Exogenous SOD Mimetic Tempol Ameliorates the Early Retinal Changes Reestablishing the Redox Status in Diabetic Hypertensive Rats

Mariana A. B. Rosales, Kamila C. Silva, José B. Lopes de Faria, and Jacqueline M. Lopes de Faria

Purpose. The purpose of this study was to investigate the efficacy of tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl), a superoxide dismutase mimetic, in preventing early retinal molecular changes in a model that combines hypertension and diabetes.

Methods. Four-week-old spontaneously hypertensive rats (SHR) were rendered diabetic by streptozotocin. Diabetic SHR rats (DM-SHR) were randomized to receive or not receive tempol treatment. After 20 days of induction of diabetes, the rats were euthanatized, and their retinas were collected.

Results. The early molecular markers of diabetic retinopathy (DR), glial fibrillary acidic protein, and fibronectin were evaluated by Western blot assays and showed an increase in DM-SHR compared with the SHR group. The oxidative balance, evaluated by superoxide production and nitric oxide end product levels estimated by a nitric oxide analyzer, and the counterpart antioxidative defense revealed an accentuated imbalance in DM-SHR compared with the SHR group. As a result, the product peroxynitrite, which was detected by immunohistochemistry for nitrotyrosine, was higher in the DM-SHR group. The retinal poly-ADP-ribose (PAR)-modified proteins, which reflect the activation of PAR polymerase (PARP), and the inducible nitric oxide synthase (iNOS) expressions were found to have increased in this group. Treatment with tempol reestablished the oxidative parameters and decreased the PAR-modified proteins, thus preventing extracellular matrix accumulation and glial reaction.

Conclusions. The administration of tempol prevented oxidative damage; decreased iNOS levels, and ameliorated the activation of PARP in the retinas of diabetic hypertensive rats. Consequently, the early molecular markers of DR, such as glial reaction (glial fibrillary acidic protein [GFAP]) and extracellular matrix accumulation (fibronectin), were prevented in tempol-treated rats. (Invest Ophthalmol Vis Sci. 2010;51:4327–4336)

DOI:10.1167/iovs.09-4690

From the Renal Pathophysiology Laboratory, Investigation on Complications of Diabetes, Department of Internal Medicine, Faculty of Medical Sciences, University of Campinas (Unicamp), São Paulo, Brazil.

Supported by the State of São Paulo Research Foundation Grants 05/58189–5 and 08/54068–7. MABR was the recipient of a scholarship from the Coordination for the Improvement of Higher Education Personnel.

Submitted for publication September 25, 2009; revised January 4 and February 17, 2010; accepted March 8, 2010.

Disclosure: M.A.B. Rosales, None; K.C. Silva, None; J.B. Lopes de Faria, None

Corresponding author: Jacqueline M. Lopes de Faria, Renal Pathophysiology Laboratory, Faculty of Medical Sciences, University of Campinas, P.O. Box 6111, Campinas, SP, Brazil; jmlfaria@fcm.unicamp.br.
induces DNA single-strand breaks and leads to overactivation of the DNA repair enzyme poly-(ADP-ribose) polymerase (PARP). PARP is the enzyme that cleaves nicotinamide adenine dinucleotide (NAD+) to form nicotinamide and a PAR polymer. Generally, the activation of PARP contributes to energy failure, transcriptional gene regulation, and the induction of apoptosis/necrosis. In DM, PARP activation contributes to endothelial cell dysfunction and appears to be central in the mechanisms by which hyperglycemia induces diabetic vascular dysfunction. In animal studies with PARP-1 knockout mice that were fed a 30% galactose diet for 2 months, Xu et al. showed that the hyperhexosemia-induced oxidative stress and increased expression of fibronectin observed in wild-type control groups were not observed in PARP-1−/− hyperhexosemic mice, suggesting that the PARP blockade in this animal model might prevent hyperhexosemia-induced effects. In addition, a previous paper addressing endothelial dysfunction in diabetes complications showed that PARP-deficient endothelial cells incubated with high glucose did not exhibit the production of reactive nitrogen and oxygen species, consequent single-strand DNA breakage, or metabolic and functional impairments. PARP activation may also cause NF-κB activation. Zheng et al. demonstrated that in streptozotocin-induced diabetes and in vitro studies, the use of a specific PARP inhibitor (PJ-34) prevented the early apoptosis of retinal vascular cells and the development of acellular capillaries and pericyte ghosts in bovine retinal endothelial cells (BRECs). It also inhibited the NF-κB activation and inflammatory markers in BRECs. By using PARP inhibitors or knocking out PARP genes, both NF-κB activation and transcription of NF-κB-dependent genes, such as inducible nitric oxide synthase (iNOS) or intracellular adhesion molecule (ICAM)-1, can be reduced. This suggests that the inhibition of PARP activation might prevent the consequences of inflammation or oxidative stress by modifying the NF-κB-dependent pathways. In our previous studies, we revealed that after 20 days of diabetes, SHR showed increased expression of the early inflammatory markers NF-κB, ICAM-1, and microglial activation. In a recent study by Drel et al., treatment with PARP inhibitors prevented apoptosis, glial reaction, and nitrosative imbalance in experimental diabetes retina. In view of the evidence that the prevalence of hypertension is very high among patients with diabetes and that antioxidants can provide protective benefits for the treatment of DR, we sought to investigate in vivo whether treatment with tempol would prevent the early molecular events in the pathogenesis of retinopathy. We have already demonstrated that the early molecular changes observed in the diabetic retina are more evident and first detected in concomitance with arterial hypertension and experimental diabetes.

**Methods**

**Research Design and Methods**

The protocol for this study complies with the guidelines of the Brazilian College for Animal Experimentation, and it was approved by the local Animal Research Ethics Committee (CEEA/IB/Unicamp, protocol number 1835-1). It is also in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Taconic (Germantown, NY) provided the SHR and the genetic control Wistar Kyoto (WKY) rats used in the present study, and we bred them in our animal facility. Experimental diabetes was induced in 4-week-old male SHR (DM-SHR) by injecting streptozotocin (STZ; 60 mg/kg; Sigma, St. Louis, MO) dissolved in a sodium citrate buffer (pH 4.5) through the tail vein after overnight fasting. The WKY control normotensive rats and the control SHR (CT-SHR) received only the citrate buffer. From the first day after diabetes induction, each DM-SHR was randomized to receive or not to receive a daily intraperitoneal injection of tempol (DM-SHR tempol; 250 mg/kg/d, 1.45 μmol/g). We used an enzymatic colorimetric GOD-PAP assay (Merck, Darmstadt, Germany) to measure plasma glucose levels 72 hours after the STZ or citrate buffer injection and on the day before euthanatizing the rats. Rats with plasma glucose values ≥15 mM were considered diabetic for the present study. We obtained systolic blood pressure by indirect tail-cuff plethysmography in unanesthetized rats with a physiograph (MK III; Narco Bio-System, Houston, TX). Twenty days after diabetes induction, the rats were euthanatized, and their retinas were detached from the retinal pigment epithelium cell layer and collected for colorimetric assays and Western blot analysis, or their eye globes were prepared for immunohistochemistry assays.

**Western Blot Analysis for GFAP, FN, Cu-Zn SOD, PARP, and iNOS**

The retinas were lysed in 300 μL of a buffer containing 2% SDS and 60 mmol Tris-HCl (pH 6.8), supplemented with a protease inhibitor cocktail (Complete; Boehringer-Mannheim, Indianapolis, IN). After centrifugation, we measured protein concentrations with the Bradford method. SDS-PAGE and Western blot analysis were performed as described. To assess the protein expression of GFAP, FN, iNOS, and Cu-Zn SOD, and to detect PARP polymers, the membrane was incubated for an additional hour with antibodies against goat polyclonal anti-GFAP (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), goat polyclonal anti-FN (1:1000; Calbiochem-Novabiochem, La Jolla, CA), rabbit polyclonal anti-iNOS (1:500; Cell Signaling Technology, Beverly, MA), rabbit polyclonal anti-Cu-Zn SOD (1:4000; AbFrontier, Seoul, Korea), or anti-PARP mouse monoclonal antibody (1:1000; Trevigen, Gaithersburg, MD), along with horseradish peroxidase–conjugated appropriate secondary antibodies and were developed by the chemiluminescence method (Super Signal CL-HRP Substrate System; Pierce, Rockford, IL). A densitometer (Bio-Rad, Hercules, CA) scanned exposed films, and image analysis software (Multi-Analyzer Macintosh Software for Image Analysis Systems; Apple, Cupertino, CA) quantitatively analyzed them. Equal loading and transfer were ascertained by reprobing the membranes for β-actin.

**Immunohistochemistry for GFAP, Nitrotyrosine, and PAR-Modified Proteins in Retinal Tissues**

After quenching endogenous peroxidase, the sections were incubated with nonfat milk. Tissue sections were then incubated with goat polyclonal anti-GFAP (Santa Cruz Biotechnology), rabbit polyclonal anti-nitrotyrosine antibody (Upstate Cell Signaling Solutions, Lake Placid, NY), and mouse monoclonal anti-PAR (Trevigen). The antibody anti-PARP detects PAR polymers. Appropriate secondary antibodies were applied to the tissue sections. Labeled nuclei were detected with a tissue staining kit (ABC Vectastain; Vector Laboratories, Burlingame, CA) and diaminobenzidine tetrahydrochloride (DAB)/chloride/hydrogen peroxide and were counterstained with hematoxylin (only for nitrotyrosine). For the negative controls, staining was performed without the primary antibody. Quantitative analyses were performed as a percentage of positive cells per square millimeter of retina with ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). An observer with no knowledge of the studied groups counted the positivity under high-power microscopic fields (1000×).
Detection of Superoxide Anion Production in Retinal Tissue

Lucigenin (bis-N-methylacridinium nitrate) (Invitrogen Inc., Eugene, OR) was used to measure superoxide anion production. The retinas were isolated and placed in tubes containing RPMI-1640 medium (Gibco/BRL, Life Technologies, Inc., Gaithersburg, MD) at 37°C in a humidified atmosphere of 95% air/5% CO2. Lucigenin (25 mM) was added, and photon emission was measured over 10 seconds; repeated measurements were made over a 3-minute period with a luminometer (TD 20-E Luminometer; Promega, Sunnyvale, CA). Superoxide production was expressed in relative luminescence units (RLU)/min/mg protein. Protein concentration was measured using the Bradford method with BSA as the standard.

Measurements of Nitric Oxide End Products (NOx; Nitrite and Nitrate) in Retina Tissue

Various cell types in the picomolar to nanomolar range produce NO, which has a very short half-life in biological fluids. Thus, the stable products of NO oxidation nitrite (NO2−) and nitrate (NO3−) are often analyzed instead to estimate the NO level in biological fluid. The NO2− and NO3− concentrations were detected using an NO chemiluminescence analyzer (NOA; Sievers Instruments Inc., Boulder, CO) in retina samples. With a syringe, 4-μL aliquots of supernatant were added to a purge chamber containing vanadium chloride (VCl3; 97°C) (Sigma-Aldrich, St. Louis, MO) in 1 N HCl under a nitrogen atmosphere. The VCl3 solution reduces nitrites and nitrates to NO gas, which the NOA detects. NO was liberated from the samples into the gaseous head space and was conducted to the NOA, where it reacted with the ozone to produce a chemiluminescent signal. The amount of light was proportional to the NO concentration, which was calculated from a standard curve of known NO3− concentrations. Each sample was analyzed in triplicate.

Measurement of Activity of Cu-Zn SOD

The enzyme activity of Cu-Zn SOD in retinal protein was measured using a kit from Cayman Chemical (Ann Arbor, MI), according to the

![Figure 1](attachment:image.png)

**FIGURE 1.** (A) Western blot of retinal lysates for GFAP from studied rats. The membranes were reprobed with anti-β-actin antibody as a control for protein loading. Bars represent mean ± SD of the band densities of the GFAP/β-actin ratio expressed in arbitrary densitometric units. 1.18 ± 0.2 vs. 0.51 ± 0.2 arbitrary densitometric units for CT-SHR vs. WKY rats, respectively; *P = 0.0001; 1.43 ± 0.2 vs. 0.51 ± 0.2 arbitrary densitometric units for DM-SHR vs. WKY rats, respectively; †P < 0.0001 vs. CT-SHR; ‡P < 0.0001 vs. CT-SHR; §P < 0.0001 vs. nontreated SHR rats. (B) Representative photomicrograph of the immunolocalization of GFAP in retinal sections. Brown: presence of immunoreactivity of GFAP. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; OPL, outer plexiform layer.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Initial Body Weight (g)</th>
<th>Final Body Weight (g)</th>
<th>SBP (mm Hg)</th>
<th>Glycemia (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY (<em>n</em> = 28)</td>
<td>117.2 ± 21.1</td>
<td>288.5 ± 26.0</td>
<td>129.2 ± 18.3</td>
<td>9.6 ± 1.2</td>
</tr>
<tr>
<td>CT-SHR (<em>n</em> = 27)</td>
<td>70.4 ± 8.4†</td>
<td>196.0 ± 20.7‡</td>
<td>152.8 ± 11.1</td>
<td>9.1 ± 2.0</td>
</tr>
<tr>
<td>DM-SHR (<em>n</em> = 51)</td>
<td>69.9 ± 10.5‡</td>
<td>125.4 ± 34.6‡</td>
<td>151.1 ± 21.4</td>
<td>29.5 ± 4.0‡</td>
</tr>
<tr>
<td>DM-SHR tempol (<em>n</em> = 30)</td>
<td>68.3 ± 8.0*</td>
<td>107.4 ± 18.5‡</td>
<td>152.6 ± 14.6</td>
<td>29.9 ± 6.0‡</td>
</tr>
</tbody>
</table>

SBP, systolic blood pressure.  
*P < 0.0001 vs. WKY.  
†P < 0.0001 vs. WKY.  
‡P < 0.0001 vs. CT-SHR.  
§P = 0.03 vs. nontreated SHR rats.  
#P = 0.003 vs. WKY.  
¶P < 0.0001 vs. nondiabetic rats.

**TABLE 1.** Physiological Characteristics of the Studied Animals

Downloaded From: http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932964/ on 09/23/2017
manufacturer’s instructions. The kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed for a 50% dismutation of the superoxide radical. The assay measures activity of Cu-Zn SOD in tissue. The standard curve was generated using a controlled SOD standard. The reaction was initiated by adding 20 μL diluted xanthine oxidase. A spectrophotometer (Elx800; BioTek, Winooski, VT) monitored the absorbance continuously at 450 nm. Protein concentrations were measured by the Bradford method.28

**Determination of Reduced Glutathione Levels in the Retina**

Retinal GSH levels were measured using the method described previously,30 with a few modifications, also described previously.16 Absorbance was read at 412 nm, and the GSH concentration was expressed as μM GSH/mg retinal protein. GSH was used as an external standard for the preparation of a standard curve.

**Statistical Analysis**

Results were expressed as mean ± SD, unless otherwise stated. We used one-way analysis of variance (ANOVA) followed by Fisher’s protected least significant difference test to assess differences among the groups. All comparisons were carried out with statistical software (StatView; SAS Institute, Cary, NC). The significance level adopted was \( P < 0.05 \).

**FIGURE 2.** Western blot of retinal lysates for FN from studied rats. The membranes were reprobed with anti–β-actin antibody as a control for protein loading. Bars represent mean ± SD of the band densities of FN/β-actin ratio expressed in arbitrary densitometric units (1.7 ± 0.6 vs. 0.7 ± 0.04 arbitrary densitometric units for DM-SHR vs. WKY rats, respectively; \( P = 0.004 \); 1.7 ± 0.6 vs. 1.02 ± 0.09 arbitrary densitometric units for DM-SHR vs. CT-SHR rats, respectively; \( P = 0.02 \); and 1.02 ± 0.2 vs. 1.7 ± 0.6 arbitrary densitometric units for DM-SHR tempol vs. DM-SHR, respectively; \( *P = 0.02 \)).

**FIGURE 3.** (A) Total superoxide generation in retinal tissue. Superoxide anion production from the retinas of control, diabetic SHR, and diabetic SHR treated with tempol was measured every 12 seconds for 5 minutes with the lucigenin-enhanced chemiluminescence method. The peak level of superoxide generation was observed around 5 minutes after lucigenin was added to the reaction buffer containing the retinas from different groups. Bars represent mean ± SD. All data shown are of \( n \) = 6 observations (6.8 ± 3.5 vs. 5.7 ± 1.2 RLU/min/mg protein for DM-SHR vs. WKY rats, respectively; \( *P = 0.05 \); and 2.1 ± 0.2 vs. 6.8 ± 3.4 RLU/min/mg protein for DM-SHR tempol vs. DM-SHR rats, respectively; \( *P = 0.01 \)). (B) Nitrite (NO\textsubscript{2}\textsuperscript{-}) and nitrate (NO\textsubscript{3}\textsuperscript{-}), the stable products of NO oxidation, are often analyzed to estimate the NO level in biological fluid by NO chemiluminescence analyzer. Results are corrected for protein concentration and are expressed as μmol NO\textsubscript{x} per milligram of protein. Each sample was analyzed in triplicate (19.2 ± 4.4 vs. 11.2 ± 0.5 μmol NO/mg protein for DM-SHR vs. WKY rats, respectively; \( *P = 0.004 \); 19.2 ± 4.4 vs. 10.6 ± 1.7 μmol NO/mg protein for DM-SHR vs. CT-SHR rats, respectively; \( *P = 0.003 \); and 10.0 ± 2.0 vs. 19.2 ± 4.4 μmol NO/mg protein for DM-SHR tempol vs. DM-SHR rats, respectively; \( #P = 0.001 \)).
results

physiological characteristics of the studied groups

the body weights of the 4-week-old SHR were lower than those of the age-matched WKY rats (P < 0.0001). the weight gain was lower in DM-SHR than in CT-SHR (P < 0.0001), and tempol treatment significantly reduced the body weights of treated DM-SHR compared with nontreated SHR (P = 0.03). Systolic blood pressure was significantly higher in the SHR groups than in the WKY rat group (P = 0.004) and was not affected by the treatment. Plasma glucose levels were higher in diabetic rats than in the nondiabetic group (P < 0.0001) and were not affected by tempol treatment (Table 1).

Glial Reaction and Fibronectin Retinal Accumulation

to assess retinal lesions in this short-term study of diabetes and hypertension, we used the retinal expression of GFAP and fibronectin. these two abnormalities have been identified as early markers of retinal lesion in diabetes.51,52 we did not use classic morphologic methods, such as retinal capillary morphology, in trypsin digest retinas because the abnormalities seen in this preparation, such as acellular capillaries and pericyte ghosts, required longer-term diabetes to be detected.53,54 GFAP levels were evaluated by Western blot analysis and immunohistochemistry. In the Western blot assay, increased GFAP levels in CT-SHR were observed compared with WKY rats (P = 0.001), and this expression was exacerbated in the DM-SHR group (P = 0.0001). Treatment with tempol prevented the enhancement of GFAP expression, maintaining levels similar to those of the control WKY levels (P = 0.7; Fig. 1A).

Evaluation of Oxidative/Antioxidative Systems and Peroxynitrite Oxidative Damage in Retinal Tissue

The redox state is dependent on the balance between free radicals and antioxidant systems that influence tyrosine nitration and oxidative damage of lipids, proteins, and nucleic acids. For estimation of the oxidative status in retinal tissue, we evaluated the production of O₂⁻ and NOX and the antioxidative system’s GSH concentrations and the activity and expression of Cu/Zn SOD.

Superoxide production in the retina, evaluated by the lucigenin-enhanced chemiluminescence method, was increased in the DM-SHR group compared with the CT-SHR group (P < 0.05), and tempol treatment reestablished superoxide produc-
tion to normal levels (P = 0.01; Fig. 3A). Additionally, retinal NO\textsuperscript{−} production was increased in the DM-SHR group compared with control groups (P = 0.004 vs. WKY and P = 0.005 vs. SHR), in which tempol treatment (P = 0.01) prevented retinal NO\textsuperscript{−} production (Fig. 3B).

Antioxidant defense was evaluated by retinal expression, Cu-Zn SOD activity, and GSH levels in retinal tissue. Two isoforms of SOD have Cu and Zn at their catalytic center and are localized to the intracellular cytoplasmic compartment and extracellularly.\textsuperscript{35} Marked reduction was observed in Cu-Zn SOD retinal expression in the DM-SHR group compared with the WKY (P = 0.0008) and the CT-SHR (P = 0.02) groups. Similarly, Cu-Zn SOD activity was significantly decreased in DM-SHR compared with WKY and CT-SHR (P = 0.002 and P = 0.001, respectively). Tempol treatment protected both the expression and the activity of Cu-Zn SOD from the effect of short-term diabetes and hypertension. The expression of Cu-Zn SOD was restored in tempol-treated DM-SHR compared with DM-SHR (P = 0.04), and the activity of Cu-Zn SOD was similar in the CT-SHR and WKY (P = 0.1) groups (Figs. 4A, 4B).

Although the presence of hypertension or short-term diabetes did not affect the levels of GSH in the retina (P = 0.4), treatment with tempol increased its levels by approximately 47% compared with all other groups (P = 0.01). This effect may be explained by the metabolism of hydrogen peroxide by the catalase-like actions of tempol, which diminish the formation of hydroxyl radicals, thus increasing the levels of GSH available in the retinal tissue (Fig. 4C).

An immunoreaction for NT in retinal tissue detected the product peroxynitrite, generated by the reaction between NO and O\textsubscript{2}\textsuperscript{−}. There was an evident increase of NT in the retinas of DM-SHR compared with WKY and SHR (P < 0.0001 and P = 0.0001, respectively). By the mechanism of a SOD mimetic, tempol competed with NO by O\textsubscript{2}\textsuperscript{−} and dismuted it to peroxide hydrogen and oxygen, leading to marked reduction of NT levels in the treated DM-SHR group similar to those observed in the SHR group (P = 0.6; Fig. 5).

Expression of Ribosylated Proteins in the Retinal Tissue of Tempol-Treated Rats

Nitrosative stress induces DNA single-strand breaks, leading to the overactivation of PARP. In the present study, the activation of PARP represented by the catalysis of the transference of ADP-ribose groups from NAD to target proteins (poly(ADP-ribose))\textsuperscript{ation), was accessed through the amount of PAR-modified proteins by Western blot and immunohistochemistry analyses. In Western blot analyses, the presence of hypertension in the CT-SHR increased the content of PAR ribosylated polymers compared with the normotensive WKY rats (P = 0.04). The concomitance of both diabetes and hypertension in the DM-SHR significantly increased these levels compared with WKY and CT-SHR (P < 0.001 and P = 0.02, respectively). Treatment with tempol in the DM-SHR reestablished this parameter to SHR levels (P = 0.3; Fig. 6A). As detected by immunohistochemistry, the distribution of PAR polymer–positive cells was homogeneous among the ganglion cells in both the inner and outer nuclear layers. The presence of hypertension solely and in concomitance with diabetes revealed a significant increase in PAR polymer–positive retinal cells compared with the WKY group (P = 0.01 and P = 0.006, respectively), suggesting the activation of PARP evaluated through the amount of PAR-modified proteins. Treatment with a SOD mimetic for 20 days reestablished this element to WKY levels (P = 0.7; Fig. 6B).

iNOS Expression Associated with Poly(ADP-ribose)\textsuperscript{ation} in Retinal Tissue

iNOS has been shown to cause DNA breaks and the subsequent activation of PARP, which was recently implicated in early diabetic retinal changes.\textsuperscript{21,56} Other investigators have shown that with specific PARP inhibitors or knocking out of the PARP gene, NF-κB activation and transcription of NF-κB–dependent genes, such as inducible nitric oxide synthase, are reduced,\textsuperscript{43,57} suggesting that the inhibition of poly(ADP-ribose)\textsuperscript{ation} might prevent the consequences of inflammation or stress by modification of NF-κB–dependent pathways. In the present work, we addressed the retinal expression of iNOS, which may be accepted as an indicator of the NF-κB–dependent pathways and PARP activation. A significant increase in retinal iNOS expression was demonstrated in the DM-SHR group compared with the WKY group (P = 0.04), and the treatment with tempol restored it to normal levels (P = 0.26; Fig. 7).

DISCUSSION

The findings presented herein demonstrate that the systemic administration of tempol reestablished the cell’s oxidative parameters and thus prevented the poly(ADP-ribose)\textsuperscript{ation of
proteins and iNOS expression in the retinas of diabetic hypertensive rats. Consequently, the early molecular changes of DR, represented here by FN accumulation and increased GFAP expression, were prevented in tempol-treated rats.

Serious retinal injuries such as retinal detachment, glaucoma, and DR can trigger retinal reactive gliosis and neurodegeneration characterized by changes in astrocyte and Müller cell morphologies and increased production of intermediate filament proteins (GFAP). Retinal Müller cells and blood vessels are in close apposition and are likely to interact with each other. It has been suggested that the increase in vascular permeability in DR may be caused by the effects of diabetes that alter the neural components of the retina, demonstrated by increased production of vascular endothelial growth factor by Müller cells and neurons leading to a breakdown in the interactions among neurons, glia, and endothelial cells. Thickening of the basal lamina that surrounds the endothelial cells and pericytes of the retinal capillaries because of extracellular matrix accumulation is another finding early in DR pathogenesis, and this is believed to be pivotal in the progression of DR. This concept was based on the findings in DM animal models in which the downregulation of FN synthesis partially prevented retinal basal lamina thickening and prevented classic early vascular changes, such as the apoptosis of pericytes and the development of acellular capillaries. In in vitro studies, Oshitari et al. downregulated several components of the extracellular matrix, such as fibronectin, laminin, and collagen type IV, and thus reduced vascular leakage in streptozotocin-induced diabetes. In light of this, fibronectin accumulation and GFAP expression might be used as molecular markers of diabetic changes in the retina in short-term experimental diabetes.

DM increases oxidative stress, which plays a key regulatory role in the development of its complications. Because oxidative stress may be the promoter of many alterations implicated in the pathogenesis of DR, representing an imbalance between excess formation and the impaired removal of reactive oxygen species (ROS), the antioxidant defense system of the cell is a crucial part of the overall oxidative stress experienced by a cell. A number of works have demonstrated beneficial effects of antioxidant treatment in the diabetic retina. Antioxidants may act at different levels, may inhibit the formation of ROS or scavengers of free radicals, or may increase the antioxidant defense enzyme capabilities. For instance, long-term lipoic acid treatment...
acid treatment attenuated the apoptosis of retinal capillary cells and decreased the levels of 8-hydroxy-2'-deoxyguanosine and NT in diabetic rats, and short-term treatment prevented the significant decrease of GSH content, normalized the mitochondrial SOD in hemizygous transgenic mice (MnSOD-Tg) and pericyte ghosts. In addition, the overexpression of mitochondrial SOD in hemizygous transgenic mice (MnSOD-Tg) prevented diabetic retinal oxidative stress, protecting the mitochondria from dysfunction. In the pathogenesis of DR, ischemia causes structural changes and a breakdown of the blood retinal barrier. Among the PARPs, nuclear PARP-1 is a DNA damage-activated enzyme and is the most abundant and commonly studied member of the family. Its enzymatic activity leads to PAR formation, and it was first described more than 40 years ago. More recently, PARP increased in importance when it was discovered to mediate nitric oxide-induced neuronal death. ROS-dependent DNA damage is thought to play a major role in triggering PARP-1 hyperactivity during ischemia. In the pathogenesis of DR, ischemia causes structural changes and a breakdown of the blood retinal barrier. PARP-1 hyperactivation causes cell death because of cellular accumulation of the PARP-1 product, PAR, which causes the translocation of the apoptosis-inducing factor from mitochondria to the nucleus and the activation of a caspase-independent programmed cell death pathway. In experimental models of brain ischemia, the use of a PARP-1 inhibitor, PJ34, preserved the endothelial tight junctions and decreased the expression of ICAM-1, thus limiting leukocyte infiltration to the ischemic brain.

Tempol is a cell membrane-permeable nitroxide and is among the most potent of the nitroxides for protecting cells and tissues from the damaging effects of ROS. Tempol has multiple antioxidant actions because of the ability of nitroxides to inhibit three or more sequential sites in an oxidative chain (for example, $\cdot OH$, $H_2O_2$, and $OH^{-}$). This may underlie its efficacy in diverse models of oxidative stress. Tempol is free of serious toxic effects in animal models. Despite these apparently beneficial effects in a wide range of animal models, tempol has yet to be developed as a drug for human use. Matsumoto et al. tested the toxic effects of five nitroxides, and tempol was the least toxic. These toxic or lethal doses of tempol are approximately 30-fold higher than the therapeutic dose for the reduction of blood pressure. In addition, tempol had no adverse effects on gene mutation in Chinese hamster. The reaction rate constant for tempol with $O_2^-$ has been determined to be $3.4 \times 10^7$ mol $\cdot$ L $\cdot$ s$^{-1}$. This makes it a much more efficient antioxidant than vitamins because the rate constant for the interaction of SOD with $O_2^-$ is approximately 1.6 to $2.4 \times 10^9$ mol $\cdot$ L $\cdot$ s$^{-1}$. For vitamin E with $O_2^-$, the rate constant is $0.59$ mol $\cdot$ L $\cdot$ s$^{-1}$. Tempol has two reaction sites with $OH^-$ that can be derived from ROS-like peroxynitrite. In cell cultures, tempol catalyzes the SOD reaction and protects against oxidative stress. In addition, exposure to conditions of oxidative stress in cultured coronary endothelial cells leads to an increase in NO end products, namely nitrite and nitrate. In the presence of tempol, these effects are reversed.

In experimental models and in postmortem retinas from diabetic patients, as well as in the vitreous fluid from diabetic patients with proliferative diabetic retinopathy, increased levels of NO end products and the upregulation of iNOS have been reported. In line with this evidence, the treatment with tempol in this study reduced the NO end products and the superoxide production observed in retinas of diabetic SHR rats. In the treatment, tempol acted as an $O_2^-$ scavenger, reducing nitric oxide end products and increasing GSH biosynthesis, thereby reestablishing the oxidative-nitrosative status of the diabetic retina. Similarly, we observed the prevention of Cu-Zn SOD retinal expression/activity reduction in treated diabetic rats. Further studies are required to address whether the variation in Cu-Zn SOD expression/activity plays a role in the effect of tempol in retinal tissue.

In conclusion, our data reveal the potential therapeutic role of tempol in the early phases of DR. In hypertensive diabetic rats, treatment with tempol reestablished the redox status by acting as an $O_2^-$ scavenger, which enhances the GSH biosynthesis and Cu-Zn SOD activity, thus preventing oxidative damage. These effects were associated with a reduction in retinal extracellular matrix accumulation and glial reaction. It may be that mechanism conferred by tempol to protect against early molecular changes in the retinas of diabetic hypertensive rats and the counteraction of oxidative-nitrosative stress, thus diminishing the amount of ADP-ribosylated proteins.

**Acknowledgments**

The authors thank Gabriela F. P. de Souza and Marcelo G. de Oliveira for their assistance in the measurements of nitric oxide end products.

**References**


