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Nephroprotective effects of TVP1022, a non-MAO inhibitor S-isomer of rasagiline, in an experimental model of diabetic renal ischemic injury

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Although the adverse renal effects of diabetes or ischemia alone were intensively studied, the mechanisms underlying the enhanced vulnerability of the diabetic kidney to ischemic injury have not been thoroughly investigated. We assume that ischemia-reperfusion in DM aggravates renal damage by worsening the status of oxidative stress and endothelial dysfunction. Previously, we have showed partial improvement of kidney function in diabetic rats that underwent ischemic renal injury attributed to the marked decline in renal parenchymal oxygen pressure, especially in the renal medulla of the diabetic kidney (3, 26, 28). Moreover, DM is characterized by endothelial dysfunction, high levels of free radicals, and a decline in antioxidant levels that may contribute to the exaggerated vulnerability of the diabetic kidney to ischemic damage (4, 29). In this context, hyperglycemia increases the production of free radicals by various biochemical pathways including glucose autoxidation, the polyol pathway, protein-advanced glycation, and increased free fatty acids levels (4). Bhatia et al. (4) have demonstrated that oxidative stress, characterizing DM, enhances lipid peroxidation, decreases antioxidant enzymatic activities due to progressive glycation of these enzymes, and reduces glutathione (GSH) levels.

Ischemia-reperfusion renal damage, as a model of AKI, is largely attributed to endothelial dysfunction (10), inflammation (16, 27), and oxidative stress (24, 30). While the former is characterized by an imbalance between the release of endothelin-1 (ET-1) and nitric oxide (NO) (17), the latter is attributed to increased free radical production via xanthine oxidase of the reperfused tissues (24). Moreover, a burst of superoxide is produced from the resumption of oxygen supply, thus enhancing oxidative stress (10, 16). In an oxidative stress environment, the majority of NO produced by the various types of nitric oxide synthase (NOS), especially inducible NOS (iNOS), converts to reactive nitrogen species (RNS) (25). iNOS is capable of generating superoxide along with NO in the proximal tubules, thus increasing nitrosative stress (16). Nitrotyrosine-modified proteins are indicative of concomitant oxidative and nitrosative stress. The latter is derived from overproduction of peroxynitrite, which leads to nitrotyrosine formation on proteins, DNA damage, and lipid peroxidation, as expressed by overexpression of 4-hydroxynonenal (4-HNE) (25).

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IT HAS BEEN RECOGNIZED THAT diabetes mellitus (DM) is a risk factor for developing acute kidney injury (AKI) in a variety of clinical settings, such as radiocontrast nephropathy or following cardiopulmonary bypass operations. This was partially
with the use of selective blockers of endothelin (ET-1) receptors and NOS inhibitors (2).

TVP1022 is the non-MAO inhibitor S-isomer of the well-known drug rasagiline (Azilect, Food and Drug Administration-approved anti-Parkinson drug), which is recognized by its ability to protect cardiac and neuronal cell cultures against apoptotic-inducing stimuli. Previously, we reported that TVP1022 prevented H₂O₂-induced damage in H9c2 (rat heart cell line) by preserving the mitochondrial membrane potential and inhibiting cytochrome c release from the mitochondria (1, 7).

In light of the antioxidative properties of TVP1022, the current study was designed to evaluate whether this agent attenuates the exaggerated vulnerability of the diabetic kidney to ischemic damage. Moreover, the efficacy of TVP1022 in this regard was compared with that of the classic antioxidant, namely, 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxo (tempol), a SOD mimetic (6, 19, 34).

**Materials and Methods**

Studies were conducted in male Sprague-Dawley rats, weighing 300–350 g (Harlan, Jerusalem, Israel), maintained on standard rodent chow (containing 0.5% NaCl, 18% protein, and 6% fat) and water ad libitum. All experiments were performed according to the guidelines of the Committee for the Supervision of Animal Experiments, Technion, IIT.

**Animal Model of Diabetes**

The animals were rendered diabetic by a single intraperitoneal injection of freshly prepared streptozotocin (STZ; 65 mg/kg body wt; Sigma) dissolved in sodium citrate buffer (pH 4.5). Diabetes was confirmed 5 days later by blood glucose sampling from the tail tip with a glucometer (Ascensia Elite XL, Milan, Italy).

**Ischemia-Reperfusion Injury (AKI)**

Five days after STZ injection, the animals were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt) and placed on a controlled heating (thermoregulated) table, the body temperature kept at 37.5°C. Unilateral renal ischemia was produced by clamping the left renal artery for 30 min. To minimize dehydration of the exposed tissues, the abdominal area was covered with saline-soaked gauze. During reperfusion, the clamp was removed and the blood flow to the kidney was reestablished with visual verification of blood supply. Subsequently, the abdomen was sutured, and the animals were returned to their cages for 48 h.

**Control Group**

To assess the effects of TVP1022, 12 male rats (normoglycemic) were subjected to the same procedure as the control group. The applied dose of TVP1022 was chosen based on previous study by our group, where the renal effects of incremental doses of TVP1022 were examined in rats with CHF induced by an aorta caval fistula (1). We found a dose-dependent beneficial effect of TVP1022 (1–7.5 mg·kg⁻¹·day⁻¹·po) on GFR and sodium excretion. Higher doses of TVP1022 (>7.5 mg·kg⁻¹·day⁻¹) didn’t yield additional effects. To avoid undesired side effects of higher doses, we applied this dose.

**Clearance Studies**

Rats were anesthetized by Inactin (100 mg/kg), put on a thermoregulated (37°C) surgical table as described above, and tracheotomized. Polyethylene tubes (PE-50) were inserted into the right carotid artery for blood pressure monitoring and for blood samples, and the jugular vein for infusion of 0.9% NaCl at a 1.5 ml/h rate and 2%ulin at a rate of 1.5 ml/h, by syringe pumps (New Era Pump Systems, Farmingdale, NY). Collectively, saline was infused at a rate of 1% of body weight/h throughout the experiment. Arterial blood pressure was continuously monitored with a pressure transducer (model 50110, Stoelting, Wood Dale, IL) connected to the carotid arterial line. Catheters were inserted after a supravesical incision into both the left ureter (PE-10), as an index of the ischemic kidney, and the bladder (PE-50), as an index of the control right kidney. After a bolus injection of 0.5 ml containing of 1%ulin, followed by an equilibration period of 60 min, urine was collected separately from each kidney in different time periods (4 collections). Between two urine collections, a reference blood sample was drawn. To minimize dehydration, the abdominal area was covered with saline-soaked gauze.

At the end of the experiment, both kidneys were removed and each one was sliced into two halves; one-half was stored in a freezer at −70°C, and the other half was paraffin embedded.

**Immunohistochemistry and Immunofluorescence Analysis**

Morphological changes were assessed in a blinded fashion regarding treatment, and the extent of necrosis, retained casts, and congestion was determined selectively in the cortical labyrinth, and in the outer and inner strips of the outer medulla. A semiquantitative analysis was performed, using a 0–4 score, with the grade 4 reflecting en block infarction of the glomeruli. Sections were incubated in 2% inulin at a rate of 1.5 ml/h, by syringe pumps (New Era Pump Systems, Farmingdale, NY). Collectively, saline was infused at a rate of 1% of body weight/h throughout the experiment. Arterial blood pressure was continuously monitored with a pressure transducer (model 50110, Stoelting, Wood Dale, IL) connected to the carotid arterial line. Catheters were inserted after a supravesical incision into both the left ureter (PE-10), as an index of the ischemic kidney, and the bladder (PE-50), as an index of the control right kidney. After a bolus injection of 0.5 ml containing of 1%ulin, followed by an equilibration period of 60 min, urine was collected separately from each kidney in different time periods (4 collections). Between two urine collections, a reference blood sample was drawn. To minimize dehydration, the abdominal area was covered with saline-soaked gauze.

At the end of the experiment, both kidneys were removed and each one was sliced into two halves; one-half was stored in a freezer at −70°C, and the other half was paraffin embedded.

**Immunohistochemistry and Immunofluorescence Analysis**

Morphological evaluation. Hematoxylin and eosin staining was performed in paraffin-embedded longitudinal 3-μm kidney sections. Morphological changes were assessed in a blinded fashion regarding treatment, and the extent of necrosis, retained casts, and congestion was determined selectively in the cortical labyrinth, and in the outer and inner strips of the outer medulla. A semiquantitative analysis was performed, using a 0–4 score, with the grade 4 reflecting en block infarction of the entire region examined, casts detected in all tubuli, extensive congestion and extravasation of red cells into the parenchyma, and extensive polymorphonuclear infiltration, respectively, as described by Kiris et al. (14).

**4-HNE**. Paraffin-embedded kidneys were cut into longitudinal 5-μm sections and mounted on glass slides precoated with polyl-lysine. The slides underwent deparaffinization in xylene and rehydration in decreased concentrations of ethanol. Sections were incubated with goat serum to prevent nonspecific binding of immunoglobulins overnight at 4°C. Antibody to 4-HNE (Alexis Biochemicals) was diluted 1:300 in goat serum (10-fold diluted), added to the slides for 24 h at 4°C, then washed three times with T-TBS 0.5% for 5 min each. Negative control slides were not incubated with the primary antibody; rather, they were incubated only with diluted goat serum (22). An immunofluorescent reaction was achieved by the addition of a 500-fold-diluted fluorescent secondary antibody, goat anti rabbit IgG-Cy3 (Enco), for 45 min for all the slides, then they were washed three times with T-TBS 0.5% for 5 min, dried, covered, and photographed by an upright fluorescent microscope [Fluorescence Microscope Axiorsp (Zeiss)]. 4-HNE immunoreactivity was analyzed by measuring fluorescence intensity by digital image analysis (Image Pro, version 5, Media Cybernetics, Bethesda, MD) of images obtained by using a high-sensitive camera (Roper Scientific Camera, Tucson, AZ).

**Nitrotyrosine**. Paraffin-embedded kidneys were cut into longitudinal 5-μm sections and mounted on glass slides precoated with polyl-lysine. Then slides underwent deparaffinization in xylene and rehydration in decreased concentrations of ethanol. Sections were incubated with a peroxidase block [Envision System HRP
(AEC) Anti-Mouse Kit, Enco) overnight at 4°C to prevent nonspecific binding of immunoglobulins. A nitrotyrosine monoclonal antibody (Alexis Biochemicals) was diluted to 1:70 in T-TBS and added to all slides overnight at 4°C. Negative control slides were incubated with negative control mouse reagent (12). An immunohistochemical reaction was performed according to the Dako Envision System HRP (AEC) Anti-Mouse Kit (Enco). All sections were counterstained with hematoxylin and photographed by an Olympus C5060W digital camera on a CH-30 microscope.

Chemical Analysis

Urine volume was determined gravimetrically. Plasma samples were separated by centrifugation, and the concentrations of sodium in plasma and urine were determined by a flame photometer (model IL 943, Instrumentation Laboratory). Concentrations of inulin in plasma and urine samples were measured by the colorimetric anthrone method (8). The glomerular filtration rate (GFR) was estimated as the infusion clearance of inulin (C\text{in} = \text{Unin} \times V/\text{Unin}). Urine flow was calculated by dividing urine volume by time of urine collection. Urinary sodium excretion (UNaV) was calculated by multiplying urinary sodium concentration (UNa) by urinary flow rate (V).

Statistical Analysis

One-way ANOVA for repeated measures, followed by the Dunnett test, was used for comparison of treatment values with baseline in each group or with corresponding values in the control group. A value of \( P < 0.05 \) was considered statistically significant. Data are presented as means of repeated measurements ± SE.

RESULTS

Effects of Ischemia-Reperfusion Injury on Renal Function in Diabetic Rats

Diabetic rats exhibited significant weight loss (−13.9 ± 1.08% compared with nondiabetic rats (+2.37 ± 0.88%; \( P < 0.0001 \)) and a dramatic increase in blood glucose levels (503 ± 15 vs. 112 ± 5 mg% in nondiabetic animals) (\( P < 0.0001 \)) (Table 1). Renal ischemia decreased GFR by 2.7-fold in nondiabetic rats (from 1.16 ± 0.04 to 0.43 ± 0.11 ml/min; \( P < 0.0001 \)) and by 45-fold in diabetic rats (from 1.70 ± 0.056 to 0.026 ± 0.010 ml/min, \( P < 0.0001 \)) (Fig. 1A). V decreased from 19.64 ± 1.62 to 1.48 ± 0.42 µl/min (\( P = 0.018 \)) in diabetic rats and from 7.22 ± 1.23 to 5.50 ± 1.53 µl/min in nondiabetic animals [\( P = \) not significant (NS)]. The percentage of decline in V following ischemia was remarkably more severe in diabetic rats compared with non-diabetic rats (% change = −92.62 ± 2.46% vs. −44.6 ± 9.2%; \( P = 0.0001 \)) (Table 2). Ischemia also decreased UNaV in both diabetic (from 0.68 ± 0.21 to 0.09 ± 0.03 µeq/min; \( P = 0.034 \)) and nondiabetic rats (from 0.74 ± 0.19 to 0.46 ± 0.18 µeq/min; \( P = 0.25 \)) (Table 2). Collectively, the decline in GFR, V, and UNaV following ischemia was extensively larger in diabetic rats than nondiabetic animals. These findings clearly suggest that the diabetic kidney is more susceptible to ischemic injury than the nondiabetic kidney.

Effect of TVP1022 Treatment on Renal Function After iAKI in Diabetic Rats

TVP1022 had no effect on the decline in body weight in diabetic animals (−11.47 ± 1.25 vs. −13.8 ± 1.08%, \( P = 0.16 \)) but mildly decreased MAP from 114.8 ± 2.9 vs. 122 ± 1, \( P = 0.031 \) (Table 1).

Administration of TVP1022 to diabetic rats decreased V by 30% (from 19.64 ± 1.62 to 13.79 ± 1.73 µl/min, \( P < 0.05 \)), increased UNaV by 30% (from 0.68 ± 0.21 to 0.89 ± 0.38 µeq/min, \( P = 0.061 \)) (Table 2) but had no effect on GFR (1.21 ± 0.16 vs. 1.17 ± 0.05 ml/min) in nonischemic kidneys (Fig. 1A).

TVP1022 treatment did not significantly affect V in ischemic diabetic kidneys (1.95 ± 0.64 vs. 1.48 ± 0.42 µl/min; \( P = 0.56 \)) and UNaV (0.07 ± 0.01 vs. 0.09 ± 0.03 µeq/min; \( P = 0.4813 \)) (Table 2). However, GFR in diabetic rats treated with TVP1022 was fourfold higher than diabetic rats without TVP1022 (from 0.026 ± 0.010 to 0.14 ± 0.06 ml/min; \( P = 0.04 \)) (Fig. 1A).

Effect of Tempol Treatment on Renal Function After iAKI in Diabetic Rats

Tempol treatment of diabetic rats attenuated the decline in body weight from −13.90 ± 1.08 to −9.30 ± 1.87%, (\( P = 0.042 \)) but didn’t have any impact on mean arterial pressure (MAP) (Table 1).

In diabetic nonischemic kidneys, tempol decreased V by 33% (from 19.64 ± 1.62 to 13.05 ± 2.43 µl/min, \( P < 0.05 \)) and UNaV by 44% (from 0.68 ± 0.21 to 0.37 ± 0.11 µeq/min, \( P = 0.3 \)) (Table 2) but had no significant effect on GFR (1.26 ± 0.14 vs. 1.17 ± 0.05 ml/min) (Fig. 1A).

In diabetic ischemic kidneys, tempol did not significantly affect V (1.33 ± 0.33 vs. 1.48 ± 0.42 µl/min; \( P = 0.8191 \)) (Table 2). However, it increased UNaV by 33% (from 0.09 ± 0.03 to 0.12 ± 0.05 µeq/min; \( P = 0.54 \)) (Table 2), and increased GFR by two fold (from 0.026 ± 0.010 to 0.045 ± 0.003 ml/min; \( P = 0.125 \)) (Fig. 1A).

Morphological Evaluation

Ischemia-reperfusion injury in normoglycemic rats was associated with necrosis and casts, mainly in the outer stripe of the renal outer medulla of both diabetic and nondiabetic rats. However, this effect was more profound in diabetic rats.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Body Weight, % Change From Baseline</th>
<th>Blood Glucose, mg/dl</th>
<th>MAP, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoglycemia (( n = 7 ))</td>
<td>+2.37 ± 0.88</td>
<td>112 ± 5</td>
<td>132.1 ± 4.2</td>
</tr>
<tr>
<td>Diabetes (( n = 8 ))</td>
<td>−13.90 ± 1.08***</td>
<td>503 ± 15***</td>
<td>121.2 ± 1.4</td>
</tr>
<tr>
<td>Diabetes+tempol (( n = 6 ))</td>
<td>−9.3 ± 1.87#</td>
<td>377 ± 33***##</td>
<td>123.0 ± 6.1</td>
</tr>
<tr>
<td>Diabetes+TVP1022 (( n = 8 ))</td>
<td>−11.47 ± 1.25***##</td>
<td>402 ± 8***###</td>
<td>114.8 ± 2.9**#</td>
</tr>
</tbody>
</table>

Values are means ± SE. Effect is shown of various treatments on body weight (expressed as % change), on blood glucose 5 days after streptozotocin (STZ) injection, and MAP on the test day of the experimental protocol. *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.0001 \) vs. normoglycemia. #\( P < 0.05 \), ##\( P < 0.01 \), ###\( P < 0.0001 \) vs. untreated diabetes.
Specifically, an extensive injury pattern in diabetic ischemic kidneys was noted, especially in the inner stripe of the outer medulla, where congestion and inflammation were remarkable [score of 2.5 out of 4] (Fig. 2). TVP1022 abolished cast formation and decreased necrosis in the cortex and medulla of ischemic diabetic kidneys. It should be emphasized that diabetes alone had no histological effects on nonischemic kidneys (data not shown).

Lipid Peroxidation

4-HNE was measured in renal tissue to determine the extent of lipid peroxidation in normoglycemic and diabetic rats that underwent renal ischemia-reperfusion injury. Immunoreactive quantification revealed an increase in renal cortical 4-HNE levels in ischemic compared with nonischemic normoglycemic kidneys. However, the increase in cortical 4-NHE was more remarkable when ischemia was induced in diabetic rats ($P = 0.03$) (Fig. 3A). Diabetes alone was associated with mild upregulation of cortical 4-HNE compared with normal rats, although this increase did not reach statistical significance (Fig. 3A). TVP1022 but not tempol decreased cortical 4-HNE in both ischemic and nonischemic diabetic kidneys.

4-HNE expression in the renal medulla exhibited a similar pattern to that observed in the cortex, where 4-HNE slightly and nonsignificantly increased in ischemic normoglycemic kidneys compared with nonischemic normoglycemic kidneys. Nevertheless, ischemic diabetic rats showed a remarkable upregulation of medullary 4-HNE compared with both ischemic normoglycemic kidneys ($P = 0.0062$) and diabetic nonischemic kidneys ($P = 0.0056$) (Fig. 3B). Tempol and TVP1022 decreased lipid peroxidation in ischemic diabetic renal medulla. Similar to the cortex, diabetes itself slightly enhanced medullary 4-HNE immunoreactivity, where tempol and TVP1022 abolished this increase. It should be emphasized that the intensity of 4-HNE staining was higher in the medulla compared with the cortex.

Nitrosative Stress

Normoglycemic nonischemic rats displayed almost no nitrotyrosine staining in renal tissue, apart from fine granulation inside the cells of the proximal convoluted tubules (Fig. 4A), where fragile staining on the brush border, besides faint staining on the luminal side of the collecting duct, were noticed (Fig. 4, B and C). Renal ischemia in normoglycemic rats induced intense staining of the brush border and the lumen of...
EFFECTS OF S-ISOMER OF RASAGILINE ON AKI

Table 2. Effects of tempol and TVP1022 treatment on kidney weight, urine flow rate (V), and sodium excretion (UNaV) in nondiabetic and diabetic rats subjected to ischemic kidney injury

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Kidney Weight, g</th>
<th>V, μl/min</th>
<th>UNaV, μeq/min</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normoglycemia</strong> (n = 7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>1.18 ± 0.11</td>
<td>7.22 ± 1.23</td>
<td>0.74 ± 0.19</td>
</tr>
<tr>
<td>I</td>
<td>1.38 ± 0.09</td>
<td>5.50 ± 1.53</td>
<td>0.46 ± 0.18</td>
</tr>
<tr>
<td><strong>Diabetes</strong> (n = 8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>1.29 ± 0.07</td>
<td>19.64 ± 1.62***</td>
<td>0.68 ± 0.21</td>
</tr>
<tr>
<td>I</td>
<td>1.68 ± 0.06</td>
<td>1.48 ± 0.42**</td>
<td>0.09 ± 0.03*</td>
</tr>
<tr>
<td><strong>Diabetes+tempol</strong> (10 mg·kg⁻¹·day⁻¹ po, 7 days; n = 6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>1.11 ± 0.04</td>
<td>13.05 ± 2.43</td>
<td>0.37 ± 0.11</td>
</tr>
<tr>
<td>I</td>
<td>1.59 ± 0.11</td>
<td>1.33 ± 0.33*</td>
<td>0.12 ± 0.05</td>
</tr>
<tr>
<td><strong>Diabetes+TVP1022</strong> (7.5 mg·kg⁻¹·day⁻¹ po, 7 days; n = 8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>1.20 ± 0.04</td>
<td>13.79 ± 1.73*#</td>
<td>0.89 ± 0.38</td>
</tr>
<tr>
<td>I</td>
<td>1.69 ± 0.16</td>
<td>1.95 ± 0.64*</td>
<td>0.07 ± 0.01*</td>
</tr>
</tbody>
</table>

Values are means ± SE. N, nonischemic kidney; I, ischemic kidney. *P < 0.05, **P < 0.01, ***P < 0.0001 vs. normoglycemia. #P < 0.05, ##P < 0.01, ###P < 0.0001 vs. untreated diabetes.

The proximal convoluted tubules with granules inside the cells compared with normoglycemic nonischemic rats (Fig. 4A). Furthermore, strong staining was detected in the sloughed and necrotic cells that resulted from the ischemic insult (Fig. 4A). Similarly, the medulla displayed a similar pattern where strong intracellular staining, on the luminal side of the collecting duct and in the medullary thick ascending limbs of Henle (mTALs) was observed (Fig. 4B and C).

Diabetic nonischemic kidneys exhibited a moderate increase in nitrotyrosine staining in the cortical tissue compared with normoglycemic rats (Fig. 4, A–C). The immunostaining was localized to basolateral proximal tubules with finely stained intracellular granules, and faint staining in the glomeruli (Fig. 4A). Noteworthy, nitrotyrosine staining was more abundant in the medullary tissue of diabetic kidneys compared with the cortex, where strong staining was detected in the luminal side of the collecting duct (CD) (Fig. 4C) and faint staining in mTALs (Fig. 4B), especially in sloughed and necrotic cells. Importantly, diabetic ischemic kidneys exhibited a very intense renal staining of nitrotyrosine in the cortical tissue especially in proximal tubules (Fig. 4A), glomerulus, and to a lesser extent in the mTALs (Fig. 4, A and B) and CDs (Fig. 4C). Moreover, proximal tubules were more sloughable, necrotic, and lacked morphological features.

TVP1022, and to a lesser extent tempol, treatment decreased nitrotyrosine staining in diabetic nonischemic kidneys (Fig. 4A), but the medulla displayed a strong intracellular staining on the luminal side of the collecting duct and mTALs (Fig. 4, B and C). Both TVP1022 and tempol remarkably reduced nitrotyrosine staining in ischemic diabetic kidneys, but didn’t prevent damage to the brush border and the sloughed and necrotic cells that resulted from the ischemic insult (Fig. 4, A–C).

DISCUSSION

The findings of the present study provide new insights into the mechanisms underlying the vulnerability of the diabetic kidney to ischemic damage, specifically the role of oxidative stress in the pathogenesis of renal dysfunction characterizing iAKI diabetic rats. We showed that induction of iAKI in diabetic animals caused a more profound reduction in renal function compared with normoglycemic animals. The exaggerated susceptibility of diabetic kidney to ischemic injury was evident by a more profound increase in necrosis and casts in the renal cortex as well as the medulla. Moreover, ischemic diabetic kidneys exhibited increased immunoreactive 4-HNE and nitrotyrosine staining. Noteworthy, these increases were to a larger extent compared with ischemic nondiabetic kidneys. These findings may be of relevance for the development of novel therapeutic approaches that rely on these mechanisms. In line with this assumption, treatment with the antioxidant TVP1022 partially ameliorated the enhanced vulnerability of the diabetic kidney to ischemic damage by improving functional and histological derangements.

It is well known that iAKI increases kidney oxidative stress (25). Moreover, similar deleterious changes in redox balance are described in long-standing diabetes (5, 21, 23). Therefore, we assume that ischemic diabetic kidneys display greater oxidative stress than each condition alone, which may contribute to the increased susceptibility of diabetic kidneys to ischemic-reperfusion injury. Accordingly, the current study examined the effect of iAKI on renal function in diabetic rats and assessed the alterations in oxidative and nitrosative stress and the beneficial effects of the novel antioxidant TVP1022 on ischemic diabetic kidneys compared with the relevant controls.

Ischemic nondiabetic kidneys showed a 60% decrease in GFR and a 38% decrease in UNaV from basal values. In contrast, induction of renal ischemia in diabetic animals resulted in more remarkable changes, i.e., a 98 and 87% decline in GFR and UNaV, respectively. These findings are in line with those reported by other groups (11, 20, 21). Melin et al. (20) showed that a 30-min occlusion of the renal artery in diabetic rats caused an 80% decrease in GFR of that measured in ischemic normoglycemic kidneys. However, the mechanisms underlying this phenomenon were not thoroughly investigated.

Ischemia increased casts and necrosis mainly in the outer medulla, but when combined with diabetes it became more intense and spread to the cortex and inner medulla. In addition, this study clearly shows that two markers of oxidative stress (4-HNE and nitrotyrosine) were remarkably increased in ischemic diabetic kidneys compared with the control groups Immunoreactivity of 4-HNE, a lipid aldehyde generated as a major end product of lipid peroxidation, was elevated in the renal cortex 48 h after iAKI in both normoglycemic and hyperglycemic rats, although this increase was markedly larger in the latter. Although Noiri et al. (25) and Nilakantan et al. (24) have showed increased immunostaining of 4-HNE in the kidney of normoglycemic rats that were subjected to renal ischemia, none of these studies
examined the status of this oxidative stress marker in combined diabetes and renal ischemia. Thus our findings extend the current knowledge and suggest that the combination of diabetes and ischemia have an additive effect on lipid peroxidation due to increased production of 4-HNE during oxidative stress.

Nitrotyrosine serves as an additional marker for cellular stress and is termed “nitrosative stress.” Under this condition, there is a combination of NO and \( \text{O}_2^– \), yielding peroxynitrite, a potent tyrosine-nitrating and -damaging species. Several in vivo and in vitro studies have suggested that NO generated by iNOS contributes to renal iAKI injury (5, 23, 30). Increased production of NO for prolonged periods during many pathological states is known to contribute to oxidative damage of critical cellular macromolecules, including nitration of tyrosine residues of proteins. We have previously reported an increase in iNOS immunoreactive levels following ischemic injury in both normoglycemic and diabetic rats (2). In our study, we found a substantial increase in nitrotyrosine in the renal cortex and medulla following ischemic insult in both normoglycemic and diabetic animals compared with the related controls. Note-worthy, the highest increase in nitrotyrosine was observed in the renal tissue of diabetic rats that underwent ischemia. Other groups have reported similar results in models of renal ischemia-reperfusion (25, 30), but none of them examined nitrosative stress in combined ischemia and diabetes.

These observations led us to examine whether treatment with antioxidants ameliorates the impaired renal function characterizing renal ischemia in diabetic rats. In this context, we investigated the protective effects of tempol and TVP1022 on renal function and histology in normoglycemic and diabetic rats following ischemic insult.
Fig. 3. Effect of tempol and TVP1022 on 4-hydroxynonenal (4-HNE) levels in diabetic kidneys. Shown are effects of the antioxidants TVP1022 and tempol on the renal abundance of 4-HNE (×20). IOD (Integrated optical density or intensity), AU (Arbitrary unit). *P < 0.05, **P < 0.01 vs. normoglycemic nonischemic kidney. #P < 0.05, vs. diabetic nonischemic kidney.
evaluated the renal effects of TVP1022, in agreement with its antioxidative and antiapoptotic properties, we found that TVP1022 administration increases urine flow and GFR in ischemic diabetic animals. Previously, we have reported similar stimulatory renal effects of TVP1022 in rats with constrictive heart failure, a clinical condition characterized by impaired renal function (1). Moreover, TVP1022 reduced casts and necrosis throughout the kidney, indicating that TVP1022 has a protective effect on renal morphology. Similarly, TVP1022 attenuated lipid peroxidation and nitrosative stress in ischemic and nonischemic diabetic kidneys. In contrast, tempol slightly and nonsignificantly improved GFR along with reduced lipid peroxidation and nitrosative stress in iAKI. The moderate efficacy of tempol in kidney function and histological alterations is to a lesser extent than the reported encouraging nephroprotective action of this agent (6, 9). These differences could be attributed to the fact that none of these studies was performed in diabetic rats, and there were the application of various doses of tempol and different methodological approaches to determine kidney dysfunction. Concerning the latter, most studies measured plasma creatinine (PCr) as a marker for kidney dysfunction. Unfortunately, creatinine is an unreliable marker during acute changes in kidney function, since serum creatinine concentrations can vary widely with muscle metabolism, medications, and hydration status. Second, serum creatinine concentrations may not change until a significant amount of kidney function

Fig. 4. Effect of tempol and TVP1022 on nitrosative stress in renal tissue. Shown are effects of the antioxidants TVP1022 and tempol on the level of protein nitrotyrosination (red; ×20) in the glomerulus and proximal tubule (A), the mTAL (B), and collecting ducts (C) as determined by immunohistochemical staining (brown color).
suggesting a role for TVP1022 therapy in the diabetic kidney.

Diabetic rats exhibited lower blood pressure compared with normoglycemic rats that could stem from the massive hyperglycemia-induced polyuria, resulting in hypovolemia (Table 1). TVP1022 administration decreased blood pressure in diabetic rats, which may contribute to the obtained beneficial renal effects of TVP1022 in iAKI diabetic rats. However, the decrease in MAP was very moderate and most likely is not the sole factor that accounts for the nephroprotective effects of TVP1022.

Interestingly, both tempol and TVP1022 significantly reduced blood glucose levels in diabetic rats. The mechanisms underlying the reduction in plasma glucose concentration by these agents are not known. However, we postulate that improvement in GFR by either tempol or TVP1022 may enhance the extension of glycosuria, thus lowering the blood glucose concentration in this model of diabetic AKI. Alternatively, since both antioxidants were given directly following diabetes induction (STZ injection), it may attenuate the destruction of the pancreatic β cells, thus improving the control of blood glucose levels compared with untreated rats. This notion is further supported by the findings of Lee et al. (18) and Kaneto et al. (13), which have shown that antioxidants protect and preserve β cells in type 2 diabetes from fibrosis and apoptosis, along with increased insulin sensitivity.

The mechanisms underlying these beneficial effects of TVP1022 are still vague. However, we have demonstrated previously that TVP1022 has antioxidative, cytoprotective, neuroprotective, and cardioprotective efficacy and antiapoptotic ability. Pretreatment of neonatal rat ventricular myocytes (NRVM) with TVP1022 or propargylamine attenuated doxorubicin- and serum starvation-induced apoptosis (15). More recently, we reported that TVP1022 increased phospho-PKC and phospho-(Ser 9) glycogen synthase kinase-3β (GSK-3β) levels in H9c2 cardiomyoblasts and NRVM and prevented H2O2-induced damage in H9c2 by preserving the mitochondrial membrane potential and inhibiting cytochrome c release from the mitochondria. In addition, TVP1022 reduced the structural and functional cardiac damage inflicted by myocardial infarction in rats (7). Although TVP1022 possesses stronger antioxidant activity than tempol, as was evident by its ability to reduce lipid peroxidation- and nitrytrosine-modified protein levels more effectively than tempol, we do not exclude that other mechanisms are underlying its nephroprotective effects.

In summary, our findings clearly show that ischemia in diabetic rats results in deleterious effects on kidney function and renal histology to a greater extent than those obtained in normoglycemic animals. This phenomenon could be attributed to the profound renal injury/oxidative and nitrosative stress characterizing the diabetic ischemic kidney. Interestingly, TVP1022, a non-MAO inhibitor, S-isomer of rasagiline, partially ameliorates the adverse effects of iAKI in diabetic rats, suggesting a role for TVP1022 therapy in the diabetic kidney under warm ischemic settings.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


