

*BRUNO SEVÁ PESSÔA*

**O BLOQUEIO DO RECEPTOR MINERALOCORTICÓIDE  
MELHORA A NEFROPATIA PELO AUMENTO DA ATIVIDADE DA  
GLICOSE-6-FOSFATO DESIDROGENASE E REDUÇÃO DO  
ESTRESSE OXIDATIVO EM RATOS DIABÉTICOS HIPERTENSOS**

*CAMPINAS  
2010*

**BRUNO SEVÁ PESSÔA**

**DISSERTAÇÃO DE MESTRADO**

**O BLOQUEIO DO RECEPTOR MINERALOCORTICÓIDE MELHORA A  
NEFROPATIA PELO AUMENTO DA ATIVIDADE DA GLICOSE-6-FOSFATO  
DESIDROGENASE E REDUÇÃO DO ESTRESSE OXIDATIVO EM RATOS  
DIABÉTICOS HIPERTENSOS**

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***Orientador: Prof. Dr. José Butori Lopes de Faria***

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**Aos meus pais e irmãos;  
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que me deram nesses últimos anos.**

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Foi demonstrado, em modelos de diabetes tipo 1 e tipo 2, que o uso de espironolactona (SPR), um antagonista do receptor mineralocorticóide, possui efeitos benéficos na lesão renal através de mecanismos anti-inflamatórios e antioxidantes. Os efeitos de um antagonista do receptor mineralocorticóide na nefropatia diabética (ND) parecem ser independentes da redução da pressão arterial (PA) e do bloqueio do sistema renina-angiotensina-aldosterona (SRAA). Em modelos *in vitro* e *in vivo* a aldosterona promoveu aumento do estresse oxidativo através da enzima glicose-6-fosfato desidrogenase (G6PD). A atividade da G6PD foi reduzida pelo aumento da aldosterona e da glicose, reduzindo a capacidade antioxidante e aumentando o estresse oxidativo.

Neste trabalho investigamos se o uso da SPR melhora a ND em animais diabéticos e hipertensos pela melhora na atividade da G6PD, reduzindo o estresse oxidativo independentemente dos efeitos na PA e na glicemia.

Ratos espontaneamente hipertensos (SHR) foram tornados diabéticos pela injeção de estreptozotocina (STZ) e os animais controles receberam apenas o veículo. Os animais diabéticos foram randomizados para receber ou não SPR na água de beber. Após 8 semanas de tratamento os animais foram eutanaziados e os rins coletados para as análises.

A glicemia foi maior nos animais diabéticos e não foi modificada pelo tratamento. Não houve alteração na PA nos grupos diabéticos e diabéticos tratados. A albuminúria e a expressão renal de fibronectina foi maior no grupo diabético em relação ao controle e esses parâmetros foram reduzidos com o tratamento. A atividade da G6PD e a relação glutatona reduzida (GSH) / glutatona oxidada (GSSG) foram reduzidas no grupo diabético e o tratamento restaurou ao nível dos controles. Os níveis urinários de 8-OHdG e TBARS foram maiores nos animais diabéticos e o tratamento reduziu ao nível dos controles. A produção de superóxido via NADPH oxidase e a expressão da subunidade p47phox da

NADPH oxidase foram maiores no grupo diabético em relação ao controle e o tratamento reduziu esses parâmetros significativamente.

Os resultados sugerem que a SPR melhora a nefropatia em ratos diabéticos e hipertensos por restaurar a atividade da G6PD, diminuindo o estresse oxidativo sem afetar a PA e a glicemia.

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**ABSTRACT**

Strict glycemic management, control of blood pressure, and use of drugs that interfere with the renin angiotensin system are the most effective interventions for prevention and treatment of diabetic nephropathy. In addition, recent studies have suggested that beneficial effects of aldosterone blockade on diabetic nephropathy seem to be independent of blood pressure reduction and renin–angiotensin blockade on diabetic nephropathy. It has been demonstrated that type 1 and type 2 diabetic animal models treated with aldosterone blocker had a beneficial effects in renal tissue through antioxidants and anti-inflammatory mechanisms. *In vitro* and *in vivo* studies showed a decreased in G6PD activity in high glucose and aldosterone levels leading to an increased in oxidative stress.

In the present study we investigated whether spironolactone improves nephropathy by increasing G6PD activity and reducing oxidative stress in hypertensive diabetic rats.

Spontaneously hypertensive rats were rendered diabetic by intravenous injection of streptozotocin. The diabetic animals were randomized to receive or not receive spironolactone for 8 weeks.

Plasma glucose levels were higher in diabetic rats and it was not modified by spironolactone. Likewise, systolic blood pressure was unaltered by diabetes or by treatment. Albuminuria and renal expression of fibronectin were higher in the diabetic group compared to control, and these parameters were reduced with aldosterone blockade. G6PD activity and the GSH / GSSG ratio were reduced in diabetic rats and the treatment restored to control levels. Urinary levels of 8-OHdG and TBARS renal cortex levels, a marker of oxidative stress, were higher in diabetic rats when compared to controls, and the treatment reduced to control levels. The production of superoxide induced by NADPH

oxidase and p47phox, an isoform of NADPH oxidase, was higher in diabetic rats when compared to controls and was significantly reduced in treated rats.

These results suggest that spironolactone ameliorates nephropathy in the diabetic hypertensive rats by restoring G6PD activity and diminishes oxidative stress without affecting blood pressure.

## *LISTA DE ABREVIATURAS*

---

8-OHdG	8-hidroxi-2'-deoxiguanosina
AGE	produtos avançados da glicosilação não enzimática
ANG II	angiotensina II
Cu/Zn SOD	cobre/zinco superóxido desmutase
DM	diabetes mellitus
ECA	enzima conversora de angiotensina
EcSOD	extracelular superóxido desmutase
ERO	espécies reativas de oxigênio
GSH	glutathiona reduzida
GSSG	glutathiona oxidada
H <sub>2</sub> O <sub>2</sub>	peróxido de hidrogênio
HA	hipertensão arterial
IRC	insuficiência renal crônica
MnSOD	manganês superóxido desmutase
ND	nefropatia diabética
NAD <sup>+</sup>	nicotinamida adenina dinucleotídeo
NADPH	nicotinamida adenine dinucleotídeo fosfato (forma reduzida)
NF-κB	fator de transcrição nuclear-κB
NO•	óxido nítrico
O <sub>2</sub> • <sup>-</sup>	superóxido
OH•	radical hidroxil
ONOO <sup>-</sup>	peroxinitrito

PA	pressão arterial
PKC	proteína quinase C
SHR	ratos espontaneamente hipertensos
SOD	superóxido desmutase
SPR	espironolactona
SRAA	sistema renina-angiotensina-aldosterona
STZ	estreptozotocina
TGF- $\beta$	fator de crescimento de transformação- $\beta$

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## **1. Introdução**

### **1.1. Nefropatia diabética**

O *diabetes mellitus* (DM) está relacionado à danos, disfunção e falência de vários órgãos, especialmente rins, olhos, nervos, coração e vasos sanguíneos (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003). Todas as formas de DM são caracterizadas por danos microvasculares nos rins, na retina e nervos periféricos e como consequência o DM é a principal causa de doença renal terminal, cegueira e neuropatia. (Brownlee, 2001).

Uma complicação importante no DM é a nefropatia diabética (ND), principal responsável pela insuficiência renal crônica (IRC) (Ritz et al., 1999), afetando mais de 50 milhões de pessoas no mundo (National Kidney Foundation, 2007). Estudo epidemiológico determinou que 41% dos pacientes diabéticos tipo 1 desenvolveram ND (Andersen et al., 1983), já os pacientes diabéticos tipo 2 estão sujeitos aos mesmos riscos quando apresentam proteinúria por mais de três anos (Hasslacher et al., 1989). Fatores genéticos também favorecem o desenvolvimento de ND. Estudo mostra que 83% dos irmãos de pacientes diabéticos com ND apresentam evidências de nefropatia (Seaquist et al., 1989).

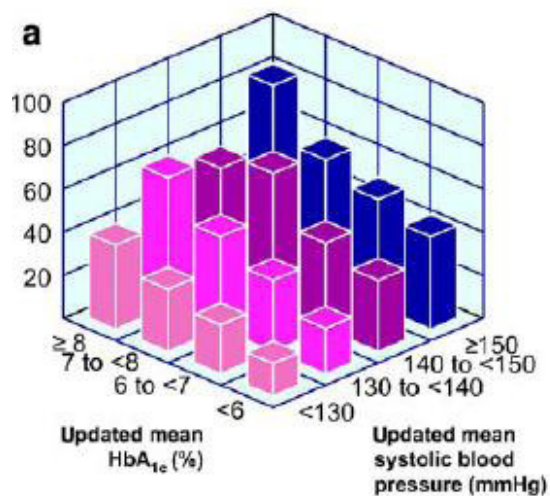
A ND é caracterizada pelo aumento na excreção urinária de albumina, parâmetro funcional utilizado como marcador precoce da doença. A doença também apresenta alterações estruturais como o espessamento da membrana basal glomerular, hipertrofia glomerular e expansão mesangial (Cooper, 1998). A alteração na expressão de proteínas de matriz extracelular como colágeno e fibronectina estão associadas ao declínio na filtração

glomerular e à redução da superfície de filtração glomerular. Essas alterações estruturais e funcionais podem causar o comprometimento da função glomerular e promover alterações progressivas culminando em IRC (Sheetz e King, 2002).

As interações entre fatores metabólicos e hemodinâmicos são responsáveis pelas alterações estruturais e funcionais da ND e clinicamente associadas ao DM e a hipertensão arterial (HA) (Adler, 2000; Stratton, 2000). A ativação da via do poli-ol, de produtos avançados da glicosilação não enzimática (AGE), o aumento do estresse oxidativo, o aumento da pressão arterial (PA) e a ativação do sistema renina-angiotensina-aldosterona (SRAA) promovem danos que podem contribuir para a progressão da ND (Cooper, 1998).

## **1.2. Hipertensão arterial**

A HA é o principal fator de risco secundário para o desenvolvimento e progressão da ND (Cooper et al., 1988). De acordo com Stratton e colaboradores, em 2006, pessoas que apresentam HA e DM estão sujeitas à maior risco de complicações do que pessoas com apenas um dos fatores, HA ou hiperglicemia. Sabe-se que a prevalência da hipertensão é elevada em pacientes diabéticos e pessoas com elevada PA apresentam 2,5 vezes mais probabilidade de desenvolver DM em 5 anos (Gress et al., 2000; Sowers e Haffner, 2002). Portanto, a diminuição da PA assim como da glicemia podem diminuir os riscos de complicações associadas ao DM. A figura 1 apresenta o risco de complicações associadas ao DM em relação à HA e à hiperglicemia.

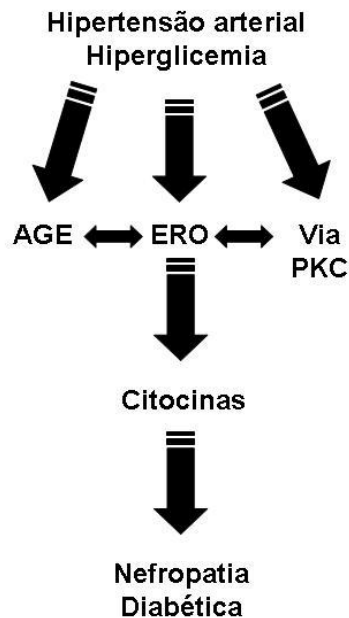


**Figura 1:** Risco de complicações associadas ao DM em relação à HA e à hiperglicemia (Stratton et al., 2006).

### 1.2.1. Interações entre hipertensão arterial e nefropatia diabética

A HA e a ND estão associados aos fatores genéticos. Famílias com histórico de HA e irmãos de pacientes com ND apresentam maior risco de doença renal (Cooper, 1998). Outro estudo demonstra que pais não diabéticos de pacientes com ND apresentavam níveis de pressão arterial maiores em relação aos pais não diabéticos de pacientes diabéticos sem ND (Viberti et al., 1987).

A HA e a hiperglicemia podem promover danos por aumentar a produção de espécies reativas de oxigênio (ERO), AGE, ativação da via da PKC e angiotensina II (ANG II), culminado na produção e ativação de vários fatores e citocinas que contribuem para a progressão da ND (Wolf, 2004). A figura 2 apresenta esquematicamente as principais interações entre HA e hiperglicemia envolvidas na progressão da ND.



**Figura 2:** Principais fatores envolvidos na progressão da ND (adaptado de Wolf, 2004).

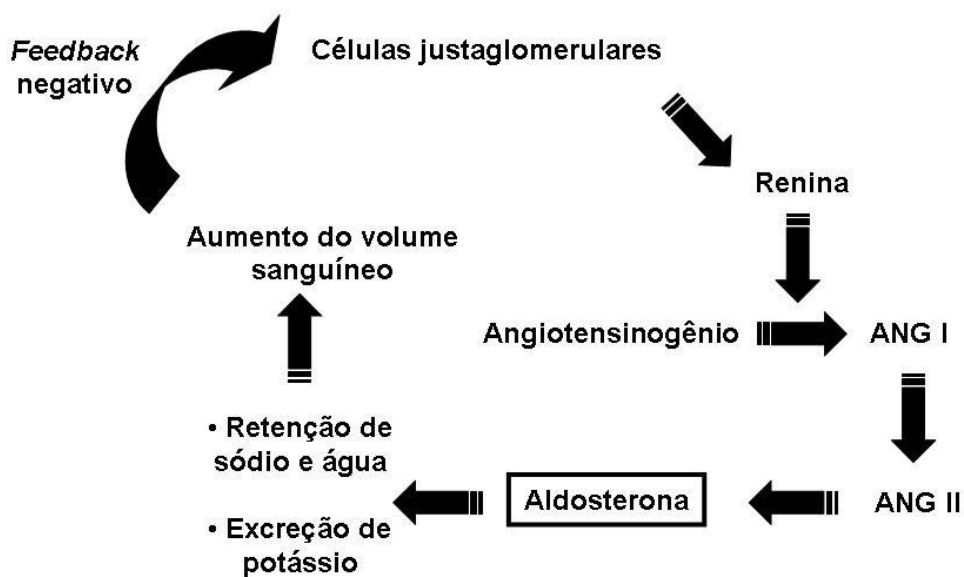
Em modelo animal de ratos de espontaneamente hipertensos (SHR) e DM, induzido pela injeção de estreptozotocina, é possível o estudo da interação entre HA e DM. Esse modelo permite o entendimento desses fatores no desenvolvimento e progressão da ND (Cooper et al., 1988). O modelo SHR com DM apresenta uma acentuada albuminúria em relação ao animal SHR não diabético e ao seu controle normotenso, o rato wistar-kyoto (WKY) (Cooper et al., 1988), além de um acúmulo renal precoce de fibronectina (Righetti et al., 2001). Já o tratamento da hipertensão com foi capaz de corrigir alterações como a albuminúria, hipertrofia glomerular e fibronectina em animais SHR diabéticos (Amazonas

e Lopes de Faria, 2006). Porém, o mecanismo fisiopatológico da interação entre DM e HA no desenvolvimento da nefropatia ainda é pouco conhecido.

### **1.3. Sistema renina-angiotensina-aldosterona**

O SRAA é o mais conhecido regulador da pressão arterial, exercendo um papel importante na doença renal (Hostetter e Ibrahim, 2003). A ativação desse sistema tem, provavelmente, origem multifatorial (Campese e Park, 2006). A renina liberada primariamente pelas células justaglomerulares atua na clivagem do angiotensinogênio formando angiotensina I (ANG I), esta, por sua vez, é convertida em ANG II pela enzima conversora de angiotensina (ECA), encontrada na membrana celular das células endoteliais renais e pulmonares. A ativação dos receptores de angiotensina promovem a síntese de aldosterona e esta se liga aos receptores mineralocorticóides expressos no rim, ocasionando a retenção de sódio e água e a excreção de potássio. Esses efeitos promovem o aumento do volume sanguíneo e, conseqüentemente, aumento da PA (Brewster e Perazella, 2004). A figura 3 resume esquematicamente o SRAA.





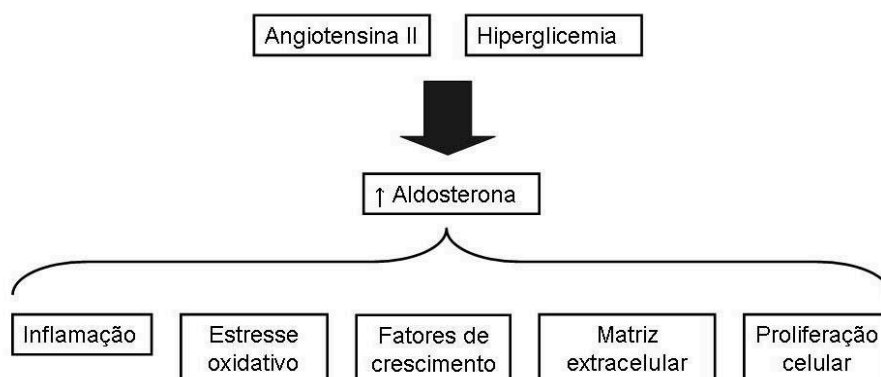
**Figura 3:** Representação esquemática do SRAA (Adaptado de Brewster e Perazella, 2004).

### 1.3.1. O sistema renina-angiotensina-aldosterona na progressão da nefropatia diabética

O controle da PA, a ativação do SRAA e a proteinúria são importantes fatores na progressão da ND (Mehdi et al., 2009). O SRAA está intimamente envolvido no processo de progressão da ND sendo a ANG II um dos principais mediadores da injúria renal. Em modelos diabéticos ocorre um aumento da síntese de aldosterona, causando maior expressão de ANG II e conseqüente aumento da produção ERO, síntese de citocinas e fatores de crescimento (Remuzzi et al., 2008). O aumento da expressão dessas moléculas contribuem para a proliferação anormal de células e fibrose renal através de níveis elevados

de fator de crescimento do endotélio vascular (VEGF), “*transforming growth factor-β*” (TGFβ), colágeno e fibronectina (Brownlee, 2001; Brewster e Perazella, 2004).

A ativação do SRAA, com aumento dos níveis de aldosterona, também promove aumento na proteinúria, contribuindo para a progressão da ND (Brewster e Perazella, 2004) através do acúmulo de matriz extracelular, fibrose, inflamação, estimulação da produção de ERO, danos endoteliais e proliferação celular (Del Vecchio et al., 2007) e induz apoptose em células tubulares (Patni et al., 2007). Outro trabalho demonstrou que a aldosterona também promove a síntese de colágeno tipo IV em cultura de células mesangiais (Wakisaka et al., 1994). Estudo com modelo animal e cultura de células demonstrou que o aumento da aldosterona prejudica a reatividade vascular por diminuição da atividade da enzima glicose-6-fosfato-desidrogenase (G6PD) (Leopld et al., 2007). Os efeitos da aldosterona no organismo são complexos e compreendem muitos mecanismos fisiopatológicos (Delyani, 2000). Resumidamente, a figura 4 apresenta os principais fatores envolvidos no aumento da aldosterona e as mais comuns consequências no tecido renal.



**Figura 4:** Efeitos da angiotensina II e da hiperglicemia no aumento da aldosterona no tecido renal (adaptado de Remuzzi et al., 2008).

#### 1.4. Estresse oxidativo

O estresse oxidativo é definido como um desbalanço entre agentes pró-oxidantes e antioxidantes, em favor da oxidação (Jones, 2006). Para melhor entender o estresse oxidativo é importante definir seus agentes. Um agente pró-oxidante é definido como uma substância capaz de gerar espécies reativas ou de induzir estresse oxidativo. Já os antioxidantes são substâncias que reduzem ou impedem a oxidação, mesmo em baixas concentrações comparadas ao substrato (Halliwell e Whiteman, 2004). Os radicais livres são agentes que contêm um ou mais elétrons não pareados e essa característica os tornam reativos à outras moléculas ou substâncias. Existem espécies reativas não radicais, ou seja, não apresentam elétrons livres mas são capazes de reagirem com moléculas ou substâncias (Halliwell, 1989). Portanto, os agentes antioxidantes são moléculas ou substâncias que agem neutralizando espécies reativas, permitindo que estas espécies sejam úteis nas funções biológicas sem causar danos (Halliwell e Gutteridge, 2006). Várias espécies reativas estão envolvidas no desenvolvimento e progressão de doenças crônicas, como o DM (Baynes e Thorpe, 1999).

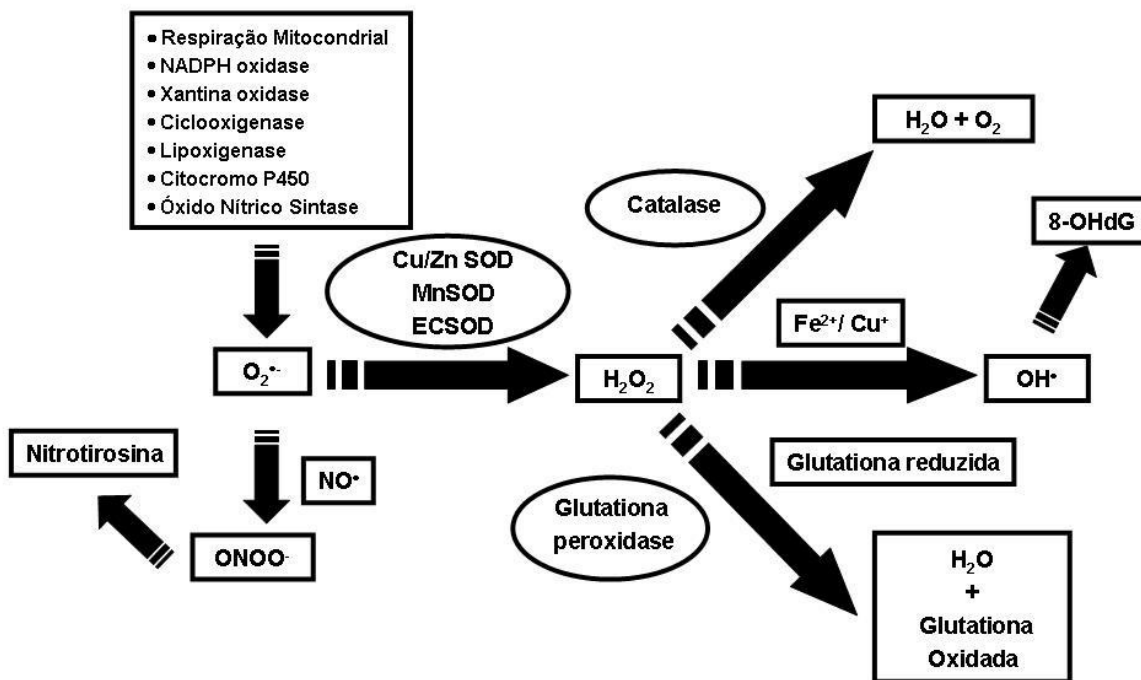
São definidas três classes de espécies reativas: espécies reativas do oxigênio (ERO), espécies reativas de nitrogênio (ERN) e espécies reativas de cloro (ERC). Em ERO estão incluídos oxigênios radicais (por exemplo, o superóxido;  $O_2^{\bullet-}$ ) e não radicais (por exemplo, o peróxido de hidrogênio;  $H_2O_2$ ) que são agentes pró-oxidantes e/ou são convertidos em radicais livres facilmente (Halliwell, 2006). Dentre as ERN, o óxido nítrico ( $NO^{\bullet}$ ) e o dióxido de nitrogênio ( $NO_2^{\bullet}$ ) são exemplos de radicais e peroxinitrito ( $ONOO^-$ ) é um

exemplo de espécie reativa não radical. Como exemplo de ERC radical temos o átomo cloro ( $\text{Cl}^\bullet$ ) e ERC não radical, o ácido hipocloroso ( $\text{HOCl}$ ) (Halliwell e Whiteman, 2004).

O desequilíbrio agentes pró-oxidantes e antioxidantes, em favor da oxidação, pode promover danos no DNA, lipídeos, proteínas e outras moléculas (Halliwell e Whiteman, 2004). O radical hidroxil ( $\text{OH}^\bullet$ ) induz a peroxidação lipídica e a hidroxilação do DNA. A reação do hidroxil com o DNA forma o radical 8-hidroxi-2'-deoxiguanosina (8-OHdG) que pode causar alterações químicas no DNA, acarretando mutações, interrupção do ciclo celular ou apoptose (Evans et al., 2004). O ânion superóxido pode reagir com o óxido nítrico e formar o peroxinitrito ( $\text{ONOO}^-$ ), um agente pró-oxidante que danifica DNA, lipídeos, proteínas (Halliwell, 2006). A reação do peroxinitrito com resíduos de tirosina gera nitrotirosina, considerada um marcador para estresse oxidativo.

Para controlar o estresse oxidativo, os sistemas biológicos apresentam alguns antioxidantes responsáveis pela neutralização de espécies reativas, reduzindo os danos oxidativos. A superóxido dismutase (SOD) é capaz de dismutar o superóxido produzido pela corrente de transporte de elétrons mitocondrial, por NADPH oxidase, óxido nítrico sintase, xantina oxidase, ciclooxigenase, lipoxigenase e citocromo p450, em peróxido de hidrogênio. A catalase e a glutatona, outros antioxidantes, podem neutralizar o peróxido de hidrogênio em água e oxigênio (Schnachenberg, 2002).

A figura 5 apresenta as principais reações pró-oxidantes e antioxidantes nos sistemas biológicos (Schnachenberg, 2002).



**Figura 5:** Resumo dos principais reações pró-oxidantes e antioxidantes nos sistemas biológicos (Adaptado de Schnachenberg, 2002).

#### 1.4.1. O estresse oxidativo na nefropatia diabética

O estresse oxidativo é considerado um fator muito importante no desenvolvimento e na progressão das complicações do DM (Baynes e Thorpe, 1999; Brownlee, 2001). A hiperglicemia está envolvida no aumento da produção de ERO através da ativação de diversas vias (Brownlee, 2001), sendo o superóxido o radical mais importante na regulação da função renal (Wilcox, 2002). Trabalhos recentes sugerem que a HA está associada ao estresse oxidativo e à inflamação renal e, conseqüentemente, está envolvida na patogênese da ND (Biswas e Lopes de Faria, 2006; Biswas e Lopes de Faria, 2007; Shah et al., 2007).

E ainda, o aumento dos níveis renais de ERO e da expressão de moléculas pró-inflamatórias estão relacionados aos mecanismos que envolvem o SRAA e contribuem para o desenvolvimento da ND (Brownlee, 2005; Del Vecchio et al., 2007).

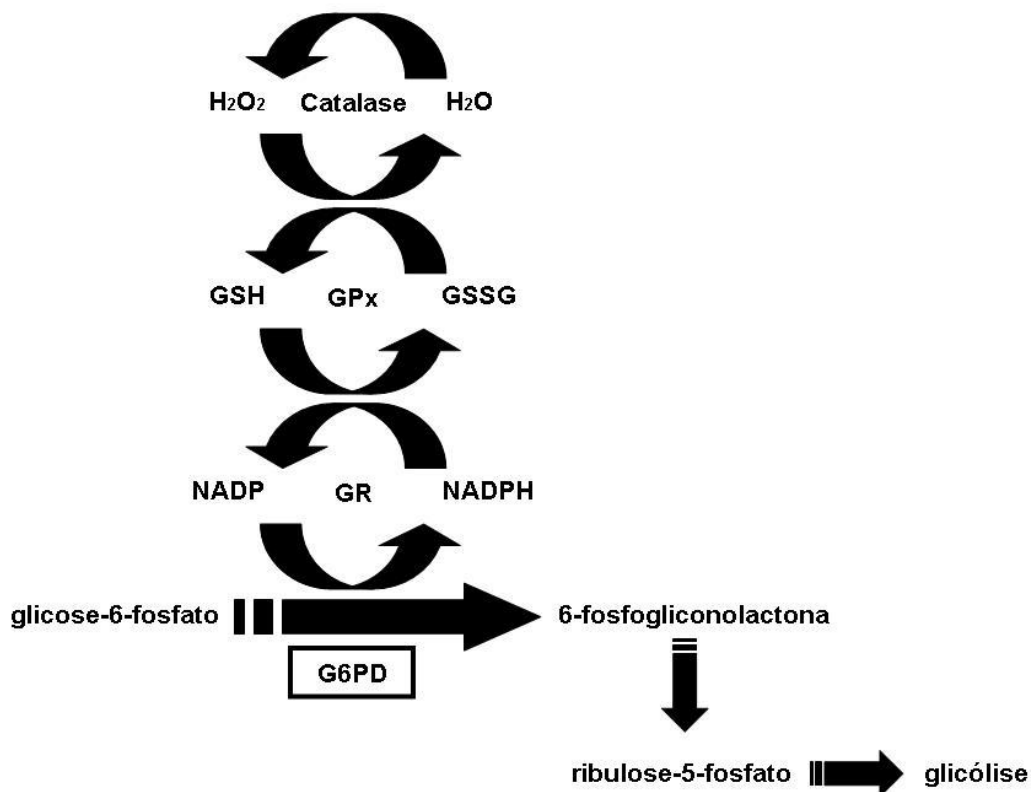
Uma importante via de estresse oxidativo é a NADPH oxidase, considerada a principal fonte de superóxido nos tecidos vascular e renal (Gill e Wilcox, 2006). Trabalhos demonstram que a expressão da NADPH oxidase está aumentada em modelos experimentais de DM, reforçando a contribuição do estresse oxidativo no desenvolvimento e na progressão da ND (Jones et al., 1995; Kitada et al., 2003; Frecker et al., 2005; Tojo et al., 2007). Outras vias também estão envolvidas no desenvolvimento e progressão da ND. A ativação da ANG II e da via PKC promove a ativação da enzima NADPH oxidase aumentando a produção de ERO e o estresse oxidativo (Onozato e Tojo, 2005; Tojo et al., 2007).

Foi demonstrado que níveis elevados de ERO estão envolvidos na progressão do dano renal provocado pela aldosterona (Nishiyama et al., 2004). Esse aumento é devido aos elevados níveis de subunidades da NADPH oxidase, gp91phox, NOX-4 e p22phox, sugerindo que a produção de ERO nos rins é mediada pela via NADPH oxidase (Nishiyama et al., 2004). Biswas e colaboradores (2008) demonstraram em modelo animal que a presença da hipertensão aumenta o estresse oxidativo via NADPH oxidase e promove diminuição das defesas anti-oxidantes ec-SOD e glutathiona reduzida (GSH). Miyata e colaboradores (2005) demonstraram que a aldosterona induz a produção do ânion superóxido, acompanhado por um aumento da atividade da enzima NADPH oxidase, em cultura de células mesangiais.

Uma importante enzima envolvida no controle oxidativo é a glicose-6-fosfato-desidrogenase (G6PD) (Leopold et al., 2003), intimamente ligada ao sistema anti-oxidante e recentemente relacionada ao acúmulo de ERO no endotélio vascular e em células musculares (Leopold et al., 2001). A G6PD é a primeira enzima da via das pentoses e promove a liberação de NADPH para várias reações celulares incluindo reciclagem de glutathiona, produção de superóxido via NADPH oxidase, síntese de óxido nítrico e colesterol (Matsui et al., 2006).

Esta enzima está expressa em vários tipos celulares e em diferentes níveis (Battistuzzi et al., 1985) e a variação na expressão e na atividade pode determinar sua contribuição relativa às defesas anti-oxidantes celulares (Leopold e Loscalzo, 2000). Em várias linhagens celulares testadas foi demonstrado que a expressão de G6PD é elevada por estresse oxidativo induzido por agentes que aumentam o superóxido intracelular ou diminuem a GSH (Salvemini et al., 1999). Em trabalhos com fibroblastos (Ho et al., 2000) e com células endoteliais (Leopold et al., 2001) G6PD deficientes houve aumento da produção de ERO e diminuição do estoque de GSH intracelular, um efeito associado à viabilidade celular. A transfeccção do gene G6PD em células endoteliais vasculares promoveu aumento dos níveis de NADPH, anulando os efeitos do estresse oxidativo e aumentando a biodisponibilidade de óxido nítrico. Esse efeito protetor deve-se ao aumento da disponibilidade de NADPH pela G6PD, permitindo que a reciclagem da glutathiona, que é dependente de NADPH, seja efetuada garantindo a ação anti-oxidante. Já a biodisponibilidade de óxido nítrico é garantida pela atividade da enzima óxido nítrico sintase endotelial (eNOS), responsável pela liberação de óxido nítrico, dependente de

NADPH (Leopold et al., 2003). A figura 6 representa as interações entre a G6PD e o sistema antioxidante.



**Figura 6:** Esquema das interações entre a G6PD e sistema antioxidante (Adaptado de Cappellini e Fiorelli, 2008). GPx = glutathiona peroxidase; GR = glutariona redutase; GSH = glutathiona reduzida; GSSG = glutathiona oxidada.

A hiperglicemia causa a inibição da atividade da G6PD por diminuição da expressão e aumento da fosforilação promovendo aumento do estresse oxidativo (Xu et al., 2005). O aumento da glicose em cultura de células endoteliais promoveu diminuição da G6PD resultando na diminuição dos níveis de NADPH, aumentando os níveis de AMPc e predispondo as células à morte (Zhang et al., 2000). Trabalho com camundongos *knockout*



para G6PD demonstrou que a deficiência nessa enzima é protetora contra aterosclerose (Matsui et al., 2006). Já a super-expressão de G6PD em células endoteliais vasculares promoveu a diminuição de ERO e mostrando um efeito protetor na disfunção endotelial (Leopold et al., 2003). Foi observado em cultura de células que a super-expressão de G6PD interfere negativamente com a ativação do fator de transcrição NF- $\kappa$ B (Salvemini et al., 1999), demonstrando uma relação entre a enzima e o processo inflamatório.

### **1.5. Efeitos benéficos do bloqueio de receptor mineralocorticóide**

Trabalhos clínicos e experimentais sugerem que a o bloqueio de receptor mineralocorticóide é benéfico na lesão renal causada pelo DM (Miric et al., 2001; Fujisawa et al., 2004; Miyata et al., 2005; Han et al., 2006; Del Vecchio et al., 2007; Yuan et al., 2007; Kang et al., 2009; Mehdi et al., 2009). Outro trabalho clínico demonstrou que a combinação de espironolactona (SPR), um bloqueador de receptor mineralocorticóide, ao tratamento com inibidores da ECA promove maior proteção renal (Mehdi et al., 2009).

Estudo demonstra que glucocorticóides específicos, mineralocorticóides e compostos esteróides podem inibir a atividade da G6PD (Criss e McKerns, 1969). Este mesmo trabalho apresenta a dehidroepiandrosterona, um composto similar à aldosterona, como um potente inibidor da G6PD. Em células endoteliais de aorta e em modelo animal foi demonstrado que a aldosterona promove a redução da atividade da enzima G6PD (Leopold et al., 2007). Animais *knockout* para G6PD apresentaram um aumento no estresse oxidativo e na albuminúria, evidenciando um efeito semelhante ao encontrado em animais diabéticos (Xu et al., 2010). Em células endoteliais expostas à alta glicose (Zhang et al.,

2000) e em modelo animal diabético (Xu et al., 2005) foi demonstrado redução na atividade da enzima G6PD via ativação da PKA e aumento do estresse oxidativo, reforçando a importância dessa enzima na ND.

A utilização de inibidores da ECA e inibidores do SRAA demonstram um efeito positivo na redução de danos cardiovasculares e renais (Karalliedde e Viberti, 2006; Del Vecchio et al., 2007). Em células endoteliais, a aldosterona promove a geração de ERO por ativação da NADPH-oxidase e o bloqueio de receptores mineralocorticóides restaura aos níveis normais (Iwashima et al., 2008). O uso de SPR ou eplerenona, bloqueadores de receptor de mineralocorticóides, promove efeitos benéficos em modelos animais de injúria renal como a redução da proteinúria (Hostetter e Ibrahim, 2003), da glomeruloesclerose e fibrose intersticial (Han et al., 2006).

A inibição de receptor de mineralocorticóide pode reduzir a geração de ERO via NADPH-oxidase em tecido renal, cardíaco, muscular, vascular e pâncreas (Manrique et al., 2009) e reverter alterações *in vivo* sem redução da pressão arterial em modelo transgênico (Lastra et al., 2008). O bloqueio da aldosterona em células endoteliais mostrou eficácia em manter normais os níveis de ERO por preservar a expressão e a atividade da G6PD (Leopold et al., 2007). Estudos comparativos em camundongos *knockout* para G6PD e o resultado de que a transfeccção do gene G6PD previne aumento na pressão arterial sugerem que os efeitos do bloqueio dos receptores de mineralocorticóides são mediados por alterações na expressão de G6PD e não por efeitos na pressão arterial (Leopold et al., 2007).

Em modelo de isquemia-reperfusão, o bloqueio da aldosterona apresenta ação redutora dos danos nas células tubulares e aumento da proteção contra ERO através do aumento da expressão das enzimas superóxido desmutase e glutationala peroxidase (Mejia-Vilet et al., 2007). A administração de espironolactona retardou significativamente o desenvolvimento da proteinúria em modelo de insuficiência renal (Patni et al., 2007).

Em ratos diabéticos, o uso da espironolactona promoveu redução da expressão de colágeno (Fujisawa et al., 2004) e TGF $\beta$  além da diminuição da infiltração de macrófagos, característica da inflamação (Fujisawa et al., 2004; Han et al., 2006). Estudo recente, em modelo animal nefrectomizado, demonstra que o bloqueio da aldosterona promove redução da inflamação, da expansão de matriz e da fibrose glomerular (Terada et al., 2008). Neste mesmo, modelo a redução nos danos renais e níveis de ERO parecem ser devida à inibição da aldosterona e não à redução da pressão arterial, tendo como principal mediador a via NADPH-oxidase (Taira et al., 2008). Em modelo de hipertensão por ingestão salina foi demonstrado que o uso da espironolactona promove a redução no número de lesões glomerulares e vasculares independentemente do efeito anti-hipertensivo (Rocha et al., 1998).

## **2. Hipótese e Objetivos**

### **2.1. Hipótese**

O bloqueio do receptor mineralocorticoide da aldosterona melhora a nefropatia em ratos hipertensos e diabéticos por restaurar a atividade da G6PD e diminuir o estresse oxidativo, independente dos seus efeitos sobre a glicemia e pressão arterial.

### **2.2. Objetivos**

- I.** Testar em ratos SHR se a indução de DM com estreptozotocina é acompanhada de redução da atividade da enzima G6PD.
- II.** Investigar se o tratamento com um bloqueador mineralocorticoide, espironolactona, restaura a atividade da enzima G6PD e se isso é acompanhado de redução do estresse oxidativo e de marcadores de nefropatia em ratos SHR diabéticos.



## **1. Metodologia**

### **1.1. Animais**

Foram utilizados ratos espontaneamente hipertensos (SHR) machos de 4 semanas de idade, linhagem produzida pela Taconic (Germantown, NY, USA), criados em biotério mantido a 22°C, expostos ao ciclo de 12 horas de luz e 12 horas de escuro e com livre acesso a água e comida. A manipulação e os experimentos foram realizados de acordo com as instruções estabelecidas pelo Colégio Brasileiro para a Experimentação Animal (COBEA). O protocolo deste estudo foi aprovado pela Comissão de Ética e Experimentação Animal (CEEA- IB- Unicamp), nº 1406-1.

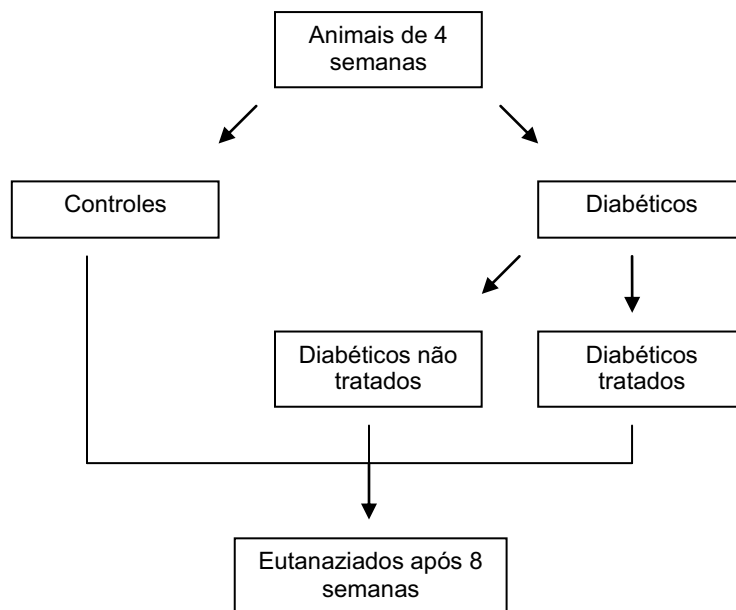
### **1.2. Protocolos e procedimentos**

Os ratos foram tornados diabéticos através da injeção endovenosa de estreptozotocina, 60mg/kg, (Sigma, St. Louis, MO, USA) e ratos controles receberam apenas o veículo da estreptozotocina (tampão citrato 0,5M, pH 4,5). Foram considerados diabéticos os animais com glicemia superior a 15mM/L.

Os animais diabéticos foram randomizados para receberem, ou não, espironolactona (50mg/kg/dia) diluída na água de beber por 8 semanas.

Após o tratamento, os animais foram eutanaziados por asfixia em câmara de CO<sub>2</sub>. Os rins foram retirados, descapsulados, pesados e cortados longitudinalmente. Um dos rins foi fixado em metacarnol para análises de imunohistoquímica. Foram retirados fragmentos

do córtex renal da outra metade para estimativa da geração de superóxido via NADPH oxidase, determinação dos níveis de TBARS, relação de GSH/GSSG, atividade da G6PD e extração de proteínas para Western blot.



### 1.2.1 Determinação da pressão arterial

A pressão arterial sistólica foi obtida através de pletismógrafo caudal (MK III physiograph, Narco Bio-System, Houston, TX, USA) como previamente descrito (Amazonas e Lopes de Faria, 2006; Biswas e Lopes de Faria, 2007). Foram feitas de 3 a 5 determinações por rato antes da indução do diabetes, 3 e 6 semanas após e no final do tratamento.

### 1.2.2 Extração de proteínas

O córtex renal foi homogeneizado em tampão (Tris-HCl 30mM, pH 7,5, EGTA 10mM, EDTA 5mM, DTT 1mM e sacarose 250mM) e coquetel de inibidor de

proteases. As amostras foram centrifugadas a 11000 rpm, 4°C por 10 minutos e o sobrenadante foi usado para Western Blot. Foi separada uma alíquota para a quantificação de proteína total pelo método Bradford.

### **1.2.3 Western blot**

Foram feitos experimentos para verificar a expressão das proteínas p47phox (sub-unidade da NADPH oxidase) e fibronectina (uma proteína de matriz extracelular).

As alíquotas de proteína total foram fracionadas por eletroforese em gel de acrilamida utilizando um Mini Protein II Dual Slab Cell (Bio-Rad Laboratories, CA, USA). Cada proteína de interesse utiliza um gel de concentração apropriada. Após a eletroforese as proteínas foram transferidas para uma membrana de nitrocelulose, a qual passou pelo bloqueio de ligações inespecíficas em leite desnatado 5%. As membranas foram incubadas com anticorpo primário para p47phox (monoclonal de camundongo anti-p47phox, BD Transduction Laboratories, BD Biosciences Pharmingen, NJ, USA) e fibronectina (policlonal de rato anti-fibronectina, Calbiochem, La Jolla, CA). Foram feitas lavagens com PBS e as membranas foram incubadas com anticorpos secundários específicos. A detecção foi feita por quimiluminescência utilizando um kit (SuperSignal CL-HRP substrate system, Pierce, Rockford, IL).

### **1.2.4 Medida da geração de superóxido via NADPH oxidase**

Um pedaço de córtex renal foi homogeneizado em tampão de lise (água, fosfato de potássio 0,1M, EGTA 0,1M, PMSF 0,1M, aprotinina e inibidor de protease) e



centrifugado. Ao sobrenadante foi adicionado tampão de reação (água, fosfato de potássio 0,1M, EGTA 0,1M, sacarose 1M), NADPH e lucigenina. Através de um método de quimiluminescência com lucigenina foi estimada a atividade da NADPH oxidase de acordo com a literatura (Biswas et al., 2008).

### **1.2.5 Determinação urinária de 8-OHdG**

A concentração urinária de 8-hydroxi-2'-deoxiguanosina (8-OHdG, um marcador de dano oxidativo ao DNA) foi determinada através de um kit de ELISA (Japan Institute for the Control of Aging, Nikken Seil Co., Shizuoka, Japan), de acordo com as instruções do fabricante.

### **1.2.6 Medida dos níveis de TBARS**

A peroxidação lipídica foi avaliada pela concentração dos níveis de TBARS utilizando o método de Ohkawa e colaboradores com pequenas modificações. Um pedaço de córtex renal foi homogeneizado em 10% solução fisiológica, 3% BHT e 1mL de TBA 1% em PBS e incubado por 2 horas a 60°C no banho-maria. Após esfriar, a solução foi centrifugada por 10 minutos a 2000rpm. A absorbância do sobrenadante foi determinada com espectrofotômetro a 532nm. A concentração de TBARS foi expressa em nano moles/mg de tecido e calculada pelo coeficiente de extinção de  $1,56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  (Wills, 1969).

### **1.2.7 Medida da atividade da enzima glicose-6-fosfato-desidrogenase**

A atividade da enzima glicose-6-fosfato-desidrogenase foi estimada por método descrito por Stanton e colaboradores (1991). Um pedaço de córtex renal foi

homogeneizado em tampão de lise (água, PBS pH 7,4, aprotinina e inibidor de protease) e centrifugado. O sobrenadante foi adicionado ao tampão de reação (50 mM Tris, 1 mM MgCl<sub>2</sub>, pH 8,1) e aos substratos (glicose-6-fosfato 200 μM, 6-fosfogluconato 200 μM e NADP<sup>+</sup> 100 μM) para a leitura no espectrofotômetro. A medida da absorbância a 340 nm da enzima 6-fosfogluconato-desidrogenase foi subtraída da medida da atividade de todas as desidrogenases para determinar a atividade da G6PD.

### **1.2.8 Medida da relação GSH/GSSG**

A relação GSH/GSSG foi estimada através do kit Bioxytech GSH-GSSG-412 enzymatic method (Oxis International, Foster City, CA, USA), de acordo com as instruções do fabricante.

### **1.2.9 Excreção urinária de albumina**

No dia anterior ao término do experimento amostras de urina foram coletadas por 24 horas através de gaiolas metabólicas individuais. A excreção urinária de albumina foi determinada por kit de ELISA (Nephrot II, Exocell, Philadelphia, PA, USA), de acordo com as instruções do fabricante.

### **1.2.10 Análise estatística**

Os resultados foram expressados na forma de média ± desvio padrão, exceto para albuminúria que foi expressa em média geométrica (variação). A comparação entre os grupos foi por ANOVA seguido de teste de Fisher utilizando-se o programa StatView<sup>®</sup>. Foram consideradas significativas as análises com p<0,05.



1. Artigo (submetido)

American Journal of Nephrology



**Mineralocorticoid receptor blockade ameliorates nephropathy by increasing glucose-6-phosphate dehydrogenase activity and reducing oxidative stress in diabetic hypertensive rats**

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Keywords:	diabetic nephropathy, oxidative stress, glucose-6-phosphate-dehydrogenase



**Mineralocorticoid receptor blockade ameliorates nephropathy by increasing glucose-6-phosphate dehydrogenase activity and reducing oxidative stress in diabetic hypertensive rats**

Running title: Aldosterone antagonist in diabetic nephropathy

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Key words: diabetic nephropathy, glucose-6-phosphate-dehydrogenase, oxidative stress

## **Abstract**

*Background/Aims:* Hyperglycemia decreases glucose-6-phosphate dehydrogenase (G6PD) activity that reduces NADPH which makes cells very sensitive to oxidant damage. Spironolactone, an mineralocorticoid receptor blocker, diminishes hyperglycemia-induced reduction in G6PD activity. In the present study we investigated whether spironolactone improves nephropathy by increasing G6PD activity and reducing oxidative stress in hypertensive diabetic rats.

*Methods:* Spontaneously hypertensive rats were rendered diabetic by streptozotocin. The diabetic animals were randomized to receive or not receive spironolactone for 8 weeks.

*Results:* Plasma glucose levels and systolic blood pressure were unaltered by diabetes or by treatment. Albuminuria and renal expression of fibronectin were higher in the diabetic group compared to control, and these parameters were reduced with mineralocorticoid receptor blocker. G6PD activity and the antioxidant GSH / GSSG ratio were reduced in diabetic rats and the treatment restored to control levels. Urinary levels of 8-OHdG and TBARS renal cortex levels, markers of oxidative stress, and the production of superoxide induced by NADPH oxidase and p47phox were all higher in diabetic rats when compared to controls, and the treatment reduced to control levels.

*Conclusions:* These results suggest that spironolactone ameliorates nephropathy in the diabetic hypertensive rats by restoring G6PD activity and diminishes oxidative stress without affecting glycemia and blood pressure.

## INTRODUCTION

Despite better management, particularly in the last two decades, diabetic nephropathy remains the leading cause of end-stage renal disease in most parts of the world. Strict glycemic management, control of blood pressure, and use of drugs that interfere with the renin angiotensin system (RAS) are the most effective interventions for prevention and treatment of diabetic nephropathy [1]. In addition, recent clinical trials have suggested that adding an inhibitor of the aldosterone system to an angiotensin-converting enzyme inhibitor-based regime in patients with diabetic nephropathy may further reduce proteinuria and thereby afford additional renal protection [2]. Beneficial effects of aldosterone blockade on diabetic nephropathy seem to be independent of blood pressure reduction [2] and renin-angiotensin blockade on diabetic nephropathy [3]. However, the mechanism by which aldosterone blockade improves diabetic nephropathy is poorly defined.

Studies in animal models have demonstrated that aldosterone can promote proteinuria, glomerular, and tubular sclerosis independent of angiotensin II [4, 5]. In this setting, inflammation and oxidative stress have been suggested as possible mediators of renal injury [4, 5]. It has been shown that the administration of spironolactone, a mineralocorticoid receptor blocker, has beneficial effects on renal lesions [4]. In both type 1 (streptozotocin-induced diabetic rats) [6] and in type 2 [7] diabetic rats, aldosterone blockade improves renal function probably through an anti-inflammatory mechanism [6, 7].

Oxidative stress (due to increased oxidant production and/or decreased antioxidant activity) has been seen as a critical underlying mechanism for microvascular complications of diabetes, including diabetic nephropathy [8]. Although angiotensin II has been proposed as an up-stream mediator of hyperglycemia-induced oxidative stress and renal disease [9], a partial

contribution of aldosterone on kidney injury has been well documented [5, 10]. In fact, both in humans and in experimental animals with diabetes mellitus (DM) it has been suggested that spironolactone ameliorates renal injury and attenuates oxidative stress [11, 12], and that this, in turn, would reduce inflammation [13].

Glucose-6-phosphate dehydrogenase (G6PD), the first and rate-limiting enzyme in the pentose phosphate pathway, is the principal intracellular source of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) [14]. NADPH is the main intracellular reductant for all cells. Consequently, a decrease in G6PD activity leads to decreased NADPH and makes cells very sensitive to oxidant damage [15, 16]. In addition, since the antioxidant system glutathione (GSH) relies on a sufficient amount of NADPH for its normal function, a decrease in NADPH supply leads to a decrease in GSH levels, aggravating oxidative stress [14, 16]. Interestingly, it has been demonstrated that G6PD activity is inhibited in endothelial cells exposed to high glucose [17]. This observation *in vitro* was confirmed *in vivo* by the demonstration that rats rendered diabetic by streptozotocin (STZ) displayed a reduction of kidney cortex G6PD via activation of protein kinase A and consequent oxidative stress [18]. That G6PD deficiency may lead to increased oxidative stress and nephropathy was recently shown by the observation that G6PD knockout mice had increased renal oxidative stress and increased albuminuria [19]. Finally, a seminal work demonstrated that aldosterone induces a G6PD-deficient phenotype that can be improved by aldosterone antagonist or gene transfer of G6PD [14].

Based on the above published information, we hypothesized that a mineralocorticoid receptor blocker, spironolactone, would improve nephropathy in hypertensive diabetic rats by restoring G6PD deficiency and improving oxidative stress, independently of its effects on



blood glucose and blood pressure. To assess this hypothesis we used an experimental model that combines genetic hypertension (spontaneously hypertensive rat, SHR) with STZ-induced DM. Presence of genetic hypertension in the diabetic rats leads to early and more severe signs of nephropathy, and an increase in kidney pro-oxidant generation and a decrease in antioxidant defense [20].

## RESEARCH DESIGN AND METHODS

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 otherwise stated. Spontaneously hypertensive rats (SHR) rats derived from animals supplied by Taconic (Germantown, NY, USA) were used in this study. Diabetes was induced in four-week-old, male SHR by injecting streptozotocin (STZ; 60 mg/kg) dissolved in sodium citrate buffer (0.5 M; pH 4.5). Control group received only vehicle (citrate buffer). Blood glucose levels were measured using an enzymatic colorimetric GOD-PAP assay (Merck, Darmstadt, Germany) 48 h after the injection of STZ or citrate buffer. Plasma glucose concentrations of >15 mM/L were considered diabetic for these experiments. The diabetic rats were randomly assigned to receive no treatment or treatment with spironolactone (SPR) (Pfizer S. R. L., Buenos Aires, Argentina), 50 mg/kg/day diluted in drinking water. Rats were sacrificed using CO<sub>2</sub> gas 8 weeks after induction of diabetes. The right kidney was removed, decapsulated, weighed, cut longitudinally into 2 halves. The left kidney was similarly removed and also cut longitudinally. One half was fixed in a 4% paraformaldehyde solution. Some pieces of the cortical tissue of the other half were homogenized and processed for Western blot, and other pieces were frozen in liquid nitrogen and preserved at -80 °C for subsequent assays.

### *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) before and in the 4<sup>th</sup> and 8<sup>th</sup> weeks after induction of diabetes. Rats were habituated to the procedure before taking blood pressure readings.

### *Albumin excretion rate (AER)*

Before induction of DM and the day before the end of treatment, urine samples were collected by placing each rat in individual metabolic cages for 24 h. AER was determined by ELISA kit (Nephrot II, Exocell, Philadelphia, PA, USA), according to the manufacturer's instructions.

#### *Determination of urinary 8-OHdG*

The urinary concentrations of 8-hydroxy-2'-deoxyguanosine (8-OHdG, a DNA-modified product) were determined using an ELISA kit (Japan Institute for the Control of Aging, Nikken Seil Co., Shizuoka, Japan), according to the manufacturer's instructions, and they were normalized by urine creatinine.

#### *Determination of TBARS levels*

Lipid peroxidation was evaluated by measuring the TBARS content according to the thiobarbituric acid (TBA) test as previously described [21] with slight modifications. To the renal cortex tissue homogenate a solution of 10% saline, 3% BHT and 1 mL TBA 1% in PBS was added and incubated at 60°C for 2 h, in a boiling water bath. After cooling, the solution was centrifuged at 2000 rpm for 10 min. The absorbance of the supernatant was determined at 532 nm using spectrophotometer against the blank. TBARS content were expressed in nano moles/mg tissue and was calculated by using an extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ .

#### *Determination of G6PD activity*

G6PD activity was determined, as previously described [22], by measuring the rate of production of NADPH. A small piece of frozen kidney cortex was homogenized in 1 ml lysis buffer (PBS, aprotinin and protease inhibitor cocktail). 50 µL renal cortex cell lysates were added to a cuvette with buffer (50 mM Tris, 1 mM MgCl, pH 8.1). Enzyme activity was determined in a spectrophotometer by measuring the rate of increase in absorbance at 341 nm due to the conversion of NADP<sup>+</sup> to NADPH by either G6PD or 6-phosphogluconate

dehydrogenase. To obtain the total dehydrogenase activity, substrates for both dehydrogenase enzymes were added to a cuvette containing 1 mL of buffer. The rate of change was measured over a 5 min observation period. In another cuvette, substrates for the second enzyme, 6-phosphogluconate dehydrogenase, were added to obtain the rate of this enzyme. Subtracting the activity of 6-phosphogluconate dehydrogenase from the total dehydrogenase activity provided the activity of the rate-limiting enzyme, G6PD. Substrate concentrations were glucose 6-phosphate (200  $\mu$ M), 6-phosphogluconate (200  $\mu$ M), and NADP<sup>+</sup> (100  $\mu$ M).

#### *Determination of GSH/GSSG ratio*

The ratio of GSH and GSSG was estimated using the kit Bioxytech GSH-GSSG-412 enzymatic method (Oxis International, Foster City, CA, USA), according to the manufacturer's instructions.

#### *NADPH oxidase activity*

NADPH oxidase activity was measured by the lucigenin-enhanced chemiluminescence method, as previously described [20, 23]. Protein concentration was measured using the Bradford method.

#### *Western blotting*

Renal cortical homogenate was used for quantification of fibronectin and NADPH oxidase subunit p47phox proteins by Western blot analysis [20]. Primary antibodies were a rat anti-fibronectin (Calbiochem, La Jolla, CA) and a mouse monoclonal anti-p47phox antibody (BD Transduction Laboratories, BD Biosciences Pharmingen, NJ, USA). To verify the uniformity of protein load and transfer efficiency across the test samples, membranes were reprobated for actin (goat polyclonal anti-actin antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

### *Statistical analysis*

The results are expressed as means  $\pm$  SD, except for albuminuria, which is expressed as geometric means and variance. Comparisons between groups were done with one-way analysis of variance (ANOVA) followed by Bonferroni test. Nonparametric data are expressed as the geometric mean and ranges, and are analyzed by Kruskal-Wallis test (for multiple groups) and Mann-Whitney U test (for 2 groups). A value of  $p < 0.05$  was considered significant. All analyses were performed using statistical software StatView (SAS Institute Inc., NC, USA).

## RESULTS

### *Physical and metabolic parameters*

Diabetic rats presented lower body weight and higher levels of kidney-to-body weight ratio compared with control group (table 1,  $p < 0.0001$ ). The blood glucose level was higher in all diabetic groups ( $p < 0.0001$ ) and was not modified by the treatment. The blood pressure was not altered by diabetes or treatment with SPR.

### *Spirolactone treatment ameliorates markers of nephropathy*

To assess nephropathy in our rats we used a functional marker (urinary albumin excretion rate, AER), and a structural parameter, the renal expression of fibronectin, a pro-fibrotic protein. The AER was significantly elevated ( $p = 0.017$ ) in diabetic rats compared to the control group, and SPR treatment reduced ( $p = 0.028$ ) this parameter to the level observed in control rats (fig. 1). Western blotting analysis showed that the expression of renal fibronectin was significantly higher in diabetic rats than in non-diabetic ( $p = 0.038$ ). This abnormality was normalized by the treatment with SPR ( $p = 0.033$ ) (fig. 2).

### *Aldosterone blocker restored G6PD activity*

G6PD is a critical determinant of the intracellular redox state [15] and its activity is modulated by aldosterone [14]. As previously observed [18], G6PD activity was reduced in the diabetic rats ( $p = 0.008$ ) and the treatment with SPR restored this parameter to the control levels ( $p = 0.032$ ) (fig. 3).

### *Aldosterone antagonist reduces oxidative stress*

To assess if the reduction in G6PD activity in DM was associated with oxidative stress and whether it could be reduced with treatment with SPR, we investigate parameters of oxidative stress, pro-oxidant, and antioxidant.

*Spironolactone decreases DNA damage induced by oxidative stress*

To investigate the influence of SPR in oxidative stress we evaluated the levels of 8-OHdG, a marker of oxidative stress on the DNA, in urinary samples. The levels of urinary 8-OHdG was markedly higher in untreated diabetic rats compared to the control group ( $p=0.019$ ), and it was significantly reduced ( $p=0.002$ ) by treatment with SPR (fig. 4a).

*Aldosterone blocker reduced lipid peroxidation levels*

TBARS levels were measured as an index of malondialdehyde (MDA) production and hence lipid peroxidation. Diabetic animals showed a significant increase of TBARS compared with control animals ( $p=0.016$ ). Treatment with SPR decreased to control levels ( $p=0.009$ ), confirming the improvement in oxidative stress (fig. 4b).

*Spironolactone increased antioxidant defense that was reduced by diabetes*

The normal function of GSH coupling relies on a sufficient supply of NADPH. Thus, a decrease in NADPH induced by a reduction in G6PD should lead to decreased GSH levels [16]. Oxygen radicals are inactivated by, among others, the glutathione system with an involvement of glutathione reductase, which regenerates GSH from GSSG at the expense of NADPH [18]. Renal cortical GSH/GSSG ratio was found reduced in diabetic rats compared to control group ( $p=0.029$ ). The treatment with SPR restored the GSH/GSSG ratio to the control levels ( $p=0.025$ , fig. 5).

*Pro-oxidant parameters were reduced by treatment with spironolactone*

The generation of superoxide by NADPH-oxidase pathway in renal cortical homogenate was higher in diabetic animals compared to control ( $p=0.009$ ). The treatment with SPR significantly ( $p=0.040$ ) reduced the production of renal cortical superoxide (fig. 6). To identify the source of superoxide production, we used diphenyleneiodonium, an inhibitor of flavin-containing oxidases, and rotenone, an inhibitor of complex I of the mitochondrial respiratory chain. Pre-incubation of the renal cortical homogenates with

diphenyleneiodonium (20  $\mu$ M final concentration) completely blocked NADPH-induced superoxide production, while pre-incubation with rotenone (20 and 100  $\mu$ M final concentration) did not produce any effect on superoxide production, indicating that the NADPH oxidase is the most likely source of the superoxide we detected. To further assess the involvement of NADPH oxidase in the exaggerated production of renal superoxide in diabetes, we also evaluated the expression of NADPH oxidase subunit, p47phox. Western blotting analysis showed that the expression of p47phox in the kidney cortex was significantly elevated in diabetic rats ( $p=0.004$ ). The treatment with SPR reduced the expression of p47phox to the control levels ( $p=0.035$ ) (fig. 7).



## DISCUSSION

Blood glucose management, blood pressure control, and use of drugs that interfere with the renin-angiotensin system are the gold standard treatment of diabetic nephropathy. Despite this, the number of patients with diabetic nephropathy that reach end stage renal disease continues to rise [24]. This observation suggests that new therapeutic intervention that can be used in addition to the gold standard treatment is necessary to reduce the epidemic of diabetic nephropathy. Clinical and experimental works have suggested that mineralocorticoid receptor blockade may be beneficial to renal disease in diabetes [2, 3, 6, 7, 10, 11, 25]. In addition, a recent clinical trial has concluded that addition of spironolactone, an aldosterone blocker, to maximal dose of ACEi affords greater renal protection, despite a similar effect on blood pressure and blood glucose [2]. Furthermore, experimental data suggest that the effect of aldosterone on glomerular and tubular sclerosis is independent of angiotensin II [3]. Therefore, the addition of aldosterone blockade to the gold standard treatment fulfills the requirement for a new treatment of diabetic nephropathy, the effects of which surpass the gold standard treatment, although a long-term, large scale, renal failure outcomes trial is still lacking [2]. However, the mechanism of how aldosterone blockade improves diabetic nephropathy is not fully understood.

In the present study we observed that spironolactone improves parameters of nephropathy, named albuminuria and renal expression of fibronectin. Since we used a model of hypertension and diabetes, we could conclude that these effects were independent of reduction in blood pressure or in blood glucose. We also observed that aldosterone treatment increases G6PD activity and that this may have led to improvement in the antioxidant system (glutathione) with consequent reduction in oxidative stress, a process at the center of pathogenesis of diabetic nephropathy [8]. To our knowledge, this is the first study to

demonstrate that mineralocorticoid receptor blockade improves nephropathy, and that this is associated with increase in G6PD activity and reduction in oxidative stress in hypertensive diabetic rats.

Clinical study in type 2 diabetic patients [2] and studies in different animal models of diabetes [3, 6, 7, 10, 11] have suggested that reduction in renal inflammation and/or oxidative stress was the main responsible for the beneficial effect of aldosterone blockade in diabetic nephropathy. It has been suggested that the primary effect of aldosterone blockade was reduction in oxidative stress where reduction in inflammation was a secondary effect [5]. However, since these two phenomena, oxidative stress and inflammation, are closely related, and one can lead to the other and vice-versa, the issue of which is the primary event on the action of aldosterone blockade is open to further investigation. Other studies have suggested that aldosterone blockade reduces transforming growth factor  $\beta$ -1 (TGF $\beta$ -1), plasminogen activator inhibitor-1 (PAI-1) and type IV collagen in both an *in vivo* and *in vitro* model of high glucose [6]. However, these findings do not exclude that these effects were secondary to a reduction in oxidative stress, an upstream event in hyperglycemia-induced TGF $\beta$ -1 and extracellular matrix accumulation [9].

Oxidative stress is a main determinant of diabetic nephropathy [8]. However, so far it has been difficult to demonstrate a beneficial effect of anti-oxidant in diabetic nephropathy in a clinical setting. Different reasons for this disappointing effect of anti-oxidant in a clinical setting are possible. Very likely, understanding how an anti-oxidant works is of great interest. In addition, since oxidative stress has many important functions in the body, including defense against different pathogens, it is possible that it would be better to improve anti-oxidant defense than to use a compound that reduces superoxide production. To this effect,

recent publications have appointed a reduction in G6PD activity as a critical step in hyperglycemia-induced oxidative stress [19]. G6PD is the main enzyme involved in production of NADPH [14, 15]. NADPH is the principal intracellular reductant and the entire antioxidant system relies on adequate supply of NADPH for its action [17]. Importance of G6PD to control oxidative stress has been demonstrated by the observation that complete or partial inhibition of G6PD activity significantly increased oxidative stress compared with the results shown in wild-type cells [14, 15]. More importantly, it has been shown that endothelial cells exposed to high glucose displayed an inhibition of G6PD activity, probably dependent on the protein kinase A phosphorylating G6PD [16]. Similarly to these *in vitro* observations, it has been shown that rats with streptozotocin-induced DM displayed an inhibition of G6PD, which contributes to oxidative stress in these rats [18]. Considering these published data, aldosterone blockade may be a good alternative to reduce oxidative stress and improve nephropathy in diabetes mellitus.

In summary, we have shown that aldosterone blockade with spironolactone improves nephropathy in diabetic hypertensive rats. This effect could not be attributed to better glycemic management or blood pressure reduction since spironolactone did not affect these parameters. However, spironolactone treated rats displayed an increase in G6PD activity accompanied by elevation in anti-oxidant system (glutathione) and consequent reduction in oxidative stress. Therefore, we conclude that in a diabetic hypertensive model, spironolactone improves nephropathy by reducing oxidative stress and improving the anti-oxidant system.

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**Table 1.** Physical and metabolic parameters of the SHR.

	Control (n=8)	Diabetic (n=8)	Diabetic + SPR (n=8)
Body weight (g)	281.5 ± 16	139.5 ± 26*	129.8 ± 22*
Systolic BP (mmHg)	187± 14	176 ± 23	177± 28
Plasma glucose (mmol/L)	11.6 ± 3.3	38.8 ± 10*	42.5 ± 12*
KW:BW ratio	0.383 ± 0.043	0.662 ± 0.088*	0.600 ± 0.094*

Data are expressed as means ± SD. \*p<0.0001 vs control group. SPR=spironolactone.

BP=blood pressure, KW=kidney weight, BW=body weight.



## LEGENDS OF FIGURES

**Figure 1.** 24-hour urinary albumin excretion rate (AER). AER was expressed as the geometric mean and variance. Experiments were performed in triplicate (n=5 in control, n=6 in diabetic and n=6 in treated group), and it was analyzed by nonparametric Kruskal-Wallis test followed by the Mann-Whitney U test for comparison between two groups. \*p=0.017 vs control; \*\*p=0.028 vs diabetic.

**Figure 2.** Fibronectin protein levels in kidney cortex of SHR. Representative Western Blot of fibronectin and actin in cortex kidney lysates (a). Densitometric analysis of renal cortical fibronectin/actin ratio (b). Actin was used as control of protein loading. Data are means  $\pm$  SD of at least 3 independent experiments (n=4 in control, n=7 in diabetic and n=6 in treated group). \*p<0.04 vs. control and diabetic + SPR.

**Figure 3.** G6PD activity in the renal cortex in control, diabetic and diabetic treated with SPR. Data are means  $\pm$  SD. Experiments were performed in triplicate (n=8 in each group). \*p=0.008 vs control; \*\*p=0.032 vs diabetic.

**Figure 4.** Urinary 8-OHdG levels, a marker of oxidative stress on the DNA (a) and TBARS levels in renal cortex, a marker of lipid peroxidation (b). Levels were expressed in ng/mL as ratio of 8-OHdG / creatinine (a) and nmol of MDA / mg protein (b). Data are means  $\pm$  SD. Experiments were performed in triplicate (n=8 in each group). \*p=0.019 vs. control and \*p=0.002 vs. diabetic + SPR (a); \*p=0.016 vs. control and \*p=0.009 vs. diabetic + SPR (b).

**Figure 5.** GSH/GSSG ratio in the renal cortex in control, diabetic and diabetic treated with SPR. Data are means  $\pm$  SD. Experiments were performed in triplicate (n=8 in each group). \*p=0.029 vs control and \*p=0.025 vs. diabetic + SPR.

**Figure 6.** NADPH-dependent superoxide generation in renal cortical homogenate. Superoxide anion generation of kidney cortex was determined by the lucigenin-enhanced chemiluminescence method and photoemission was measured every 20 s for 10 min. The

peak level of superoxide generation was observed around 10 min after NADPH addition to reaction buffer containing renal cortical homogenates from different groups (a). Average NADPH oxidase activities during the last 5 min was used for comparison among the groups and expressed as relative luminescence units (RLU)/20 s/mg protein. Bars represent the means  $\pm$  SD. Experiments were performed in triplicate (n=5 in control, n=8 in diabetic and n=6 in treated group) (b). Data were analyzed by nonparametric Kruskal-Wallis test followed by the Mann-Whitney U test for comparison between two groups. \*p=0.009 vs control; \*\*p=0.040 vs diabetic.

**Figure 7.** p47phox protein level in the renal cortex. Representative Western Blot analysis of p47phox and actin in SHR control, diabetic and diabetic treated with SPR (a). Densitometric analysis of p47phox/actin ratio in different group of rats. Actin was used as control of protein loading. Data are means  $\pm$  SD. Experiments were performed in triplicate (n=5 in control, n=6 in diabetic and n=5 in treated group) (b). \*p=0.004 vs control; \*\*p=0.035 vs diabetic.

Figure 1

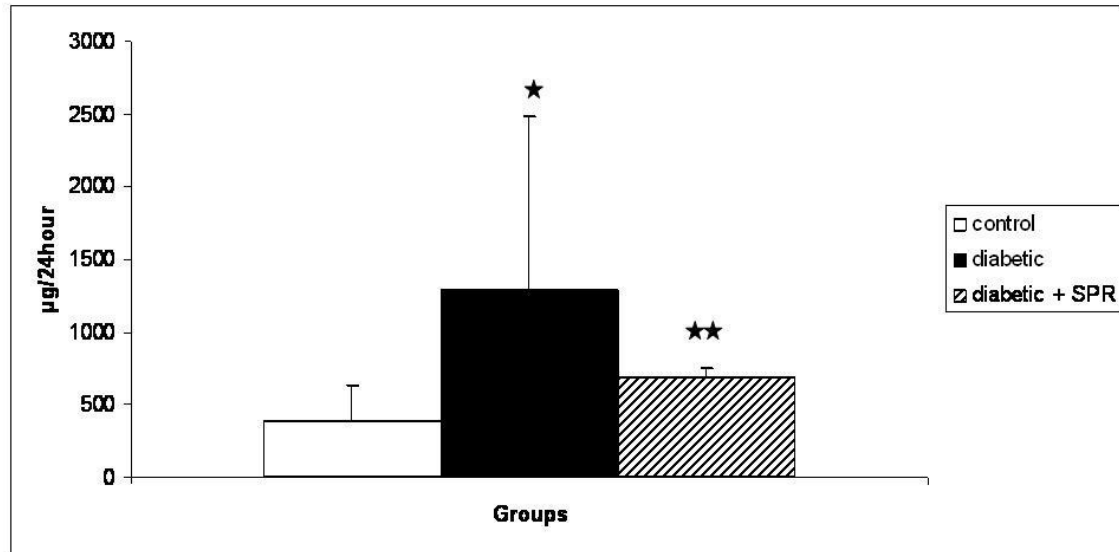


Figure 2

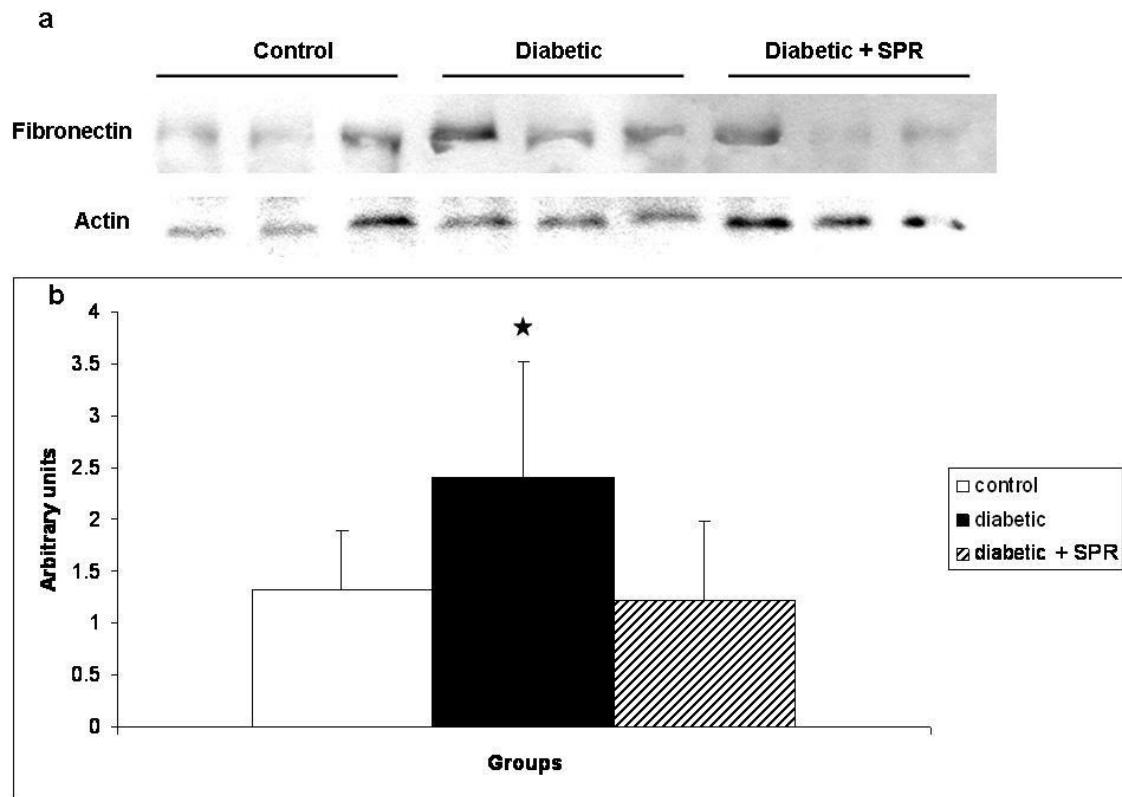


Figure 3

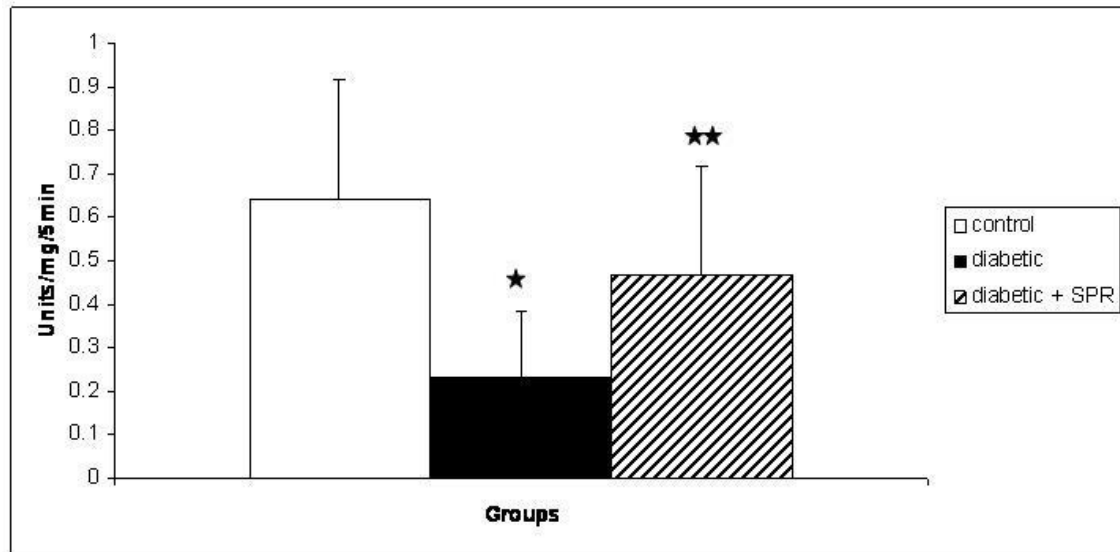


Figure 4

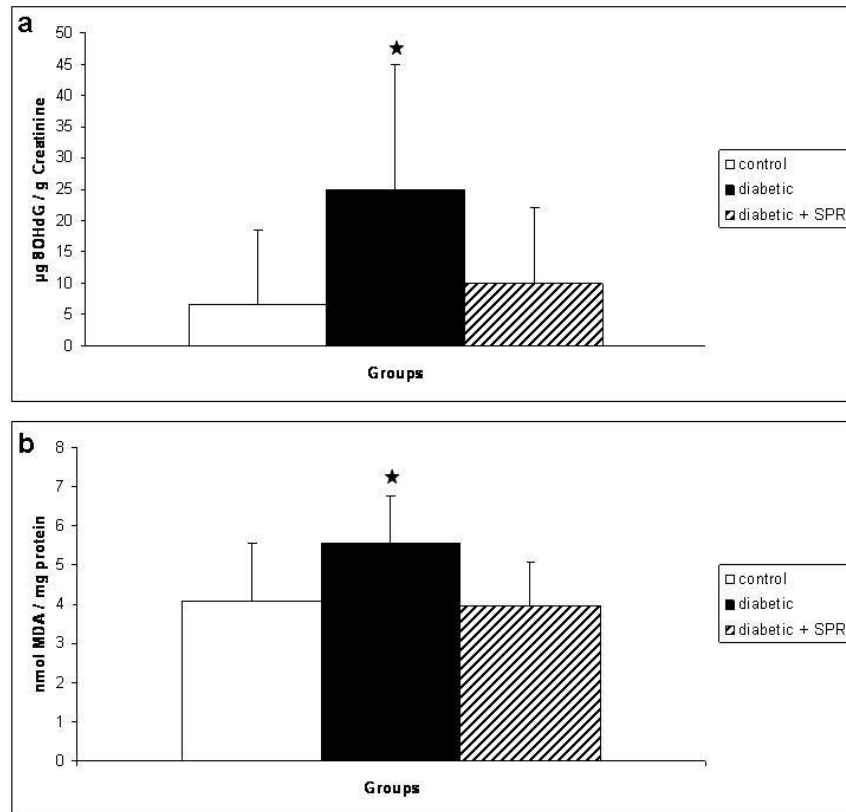


Figure 5

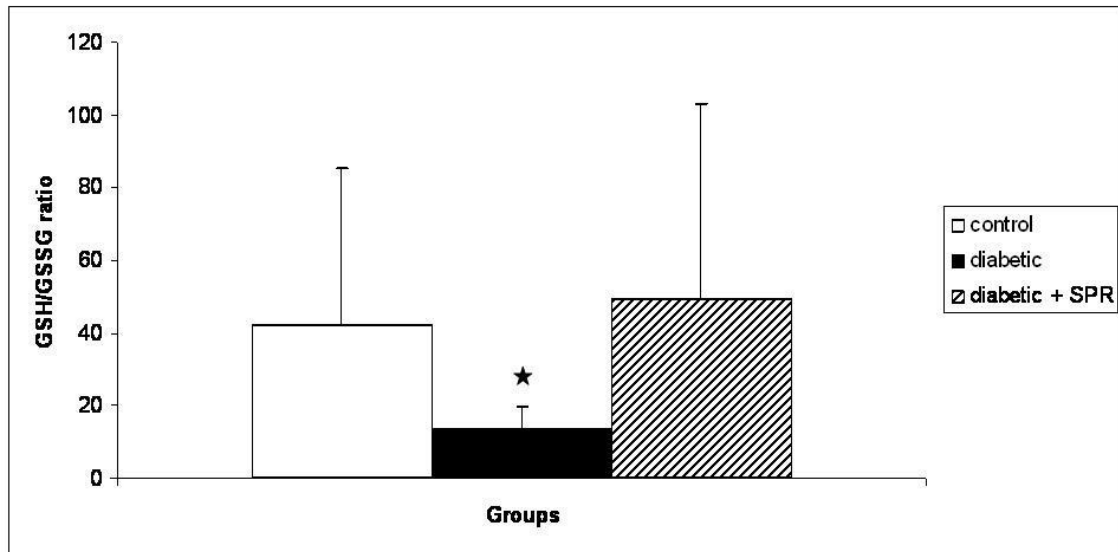


Figure 6

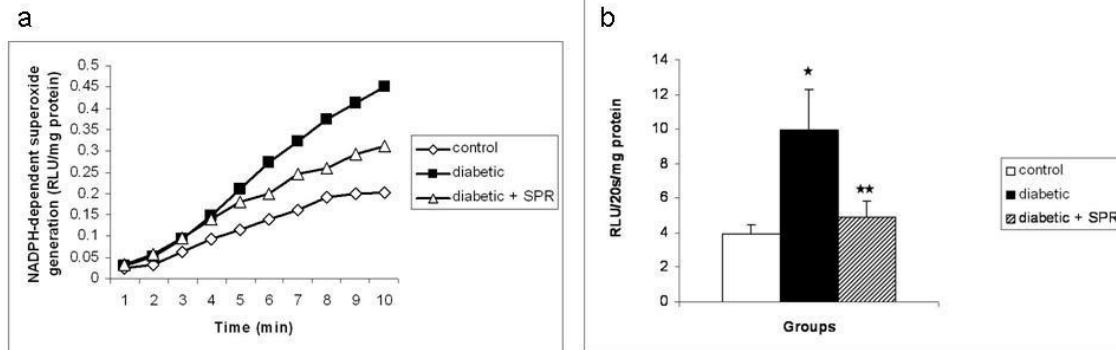
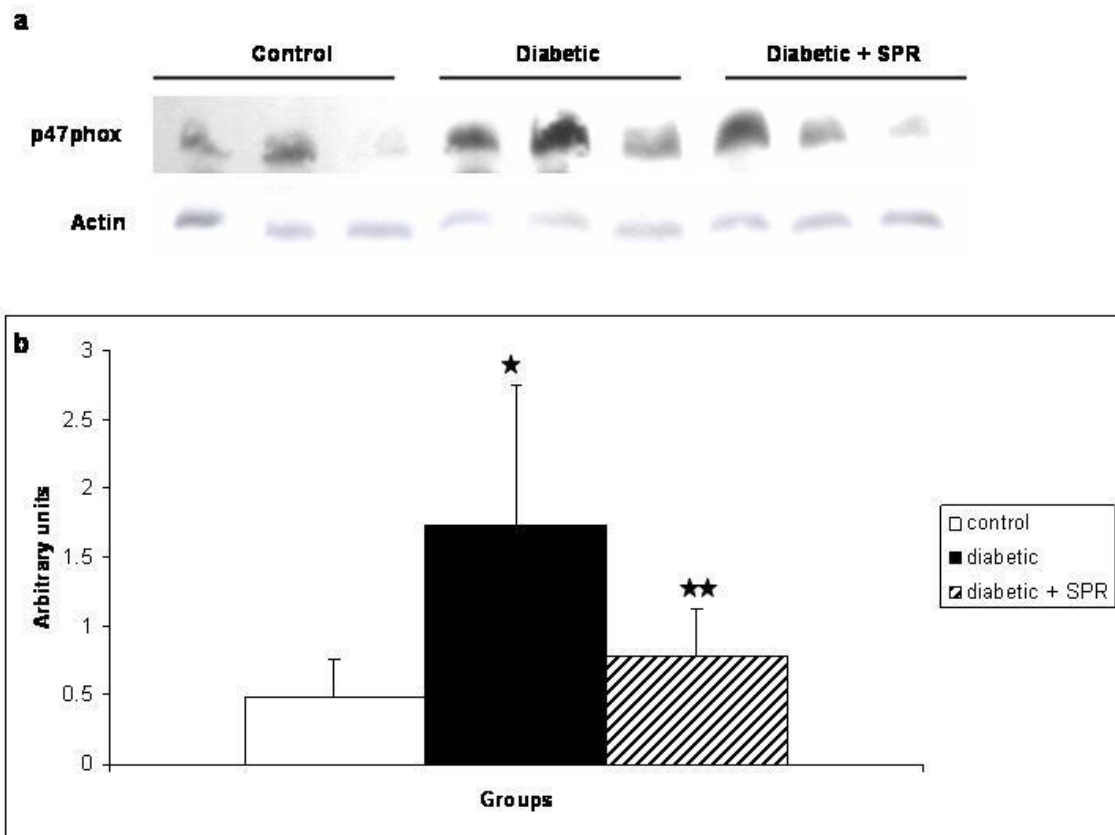




Figure 7





## **1. Sumário**

- O tratamento com espironolactona melhora a nefropatia através da redução do estresse oxidativo;
- O bloqueio da aldosterona promove redução da geração de superóxido via NADPH oxidase;
- A melhora na atividade da enzima G6PD com o tratamento promove melhora do sistema antioxidante glutatona;

## **2. Conclusão geral**

- O bloqueio da aldosterona melhora a nefropatia diabética em animais diabéticos e hipertensos.
- O tratamento com espironolactona restaura a atividade da G6PD, o que promove melhora da atividade antioxidante com redução do estresse oxidativo, sem efeitos na glicemia e pressão arterial.



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## Antioxidant SOD Mimetic Prevents NADPH Oxidase-Induced Oxidative Stress and Renal Damage in the Early Stage of Experimental Diabetes and Hypertension

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### Key Words

Albuminuria · Diabetic nephropathy · Extracellular matrix · Hypertension · Oxidative stress · Spontaneously hypertensive rats · Tempol

### Abstract

**Aims:** The presence of hypertension increases renal oxidative stress by increasing NADPH oxidase-dependent superoxide production and by decreasing antioxidant defense in the early stage of experimental diabetes mellitus (DM). In the present study, we investigated whether the administration of an antioxidant mimetic of the superoxide dismutase (SOD) (tempol) corrects the oxidative imbalance and oxidative stress-induced renal injury in the presence of DM and hypertension. **Methods:** DM was induced in spontaneously hypertensive rats (SHR) by streptozotocin at 4 weeks of age. The diabetic rats either did or did not receive tempol for 20 days. Oxidative-stress parameters and indices of renal injury were evaluated. **Results:** Tempol reestablished the imbalance in redox status induced by DM. It elevated the expression of renal antioxidant extracellular SOD,  $p < 0.0001$ ; decreased ( $p = 0.049$ ) the production of renal NADPH-dependent superoxide production, and diminished ( $p = 0.016$ ) a marker of oxidative stress-induced DNA damage, 8-hydroxy-2'-deoxyguanosine. Reduction of oxidative stress markers was associated with reduction in renal damage parameters associated with DN. DM-induced albuminuria and elevation in renal expression of collagen IV were reduced to the level ob-

served in control rats. **Conclusion:** We conclude that an imbalance in renal redox status is associated with markers of renal injury in the early stage of DM and hypertension. Antioxidant treatment reestablished the redox status and prevented oxidative stress-induced renal damage.

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### Introduction

Hypertension is common in patients with diabetes mellitus (DM) [1], and the combination of these conditions increases the frequency and severity of nephropathy in an additive manner [2]. Accordingly, besides glycemic control, antihypertensive treatment is the most effective maneuver for prevention and treatment of diabetic nephropathy (DN) [3, reviewed in 4]. However, the underlying mechanistic basis of how the combination of diabetes and hypertension aggravates renal disease is unclear.

DN appears to be multifactorial in origin, involving genetic, hemodynamic and metabolic factors [reviewed in 5, 6]. A number of recent studies, in humans and animal models, strongly implicate the contribution of renal oxidative stress to the pathogenesis of DN [7–11]. Likewise, renal oxidative stress may not only be the consequence of hypertension, but also it may contribute to the pathogenesis of elevated blood pressure [12, reviewed in 13]. Oxidative stress has been proposed as an imbalance between oxidants and antioxidants in favor of the oxi-

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dants, leading to a disruption of redox signaling and control and/or molecular damage [14]. The antioxidant system provides major protection against oxidative stress by neutralizing or scavenging reactive oxygen species (ROS) or by breaking the chain reactions [15]. Extracellular superoxide dismutase (EC-SOD), the first-line endogenous defense against superoxide production, converts superoxide ( $O_2^-$ ) to hydrogen peroxide ( $H_2O_2$ ) that is degraded to water and molecular oxygen by catalase and glutathione peroxidase [15]. Therefore, improvement of the antioxidant system could be a potential therapeutic target in DN.

In the kidney, it has been demonstrated that the activation of the enzyme NADPH oxidase is an important source of superoxide production in both hyperglycemia and hypertension [16, 17]. We have recently demonstrated that the presence of hypertension increases renal oxidative stress by increasing NADPH oxidase-dependent superoxide production and by decreasing antioxidant defense, named EC-SOD and reduced glutathione, in early stage of experimental diabetes [9]. Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) is a SOD mimetic that in streptozotocin (STZ)-induced diabetic rats has been shown to reduce superoxide production, renal extracellular matrix and the expression of transforming growth factor ( $TGF-\beta$ ) [18]. An *in vitro* study demonstrated that tempol treatment in mesangial cells reduced ROS and collagen IV production [19]. In the present study, we investigated whether early renal abnormalities could be prevented by an antioxidant intervention, treatment with tempol, in the context of diabetes and hypertension. Our findings indicate that tempol reestablished the redox status, and it was associated with prevention of early renal injury in diabetic hypertensive rats.

## Methods

### *Animals and Experimental Protocol*

The protocol for this study complied with the guidelines established by The Brazilian College for Animal Experimentation (COBEA) and was approved by the Institutional Ethical Committee. Spontaneously hypertensive rats (SHR) derived from rats 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. Experimental diabetes was induced in 4-week-old, prehypertensive male SHR by injecting 60 mg/kg STZ (Sigma, St. Louis, Mo., USA) dissolved in sodium citrate buffer (0.5 M; pH 4.5) via a tail vein after an overnight fast. The control group received only vehicle (citrate buffer). Blood glucose levels were measured using an enzymatic colorimetric GOD-PAP assay (Merck, Darmstadt, Germany)

48 h after the injection of STZ or citrate buffer. Plasma glucose concentrations of  $>15$  mM were considered diabetic for these experiments. The diabetic rats were randomly assigned to receive no treatment or treatment with tempol (Calbiochem, La Jolla, Calif., USA) at a dose of 250 mg/kg diluted in saline solution and administered intraperitoneally [12]. Control, untreated and treated diabetic rats were sacrificed using  $CO_2$  gas 20 days after induction of diabetes. The right kidney was removed, decapsulated, weighed, cut longitudinally into two halves and fixed by immersion in a solution of methacarn (60% methanol, 30% chloroform and 10% glacial acetic acid). The left kidney was similarly removed and also cut longitudinally. One half was fixed in a 4% paraformaldehyde solution. Some pieces of the cortical tissue of the other half were homogenized and processed for Western blot, and other pieces were frozen in liquid nitrogen and preserved at  $-80^\circ C$ .

### *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) before and on days 10 and 20 after induction of diabetes. Rats were habituated to the procedure before taking blood pressure readings.

### *Albumin Excretion Rate (AER)*

Before induction of DM and the 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 AER, as described previously [9].

### *Urinary Excretion of Nitrate ( $NO_3^-$ ) and Nitrite ( $NO_2^-$ )*

$NO_3^-$  and  $NO_2^-$  were determined in 24-hour urine samples by the Griess reaction [20]. Briefly, after centrifugation, urine samples were deproteinized by incubation overnight with cold acetone. Since the Griess reaction detects only  $NO_2^-$ , all the  $NO_3^-$  was converted to  $NO_2^-$  by an enzymatic method [21]. Urine samples were mixed with Griess reagent and the absorbance was read at 540 nm. A sodium nitrate ( $NaNO_3$ ) calibration curve was developed utilizing different  $NaNO_3$  concentration in distilled water.  $NO_2^-$  excretion was expressed as  $NO_2^-/creatinine$  ratio. Urinary creatinine was determined by colorimetric creatinine kit assay (CELM, Barueri, SP, Brazil).

### *NADPH Oxidase Activity*

NADPH oxidase activity was measured by the lucigenin-enhanced chemiluminescence method, as previously described [9, 17]. Briefly, a small piece of frozen kidney cortex was homogenized in 1 ml lysis buffer (20 mM  $KH_2PO_4$ , pH 7.0; 1 mM EGTA; 1 mM phenylmethylsulfonyl fluoride (PMSF); 10  $\mu$ l/ml aprotinin and a cocktail of protease inhibitors 40  $\mu$ l/ml) using a Dounce homogenizer (100 strokes on ice). Homogenates were subjected to low-speed centrifugation at 800 g for 10 min at 4°C to remove unbroken cells and debris. Aliquots of homogenates supernatant (50  $\mu$ l) were added to 450  $\mu$ l of reaction buffer (50 mM phosphate buffer, pH 7.0; 1 mM EGTA; 150 mM sucrose, 5  $\mu$ M lucigenin and 100  $\mu$ M NADPH). Photon emission in terms of relative luminescence units (RLU) was measured every 20 s for 10 min in a Turner TD-20e luminometer. To identify which pathway contributes superoxide production, we preincubated with diphenyleiiodonium

**Table 1.** Physical and metabolic parameters of the experimental groups following 20 days of diabetes

	SHR control (n = 19)	SHR diabetic (n = 24)	SHR diabetic + tempol (n = 11)
Body weight, g	171 ± 16	94 ± 12*	86 ± 30*
Systolic blood pressure, mm Hg	159 ± 11	156 ± 7	152 ± 9
Urinary NO <sub>2</sub> /creatinine ratio, μmol/g	619 ± 423	266 ± 142**	253 ± 105***
Plasma glucose, mg/dl	169 ± 24	482 ± 52*	444 ± 276*
Kidney weight:body weight ratio, %	0.48 ± 0.06	0.69 ± 0.10*	0.66 ± 0.09*

Data are expressed as means ± SD. These parameters were analyzed by the ANOVA test followed by Bonferroni's test.

\* p < 0.0001 vs. SHR control group; \*\* p = 0.0146 vs. SHR control group; \*\*\* p = 0.0117 vs. SHR control group.

(DPI, an inhibitor of NADPH oxidase pathway) and rotenone (an inhibitor of mitochondrial pathway), at 20 and 100 μM final concentration, respectively, for 10 min [9]. Superoxide production was expressed as RLU/20 s/mg protein. Protein concentration was measured using the Bradford method.

#### Immunohistochemistry

To detect extracellular matrix expansion and oxidative stress-induced DNA damage, immunohistochemistry was done for collagen IV and 8-hydroxy-2'-deoxyguanosine (8-OHdG, a DNA base-modified product), respectively. Renal tissues were fixed in 4% paraformaldehyde and methacarn solution for collagen IV and 8-OHdG, respectively, and then they were embedded in paraffin and sectioned at 4 μm. Sections were deparaffinized and rehydrated. After microwave exposure, endogenous peroxidase was blocked by incubating the slides in 3% H<sub>2</sub>O<sub>2</sub> for 10 min. The sections were then incubated overnight at 4°C with a 1:20 dilution of goat anti-type IV collagen antibody (Southern Biotech, Birmingham, Ala., USA) and with a 1:50 dilution of mouse monoclonal anti-8-OHdG antibody (N45.1; Japan Institute for the Control Aging, Japan). The sections were then incubated at room temperature for 1 h with biotinylated secondary anti-goat IgG antibody (Vector, Burlingame, Calif., USA) diluted 1:200 or with anti-mouse IgG antibody (Vector) diluted 1:200. Subsequently, slides were incubated with avidin-biotin complex (ABC) reagent (Dako, Glostrup, Denmark) for 30 min followed by the addition of diaminobenzidine tetrahydrochloride (Sigma). After dehydration and counterstaining with hematoxylin, the slides were mounted in Entellan (Merck). For negative controls, staining was performed omitting the primary antibody. Collagen IV intensity was evaluated by a semiquantitative method as described previously [22]. Tubulointerstitial cells containing 8-OHdG-positive nuclei were counted in 50 sequential high-power microscopic fields (×400), as previously described [9].

#### Western Blotting

Renal cortical homogenate was used for quantification of collagen IV and EC-SOD proteins by Western blot analysis. Molecular weight markers (PageRuler™, Fermentas Life Sciences) were used as standards. Cortical proteins at concentrations of 100 and 140 μg were separated on 6 and 10% SDS-polyacrylamide gel for collagen IV and EC-SOD, respectively. Proteins were electropho-

retically transferred to nitrocellulose membranes. To block non-specific binding and antibody incubation, 5% non-fat milk was used in phosphate-buffered saline or Tris-buffered saline containing 0.1% Tween-20. After blocking non-specific binding, membranes were incubated with a rabbit polyclonal anti-EC-SOD antibody (diluted 1:1,000; SOD-105, Stressgen Bioreagents Corp., Victoria, B.C., Canada) and with a goat anti-type IV collagen antibody (diluted 1:500; Southern Biotech). Following primary antibodies, membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (diluted 1:10,000; Santa Cruz). Immunoreactive bands were made visible using the enhanced chemiluminescence method (Super Signal Cl-HRP Substrate System; Pierce, Rockford, Ill., USA). To verify the uniformity of protein load and transfer efficiency across the test samples, membranes were re probed with actin (goat polyclonal anti-actin antibody, diluted 1:1,000, Santa Cruz) or β-tubulin (mouse polyclonal anti-β-tubulin antibody, diluted 1:500, Calbiochem). 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).

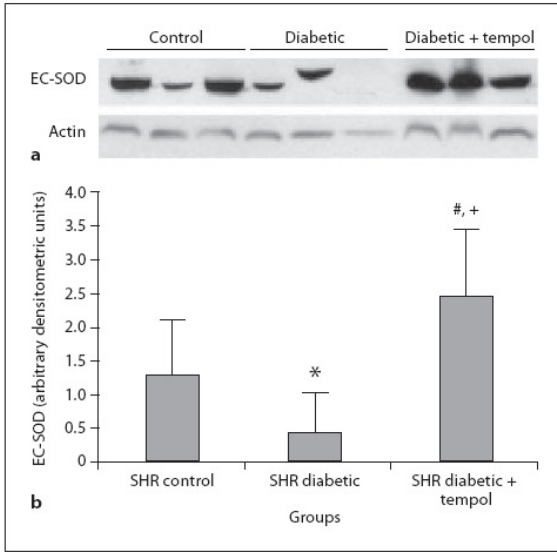
#### Statistical Analysis

The results are expressed as means ± SD, except for albuminuria, which is expressed as geometric means and variance. Comparisons between groups were done with one-way analysis of variance (ANOVA) followed by Bonferroni test. Non-parametric data are expressed as the geometric mean and ranges, and are analyzed by Kruskal-Wallis test (for multiple groups) and Mann-Whitney U test (for two groups). A value of p < 0.05 was considered significant. All analyses were performed using statistical software StatView (SAS Institute Inc., Cary, N.C., USA).

## Results

### Physical and Metabolic Parameters

Body weight gain was significantly reduced, and kidney-to-body weight ratio increased markedly both in untreated and treated diabetic rats, when compared with control group (p < 0.0001, table 1). The systolic blood

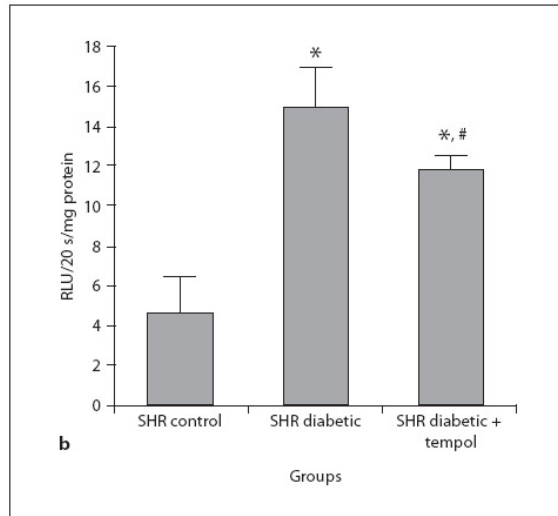
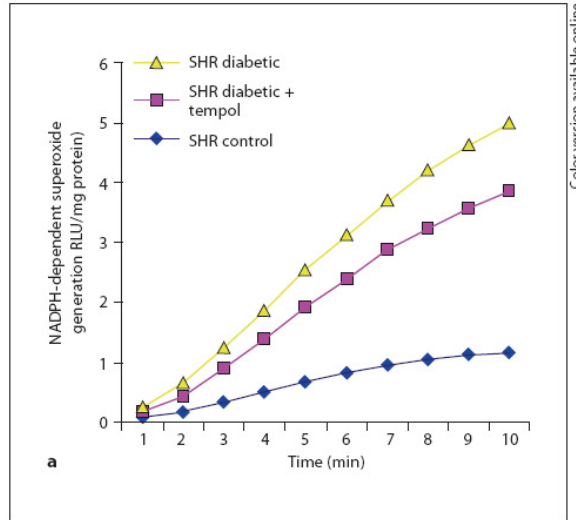


**Fig. 1.** EC-SOD protein level in the renal cortex. **a** Representative Western blot analysis of EC-SOD in SHR control, diabetic and diabetic treated with tempol. **b** Densitometric analysis of EC-SOD/actin ratio in different group of rats. Actin was used as control of protein loading. Bars represent means  $\pm$  SD of band densities (arbitrary densitometric units) from at least two independent experiments (n = 4 in each group), \* p < 0.0423 vs. SHR control group; # p = 0.0221 vs. SHR control group; + p < 0.0001 vs. SHR diabetic untreated group.

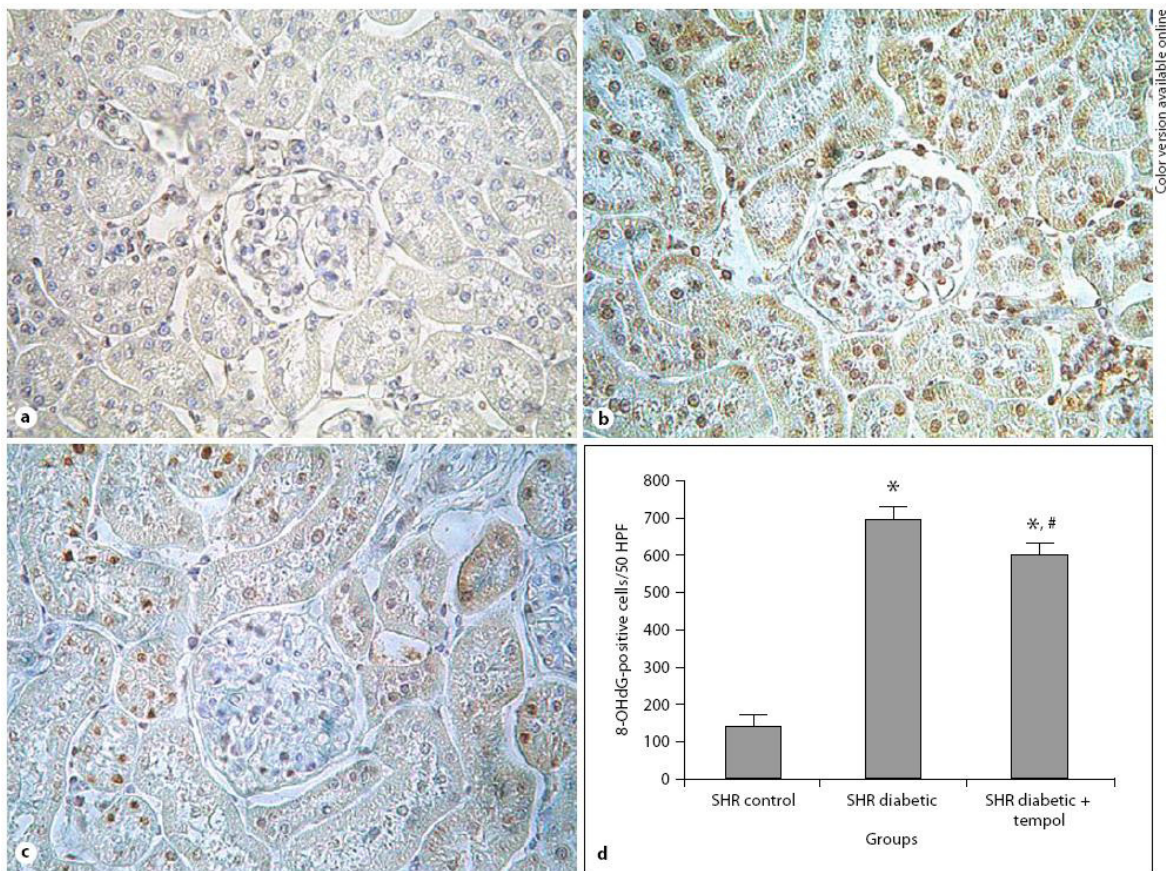
pressure was neither altered by diabetes nor by treatment with tempol. The excretion of  $\text{NO}_2^-$  expressed as  $\text{NO}_2^-/\text{creatinine}$  ratio, an indirect estimation of nitric oxide (NO) synthesis, decreased significantly in both untreated (p = 0.0146) and tempol-treated diabetic rats (p = 0.0117), compared with the control group. The blood glucose level was higher in the diabetic group, and tempol treatment did not affect this parameter (p < 0.0001, table 1).

#### Upregulation of Renal Expression of EC-SOD Was Observed with Tempol Treatment

Western blotting analysis showed that the expression of EC-SOD in the kidney cortex was significantly reduced in diabetic rats ( $0.418 \pm 0.605$  arbitrary units; n = 4) when compared to the control group ( $1.274 \pm 0.823$ ; n = 4, p = 0.0423). However, the treatment with tempol markedly elevated the expression of antioxidant EC-SOD ( $2.450 \pm 1.015$ ; n = 4) compared with control (p = 0.0221) and non-treated diabetic rats (p < 0.0001) (fig. 1).



**Fig. 2.** NADPH-dependent superoxide generation in renal cortical homogenate. **a** Superoxide anion generation of kidney cortex was determined by the lucigenin-enhanced chemiluminescence method and photoemission was measured every 20 s for 10 min. The peak level of superoxide generation was observed around 10 min after NADPH addition to reaction buffer containing renal cortical homogenates from different groups. **b** Average NADPH oxidase activities during the last 5 min was used for comparison among the groups and expressed as relative luminescence units (RLU)/20 s/mg protein. Bars represent means  $\pm$  SD. Data were analyzed by non-parametric Kruskal-Wallis test followed by the Mann-Whitney U test for comparison between two groups. \* p = 0.0167 vs. SHR control group; # p = 0.0495 vs. SHR diabetic untreated group (at least 5 rats were used in each group).



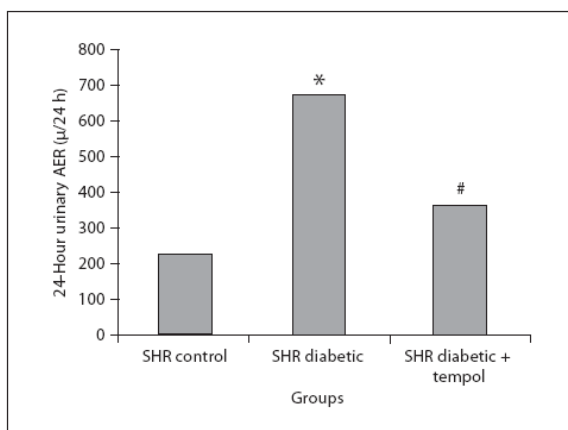
**Fig. 3.** Immunohistochemical detection of oxidative stress-induced DNA damage. Cells in the kidney cortex containing 8-OHdG-stained were identified by their brown-colored nuclei from SHR control (n = 4) (a), diabetic (n = 5) (b) and diabetic treated with tempol (n = 5) (c). At least three sections were stained and evaluated for each rat. Orig. magnif.  $\times 400$ . The median number of 8-OHdG-containing cells per 50 high-power fields (HPF) (d). \* p = 0.014 vs. SHR control group; # p = 0.014 vs. SHR diabetic untreated group. Data were analyzed by the Kruskal-Wallis test followed by the Mann-Whitney U test.

#### *Elevation of NADPH-Dependent Superoxide Generation in DM Was Reduced by Tempol Treatment*

The exaggerated production of renal cortical superoxide observed in the kidney in diabetes is mainly generated by stimulation of NADPH oxidase pathway. Therefore, we investigated the influence of treatment with antioxidant tempol on NADPH oxidase activity using the lucigenin-enhanced chemiluminescence method. Superoxide production via NADPH oxidase in the diabetic renal cortical homogenate ( $15.01 \pm 2.01$  RLU/20 s/mg protein) was significantly (p = 0.0167) higher than in the con-

trol rats ( $4.58 \pm 1.89$ ). Treatment with tempol ( $11.84 \pm 0.73$ ) led to a significant (p = 0.0495) reduction in NADPH-dependent superoxide generation when compared with untreated diabetic rats (fig. 2). To identify the source of superoxide production, we used DPI, an inhibitor of flavin-containing oxidases, and rotenone, an inhibitor of complex I of mitochondrial respiratory chain. Preincubation of the renal cortical homogenates with DPI (20  $\mu$ M final concentration) completely blocked NADPH-induced superoxide production, while preincubation with rotenone (20 and 100  $\mu$ M final concentration) did not





**Fig. 4.** 24-Hour urinary AER. Albuminuria is expressed as the geometric mean, and it was analyzed by non-parametric Kruskal-Wallis test followed by the Mann-Whitney U test for comparison between two groups. \*  $p = 0.012$  vs. SHR control; #  $p = 0.052$  vs. SHR diabetic untreated group. At least three experiments were evaluated for each rat ( $n = 6$  for control group;  $n = 10$  for diabetic group, and  $n = 11$  for diabetic + tempol group).

produce any effect on superoxide production (data not shown), indicating that the NADPH oxidase is the most likely source of the superoxide we detected.

#### *Tempol Decreased 8-OHdG Levels in Diabetic Kidney*

To investigate the influence of tempol in oxidative stress-induced kidney damage, we evaluated the effect of oxidative stress on the DNA by immunohistochemical identification of 8-OHdG-containing cells in the kidney. It was verified that renal cells containing the modified base 8-OHdG in the nucleus were localized mainly in the cortical tubular cells. The number of tubulointerstitial 8-OHdG cells staining (per 50 high-power field) was markedly higher in untreated diabetic rats (695 (642–754);  $n = 5$ ) compared to the control group (159 (130–206);  $p = 0.014$ ,  $n = 4$ ), and it was significantly reduced by treatment with tempol (595 (555–659);  $p = 0.016$ ,  $n = 5$ ) (fig. 3). These findings indicate that antioxidant treatment with tempol attenuates the oxidative stress-induced DNA damage in the kidney in the early stage of experimental diabetes and hypertension.

#### *Tempol Treatment Normalized the Urinary AER after 20 Days of Diabetes*

The urinary AER was significantly elevated ( $p = 0.012$ ) in diabetic rats (680 (180–1,815)  $\mu\text{g}/24\text{h}$ ) compared to the

control group (229 (109–540)), and tempol treatment (366 (109–1,342)) significantly reduced this parameter to the levels seen in control rats ( $p = 0.052$  compared to untreated diabetic rats; fig. 4). This finding shows that the treatment with an antioxidant prevents the development of albuminuria, a hallmark of DN, in early diabetes and hypertension.

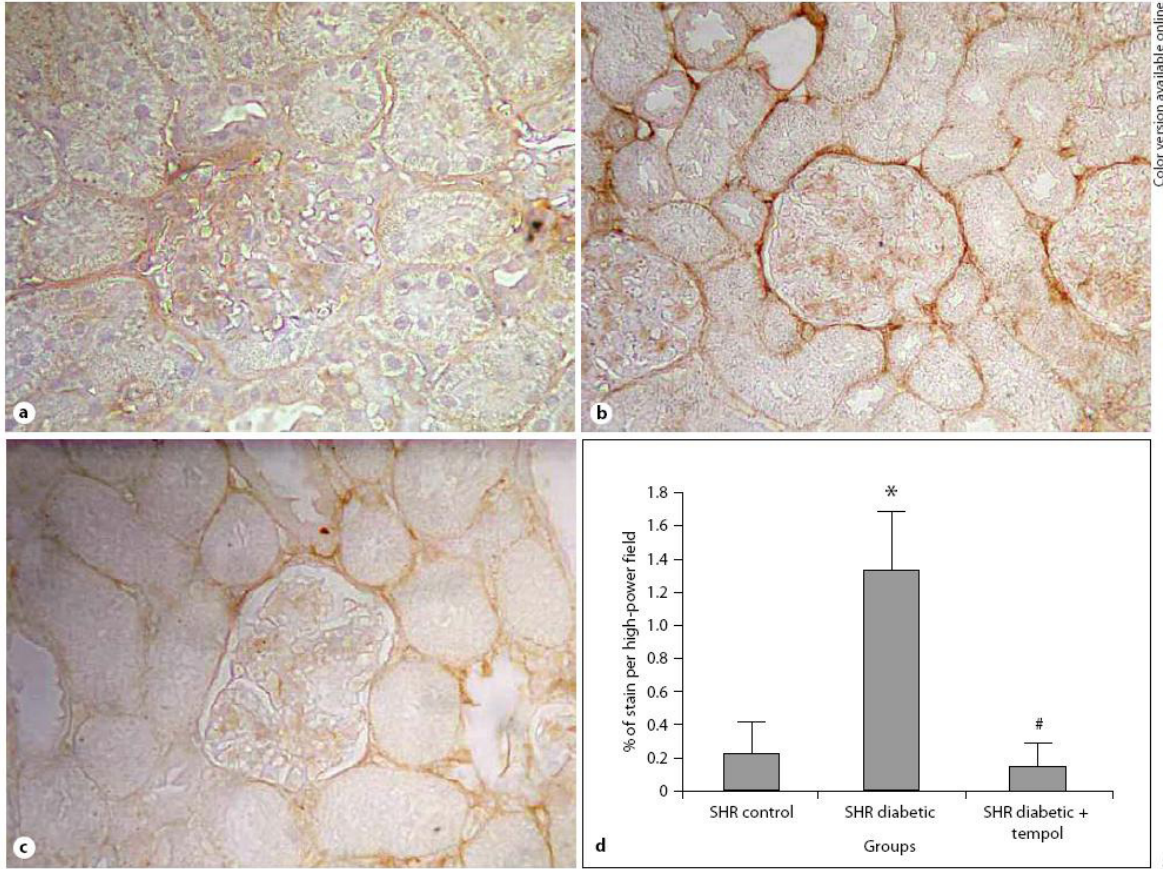
#### *Elevation in Collagen IV Expression Induced by Diabetes Was Prevented by Tempol Treatment*

Since the extracellular matrix accumulation is an important mark of kidney damage associated with early DN, we investigated the expression of collagen IV in our studied rats. By immunohistochemistry, diabetic rats showed higher glomerulus and tubulointerstitial expressions of collagen IV (fig. 5b, d), compared to the control group ( $p = 0.0005$ ; fig. 5a, d) and tempol treatment restored this expression to the levels seen in the control group (fig. 5c, d). Similarly, Western blot analysis (fig. 6) showed an elevated collagen IV protein expression in diabetic kidney cortex ( $4.247 \pm 2.222$  arbitrary units;  $n = 4$ ) compared to the control ( $1.020 \pm 0.744$ ;  $n = 5$ ,  $p = 0.0196$ ), and tempol treatment ( $1.550 \pm 1.513$ ;  $n = 5$ ) significantly reduced this expression to levels similar to those observed in control rats ( $p = 0.0420$ , when compared to the untreated diabetic group). So, an early profibrotic response injury observed in DN was prevented by an antioxidant treatment, i.e. tempol.

## Discussion

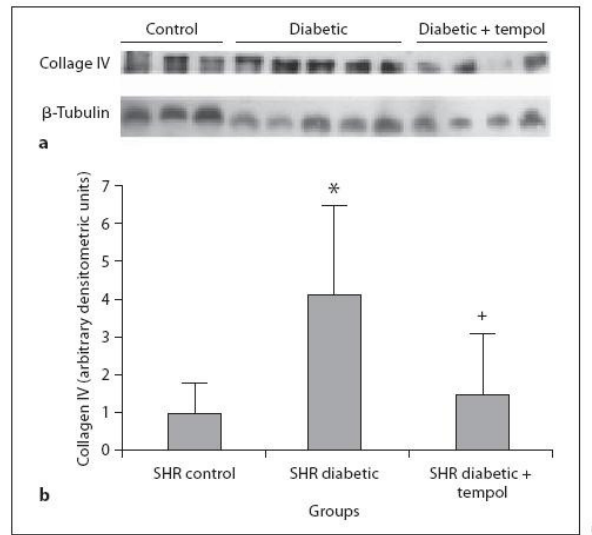
Findings of the present study confirm our previous observations that short-term induction of diabetes in SHR rats led to renal oxidative stress by increasing prooxidant generation and decreasing antioxidant defense [9]. In addition, we observed that this imbalance in oxidative stress status was accompanied by indices of renal injury, both functional (increased albuminuria) and structural (renal collagen IV accumulation). Finally, we found that use of an antioxidant, SOD mimetic (tempol), not only restored the redox imbalance but also prevented renal abnormalities. These observations suggest that an antioxidant therapy may be useful for prevention of renal disease in the presence of diabetes and hypertension.

To our knowledge, no study has previously investigated the use of tempol in diabetic hypertensive rats. Investigation of renal disease in this animal model may be of great importance since hypertension and diabetes frequently coexist in humans [1]. We and others have demonstrated



**Fig. 5.** Immunohistochemical detection of collagen IV protein expression. Immunostain for collagen IV was detected in the kidney cortex of SHR control (a), diabetic (b) and treated with tempol (c), after 20 days of diabetes. The quantification of collagen IV expression in the groups from at least three independent experiments (d). \*  $p = 0.0005$  vs. control group; #  $p = 0.0002$  vs. SHR diabetic untreated group ( $n = 4$  in each group). Data were analyzed by the ANOVA followed by the Bonferroni test.

**Fig. 6.** Expression of collagen IV protein in the renal cortex. **a** Representative Western blot analysis of collagen IV in SHR control, diabetic and diabetic treated with tempol. **b** Densitometric analysis of collagen IV/ $\beta$ -tubulin ratio in different group of rats.  $\beta$ -Tubulin was used as control of protein loading. Bars represent means  $\pm$  SD of band densities (arbitrary densitometric units) from at least three independent experiments (mean of  $n = 5$  in each group). \*  $p = 0.0196$  vs. SHR control group, +  $p = 0.0420$  vs. SHR diabetic untreated group.



that the presence of genetic hypertension significantly aggravates indices of renal injury in diabetic rats [23–25]. Particularly, we have demonstrated that induction of diabetes for 20 days in 4-week-old SHR (same duration of DM and age in the present study) increased albuminuria, glomerular expression of fibronectin and TGF- $\beta_1$  receptor and decreased glomerular expression of nephrin [26, 27]. It has also been demonstrated that in these rats different classes of antihypertensive medications effectively improved nephropathy [26–31]. As expected, however, these drugs invariably reduced blood pressure, which suggests that this was the main determinant for the beneficial effect in nephropathy [26–31], although specific renal protection has been claimed for some group of drugs [32]. Interestingly, in the present study, treatment with an antioxidant (tempol) did not affect blood pressure or glycemic control, further supporting the importance of oxidative stress in mediating renal abnormalities in these rats and the potential of antioxidant therapy in the presence of diabetes and hypertension.

It has been demonstrated that administration of tempol to SHR leads to a significant amelioration of hypertension [33]. Reduction of oxidative stress with subsequent increase in bioavailability of NO has been suggested as the mechanism responsible for the effect of tempol in SHR [34]. However, as observed in the present study, it has been demonstrated that in diabetic rats NO production is diminished [35, 36], a factor that may render blood pressure levels in diabetic SHR insensitive to tempol.

Our present finding clearly demonstrates that administration of tempol to diabetic hypertensive rats reduces renal NADPH-induced superoxide generation and elevation of renal EC-SOD expression. These findings, therefore, support previous data showing that tempol is a SOD mimetic, with additional ROS-scavenging activities [37, 38] that may also reduce NADPH oxidase activation [39]. However, the magnitude of increase in the expression of EC-SOD (fig. 1) in rats receiving tempol was smaller than the reduction of NADPH-induced superoxide generation and accumulation of immunoreactive 8-OHdG (fig. 2, 3). This dissociation allows different interpretations. One possibility is that the expression of EC-SOD does not accurately reflect the enzymatic activity of this protein. Another explanation is that the protection of SOD has been diminished due to its higher concentration. Such a ‘bell-shaped’ profile of protection is a recognized feature of SOD and SOD mimetic, as increasing SOD concentrations in mammalian cells can produce a paradoxical pro-oxidant action by which protection against oxidative stress begins to decline [40, 41]. Furthermore, the activities of

SOD are limited to a narrow concentration range in which superoxide cytotoxicity is prevented, yet superoxide-dependent termination events are allowed to occur [42].

SOD are very important enzymatic antioxidants that rapidly catalyze the dismutation of superoxide, and promote its removal [43]. In case of a deficiency in SOD (or increased production of superoxide) the superoxide preferentially reacts with NO and produces peroxynitrite, a powerful oxidizing and nitrating agent that can directly damage proteins, lipids, and DNA [44]. Among the three mammalian isozymes of SOD, EC-SOD (the other two are Cu<sup>2+</sup>, Zn<sup>2+</sup>-containing intracellular SOD and Mn-containing mitochondrial SOD) constitutes as much as 70% of the total SOD activity in human and baboon aorta [45], and high levels of EC-SOD expression have been observed in the lungs and kidneys [46]. Recent studies indicate that renal cortical EC-SOD expression is around 50% lower in SHR compared with normotensive Wistar-Kyoto rats [47], and that induction of DM in SHR markedly reduces its expression [9]. The mechanism by which diabetes and/or hypertension reduces EC-SOD is not completely understood. However, the observation that expression of EC-SOD in mice is regulated by NO has been made [48]. In addition, it has been shown that TGF- $\beta$  inhibits the antioxidative capacity of SOD [49, 50]. Since we have previously demonstrated, in the same model and conditions of the current study, that the expression of kidney TGF- $\beta$  is elevated in these rats [26], it is possible that in the current study the reduction in the expression of EC-SOD in the diabetic rats is secondary to higher expression of TGF- $\beta$ . It can also be speculated that tempol has determined an elevation of EC-SOD because it has decreased the expression of TGF- $\beta$ . That tempol decreases the expression of TGF- $\beta$  has recently been demonstrated by Zhao et al. [51] in hypertensive rats that have received angiotensin II.

The importance of SOD in the pathogenesis of diabetic renal lesions has been elegantly demonstrated by DeRubertis and co-workers [52, 53] in genetically modified animals. These authors have shown that overexpression of cytosolic Cu<sup>2+</sup>, Zn<sup>2+</sup>-containing SOD (SOD1) attenuates oxidative stress and glomerular injury in mouse models of type 1 (streptozotocin diabetic mouse) and type 2 (db/db mouse) DM. Concordantly, acceleration of renal injury was observed in the SOD1 knockout mouse (KO) rendered diabetic by streptozotocin [54]. In the latter study, treatment of diabetic KO with tempol for 4 weeks suppressed albuminuria, decreased glomerular TGF- $\beta$ , collagen  $\alpha 1$  (IV), nitrotyrosine, and glomerular peroxide. A similar observation was obtained when exogenous SOD was administered intraperitoneally to young

streptozotocin-induced diabetic rats. This treatment leads to reduction in superoxide levels, albuminuria and renal expression of TGF- $\beta_1$ , fibronectin and 8-OHdG [55]. However, Asaba et al. [18] were unable to demonstrate reduction in albuminuria when tempol was given to STZ-induced diabetic rats, though the authors did observe a reduction in renal expression of TGF- $\beta$  and mesangial expansion.

In the present study the effects of tempol on indices of oxidative stress and renal injury were not concurrently examined in the non-diabetic SHR or the diabetic genetically normotensive control Wistar-Kyoto rats. However, we have previously shown that induction of diabetes leads to an increase in the production of NADPH oxidase-dependent superoxide generation in both normo- and hypertensive rats [9]. Interestingly enough, antioxidant parameters such as EC-SOD and the reduced form of glutathione that was unchanged in diabetic normotensive rats were significantly reduced only in the diabetic hypertensive rats. Consequently, oxidative stress-induced DNA damage and protein nitration, estimated by immunohistochemistry for 8-OHdG and by Western blot for nitrotyrosine, respectively, was elevated only in the diabetic hypertensive rats. In the present study we aimed to assess, firstly, whether the abnormalities observed in oxidative stress parameters in the kidney of diabetic hypertensive rats were associ-

ated with indices of renal injury, and secondly, whether correction of oxidative stress imbalance with tempol leads to prevention of renal injury. We have observed that tempol has in fact restored the redox imbalance and decreased renal injury without modifying blood glucose and arterial pressure, suggesting a link between oxidative stress and renal injury in the diabetic hypertensive rats.

We conclude that antioxidant therapy with tempol reduces oxidative stress and indices of renal injury in the presence of diabetes and hypertension. This finding strengthens the concept that intervention aimed at increasing the antioxidant defense system may be useful for treating nephropathy in diabetic hypertensive patients [56, 57].

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