Free Radical Research, October 2007; 41(10): 1151–1158

informa
healthcare

Arterial hypertension exacerbates oxidative stress in early diabetic retinopathy

CAMILA C. PINTO, KAMILA C. SILVA, SUBRATA K. BISWAS, NATÁSSIA MARTINS, JOSÉ B. LOPES DE FARIA, & JACQUELINE M. LOPES DE FARIA

Renal Pathophysiology Laboratory, Department of Internal Medicine, Faculty of Medical Sciences (FCM), State University of Campinas (UNICAMP), 13084-971, Campinas, São Paulo, Brazil

Accepted by Professor F. Kelly

(Received 3 May 2007; in revised form 12 August 2007)

Abstract

The present study was undertaken to investigate the redox status in the retina of an experimental model that combines hypertension and diabetes. Spontaneously hypertensive rats (SHR) and their control Wystar Kyoto (WKY) rats were rendered diabetic and, after 20 days, the rats were sacrificed and the retinas collected. The superoxide production was higher in diabetic than in control WKY ($p < 0.03$) and SHR rats showed elevated superoxide production compared with WKY groups ($p < 0.009$). The glutathione antioxidant system was diminished only in diabetic SHR ($p < 0.04$). Tirosyne nitration was higher in diabetic WKY and control SHR compared with control WKY ($p < 0.03$), and further increment was observed in diabetic SHR ($p < 0.02$). The DNA damage estimated by immunohystochemistry for 8-OHdG was higher in control SHR than in WKY, mainly in diabetic SHR ($p < 0.0001$). Hypertension aggravates oxidative-induced cytotoxicity in diabetic retina due to increasing of superoxide production and impairment of antioxidative system.

Keywords: *Diabetic retinopathy, hypertension, oxidative stress, superoxide, nitrotyrosine, 8-hydroxy-2′-deoxyguanosine, glutathione*

Abbreviations: *8-OHdg, 8-hydroxy-2′-deoxyguanosine; $ONOO^-$, Peroxynitrite; ROS, Reactive oxygen species; SHR, Spontaneously hypertensive rat; SOD, Superoxide dismutase; WKY, Wistar-Kyoto*

Introduction

The long-term eye complications of diabetes are the leading cause of blindness in patients aged 20–64 years [1]. Hyperglycaemia is needed to initiate diabetic retinopathy (DR) but the sequence of the events, which lead to early retinal lesion, is not yet clear. In addition to hyperglycaemia, epidemiological studies clearly identify hypertension as the most important independent risk factor for DR [2,3]. The UK Prospective Diabetes Study (UKPDS) have convincingly demonstrated that, in patients with type 2 diabetes, the strict metabolic control leads to a 25% reduction in the risk of development and progression of DR. Interestingly, the effect of rigorous control of blood pressure was higher than glycemic control because of its protective impact of up to 37% reduction in the risk of development and progression of DR [4]. However, the mechanism of interaction between hyperglycaemia and hypertension exacerbating DR is not fully understood. Various hyperglycaemia-induced metabolic abnormalities have been postulated to contribute to the

Correspondence: Jacqueline M. Lopes de Faria, Renal Pathophysiology Laboratory, Faculty of Medical Sciences, University of Campinas (UNICAMP), PO Box 6111, Campinas, SP, Brazil 13084-971. Tel: +55-19-35217499. Fax: +55-19-35217366. E-mail: jmlfaria@fcm.unicamp.br

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760701632816

development of the diabetic retinal disease, including increased oxidative stress, non-enzymatic glycation, polyol pathway and protein kinase C (PKC) activation [5–7]. The possible role of oxidative stress in the mechanism of interaction between diabetes and hypertension in the pathogenesis of diabetic retinopathy is not known.

Under diabetic conditions, an imbalance exists between oxidant stress and antioxidative defense mechanisms that favour the former. The retina is susceptible to oxidative stress due to its high consumption of oxygen, high proportion of polyunsaturated fatty acids and exposure to visible light [8]. Oxidative stress can influence the expression of multiple genes, including signalling molecules. The over-expression of these genes may cause mitochondrial dysfunction and peroxidization of the lipid and protein structure which further induce a variety of cellular dysfunctions [8]. Oxidative stress develops in the retina of diabetic animals indicating that oxidative stress is associated with the development of retinopathy [6]. Consistent with this, Armstrong et al. [5] reported a correlation between increased serum lipid hydroperoxides and the prevalence of retinopathy in diabetic patients. Superoxide is of particular interest as it can react with nitric oxide (NO) to produce the highly reactive peroxynitrite, which can result in cytotoxicity due to lipid peroxidation, inactivation of enzymes by oxidation of protein sulphydryls and nitration of tyrosines and damage to DNA and mitochondria. Nitrotyrosine has been detected on retinal proteins of diabetic animals [9]. In the present study, we examined whether the presence of hypertension stimulates an accentuated oxidative stress changes in the retina of diabetic rats.

Methods

Research design and methods

The protocol for this study complies with the guidelines of the Brazilian College for Animal Experimentation (COBEA) and it was approved by the local Committee for Ethics in Animal Research and in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Spontaneously hypertensive rats (SHR) and their normotensive control Wistar-Kyoto (WKY) rats used in the present study were derived from animals supplied by Taconic (Germantown, NY) and bred in our animal facility. Experimental diabetes was induced in 4-week-old pre-hypertensive male SHR and WKY rats by injecting streptozotocin (STZ, 60 mg/kg) (Sigma, St. Louis, MO) dissolved in sodium citrate buffer (pH 4.5) via a tail vein after an overnight fast. Control groups received only vehicle (citrate buffer). Plasma glucose levels were measured using an enzymatic colourimetric GOD-PAP assay (Merck, Darmstadt, Germany) 72 h after the injection of STZ or citrate buffer and on the day before sacrificing the rats. Glucose values ≥ 15 mmol/L were considered diabetic for the present study. Systolic blood pressure (three-to-five determinations per rat) was obtained by indirect tail-cuff plethysmography in unanaesthetized rats using an MK III physiograph (Narco Bio-System, Houston, TX). Twenty days after the induction of diabetes, the rats were sacrificed and the retinas were detached from the retinal pigmented epithelium cell layer and collected for protein extraction and colourimetric assays. In the retina, redox status markers were evaluated through production of superoxide anion, immunolocalization of nitrotyrosine and 8-hydroxy-2′-deoxyguanosine (8-OhdG) and by the measurement of reduced glutathione levels which is an important antioxidant defense system present in the retina.

Isolation of retina

The eyes were enucleated and the retinas were dissected and isolated from the pigmented epithelium and were lysed directly on ice in 300 μl of a buffer containing 2% SDS and 60 mmol Tris-HCl (pH 6.8) supplemented with a cocktail of protease inhibitors (Complete: contains antipain-HCl, chymostatin, leupeptin, bestatin, pepstatin, phosphoramidon, aprotinin and EDTA; Boehringer) [10]. After centrifugation, the supernatants were transferred to new tubes and the protein concentrations were measured by the Bradford method [11], using BSA as a standard.

Detection of superoxide anion

As previously described by Du et al. [9], the lucigenin (bis-N-methylacridinium nitrate), an acridylium dinitrate compound, was used to measure superoxide anion production. On the day of the study, rats were killed and both retinas were rapidly isolated and placed in polypropylene tubes containing 0.2 ml Krebs-HEPES buffer and incubated in the dark at 37°C in 95% O_2/5% CO_2 for a 30 min equilibration period. After equilibration, 0.5 mM lucigenin was added to the tube and photon emission was measured over 10 s, repeated measurements were made over a 10 min period in a luminometer (TD 20-E Luminometer Turner, USA). Reaction blanks (vials containing all components except retinas) were counted and these blank values subtracted from all other readings.

Determination of reduced glutathione (GSH) levels

Retinal GSH level was measured by the method described by Beutler et al. [12] with few modifications. The eyes were enucleated and the retinas were dissected and isolated from the pigmented epithelium and were lysed directly on ice in 300 μl of a buffer containing 10% tricloroacetic acid and 5 mM EDTA. The homogeneized was centrifugated at 3000 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 μM GSH per μg of retinal protein. GSH was used as an external standard for preparation of a standard curve.

Immunohistochemistry for nitrotyrosine and 8-hydroxy-2′-deoxyguanosine (8-OHdG) in retinal tissues

As previously described [13], paraformaldehyde 4%-fixed paraffin-embedded eye sections (4 μm) were dewaxed and rehydrated. Then the slides were put in citrate buffer (10 mM, pH 6) and exposed to microwave in a domestic oven (Panasonic Junior, Panasonic) for two cycles of 10 min at maximum potency. Slides were cooled in ice-cold water for 20 min, maintaining the slides in citrate buffer. Thereafter, the slides were put in 3% H_2O_2 methanol for 10 min to block endogenous peroxidase. The slides were incubated in 1% non-fat milk in PBS for 1 h at room temperature to block non-specific staining. Tissue sections were then incubated with a 1:200 dilution of a polyclonal rabbit anti-Nitrotyrosine antibody (Upstate Cell Signaling Solutions, Lake Placid, NY) and 1:50 dilution of a mouse monoclonal anti-8-OhdG antibody (N45.1; Japan Institute for the Control of Ageing, Japan) overnight at 4°C. After washing in PBS, alkaline phosphatase labelled polymer conjugated with 1:200 dilution of secondary anti-rabbit antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) for nitrotyrosine and 1:200 dilution of secondary anti-mouse antibody IgG antibody (Vector, Burlingame, CA) for 8-OhdG were applied for 1 h at room temperature. After washing in PBS, slides were incubated with 1:50 avidin-biotin complex (ABC) reagent (Dako, Glostrup, Denmark) for 30 min at room temperature. Then, 2 mg of diaminobenzidine tetrahydrochloride (DAB) in 5 mL of 1 M Tris-HCl, pH 7.5 plus 2.5 μL H_2O_2 were put in slides for 2 min. The slides were then counterstained with haematoxylin (only for Nitrotyrosine) and mounted with entellan and coverslip. For negative controls, staining was performed omitting the primary antibody or by using an irrelevant immunoglobulin (an immunoglobulin G which does not bind with the specific protein). The semi-quantitative analyses of the images of immunohistochemistry for nitrotyrosine were determined using the public domain program Image J (National Institutes of Health, http://rsb.info.nih.gov/ij). For 8-OHdG, the quantitative analyses were performed as a percentage of positive cells in the ganglion cell and inner nuclear layers scored as 0 (no positivity), 0.5 (up to 10% positivity), 1 (11–25% positivity), 1.5 (26–40% positivity), 2 (41–53% positivity), 2.5 (54–66% positivity), 3 (67–80% positivity) to 4 (>80% positivity) [14,15]. The positive cellular nuclei were counted by an observer with no knowledge of the studied groups in eight non-consecutive retinal sections distanced ~24 μm from each other under high power microscopic fields (×1000).

Western blotting for gp91phox and Nox-4

The role of the NADPH oxidase in the generation of superoxide anion was evaluated by its sub-units gp91phox and NOX-4 expressions in total retinal lysated.

NADPH oxidase is an enzyme system that source pro-oxidant superoxide in the vascular tissue and is also been linked to hypertension and inflammation [16]. For Western blot analysis [17], 100 μg for gp91phox and 50 μg for Nox-4 of total retinal protein in 5% glycerol/0.03% bromophenol blue/10 mmol dithiothreitol were loaded into 8% SDS polyacrylamide gels. Molecular weight markers (Rainbow; Amersham Pharmacia) were used as standards. Proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) in transfer buffer (50 mml Tris-HCl, pH 7.0, 380 mmol glycine, 0.1% SDS and 20% methanol). Membranes were blocked (for 1 h at room temperature in 2.5% non-fat milk) and incubated with rabbit polyclonal IgG gp91phox antibody (1:750; Upstate Cell Signaling Solutions, Lake Placid, NY) and rabbit polyclonal IgG Nox-4 antibody (1:2500 donated by Dr Karen Block, University of Texas Health Science Center at San Antonio, CA) for 1 h at room temperature. The blots were subsequently incubated with horseradish peroxidase HRP-conjugated anti-rabbit secondary antibody (1:10 000, Santa Cruz Biotechnologies, Santa Cruz, CA) and developed using chemiluminescence method (Super Signal CL-HRP Substrate System; Pierce, Rockford, IL). Exposed films were scanned with a densitometer (Bio-Rad) and analysed quantitatively with Multi-Analyst Macintosh Software for Image Analysis Systems. Western blots were repeated three-to-five times and gave qualitatively similar results. Equal loading and transfer was ascertained by reprobing the membranes for β-actin.

Statistical analysis

The results were expressed as the mean ± SD, unless otherwise stated. One-way analysis of variance (ANOVA) followed by Fisher's protected least-significant difference test was used to assess differences among the groups. All comparisons were carried out using the StatView statistical package software. A value of $p < 0.05$ indicated significance.

Results

Physiological characteristics of the studied groups

The weight at the sacrifice was lower following streptozotocin injection in both WKY and SHR rats

($p < 0.0001$). As expected, systolic blood pressure (SBP) was significantly higher in SHR than the WKY rats ($p < 0.005$) and blood glucose levels were higher in diabetic rats compared with non-diabetic groups ($p < 0.0001$) (Table I).

Superoxide anion production, reduced glutathione (GSH) and nitrotyrosine concentrations

As the redox state is largely dependent upon glutathione levels, which in turn influence nitration, we measured the production of superoxide anion production, reduced glutathione concentrations and protein tyrosine nitration in retinal tissue. We detected a significant increment of superoxide production in the diabetic WKY group ($p = 0.03$) and further increase was observed in both control and diabetic SHR groups compared with control WKY ($p < 0.0009$). This finding indicates that the presence of diabetes or hypertension solely induced an early increase of superoxide anion production, but the concomitance of hypertension and diabetes did not further increase the superoxide anion production in the retina tissue (Figure 1). Along with the superoxide production, an antioxidant defense system was accessed through the quantitative measurement of GSH levels in the retina tissue. After 20 days of diabetes, the GSH concentrations were slightly decreased in control SHR (by $\sim 4\%$) compared with WKY groups and markedly diminished in the presence of diabetes (by 15%, $p = 0.04$) (Figure 2). The nitrosative stress-induced protein modification was assessed in retinal tissue by immunohistochemistry for nitrotyrosine. We detected a moderate increase in both diabetic WKY and control SHR ($p = 0.03$) in comparison to control WKY. The concomitance of diabetes and hypertension lead to a marked increment of nitrotyrosine in all retinal layers ($p = 0.004$) (Figures 3A and B). Similarly, in the hypertensive rats, the redox state shows a tendency to unbalance the oxidative stress resulting in a moderate level of tyrosine nitration in the retinal tissue. Diversely, in the hypertensive and diabetic rats, the increment of the superoxide anion production with a significant diminishing in reduced glutathione levels lead to a profound tyrosine nitration in the retina tissue, as demonstrated in Figure 3.

Table I. Physiological characteristics of studied rats.

	Wc	Wd	Sc	Sd
n	15	19	18	19
Final body weight (g)	245±16	173±13$^{\pi}$	181±12	116±16$^{\pi}$
SBP (mmHg)	127±2.7	127±2.6	150±17.6$^{\blacklozenge}$	157±14.2$^{\blacklozenge}$
Glycaemia (mmol/L)	10.5±1.4	29.9±1.9$^{\pi}$	9.44±0.46	30.38±2.9$^{\pi}$

Wc: control WKY; Wd: diabetic WKY; Sc: control SHR; Sd: diabetic SHR; SBP: systolic blood pressure.
$^{\pi}p < 0.0001$ vs respective control groups, $^{\blacklozenge}p < 0.005$ vs WKY groups.

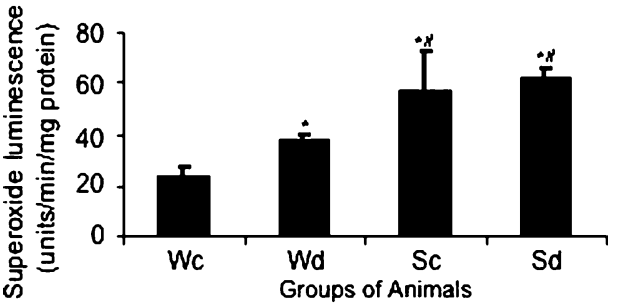

Figure 1. Production of superoxide anion using the lucigenin enhanced method from control and diabetic WKY and SHR rat retinas. Diabetes or hypertension increased superoxide production (units/min/mg protein) in retina. Bars represent mean±SD. $^{*}p < 0.03$ vs Wc, $^{\#}p < 0.009$ vs WKY groups. All data shown are of $n \geq 6$ observations.

Oxidative damage of nucleic acids

We studied the expression of 8-OHdG, a modification of DNA base guanine-containing nucleoside 2′-deoxyguanosine, by immunohistochemical assay. We demonstrated a moderate increase in both diabetic WKY and control SHR ($p = 0.2$) in comparison to control WKY. The concomitance of short-term diabetes and genetic hypertension resulted in distinguishable DNA damage in the retinal cells ($p < 0.0001$) (Figures 4A and B). Accordingly, both evaluation parameters of oxidative citotoxicity in the retina, nitration of tyrosine and 8OHdG positivity indicate that the concomitance of diabetes and hypertension are deleterious to retinal cells leading to diffuse cell damage in retinal tissue.

The role of NADPH oxidase in production of anion superoxide

To study the participation of this enzyme system in the pathogenesis of retinal oxidative stress, we investigated the expressions of its sub-units gp91phox and Nox4 in total retinal lysates. The expressions of

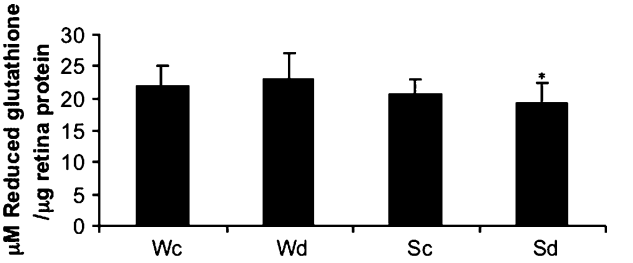

Figure 2. Concentration of reduced glutathione (GSH) from retina of control and diabetic WKY and SHR rats (μM glutathione/μg of retinal protein). There was a significant decreasing in reduced glutathione levels only in diabetic SHR retina rats. Bars represent mean±SD. $^{*}p < 0.04$ vs Wd.

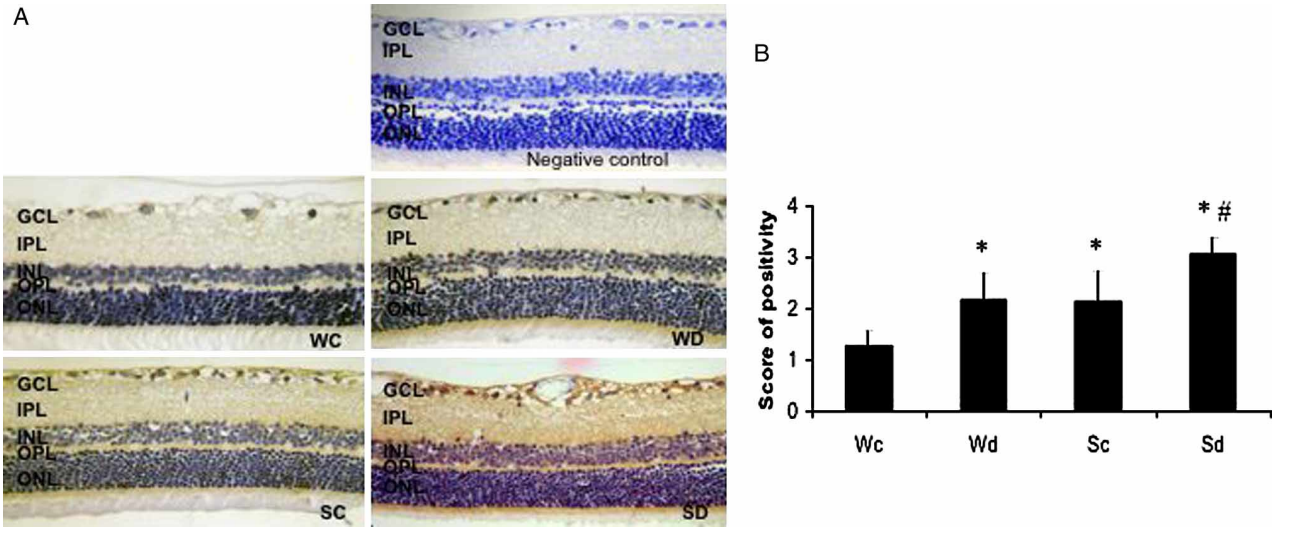

Figure 3. (A) Representative photomicrograph of immunolocalization of nitrotyrosine in control and diabetic WKY and SHR rat retina. Presence of nitrotyrosine is indicated in brown colour. (B) Bars represent mean ± SD of intensity of nitrotyrosine per retinal section as defined in the Methods section. Diabetes and hypertension solely increased tirosyne nitration in retinal tissue and further nitration was observed when both were present. $^{\star}p < 0.03$ vs Wc, $^{\#}p < 0.02$ vs other groups.

both, evaluated by Western blot assay, were not different among different groups (Figures 5A and B).

Discussion

These evidences suggest that the presence of hypertension may exacerbate the hyperglycaemia-induced oxidative damage in the retina.

In the present study, we investigated the possible effects of arterial hypertension on early oxidative stress phenomena in the pathogenesis of DR. Experimental diabetes was simultaneously induced in genetic hypertensive SHR rats and its normotensive control WKY and the superoxide production, reduced glutathione levels, tyrosine nitration and 8-OHdG in the retinal tissue were analysed after 20 days. We demonstrated that, in normotensive short-term diabetic rats, there was a significant increment in superoxide production, which was at least in part equilibrated by the retinal antioxidant defense system resulting in a moderate tyrosine nitration, a marker of nitrosative stress. The concomitance of both diabetes and hypertension clearly promoted an extensive and profound tyrosine nitration and DNA damage in the diabetic retina due to increment of superoxide production and significant

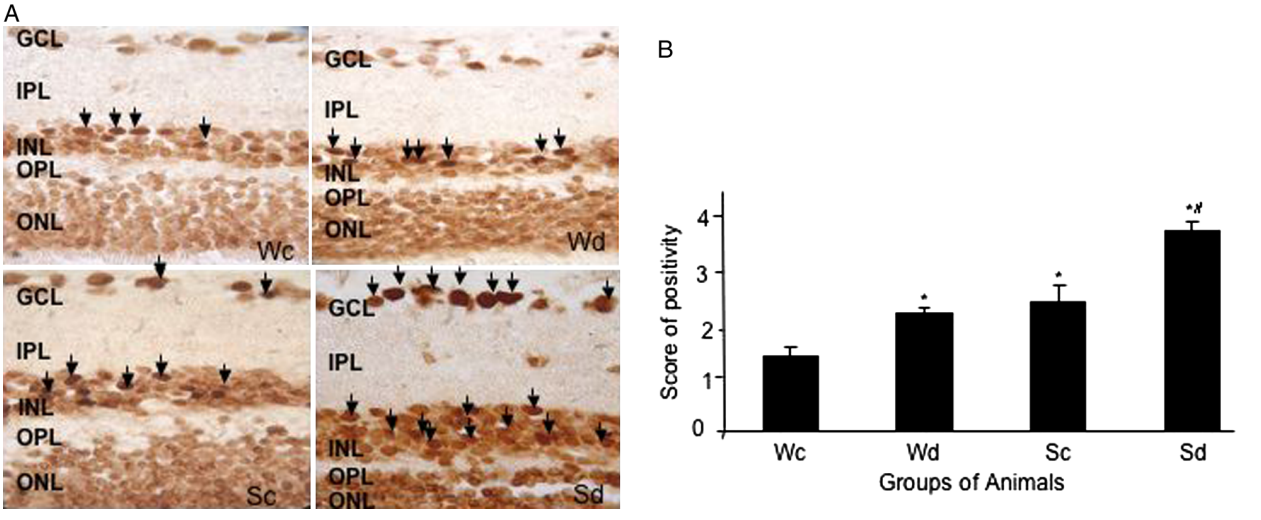

Figure 4. (A) Representative photomicrograph of immunohistochemistry for 8-hydroxi-2′-deoxiguanosine (8OHdG) from control and diabetic WKY and SHR rat retinas. The 8OHdG positive cells are indicated by arrows. (B) Bars represent mean ± SD of score of positivity of 8OHdG per retinal section as defined in Methods section. Similarly, as observed in Figure 4, diabetes or hypertension solely induced DNA damage by oxidative stress and further damage was observed in concomitance of both factors; $^{\star}p = 0.02$ vs Wc; $^{\#}p < 0.0001$ vs Wc.

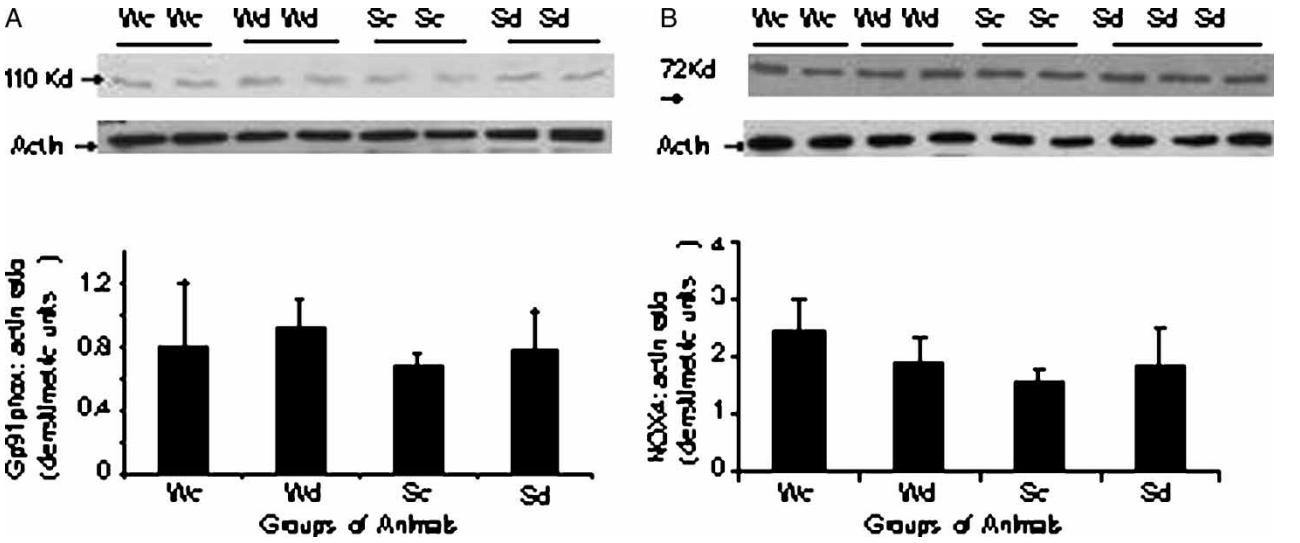

Figure 5. (A) Western blot analysis of gp91phox from control and diabetic WKY and SHR rat retinas. The film represents the western blot assay for gp91phox and the bars represent mean ± SD of band densities expressed in arbitrary densitometric units. (B) The membranes were incubated with NOX 4 antibody. The bars represent mean ± SD of band densities expressed in arbitrary densitometric units. The membranes were reprobed with anti-β-actin antibody as a control for protein loading. Wc: control WKY, Wd: diabetic WKY, Sc: control SHR, Sd: diabetic SHR.

diminishing retinal level of reduced glutathione. Of interest, the presence of diabetes did not reveal an additional effect on superoxide production among hypertensive rats, which was already significantly higher than the normotensive rats. One can speculate that the superoxide production was too high in the retina from hypertensive rats so that no additional production could be possible in retinal tissue. These findings provided experimental evidence that hypertension may contribute to early aggravation of DR through oxidative stress unbalance.

Diabetes-related complications promote an imbalance between the production and defense against free radical species in the retina. The term free radical species encompasses several highly reactive molecules that include reactive oxygen species and reactive nitrogen species, superoxide and NO being prominent members of those groups. The diabetes-induced increase in nitration of retinal proteins [18] (presumably via peroxynitrite) suggests that both superoxide and NO are present in excessive amounts. Our studies demonstrate that the concomitance of diabetes and hypertension results in an increase of superoxide production in the retina accompanied by an impaired antioxidant defense system leading to massive tyrosine nitration and DNA damage. Additionally, in the present paper, the role of NADPH oxidase to retinal production of superoxide in short-term diabetes was minor or did not contribute to oxidative stress in the retina. The contribution of these enzymes to superoxide production might differ between specific cell types.

Mitochondria are important endogenous source of superoxides and hydroxyl radicals [9] and are pivotal in the mechanism for diabetic complications [19]. Reactive oxidant species can trigger mitochondria to release cytochrome *c*, resulting in activation of apoptotic cascade [20,21]. Mitochondrial changes are associated with the activation of apoptotic pathways resulting in impaired kidney function [22] and myocardial abnormalities [23]. However, the involvement of the mitochondrial dysfunction on an experimental model of hypertension and DM was never addressed.

As previously demonstrated by Nishikawa et al. [24], the excessive production of superoxide has been reported to stimulate several biochemical sequelae of hyperglycaemia (protein kinase C, polyol pathway and advanced glycation pathways). Nevertheless, superoxide generation can have deleterious effects on cells even independent of these pathways. Superoxide also can react with NO to form peroxynitrite, a highly reactive oxidant [25,26], which can result in cytotoxicity due to lipid peroxidation, inactivation of enzymes by oxidation of protein sulphydryls and nitration of tyrosines and damage to DNA. The hydroxyl free radical (OH^-) induces lipid peroxidation and DNA damage by reacting with the ring structure of guanine in DNA. As a result the 8-hydroxy-2′-deoxyhuanosine (8OHdG) formed causes chemical alterations of the bases and DNA breakage. Imperfect repair of DNA can lead to mutations, arrest cell cycle or induce apoptosis [27]. Of interest, apoptosis is now described as the main mechanism by which neuro-glial and vascular cells from diabetic retina are lost in the early pathogenesis of DR [28,29].

In the literature, there is no data of the possible effects of arterial hypertension on the redox state of the retina, one of its target organs for microvascular complications. Hypertension has been shown to cause oxidative stress and inflammation in renal and cardiovascular tissues in experimental animals. Our recent paper examining whether the presence of hypertension stimulates early inflammatory changes in the retina in diabetic rats revealed that the combination of diabetes and hypertension markedly increases the retinal inflammatory events that could be completely prevented by the prevention of development of hypertension. It is suggested that the inflammatory events may be involved in the mechanism by which hypertension exacerbates retinopathy in patients with diabetes [30]. Previous experimental studies using a salt-sensitive hypertension model demonstrated that enhancing the glutathione system with dietary N-acetylcysteine administration attenuates the renal damage that normally occurs in this model, indicating that the oxidative stress acts upon the development of hypertension-related microvascular complications [31].

Our findings demonstrated that NADPH oxidase did not play a significant role in superoxide production in the retina from diabetic rats. Mitochondrial dysfunction associated with membrane depolarization, superoxide production and induction of programmed cell death (apoptosis) are under investigation in a model that combines diabetes and hypertension.

In conclusion, we first demonstrated that, in an experimental animal model that combines diabetes and hypertension, there is an early and deep imbalance in the redox status, leading to nitration of tyrosine and DNA damage of retinal cells. The redox imbalance is due to increasing the superoxide production in the presence of an inadequate antioxidative system. These evidences suggest that the presence of hypertension may exacerbate the hyperglycaemia-induced oxidative damage in the retina and may contribute to a better understanding of the mechanism of exacerbation of diabetic retinopathy in the presence of hypertension.

Acknowledgements

This work was supported by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) and Camila Cerboni Pinto received a scholarship from CAPES. The authors wish to thank Dr Karen Block from University of Texas Health Science Center, San Antonio, CA, USA, for donation of Nox-4 antibody and Bruno Sevá Pessoa, Elisa B. M. I. Peixoto and Denise Silvia de Souza for assistance in laboratory procedures and Sérgio Magalhães for technical assistance.

References

[1] Patz A, Smith RE. The ETDRS and diabetes 2000. Ophthalmology 1991;98:739–740.
[2] Klein R, Klein BAK, Davis MD. Is cigarette smoking associated with diabetic retinopathy? Am J Epidemiol 1983;118:228–238.
[3] Janka HU, Ziegler AG, Valsania P, Warram JH, Krolewski AS. Impact of blood pressure on diabetic retinopathy. Diabete Metab 1989;15:333–337.
[4] United Kingdom Prospective Diabetes Study Group. Tight blood pressure control and risk of macrovascular and microvascular complications in type 2 diabetes: UKPDS 38. BMJ 1998;317:703–713.
[5] Armstrong D, Abdella N, Salman A, Miller N, Rahman EA, Bojancyzk M. Relationship of lipid peroxides to diabetic complications. Comparison with conventional laboratory tests. J Diabetes Complications 1992;6:116–122.
[6] Kowluru RA, Tang J, Kern TS. Abnormalities of retinal metabolism in diabetes and experimental galactosemia: VII. Effect of long-term administration of antioxidants on the development of retinopathy. Diabetes 2001;50:1938–1942.
[7] Kowluru RA, Odenbach S. Effect of long-term administration of α-Lipoic acid on retinal capillary cell death and the development of retinopathy in diabetic rats. Diabetes 2004;53:3233–3238.
[8] Jain SK. Superoxide dismutase overexpression and cellular oxidative damage in diabetes: a commentary on 'Overexpression of mitochondrial superoxide dismutase in mice protects the retina from diabetes-induced oxidative stress'. Free Radic Biol Med 2006;41:1187–1190.
[9] Du Y, Miller CM, Kern TS. Hyperglycemia increases mitocondrial superoxide in retina and retinal cells. Free Radic Biol Med 2003;35:1491–1499.
[10] Silveira LA, Bacchi CE, Pinto GA, De Faria JB. The genetics of hypertension modifies the renal cell replication response induced by experimental diabetes. Diabetes 2002;51:1529–1534.
[11] Bradford MM. A rapid and sensive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 1976;72:248–254.
[12] Beutler E, Duron O, Kelly BM. Improved method for the determination of blood glutathione. J Lab Clin Med 1963;16:882–888.
[13] 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.
[14] Mezzano S, Droguett A, Burgos ME, Ardiles LG, Flores CA, Aros CA, Caorsi I, Vio CP, Ruiz-Ortega M, Egido J. Renin-angiotensin system activation and interstitial inflammation in human diabetic nephropathy. Kidney Int Suppl 2003;86:S64–S70.
[15] Nangaku M, Miyata T, Sada T, Mizuno M, Inagi R, Ueda Y, Ishikawa N, Yuzawa H, Koike H, van Ypersele de Strihou C, Kurokawa K. Anti-hypertensive agents inhibit *in vivo* the formation of advanced glycation end products and improve renal damage in a type 2 diabetic nephropathy rat model. J Am Soc Nephrol 2003;14:1212–1222.
[16] Griendling KK, Sorescu D, Ushio-Fukai M. NADPH oxidase: role in cardiovascular biology and disease. Circ Res 2000;86:494–501.
[17] Biswas SK, Lopes de Faria JB. Which comes first: renal inflammation or oxidative stress in spontaneously hypertensive rats? Free Rad Res 2007;41:216–224.
[18] Du Y, Smith MA, Miller CM, Kern TS. Diabetes-induced nitrative stress in the retina, and correction by aminoguanidine. J Neurochem 2002;80:771–779.

[19] Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. Diabetes 2005;54:1615–1625.
[20] Phaneuf S, Leeuwenburg C. Cytochrome c release from mitochondria in the aging heart: a possible mechanism for apoptosis with age. Am J Physiol 2002;282:R423–R430.
[21] De Giorgi F, Lartigue L, Bauer MK, Schubert A, Grimm S, Hanson GT, Remington SJ, Youle RJ, Ichas F. The permeability transition pore signals apoptosis by directing Bax translocation and multimerization. FASEB J 2002;16:607–609.
[22] Verzola D, Bertolotto MB, Villaggio B, Ottonello L, Dallegri F, Frumento G, Berruti V, Gandolfo MT, Garibotto G, Deferran G. Taurine prevents apoptosis induced by high ambient glucose in human tubule renal cells. J Invest Med 2002;50:443–451.
[23] Cai L, Li W, Wang G, Guo L, Jiang Y, Kang YJ. Hyperglycemia-induced apoptosis in mouse myocardium: mitochondrial cytochrome c-mediated caspase-3 activation pathway. Diabetes 2002;51:1938–1948.
[24] Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 2000;404:787–790.
[25] Tamir S, Burney S, Tannenbaum SR. DNA damage by nitric oxide. Chem Res Toxicol 1996;9:821–827.
[26] Burney S, Caulfield JL, Niles JC, Wishnok JS, Tannenbaum SR. The chemistry of DNA damage from nitric oxide and peroxynitrite. Mutat Res 1999;424:37–49.
[27] Evans MD, Dizdaroglu M, Cooke MS. Oxidative DNA damage and disease: induction, repair and significance. Mutat Res 2004;567:1–61.
[28] Barber AJ, Lieth E, Khin SA, Antonetti DA, Buchanan AG, Gardner TW. Neural apoptosis in the retina during experimental and human diabetes. Early onset and effect of insulin. J Clin Invest 1998;102:783–791.
[29] Joussen AM, Poulaki V, Mitsiades N, Cai WY, Suzuma I, Pak J, Ju ST, Rook SL, Esser P, Mitsiades CS, Kirchhof B, Adamis AP, Aiello LP. Suppression of Fas-FasL-induced endothelial cell apoptosis prevents diabetic blood-retinal barrier breakdown in a model of streptozotocin-induced diabetes. FASEB J 2003;17:76–78.
[30] Silva KC, Pinto CC, Biswas SK, Souza DS, Lopes de Faria JB, Lopes de Faria JM. Prevention of hypertension abrogates early inflammatory events in the retina of diabetic hypertensive rats. Exp Eye Res 2007;85(1):123–129.
[31] Tian N, Rose RA, Jordan S, Dwyer TM, Hughson MD, Manning Jr RD. N-Acetylcysteine improves renal dysfunction, ameliorates kidney damage and decreases blood pressure in salt-sensitive hypertension. J Hypertension 2006;24:2263–2270.