ELISA BOUÇADA MAURO INÁCIO PEIXOTO

TEMPOL É RENOPROTETOR NA NEFROPATIA DIABÉTICA EXPERIMENTAL

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

Orientador: Prof. Dr. José Butori Lopes de Faria

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Á toda minha família e ao Caio

pelo amor e apoio

incondicionais

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ÍNDICE

8-OHdG	8-hidroxi-2'-deoxiguanosina
ADP	adenosina fosfato
AGE	produtos finais da glicosilação avançada
ANG II	angiotensina II
CDK	cinase dependente de ciclinas
CTGF	fator de crescimento de conexão tecidual
Cu/Zn SOD	cobre/zinco superóxido desmutase
DIN1	domínio intracelular de Notch 1
DM	diabetes mellitus
DRC	doença renal crônica
EcSOD	extracelular superóxido desmutase
ERC	espécies reativas de cloro
ERN	espécies reativas de nitrogênio
ERO	espécies reativas de oxigênio
ESL	superfície celular endotelial
GAPDH	gliceraldeído-3-fosfato dehidrogenase
GESF	glomeruloesclerose focal e segmentar
GSH	glutationa reduzida
H_2O_2	peróxido de hidrogênio
HAS	hipertensão arterial sistêmica
HOCL	ácido hipocloroso
IRC	insuficiência renal crônica
MAPK	proteína quinase ativadora da mitogênese
MbFeIII	metmioglobina
MBG	membrana basal glomerular
MnSOD	manganês superóxido desmutase
NAD^+	nicotinamida adenina dinucleotídeo
NADPH	nicotinamida adenine dinucletídeo fosfato (forma reduzida)
NF-κB	fator de transcrição nuclear-κB

NO	óxido nítrico
O_2^-	superóxido
$OH^\bullet$	radical hidroxil
$ONOO^-$	peroxinitrito
PA	pressão arterial
PARP-1	poli (ADP-ribose) polimerase
PARs	poli (ADP-riboses)
PKC	proteína quinase C
RFG	ritmo de filtração glomerular
SHR	ratos espontaneamente hipertensos
SOD	superóxido desmutase
TGF-β	fator de crescimento de transformação-β
WT-1	proteína de tumor de Wilms

Hipertensão arterial (HA) e *diabetes mellitus* (DM) são os dois maiores fatores de risco independentes para o desenvolvimento da doença renal crônica. Porém, HA e DM frequentemente coexistem em humanos e sua combinação acarreta aumento na incidência e gravidade da nefropatia e conseqüente progressão para doença renal terminal. Tanto a hiperglicemia quanto a HA induzem estresse oxidativo no rim, que está implicado na patogênese da nefropatia diabética. O aumento do estresse oxidativo, pela produção de superóxido dependente da via NADPH oxidase e pela diminuição da defesa antioxidante, acarreta danos renais, aumento da albuminúria e progressão da nefropatia diabética. Na presente série de estudos, investigamos os efeitos do tempol, um antioxidante mimético da superóxido desmutase endógena (SOD), no estado redox via NADPH oxidase e em danos renais induzidos pelo estresse oxidativo em um modelo experimental que combina HA genética (ratos espontaneamente hipertensos – SHR) e DM induzido por estreptozotocina. Os estudos foram descritos nos seguintes artigos:

Artigo I: Neste artigo, investigamos se o tratamento com o antioxidante tempol melhora o estresse oxidativo e os conseqüentes danos renais, na presença de HA e fase inicial de DM. Parâmetros de estresse oxidativo e índices de lesão renal foram avaliados em ratos SHR pré-hipertensos de 4 semanas de idade, com 20 dias de DM. Ratos diabéticos receberam ou não tempol intraperitonealmente por 20 dias. O tratamento com tempol proporcionou o aumento da expressão renal do antioxidante SOD extracelular, diminuição da produção renal de superóxido via NADPH oxidase e redução na expressão de 8-hidroxi-2'-deoxiguanosina (8-OHdG), marcador de dano ao DNA induzido por estresse oxidativo, comparado com os animais diabéticos não-tratados. Além disso, o tratamento com tempol melhorou danos renais característicos da nefropatia diabética, como albuminúria e expressão de colágeno IV. Portanto, concluímos que o tratamento com o antioxidante tempol reestabelece o estado redox e previne danos renais induzidos pelo estresse oxidativo em estágio precoce de DM e HA.

Artigo II: No segundo artigo investigamos como o tempol melhora a albuminúria em ratos diabéticos e hipertensos. Córtex renal e glomérulos isolados foram coletados de ratos SHR com 4 semanas de idade, após 20 dias de DM e tratamento com tempol. O

tratamento com tempol restaurou os componentes da barreira de filtração glomerular em ratos SHR diabéticos, pois preveniu a depleção de podócitos por apoptose, além de evitar a redução da expressão de nefrina induzidas pelo DM. Tempol também reduziu a expressão da proteína poli(ADP-ribose) polimerase (PARP-1) ribosilada em glomérulos de SHR diabéticos, uma enzima nuclear ativada por danos ao DNA induzidos por estresse oxidativo, sugerindo redução na atividade de PARP-1. *In vitro*, glomérulos isolados incubados com H_2O_2 (espécies reativas de oxigênio) apresentaram elevado número de células em apoptose, e os tratamentos com tempol e com inibidor de PARP-1 evitaram a apoptose de células glomerulares. Portanto, concluímos nesse artigo que o tempol reduz albuminúria em ratos diabéticos e hipertensos pela diminuição da apoptose de podócitos induzida, em parte, por PARP-1.

Em resumo, a presente tese fornece evidência de que o antioxidante tempol é renoprotetor na nefropatia diabética experimental, uma vez que restaura o estado redox, reduz o acúmulo de matriz extracelular, previne apoptose de podócitos induzida por PARP-1 e reduz albuminúria, sem alterar a glicose sangüínea e a pressão arterial.

ABSTRACT

Hypertension (HTN) and *diabetes mellitus* (DM) are the two major independent risk for development of chronic renal disease. However, HTN and DM frequently coexist in human and the combination of these conditions increases the frequency and severity of nephropathy and end-stage renal disease. Both, hyperglycemia and HTN, can induce renal oxidative stress, which strongly contributes to the pathogenesis of diabetic nephropathy. Elevation in oxidative stress, by increasing NADPH oxidase-dependent superoxide production and by decreasing antioxidant defense, leads to renal injury, albuminuria and diabetic nephropathy progression. The present series of studies were therefore undertaken to investigate the effect of tempol, an antioxidant mimetic of the superoxide dismutase (SOD), in the redox status via NADPH oxidase and in oxidative stress-induced renal injury in an experimental model that combines genetic HTN (spontaneously hypertensive rats – SHR) and streptozotocin-induced DM. The studies were described in the following papers:

Paper I: In this study we investigated whether the treatment with tempol improve the redox imbalance and consequent renal injury, in the presence of HTN and early stage of DM. Oxidative-stress parameters and indices of renal injury were evaluated in 4-week-old prehypertensive SHR with 20 days of DM. The diabetic rats either did or did not receive tempol intraperitoneally for 20 days. Treatment with tempol provided an elevation on the expression of renal antioxidant extracellular SOD, decreased the production of renal NADPH-dependent superoxide production and diminished 8-hydroxy-2'-deoxyguanosine (8-OHdG) expression, a marker of oxidative stress-induced DNA damage, compared with untreated diabetic group. Additionally, tempol treatment reduced renal damage parameters associated with diabetic nephropathy, such as albuminuria and collagen IV expression. Therefore, in the paper I we concluded that treatment with an antioxidant tempol reestablished the redox status and prevented oxidative stress-induced renal damage in early stage of DM and HTN.

Paper II: In this study we investigated the mechanism by which tempol corrects albuminuria in early stage of experimental DM and HTN. Renal cortex and isolated glomeruli of 4-week-old prehypertensive SHR were collected after 20 days of DM and treatment with tempol. Tempol ameliorates the components of glomerular filtration barrier

in the diabetic SHR by reducing podocyte apoptosis and depletion, and by restoring nephrin expression. Tempol also diminished the amount of ribosilated poly(ADP-ribose) polimerase PARP-1, a nuclear enzyme activated by DNA strand breaks due to oxidative stress, in the diabetic kidney of SHR, suggesting reduction of PARP-1 activity. In vitro, isolated glomeruli exposed to reative oxygen species by incubation with hydrogen peroxide showed a higher number of apoptotic cells that was prevented by tempol or a PARP-1 inhibitor. Therefore, in the paper II we concluded that tempol reduces albuminuria in experimental model of DM and HTN at least in part by diminish PARP-induced podocyte apoptosis and oxidative stress.

In summary, the present thesis provides evidence that the antioxidant tempol is renal protective in experimental diabetic nephropathy by reestablishing redox status, reducing accumulation of kidney extracellular matrix, reducing PARP-1-induce podocyte apoptosis and albuminuria. These effects were independent of blood glucose and blood pressure reduction, the two most effective approaches for the treatment of diabetic nephropathy.

CAPÍTULO 1

1. INTRODUÇÃO

1.1. Considerações gerais

1.1.1 Hipertensão arterial

A hipertensão, ou aumento na pressão arterial sistêmica, é um importante desafio da saúde pública em todo o mundo devido à sua alta freqüência, e concomitante risco de doenças cardiovasculares e renais (He e Whelton, 1997). Estudo recente demonstra que o aumento na pressão arterial (PA) em adultos está associado com significativa redução na expectativa de vida, de aproximadamente 5 anos, comparado com indivíduos normotensos (Franco et al., 2005). "The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure (JNC-7)" (2003) definiu hipertensão como pressão sanguínea sistólica $\geq$ 140 mmHg e pressão diastólica $\geq$ 90 mmHg. A condição de pré-hipertensão (pressão sistólica 120-139 mmHg ou pressão diastólica 80-89 mmHg) requer modificações no estilo de vida do indivíduo a fim de evitar o aumento progressivo na PA e conseqüente doença cardiovascular (Chobanian et al., 2003). A prevalência da hipertensão no mundo foi estimada em torno de 1 bilhão de indivíduos em 2000-2002, e aproximadamente 7,1 milhões de mortes por ano foram atribuídas à hipertensão (World Health Report, 2002; Kearney et al., 2005). Segundo pesquisa de Kearney e colaboradores (2005), o número de adultos com hipertensão em 2025 foi prognosticado a aumentar em 60%. A hipertensão não só parece afetar mais pessoas em países em desenvolvimento econômico, em comparação aos países desenvolvidos, como há maior incidência de doenças cardiovasculares em indivíduos jovens nestes países (Pearson, 1999; Kearney et al., 2005). A prevalência da hipertensão é semelhante entre homens e mulheres, sendo que sua incidência aumenta com a idade consistentemente em cada região do mundo.

A hipertensão essencial, primária ou hipertensão de causa desconhecida representa 95% de todos os casos de hipertensão e é considerada uma doença heterogênea, em que os diversos pacientes possuem diferentes fatores que acarretam o aumento na PA. A hipertensão primária é resultado de uma complexa interação entre fatores genéticos e

ambientais (Carretero e Oparil, 2000; Oparil et al., 2003). Diversos fatores fisiopatológicos têm sido implicados na gênese da hipertensão primária, como por exemplo: aumento na atividade do sistema nervoso simpático; superprodução de hormônios que retém sódio ou vaso constritores; dieta inadequada de potássio e cálcio; secreção aumentada ou inapropriada de renina, resultando em superprodução de angiotensina II e aldosterona; deficiência de vasodilatadores como prostaciclina, óxido nítrico e peptídeos natriuréticos; alterações na resistência vascular, incluindo lesões na microvasculatura renal; *diabetes mellitus* (DM); resistência à insulina; obesidade; dentre outros (Calhoun et al., 2000; Oparil et al., 2003). Em contrapartida, a hipertensão secundária constitui apenas 5% de todos os casos de hipertensão e é resultante de causas conhecidas como doença renovascular, falência renal, hiperaldosteronismo ou outras implicações monogênicas (Carretero e Oparil, 2000).

Tem sido demonstrado que há uma correlação positiva e contínua entre o aumento na PA e o risco de doenças cardiovasculares como infarto do miocárdio, falência cardíaca e acidente vascular cerebral, doenças renais e mortalidade (Carretero e Oparil, 2000; Chobanian et al., 2003). Esta correlação parece ser mais intensa com a pressão sistólica do que a diastólica (Cutler, 1996). A hipertensão é considerada a segunda causa de estágio dialítico da doença renal, perdendo apenas para o DM (United States Renal Data System, 2006). O número de indivíduos que possuem hipertensão e diabetes é muito grande na população mundial. A prevalência da hipertensão é muito maior em diabéticos, enquanto que pessoas com elevada pressão arterial apresenta 2,5 vezes mais probabilidade de desenvolver DM em 5 anos (Gress et al., 2000; Sowers e Bakris, 2000; Sowers e Haffner, 2002). A coexistência de hipertensão e DM é particularmente perigosa devido à alta relação destas duas condições com todas as doenças cardiovasculares, acidente vascular cerebral, progressão da doença renal e retinopatia diabética (Chobanian et al., 2003).

Geralmente são recomendados tratamentos antihipertensivos genéricos, com pouca ênfase em terapia seletiva baseada na fisiopatologia do aumento da PA. A identificação das causas específicas da hipertensão individualmente, por meio de novas tecnologias em genética, genômica e proteômica, integradas com estudos em fisiologia proporcionará o

desenvolvimento de terapias seletivas para mecanismos fisiopatológicos distintos, com poucos efeitos colaterais, resultando em redução mais efetiva da PA e prevenção das conseqüentes complicações (Chobanian et al., 2003; Oparil et al., 2003).

1.1.2. *Diabetes mellitus*

O *diabetes mellitus* (DM) é uma doença metabólica crônica caracterizada por hiperglicemia resultante, principalmente, de uma deficiência na secreção de insulina e/ou na inabilidade da insulina de exercer adequadamente seus efeitos (American Diabetes Association, 2004; Balakumar et al., 2009; Oliveira et al., 2009). O DM é classificado em tipo1, tipo 2 e gestacional (The Expert Committee on the Diagnosis Classification of Diabetes Mellitus, 2003). O diabetes tipo 1 provem da destruição auto-imune das células beta no pâncreas, acarretando deficiência absoluta de insulina e levando a completa dependência de insulina exógena para regular o nível de glicose sanguínea (Atkinson e Maclaren, 1994; Genuth et al., 2003; The Expert Committee on the Diagnosis Classification of Diabetes Mellitus, 2003). Embora o diabetes tipo 1 seja reconhecido como uma doença que acomete crianças e adultos jovens, estudos epidemiológicos tem indicado uma incidência comparável em adultos (Onkamo et al., 1999). O diabetes tipo 2 acomete aproximadamente 85 a 95% dos casos diagnosticados de diabetes. As células beta pancreáticas tornam-se progressivamente menos capazes de secretar insulina suficiente para manter a homeostase normal de carboidrato e lipídeo (Bell e Polonsky, 2001). Além do que, o diabetes tipo 2 é usualmente caracterizado pela resistência à insulina, de maneira que os tecidos alvos não usam adequadamente a insulina com o efeito da idade, obesidade e sedentarismo (Saltiel e Kahn, 2002). O terceiro tipo, o diabetes gestacional, desenvolve-se durante a gravidez (The Expert Committee on the Diagnosis Classification of Diabetes Mellitus, 2003).

O DM é um sério e crescente problema de saúde pública, com características epidêmicas, que resulta na redução da expectativa de vida e aumento da mortalidade devido as complicações vasculares específicas da doença (Ceriello et al., 2009). A prevalência do DM em todo o mundo foi estimada de 2,8% da população em 2000 para 4,4% em 2030. O número de pessoas com DM está projetado para um aumento de 171 milhões em 2000 para

366 milhões em 2030 (Wild et al., 2004). No Brasil, a frequência do DM é comparável com países mais desenvolvidos e é estimado que o DM acomete aproximadamente 8% da população com idade entre 30 e 69 anos, com maior prevalência (17,4%) entre 60 e 69 anos (Malerbi e Franco, 1992). O DM atinge todas as idades e raças, e é causado tanto por fatores ambientais quanto genéticos. Evidências atuais mostram que a magnitude da contribuição genética para o diabetes tipo 1 e 2 varia dramaticamente, sendo a taxa do risco genético para o tipo 2 maior que para o tipo 1 (Weijnen et al., 2002; Rich, 2006). Vários fatores ambientais estão associados com o aumento do número de pessoas com DM, incluindo crescimento populacional, idade, urbanização, drogas, agentes tóxicos, infecção viral e aumento da prevalência da obesidade e sedentarismo (World Health Organization, 2004; Wild et al., 2004). A população urbana em países em desenvolvimento está projetada para duplicar entre 2000 e 2030 (Wild et al., 2004) e a progressiva urbanização leva a mudanças no estilo de vida, hábitos culturais e estresse nas grandes cidades, representando fatores para o desencadeamento do DM em indivíduos com susceptibilidade genética (Nayaran et al., 2000).

1.2. A nefropatia diabética

1.2.1. Estimativas e Definição

As complicações relacionadas ao DM incluem doenças microvasculares (por exemplo, retinopatia, cegueira, nefropatia e falência renal, neuropatia) e doenças macrovasculares (doença coronariana, infarto, doença vascular periférica e cerebrovascular) (Nathan, 1993; Rich, 2006).

A nefropatia associada ao DM é uma das mais sérias e comuns complicações microvasculares do diabetes tipo 1 e tipo 2, sendo considerada a principal causa de insuficiência renal crônica (IRC) e principal indicação para diálise e transplante (estágio terminal ou estágio dialítico da doença renal). Aproximadamente 30% dos pacientes com DM desenvolve nefropatia (USRDS, 2006). Na maior parte do mundo, essa complicação crônica do DM acomete cerca de 25% dos indivíduos com diabetes tipo 1 e de 5% a 10% com diabetes tipo 2 (USRDS, 2006). Há uma considerável variabilidade étnica-racial no desenvolvimento da nefropatia diabética e sua conseqüente progressão para insuficiência

renal crônica, particularmente em pacientes com DM tipo 2 (Molitch et al., 2003). A prevalência da nefropatia diabética tem aumentado, portanto, há urgência na definição dos mecanismos fisiopatológicos envolvidos na gênese desta doença epidêmica, o que poderá acarretar a adição de medidas preventivas e terapêuticas mais eficazes.

Observações clínicas, principalmente em pacientes com DM tipo 1, indicam que há um período latente de vários anos após o início do diabetes que é caracterizado por nefromegalia e aumento da taxa de filtração glomerular, sem outros sinais e sintomas clínicos (Susztak et al., 2006; Wolf e Ziyadeh, 2007). A primeira manifestação clínica típica da nefropatia diabética em humanos é o aumento da excreção urinária de albumina, microalbuminúria ou nefropatia incipiente (30 a 300 mg/d), que geralmente aparece cerca de 5-15 anos após o diagnóstico do DM (Cooper 2001; Molitch et al., 2003; Susztak et al., 2006; Alsaad e Herzenberg, 2007) acompanhada por aumento na pressão arterial sistêmica e glomerular, e posterior redução na filtração glomerular. Aumento na excreção de albumina, considerado como macroalbuminúria ou nefropatia clínica (≥300 mg/d, ≥200 μg/min ou >200 mg/g de creatinina na urina), pode progredir para insuficiência renal (taxa de filtração glomerular <60 ml/min, correspondendo a creatinina sérica >1,5 mg/dl em homens e >1,3 mg/dl em mulheres), definindo a presença de doença renal crônica em pacientes diabéticos (Chobanian et al., 2003; Molitch et al., 2003; Susztak et al., 2006; Wolf e Ziyadeh, 2007). Além do que, albuminúria, em vários graus (2-20 mg/d a 30-300 mg/d), é um fator de risco independente para doenças cardiovasculares, em indivíduos com ou sem DM (Gerstein et al., 2001).

É postulado que a nefropatia diabética deve resultar da interação entre susceptibilidade genética e fatores ambientais, de origem metabólica e hemodinâmica (Giunti et al., 2006). Vias metabólicas dependentes de glicose, como aumento do estresse oxidativo, formação de poliol e acúmulo de produtos finais da glicosilação avançada (AGE), são ativadas dentro do rim diabético. Os fatores hemodinâmicos incluem aumento na pressão sistêmica e intraglomerular e ativação de várias vias de hormônios vasoativos como o sistema renina-angiotensina e endotelina (Cooper, 2001). Os fatores hemodinâmicos, independentemente ou associados com vias metabólicas, ativam

mensageiros secundários como isoformas da proteína quinase C (PKC) e MAPK (proteína quinase ativadora da mitogênese), fatores de transcrição nuclear como NF-κB, vários fatores de crescimento como "transforming growth factor" (TGF-β), fator de crescimento vascular endotelial (VEGF) e "connective tissue growth factor" (CTGF). Em resposta à hiperglicemia, aos AGEs e PKC, as citocinas pró-fibróticas TGF-β e CTGF, aumentam significativamente no DM exercendo importante papel na progressão da nefropatia diabética, uma vez que aumentam o acúmulo de matriz extracelular por meio do estímulo de produção de colágeno IV e fibronectina, resultando em fibrose intersticial e glomeruloesclerose (Rocco et al. 1992; Cooper, 2001; Zhou et al., 2004).

1.2.2. Características morfológicas

Alguns estudos mostram que a albuminúria é resultante de anormalidades nas funções tanto tubular quanto glomerular. O grau de proteinúria se correlaciona com a progressão da glomerulosclerose e fibrose tubulointersticial. Alterações na estrutura e função tubulointersticial renal com o DM alteram o transporte tubular e a atividade metabólica, contribuindo para a proteinúria e alteração hemodinâmica renal (Ziyadeh e Goldfarb, 1991). Porém, a maioria dos estudos demonstra que a nefropatia diabética é conseqüência de alterações na barreira de filtração glomerular, uma desordem estrutural primária glomerular, o que acarreta a perda da permeabilidade seletiva à passagem de proteínas para o espaço da cápsula de Bowman e conseqüente proteinúria (Wolf e Ziyadeh, 2007). Na progressão da nefropatia, a disfunção grave da barreira de filtração glomerular a proteínas circulantes acarreta a sobrecarga de proteínas para as células epiteliais tubulares, ativação intrarenal do sistema complemento que é responsável pelo espalhamento da lesão ao tubulointersticio, como inflamação tubulointersticial e fibrose tubular (Abbate et al., 2006) (Figura 1).

A barreira de filtração glomerular é um complexo biológico de estruturas com propriedades que permitem a alta taxa de filtração de água, a passagem irrestrita de moléculas de tamanhos pequeno e médio, e restrição à passagem de albumina e proteínas maiores (Haraldsson et al., 2008). A barreira de filtração glomerular é composta pelo endotélio fenestrado glomerular, a membrana basal glomerular (MBG) e pelas células

epiteliais viscerais glomerulares (podócitos) (Figura 2). As principais lesões morfológicas do aparato de filtração glomerular características da nefropatia diabética são a expansão da matriz mesangial e aumento na espessura da membrana basal glomerular (MBG), o que acarreta em redução na área de filtração glomerular contribuindo, portanto, para a proteinúria, progressiva perda da função renal e consequente falência renal (Mauer, 1994; Wolf, 2004). Essas alterações patológicas são causadas por aumento na deposição de matriz extracelular, principalmente colágeno IV e fibronectina, e hipertrofia das células mesangiais (Wolf e Ziyadeh, 1999). Tanto o aumento da síntese de proteína da matriz extracelular quanto a diminuição na degradação protéica contribuem para o espessamento da MBG. Em adição ao aumento de deposição de colágeno IV, ocorre a diminuição da expressão de heparan sulfato proteoglicanas, demonstrando que alterações na arquitetura e composição da MBG acarretam perda na seletividade da filtração glomerular (Kefalides, 1981; Isogai et al., 1999; Wolf e Ziyadeh, 1999).

Porém, pouco se sabe sobre o mecanismo patológico celular e molecular que inicia a nefropatia diabética e que precede a albuminúria clínica. Atualmente, estudos confirmam que a proteinúria na nefropatia diabética não pode ser totalmente explicada pela associação com expansão de matriz mesangial e espessamento da membrana basal glomerular (Wolf e Ziyadeh, 2007; Tejada et al., 2008). Estudos recentes evidenciam que alterações nos podócitos e células endoteliais glomerulares diminuem a barreira de filtração glomerular, estando fortemente envolvidas na manifestação da proteinúria e progressão ao estágio dialítico da doença renal na nefropatia diabética (Hoshi et al., 2002; Jefferson et al., 2008; Satchell e Braet, 2009). Lesões podocitárias (podocitopatia) e diminuição no número de podócitos (podocitopenia) têm sido reportados em pacientes com DM tipo 1 e tipo 2, e na nefropatia experimental, questionando o "dogma mesangiocêntrico" da doença glomerular diabética (Pagtalunan et al., 1997; Wolf et al., 2005; Susztak et al., 2006; Wolf e Ziyadeh, 2007).

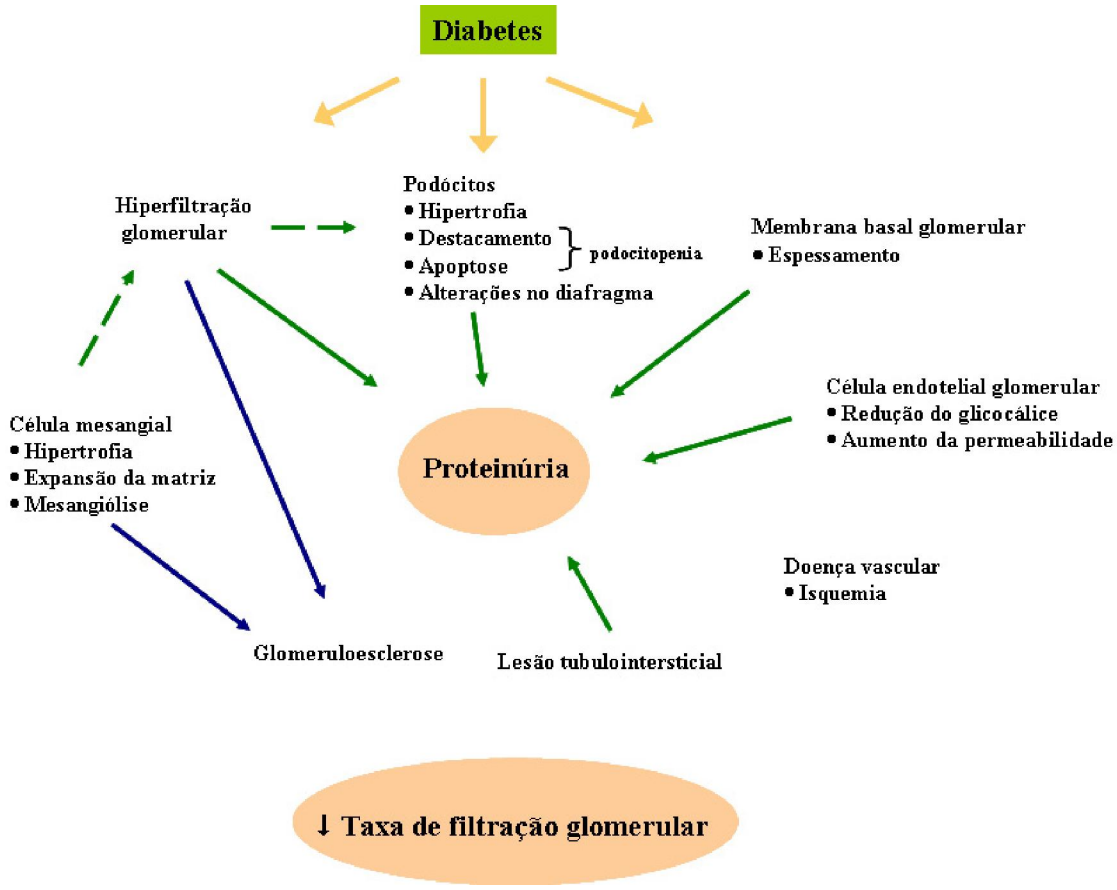

Figura 1: Esquema que propõe a unificação de mecanismos de proteinúria (representado pelas setas verdes) e diminuição da taxa de filtração glomerular (representado pelas setas azuis) na nefropatia diabética (Adaptado de Jefferson et al., 2008). A hiperglicemia tem efeito em todos os tipos celulares renais, contribuindo, portanto, primariamente ou secundariamente para o desenvolvimento da albuminúria e redução na taxa de filtração glomerular. Tanto efeitos hemodinâmicos quanto agressões aos componentes da barreira de filtração glomerular (podócitos, membrana basal glomerular e células endoteliais glomerulares), ocasionadas pela hiperglicemia, levam à proteinúria. Além disso, lesão tubulointersticial diminui a absorção de proteína. Lesão nas células mesangiais contribui secundariamente para a proteinúria por meio da expansão mesangial que causa a perda da área de filtração glomerular. Lesões nas células mesangiais e aos podócitos levam primariamente à glomeruloesclerose e conseqüente redução na filtração glomerular.

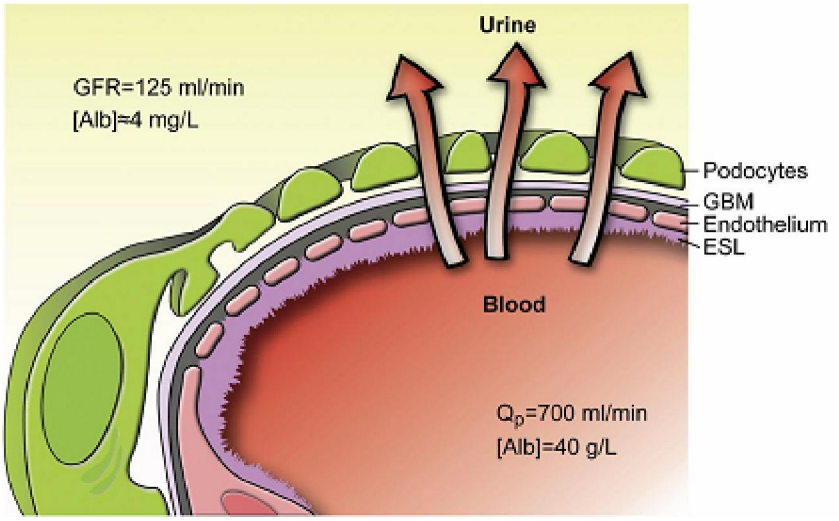

Figura 2: Desenho esquemático da barreira de filtração glomerular (Modificado de Haraldsson et al., 2008). Podocytes, podócitos; GBM, membrana basal glomerular; Endotelium, células endotelias fenestradas; ESL, superfície celular endotelial (refere-se ao glicocálix na superfície apical do endotélio). As setas indicam a filtração do plasma através da barreira glomerular, formando urina a uma taxa de filtração glomerular (GFR) de 125 ml/min em humanos. O fluxo plasmático renal é de 700 ml/min. O esquema também mostra a concentração de albumina ([Alb]) no soro sendo de 40 g/l e da urina de 4 mg/l (0,1% do plasma total). O coeficiente de albumina que atravessa a barreira glomerular é estimado em 10% do existente nos roedores.

1.3. Podócitos

1.3.1. Morfologia dos podócitos

As células epiteliais dos glomérulos, denominadas podócitos, são células especializadas, altamente diferenciadas e que se localizam no glomérulo externamente aos capilares, sendo consideradas o principal componente da barreira glomerular. Os podócitos possuem um corpo celular volumoso e estendem longos prolongamentos citoplasmáticos, conhecidos como processos podais ou pés podocitários primários e secundários (Boer e Gontijo, 2005; Haraldsson et al., 2008). Os processos primários se estendem ao redor dos capilares glomerulares e emitem processos secundários ou pedicelos que serão ancorados à MBG. Os pedicelos se interdigitam aos das células vizinhas e, entre eles, são formadas as fendas de filtração glomerulares. Próximo à MBG a distância entre os pedicelos varia entre 25-60 nm. Neste estreito espaço pode ser observada uma estrutura que interconecta os

pedicelos, conhecida como diafragma da fenda de filtração (Figura 3 A). O diafragma é composto por um filamento central, paralelo à membrana dos pedicelos, que está ancorado às fibras que o conectam à membrana dos pedicelos. Estas fibras têm 14 nm de comprimento e se apresentam espaçadas regularmente, formando poros com aproximadamente 7 nm de diâmetro. Este arranjo origina uma estrutura parecida com um zíper (Boer e Gontijo, 2005). Pode-se afirmar que o diafragma é uma junção celular modificada. Os poros são totalmente permeáveis à água e a pequenos solutos, mas relativamente impermeáveis a proteínas plasmáticas. Portanto, a integridade do diafragma é um dos principais determinantes da propriedade da permeabilidade seletiva da barreira de filtração glomerular (Wolf et al., 2005; Haraldsson et al., 2008). Os componentes moleculares do diafragma têm sido extensivamente estudados e algumas proteínas são vitais na manutenção da permeabilidade seletiva glomerular.

A primeira e principal proteína associada ao diafragma descoberta foi a nefrina. A nefrina é uma proteína transmembrana que exibe uma interação homofílica que pode promover o contato celular através da interação direta nefrina-nefrina, formando o arranjo molecular como um zíper (Khoshnoodi et al., 2003; Pavenstadt et al., 2003). Outras proteínas constituintes do diafragma como "zonula occludens" (ZO-1), FAT, podocina, proteína associada ao CD-2 e P-caderina estão envolvidas na manutenção da forma do diafragma e exercem importante papel na sua integridade (Pavenstadt et al., 2003; Boer e Gontijo, 2005). Podocina e proteína associada ao CD-2 interagem com a nefrina ancorando-a aos filamentos de actina do citoesqueleto podocitário. Além da nefrina, podocina e CD2AP, foram identificadas outras proteínas específicas dos podócitos como sinaptopodina (proteína associada aos filamentos de actina), podoplanina (glicoproteína transmembrana presente na superfície dos podócitos) e podocalexina (glicoproteína integral constituinte do glicocálix e que mantém as cargas negativas da superfície podocitária) (Saleem et al., 2002; Pavenstadt et al., 2003; Boer e Gontijo, 2005) (Figura 3 B).

A diferenciação das células epiteliais primárias em podócitos maduros é correlacionada com o aumento da expressão da proteína WT-1 (tumor de Wilms), portanto,

a proteína WT-1 permanece expressa como um marcador específico de podócitos durante toda a ontogenia e nos adultos (Pavenstadt et al., 2003; Menini et al., 2007).

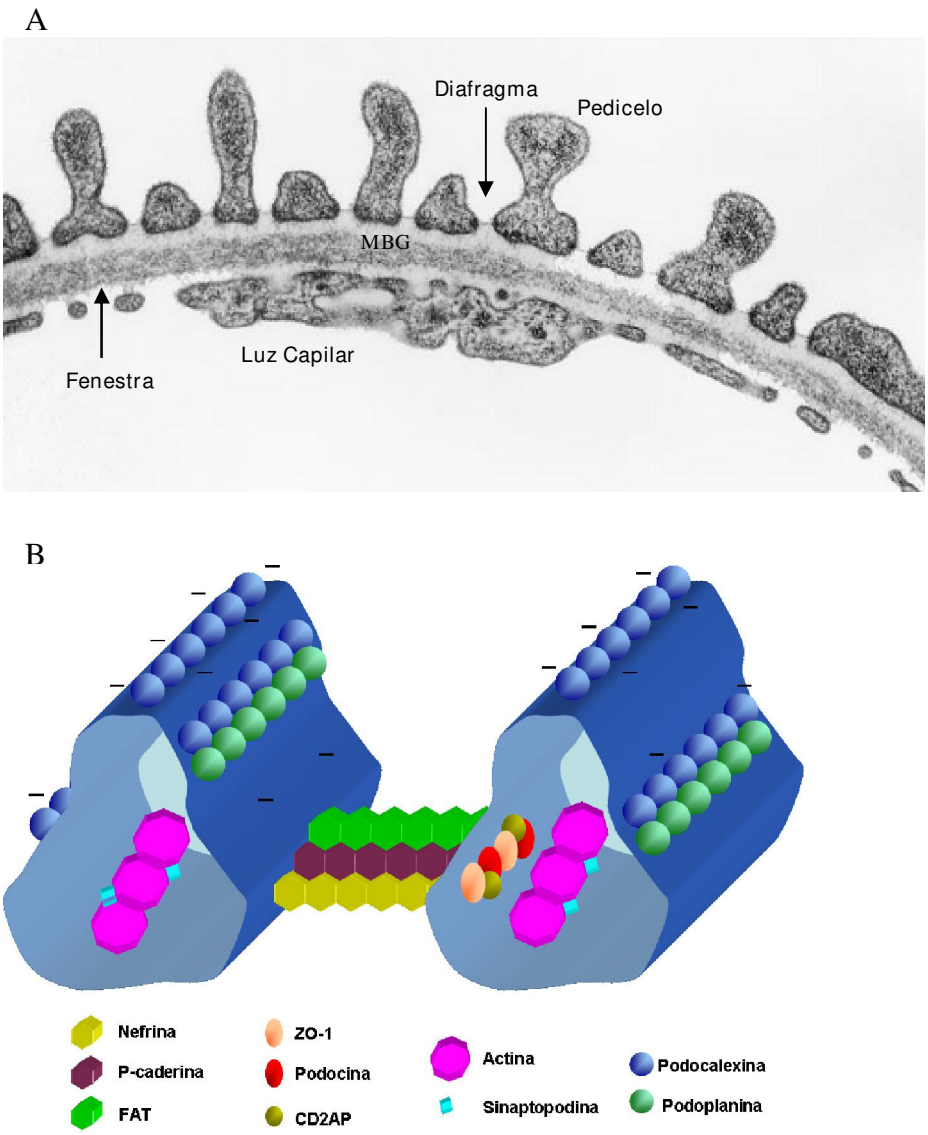

Figura 3: (A) Micrografia eletrônica mostrando os componentes da barreira de filtração glomerular: fenestrações endoteliais, membrana basal glomerular (MBG) e pedicelos de podócitos sobre a MBG. Entre os pedicelos são formadas as fendas de filtração onde pode ser observado o diafragma (modificado de Pavenstädt et al., 2003). (B) Representação esquemática de dois pedicelos e algumas proteínas características dos podócitos, importantes na manutenção das propriedades dos podócitos (Modificado de Boer e Gontijo, 2005).

1.3.2. Podócitos e nefropatia diabética

Estudos recentes indicam que agressões aos podócitos representam um importante fator no desencadeamento de todas as formas de glomerulopatia (Wiggins et al., 2005; Wolf et al., 2005; LeHir e Kriz, 2007; Wiggins, 2007; Barisoni et al., 2009) e apresentam papel essencial no desenvolvimento da ND (Li et al., 2007; Marshall, 2007; Reddy et al., 2008; Ziyadeh e Wolf, 2008). Tanto em humanos, quanto em modelos animais de DM, alterações na função e estrutura dos podócitos já são observadas em estágios iniciais do dano glomerular (Pagtalunan et al., 1997;Coimbra et al., 2000; Dalla Vestra et al., 2003; Doublier et al., 2003; Siu et al., 2006; Susztak et al., 2006).

O processo degenerativo dos podócitos pode se iniciar com alterações moleculares na estrutura do diafragma, especificamente alterações nas proteínas associadas aos podócitos. Em estudo com diabetes tipo 1, a excreção de nefrina na urina (nefrinúria) foi presente em 30% dos pacientes com normoalbuminúria, 17% com microalbuminúria e 28% com macroalbuminúria, embora nenhum paciente não diabético apresentou nefrinúria, sugerindo que o aumento da nefrina na urina corresponde a uma lesão inicial podocitária, mesmo antes do estabelecimento da microalbuminúria (Patari et al., 2003). Essas observações se correlacionam com redução na expressão de nefrina em biópsias renais de pacientes com DM tipo 1, além de estudos revelarem diminuição na expressão protéica e de mRNA da nefrina em DM tipo 2, comparado com indivíduos não diabéticos (Langham et al., 2002; Doublier et al., 2003; Koop et al., 2003; Benigni et al., 2004). A redução na nefrina, característica de um estado proteinúrico no DM, também se relaciona com modificações na morfologia característica dos podócitos, uma vez que se observa a perda dos processos citoplasmáticos, convertendo os podócitos em simples células epiteliais.

Dentre várias características morfológicas, a redução no número de podócitos por glomérulo (podocitopenia) é considerada um forte preditor da progressão da nefropatia diabética (Pagtalunan et al., 1997; Meyer et al., 1999). A depleção de podócitos foi observada em pacientes com nefropatia diabética inicial e tardia, assim como em modelos de diabetes animal (Pagtalunan et al., 1997; Steffes et al., 2001; Susztak et al., 2006). Estudos em pacientes diabéticos revelaram um declínio no número de podócitos

correlacionado positivamente com o grau de proteinúria e progressão da doença renal, sendo o número de podócitos um prognóstico do aumento da albuminúria (Pagtalunan et al., 1997; Meyer et al., 1999; Steffes et al., 2001;White et al., 2002).

A etiologia exata da perda de podócitos no DM permanece especulativa. Muitos estudos recentes com cultura de podócitos exposta a alta glicose e com modelos experimentais de diabetes indicam a apoptose como o principal mecanismo celular da depleção de podócitos na progressão da nefropatia diabética (Susztak et al., 2006; Szabó et al., 2006; Menini et al., 2007; Niranjan et al., 2008; Eid et al., 2009), embora exista controvérsia se a apoptose ocorre precocemente (Susztak et al., 2006) ou tardiamente (Menini et al., 2007) no curso da glomerulopatia diabética. A cascata apoptótica no DM parece ser especificamente induzida pela hiperglicemia. Alta glicose e angiotensina II (ANG II) induzem apoptose em cultura de podócitos, mediada pela ação de TGF-β (Schiffer et al., 2001; Ding et al., 2002). Há evidências que a formação de AGEs, de espécies reativas de oxigênio (ERO) e ativação de NF-κB contribuem para apoptose de podócitos *in vitro* e *in vivo* (Steffes et al., 1989; Pesce et al., 2002; Menini et al., 2006; Sustak et al., 2006; Szabó et al., 2006).

Outro mecanismo de perda de podócitos no DM se refere ao destacamento dos podócitos da membrana basal glomerular (Peterman et al., 2003). No rim maduro, os podócitos são incapazes de se replicar (Kriz, 1996). No intuito de manter a monocamada intacta e funcional, os podócitos aumentam de tamanho, enquanto a densidade de podócitos por glomérulo diminui. No DM, porém, o alargamento glomerular (em função da deposição de matriz extracelular) excede o limite dos podócitos, de maneira que os podócitos não são capazes de se manterem próximos. Assim, a hipertrofia compensatória é acompanhada por mudanças na morfologia dos podócitos, alteração na expressão de proteínas, piora no funcionamento e susceptibilidade ao destacamento da membrana basal (Kim, 2005; Wiggins et al., 2005;White, 2006; Davis et al., 2007; Lemley, 2008). Esse cenário de destacamento não exclui o papel da apoptose, uma vez que células sem a interação matriz-célula podem entrar em apoptose. Alternativamente, células destacadas podem ser liberadas na urina como podócitos vivos (Volgemann et al., 2003).

1.4. Hipertensão arterial e nefropatia diabética

A hipertensão arterial sistêmica (HAS) acelera a lesão renal associada ao DM. Hipertensão arterial e DM são os dois maiores fatores de risco independentes para a doença renal crônica (DRC) (USRDS 2006), sendo que esses dois fatores frequentemente coexistem e sua combinação acarreta um aumento na incidência e severidade das complicações renais (Cooper et al., 1988). A presença da HAS associada ao DM determina aumento da albuminúria e na espessura da membrana basal glomerular (Cooper, 2001). Clinicamente, a redução significativa na pressão arterial parece ser a mais importante e simples intervenção para retardar a progressão da nefropatia diabética tanto no DM tipo 1 quanto no tipo 2 (Giunti et al., 2006). Pacientes diabéticos com pressão correspondente a 130/80 mmHg raramente desenvolvem microalbuminúria e demonstram um declínio anual no ritmo de filtração glomerular (RFG), semelhante ao observado na população normal. Porém, pacientes diabéticos com pressão entre 130/80 e 140/90 mmHg apresentam declínio mais acentuado no RFG, sendo que 30% dos pacientes desenvolvem microalbuminúria e proteinúria (Giunti et al, 2006). Modelos experimentais de ratos que combinam HA genética (SHR) com DM induzido por estreptozotocina contribuem para o entendimento do mecanismo da interação entre HA genética e DM na progressão e desenvolvimento da doença renal (Cooper et al., 1988). O modelo SHR diabético apresenta características de nefropatia acelerada, evidenciada por elevada albuminúria e espessamento da membrana basal glomerular (Cooper et al., 1988). Estudos recentes demonstram que ratos diabéticos, jovens, pré-hipertensos SHR apresentam uma redução na proliferação celular renal devido a um aumento na expressão de inibidores de cinase dependente de ciclinas (Cdk) como o $p27^{Kip1}$ e conseqüente hipertrofia renal (Silveira et al., 2002). O DM experimental na presença de HA genética promove um acúmulo precoce de fibronectina renal (Righetti et al., 2001). Foi demonstrado também que, em SHR diabético, o tratamento com anti-hipertensivos é capaz de corrigir alterações renais precoces como hipertrofia glomerular, diminuição na replicação das células renais e acúmulo de matriz extracelular (Amazonas e Lopes de Faria, 2006). Porém, o mecanismo fisiopatológico da interação entre DM e HA no desenvolvimento da nefropatia ainda é pouco conhecido.

1.5. Estresse oxidativo

1.5.1. Definições básicas

Atualmente, o estresse oxidativo é definido como um desbalanço entre agentes pró-oxidantes e defesa antioxidante, em favor da oxidação (remoção de elétrons), levando a uma alteração da sinalização redox (ganho de elétrons) e/ou dano molecular (Jones, 2006). O termo pró-oxidante define qualquer substância que pode gerar espécies reativas ou capaz de induzir estresse oxidativo. Radicais livres e outras espécies reativas contribuem para o desenvolvimento de doenças crônicas como Alzheimer, câncer, aterosclerose, doenças neurodegenerativas e diabetes, pela ativação do estresse oxidativo e danos oxidativos teciduais (Baynes e Thorpe, 1999; Chowienczyk, et al., 2000; Halliwell, 2001; Butterfield, 2002). Antioxidantes são substâncias que, quando presentes em baixas concentrações comparadas com o substrato oxidável, retarda ou impede a oxidação deste substrato (Halliwell e Whiteman, 2004). É importante ressaltar que espécies reativas (radicais livres ou não radicais) não são totalmente ruins e os antioxidantes não são totalmente bons. A vida é um balanço entre ambos: antioxidantes atuam mantendo baixos os níveis de espécies reativas, permitindo que estas espécies sejam úteis nas funções biológicas sem causar danos (Halliwell e Gutteridge, 2006).

Radicais livres são espécies que contém um ou mais elétrons não pareados, e uma vez que elétrons são mais estáveis quando pareados em orbitais, radicais livres são geralmente reativos com outras espécies. Portanto, um radical deve doar seu elétron não pareado para outra molécula, ou deve receber um elétron para se tornar estável (Halliwell, 1989). Existem três diferentes classes de espécies reativas: espécies reativas de oxigênio (ERO), espécies reativas de nitrogênio (ERN) e espécies reativas de cloro (ERC). ERO é um termo coletivo que inclui tanto oxigênios radicais e não radicais que são agentes oxidativos e/ou são facilmente convertidos em radicais livres (Halliwell, 2006). Superóxido ($O_2^{\bullet -}$) e peróxido de hidrogênio (H_2O_2) são exemplos de ERO radical e não radical, respectivamente (Halliwell, 2006). Da mesma maneira, ERN e ERC também são considerados termos gerais, sendo óxido nítrico ($NO^{\bullet}$), dióxido de nitrogênio ($NO_2^{\bullet}$) e átomo cloro ($Cl^{\bullet}$) exemplos de radicais; e peroxinitrito ($ONOO^-$) e ácido hipocloroso (HOCL) caracterizados como não radicais (Halliwell e Whiteman, 2004) (Tabela 1).

Superóxido, $O_2^{\bullet-}$, é a primeira espécie produzida nas células e muitas outras espécies reativas de importância fisiológica são derivadas do ânion superóxido, como H_2O_2, radical hidroxil ($OH^{\bullet}$) e peroxinitrito ($ONOO^-$) (Munzel et al., 2002). As principais vias de produção de superóxido são pela cadeia transportadora de elétrons mitocondrial, pela enzima NADPH oxidase ("reduced nicotinamide adenine dinucleotide phosphate"), xantina oxidase, ciclooxigenase, lipooxigenase, óxido nítrico sintase e citocromo P450 (Schnachenberg, 2002).

Danos oxidativos são considerados danos biomoleculares causados diretamente por espécies reativas durante estresse oxidativo. O estresse oxidativo pode causar danos em moléculas como lipídeos, DNA, proteínas, carboidratos, dentre outras (Halliwell e Whiteman, 2004). A rápida interação do ânion superóxido com óxido nítrico resulta na produção de peroxinitrito ($ONOO^-$), um potente agente oxidativo e nitrativo que danifica proteínas, lipídeos e DNA (Halliwell, 2006). A nitração de resíduos de tirosina de proteínas gera nitrotirosina, considerada um biomarcador para estresse oxidativo. O radical hidroxil ($OH^{\bullet}$) induz peroxidação lipídica e hidroxilação de DNA, sendo outros importantes danos oxidativos ao organismo. $OH^{\bullet}$ pode reagir com o DNA e formar 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).

Tabela 1: Nomenclatura de espécies reativas (Halliwell e Whiteman, 2004)

Radicais Livres	Não Radicais
Espécies Reativas de Oxigênio (ERO)	
Superóxido, $O_2^{\bullet-}$	Peróxido de hidrogênio, H_2O_2
Hidroxil, $OH^{\bullet}$	Ácido hipobromoso, HOBr
Hidroperoxil, $HO_2^{\bullet}$	Ácido hipocloroso, HOCl
Peroxil, $RO_2^{\bullet}$	Ozônio, O_3
Alcoxil, $RO^{\bullet}$	Oxigênio livre, $O_2{}^1\Delta g$
Carbonato, $CO_3^{\bullet-}$	Peróxidos orgânicos, ROOH
Dióxido de carbono, $CO_2^{\bullet-}$	Peroxinitrito, $ONOO^-$
	Ácido peroxinitroso, ONOOH
Espécies Reativas de Cloro (ERC)	
Átomo de cloro, $Cl^{\bullet}$	Ácido hipocloroso, HOCl
	Cloro Nitril (nitronium), NO_2Cl
	Cloraminas
	Gás Cloro, Cl_2
Espécies Reativas de Nitrogênio (ERN)	
Óxido Nítrico, $NO^{\bullet}$	Ácido nitroso, HNO_2
Dióxido de nitrogênio, $NO_2^{\bullet}$	Cátion nitrosil, NO^+
	Ânion nitroxil, NO^-
	Tetróxido de dinitrogênio, N_2O_4
	Trióxido de dinitrogênio, N_2O_3
	Peroxinitrito, $ONOO^-$
	Ácido peroxinitroso, ONOOH
	Cátion nitronio (nitril), NO_2^+
	Alquil peroxinitrito, ROONO
	Cloro Nitril (nitronium), NO_2Cl

1.5.2. Estresse oxidativo e nefropatia diabética

Estresse oxidativo é amplamente reconhecido como um componente chave no desenvolvimento e progressão de complicações diabéticas, como a nefropatia diabética (Giugliano et al., 1996; Baynes e Thorpe, 1999; Browlee, 2001). Estudos recentes revelam a importante contribuição do estresse oxidativo renal na patogênese da nefropatia diabética e na doença renal crônica (Beisswenger et al., 2005; Wardle, 2005). A hiperglicemia é conhecida por induzir estresse oxidativo por meio da elevada produção de espécies reativas de oxigênio (ERO) (Brownlee, 2005). Dentre as ERO, o anion superóxido ($O_2^{\bullet-}$) apresenta crítica importância na regulação da função renal (Wilcox, 2002).

A maior fonte de superóxido nos tecidos vasculares e no rim é pela via da NADPH oxidase (Gill e Wilcox, 2006; Yang et al., 2006), um complexo enzimático que consiste de seis subunidades: subunidades de membrana $p22^{phox}$ e $gp91^{phox}$, componentes citosólicos como $p40^{phox}$, $p47^{phox}$ e $p67^{phox}$, e uma proteína G de baixo peso molecular Rac1 ou Rac2 (Yang et al., 2006). NADPH oxidase fagocítica foi o primeiro exemplo identificado de um sistema de geração de ROS não como um produto secundário de reações biológicas, mas como uma função primária de um sistema enzimático. Uma série de observações sugeriu que um sistema similar à NADPH oxidase fagocítica, composta por proteínas denominadas Nox, está presente em muitos outros tipos celulares como fibroblastos, células tumorais, dentre outras. Existe uma similaridade dos componentes da NADPH oxidase fagocítica com a família Nox, por exemplo, na terminologia Nox, $gp91^{phox}$ é denominado Nox 2 (Bedard e Krause, 2007) (Figura 4). Pelo menos três diferentes isoformas de Nox são expressas no córtex renal: Nox 1, Nox 2 e Nox 4 (Bedard e Krause, 2007). De maneira interessante, Nox 4 tem sido o principal pivô na produção de superóxido via NADPH oxidase nos músculos lisos vasculares e rim (Gorin et al., 2005; Touyz et al., 2002). NADPH oxidase tem uma localização celular distinta no rim. ERO são produzidas no rim por fibroblastos, células endoteliais, células musculares lisas vasculares, células mesangiais, células tubulares e podócitos (Gill e Wilcox, 2006). A expressão de NADPH oxidase está aumentada nos glomérulos e túbulos distais de modelos experimentais com nefropatia diabética (Tojo et al., 2007). Células mesangiais expressam $p22^{phox}$ e $p47^{phox}$, e a atividade destas subunidades em alta concentração de glicose tem sido implicada na

contribuição do estresse oxidativo associado com a patogênese da nefropatia diabética (Frecker et al., 2005; Jones et al., 1995; Kitada et al., 2003).

Em resposta à hiperglicemia, a interação AGE com seu receptor RAGE está presente em grande quantidade em glomérulos renais diabéticos (Horie et al., 1997), possuindo, portanto, um papel crítico no desenvolvimento da nefropatia diabética, uma vez que alteram a estrutura e função da matriz tecidual, acarretam lesão glomerular e fibrose tubulointersticial (Browlee et al., 1988; Soulis-Liparota et al., 1991; Beisswenger et al., 1993; Horie et al., 1997; Suzuki et al., 1999; Jerums et al., 2003). A via de ação AGE-RAGE parece ser a ativação da produção de espécies reativas de oxigênio, PKC, citocinas inflamatórias, como NF-κB, e fatores de crescimento como TGF-β e CTGF (Yan et al., 1994; Tsuchida et al., 1999; Kelly et al., 2001). PKC representa uma via importante na patogênese da nefropatia diabética (Cooper, 2001). A geração de ROS induzida pela hiperglicemia em células mesangiais parece ser dependente de PKC, de maneira que a elevada glicose ativa isoformas de PKC indiretamente por meio da ligação de receptores de AGE e aumento da atividade da via do poliol (Inoguchi et al., 2003; Brownlee, 2001). Estudo recente demonstra que AGEs induzem estresse oxidativo e ativa PKC em células mesangiais neonatal (Scivittaro et al., 2000). PKC tem demonstrado exercer importante papel na ativação tanto da NADPH oxidase fagocítica quanto não fagocítica (Inoguchi et al., 2000). A ativação de PKC ou a sinalização da angiotensina II renal induz ativação da NADPH oxidase aumentando a translocação de $p47^{phox}$ do citosol para a membrana e, portanto, há a produção de pró-oxidantes e estresse oxidativo no DM (Onozato e Tojo, 2005; Tojo et al., 2007). Estudo recente demonstra que células mesangiais expostas à alta concentração de glicose, *in vitro*, geram ERO predominantemente através da ativação da NADPH oxidase, sendo a super-regulação das subunidades $p22^{phox}$ e $p47^{phox}$ dependente de PKC-α e -β (Xia et al., 2006). A ativação de PKC está ligada à patogênese da nefropatia diabética por meio da indução de TGF-β1, que estimula o aumento da síntese de fibronectina e colágeno IV levando ao acúmulo de matriz extracelular e conseqüente redução na área de filtração glomerular e albuminúria. Além do que, PKC diminui a produção de óxido nítrico, pela diminuição da expressão de eNOS, e aumenta a atividade

de endotelina-1, levando a alterações na pressão intraglomerular renal com o DM (Brownlee, 2001).

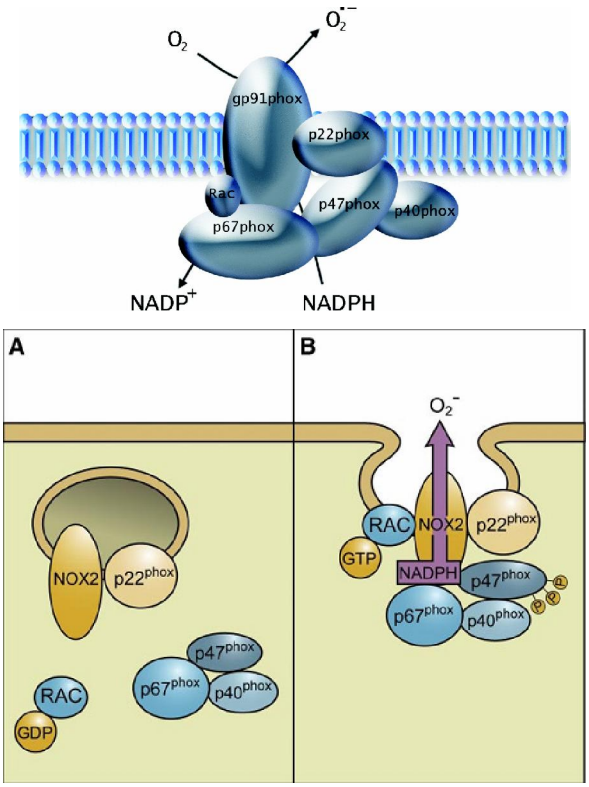

Figura 4: Ativação do complexo NADPH oxidase. NOX 2/gp91^{phox} está associado com a subunidade p22^{phox}, na membrana plasmática. Após estímulo, alguns sinais induzem a ativação das subunidades citosólicas, como a fosforilação de p47^{phox}, p67^{phox} e p40^{phox}, além da ligação de GTP a Rac, e translocação dos fatores citosólicos para a fração de membrana para formar um complexo com as proteínas de membrana NOX 2/gp91^{phox} e p22^{phox}. Uma vez que as subunidades interagem, o complexo está ativado e gera superóxido pela transferência de elétrons da NADPH no citosol para oxigênio presente no espaço extracelular (Modificado de Ray and Shah, 2005; Bedard e Krause, 2007).

1.5.3. Estresse oxidativo e apoptose de podócitos

O estresse oxidativo é considerado o fator principal que ocasiona a apoptose de podócitos, consequentemente levando albuminúria, danos renais e progressão da nefropatia diabética. Susztak e colaboradores (2006) demonstraram, pela primeira vez, que a geração

de ERO-dependente de NADPH oxidase constitui a principal via de apoptose de podócitos induzida pela hiperglicemia/diabetes, *in vitro* e *in vivo*. Este trabalho sugere que a apoptose de podócitos mediada por ERO representa um dano celular glomerular precoce associado com subseqüente glomerulopatia em DM do tipo 1 e tipo 2 experimental. Portanto, é sugerido que novos estudos focados na inibição de espécies reativas de oxigênio e da via de apoptose, incluindo NADPH oxidase sejam desenvolvidos. De fato, diversos estudos experimentais têm revelado a influência de antioxidantes na prevenção de apoptose de podócitos e conseqüente progressão da nefropatia diabética (Oba et al., 2007; Zheng et al., 2008; Lee et al., 2009). Inibidores de NADPH oxidase, como apocinina, revelaram diminuir, mas não completamente normalizaram a excreção urinária de albumina em DM tipo 2 (camundongo db/db) (Asaba et al., 2005). Este resultado parece indicar a influência de vias independentes de NADPH oxidase, como por exemplo a via mitocondrial e citocromo P450 na patogênese da nefropatia diabética (Ziyadeh et al., 2000; Eid et al., 2009). Eid e colaboradores (2009) demonstraram que a geração de ERO, via citocromo P450 e via NADPH oxidases Nox 1 e Nox 4, está envolvida na apoptose de podócitos em cultura exposta a alta glicose e depleção de podócitos e proteinúria em modelo experimental de DM tipo 1.

Recente estudo de Niranjan e colaboradores (2008) revelou a via de sinalização da Notch, proteína transmembrana presente na superfície celular, como importante mecanismo para danos aos podócitos e subseqüente falência renal, em humanos e modelos de roedores com nefropatia diabética ou glomeruloesclerose focal e segmentar (GESF). A expressão do domínio intracelular de Notch 1 (DIN1) está aumentada em podócitos na nefropatia diabética e GESF. Estudos *in vitro* e *in vivo* mostraram que DIN1 induz apoptose de podócitos através da ativação de p53. A inativação da sinalização de Notch por deleção genética ou inibição farmacológica evita e reverte os danos aos glomérulos característicos das doenças renais proteinúricas.

1.5.4. Estresse oxidativo, nefropatia diabética e PARP

Outra via que tem sido revelada como importante mecanismo na patogênese da nefropatia diabética é a ativação da poli(ADP-ribose) polimerase (PARP-1) (Szabó et al.,

2006). A PARP-1 é a principal proteína da família das enzimas PARP encontrada nos mamíferos, é uma proteína nuclear que tem papel importante no reparo do DNA, sendo ativada por danos ao DNA. PARP-1 ativada cliva NAD^+ em nicotinamida e ADP-riboses que são posteriormente polimerizadas formando poli (ADP-riboses) ou PARs. O polímero se liga a diversos receptores protéicos nucleares e está envolvido na regulação de diversos processos celulares como reparo do DNA, organização da cromatina, transcrição, replicação, degradação de proteínas, dentre outros (Erdélyi et al., 2005; Yam-Canul et al., 2008; Chandak et al., 2009). PARs interage com diversas proteínas alvo, incluindo histonas e a própria enzima PARP. Paradoxalmente, a ativação constante e excessiva de PARP pode ser deletéria, pois, uma vez que PARs se liga à PARP ocorre retroalimentação de inibição da enzima PARP, impedindo o reparo do DNA e levando ao dano celular e morte celular. Sabe-se que o extresse oxidativo, via espécies reativas de oxigênio e nitrogênio, acarreta lesões severas ao DNA, induzindo ativação excessiva do reparo ao DNA pela enzima PARP (Devalaraja-Narashimha et al., 2005; Yam-Canul et al., 2008; Chandak et al., 2009).

Estudos demonstram que a hiperglicemia induz a ativação de PARP-1 como conseqüência da geração elevada de ERO intracelular e subseqüente dano ao DNA (Brownlee, 2005; Du et al., 2003). A ativação de PARP-1, ocasionada pelo aumento na produção de superóxido induzido por hiperglicemia, altera a atividade da enzima glicolítica GAPDH intracelular, consequentemente ocorre a ativação da via de poliol, ativação de PKC, aumento na formação de AGE, ativação de NF-κb e subseqüente danos teciduais e desenvolvimento de doenças como nefropatia diabética e retinopatia diabética (Brownlee, 2005; Erdelyi et al., 2005; Xu et al., 2008). Estudos recentes demonstraram que a ativação da PARP exerce um importante papel na patogênese da glomerulopatia diabética, em modelo experimental de DM tipo 1 e tipo 2 (Szabó et al, 2006; Drel et al., 2009). Foi comprovada a influência de PARP-1 na apoptose de podócitos, sendo que a administração de inibidores de PARP preveniu a depleção podocitária, além de diminuir o desenvolvimento de lesões associadas a nefropatia diabética como expansão glomerular e albuminuria.

Estudos baseados na utilização de diversos inibidores de PARP-1 (como por exemplo INO 1001 e PJ-34) e de camundongos knockout para PARP-1 têm elucidado a função da PARP-1 no desenvolvimento de múltiplas doenças, em modelos experimentais e cultura de células, como diabetes, cancer, neuropatia, nefropatia, isquemia cerebral, infarto do miocárdio, disfunção vascular, lesão renal, dentre outras (Masutani et al., 1999; Abdelkarim et al., 2001; De Blasio et al., 2003; Du et al., 2003; Chatterjee et al., 2004; Brownlee, 2005; Obrosova et al., 2005; Zheng et al., 2005; Haddad et al., 2006; Szabó, 2006).

1.5.5. Estresse oxidativo e hipertensão arterial

Assim como o DM, a HA também induz estresse oxidativo no rim. NADPH oxidase renal apresenta um importante papel fisiopatológico no desenvolvimento da HA, a atividade da NADPH oxidase é aumentada pela infusão prolongada de angiotensina II ou dieta com alta dose de sal (Gill e Wilcox, 2006). Em particular, em modelo de rato espontaneamente hipertenso (SHR), a contribuição do estresse oxidativo para o desenvolvimento da HA tem sido sugerida em estudos que demonstram a melhora ou reversão da HA com o uso de antioxidantes (Schnackenberg e Wilcox, 1999; Schnackenberg et al., 1998). Adler e Huang (2004) demonstraram que o aumento do estresse oxidativo em SHR, levando a uma diminuição de NO e possivelmente aumento da HA, é decorrente do aumento da expressão e ativação de elementos do complexo NADPH e concomitante diminuição do antioxidante SOD-3 contribuindo para o estresse oxidativo nesses animais. Alguns estudos sugerem que o aumento do estresse oxidativo via NADPH oxidase possa representar o fenômeno primário na patogênese de HA em ratos SHR (Biswas e Lopes de Faria, 2007). Além do que, a presença da HA aumenta o estresse oxidativo no rim de SHR em estágio inicial do DM experimental, por meio da excessiva produção de pró-oxidantes e diminuição na defesa antioxidante (Biswas et al., 2007; Biswas e Lopes de Faria, 2006).

1.6. Sistema antioxidante

1.6.1. Antioxidantes endógenos

Com o intuito de minimizar o dano oxidativo, o sistema antioxidante do organismo é ativado. Antioxidantes enzimáticos como a superóxido dismutase (SOD), catalase e glutationa peroxidase, e antioxidantes não-enzimáticos como vitamina C, E, glutationa reduzida (GSH) e beta-caroteno fornecem a principal proteção contra estresse oxidativo pela neutralização ou "scavenging" de espécies reativas (Scandalios, 2005). A SOD representa a principal enzima antioxidante que atua na remoção do superóxido (Fridovich, 1995). SOD converte $O_2^{\bullet-}$ a H_2O_2 que, então, é degradado a água e oxigênio molecular pela catalase e glutationa peroxidase (Schnachenberg, 2002; Asaba et al., 2007).

Nos mamíferos, existem 3 isoformas de SODs: Cu/Zn SOD (SOD1), MnSOD (SOD2) e SOD extracelular (ecSOD ou SOD3). Cada isoforma é derivada de genes distintos, porém catalizam a mesma reação (Qin et al., 2008). SOD1 é uma proteína que contém cobre e zinco, presente no citosol, núcleo, peroxissomos e membrana interna mitocondrial. É a isoforma da SOD predominante no córtex renal e no glomérulo, representando mais de 90% da atividade total de SOD nestes tecidos (Marklund, 1984; Fridovich, 1995; Craven et al., 2001; DeRubertis et al., 2004). SOD2 é uma enzima mitocondrial que dismuta superóxidos gerados pela cadeia transportadora de elétrons. EcSOD, outra dismutase que contém cobre e zinco, é a primeira enzima antioxidante secretada no espaço extracelular sendo a principal forma extracelular da SOD e está altamente expressa nos vasos sanguíneos, coração, pulmão, rim, placenta e fluidos extracelulares (Marklund, 1982; Qin et al., 2008). EcSOD possui resíduos de aminoácidos com elevada homologia a seqüências da Cu/Zn SOD, ao mesmo tempo em que não há seqüências homólogas à MnSOD (Hjalmarsson et al., 1987; Folz e Crapo, 1994). Uma característica estrutural importante para a função da ecSOD é sua afinidade por glicosaminoglicanas sulfato, que localiza esta enzima em regiões específicas extracelulares, como a matriz extracelular dos tecidos (Folz e Crapo, 1994; Marklund, 1984a; Marklund, 1984b; Oury et al., 1996). EcSOD é considerada também um eficiente "scavenger" de óxido nítrico (Oury et al., 1996). EcSOD exerce um importante papel na regulação da pressão arterial e contração vascular por meio da modulação da função endotelial pelo

controle de níveis extracelulares de superóxido e biodisponibilidade do óxido nítrico na vasculatura (Junge et al., 2003; Adler e Huang, 2004).

1.6.2. SOD e nefropatia diabética

Existem evidências substanciais que a atividade da SOD em células sanguíneas periféricas está reduzida em pacientes diabéticos com nefropatia diabética, comparado com aqueles sem complicações decorrentes do DM (Bhatia et al., 2003; Hodgkinson et al., 2003). Além disso, estudos recentes têm implicado um polimorfismo nos genes para SOD1 e SOD2 em humanos com risco de desenvolverem nefropatia diabética (Möllsten et al., 2007; Al-Kateb et al., 2008). Tem sido demonstrado que camundongos transgênicos para SOD1 ou SOD2 são resistentes as lesões vasculares induzidas pelo DM, incluindo nefropatia (Craven et al., 2001; DeRubertis et al., 2004; DeRubertis et al., 2007). Estudo recente demonstrou que modelo de camundongo com DM espontânea e progressiva nefropatia diabética exibiu uma redução na expressão de SOD1 e SOD3, mas não de SOD2 (Fujita et al., 2009). Além do que, o aumento na expressão de SOD1 atenua lesões glomerulares e estresse oxidativo em modelos experimentais de DM tipo 1 e tipo 2 (Craven et al., 2001; DeRubertis et al., 2004). Portanto, essas descobertas sugerem um papel primordial da SOD na patogênese da nefropatia diabética.

Como a SOD endógena possui elevado peso molecular e limitada permeabilidade a membrana, diversos agentes farmacológicos tem sido desenvolvidos para mimetizar SOD, incluindo o tempol (Schnackenberg, 2002).

1.6.3. Tratamento com antioxidantes

A atividade da SOD e da catalase está diminuída no DM, portanto, a melhora do sistema antioxidante pode representar uma importante terapia para a nefropatia diabética (Sindhu et al., 2004). Recentemente, estudos vêm demonstrando a importância do tratamento da HA ou DM com antioxidantes, como apocinina, tempol e flavonóides.

A apocinina é considerada um inibidor específico da NADPH oxidase, de maneira que impede a translocação das subunidades citosólicas $p47^{phox}$ e $p67^{phox}$ para a membrana,

um evento que é essencial para a ligação funcional do complexo NADPH oxidase (Engels et al., 1992). Recentemente, foi demonstrado que a apocinina inibe o aumento da produção de superóxido, via NADPH oxidase, em ratos hipertensos DOCA-salt e Sprague-Dawley tratados com aldosterona também por meio da diminuição da expressão do RNAm da subunidade $p22^{phox}$ em segmentos aórticos (Park et al., 2004). É observada uma diminuição significativa na pressão arterial de ratos com HA induzida por mineralocorticóide (Beswick et al., 2001), angiotensina II (Virdis et al., 2004), dexametasona (Hu et al., 2006) e leptina (Beltowski et al., 2005) após tratamento com apocinina. Em modelo de nefropatia diabética, foi demonstrado que a apocinina inibiu estresse oxidativo em todo rim e melhorou a proteinúria e expansão de matriz mesangial glomerular (Asaba et al., 2005).

Flavonóides, como epicatequinas e catequinas, representam a principal classe de metabólitos polifenólicos e estão comumente presentes em plantas como *Vitis vinifera* (vinho), *Camellia sinensis* (chá) e *Theobroma cacao* (cacau) que são especialmente notáveis no contexto da nutrição humana (Schroeter et al., 2006). Diversas são as bioatividades mediadas pelos flavonóides observadas através de intervenção por dieta em humanos e em modelos experimentais de ratos hipertensos (SHR): melhora da função endotelial (Potenza et al., 2007), redução da pressão sanguínea (Potenza et al., 2007; Taubert et al., 2003), melhora da resistência à insulina e tolerância à glicose (Potenza et al., 2007; Grassi et al., 2005), atenuação da reatividade plaquetária (Holt et al., 2002), melhora da resposta imune e sistema de defesa antioxidante (Sies et al., 2005; Keen et al., 2005). Estudos com administração de chá-verde para ratos diabéticos demonstraram melhora na função renal, redução da glicemia e da concentração de proteínas glicadas, assim como atenuou a retinopatia e produção de ERO mitocondrial renal (Mustata et al., 2005; Renno et al., 2008). Ribaldo e colaboradores (2009) demonstraram que o tratamento de ratos hipertensos (SHR) diabéticos com chá-verde melhora a albuminúria e acúmulo de colágeno IV renal, além de reduzir marcadores de estresse oxidativo renal. O mecanismo desses efeitos benéficos parece envolver a diminuição na expressão da subunidade da NADPH oxidase, Nox 4. O tratamento com chá-verde não alterou PA nem concentração de glicose plasmática nos SHR, evidenciando a importância do estresse oxidativo na lesão renal e o efeito do chá-verde no tecido renal sendo mediado pela restauração do estado redox.

1.6.4. Tempol

Segundo Brownlee e colaboradores (2005), enquanto a elevada produção de superóxido, induzida pela hiperglicemia, é um processo contínuo, antioxidantes convencionais neutralizam moléculas reativas de oxigênio numa razão 1:1. Portanto, é necessária a atuação dos chamados antioxidantes catalíticos, como miméticos da SOD/catalase, que trabalham continuamente, como enzimas. Um exemplo característico deste tipo de antioxidante é o tempol.

. O tempol (4-hidroxi-2,2,6,6-tetrametil-piperidine-1-oxil) é considerado um nitróxido, ou seja, um radical livre que pode passar por uma ou duas reações de redução (ganho de elétrons) se transformando em cátions e fornecendo, portanto, ações metabólicas redox (Wilcox e Pearlman, 2008). O tempol é caracterizado como uma molécula de baixo peso molecular, estável, metal-independente, com excelente permeabilidade celular, e ativo tanto *in vivo* quanto *in vitro* (Beltowski et al., 2005; Asaba et al., 2007; Schnackenberg, 2002). Tempol dismuta superóxido catalíticamente, sendo considerado, portanto, um mimético da SOD. Outra característica do tempol é que *in vitro* ele aumenta a atividade catalase-mimética da metmioglobina (MbFeIII), facilitando, portanto, a dismutação do peróxido de hidrogênio.

$$MbFe^{III} + H_2O_2 \xrightarrow{k_1} {}^{\cdot}MbFe^{IV} + H_2O^{\cdot}$$

Dessa maneira, o tempol é um SOD mimético que não apenas reduz os efeitos diretos do superóxido, mas também dirige o superóxido à reação de Fenton. Tempol facilita o metabolismo de H_2O_2 pela atuação semelhante à da catalase, mas não é um mimético da catalase por si (Schnackenberg, 2002; Wilcox e Pearlman, 2008) (Figura 5). Além do que, pode-se afirmar que o tempol limita a formação de radicais hidroxil tóxicos produzidos pela reação de Fenton, uma vez que atua pela oxidação de metais reduzidos de transição como ferro, cobre, cádmio ou cromo. Portanto, diminui a viabilidade de espécies reduzidas da reação de Fenton (Wilcox e Pearlman, 2008).

Reação de Fenton:

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$

(íon ferroso (II) é oxidado por peróxido de hidrogênio a íon férrico (III), radical hidroxil e ânion hidroxil).

Portanto, tempol pode metabolizar ânion superóxido e muitas outras ERO, sendo considerado um importante nitróxido que protege células e tecidos de danos provenientes de ERO.

Estudos mostram que o tempol normaliza a pressão arterial em vários modelos animais de hipertensão como ratos espontaneamente hipertensos (Schnackenberg and Wilcox, 1999), ratos Dahl-sensíveis a sal (Zicha et al, 2001), HA induzida por angiotensina II (Nishiyama et al., 2001), por acetato de deoxicorticosterona e dieta com sal (Beswick et al., 2001), pela endotelina (Sedeek et al., 2003), pela dexametasona (Zhang et al., 2004) e dieta com alta frutose (Onuma e Nakanishi, 2004). Segundo trabalho de Asaba et al. (2007), o tratamento de ratos com DM induzida por estreptozotocina, a fim de verificar a influencia desse mimético da SOD na nefropatia diabética, inibiu a expansão de matriz glomerular via supressão de produção de superóxido e TGF-β, porém não reduziu a proteinúria. Estudo *in vitro* demonstrou que o tratamento com tempol de células mesangiais extraídas de glomérulos de ratos Sprague-Dawley e expostas à alta concentração de glicose evitou a produção de ROS e o aumento na expressão da proteína colágeno IV (Xia et al., 2006). Estudo recente demonstrou que o tratamento com tempol reduziu albuminúria, taxa de filtração glomerular, hipertrofia renal e expansão da matriz mesangial em modelo experimental com avançada nefropatia diabética, sem alterar o nível de hiperglicemia (Fujita et al., 2009). O tratamento com tempol de camundongos diabéticos knockout para SOD1 suprimiu albuminúria, reduziu superóxido glomerular, diminuiu a expressão de TGF-β, de nitrotirosina e colágeno IV (DeRubertis et al., 2007).

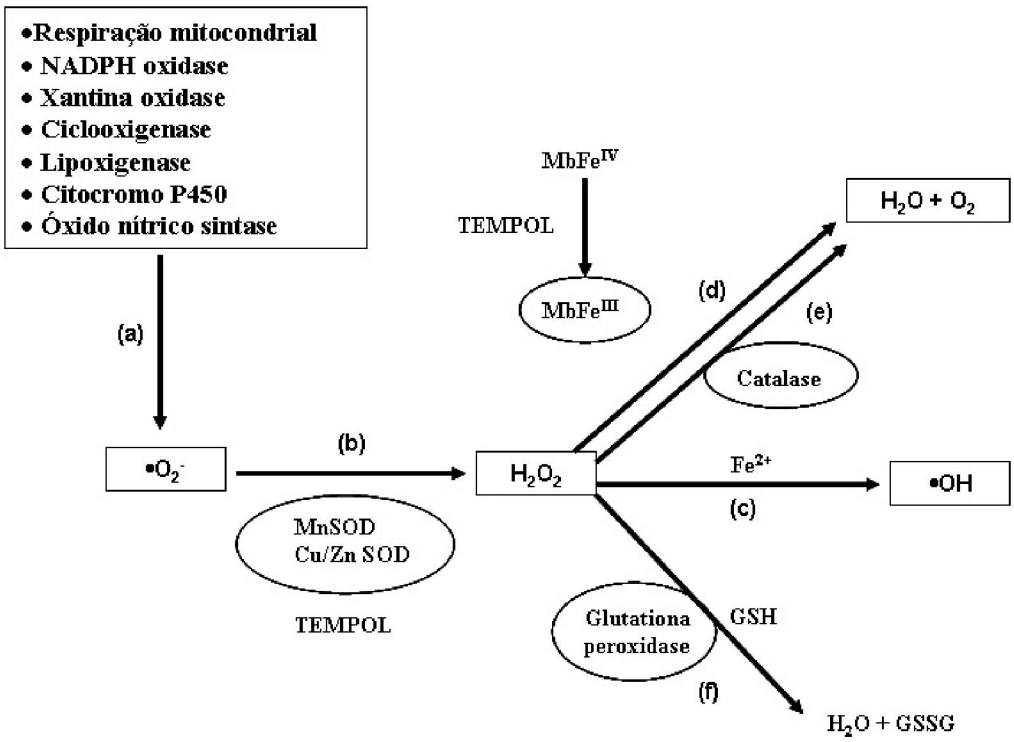

Figura 5: Produção e degradação de superóxido – atuação do tempol (Modificado de Schnackenberg, 2002). a) O ânion superóxido pode ser produzido por uma variedade de processos celulares. b) O ganho espontâneo de um elétron para formar H_2O_2 pode ser catalisado por isoformas de SOD (Mn, manganês; Cu, cobre; Zn, zinco) e por miméticos farmacológicos da SOD, como o tempol. c) Na presença de ferro catalítico, o radical hidroxil ($OH^{\bullet}$) é formado a partir de H_2O_2, via reação de Fenton. H_2O_2 pode ser decomposto em água pela metmioglobina (MbFe) (d), catalase (e) ou glutationa peroxidase (f). Os oxigênios radicais estão representados em retângulos e as enzimas catalíticas em círculos. GSH, glutationa reduzida; GSSG, glutationa oxidada.

2. HIPÓTESE E OBJETIVOS

A combinação da HA com o DM aumenta a incidência e severidade da lesão renal associada ao DM. Porém, o mecanismo fisiopatológico pelo qual a HA exarceba o risco de desenvolver a nefropatia diabética ainda é pouco conhecido. Tanto a hiperglicemia quanto a HA induzem estresse oxidativo no rim, um dos importantes mecanismos envolvidos na patogênese da nefropatia diabética. É sugerido que o aumento do estresse oxidativo, principalmente pela produção de espécies reativas de oxigênio via NADPH oxidase, ocasiona a apoptose de podócitos, consequentemente levando albuminúria, danos renais e progressão da nefropatia diabética. Além do que, a atividade antioxidante como a da superóxido dismutase (SOD) está diminuída no DM, indicando que a melhora do sistema antioxidante pode representar importante terapia para a nefropatia diabética. No presente trabalho, testamos os efeitos do antioxidante tempol, um mimético da SOD endógena, no estado redox via NADPH oxidase e em conseqüentes danos renais em um modelo de HA genética e DM induzido pela estreptozotocina.

Hipótese: O tratamento com antioxidante tempol protege o rim de lesões decorrentes do estresse oxidativo via NADPH oxidase em um modelo experimental que combina HA genética e DM.

Objetivos específicos da presente tese foram:

1. Investigar se a administração de um antioxidante mimético da SOD, o tempol, corrige o desbalanço oxidativo e melhora as lesões renais precoces induzidas pelo estresse oxidativo na presença de DM e HA.
2. Investigar a hipótese de que o tempol melhora a albuminúria em modelo de DM associado à HA pela redução da apoptose de podócitos via inibição de PARP-1.

CAPÍTULO 2

Artigo I

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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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 observed 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.

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 endogen 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-tetrametylpiperidine-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-β) [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°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 ($NaNO_2$) calibration curve was developed utilizing different $NaNO_2$ 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 μl/ml aprotinin and a cocktail of protease inhibitors 40 μl/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 μl) were added to 450 μl of reaction buffer (50 mM phosphate buffer, pH 7.0; 1 mM EGTA; 150 mM sucrose, 5 μM lucigenin and 100 μM 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 diphenyleneiodonium

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_2^-/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_2O_2 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 electrophoretically 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 reprobed 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 significative. 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

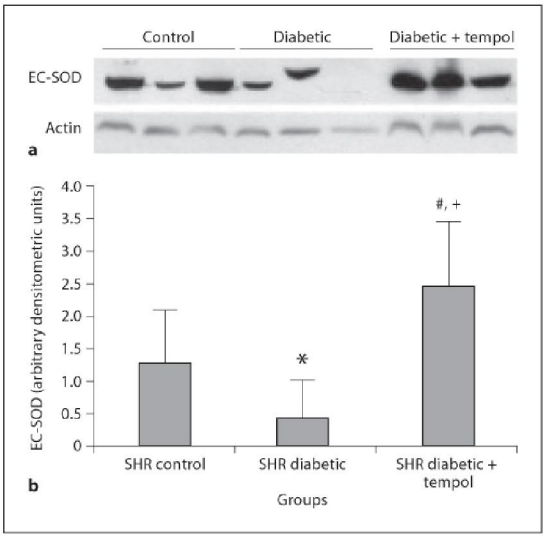
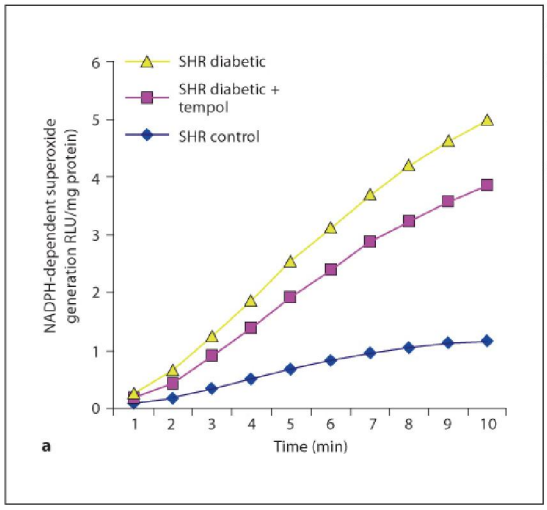


Fig. 1. EC-SOD protein level in the renal cortex. **a** Representative Western blot analysis of EC-SOD in SHR control, diabetic and diabetic treated with tempol. **b** Densitometric analysis of EC-SOD/actin ratio in different group of rats. Actin was used as control of protein loading. Bars represent means ± 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.



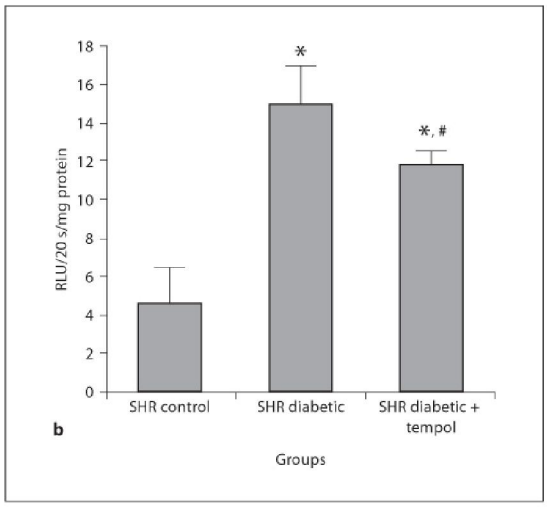


Fig. 2. NADPH-dependent superoxide generation in renal cortical homogenate. **a** Superoxide anion generation of kidney cortex was determined by the lucigenin-enhanced chemiluminescence method and photoemission was measured every 20 s for 10 min. The peak level of superoxide generation was observed around 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 ± 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).

pressure was neither altered by diabetes nor by treatment with tempol. The excretion of NO_2^- expressed as NO_2^-/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 ± 0.605 arbitrary units; n = 4) when compared to the control group (1.274 ± 0.823; n = 4, $p = 0.0423$). However, the treatment with tempol markedly elevated the expression of antioxidant EC-SOD (2.450 ± 1.015; n = 4) compared with control ($p = 0.0221$) and non-treated diabetic rats ($p < 0.0001$) (fig. 1).

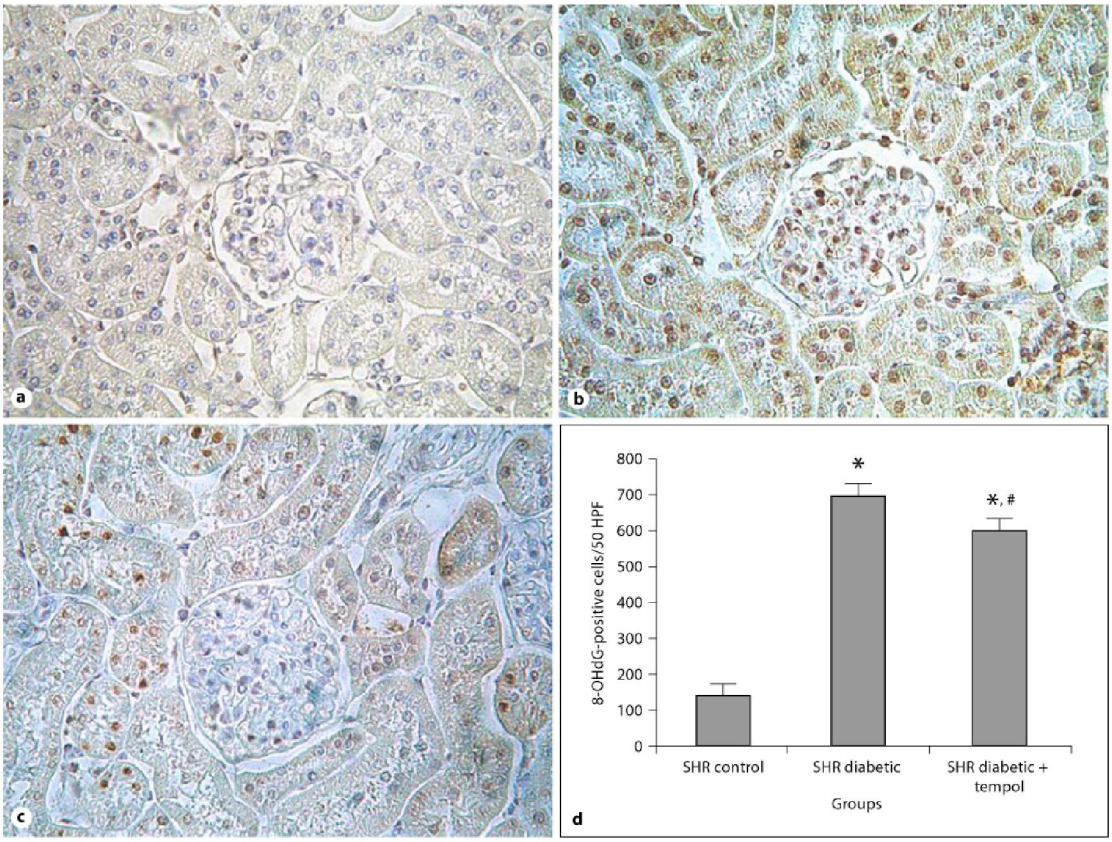


Fig. 3. Immunohistochemical detection of oxidative stress-induced DNA damage. Cells in the kidney cortex containing 8-OHdG-stained were identified by their brown-colored nuclei from SHR control (n = 4) (**a**), diabetic (n = 5) (**b**) and diabetic treated with tempol (n = 5) (**c**). At least three sections were stained and evaluated for each rat. Orig. magnif. ×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 ± 2.01 RLU/20 s/mg protein) was significantly (p = 0.0167) higher than in the control rats (4.58 ± 1.89). Treatment with tempol (11.84 ± 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 μM final concentration) completely blocked NADPH-induced superoxide production, while preincubation with rotenone (20 and 100 μM final concentration) did not

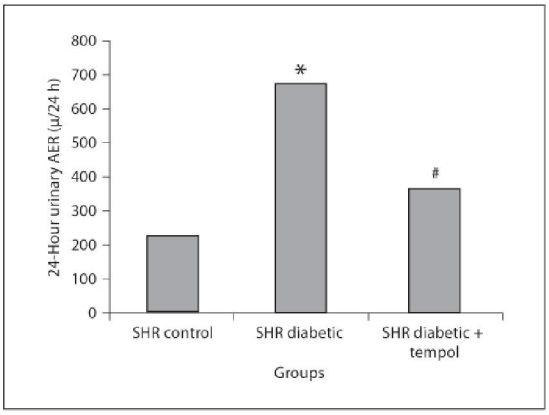


Fig. 4. 24-Hour urinary AER. Albuminuria is expressed as the geometric mean, and it was analyzed by non-parametric Kruskal-Wallis test followed by the Mann-Whitney U test for comparison between two groups. * p = 0.012 vs. SHR control; # p = 0.052 vs. SHR diabetic untreated group. At least three experiments 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) μg/24 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.

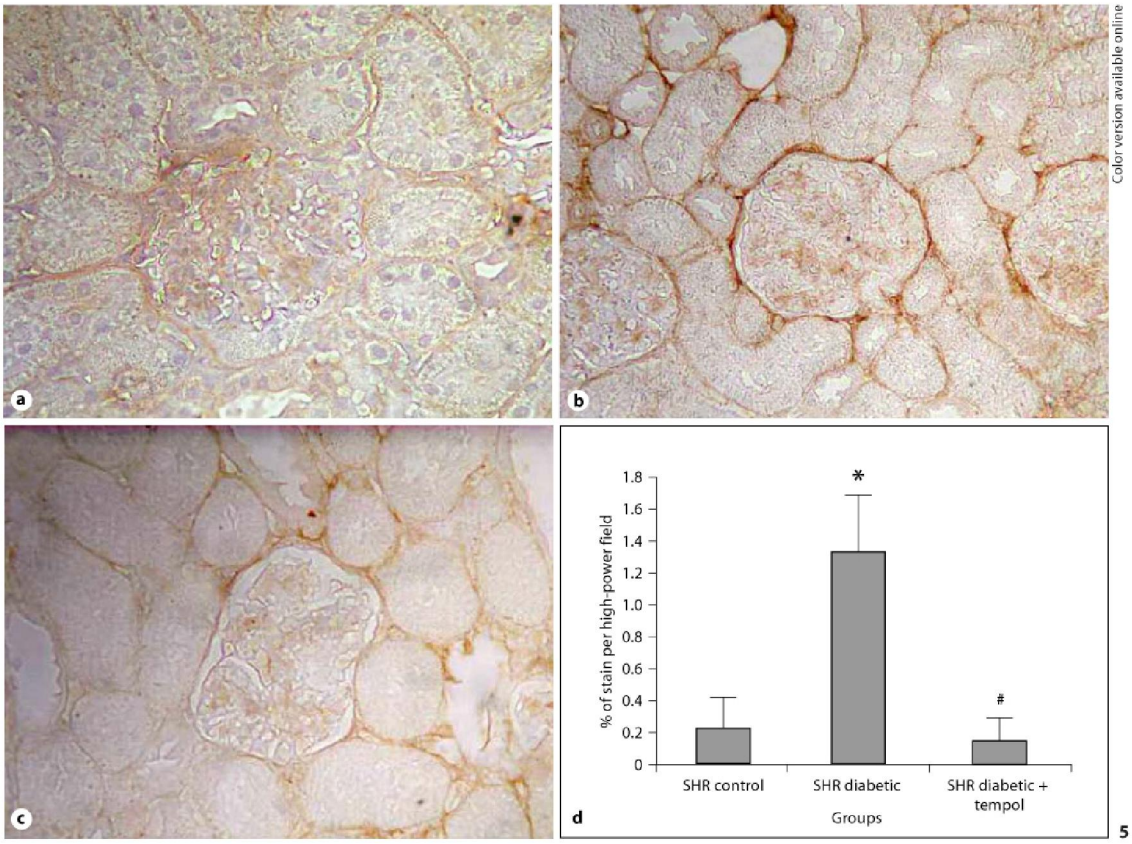
Elevation in Collagen IV Expression Induced by Diabetes Was Prevented by Tempol Treatment

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 ± 2.222 arbitrary units; n = 4) compared to the control (1.020 ± 0.744; n = 5, p = 0.0196), and tempol treatment (1.550 ± 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



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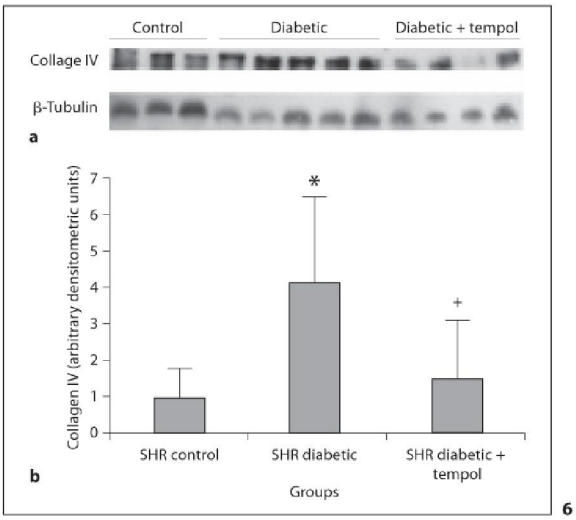

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Fig. 5. Immunohistochemical detection of collagen IV protein expression. Immunostain for collagen IV was detected in the kidney cortex of SHR control (**a**), diabetic (**b**) and treated with tempol (**c**), after 20 days of diabetes. The quantification 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/β-tubulin ratio in different group of rats. β-Tubulin was used as control of protein loading. Bars represent means ± 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-β_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^{2+}, Zn^{2+}-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-β 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-β 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-β. It can also be speculated that tempol has determined an elevation of EC-SOD because it has decreased the expression of TGF-β. That tempol decreases the expression of TGF-β 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^{2+}, Zn^{2+}-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-β, collagen α1 (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-β_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-β 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 associated 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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CAPÍTULO 3

Artigo II

Tempol is renal protective in experimental diabetic nephropathy

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Abstract

Background: In diabetic hypertensive rats a superoxide desmutase mimetic, tempol, reduces albuminuria by restoring the redox imbalance. Increased formation of reactive oxygen species (ROS) leading to activation of poly(ADP-ribose) polymerase (PARP)-1 and podocyte loss by apoptosis contributes to albuminuria in diabetes mellitus (DM). In the present study we investigate the hypothesis that in DM tempol is renal protective by reducing podocyte apoptosis through inhibition of PARP. *Methods:* DM was induced in 4 week of age spontaneously hypertensive rats (SHR) by streptozotocin. Plasma glucose levels were higher in diabetic rats and it was not affected by treatment with tempol. Systolic blood pressure was unaltered by diabetes or tempol treatment. *Results:* Albuminuria was significantly higher in diabetic rats ($p=0.004$) and it was reduced by tempol ($p=0.006$). DM leads to an elevation of glomerular apoptotic cells and to podocyte loss. These alterations were prevented by tempol treatment. In vivo, DM increases the expression of PARP-1 in isolated glomeruli ($p=0.037$), and it was markedly reduces ($p=0.026$) by tempol treatment. In vitro, the incubation of isolated glomeruli with H_2O_2 leads to higher number of apoptotic cells that was prevented by tempol or a PARP-1 inhibitor. *Conclusion:* We conclude that in DM tempol reduces albuminuria by diminish PARP-induced podocyte apoptosis and oxidative stress.

INTRODUCTION

In patients with diabetes mellitus (DM) elevation of albumin excretion rate is an early sign of renal disease, and it is often associated with progression to end stage renal disease [1]. Integrity of glomerular filtration barrier is an important determinant of retention of essential proteins such as albumin. Podocytes or glomerular visceral epithelial cells are important components of integrity of the glomerular filtration barrier [2-6]. To this, in patients with DM it has been demonstrated that podocyte depletion is not only associated with overt diabetic nephropathy but it also precedes the onset of microalbuminuria [2, 7]. In addition, reduction in podocyte number is the strongest morphological finding predictor of progression of diabetic nephropathy, where fewer cells predict more rapid progression [7, 8]. Experimental studies have also shown that podocyte depletion is one of the earliest cellular lesions affecting diabetic kidney [3-6]. The mechanisms that underlie the loss of podocytes in diabetic nephropathy are under intense investigation.

Several lines of evidence suggest that in diabetic nephropathy podocyte apoptosis contributes to reduced podocyte number [3, 4, 6, 9]. In cultured podocytes not only high glucose, but also downstream mediators of hyperglycemia-induced renal disease such as transforming growth factor (TGF-ß) and angiotensin II, induce apoptosis of these cells [3, 9, 10]. It has been suggested that increased reactive oxygen species (ROS) may be a trigger mechanism in podocyte apoptosis and loss in DM [3, 11]. Upregulation of NADPH oxidases and cytochrome P450 have been implicated as major sources of ROS in the podocytes in DM [11]. In addition, it has been suggested that the activation of the poly(ADP-ribose) polymerase (PARP) 1 is involved in hyperglycemia-induced ROS and podocyte apoptosis since PARP inhibitors blocked these later events [4]. PARP-1 inhibition also ameliorated hallmarks of diabetic nephropathy, named albuminuria and mesangial expansion [4]. These observations lead to the suggestion that PARP inhibitors could be used as a therapeutic strategy in diabetic nephropathy.

Oxidative stress has been proposed as an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage [12]. The antioxidant system provides major protection against oxidative stress by neutralizing or scavenging ROS or by breaking the chain reactions [13]. Superoxide dismutase (SOD), the first line endogen defense against superoxide production, converts superoxide ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) that is degraded to water and molecular oxygen by catalase and glutathione peroxidase [13]. We and other have demonstrated that in experimental DM down-regulation of SOD contributes to oxidative stress and consequent development of renal disease [14, 15]. Furthermore, we have shown that administration of tempol (4-hydroxy-2,2,6,6-tetrametylpiperidine-1-oxyl), a SOD mimetic, to streptozotocin (STZ)-induced diabetic rats reestablished the imbalance in redox status [16]. It elevated the renal expression of renal antioxidant extracellular SOD, decreased the production of NADPH-dependent superoxide production and reduced markers of tissue oxidative stress. It also reduces renal expression of collagen IV and albuminuria. The mechanism by which tempol reduced albuminuria in the diabetic rats remains unknown. Animal studies have shown that tempol is free of serious toxic effects and it may be developed as a drug for human use [17].

Here, we demonstrate that tempol ameliorates the components of glomerular filtration barrier in the diabetic spontaneously hypertensive rats (SHR). It reduces podocyte apoptosis and depletion and restored nephrin expression. Tempol also diminished the amount of PARP-1 ribosilated proteins in the diabetic kidney of SHR suggesting reduction PARP-1 activity. In vitro, tempol and PARP-1 inhibitor reduced hydrogen peroxide induced-apoptosis in isolated glomeruli. We conclude that in DM tempol reduces albuminuria by diminish PARP-induced podocyte apoptosis and oxidative stress.

MATERIALS AND METHODS

Experimental animals and protocol

All experiments complied with the guidelines established by The Brazilian College for Animal Experimentation (COBEA) and were approved by the Institutional Ethical Committee. Experiments were performed on male spontaneously hypertensive rats (SHR) derived from rats supplied by Taconic (Germantown, NY, USA) that were housed in a room temperature maintained at around 22 ºC, exposed to a 12-hour dark/light cycle, and allowed free access to food and tap water. DM was induced in prehypertensive 4-week-old SHR by injection of 60 mg/kg streptozotocin (STZ, Sigma-Aldrich, 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. Blood glucose levels were measured using an enzymatic colorimetric assay (Glicose PAP Liquiform, Labtest; Lagoa Santa, MG, Brazil) 48 h after injection of STZ or vehicle. Animals having a blood glucose of >270 mg/dL were considered diabetic. The diabetic rats were randomly divided to receive or not tempol treatment (Calbiochem, La Jolla, CA, USA) at a dose of 250 mg/kg diluted in saline solution and administered intraperitoneally [16]. All groups, control, untreated and treated diabetic rats, were euthanized using CO_2 20 days after induction of diabetes. Kidneys were removed from the animal, decapsulated and cut longitudinally into two halves. Halves were fixed in a 4% paraformaldehyde solution for immunohistochemistry, and/or were frozen in Tissue-Tek O.C.T Compound embedding medium (Sakura Finetek, Torrance, CA, USA) for immunofluorescence assay, and/or processed to isolation of glomeruli.

Blood pressure determination

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 DM, as previously reported [16].

Albumin excretion rate (AER)

AER was determined by single radial immunodiffusion, as described previously [16]. Urine samples were collected by placing each rat in individual metabolic cage for 24 h, before DM induction and the day before euthanasia.

Glomeruli isolation

Glomeruli were isolated using established sieving technique [18]. Kidneys were decapsulated, fragments of 1 to 2 mm from the outer renal cortex were prepared and passed through consecutive screens of 250, 125 and 63 mesh in Hank's solution (Gibco, Grand Island, NY, USA). Glomeruli were recovered from the top of 63 mesh screen. Solution with isolated glomeruli was centrifuged at 4 ºC, 1500 rpm, for 10 min. The pellet was sonicated in homogenization buffer (30 mM Tris-HCl; 10 mM EGTA; 5 mM EDTA; 1 mM DTT; 250 mM sucrose; 10 µg/ml aprotinine; protease inhibitor), centrifuged at 4 ºC, 11000 rpm for 10 min and the supernatant was prepared for Western blot analysis.

Western blot

Isolated glomeruli homogenate was used for quantification of PARP ribosilated protein expression by Western blot analysis. Molecular weight markers (PageRuler^{TM}, Fermentas Life Sciences) were used as standards. Proteins at concentration of 50 µg were separated on 10% SDS-polyacrylamide gel and were electrophoretically transferred to nitrocellulose membranes. To block nonspecific binding and antibody incubation, 2% bovine serum albumin (BSA; Sigma-Aldrich) was used in tris-buffered saline containing 0.1% Tween-20 (TBST). After blocking nonspecific binding, membranes were incubated overnight with an anti-Poly (ADP-ribose) mouse monoclonal antibody (diluted 1:1000; Trevigen, Gaithersburg, MD, USA). Following primary antibodies, membranes were incubated with anti-mouse horseradish peroxidase-conjugated antibody (diluted 1:5000 in TBST + 5% non-fat milk; Santa Cruz, CA, USA). Immunoreactive bands were made visible using the enhanced chemiluminescence method (Super Signal Cl-HRP Substrate System; Pierce, Rockford, USA). To verify the uniformity of protein load and transfer efficiency across the test samples, membranes were reprobed for actin (goat polyclonal anti-actin antibody, diluted 1:1000, Santa Cruz). Exposed films were scanned with a laser densitometer (Bio-

Rad; Hercules, CA, USA) and were analyzed quantitatively with Multi-Analyst Macintosh Software for Image Analysis Systems (Bio-Rad).

In vitro experiment

Isolated glomeruli from SHR control with 7 weeks old were ressuspended in RPMI 1640 (Gibco) containing 15 mM Hepes, 1% fetal bovine serum, 60 U/ml penicillin, 60 µg/ml streptomycin and 50 µg/ml fungizone and incubated at 37 ºC in 5% CO_2 and 95% air. To mimic oxidative stress *in vitro*, 500 glomeruli/well were incubated with 1 mM of hydrogen peroxide (H_2O_2). H_2O_2 treatment was performed in the presence or absence of 100 mM tempol, 1000 nM PJ34 (a specific inhibitor of PARP; Sigma-Aldrich) or tempol + PJ34. After 5 h of incubation, glomeruli were harvested and glomerular apoptosis was detected by TUNEL method.

TUNEL (terminal deoxynucleotidyl transferase-mediated nick-end labeling)

To determine whether glomerular cell apoptosis was modified by diabetes, H_2O_2 and treatment with tempol or PARP inhibitor, the TUNEL method for detecting DNA breaks in situ was applied to cortex tissue or isolated glomeruli from the studied group [18]. Cortex section and isolated glomeruli were incubated with proteinase K (9.3 µg/ml; Boehringer Mannheim, Indianapolis, IN) for 15 min at room temperature. After quenching endogenous peroxidase, the slides were rinsed in One-Phor-All buffer (100 mM Tris-acetate buffer, 100 mM magnesium acetate, 500 mM potassium acetate, pH 7.5) and incubated with TdT (Amersham Pharmacia, Buckinghamshire, England) and biotinylated-dUTP (Gibco) for 60 min. Labeled nuclei were detected with ABC Vectastain (Vector Laboratories, Burlingame, CA, USA) in PBS. Afterwards, 2.5 mg of diaminobenzidine tetrahydrochloride (DAB) in 5 mL of 1 M Tris-HCl, pH 7.5 plus 2.5 uL H_2O_2 were put in slides for 4 minutes and counterstained with hematoxylin. As a positive control, some slides were treated with DNAse (20 Kunitz units/ml; Sigma). The quantitative analysis for TUNEL was done by counting positive cells per 100 glomeruli of cortex from studied group (n=4 in each group). For isolated glomeruli, intensity of TUNEL staining was evaluated by a semiquantitative method (Bio ColorScanner program) and was expressed as the mean of percentage of

positive cells per glomerulus, in each treatment (n=20 glomeruli per treatment, 3 independent experiments).

Immunohistochemistry

Identification and quantification of podocytes were performed by immunohistochemistry for Wilm's tumor protein (WT-1), a marker of podocytes [19]. WT-1 expression was detected in 4% paraformaldehyde fixed, paraffin-embedded kidney sections. Antigenic recuperation was done with microwave exposure and endogenous peroxidase was blocked by incubating the slides in 3 % H_2O_2 in methylic alcohol for 10 min. The sections were then incubated overnight at 4 °C with a 1:50 dilution of rabbit anti-WT-1 antibody (Santa Cruz). Staining was visualized using peroxidase-conjugated antibody to rabbit immunoglobulin (diluted 1:200; Santa Cruz) using avidin-biotin complex ABC Vectastain (Vector Laboratories) and 3,3-diaminobenzidine tetrahydrochloride (DAB) (Sigma). After dehydration and counterstaining with hematoxylin, the slides were mounted in Entellan (Merck, Darmstadt, Germany). For negative controls, staining was performed omitting the primary antibody. Glomerular WT-1 positive cells were counted per glomerulus in a total of 20 glomeruli for each animal (n=4 animals in each group).

Double immunofluorescence for WT-1 and caspase-3

Kidney halves were fixed in 4% paraformaldehyde in ice for 1 h, embedded overnight in 30% sucrose at 4 ˚C, and then were frozen in Tissue-Tek O.C.T, compound embedding medium. Frozen sections were blocked with 1% bovine serum albumin (BSA) for 1 h and incubated with both primary antibodies to WT-1 (diluted 1:10, Santa Cruz), and cleaved caspase-3 (diluted 1:50, Cell Signaling, Beverly, MA, USA), a marker of apoptosis. Staining were developed using FITC-conjugated anti-mouse (diluted 1:100, Santa Cruz) and rhodamine conjugated anti-rabbit (diluted 1:100, Santa Cruz) secondary antibodies. Then, the sections were cover-slipped with Vectashield antifading medium (Vector Laboratories). The double-staining was examined using a Nikon-E800 fluorescence microscope. Digital images were captured using specific software (Image Pro-Plus).

Immunofluorescence for nephrin

For nephrin fluorescence labeling, kidney halves were prepared as described above in double immunofuorescence staining assay. Frozen sections were blocked with 2% BSA for 30 min, incubated with primary antibody to nephrin (1:2000, provided by Dr. Hiroshi Kawachi, University Graduate School of Medical and Dental Sciences, Niigata, Japan) for 2 h, and then incubated with FITC-conjugated anti-mouse secondary antibody (1:200, Santa Cruz). The sections were examined using a Nikon-E800 fluorescence microscope and images captured using Image Pro-Plus specific software. The semiquantitative analyses were performed using Bio ColorScanner program and the fluorescence of nephrin was expressed in percentage of fluorescence per glomerulus.

Statistical Analysis

The results are expressed as means ± SD, except for albuminuria that is expressed as geometric means and confidential interval. Comparisons between groups were done with one-way analysis of variance (ANOVA) followed by Bonferroni test. Albuminuria was analyzed by nonparametric tests, Kruskal-Wallis followed by Mann-Whitney U test. A value of $p<0.05$ was considered significant. All analyses were performed using statistical software StatView (SAS Institute Inc., Cary, NC, USA).

RESULTS

Tempol treatment did not alter blood pressure and glycemia levels

As expected, diabetic rats showed impaired body weight gain and elevated blood glucose levels compared with control rats, and treatment with tempol did not affect these parameters ($p < 0.0001$, table). The systolic blood pressure was neither altered by diabetes nor by treatment with tempol.

Urinary albumin excretion rate (AER) was normalized by tempol treatment

Untreated diabetic rats exhibited a marked increase in urinary AER ($p=0.004$) compared to the control rats, and treatment with tempol significantly reduced AER ($p=0.006$) (figure 1).

Tempol treatment prevented podocyte apoptosis and depletion induced by diabetes

By TUNEL method, we observed that glomerular apoptotic cells number was significantly higher in untreated diabetic rats compared to the control ($p=0.0014$) and tempol treatment significantly ($p=0.01$) reduced this number (Figure 2 A, B). Afterwards, we demonstrated that apoptotic glomeruli cells were characterized as podocytes using double immnuofluorescence for cleaved caspase-3, a marker of apoptosis, and WT-1, a transcription factor participating in podocyte differentiation and maintenance of mature phenotype [19] (Figure 2 C). Immunohistochemistry for WT-1 (Figure 3) showed that diabetic untreated rats exhibits a marked reduction in the podocyte number compared to the control group ($p=0.014$), and treatment with tempol increase this number to the levels seen in control rats ($p=0.0369$ compared to the untreated diabetic group).

The reduction in nephrin expression induced by diabetes was prevented by tempol treatment

Studies demonstrated that mutations or deletion of several slit diaphragm-associated proteins in glomerulus, such as nephrin, contribute to podocyte dysfunction, leading to defects in glomerular filtration and proteinuria in animal models and patients with diabetic nephropathy [20, 21]. Theferore, we assessed the effect of antioxidant tempol in nephrin expression in experimental model of early diabetes associated with hypertension. Immunofluorescence (Figure 4) revealed a significantly reduction in nephrin expression in diabetic glomerulus, when compared to the control ($p<0.0001$), and treatment with tempol significantly increase this expression ($p=0.0380$), establishing an important amelioration in integrity of glomerular filtration barrier by antioxidant tempol in diabetes and hypertension.

Diabetes-induced elevation in PARP-1 expression was prevented by tempol treatment

By Western blotting analysis, we observed that the expression of ribosilated PARP-1 in isolated glomeruli was significantly elevated in diabetic rats ($p=0.0379$). The treatment with tempol markedly reduced the expression of PARP-1 ($p=0.0262$, compared with untreated diabetic rats). Estimation of ribosilated PARP-1 is indicative of PARP-1 activity [4].

Glomerular cells apoptosis were prevented by tempol treatment and PARP inhibition in vitro in isolated glomeruli

We investigated the participation of PARP-1 in tempol-induced reduction of podocyte apoptosis by in vitro study. Isolated glomeruli were exposed to ROS by incubation with hydrogen peroxide (H_2O_2) with tempol and/or a specific PARP-1 inhibitor (PJ 34). TUNEL method showed that H_2O_2 increased markedly the number of apoptotic cells, compared with control ($p<0.0001$) (Figures 6 a, b, f). Treatment with tempol and with PARP-1 inhibitor PJ34 reduced significantly the number of glomerular apoptotic cells, when compared with H_2O_2 ($p<0.0001$ and $p=0.0057$, respectively) (Figures 6 c, d, f). However, treatment with tempol was more effective in reducing apoptosis than treatment with PJ34 and there were no additive effect on glomerular cell apoptosis with concomitant use of tempol and PJ34 (Figures 6 e, f). These findings suggest that tempol treatment prevented H_2O_2-induced glomerular cell apoptosis at least partly by PARP-1 inhibition.

DISCUSSION

In patients with diabetes mellitus increased albumin excretion rate is an early sign of renal disease and it is associated with progression to renal insufficiency [1]. There are compelling evidences that in DM podocyte apotosis and loss contribute to albuminuria [3-5, 7-9]. Podocyte depletion can also cause glomerulosclerosis [22]. Oxidative stress is involved in the pathogenesis of diabetes complications [23], including podocyte apoptosis [3]. It has been suggested that poly(ADP ribose) polimerase (PARP) signaling participates in hyperglycemia-induced reactive oxygen species (ROS) and podocyte apoptosis since a specific PARP inhibitor blocked podocytes apoptosis [4]. Herein, we demonstrated that in diabetic hypertensive rats podocytes apoptosis and loss are associated with generation of ROS and activation of PARP. These changes that were accompanied of nephrin loss and albuminuria were prevented by a SOD mimetic, tempol. Attenuation of ROS-induced podocyte apoptosis by tempol was confirmed in isolated glomeruli exposed to hydrogen peroxide, and this anti-apoptotic effect was also observed with a PARP inhibitor. Collectively these observations suggest, for the first time, that in experimental diabetes tempol reduces podocyte apoptosis and albuminuria through PARP signaling.

Susztak and collaborators have elegantly demonstrated that podocyte apoptosis may be casually involved in albuminuria since in two murine models of type 1 and type 2 diabetes it precedes significant levels of albuminuria, podocyte depletion, and mesangial expansion [3]. Therefore, it is currently acknowledge that inhibition of podocyte apoptosis may be a useful strategy to the treatment of diabetic nephropathy. This can be obtained in experimental diabetic nephropathy by reduction in NADPH-induced ROS generation and by PARP inhibition [3, 4, 11]. To this, Susztak and collaborators have shown that the administration of apocynin, an inhibitor of ROS generation via NADPH oxidase, was able to prevent several renal abnormalities including podocyte apoptosis in a murine model of diabetes [3]. Concordantly, Eid and coworkers have demonstrated that inhibition of cytochrome P450 prevented NADPH-dependent superoxide anion generation and podocyte apoptosis in vitro and in vivo [11]. In the present study we have shown that tempol reduces podocyte apoptosis and albuminuria. Tempol is a SOD mimetic, with additional ROS-scavenging activities [24, 25] that may also reduce NADPH oxidase activity [26]. In fact, we have previously shown that in streptozotocin-induced diabetic rats tempol reduces NADPH oxidase-dependent superoxide production [16].

It has been hypothesized that one consequence of high glucose-induced increased intracellular ROS and subsequent DNA breakage is activation of PARP-1 [4]. PARP-1 is one of the most abundant nuclear proteins that has been involved in the development of multiple disease conditions including stroke, myocardial infarction, heart failure, vascular dysfunction, and mesenteric, muscle, or renal ischemia reperfusion injury [27]. PARP has also been suggested to play a role in the development of type 1 diabetes and in diabetic complication such as retinopathy [28, 29]. More recently, it has been demonstrated that PARP inhibition attenuates the development of albuminuria and podocyte apoptosis and depletion in diabetic mice [4]. The authors of this last study have hypothesized that inhibition of ROS generation and normalization of mitochondrial function was the likely mode of PARP inhibitor protective action. In the present study, reduction in podocyte apoptosis and depletion obtained with tempol was associated with inhibition of PARP-1.

In addition, induction of apoptosis in isolated glomeruli with H_2O_2 was attenuated by tempol and PARP-1 inhibitor. There was no additive effect on attenuation of apoptosis with the concomitant use of tempol and PARP-1 inhibitor. Current observation that tempol reduced PARP-1-induced podocyte apoptosis and albuminuria, and our previous observations that tempol reestablished the redox status [16], suggest that in diabetic nephropathy tempol is renal protective.

Recently, a seminal work has demonstrated that Notch activation may be involved in podocyte apoptosis and albuminuria in glomerular diseases, including diabetic nephropathy [5]. Notch pathway is crucial in podocyte development, and its activation in glomerular disease in mature kidney may lead to podocyte damage. In that study, remarkably, inactivation of Notch signaling via genetic or pharmacological intervention was sufficient to prevent podocyte apoptosis and damage to glomeruli [5]. Interesting Notch activation was accompanied of PARP activation. Contribution of oxidative stress to Notch and PARP activation and consequent podocyte apoptosis was not investigated.

In summary, in experimental diabetic nephropathy tempol is renal protective by reducing PARP-1-induced podocyte apoptosis and albuminuria. In previous study we also demonstrated that tempol reestablished the redox status and reduces the accumulation of kidney extracellular matrix in experimental diabetes mellitus [16]. These effects were independent of blood glucose and blood pressure reduction, the two most effective approaches for the treatment of diabetic nephropathy [17]. Collectively, these observations and the fact that animal studies have shown that tempol is free of serious toxic effects [30] would suggest that tempol may have clinical benefits in human diabetic nephropathy.

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LEGENDS OF FIGURES

Fig.1. Tempol treatment reduced albumin excretion rate (AER) in diabetic SHR. AER was expressed as geometric mean. *p=0.004 vs. SHR control group; $^{\#}$p=0.006 vs. SHR diabetic untreated group.

Fig.2. Tempol treatment blocks podocyte apoptosis in diabetic SHR. Glomeruli apoptotic cells were identified by TUNEL method (arrow, A) and quantified as described in method (B). Apoptotic cells within glomeruli were identified as podocyte by labeling these cells for specific podocyte marker (WT-1) and apoptosis marker (caspase-3) (arrows) (C). Orig. magnif. X400. *p=0.0014 vs. SHR control group; $^{\#}$p=0.01 vs. SHR diabetic untreated group.

Fig.3. Tempol treatment blocks podocyte depletion in diabetic SHR. Positive immunostain for WT-1, a specific podocyte marker, was determined by brown-colored nuclei in control (a), diabetic (b) and tempol treated diabetic (c) SHR (arrows). The quantification of WT-1 expression was assessed in 4 independent experiments (d). Orig. magnif. X1000. *p=0.014 vs. SHR control group; $^{\#}$p=0.036 vs. SHR diabetic untreated group.

Fig.4. Tempol treatment impedes diabetes-induced decrease in nephrin expression. Expression of nephrin was estimated by immunofluorescence assay in control (a), diabetic (b) and treated (c) SHR groups. Glomerular nephrin intensity was evaluated by a semiquantitative method and mean of glomeruli intensity of each animals was expressed as percentage of control mean (d) from at least four independent experiments (n = 4 in each

group). Orig. magnif. X400. * $p<0.0001$ vs SHR control group; $^{\#}$ $p=0.0380$ vs SHR diabetic untreated group.

Fig.5. Tempol blocks the diabetes-induced elevation in poly (ADP-ribose) polymerase (PARP-1) expression in renal cortex. A. Representative Western blot analysis of PARP expression in control, diabetic and tempol treated diabetic rats. B. Densitometric analysis of PARP/actin expression in different groups. Actin was used as control of protein loading. Bars represent means ± SD of band densities of two independent experiments (mean of 5 rats in each group). *$p=0.0379$ vs. SHR control group; $^{\#}p=0.0262$ vs. SHR diabetic untreated group. PC = positive control of PARP.

Fig.6. Tempol and PARP inhibitor block podocyte apoptosis. In vitro estimation of apoptosis by TUNEL method was performed in isolated glomeruli after incubation with (b) or without H_2O_2 (1mM) (a). The treatment with H_2O_2 was performed in the presence of tempol (100 mM, c), PJ34 (a PARP inhibitor, 1000 nM, d) and tempol 100 mM + PJ34 1000 nM (e). Orig. magnif. X400. TUNEL positive stain was determined by brown-colored nuclei and was quantified by a semiquantitative method in at least 25 glomerulus from three independent experiments (f). *$p<0.0001$ vs control; **$p<0.0001$ vs H_2O_2; $^{\&}p=0.0045$ vs control; $^{\#}p=0.0057$ vs H_2O_2; ***$p=0.0370$ vs H_2O_2 + tempol.

Table 1. Physical and metabolic parameters in control and diabetic rats with and without tempol treatment.

	Body weight (g)	Systolic blood pressure (mmHg)	Plasma glucose (mg/dl)
SHR control (n=15)	194 ± 15	158± 10	153 ± 23
SHR diabetic (n=15)	119 ± 34*	157 ± 11	547 ± 82*
SHR diabetic + tempol (n=15)	109 ± 18*	149 ± 07	545 ± 89*

Data were expressed as means ± SD. *p<0.0001 vs. SHR control group.

FIGURE 1

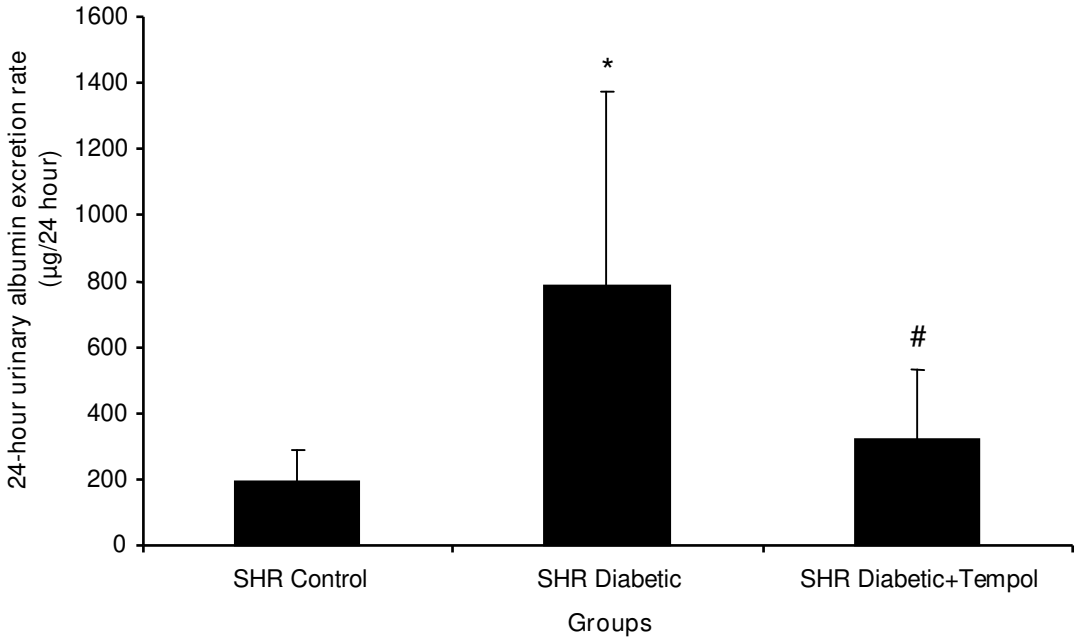

24-hour urinary albumin excretion rate (µg/24 hour)
1600
1400
1200
1000
800
600
400
200
0
*
#
SHR Control
SHR Diabetic
SHR Diabetic+Tempol
Groups

FIGURE 2

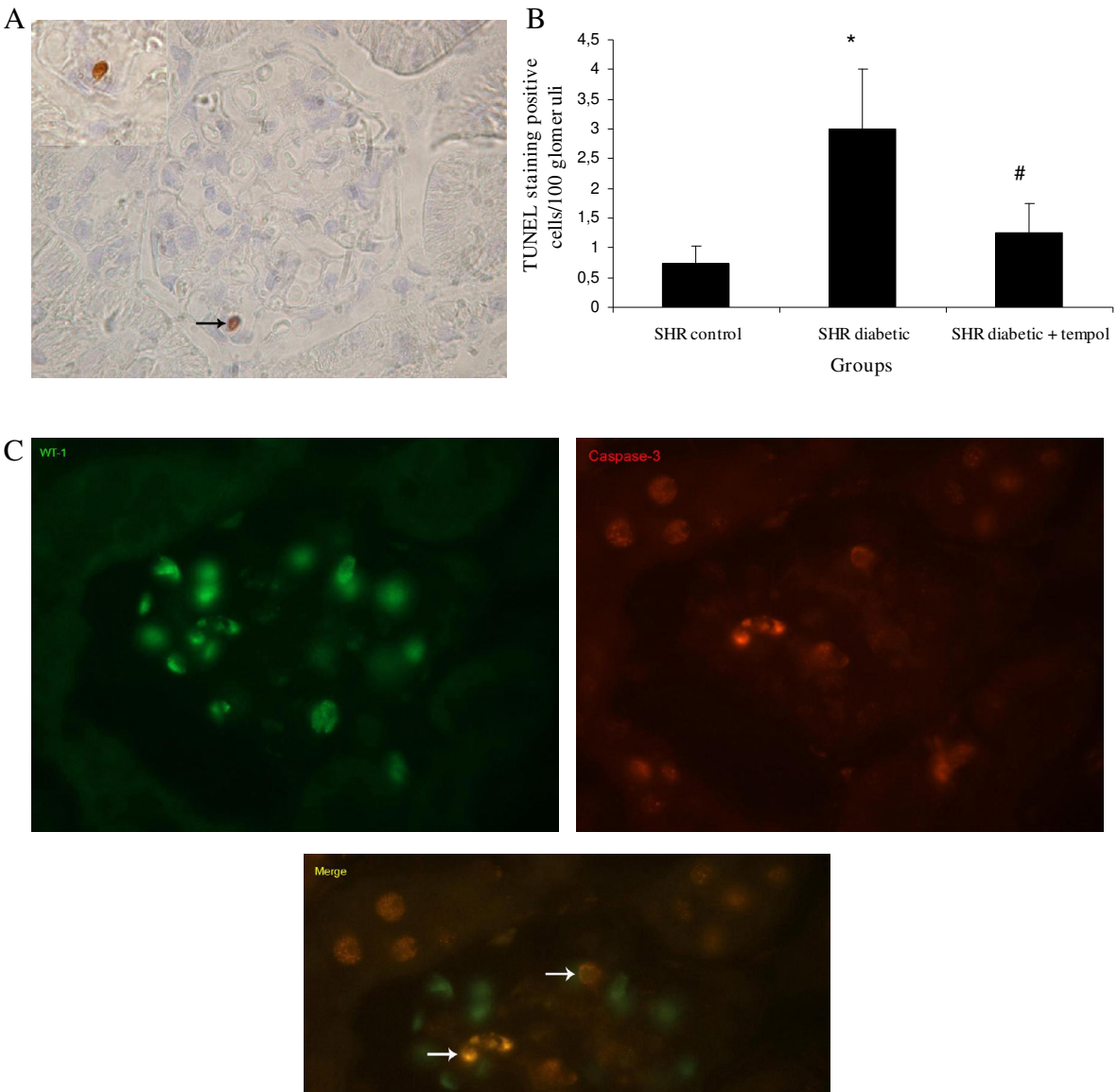
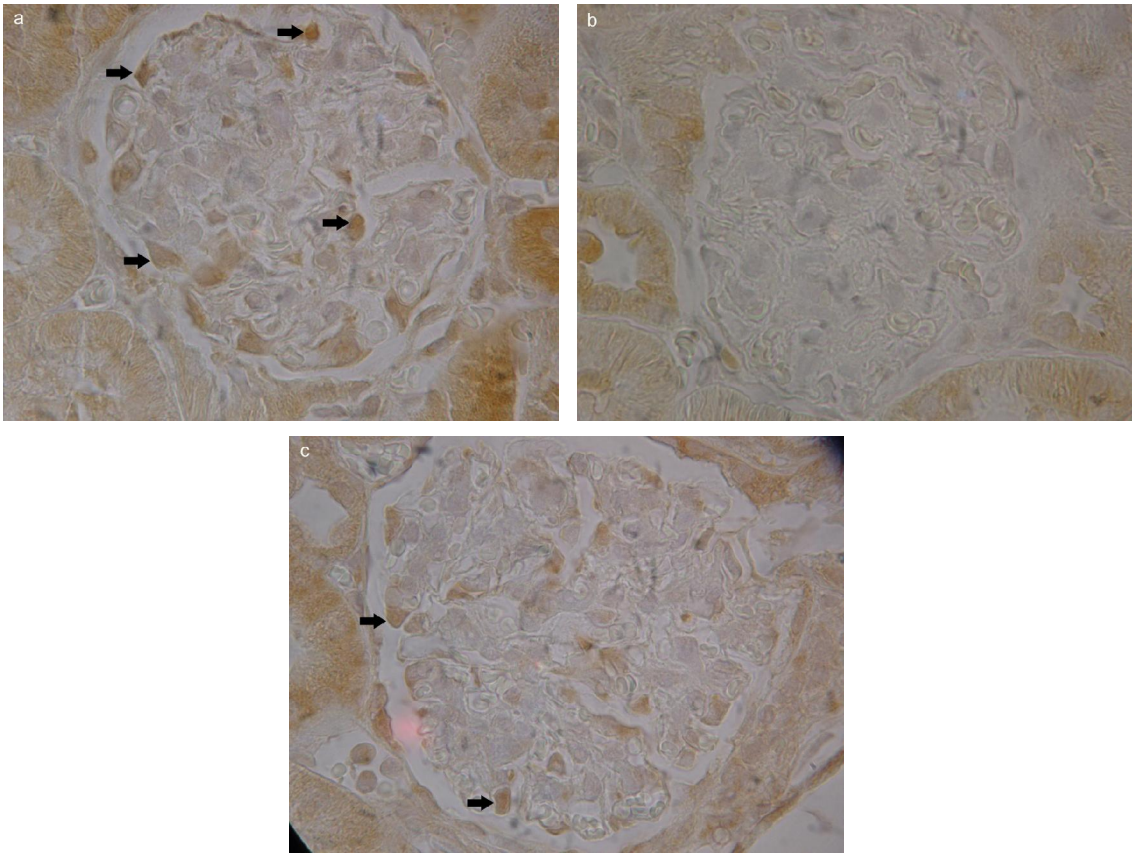

A
B
TUNEL staining positive cells/100 glomeruli
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
*
#
SHR control
SHR diabetic
SHR diabetic + tempol
Groups
C
WT-1
Caspase-3
Merge

FIGURE 3


a
b
c

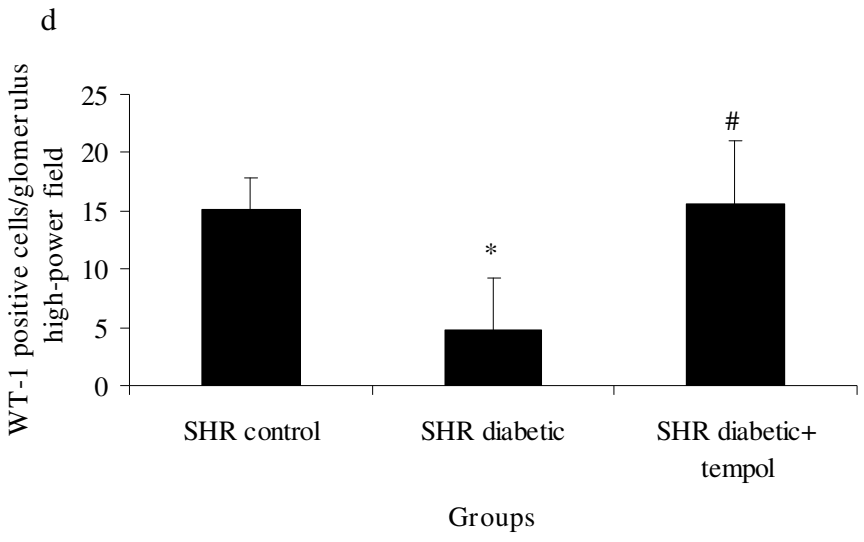

d
WT-1 positive cells/glomerulus high-power field
25
20
15
10
5
0
*
#
SHR control
SHR diabetic
SHR diabetic+ tempol
Groups

FIGURE 4

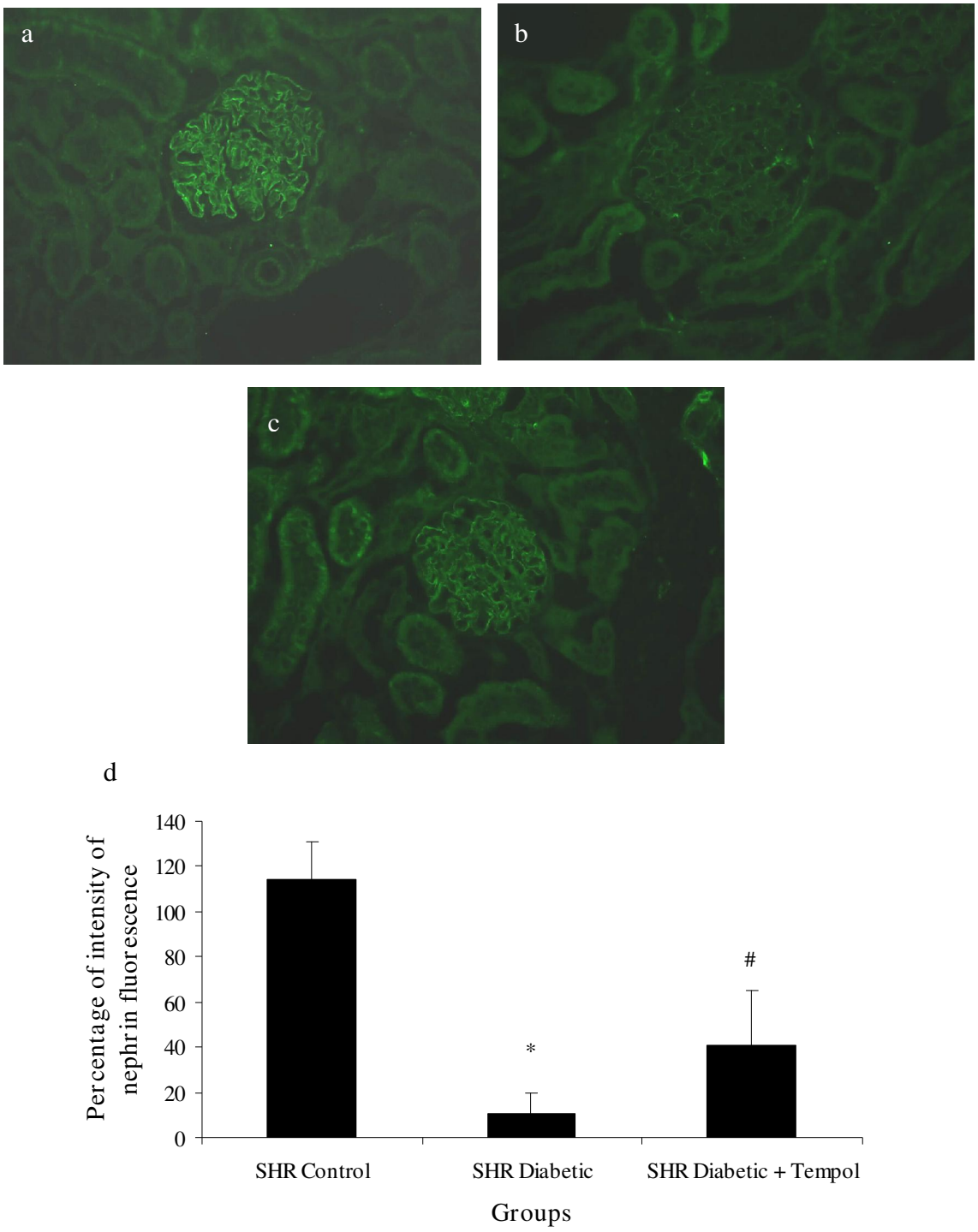

a
b
c
d
Percentage of intensity of nephrin fluorescence
140
120
100
80
60
40
20
0
*
#
SHR Control
SHR Diabetic
SHR Diabetic + Tempol
Groups

FIGURE 5

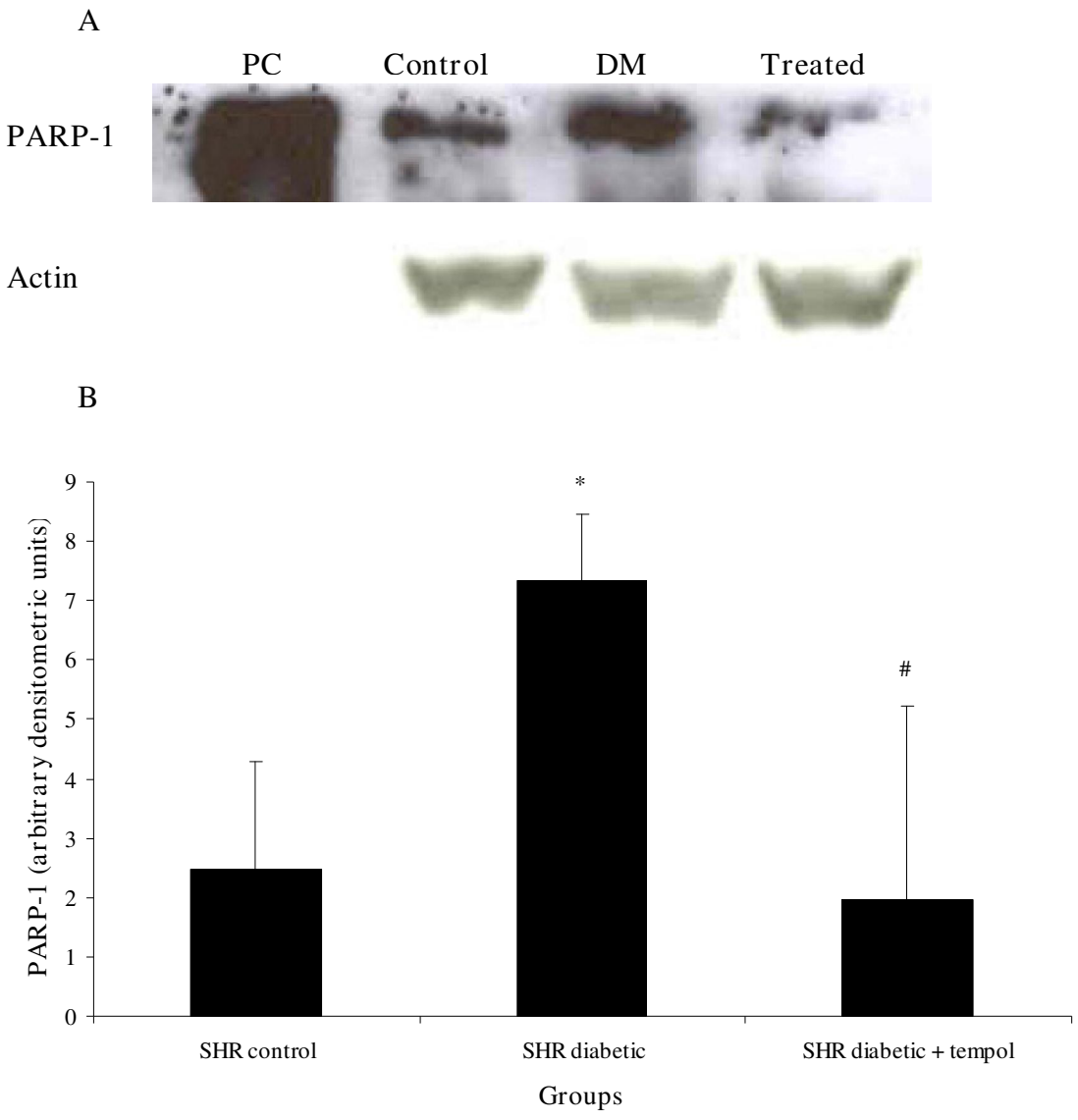
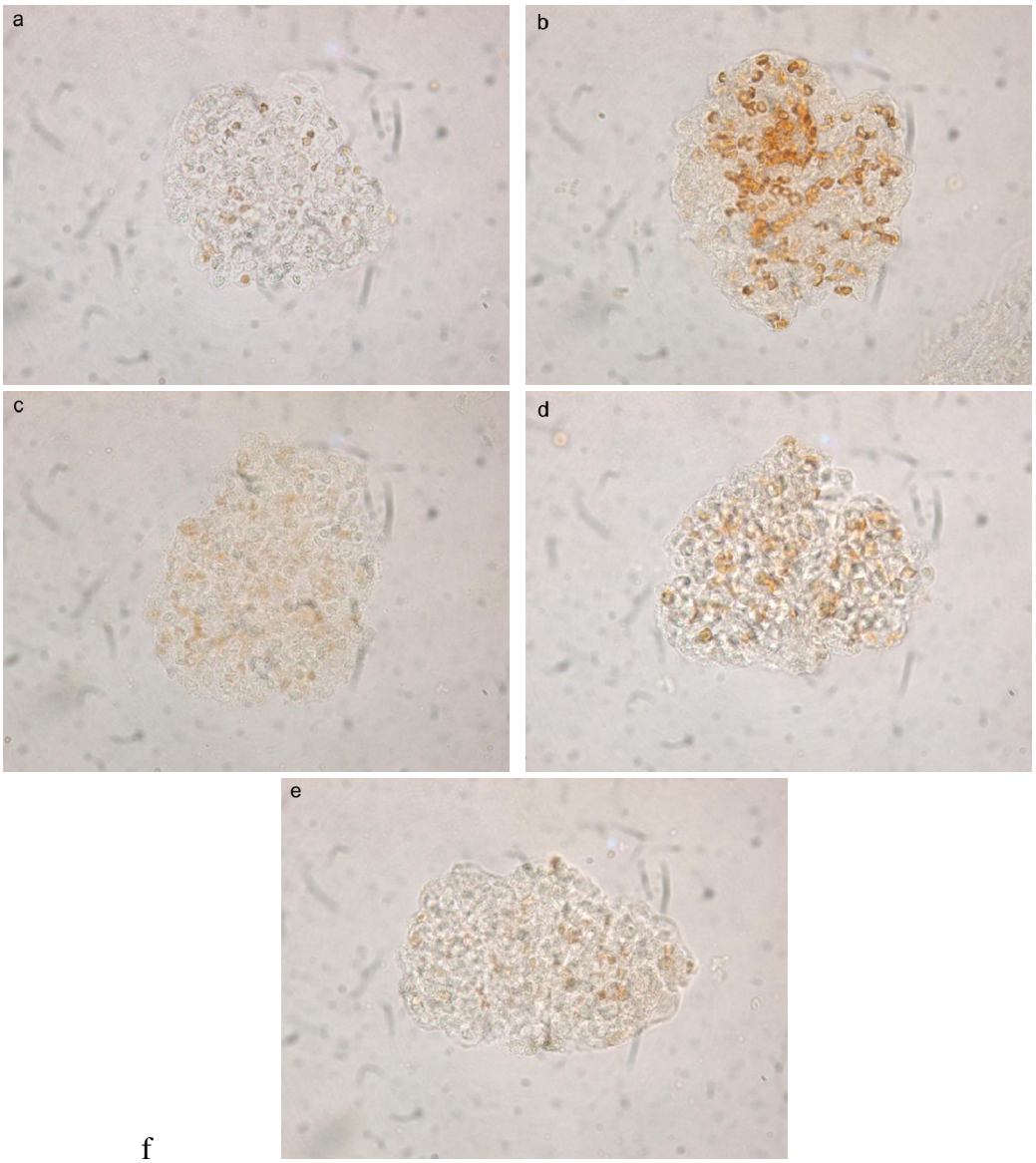
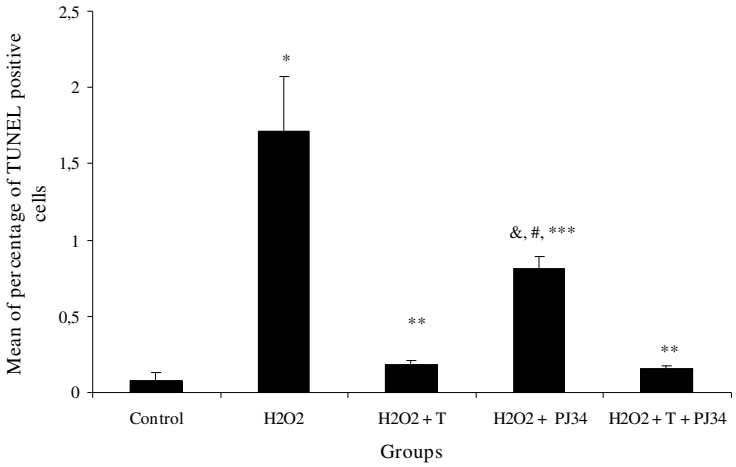

A
PC
Control
DM
Treated
PARP-1
Actin
B
PARP-1 (arbitrary densitometric units)
0
1
2
3
4
5
6
7
8
9
*
#
SHR control
SHR diabetic
SHR diabetic + tempol
Groups

FIGURE 6


a
b
c
d
e

f


Mean of percentage of TUNEL positive cells
2,5
2
1,5
1
0,5
0
*
**
&, #, ***
**
Control
H2O2
H2O2 + T
H2O2 + PJ34
H2O2 + T + PJ34
Groups

CAPÍTULO 4

1. SUMÁRIO

1. O tratamento com antioxidante tempol, mimético da SOD endógena, restaura o desbalanço redox e previne lesões renais na presença concomitante de DM e HA.

2. O tratamento com tempol não altera a glicemia nem a pressão arterial.

3. Tempol reduz albuminúria em ratos diabéticos SHR pela melhora em componentes da barreira de filtração glomerular, como redução na apoptose de podócitos e aumento da expressão de nefrina.

4. Tempol reduziu a atividade de PARP-1 em rins de ratos SHR diabéticos, além de, concomitante com um inibidor de PARP-1, diminuiu a apoptose induzida por peróxido de hidrogênio em glomérulos isolados *in vitro*, comprovando que tempol reduz apoptose de podócitos induzida, em parte, por PARP-1.

2. CONCLUSÃO GERAL

Pode-se considerar o antioxidante tempol como um renoprotetor na nefropatia diabética experimental, uma vez que restaura o estado redox, reduz o acúmulo de matriz extracelular, restaura a expressão de nefrina, previne apoptose de podócitos induzida parcialmente por PARP-1 e melhora albuminúria, sem alterar a glicose sangüínea e a pressão arterial.

3. PERSPECTIVAS FUTURAS

A nefropatia diabética é considerada a principal causa de insuficiência renal crônica e estágio final da doença renal. A prevalência da nefropatia diabética tem aumentado significativamente, portanto, é importante a definição de seus mecanismos fisiopatológicos e, consequentemente, o desenvolvimento de medidas preventivas e terapêuticas.

O uso de antioxidantes, como o tempol, tem tido bastante sucesso na prevenção e melhora de alterações renais em diversos modelos de nefropatia diabética experimental. A presente tese demonstrou pela primeira vez a influência do antioxidante tempol na prevenção da nefropatia diabética em modelo de DM e HA, diminuindo o estresse oxidativo e melhorando alterações renais e albuminúria. Portanto, é preciso avaliar mais profundamente os mecanismos de atuação do tempol na melhora da nefropatia diabética neste modelo, especificamente a influência do tempol na integridade da barreira de filtração glomerular por meio de estudos das diversas vias que poderiam estar envolvidas na apoptose de podócitos e a influência de proteínas de junções intercelulares pertencentes à barreira de filtração glomerular. Para isso, poderiam ser utilizados métodos com cultura de células de podócitos, RNA de interferência, camundongos knockout, dentre outras metodologias moleculares.

Além do que, uma vez que os estudos com animais demonstraram que o tempol não possui efeito tóxico, o aumento da defesa antioxidante com o tratamento com tempol pode ser uma medida terapêutica para nefropatia em pacientes diabéticos e hipertensos.

CAPÍTULO 5

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CAPÍTULO 6

1. APÊNDICE (Publicações adicionais)

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

Biswas SK, Peixoto EB, Souza DS, Lopes de Faria JB. Hypertension increases pro-oxidant generation and decreases antioxidant defense in the kidney in early diabetes. Am J Nephrol 2008; 28: 133–142.

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

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

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

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

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

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

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

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Hypertension Increases Pro-Oxidant Generation and Decreases Antioxidant Defense in the Kidney in Early Diabetes

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Key Words
Diabetes · Hypertension · Nephropathy · Pro-oxidants · Antioxidants · Oxidative stress

Abstract
Aims: The combination of hypertension and diabetes exacerbates renal oxidative stress. The aim of the present study was therefore to evaluate the pro-oxidant and antioxidant mechanisms responsible for the induction of renal oxidative stress in the presence of hypertension and diabetes mellitus. ***Methods:*** Diabetes was induced in spontaneously hypertensive rats (SHR) and their genetically normotensive control Wistar-Kyoto (WKY) rats by streptozotocin at 12 weeks of age. After 10 days, pro-oxidant, antioxidant and oxidative stress parameters were evaluated in the renal tissue. ***Results:*** NADPH oxidase-dependent superoxide generation in the renal cortex was significantly elevated in WKY and SHR diabetic (D) groups compared to the respective control (C) groups ($p < 0.005$, $n = 5$). However, the highest level of superoxide generation was observed in the SHR-D group compared to all other groups. The expression of the gp91phox subunit of NADPH oxidase was significantly elevated in the SHR-D ($p < 0.05$, $n = 5$), but not in the WKY-D group, compared to the respective control groups. The renal cortical extracellular-superoxide dismutase level was found to be markedly decreased in the SHR groups compared to the WKY groups ($p < 0.05$, $n = 5$). The antioxidant glutathione level was found to be lower in the SHR-D ($p = 0.03$, $n = 15$), but not in the WKY-D group, compared to the respective control groups. Finally, nitrotyrosine and 8-hydroxy-2′-deoxyguanosine, markers of oxidative stress, were found to be similar in the kidneys of WKY-C and WKY-D, but were elevated in the SHR-D compared to the SHR-C group. ***Conclusion:*** We therefore conclude that hypertension increases pro-oxidant generation and decreases antioxidant defense, and thereby induces renal oxidative stress in early diabetes.

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Introduction

Diabetes mellitus (DM) and hypertension are the most important causes of nephropathy and end-stage renal disease (ESRD). A recent report shows that DM constitutes about half and hypertension constitutes about one third of the new cases of ESRD [1]. However, the underlying pathophysiological mechanism in the development of nephropathy and ESRD resulting from DM or hypertension are not clear. Genetic susceptibility and a complex interaction among a number of metabolic and hemody-

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namic factors, including reactive oxygen species (ROS), advanced glycation end-products, polyols, protein kinase C (PKC) isoforms, systemic and intraglomerular hypertension, and various vasoactive hormones are involved in the pathogenesis of nephropathy and its progression to ESRD [for review see 2–4]. However, recent studies strongly implicate the contribution of renal oxidative stress to the pathogenesis of nephropathy and ESRD [5–7]. In fact, oxidative stress has been demonstrated in the kidney in almost all types of nephropathy whatever the primary disease of its origin might be [6, 7].

Hyperglycemia is well known to induce oxidative stress through exaggerated generation of ROS from the mitochondrial electron transport chain [8]. Mitochondrial superoxide can inhibit the glycolytic enzyme glycerol-3-phosphate dehydrogenase and thereby can activate several pathways of hyperglycemic damage, including advanced glycation end-products, PKC, polyol, hexosamine and nuclear factor-κB pathways [8, 9]. PKC-induced NADPH oxidase activation further enhances pro-oxidant generation and oxidative stress in hyperglycemia [9, 10]. Moreover, NADPH oxidase Nox4 is also directly involved in enhanced superoxide generation in the kidney in early diabetes [11]. Furthermore, neutralization of ROS or alleviation of renal oxidative stress in diabetes has been found to improve the functional and structural features of diabetic nephropathy, including albuminuria, renal hypertrophy, mesangial expansion and glomerular accumulation of fibronectin and collagen IV [11–14]. As in the case of DM, hypertension also induces oxidative stress in the kidney. In fact, exaggerated oxidative stress in the kidney has been shown in almost all animal models of hypertension, including spontaneously hypertensive rats (SHR), a rat model of human essential hypertension [15, 16]. Therefore, DM- or hypertension-induced oxidative stress in the kidney may participate in the pathogenesis of nephropathy and ESRD.

DM and hypertension frequently coexist in humans [17] and their combination increases the frequency and severity of nephropathy, sometimes in an additive manner [18, 19]. However, the underlying mechanistic basis of how the combination of diabetes and hypertension aggravates renal disease is not clear. In a recent study, we demonstrated that the presence of hypertension increases oxidative stress in the kidney in the early stage of experimental diabetes [20]. We have extended our findings in the present study with the aim of identifying specific abnormalities by which hypertension leads to early and enhanced oxidative stress in the kidneys of DM subjects.

Subjects and Methods

Animals and Experimental Protocol

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

Control and diabetic rats from each group were sacrificed using CO_2 gas 10 days after induction of diabetes. The abdomen was opened via a midline incision and the right kidney was immediately removed, decapsulated, weighed and further processed for homogenization of the cortical tissue. The left kidney was similarly removed and cut longitudinally into 2 halves. Part of the cortical tissue of one half was frozen in liquid nitrogen and preserved at −80°C, and the other half was fixed by immersion in a solution of methacarn (60% methanol, 30% chloroform and 10% glacial acetic acid). One day before sacrifice, urine samples were collected by placing each rat in individual metabolic cages for 24 h. Urine samples were analyzed by single radial immunodiffusion to determine the albumin excretion rate (AER) as described previously [20].

Blood Pressure Determinations

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

Preparation of Renal Cortical Extract

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

NADPH Oxidase Activity

NADPH oxidase activity was measured by the lucigenin-enhanced chemiluminescence method as previously described by Gorin et al. [11] with a few modifications. A small piece (around 50 mg) 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, 10 μg/ml aprotinin, and a cocktail of protease inhibitors 40 μl/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, and aliquots were used immediately. To start the assay, 50 µl of homogenates were added to 450 µl reaction buffer (50 mM phosphate buffer, pH 7.0, containing 1 mM EGTA, 150 mM sucrose, 5 µM lucigenin, and 100 µM NADPH). Photon emission in terms of relative luminescence units (RLU) was measured every 20 s for 10 min in a Turner TD-20e luminometer. There was no measurable activity in the absence of NADPH or tissue homogenate. To identify which pathway contributes superoxide production, tissue homogenates were pre-incubated on ice with diphenyleneiodonium and rotenone, at 20 and 100 µM final concentration for 10 min. Superoxide production was expressed as RLU/20 s/mg protein. Protein concentration was measured using the Bradford method and BSA as standard.

Western Blotting

Renal cortical homogenate was used for quantification of gp-91phox, Nox4, extracellular superoxide dismutase (EC-SOD), heme oxygenase-2 (HO-2) and nitrotyrosine by Western blot analysis. Molecular weight markers (PageRuler™, Fermentas Life Sciences) were used as standards. To block nonspecific binding and antibody incubation, 1–5% non-fat milk was used in phosphate-buffered saline containing 0.1% Tween-20 (PBST). Fifty grams of cortical protein was separated on 10% SDS-polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane using Mini-Protean II Dual Slab Cell apparatus (Bio-Rad Laboratories, Hercules, Calif., USA). After blocking nonspecific binding, the membranes were incubated with a rabbit polyclonal anti-gp91phox IgG (1:750; Upstate, Lake Placid, N.Y., USA), a rabbit polyclonal anti-Nox4 (1:2,000; a gift from Dr. Karen Block), a rabbit polyclonal anti-EC-SOD (1:2,000; SOD-105, Stressgen Bioreagents Corp., Victoria, B.C., Canada), a rabbit polyclonal anti-HO-2 (1:3,000; Stressgen), or a mouse monoclonal anti-nitrotyrosine antibody (1:2,000; clone 1A6, Upstate). After washing with PBST, the membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (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 reprobed for actin (goat polyclonal anti-actin antibody, 1:1,000, Santa Cruz). 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).

Immunohistochemistry

To detect oxidative stress-induced DNA base modification, immunohistochemistry was done for 8-hydroxy-2′-deoxyguanosine (8-OHdG, a DNA base-modified product) in methacarn-fixed paraffin-embedded renal tissue sections (4 µm). After microwave exposure and blocking of endogenous peroxidase with 3% H_2O_2, slides were incubated with a 1:50 dilution of a mouse monoclonal anti-8-OHdG antibody (N45.1; Japan Institute for the Control of Aging, Japan), and subsequently a 1:200 dilution of a biotinylated secondary anti-mouse IgG antibody (Vector, Burlingame, Calif., USA). After incubation with avidin-biotin complex reagent (Dako, Glostrup, Denmark), slides were developed in diaminobenzidine tetrahydrochloride and counterstained with hematoxylin. For negative controls, staining was performed omitting the primary antibody or by using an irrelevant immunoglobulin. Tubulointerstitial cells containing 8-OHdG-positive nuclei were counted in 50 sequential high power microscopic fields (×400).

Determination of Reduced GlutathioneConcentration

Renal cortical glutathione (GSH) level was measured by the method of Beutler et al [22] as described previously [21]. Briefly, a small piece of frozen kidney cortex was weighed and directly homogenized in cold 10% trichloroacetic acid on ice. Homogenate was centrifuged at 3,000 rpm for 15 min at 4°C. The supernatant was reacted with 0.3 M phosphate buffer and 0.04% 5,5′-dithiobis-(2-nitrobenzoic acid). Absorbance was read at 412 nm and the GSH concentration was expressed as micromoles of GSH per gram of frozen tissue from a standard curve. GSH (reduced form) was used as an external standard for preparation of a standard curve.

Statistical Analysis

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

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

Results

Physical and Metabolic Parameters

WKY rats and SHR were used in this study as normotensive and hypertensive animal models, respectively. Diabetes was induced for 10 days in 12-week-old WKY and SHR. Body weights of the diabetic rats were significantly reduced compared to the control rats in both rat strains (table 1). The SHR were hypertensive, and the systolic blood pressure of SHR was significantly higher than that of WKY ($p < 0.001$; table 1). However, STZ-induced diabetes of 10 days duration had no effect on systolic blood pressure in either WKY or SHR. Fasting plasma glucose levels were significantly elevated ($p < 0.001$; $n = 15$ in each group) in the WKY-diabetic (WKY-D) and SHR-diabetic (SHR-D) groups compared to the respective control (C) groups (table 1). However, there was no difference in fasting plasma glucose levels between the WKY-D and SHR-D groups. Diabetic rats showed hypertrophy of the kidney as evidenced by significantly higher kidney weight ($p < 0.02$; $n = 15$) and kidney weight/body weight ratio ($p < 0.001$; $n = 15$) in the diabetic groups than in the respective control groups (table 1). In addition, urinary AER was similarly elevated in both rat strains after 10 days of experimental diabetes ($p < 0.02$).

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

Group	Body weight g	Systolic BP mm Hg	Plasma glucose mM	Kidney weight g	KW:BW ratio, %	AER mg/day
WKY-C	415 ± 30	131 ± 9	8.7 ± 0.80	1.37 ± 0.16	0.33 ± 0.03	0.42 (0.30–0.91)
WKY-D	318 ± 30[a]	130 ± 11	24.9 ± 2.19[a]	1.65 ± 0.18[a]	0.50 ± 0.05[a]	1.15 (0.89–2.09)[a]
SHR-C	254 ± 37[b]	187 ± 15[d]	8.0 ± 0.95	0.90 ± 0.10	0.36 ± 0.08	0.41 (0.34–0.61)
SHR-D	201 ± 26[a–c]	188 ± 16[d]	25.69 ± 1.42[a]	1.05 ± 0.12[a]	0.51 ± 0.03[a]	1.30 (0.74–2.23)[a]

Data are means ± SD and n = 15 per group. The 24-hour urinary albumin excretion rate (AER) is expressed as the median (range) and was analyzed using the Kruskal-Wallis test followed by the Mann-Whitney test.

KW = Kidney weight; BW = body weight; C = control; D = diabetic.

[a] $p < 0.02$ vs. the respective control group; [b] $p < 0.001$ vs. WKY-C; [c] $p < 0.001$ vs. WKY-D; [d] $p < 0.001$ vs. WKY-C/D.

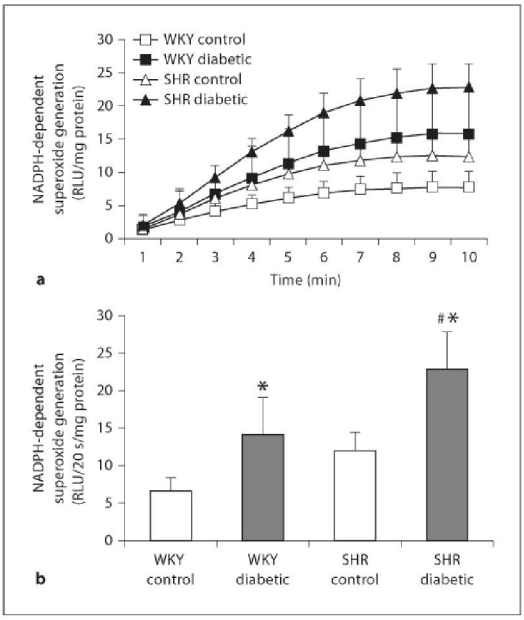

Fig. 1. NADPH oxidase activity in renal cortical homogenates. **a** Superoxide anion generation was determined by the lucigenin-enhanced chemiluminescence method and photoemission was measured every 20 s for 10 min. The peak level of superoxide generation was observed around 10 min after addition of NADPH to the reaction mixture containing renal cortical homogenates from different groups of rats. **b** Average NADPH oxidase activities during the last 5 min was used for comparison among the groups. NADPH-dependent superoxide generation was expressed as relative luminescence units (RLU)/20 s/mg protein. Bars represent the means ± SD of the activities from the kidney cortex of more than 5 rats per group. * $p < 0.005$ versus the respective control group; # $p = 0.002$ versus the WKY diabetic group.

Pro-Oxidant Parameters

NADPH oxidase is the major source of pro-oxidant superoxide in the vascular tissue [23]. We evaluated NADPH oxidase activity in the kidney cortex using the lucigenin-enhanced chemiluminescence method. Figure 1a shows the changes in the rate of reaction over 10 min after the addition of NADPH into the reaction mixture containing renal cortical homogenate, and figure 1b shows the average NADPH oxidase activity during the last 5 min of the measuring time. NADPH-dependent superoxide production was significantly increased in the renal cortical homogenates of diabetic animals compared with controls (WKY-C 6.54 ± 1.90 vs. WKY-D 14.05 ± 5.02 RLU/20 s/mg protein, $p = 0.001$; SHR-C 11.90 ± 2.56 vs. SHR-D 22.90 ± 5.04, $p < 0.001$; n = 5 per group; fig. 1b). However, the highest level of superoxide generation was observed in the SHR-D group, which was significantly higher than the WKY-D group ($p = 0.002$). To identify the source of superoxide production, we used diphenyleneiodonium, an inhibitor of flavin-containing oxidases, and rotenone, an inhibitor of complex I of mitochondrial respiratory chain. Pre-incubation of the renal cortical homogenates with diphenyleneiodonium (20 μM final concentration) completely blocked NADPH-induced superoxide production, while pre-incubation with rotenone (20 and 100 μM 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 establish the involvement of the NADPH oxidase system in the exaggerated production of renal cortical superoxide in diabetes, we also evaluated the expression of NADPH oxidase subunit gp91phox in the renal cortex. We observed that the gp91phox expression did not alter in WKY groups after induction of diabetes (WKY-C 0.74

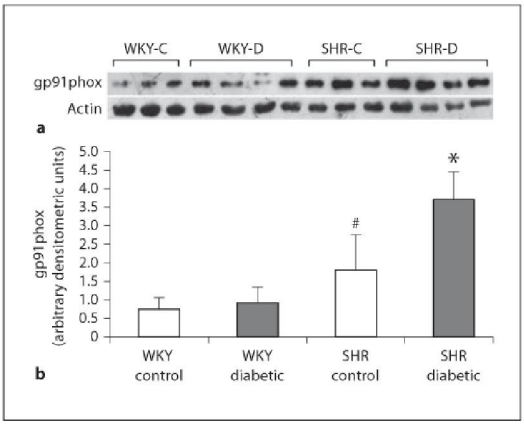

Fig. 2. a Western blot analysis of renal cortical gp91phox. Expression of the NADPH oxidase subunit gp91phox was determined by Western blot analysis of renal cortical homogenate. Representative Western blots from different groups of rats. Actin was used as a control of protein loading. **b** Densitometric analysis of the gp91phox/actin ratio in different groups of rats. Bars represent the means ± SD of at least 3 independent experiments; n = 5 in each group. * $p < 0.05$ versus all other groups; # $p < 0.05$ versus WKY control. C = Control; D = diabetic.

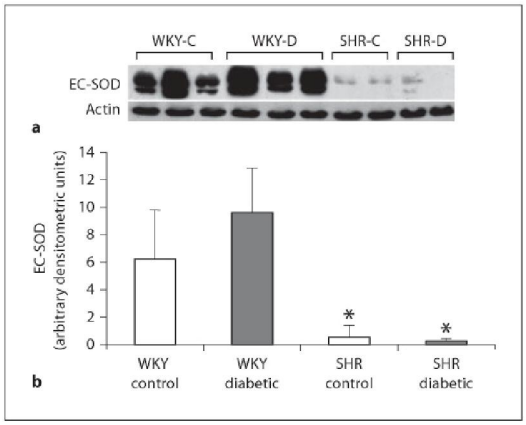

Fig. 3. a Western blot analysis of renal cortical extracellular superoxide dismutase (EC-SOD). Representative Western blots from different groups of rats. EC-SOD protein appeared as a double-band at the 34- and 32-kDa positions. Actin was used as control of protein loading. **b** Densitometric analysis of both bands of EC-SOD/actin ratio in different groups of rats. Bars represent the means ± SD of at least 3 independent experiments; n = 5 in each group. * $p < 0.05$ versus WKY control/diabetic. C = Control; D = diabetic.

± 0.31 vs. WKY-D 0.93 ± 0.41 expressed as a ratio of gp91phox/actin in densitometric units; fig. 2). However, the expression of gp91phox was significantly increased ($p < 0.05$; n = 5) in the SHR-D group (3.70 ± 0.76) compared with the SHR-C group (1.82 ± 0.95). In fact, the highest level of the expression of gp91phox was observed in the SHR-D group compared with all other groups ($p < 0.05$; n = 5). We also noticed that the expression of gp91phox was significantly higher in the SHR-C than the WKY-C group ($p < 0.05$; n = 5; fig. 2).

We also evaluated Nox4, a homologue of gp91phox, which has recently been implicated in the generation of superoxide and induction of oxidative stress in the kidney in early diabetes [11]. However, in our situation, Nox4 protein expression in the renal cortex was found unaltered in both the WKY (WKY-C 1 ± 0.18 vs. WKY-D 0.89 ± 0.26 densitometric units; n = 5) and SHR (SHR-C 1 ± 0.30 vs. SHR-D 1.25 ± 0.55; n = 5) groups after induction of diabetes for 10 days.

Antioxidant Parameters

As a measure of the protection against pro-oxidant stress in diabetes, we evaluated the antioxidant system in the renal cortical tissue. EC-SOD, one of the most important enzymatic antioxidants that provides defense against superoxide, was measured by Western blotting. As was demonstrated previously [24], two bands at around the 34- and 32-kDa positions were identified for EC-SOD in the renal cortical tissue. In spite of large intra-group variation in EC-SOD protein expression, we observed that the expression of EC-SOD in the renal cortex was extremely low in the SHR groups compared with the WKY groups ($p < 0.05$; n = 5 in each group; fig. 3). However, the induction of diabetes for 10 days did not significantly modify EC-SOD expression in either the WKY group (WKY-C 6.21 ± 3.60 vs. WKY-D 9.65 ± 3.19 expressed as a ratio of EC-SOD/actin in densitometric units) or the SHR group (SHR-C 0.56 ± 0.85 vs. SHR-D 0.27 ± 0.14).

We also assessed the non-enzymatic antioxidant GSH (reduced form), which provides important protection against the pro-oxidant molecule hydrogen peroxide. As we demonstrated previously [20], the GSH level did not show any significant difference between the WKY-C and SHR-C groups. However, the induction of diabetes for 10 days significantly decreased the GSH level in the SHR

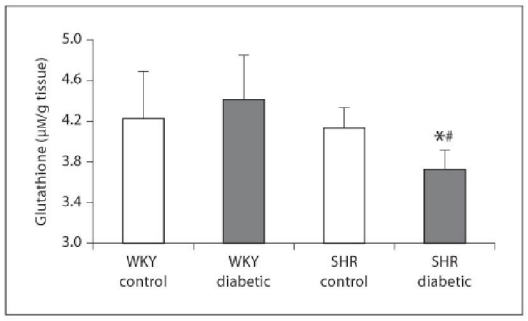

Fig. 4. Renal cortical reduced glutathione (GSH) level. The GSH concentration is expressed in μM/g frozen tissue. Data are means ± SD. * p = 0.03 versus SHR-C; # p < 0.05 versus all other groups. n = 15 in each group.

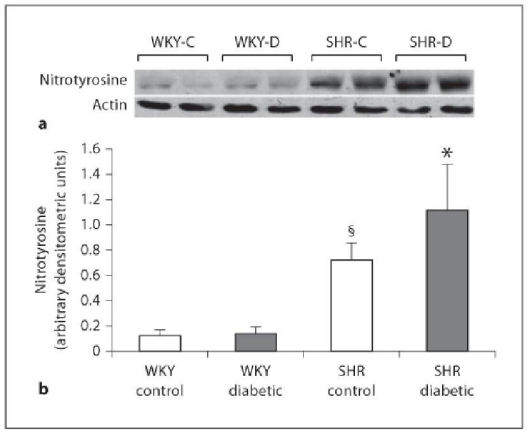

Fig. 5. a Western blot analysis of renal cortical nitrotyrosine. Representative Western blots from different groups of rats. Actin was used as a control of protein loading. **b** Densitometric analysis of nitrotyrosine/actin ratio in different groups of rats. Bars represent means ± SD of at least 3 independent experiments; n = 5 in each group. * p = 0.042 versus SHR control; § p = 0.001 versus WKY control. C = Control; D = diabetic.

group (SHR-C 4.13 ± 0.20 vs. SHR-D 3.73 ± 0.19 μM/g tissue; p = 0.03; n = 15), but not in the WKY group (WKY-C 4.22 ± 0.47 vs. WKY-D 4.41 ± 0.44; n = 15; fig. 4). Consequently, the lowest level of GSH was observed in the SHR-D group compared with all other groups ($p < 0.05$).

Another antioxidant, HO-2, is involved in bilirubin metabolism and provides protection against pro-oxidant stress particularly in the context of kidney tissue [25]. However, we did not observe any alteration in HO-2 expression in the kidney tissue after induction of diabetes for 10 days in either the WKY (WKY-C 0.56 ± 0.53 vs. WKY-D 0.82 ± 0.67 expressed as a ratio of HO-2/actin in densitometric units; n = 5) or the SHR (SHR-C 0.93 ± 0.53 vs. SHR-D 0.48 ± 0.40) groups.

Oxidative Stress Parameters

To identify whether the altered balance between pro-oxidant stress and antioxidant defense after a short-term of experimental diabetes culminates in oxidative tissue injury, we measured nitrotyrosine, a marker of oxidative/nitrosative stress-induced protein modification, and 8-OHdG, a marker of oxidative stress-induced DNA base modification, in renal cortical tissue. The nitrotyrosine level was found to be significantly elevated in the SHR group (SHR-C 0.72 ± 0.14 vs. SHR-D 1.11 ± 0.37 expressed as a ratio of nitrotyrosine/actin in densitometric units; p = 0.042, n = 5), but not in the WKY group (WKY-C 0.12 ± 0.05 vs. WKY-D 0.14 ± 0.05; n = 5), after the induction of diabetes for 10 days (fig. 5). We also noticed a markedly elevated level of renal cortical nitrotyrosine expression in the SHR-C group compared with the WKY-C group (p = 0.001; n = 5). In case of an oxidative stress-induced DNA base modification, we detected renal cortical cells containing the modified base 8-OHdG in the nucleus involving mainly the tubular cells of inner cortical region, as described previously [20]. The number of renal cortical cells containing 8-OHdG-positive nuclei (per 50 high power fields) was found to be elevated (p = 0.001; n = 6) in the SHR-D group (median 335 range 295–445) compared with the SHR-C group (median 155, range 31–308). However, there was no difference in the number of cells containing 8-OHdG-positive nuclei between the WKY-C (median 85, range 26–297) and the WKY-D (median 97, range 39–178) groups (n = 6; fig. 6).

Discussion

In the present study, we found that the presence of hypertension increases renal oxidative stress by increasing pro-oxidant generation and decreasing antioxidant defense in the early stage of experimental diabetes. It is known that both hypertension and diabetes individually increase oxidative stress [8, 9, 11–16]. However, identification of their individual contribution to oxidative stress

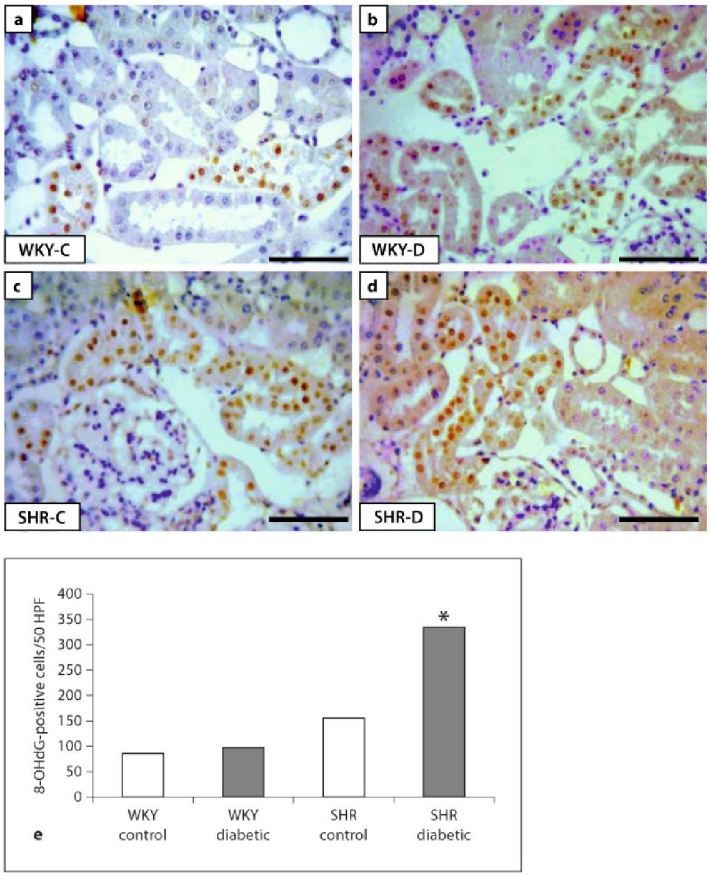

Fig. 6. Immunohistochemical detection of oxidative stress-induced DNA damage. **a–d** Photomicrographs of the immunohistochemical identification of 8-OHdG-containing cells in the kidney cortex. Tubulointerstitial cells containing 8-OHdG were identified by their brown-colored (darker) nuclei. Original magnification ×400. Counterstained with hematoxylin. Scale bar = 50 μm. **e** The median number of tubulointerstitial 8-OHdG-containing cells per 50 HPF. At least 3 sections were stained and evaluated for each rat. * $p = 0.001$ versus SHR-C. $n = 6$ in each group. Data were analyzed by the Kruskal-Wallis test followed by the Mann-Whitney U test. HPF = High-power field; C = control; D = diabetic.

is difficult when both hypertension and diabetes exist concomitantly. Here, we circumvent this problem by using a duration factor, because we demonstrated previously that oxidative stress does not increase in the kidney with a short duration of diabetes of 10 days, but it increases in the presence of hypertension with the same duration of diabetes [20]. Therefore, in the present study, we tried to identify the influence of hypertension on pro-oxidant generation and antioxidant defense in the kidney 10 days after induction of experimental diabetes.

The free radical superoxide is a primary reactive species that generates several other reactive species (or pro-oxidants) of physiological significance as products of the downstream reaction cascade [26]. The superoxide is produced by the mitochondrial respiratory chain and by NADPH oxidase, xanthine oxidase, cycloxygenase and lipoxygenase, nitric oxide synthase and cytochrome p450 [27]. In the present study, however, we did not evaluate mitochondrial superoxide generation despite its well-documented involvement in the pathogenesis of diabetic complications [8, 9]. Elegant studies performed by Nishikawa et al. [8] showed that a high level of glucose rapidly increases superoxide generation in bovine aortic endothelial cells through the mitochondrial respiratory chain. We therefore hypothesized that a similar level of plasma glucose, as observed in our WKY-D and SHR-D groups, would produce a similar extent of superoxide through the mitochondrial respiratory chain. As the main focus of the

present study was to identify the contribution of hypertension to renal oxidative stress in the presence of hyperglycemia, we rather evaluated the NADPH oxidase system. Because NADPH oxidase-mediated renal oxidative stress has been demonstrated in hypertension [28], and high blood pressure has been shown to promote the expression of NADPH oxidase subunits in the arterial wall [29]. Furthermore, all or most of the components of the NADPH oxidase system have been reported to be present in different cell types in the kidney [30]. Therefore, we considered that the NADPH oxidase system might be more pertinent to the question we intended to answer in the present study.

Our present finding clearly demonstrates that the NADPH-induced superoxide generation increases in the kidney in the early stage of diabetes. This finding, therefore, supports many previous data showing that hyperglycemia in animals, or high glucose in cell culture media, rapidly induces pro-oxidant generation [8, 11–13]. However, a more important part of our data is that the hypertensive diabetic rats showed the highest level of superoxide production compared with normotensive diabetic rats or non-diabetic hypertensive rats. This finding is supported by the expression of gp91phox, the catalytic subunit of NADPH oxidase, the expression of which was found to be highest in the hypertensive diabetic group compared with all other groups. However, the expression of gp91phox increased in hypertensive SHR, but not in normotensive WKY, after the induction of diabetes. Apparently this finding does not correlate with NADPH-induced superoxide generation which was increased in both normotensive and hypertensive diabetic rats. It seems that the NADPH oxidase activity could be increased up to a certain level without an increase in gp91phox expression; however, for a greater increase in this enzyme activity, as seen in hypertensive diabetic groups, a significant elevation in the expression of gp91phox is needed. In fact, the NADPH oxidase enzyme complex is composed of membrane-bound subunits (gp91phox and p22phox) and cytosolic subunits (p47phox, p67phox and p40phox). Upon activation, cytosolic subunits are phosphorylated and translocated to the membrane and participate in superoxide generation [31]. Thus, the enzymatic activity of NADPH oxidase depends on the coordinated participation of several subunits, rather than on the expression of a particular subunit.

Among the other sources of pro-oxidant generation in diabetes, Gorin et al. [11] demonstrated that the Nox4, a homologue of the gp91phox subunit of NADPH oxidase, is a major source of renal cortical superoxide production in early diabetes. They observed an overexpression of Nox4, and Nox4-mediated exaggerated renal cortical superoxide production in Sprague-Dawley rats 14 days after the induction of diabetes [11]. However, in the present study, we found that the renal cortical Nox4 expression did not alter after 10 days of induction of diabetes, either in WKY or in SHR. This discrepant finding could, at least partly, be explained by rat strain and duration of diabetes.

SODs are very important enzymatic antioxidants that rapidly catalyze the dismutation of superoxide. In case of a deficiency in SOD (or increased production of superoxide), the superoxide preferentially reacts with nitric oxide and produces peroxynitrite, a powerful oxidizing and nitrating agent that can directly damage proteins, lipids, and DNA [32]. Among the three mammalian isozymes of SOD, EC-SOD (the other two are Cu,Zn-containing intracellular SOD and Mn-containing mitochondrial SOD) constitutes as much as 70% of the total SOD activity in human and baboon aorta [33], and high levels of EC-SOD expression have been observed in the lungs and kidneys [34]. Recent studies indicate that renal cortical EC-SOD expression is around 50% lower in hypertensive SHR compared with normotensive WKY [35], and the gene transfer of EC-SOD can reduce blood pressure in SHR [36]. We therefore considered that the EC-SOD imbalance might be an important mechanistic aspect of hypertension-induced renal oxidative stress in early diabetes.

In the present study, we observed an abundant expression of renal cortical EC-SOD in normotensive WKY, which was found markedly reduced in hypertensive SHR. In addition, the non-enzymatic antioxidant GSH level was significantly reduced in the SHR group, but not in the WKY group, after 10 days of experimental diabetes. Therefore, taking together the results of pro-oxidant, antioxidant and oxidative stress markers, we could summarize that the reduced level of antioxidant EC-SOD and GSH in SHR, particularly in diabetic SHR, in the face of exaggerated superoxide generation rapidly induces oxidative damage to proteins and DNA, as evidenced by increased protein nitration (nitrotyrosine) and DNA base modification (8-OHdG), respectively. On the other hand, normotensive WKY group showed a relatively lower level of superoxide generation as well as higher levels of antioxidants, the levels of which were maintained in diabetes. Consequently, no evidence of oxidative protein or DNA damage was detected in the renal cortical tissue in WKY group after 10 days of experimental diabetes. We consider that this finding is of profound clinical importance. Because diabetes and hypertension frequently co-

exist in humans, and the combination of diabetes and hypertension increases the frequency and severity of nephropathy [17–19]. Thus, the findings of the present study suggest that the increased susceptibility to renal damage in the presence of hypertension and diabetes could be mediated through enhanced oxidative stress in the kidney.

In the present study, however, urinary AER similarly increased in both normotensive and hypertensive rats indicating that the hypertension or hypertension-induced renal oxidative stress does not affect urinary albumin excretion in the very early stage of diabetes [20]. This early finding, of course, does not exclude the important contribution of hypertension and/or oxidative stress in the pathogenesis of diabetic nephropathy [1, 5–7]. In fact, in a previous paper, we showed that long-term diabetes along with hypertension increased albuminuria and renal fibronectin expression, which was completely prevented by normalization of blood pressure [37]. Future studies with specific objectives are definitely needed to identify the contribution of hypertension-induced oxidative stress to the structural and functional renal damage in long-term diabetes.

We consider that the findings obtained in the present study could not be attributed to differences in metabolic control, because the levels of glycemia were similar between the WKY-D and SHR-D groups. Similarly, the oxidative imbalance observed in the SHR-D group could not be attributed to STZ toxicity, because the same dosage of STZ was used in the WKY and SHR groups to induce diabetes. However, we are aware that the control groups used in the present study were not truly comparable, because renal oxidative stress was already elevated in the hypertensive control group compared with the normotensive control. We do admit that this difference might have partly confounded our findings, but we did not avail any truly comparable model since hypertension is almost invariably associated with renal oxidative stress [15, 16]. However, comparing all 4 groups together with appropriate statistical techniques, we observed that the hypertensive, but not normotensive, diabetic group exhibits the highest level of pro-oxidant generation with impaired antioxidant protection leading to increased renal oxidative stress. Furthermore, pair-wise comparison between normotensive control and diabetic groups, and then between hypertensive control and diabetic groups provided similar conclusions. Therefore, despite the limitation of the model used in the present study, our data strongly suggest that the presence of hypertension disrupts renal oxidative balance in the very early stage of experimental diabetes.

We conclude that the presence of hypertension increases pro-oxidant generation and decreases antioxidant defense, and thereby induces oxidative stress in the kidney in the early stage of diabetes. This finding strengthens the concept that hypertension contributes to diabetic nephropathy, and highlights the importance of blood pressure control to prevent and treat renal complications in diabetes.

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