65:116-128
7. Nguyen D, Ping F, Mu W, Hill P, Atkins RC, Chadban SJ (2006) Macrophage accumulation in human progressive diabetic nephropathy. Nephrology 11:226-231
8. Biswas SK, Loped de Faria JB (2006) Hypertension induces oxidative stress but not macrophage infiltration in the kidney in the early stage of experimental diabetes mellitus. Am J Nephrol 26:415-422

9. Tone A, Shikata K, Sasaki M, Ohga S, Yozai K, Nishishita S, Usui H, Nagase R, Ogawa D, Okada S, Shikata Y, Wada J, Makino H (2005) Erythromycin ameliorates renal injury via anti-inflammatory effects in experimental diabetic rats. Diabetologia 48:2402-2411

10. Lane PH (2002) Diabetic kidney disease: impact of puberty. Am J Physiol Renal Physiol 283:589-600

11. Lane PH, Snelling DM, Hollman A, Langer WJ (2001) Puberty permits increased expression of renal transforming growth factor-β1 in experimental diabetes. Pediatr Nephrol 16:1033-1039

12. Ziyadeh FN, Hoffman BB, Han DC, Iglesias-De La Cruz MC, Hong SW, Isono M, Chen S, McGowan TA, Sharma K (2000) Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal antitransforming growth factor-β antibody in db/db diabetic mice. Proc Natl Acad Sci USA 97:8015-8020

Table

Table 1. Physiological parameters and macrophage infiltration in the kidney of control and diabetic rats

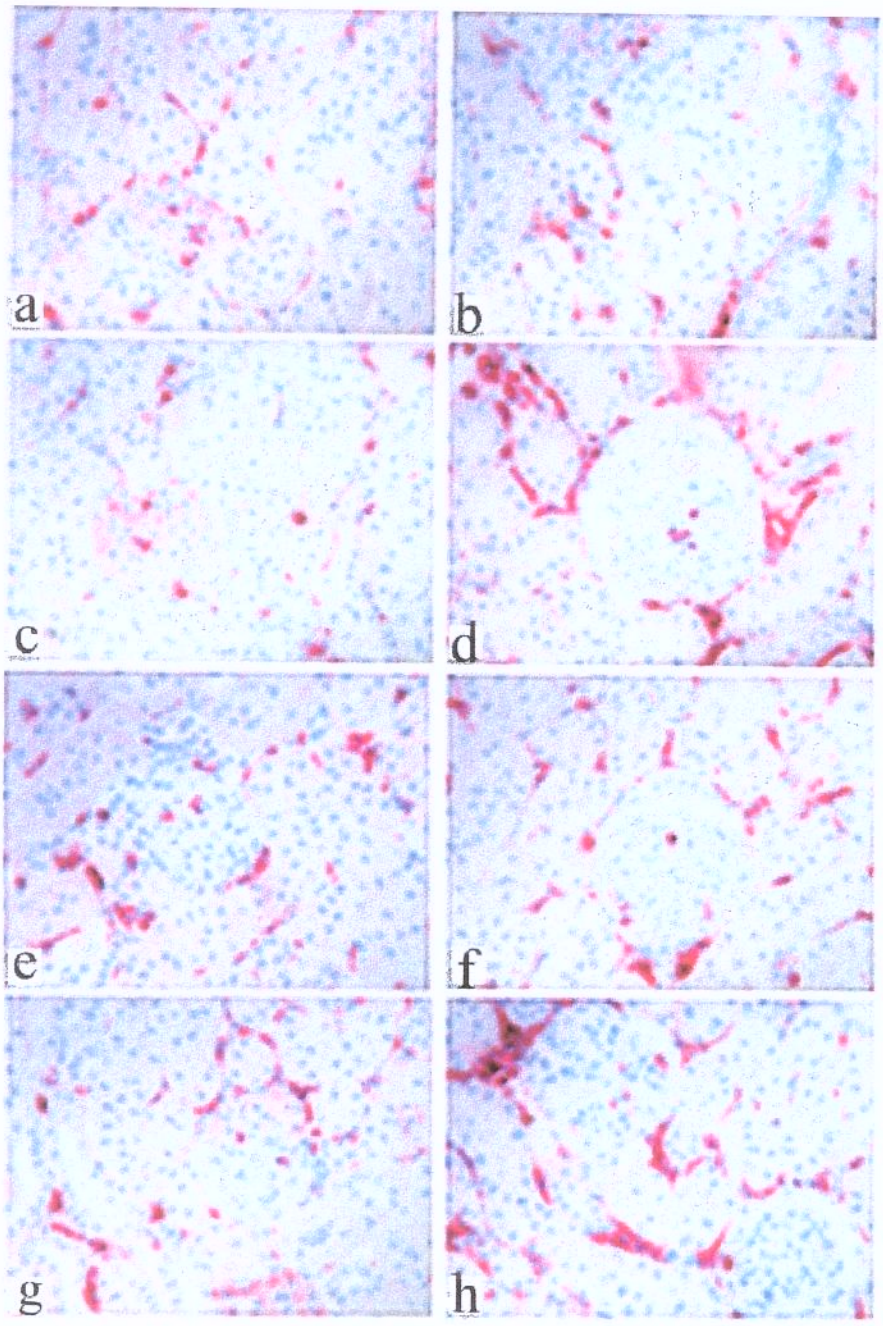
	Prepubertal		Adult	
	WC (n=5)	WD (n=5)	WC (n=7)	WD (n=5)
Body wt (g)	188±9#	148±22§	420±38	321±32*
Systolic BP (mmHg)	108±6#	110±8	124±6	119±8
Plasma glucose (mM/L)	9.1±0.5	27.0±1.1*	9.2±0.4	25.9±0.9*
Macrophage/GCS	1.18±0.27	1.26±0.33	1.16±0.26	1.83±0.50*
Macrophage/HPF	11.92±1.05	12.54±2.60§	7.86±2.16	22.86±3.93*
	Prepubertal		Adult	
	SC (n=5)	SD (n=7)	SC (n=7)	SD (n=6)
Body wt (g)	112±13#	89±10*§	281±16	192±17*
Systolic BP (mmHg)	123±6#	126±7§	180±13	175±9
Plasma glucose (mM/L)	7.6±1.8	26.6±2.6*	8.1±1.1	26.8±1.3*
Macrophage/GCS	2.42±0.62	2.72±0.78	1.95±0.35	2.52±0.34
Macrophage/HPF	17.35±2.60	21.24±5.23	11.48±1.23	26.41±5.91*

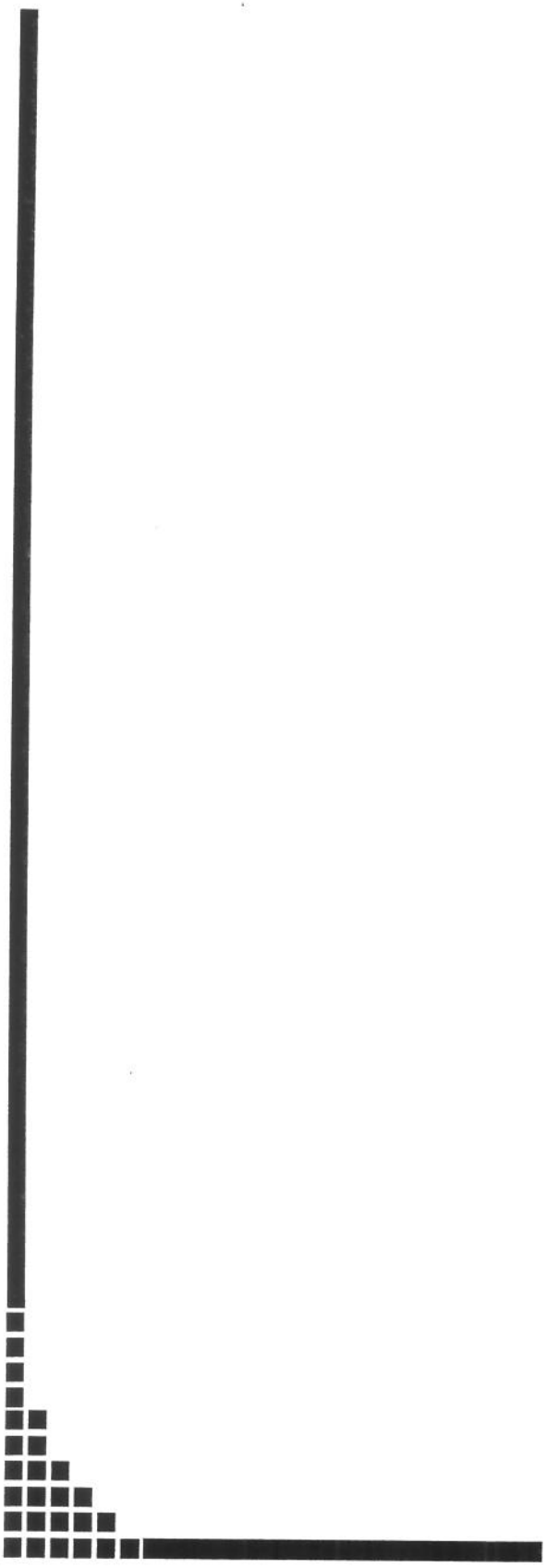
Values are expressed in means±SD. *p<0.03 vs. age-matched control; #p<0.02 vs. adult control; §p<0.001 vs. adult diabetic. WC = WKY control; WD = WKY diabetic; SC = SHR control; SD = SHR diabetic; GCS = glomerular cross section; HPF = high power field.

Legends to figures

Figure 1: Macrophage infiltration in the kidney as detected by ED1 immunohistochemistry. ED1 positive cells are showing bright red granular staining predominantly in the cytoplasm surrounding or overlapping the nucleus. Induction of diabetes did not induce macrophage infiltration in prepubertal rats of WKY (**a**, control; **b**, diabetic) and SHR (**e**, control; **f**, diabetic) strains. Exaggerated renal macrophage infiltration observed after induction of diabetes in adult rats of WKY (**c**, control; **d**, diabetes) and SHR (**g**, control; **h**, diabetes) strains. Original magnification x400, counterstained with hematoxylin. Scale bar=50 μm.

Figure 1





CHAPTER 5

Discussão

Uma associação estreita entre HAS, inflamação renal e estresse oxidativo tem sido repetidamente demonstrada em diferentes modelos animais de HAS. Entretanto, é difícil demonstrar a exata relação entre esses eventos uma vez que os mesmos são interdependentes e interrelacionados. Além disso, no DM, como a inflamação renal e o estresse oxidativo poderiam influenciar a presença de variáveis com relevância clínica, tais como HAS e idade, é ainda um ponto crítico. Na presente tese, algumas relações importantes entre inflamação, estresse oxidativo, HAS e DM forma identificadas.

Na presente tese o estresse oxidativo renal foi identificado como uma alteração primária em ratos SHR jovens, sendo possível que o mesmo tenha papel no desenvolvimento da inflamação e da HAS nos ratos SHR adultos. Entretanto, a introdução de dietas, iniciadas ao nascimento ou mesmo no período pré-natal, que visavam aumentar a proteção anti-oxidante demonstraram correção apenas parcial da HAS e da inflamação (Rodriguez-Iturbe et al., 2003; Zhan et al., 2004; Noyan-Ashraf et al., 2006). Os agentes anti-oxidantes utilizados nestes estudos parecem inadequados ou inespecíficos para combaterem as espécies reativas de oxigênio presentes no SHR. Portanto, o bloqueio específico das espécies reativas de oxigênio, ao invés da neutralização com anti-oxidantes, poderia ser uma alternativa mais eficaz. Para isso a identificação do mecanismo primário do estresse oxidativo no SHR é um passo essencial. As observações incluídas na presente tese que sugerem que o sistema NADPH oxidase possa ser responsável pelo aumento do estresse oxidativo no SHR precisam ser confirmadas, assim como outras vias pró-oxidantes precisam ser investigadas.

O conhecimento sobre a influência da HAS ou da idade no estresse oxidativo ou infiltração de macrófagos no DM pode ajudar no melhor entendimento dos mecanismos fisiopatológicos que participam do desenvolvimento da nefropatia diabética. Os estudos incluídos na presente tese enfatizam a importância do controle precoce da pressão arterial no DM uma vez que a presença da HAS exacerba o estresse oxidativo renal. Esta tese também sugere um possível mecanismo para a proteção relativa à nefropatia diabética no

DM com início pré-púbere. Entretanto, estudos adicionais são necessários para definirem os mecanismos básicos das observações relatadas na presente tese.

1. SUMMARY AND CONCLUSION

A tight association among HTN, renal inflammation and oxidative stress has been repeatedly demonstrated in different animal models of experimental HTN. However, it is difficult to identify an exact relationship between them, as the events are interdependent and interrelated. Furthermore, in DM, how renal inflammation or oxidative stress could be influenced by the presence of clinically relevant covariates, like HTN or age, is still a critical issue. In the present thesis, some important relationships among renal inflammation, oxidative stress, HTN and DM have been identified. Major conclusions that can be drawn from the present thesis are:

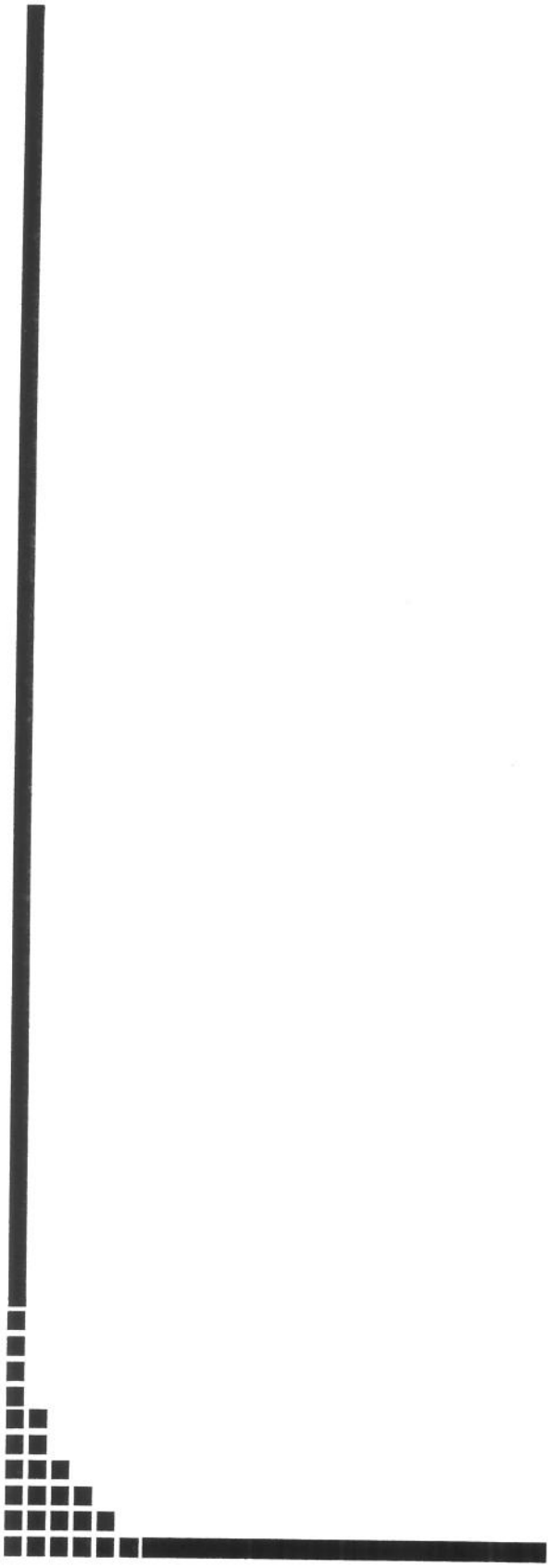
1. Oxidative stress appears as a primary abnormality in the kidney before development of renal inflammation or systemic HTN in SHR.
2. The presence of HTN adversely affects renal oxidative stress in the early stage of experimental diabetes.
3. Renal macrophage infiltration increases markedly in the early stage of experimental diabetes, regardless of the presence or absence of HTN but depending on the age of induction of diabetes.
4. Prepubertal or young age of induction of experimental diabetes appears to be relatively protected against early renal macrophage infiltration compared with adult age of induction of diabetes.

2. PERSPECTIVE

Renal oxidative stress has been identified, in the present thesis, as an important primary abnormality in prehypertensive young SHR that may have some role in the development of renal inflammation and systemic HTN in adult SHR. However, dietary approaches to enhance antioxidant protection in SHR, starting from birth or even from prenatal stage, showed only a partial correction of HTN or renal inflammation (Rodriguez-Iturbe et al., 2003; Zhan et al., 2004; Noyan-Ashraf et al., 2006). The antioxidant agents used in these studies appear to be inadequate or non-specific to combat against the prevailing reactive species in SHR. Therefore, blocking the production of reactive species by specific inhibitors, instead of neutralization by antioxidants, could be a reasonable alternative approach. Identification of the primary mechanism of oxidative stress in SHR is an essential next step in that direction. The data included in the present thesis suggest that the NADPH oxidase system may be responsible for the exaggerated renal oxidative stress in SHR, which need to be confirmed as well as other pathways of pro-oxidant generation should be examined.

The knowledge of the specific influence of HTN or age on renal oxidative stress or macrophage infiltration in DM could help better understand the pathophysiological mechanism of development of diabetic nephropathy. The studies included in the present thesis emphasize the importance of early control of BP in diabetes as the presence of HTN exaggerates early renal oxidative stress. This thesis also suggests a possible mechanism of relative protection against diabetic nephropathy in prepubertal onset of type 1 diabetes in humans. However, further studies are needed to identify the underlying mechanistic basis of the findings obtained in the present thesis. The author is actively engaged in detail investigation including the measurement of activities and expressions of suspected sources of pro- and antioxidant systems in the kidney in diabetes. Furthermore, studies to identify the differences in renal pathology between prepubertal and postpubertal induction of diabetes are under active consideration.

The studies included in the present thesis are performed in animal models, as these direct experiments cannot be done in humans. It is expected that the knowledge obtained in animals will provide new directions for further studies in human. In fact, renal inflammation or oxidative stress could be evaluated in humans by some indirect markers in urine and serum. When such studies will be fruitful to bring about human well-being, only then this work will be considered successful and the sacrifice of the animals will be valid in true sense.



CHAPTER 6

1. REFERENCES

American Diabetic Association: Nephropathy in diabetes (position statement). Diabetes Care 2003; 27: S79-S83.

Anderson MT, Staal FJ, Gitler C, Herzenberg LA, Herzenberg LA. Separation of oxidant-initiated and redox-regulated steps in the NF-κB signal transduction pathway. Proc Natl Acad Sci USA 1994; 91: 11527-11531.

Asaba K, Tojo A, Onozato ML, Goto A, Quinn MT, Fujita T, et al. Effects of NADPH oxidase inhibitor in diabetic nephropathy. Kidney Int 2005; 67: 1890-1898.

Babior BM. NADPH oxidase: an update. Blood 1999; 93: 1464-1476.

Barnes PJ, Karin M. mechanisms of disease: nuclear factor-κB – a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 1997; 336: 1066-1071.

Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol 1996; 271: C1424-C1437.

Bierhaus A, Schiekofer S, Schwaninger M, Andrassy M, Humpert PM, Chen J, et al. Diabetes-associated sustained activation of the transcription factor nuclear factor-kappaB. Diabetes 2001; 50: 2792-2808.

Bierhaus A, Humpert PM, Morcos M, Wendt T, Chavakis T, Arnold B, et al. Understanding RAGE, the receptor for advanced glycation end products. J Mol Med 2005; 83: 876-886.

Biswas SK, Lopes de Faria JB. Does peroxynitrite sustain nuclear factor-κB? Cardiovasc Res 2005; 67: 745-746.

Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature 2001; 414: 813-820.

Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. Diabetes 2005; 54: 1615-1625.

Burlando G, Sanchez RA, Ramos FH, Mogensen CE and Zanchetti A, on behalf of the Latin American Experts Group. Latin American Consensus on diabetes mellitus and hypertension. J Hypertens 2004; 22: 2229-2241.

Canani LH, Gerchman F, Gross JL. Familial clustering of diabetic nephropathy in Brazilian type 2 diabetic patients. Diabetes 1999; 48: 909-913.

Carretero OA, Oparil S. Essential hypertension- part I: definition and etiology. Circulation 2000; 101: 329-335.

Chen F, Castranova V, Shi X, Demers LM. New insights into the role of nuclear factor-κB, a ubiquitous transcription factor in the initiation of diseases. Clin Chem 1999; 45: 7-17.

Chabrashvili T, Tojo A, Onozato ML, Kitiyakara C, Quinn MT, Fujita T, et al. Expression and cellular localization of classic NADPH oxidase subunits in the spontaneously hypertensive rat kidney. Hypertension 2002; 39: 269-274.

Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL, Jr, et al. The seventh report of the joint national committee on prevention, detection, evaluation, and treatment of high blood pressure: The JNC 7 Report. JAMA 2003; 289: 2560-2572.

Chow FY, Nikolic-Paterson DJ, Ozols E, Atkins RC, Rollin BJ, Tesch GH. Monocyte chemoattractant protein-1 promotes the development of diabetic renal injury in streptozotocin-treated mice. Kidney Int 2006; 69: 73-80.

Chow FY, Nikolic-Paterson DJ, Ozols E, Atkins RC, Tesch GH. Intercellular adhesion molecule-1 deficiency is protective against nephropathy in type 2 diabetic db/db mice. J Am Soc Nephrol 2005; 16: 1711-1722.

Chow F, Ozols E, Nikolic-Paterson DJ, Atkins RC, Tesch GH. Macrophages in mouse type 2 diabetic nephropathy: correlation with diabetic state and progressive renal injury. Kidney Int 2004; 65: 116-128.

Collins T. Acute and chronic inflammation. In: *Robbins Pathologic Basis of Disease*, edited by Cotran RS, Kumar V, Collins T. Philadelphia, W.B. Saunders Company: Philadelphia. 1999: 50-88.

Cooper ME, Allen TJ, O'Brien RC, Macmillan PA, Clarke B, Jerums G, et al. Effects of genetic hypertension on diabetic nephropathy in the rat—functional and structural characteristics. J Hypertens 1988; 6: 1009-1016.

Cooper ME. Pathogenesis, prevention, and treatment of diabetic nephropathy. Lancet 1998; 352: 213-219.

Cuzzocrea S, Mazzon E, Dugo L, Di Paola R, Caputi AP, Salvemini D. Superoxide: a key player in hypertension. FASEB J 2004; 18: 94-101.

DeRubertis FR, Craven PA, Melhem MF, Salah EM. Attenuation of renal injury in *db/db* mice overexpressing superoxide dismutase: evidence for reduced superoxide-nitric oxide interaction. Diabetes 2004; 53: 762-768.

Evans MD, Dizdaroglu M, Cooke MS. Oxidative DNA damage and disease: induction, repair and significance. Mutat Res 2004; 567: 1-61.

Finne P, Reunanen A, Stenman S, Groop PH, Gronhagen-Riska C. Incidence of end-stage renal disease in patients with type 1 diabetes. JAMA 2005; 294: 1782-1787.

Flohe L, Brigelius-Flohe R, Saliou C, Traber MG, Packer L. Redox regulation of NF-κB activation. Free Radic Biol Med 1997; 22: 1115-1126.

Furuta T, Saito T, Ootaka T, Soma J, Obara K, Abe K, et al. The role of macrophages in diabetic glomerulosclerosis. Am J Kidney Dis 1993; 21: 480-485.

Giunti S, Barit D, Cooper ME. Mechanisms of diabetic nephropathy: role of hypertension. Hypertension 2006; 48: 519-526.

Griendling KK, Sorescu D, Ushio-Fukai M. NADPH oxidase: role in cardiovascular biology and disease. Circ Res 2000; 86: 494-501.

Gorin Y, Block K, Hernandez J, Bhandari B, Wagner B, Barnes JL, et al. Nox4 NAD(P)H oxidase mediates hypertrophy and fibronectin expression in the diabetic kidney. J Biol Chem 2005; 280: 39616-39626.

Guyton AC. Blood pressure control – special role of the kidneys and body fluids. Science 1991; 252: 1813-1816.

Halliwell B. Tell me about free radicals, doctor: a review. J Roy Soc Med 1989; 82: 747-752.

Halliwell B. What nitrates tyrosine? Is nitrotyrosine specific as a biomarker of peroxynitrite formation in vivo? FEBS Lett 1997; 411: 157-160.

Halliwell B. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol 2006; 141: 312-322.

Halliwell B, Whiteman M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Brit J Pharmacol 2004; 142: 231-255.

Imig JD, Falck JR, Gebremedhin D, Harder DR, Roman RJ. Elevated renovascular tone in young spontaneously hypertensive rats. Role of cytochrome p-450. Hypertension 1993; 22: 357-364.

Johnson RJ, Rodriguez-Iturbe B, Kang DH, Feig DI, Herrera-Acosta J. A unifying pathway for essential hypertension. Am J Hypertens 2005; 18: 431-440.

Jones DP. Redefining oxidative stress. Antioxid Redox Signal 2006; 8: 1865-1879.

King H, Aubert RE, Herman WH. Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. Diabetes Care 1998; 21: 1414-1431.

Kiritoshi S, Nishikawa T, Sonoda K, Kukidome D, Senokuchi T, Matsuo T, et al. Reactive Oxygen species from mitochondria induce cyclooxygenase-2 gene expression in human mesangial cells: potential role in diabetic nephropathy. Diabetes 2003; 52: 2570-2577.

Lopes de Faria JB, Zoukhri D, Lorenzi M. Mesangial cell abnormalities in spontaneously hypertensive rats before the onset of hypertension. Kidney Int 1997; 52: 387-392.

Lopes de Faria JB, Bittencourt ZZ, Ribeiro Alves MA. Prevalence of diabetic nephropathy in adult patients with chronic kidney failure. Rev Assoc Med Bras 1995; 41: 353-355.

Makino A, Skelton MM, Zou AP, Cowley AW Jr. Increased renal medullary H2O2 leads to hypertension. Hypertension 2003; 42: 25-30.

Malerbi DA, Franco LJ. Multicenter study of the prevalence of diabetes mellitus and impaired glucose tolerance in the urban Brazilian population aged 30-69 yr. The Brazilian

Cooperative Group on the Study of Diabetes Prevalence. Diabetes Care 1992; 15: 1509-1516.

Mayes PA, Botham KM. Biologic oxidation. In: *Harper's Illustrated Biochemistry*, edited by Murray RK, Granner DK, Mayes PA, Rodwell VW. McGraw-Hill: India 2003; 86-91.

Munzel T, Afanas'ev IB, Kleschyov AL, Harrison DG. Detection of superoxide in vascular tissue. Arterioscler Thromb Vasc Biol 2002; 22: 1761-1768.

Nava M, Quiroz Y, Vaziri ND, Rodriguez-Iturbe B. Melatonin reduces renal interstitial inflammation and improves hypertension in spontaneously hypertensive rats. Am J Physiol Renal Physiol 2003; 284: F447-F454.

Nguyen D, Ping F, Mu W, Hill P, Atkins RC, Chadban SJ. Macrophage accumulation in human progressive diabetic nephropathy. Nephrology 2006; 11: 226-231.

Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, et al. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 2000; 404: 787-790.

Noyan-Ashraf MH, Wu L, Wang R, Juurlink BH. Dietary approaches to positively influence fetal determinants of adult health. FASEB J 2006; 20: 371-373.

Obrosova IG, Fathallah L, Liu E, Nourooz-Zadeh J. Early oxidative stress in the diabetic kidney: effect of DL-alpha-lipoic acid. Free Radic Biol Med 2003; 34: 186-195.

Okada S, Shikata K, Matsuda M, Ogawa D, Usui H, Kido Y, et al. Intercellular adhesion molecule-1—deficient mice are resistant against renal injury after induction of diabetes. Diabetes 2003; 52: 2586-2593.

Okamoto K, Aorki K: Development of a strain of spontaneously hypertensive rats. Jpn Circ J 1963; 27: 282-293.

Oparil S, Zaman MA, Calhoun DA. Pathogenesis of hypertension. Ann Intern Med 2003; 139: 761-776.

Osicka TM, Russo LM, Qiu ML, Brammar GC, Thallas V, Forbes JM, et al. Additive effects of hypertension and diabetes on renal cortical expression of PKC-α and -ε and α-tubulin but not PKC-β1 and β2. J Hypertens 2003; 21: 2399-2407.

Righetti AE, Boer-Lima PA, Lopes de Faria JB. The presence of genetic hypertension stimulates early renal accumulation of fibronectin in experimental diabetes mellitus. Diabetologia 2001; 44: 2088-2091.

Rodriguez-Iturbe B, Quiroz Y, Nava M, Bonet L, Chavez M, Herrera-Acosta J, et al. Reduction of renal immune cell infiltration results in blood pressure control in genetically hypertensive rats. Am J Physiol Renal Physiol 2002; 282: F191-F201.

Rodriguez-Iturbe B, Zhan CD, Quiroz Y, Sindhu RK, Vaziri ND. Antioxidant-rich diet relieves hypertension and reduces renal immune infiltration in spontaneously hypertensive rats. Hypertension 2003; 41: 341-346.

Rodriguez-Iturbe B, Vaziri ND, Herrera-Acosta J, Johnson RJ. Oxidative stress, renal infiltration of immune cells, and salt-sensitive hypertension: all for one and one for all. Am J Physiol Renal Physiol 2004a; 286: F606-F616.

Rodriguez-Iturbe B, Quiroz Y, Ferrebuz A, Parra G, Vaziri ND. Evolution of renal interstitial inflammation and NF-κB activation in spontaneously hypertensive rats. Am J Nephrol 2004b; 24: 587-594.

Rodriguez-Iturbe B, Ferrebuz A, Vanegas V, Quiroz Y, Mezzano S, Vaziri ND. Early and sustained inhibition of nuclear factor-κB prevents hypertension in spontaneously hypertensive rats. J Pharmacol Exp Ther 2005; 315: 51-57.

Sassy-Prigent C, Heudes D, Mandet C, Belair MF, Michel O, Perdereau B, et al. Early glomerular macrophage recruitment in streptozotocin-induced diabetic rats. Diabetes 2000; 49: 466-475.

Scandalios JG. Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. Braz J Med Biol Res 2005; 38: 995-1014.

Schnachenberg CG. Oxygen radicals in cardiovascular-renal disease. Curr Opin Pharmacol 2002; 2: 121-125.

Stratton IM, Adler AI, Neil HA, Matthews DR, Manley SE, Cull CA, et al. Association of glycemia with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study. BMJ 2000; 321: 405-412.

Stratton IM, Cull CA, Adler AI, Matthews DR, Neil HA, Holman RR. Additive effects of glycemia and blood pressure exposure on risk of complications in type 2 diabetes: a prospective observational study (UKPDS 75). Diabetologia 2006; 49: 1761-1769.

Susztak K, Raff AC, Schiffer M, Bottinger EP. Glucose-induced reactive oxygen species cause apoptosis of podocytes and podocyte depletion at the onset of diabetic nephropathy. Diabetes 2006; 55: 225-233.

Svensson M, Nystrom L, Schon S, Dahlquist G. Age at onset of childhood-onset type 1 diabetes and the development of end-stage renal disease: a nationwide population-based study. Diabetes Care 2006; 29: 538-542.

Tak PP, Firestein GS. NF-κB: a key role in inflammatory diseases. J Clin Invest 2001; 107: 7-11.

Tarnow L, Rossing P, Gall MA, Nielsen FS, Parving HH. Prevalence of arterial hypertension in diabetic patients before and after JNC-V. Diabetes Care; 17: 1247-1251.

The Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med 1993; 329: 977-986.

The expert committee on the diagnosis and classification of diabetes mellitus. Report of the expert committee on the diagnosis and classification of diabetes mellitus. Diabetes Care 2003; 26: S5-S20.

Tone A, Shikata K, Sasaki M, Ohga S, Yozai K, Nishishita S, et al. Erythromycin ameliorates renal injury via anti-inflammatory effects in experimental diabetic rats. Diabetologia 2005; 48: 2402-2411.

United Kingdom Prospective Diabetes Study Group. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). Lancet 1998; 352: 837-853.

US Renal Data System, USRDS 2006 Annual Data Report: Atlas of End-Stage Renal Disease in the United States, National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, 2006.

Utimura R, Fujihara CK, Mattar AL, Malheiros DM, Noronha IL, Zatz R. Mycophenolate mofetil prevents the development of glomerular injury in experimental diabetes. Kidney Int 2003; 63: 209-216.

Vasan RS, Beiser A, Seshadri S, Larson MG, Kannel WB, D'Agostino RB, et al. Residual lifetime risk for developing hypertension in middle-aged women and men: The Framingham Heart Study. JAMA 2002; 287: 1003-1010.

Vaziri ND, Wang XQ, Oveisi F, Rad B. Induction of oxidative stress by glutathione depletion causes severe hypertension in normal rats. Hypertension 2000; 36: 142-146.

Vaziri ND. Roles of oxidative stress and antioxidant therapy in chronic kidney disease and hypertension. Curr Opin Nephrol Hypertens 2004; 13: 93-99.

Vaziri ND, Ni Z. Expression of NOX-I, gp91phox, p47phox and p67phox in the aorta segments above and below coarctation. Biochim Biophys Acta 2005; 1723: 321-327.

Vaziri ND, Rodriguez-Iturbe B. Mechanisms of disease: oxidative stress and inflammation in the pathogenesis of hypertension. Nat Clin Pract Nephrol 2006; 2: 582-593.

Whiteman M, Armstrong JS, Cheung NS, Siau JL, Rose P, Schantz JT, et al. Peroxynitrite mediates calcium-dependent mitochondrial dysfunction and cell death via activation of calpains. FASEB J 2004; 18: 1395-1397.

Wilcox CS. Oxidative stress and nitric oxide deficiency in the kidney: a critical link to hypertension? Am J Physiol Regul Integr Comp Physiol 2005; 289: 913-935.

Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000and projections for 2030. Diabetes Care 2004; 27: 1047-1053.

Wolf G. New insights into the pathophysiology of diabetic nephropathy: from hemodynamics to molecular pathology. Eur J Clin Invest 2004; 34: 785-796.

World Health Report 2002: Reducing risks, promoting healthy life. Geneva, Switzerland: World Health Organization. 2002 http://www.who.int/whr/2002/

Young BA, Johnson RJ, Alpers CE, Eng E, Gordon K, Floege J, et al. Cellular events in the evolution of experimental diabetic nephropathy. Kidney Int 1995; 47: 935-944.

Yozai K, Shikata K, Sasaki M, Tone A, Ohga S, Usui H, et al. Methotrexate prevents renal injury in experimental diabetic rats via anti-inflammatory actions. J Am Soc Nephrol 2005; 16: 3326-3338.

Zhan CD, Sindhu RK, Vaziri ND. Up-regulation of kidney NAD(P)H oxidase and calcineurin in SHR: reversal by lifelong antioxidant supplementation. Kidney Int 2004; 65: 219-227.

Zimmet P, Alberti KG, Shaw J. Global and Societal implications of the diabetes epidemic. Nature 2001; 414: 782-787.

CHAPTER 7

1. APPENDIX (Additional publications)

Biswas SK, Lopes de Faria JM, Lopes de Faria JB. -to: Gardiner TA, Anderson HR, Degenhardt T et al. (2003) Prevention of retinal capillary basement membrane thickening in diabetic dogs by a non-steroidal anti-inflammatory drug. Diabetologia 46:1269-1275. Diabetologia: 2004; 47: 763.

Biswas SK, Lopes de Faria JM, Lopes de Faria JB. -to: Davis BJ, Forbes JM, Thomas MC et al. (2004) Superior renoprotective effects of combination therapy with ACE and AGE inhibition in the diabetic spontaneously hypertensive rat, Diabetologia 47:89-97. Diabetologia 2004; 47: 1471.

Biswas SK, Lopes de Faria JB. Does peroxynitrite sustain nuclear factor-κB? Cardiovasc Res 2005; 67: 745-746.

Biswas SK, Peixoto EB, de Faria JB. Do advanced glycation end products and glucose induce similar signaling events in mesangial cells? Kidney Int 2006; 70: 1523.

Table 1. Physical and metabolic parameters of the experimental groups following 10 days of diabetes

	WKY-control	WKY-diabetic	SHR-control	SHR-diabetic
Number	14	13	13	11
Body weight, g	417 ± 30	323 ± 34[a]	279 ± 23[b]	199 ± 20[a–c]
Systolic BP, mm Hg	128 ± 9	130 ± 10	193 ± 11[d]	182 ± 14[d]
Plasma glucose, mM	8.7 ± 0.73	25.4 ± 0.86[a]	8.1 ± 0.67	26.3 ± 1.26[a]
KW:BW ratio, %	0.34 ± 0.02	0.51 ± 0.04[a]	0.32 ± 0.01	0.52 ± 0.03[a]
AER, mg/day	0.49 (0.28–0.83)	1.19 (0.93–2.28)[a]	0.43 (0.35–0.55)	1.2 (0.78–1.82)[a]

Data are means ± SD. The 24-hour urinary albumin excretion rate (AER) is expressed as the median (range) and was analyzed by the Kruskal-Wallis test followed by the Mann-Whitney U test. KW = Kidney weight; BW = body weight.

[a] $p < 0.01$ vs. respective control group; [b] $p < 0.001$ vs. WKY control; [c] $p < 0.001$ vs. WKY diabetic; [d] $p < 0.001$ vs. WKY control/diabetic.

controls, staining was performed omitting the primary antibody or by using an irrelevant immunoglobulin. ED1-positive cells were counted in 50 sequential cortical glomeruli and 20 high power microscopic fields (×400) for the evaluation of glomerular and tubulointerstitial macrophage infiltration, respectively. Tubulointerstitial cells containing 8-OHdG-positive nuclei were counted in 50 sequential high power microscopic fields (×400).

Determination of Reduced Glutathione (GSH) Concentration

The renal cortical GSH level was measured by the method of Beutler et al. [22] with a few modifications. A small piece of frozen kidney cortex was weighed and directly homogenized in cold 10% trichloroacetic acid on ice. The homogenate was centrifuged at 3,000 rpm for 15 min at 4°C. The supernatant was reacted with 0.3 M phosphate buffer and 0.04% 5,5'dithio-bis-2-nitrobenzoic acid. Absorbance was read at 412 nm and the GSH concentration expressed as micromoles GSH per gram frozen tissue from a standard curve. GSH (reduced form) was used as an external standard for preparation of a standard curve.

Western Blotting

Renal cortical homogenate was used to quantify nitrotyrosine by Western blot analysis. Molecular weight markers (PageRuler™, Fermentas Life Sciences) were used as standards. For blocking of nonspecific binding and antibody incubation, 5% nonfat milk was used in phosphate-buffered saline containing 0.1% Tween-20 (PBST). 50 μg of cortical protein was separated on 10% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose membrane using Mini-Protean II Dual Slab Cell apparatus (Bio-Rad Laboratories, Hercules, Calif., USA). After blocking nonspecific binding, the membrane was incubated with a mouse monoclonal anti-nitrotyrosine antibody (1:2,000; clone 1A6, Upstate, Lake Placid, N.Y., USA). After washing with PBST, the membrane was incubated with anti-mouse horseradish peroxidase-conjugated secondary antibody (Santa Cruz). Immunoreactive bands were made visible using the enhanced chemiluminescence method (Super Signal CL-HRP Substrate System; Pierce, Rockford, Ill., USA). Exposed films were scanned with a laser densitometer (Bio-Rad) and analyzed quantitatively with Multi-Analyst Macintosh Software for Image Analysis Systems (Bio-Rad).

Statistical Analysis

The results are expressed as means ± SD. Comparisons between groups were done with ANOVA followed by Bonferroni corrected t test.

Nonparametric data are expressed as median (range), and were analyzed by Mann-Whitney U test (for 2 groups) and Kruskal-Wallis test (for multiple groups). Statistical significance was set at $p < 0.05$. All analyses were performed using statistical software StatView.

Results

Physical and Metabolic Parameters

Diabetes was induced in 12-week-old WKY and SHR rats for 10 days. As seen in table 1, the body weights of the diabetic rats were significantly reduced as compared to the control rats in both rat strains. The SHR rats used in the study were hypertensive, and their systolic blood pressure was significantly higher than the WKY rats ($p < 0.001$, $n > 10$ in each group; table 1). However, STZ-induced diabetes of 10 days duration did not alter systolic blood pressure in WKY or SHR rats. Fasting plasma glucose levels were significantly elevated ($p < 0.001$) in WKY-diabetic (WKY-D) and SHR-diabetic (SHR-D) groups compared to the respective control groups (table 1). Diabetic rats showed hypertrophy of the kidney as evidenced by a significantly higher ($p < 0.001$) kidney weight/body weight ratio in diabetic groups than in control groups. Urinary AER was elevated after 10 days of diabetes in both rat strains. However, the AER was not different between SHR-control (SHR-C) and WKY-control (WKY-C) groups, nor was it different between SHR-D and WKY-D groups (table 1).

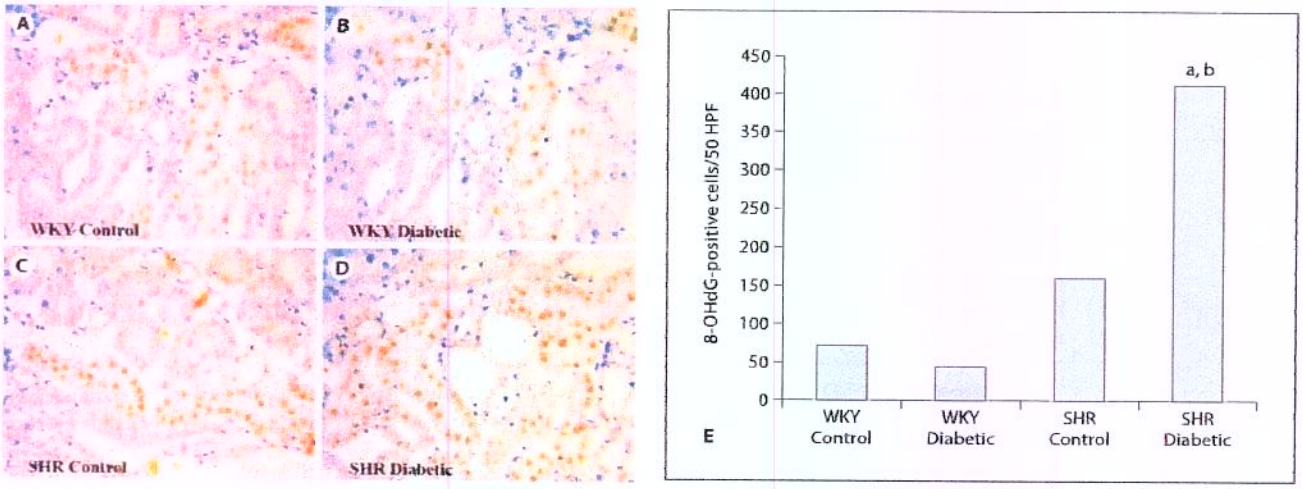


Fig. 1. Immunohistochemical detection of oxidative stress-induced DNA damage. **A–D** Photomicrographs of the immunohistochemical identification of 8-OHdG-containing cells in the kidney cortex. Tubulointerstitial cells containing 8-OHdG were identified by their brown colored nuclei. Original magnification ×400. Counterstained with hematoxylin. **E** Median number of tubulointerstitial 8-OHdG-containing cells per 50 high power fields (HPF). At least 3 sections were stained and evaluated for each rat. [a] $p = 0.014$ vs. SHR-C; [b] $p < 0.05$ vs. all other groups ($n = 5$ in each group). Data were analyzed by the Kruskal-Wallis test followed by the Mann-Whitney U test.

Oxidative Stress Parameters

To identify the potentially damaging effect of oxidative stress on the nucleic acids, we studied 8-OHdG in the kidney tissue. The DNA base guanine-containing nucleoside 2′-deoxyguanosine can be modified to 8-OHdG by excessive oxidative stress. We detected renal cortical cells containing the modified base 8-OHdG in the nucleus by immunohistochemistry using monoclonal antibody against 8-OHdG. The staining pattern was heterogeneous, mainly involving the tubular cells of the inner cortical region (fig. 1). The number of tubulointerstitial cells containing 8-OHdG (per 50 high power fields) was not different between WKY-C (median (range): 72 (12–331)) and WKY-D (45 (10–141)9) groups ($n = 5$). However, the SHR-D group showed a significantly higher number of 8-OHdG-containing cells in the tubulointerstitial area compared to the SHR-C group (SHR-C, 161 (24–348); SHR-D, 411 (356–472); $p = 0.014$, $n = 5$; fig. 1). This finding indicates that the presence of genetic hypertension induces oxidative stress-induced DNA damage in the kidney in the early stage of experimental diabetes.

Oxidative stress-induced protein modification was assessed by Western blot analysis of renal cortical nitrotyrosine. Although nitrotyrosine is not a specific marker for the in vivo generation of peroxynitrite, it has widely been used as a marker of oxidative and nitrosative stress [23]. Renal cortical nitrotyrosine levels were not different between the WKY-C (1.0 ± 0.58 arbitrary units) and WKY-D (0.83 ± 0.33) groups ($n = 5$). However, the nitrotyrosine level was found to be elevated in the SHR-D group (1.9 ± 1.1) compared to the SHR-C group (1.0 ± 0.5), although the elevation failed to reach the conventional level of significance ($p = 0.068$, $n = 5$; fig. 2).

We analyzed the renal cortical reduced GSH concentration as a measure of antioxidant defense. Diabetes mellitus was associated with a small, nonsignificant elevation in the GSH concentration (μmol/g frozen tissue) in the normotensive rats (WKY-C, 4.34 ± 0.36; WKY-D, 4.60 ± 0.33; $n = 10$). However, the presence of hypertension significantly reduced the renal cortical GSH level after 10 days of experimental diabetes (SHR-C, 4.16 ± 0.25; SHR-D, 3.70 ± 0.25; $p = 0.034$, $n = 10$; fig. 3).

Inflammatory Parameter

Macrophages are one of the central mediators of renal vascular inflammation, and their accumulation is a characteristic feature of diabetic nephropathy [9, 15–17, 24]. In the present study, macrophage infiltration in the kidney tissue was evaluated by immunohistochemical staining for ED1. The ED1-positive cells (macrophages) showed

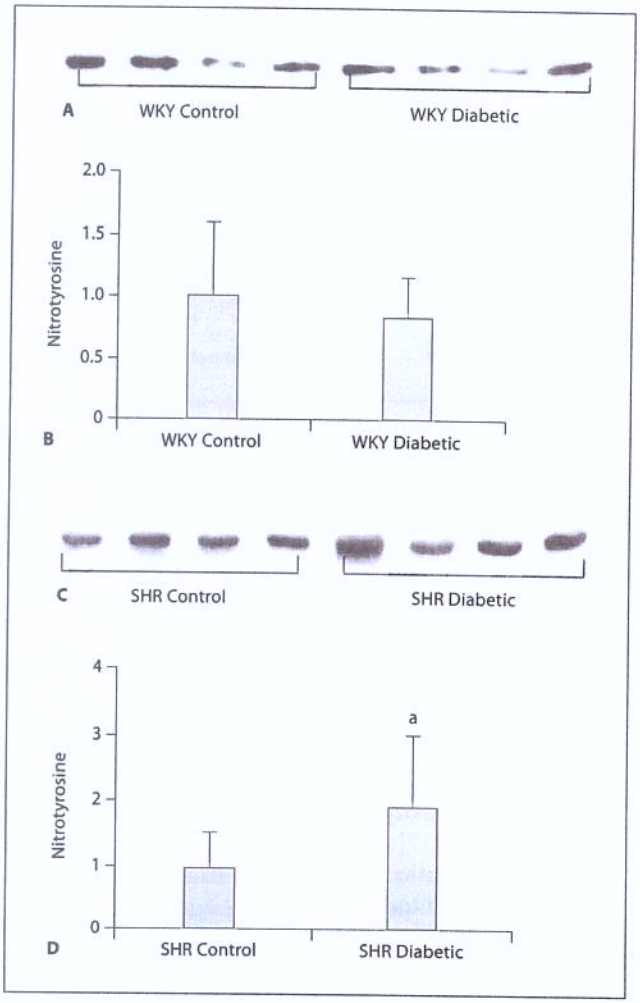


Fig. 2. Western blot analysis of renal cortical nitrotyrosine. Representative Western blots and densitometric analysis of nitrotyrosine in the WKY (**A**, **B**) and SHR (**C**, **D**) groups. Values obtained in the diabetic groups are expressed relative to those of the control groups, which are arbitrarily assigned a value of 1.0. Bars represent means ± SD of band densities (arbitrary densitometric units) from at least 3 independent experiments (n = 5 in each group). [a] p = 0.068 vs. SHR-C.

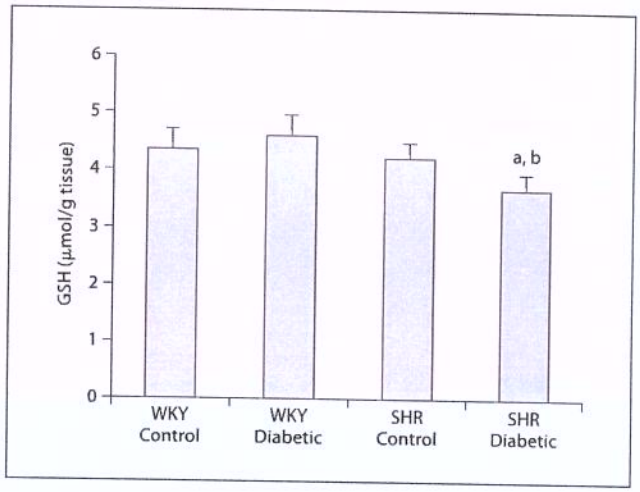


Fig. 3. Renal cortical reduced glutathione (GSH) level. The GSH concentration is expressed in micromoles per gram frozen tissue. Data are means ± SD. [a] p = 0.034 vs. SHR-C; [b] p < 0.05 vs. all other groups (n = 10 in each group).

intense cytoplasmic staining surrounding or overlapping the nucleus, and were distributed both in the glomerular and tubulointerstitial areas (fig. 4). Renal cortical macrophage infiltration was expressed as the number of ED1-positive cells per glomerular cross section (GCS) and per high power field (HPF) for the glomerular and tubulointerstitial areas, respectively. As shown in figure 4, glomerular macrophage infiltration was significantly higher in the WKY-D (WKY-C, 1.04 ± 0.26 cells/GCS; WKY-D, 1.81 ± 0.51 cells/GCS; p = 0.03, n = 5) and SHR-D (SHR-C, 1.76 ± 0.32 cells/GCS; SHR-D, 2.65 ± 0.22 cells/GCS; p = 0.01, n = 5) groups than in the respective control groups (fig. 4). Tubulointerstitial macrophage infiltration was also higher in the WKY-D (WKY-C, 8.88 ± 2.15 cells/HPF; WKY-D, 22.61 ± 4.45 cells/HPF; p = 0.003, n = 5) and SHR-D (SHR-C, 11.33 ± 1.04 cells/HPF; SHR-D, 24.05 ± 8.12 cells/HPF; p = 0.005, n = 5) groups than in the respective non-diabetic control groups (fig. 4). Therefore, this finding shows that macrophage infiltration in the kidney cortex increases both in normotensive and hypertensive rats after the induction of diabetes for a short duration.

Discussion

In the present study, we investigated the effects of experimental diabetes of short duration on renal inflammation and oxidative stress in normotensive and genetically hypertensive rats. The simultaneous induction of diabetes in normotensive and hypertensive rats allowed us to compare the effects of diabetes and the effects of the combination of diabetes and hypertension. Since oxidative stress and inflammatory processes are partially interdependent [19], we decided to study short-duration diabetes of only 10 days; otherwise, with a longer duration of diabetes, it would have been difficult to see a dif-

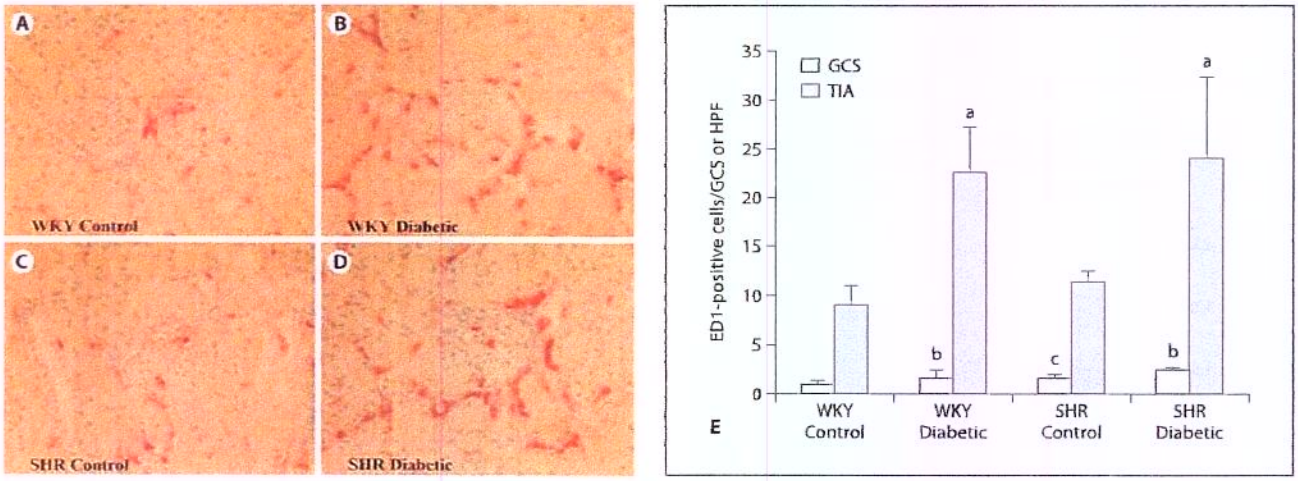


Fig. 4. Macrophage infiltration as detected by ED1 immunohistochemistry. **A–D** Photomicrographs of immunohistochemical identification of ED1-positive cells in the kidney cortex. ED1-positive cells were identified by the bright red granular staining predominantly in the cytoplasm surrounding or overlapping the nucleus. Original magnification ×400. Counterstained with hematoxylin. **E** Numbers of glomerular and tubulointerstitial macrophage infiltrations expressed per glomerular cross-section (GCS) and high power field (HPF), respectively. At least 3 sections were stained and evaluated for each rat. Data are means ± SD (n = 5 in each group). [a] $p < 0.01$, [b] $p < 0.05$ vs. respective control group; [c] $p < 0.05$ vs. WKY-C. TIA = Tubulointerstitial area.

ference between the effects of diabetes and those of the combination of diabetes and hypertension on renal inflammation and oxidative stress. In the present study, we demonstrated that the short duration of diabetes clearly induced oxidative stress in the kidney of hypertensive rats, while there was no evidence of renal oxidative stress in normotensive rats with the same duration of diabetes. However, macrophage infiltration in the kidney was similarly elevated in both normotensive and hypertensive rats with the same duration of diabetes.

Hyperglycemia-induced oxidative stress and pro-oxidant generation have been consistently shown in many studies, particularly in in vitro experiments. However, discrepancy exists in published data on renal oxidative stress in intact animals during early diabetes [12]. Elevated pro-oxidant generation with or without biomolecular damage or modification (lipoperoxide, nitrotyrosine) associated with unchanged/elevated/decreased activity of enzymatic antioxidants (superoxide dismutase, glutathione peroxidase, glutathione reductase, glutathione transferase, catalase and heme oxygenase) and reduction of non-enzymatic antioxidants (GSH, ascorbate) have been demonstrated in various combinations in the kidney during the early stage of experimental diabetes [12–14]. Discrepancy also exists in the case of inflammatory cellular infiltration in the kidney in early diabetes. There are studies demonstrating elevated macrophage infiltration in the kidney as early as 3 days [15] or 8 days [16] or as late as 2 months [17] after the induction of diabetes. The method of evaluation of oxidative stress or inflammation, the animal model studied, the age at induction of diabetes, the duration of diabetes and the presence or absence of insulin treatment may partly explain the discrepancies observed from one study to another.

Hyperglycemia is the major determinant of pro-oxidant generation in diabetes [6, 7]. Therefore, the findings of the present study raise the question why there was no evidence of oxidative stress in the kidneys of normotensive rats in spite of similar levels of plasma glucose in normotensive and hypertensive rats. We consider that the issue could be explained on a valid scientific basis, because pro-oxidant generation is not always synonymous with oxidative stress, particularly in in vivo situations where multiple antioxidant protective mechanisms are operative. In fact, oxidative stress is typically defined as a disturbance in the pro-oxidant/antioxidant balance in favor of the former, leading to potential damage [25]. Therefore, in the present study, it is most likely that the pro-oxidant/antioxidant balance was well maintained in normotensive WKY rats, and the balance was disrupted

Comment

—to: Gardiner TA, Anderson HR, Degenhardt T et al. (2003) Prevention of retinal capillary basement membrane thickening in diabetic dogs by a non-steroidal anti-inflammatory drug. Diabetologia 46:1269–1275

To the Editor: We read with interest the recent paper by Gardiner et al. [1] reporting the effect of a non-steroidal anti-inflammatory drug Sulindac on the early vascular pathology of diabetic retinopathy and some insights into the mechanism by which Sulindac acts in this respect. Gardiner et al. found that Sulindac treatment significantly reduced retinal capillary basement membrane thickening, a common early lesion of diabetic retinopathy, in experimental diabetic dogs. Their study thus reproduced 13 years later the finding of a previous study on diabetic cats [2]. In this previous study the authors had suggested that the effect of treatment with Sulindac may be mediated by inhibition of aldose reductase activity, as Sulindac is a known aldose reductase inhibitor [3].

Whilst investigating the mechanism of the beneficial effects of Sulindac, Gardiner et al. concluded that the treatment benefit is not derived from the inhibition of advanced glycation, the reduction of aldose reductase activity or the improvement of anti-oxidant status. However, they came to this conclusion by assessing the markers of the polyol pathway, advanced glycation and oxidative stress in plasma, serum, erythrocytes and skin collagen. We believe that the conclusion could have been different, if they had investigated these pathological processes in the tissue affected by diabetes, i.e. in the retina [4].

Joussen et al. [5] have previously shown that non-steroidal anti-inflammatory drugs prevent early diabetic retinopathy via TNF-α suppression. They have shown that aspirin and selective cycloxigenase-2 inhibitor meloxicam can reduce blood–retinal barrier breakdown and leucocyte adhesion to diabetic retinal vasculature, at least in part, through the inhibition of TNF-α. Unfortunately, in the study by Gardiner et al. there is no discussion of the possibility of TNF-α-mediated retinal effects of Sulindac. At the end of their discussion the authors mention that the treatment benefit of aspirin in the study by Joussen et al. [5] was clearly derived from its classic anti-inflammatory role as a non-steroidal anti-inflammatory drug. In fact this is only partially true, since the classic mechanism of aspirin as a non-steroidal anti-inflammatory drug involves inhibition of the enzyme cycloxigenase [6]. Moreover, TNF-α suppression is not supposed to be a part of the classic anti-inflammatory mechanism of non-steroidal anti-inflammatory drugs, a factor that has not been appreciated in the study by Gardiner et al.

S. K. Biswas · J. B. Lopes de Faria
Department of Internal Medicine, Faculty of Medical Sciences, Campinas, São Paulo, UNICAMP, Brazil

J. M. Lopes de Faria
Department of Ophthalmology, Faculty of Medical Sciences, University of Campinas, São Paulo, Brazil

References

1. Gardiner TA, Anderson HR, Degenhardt T et al. (2003) Prevention of retinal capillary basement membrane thickening in diabetic dogs by a non-steroidal anti-inflammatory drug. Diabetologia 46:1269–1275
2. Mansour SZ, Hatchell DL, Chandler D, Saloupis P, Hatchell MC (1990) Reduction of basement membrane thickening in diabetic cat retina by sulindac. Invest Ophthalmol Vis Sci 31:457–463
3. Jacobson M, Sharma YR, Cotlier E, Hollander JD (1983) Diabetic complications in lens and nerve and their prevention by sulindac or sorbinil: two novel aldose reductase inhibitors. Invest Ophthalmol Vis Sci 24:1426–1429
4. Lorenzi M, Gerhardinger C (2001) Early cellular and molecular changes induced by diabetes in the retina. Diabetologia 44:791–804
5. Joussen AM, Poulaki V, Mitsiades N et al. (2002) Non-steroidal anti-inflammatory drugs prevent early diabetic retinopathy via TNF-alpha suppression. FASEB J 16:438–440
6. Vane JR (1971) Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nat New Biol 231:232–235

J. B. Lopes de Faria (✉)
Department of Internal Medicine, Faculty of Medical Sciences, CEP: 13084-971, Caixa Postal: 6111, Campinas, São Paulo, UNICAMP, Brazil
E-mail: jblfaria@fcm.unicamp.br
Tel.: +55-19-37887499
Fax: +55-19-37887366

DOI 10.1007/s00125-004-1361-3
Received: 25 November 2003 / Accepted: 26 January 2004
Published online: 10 March 2004

Diabetologia (2004) 47:1471

Diabetologia

Comment

—to: Davis BJ, Forbes JM, Thomas MC et al. (2004) Superior renoprotective effects of combination therapy with ACE and AGE inhibition in the diabetic spontaneously hypertensive rat. Diabetologia 47:89–97

To the Editor: We read with interest the recent paper by Davis et al. [1] reporting the renoprotective effects of an ACE inhibitor, an AGE inhibitor and a combined ACE and AGE inhibitor on streptozotocin-induced diabetic spontaneously hypertensive rats. The level of renoprotection obtained by the ACE inhibitor, perindopril, was similar to that obtained by the AGE inhibitor, aminoguanidine, as measured by the prevention of albuminuria, glomerular and tubulointerstitial injury and TGFβ1 overexpression, and by the suppression of nephrin expression. Davis et al. claimed that they obtained superior renoprotection with combination therapy using ACE and AGE inhibitors. However, the mechanism by which it happened is unclear for several reasons. Firstly, the ACE inhibitor perindopril is not a specific inhibitor of ACE, but a combined ACE and AGE inhibitor [2]. A significant degree of inhibition of AGE formation by perindopril is also clear from the study by Davis et al. In spite of this, the authors were unable to demonstrate superior renoprotection of perindopril over AGE inhibitor aminoguanidine. Moreover, the addition of aminoguanidine with perindopril had no additional inhibitory effect on AGE formation. Therefore, it is not clear that the superiority of renoprotection after addition of aminoguanidine was a result of the inhibition of AGE formation. Secondly, ACE inhibitor perindopril was not used at a sufficient dose in the study by Davis et al., and the rats were still hypertensive after 24 weeks of therapy. Davis et al. argued that a maximal dose of ACE inhibitor would have prevented assessment of the superiority of dual blocking. We, however, are in doubt about the validity of this argument in this respect, as the superiority of combination therapy cannot be claimed over monotherapy without assessing the full potential of monotherapy. It is most likely that tight control of blood pressure by adequate dosing of ACE inhibitor would have abolished the superiority of combination therapy. This was taken into consideration, in part, by Davis et al. Tight control of blood pressure, as an effective measure of renoprotection, has recently been reported by our group in diabetic spontaneously hypertensive rats [3, 4]. In our studies, ACE inhibitor and non-ACE inhibitor antihypertensive agents showed similar levels of renoprotection. In fact, the beneficial effects of blood pressure reduction, independent of the renin–angiotensin system inhibition, had previously been suggested by the group of Davis et al. in studies of diabetic spontaneously hypertensive rats [5, 6].

S. K. Biswas, J. B. Lopes de Faria
Department of Internal Medicine, Faculty of Medical Sciences, University of Campinas (UNICAMP), Campinas, SP, Brazil

J. M. Lopes de Faria
Department of Ophthalmology, Faculty of Medical Sciences, University of Campinas (UNICAMP), São Paulo, Brazil

References

1. Davis BJ, Forbes JM, Thomas MC et al. (2004) Superior renoprotective effects of combination therapy with ACE and AGE inhibition in the diabetic spontaneously hypertensive rat. Diabetologia 47:89–97
2. Miyata T, Ypersele de Strihou C van, Ueda Y et al. (2002) Angiotensin II receptor antagonists and angiotensin-converting enzyme inhibitors lower in vitro the formation of advanced glycation end products: biochemical mechanisms. J Am Soc Nephrol 13:2478–2487
3. Pavan MV, Ghini B, Castro M, Lopes De Faria JB (2003) Prevention of hypertension attenuates albuminuria and renal expression of fibronectin in diabetic spontaneously hypertensive rats. Am J Nephrol 23:422–428
4. Lehfeld LS, Silveira LA, Ghini B, Lopes De Faria JB (2004) Early blood pressure normalization independent of the class of antihypertensive agent prevents augmented renal fibronectin and albuminuria in experimental diabetic nephropathy. Kidney Blood Press Res 27:114–120
5. Cooper ME, Allen TJ, O'Brien RC et al. (1990) Nephropathy in model combining genetic hypertension with experimental diabetes. Enalapril versus hydralazine and metoprolol therapy. Diabetes 39:1575–1579
6. Cooper ME, Rumble JR, Allen TJ, O'Brien RC, Jerums G, Doyle AE (1992) Antihypertensive therapy in a model combining spontaneous hypertension with diabetes. Kidney Int 41:898–903

J. B. Lopes de Faria (✉)
Department of Internal Medicine, Faculty of Medical Sciences, University of Campinas (UNICAMP), CEP: 13084-971, Caixa Postal: 6111, Campinas, SP, Brazil
E-mail: jblfaria@fcm.unicamp.br
Tel.: +55-19-37887499, Fax: +55-19-37887366

DOI 10.1007/s00125-004-1469-5
Received: 18 May 2004 / Accepted: 9 June 2004
Published online: 22 July 2004

ELSEVIER

Cardiovascular Research 67 (2005) 745 – 746

Cardiovascular Research

www.elsevier.com/locate/cardiores

Letter to the Editor

Does peroxynitrite sustain nuclear factor-κB?

Subrata K. Biswas *, Jose B. Lopes de Faria

Laboratorio de Fisiopatologia Renal, NMCE, Division of Nephrology, FCM, University of Campinas, Cidade Universitaria, Campinas 13083-970, Sao Paulo, Brazil

Received 4 April 2005; accepted 15 June 2005
Available online 1 July 2005

We read with interest the article by Hattori et al. [1] published in *Cardiovascular Research* in July, 2004. The authors showed almost complete suppression of lipopolysaccharide (LPS)-induced nuclear factor-κB (NF-κB) activation by nitric oxide (NO) in vascular smooth muscle cells. They showed that this effect of NO was not mediated through the second messenger cGMP or through peroxynitrite, an NO-derived reactive nitrogen species (RNS). Instead, the authors demonstrated sustained activation of LPS-induced NF-κB by peroxynitrite. Although the molecular mechanisms were not very clear, we considered this finding as a further step to explain the existing controversies regarding NO-mediated regulation of NF-κB activity. Actually, we are trying to explain some of our in vivo findings in the light of this in vitro evidence. However, a detailed report by Park et al. [2] published in March, 2005, compelled us to reanalyze the article by Hattori et al. [1] and to mention some essential points.

Park et al. [2] also showed suppression of NF-κB activity (both constitutively active and cytokine-activated) by NO. In striking contrast to the finding of Hattori et al. [1], however, Park et al. showed that NF-κB could also be suppressed by peroxynitrite. Actually, Park et al. demonstrated that NO-mediated suppression of NF-κB activation was mediated through peroxynitrite by using a peroxynitrite scavenger and also by performing a transfection study. By detailed investigation, Park et al. identified tyrosine nitration of the p65 subunit as the factor responsible for the NO-mediated inactivation of active NF-κB. Since peroxynitrite and other RNS play a major role in tyrosine nitration [3,4], the finding of Park et al. seems scientifically conceivable. On the other hand, sustained activation of NF-κB by peroxynitrite as shown by Hattori et al. could also be explained by its strong oxidant property [5]. However, this finding of Hattori et al. has become questionable for the following reasons.

The experiments from which Hattori et al. [1] have claimed that both pure peroxynitrite and peroxynitrite donor sustained, rather than suppressed, LPS-induced NF-κB activation were not perfectly designed: no control or neutral substance was used in these experiments to show if LPS-induced NF-κB activation is normally sustained in the absence of any potential interference. Thus, the sustained activation of NF-κB observed with pure peroxynitrite and peroxynitrite donor might be a normal response to LPS and is, therefore, not necessarily an effect of peroxynitrite. As admitted by the authors [1] that high reactivity would consume all added peroxynitrite within seconds of addition to cells, we also are in doubt if the added peroxynitrite was active or effective under the conditions used in the study. This suspicion becomes stronger due to the fact that the yields of nitrotyrosine are very low in in vitro reactions when peroxynitrite is slowly infused or slowly generated from chemical precursors [6,7]. Whether added peroxynitrite, either in pure form or generated from a donor, was optimally effective or not, is, therefore, a pertinent point to consider regarding the study by Hattori et al.

Whether the suppression of NF-κB by NO in the study by Hattori et al. [1] was mediated through peroxynitrite could not be confirmed, but the finding of Park et al. [2] leads us to think that peroxynitrite may be the candidate molecule. Since Hattori et al. did not use any peroxynitrite scavenger, it is difficult to ascertain if NO-mediated suppression of NF-κB was independent of peroxynitrite in their study.

Hattori et al. [1] also observed suppression of LPS/interferon γ-induced NF-κB activation by antioxidants, *N*-

* Corresponding author. Tel.: +55 19 37887499; fax: +55 19 37887366.
E-mail address: subrata@fcm.unicamp.br (S.K. Biswas).

0008-6363/$ - see front matter
doi:10.1016/j.cardiores.2005.06.003

acetylcysteine (NAC) and pyrrolidine dithiocarbamate (PDTC), in addition to NO. From this finding, the authors [1] postulated that the NF-κB-suppressing effect of NO might be explained by its antioxidant property through scavenging of superoxide radical, which was also supported by Tritto and Ambrosio [8] in the editorial accompanying the article by Hattori et al. Although this explanation seems scientifically valid, it may not be the case in the study by Hattori et al. because superoxide dismutase, a specific scavenger of superoxide radical, was unable to suppress LPS-induced NF-κB activity. However, it is very strange since NAC and PDTC markedly suppressed LPS-induced NF-κB activity.

In conclusion, that NO may inactivate active NF-κB by tyrosine nitration of p65 through peroxynitrite or other RNS, as shown by Park et al. [2], seems more convincing to us. The partially contradictory finding of Hattori et al. [1] might be explained by considering the suboptimum activity/effect of the peroxynitrite used in their study. This consideration suggests a unified, underlying molecular mechanism, i.e. tyrosine nitration of p65 by RNS, which will need further study to be proved. However, this discussion is important to avoid misleading readers by apparently contradictory findings.

References

[1] Hattori Y, Kasai K, Gross SS. NO suppresses while peroxynitrite sustains NF-κB: a paradigm to rationalize cytoprotective and cytotoxic actions attributed to NO. Cardiovasc Res 2004;63:31–40.
[2] Park SW, Huq MD, Hu X, Wei LN. Tyrosine nitration on p65: a novel mechanism to rapidly inactivate nuclear factor-κB. Mol Cell Proteomics 2005;4:300–9.
[3] Beckman JS. Oxidative damage and tyrosine nitration from peroxynitrite. Chem Res Toxicol 1996;9:836–44.
[4] Halliwell B. What nitrates tyrosine? Is nitrotyrosine specific as a biomarker of peroxynitrite formation in vivo? FEBS Lett 1997;411:157–60.
[5] Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci U S A 1990;87:1620–4.
[6] Jourd'heuil D, Jourd'heuil FL, Kutchukian PS, Musah RA, Wink DA, Grisham MB. Reaction of superoxide and nitric oxide with peroxynitrite. Implications for peroxynitrite-mediated oxidation reactions in vivo. J Biol Chem 2001;276:28799–805.
[7] Hurst JK. Whence nitrotyrosine? J Clin Invest 2002;109:1287–9.
[8] Tritto I, Ambrosio G. The multi-faceted behavior of nitric oxide in vascular "inflammation": catchy terminology or true phenomenon? Cardiovasc Res 2004;63:1–4.

Do advanced glycation end products and glucose induce similar signaling events in mesangial cells?

Kidney International (2006) **70,** 1523. doi:10.1038/sj.ki.5001751

To the Editor: In a recent issue of *Kidney International*, Lin *et al.*[1] demonstrated that the advanced glycation end products (AGE) induce the same signaling events in rat mesangial cells as induced by high glucose culminating in increased expression of fibronectin and transforming growth factor-β1. They found that both high glucose and AGE increase Ras-dependent and nicotinamide adenine dinucleotide phosphate (reduced form) oxidase-mediated superoxide production and subsequently induce cytosolic extracellular signal-regulated protein kinase and nuclear c-Jun activation, leading to extracellular matrix accumulation.

The cellular handling of glucose is different from that of AGE, because the involved receptors and metabolic pathways are completely different. Therefore, it is surprising that glucose and AGE induce exactly the same signaling pathways in mesangial cells. As high glucose can rapidly form intracellular AGE,[2] one probable explanation could be that the high glucose-induced signaling events in the mesangial cells were actually mediated by AGE derived from glucose in the study by Lin *et al.*[1] However, the experimental conditions utilized by Lin *et al.* raise some serious concerns. Mesangial cells were treated with very high concentrations of D-glucose and AGE (35 mM and 100 μg/ml, respectively), and the findings were compared with those of the vehicle-treated condition. Lin *et al.*[1] neither included a control substance, like L-glucose or mannitol as a control for D-glucose, or bovine serum albumin as a control for AGE in the experiments, nor did they test the effects of 5 mM glucose in the mesangial cells. Therefore, it is uncertain whether the signaling pathways induced by high glucose and AGE reflect the characteristics of these substances or the same signaling pathways could be induced simply by osmotic/toxic effect of any substance. Another methodological issue that has also drawn our attention is the measurement of 8-hydroxy-2′-deoxyguanosine (8-OHdG) by immunoblotting. To our knowledge, the antibody against 8-OHdG has not been tested for immunoblotting, and the DNA fragments containing the modified base 8-OHdG in tissue homogenate is unlikely to be detected as a specific band in immunoblot. Lin *et al.*[1] demonstrated a single band, but did not mention the approximate molecular weight of the band they considered for 8-OHdG.

1. Lin CL, Wang FS, Kuo YR *et al.* Ras modulation of superoxide activates ERK-dependent fibronectin expression in diabetes-induced renal injuries. *Kidney Int* 2006; **69**: 1593–1600.
2. Schiekofer S, Andrassy M, Chen J *et al.* Acute hyperglycemia causes intracellular formation of CML and activation of ras, p42/44 MAPK, and nuclear factor κB in PBMCs. *Diabetes* 2003; **52**: 621–633.

SK Biswas[1], EBMI Peixoto[1] and JB de Faria[1]
[1]*Renal Pathophysiology Laboratory, Department of Internal Medicine, Division of Nephrology, Faculty of Medical Sciences, State University of Campinas (UNICAMP), Campinas, Sao Paulo, Brazil*
Correspondence: *SK Biswas, Renal Pathophysiology Laboratory, Department of Internal Medicine, Division of Nephrology, Faculty of Medical Sciences, State University of Campinas (UNICAMP), 13084-971 Campinas, Sao Paulo, Brazil.*
E-mails: subrata@fcm.unicamp.br or su.biswas@yahoo.com

Response to 'Do advanced glycation end products and glucose induce similar signaling events in mesangial cells?'

Kidney International (2006) **70,** 1523–1524. doi:10.1038/sj.ki.5001774

We thank Dr Biswas *et al.*[1] for their interest in our recently published work of high glucose and advanced glycation end products raise Ras- and extracellular signal-regulated kinases-dependent fibronectin accumulation of mesangial cells.[2] Their comments reflect the multiple signal transduction pathways in high glucose and advanced glycation end products induction of oxidative stress and renal fibrosis factor expression in mesangial cells. To this interesting issue, the protocols of *in vitro* advanced glycation end products (100 μM)- and high glucose (35 mM)-stressed mesangial cells have been well established. We would refer them to some recently published articles.[3–5] It is not surprising then that high glucose and advanced glycation end products share similar molecular mechanisms to induce fibrosis factor expression when basal medium (10% fetal bovine serum and Dulbecco's modified Eagle's medium constituting 5 mM D-glucose) with or without 35 mM mannitol do not raise superoxide burst in our study model.

We note immunohistochemically and by immunoblotting exogenous superoxide dismutases alleviation of 8-hydroxy-2′-deoxyguanosine levels in diabetic kidney *in vivo*. The provided protein band corresponding to 26 kDa is one of the evident oxidative damaged molecules containing 8-hydroxy-2′-deoxyguanosine immunoreactivities in the diabetic kidney homogenate. The study emphasizes the early control of oxidative stress in preventing diabetes-induced fibrosis-promoting factor accumulation of renal tissue.

1. Biswas SK, Peixoto EBMI, de Faria JBL. Do advanced glycation end products and glucose induce similar signaling events in mesangial cells?. *Kidney Int* 2006 (in press).
2. Lin CL, Wang FS, Kuo YR *et al.* Ras modulation of superoxide activates ERK-dependent fibronectin expression in diabetes-induced renal injuries. *Kidney Int* 2006; **69**: 1593–1600.
3. Singh R, Singh AK, Alavi N, Leehey DJ. Mechanism of increased angiotension II levels in glomerular mesangial cells cultured in high glucose. *J Am Soc Nephrol* 2003; **14**: 873–880.