Kidney

Tempol Prevents Altered K+ Channel Regulation of Afferent Arteriolar Tone in Diabetic Rat Kidney

Carmen M. Troncoso Brindeiro, Pascale H. Lane, Pamela K. Carmines

Abstract—Experiments were performed to test the hypothesis that oxidative stress underlies the enhanced tonic dilator impact of inward-rectifier K+ channels on renal afferent arterioles of rats with streptozotocin-induced diabetes mellitus. Sham and diabetic rats were left untreated or provided Tempol in their drinking water for 26±1 days, after which afferent arteriolar lumen diameter and its responsiveness to K+ channel blockade were measured using the in vitro blood-perfused juxtamedullary nephron technique. Afferent diameter averaged 19.4±0.8 μm in sham rats and 24.4±0.8 μm in diabetic rats (P<0.05). The decrease in diameter evoked by Ba2+ (inward-rectifier K+ channel blocker) was 3 times greater in diabetic rats than in sham rats. Glibenclamide (KATP channel blocker) and tertiapin-Q (Kir1.1/Kir3.x channel blocker) decreased afferent diameter in diabetic rats but had no effect on arterioles from sham rats. Chronic Tempol treatment prevented diabetes mellitus–induced increases in both renal vascular dihydroethidium staining and baseline afferent arteriolar diameter. Moreover, Tempol prevented the exaggeration of afferent arteriolar responses to Ba2+, tertiapin-Q, and glibenclamide otherwise evident in diabetic rats. Preglomerular microvascular smooth muscle cells expressed mRNA encoding Kir1.1, Kir1.2, Kir1.6, or SUR2B protein levels in renal cortical microvessels. To the extent that the effects of Tempol reflect its antioxidant actions, our observations indicate that oxidative stress contributes to the exaggerated impact of Kir1.1, Kir2.1, and KATP channels on afferent arteriolar tone during diabetes mellitus and that this phenomenon involves posttranslational modulation of channel function. (Hypertension. 2012;59:657-664.) ● Online Data Supplement

Key Words: diabetes mellitus ▪ inward-rectifier K channels ▪ Tempol ▪ ROMK ▪ ATP-sensitive K channels

Long-term renal microvascular dysfunction is one of many complications of type 1 diabetes mellitus (T1D) that are thought to contribute to nephropathy.1 In the early stages of the disease, the main alteration that occurs in the renal microvasculature is afferent arteriolar dilatation; however, the events underlying tonic dilation of this vessel are poorly understood. Many studies have demonstrated an increase in reactive oxygen species (ROS) generation during diabetes mellitus,2–3 as well as altered activity of major antioxidant enzymes responsible for maintaining redox balance (ie, superoxide dismutase and catalase).3–5 Treatment of diabetic rats with the membrane-permeable antioxidant Tempol has been shown to decrease ROS accumulation and to increase the activity of antioxidant enzymes.3–4 Tempol has also been shown to recover NO-dependent vasodilator function in afferent arterioles from diabetic rabbits,6 likely reflecting the effect of reduced superoxide anion (O2•−) levels to decrease peroxynitrite generation, thereby restoring NO bioavailability.

Preglomerular microvascular resistance is coupled to vascular smooth muscle membrane potential, and K+ channels are important determinants of membrane potential. In vascular smooth muscle, opening of K+ channels allows efflux of K+ from the cell resulting in membrane hyperpolarization, which causes closure of voltage-gated Ca2+ channels and reduced Ca2+ influx, thereby evoking vasodilation. We reported previously that members of the inward-rectifier K+ channel family (Kir channels), including Kir2.1, Kir1.1/3.x, and ATP-sensitive K+ (KATP) channels, exert an exaggerated tonic dilator influence on afferent arteriolar tone in rats with streptozotocin (STZ)-induced T1D.7,8 Moreover, afferent arteriolar dilator responses to KATP activation (pinacidil) are accentuated during T1D,7 whereas responses to Kir activation (20 mmol/L K+) are attenuated.8 The mechanism underlying altered K+ channel control of afferent arteriolar function during T1D remains unclear. Because ROS can alter the function of several K+ channels in vascular smooth muscle,9 we hypothesized that oxidative stress underlies the changes in K+ channel function that arise in the afferent arteriole during T1D. The goal of the present study was to determine whether chronic treatment with the membrane-permeable antioxidant

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Tempol would prevent the exaggerated tonic impact of Kir channel family members on lumen diameter of the afferent arteriole in rats with STZ-induced T1D. We also explored the postulate that the changes in Kir channel regulation of afferent arteriolar function might reflect altered expression of the channel at the mRNA and/or protein levels.

**Methods**

For an expanded Methods section, see the online-only Data Supplement.

**Animals**

Animals in this study (male Sprague-Dawley rats weighing 275–300 g; n=135) were treated according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals using procedures approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. T1D was induced by injection of STZ (65 mg/kg IV; STZ rats), and moderate hyperglycemia was maintained by partial insulin replacement, as described previously.8 Sham rats received vehicle treatments. Half of the rats in each group received chronic antioxidant therapy, achieved by the addition of Tempol to the drinking water. Because STZ rats are polydipsic, the concentration of Tempol provided in the water was lower in the STZ+Tempol group (0.4 mmol/L) than in the sham+Tempol group (1 mmol/L). In preliminary experiments, these concentrations resulted in similar daily Tempol doses in the 2 groups of rats. Terminal experiments were performed 26±1 days after STZ (or vehicle) injection. Each rat was housed in a metabolic cage for the 3 days preceding the terminal experiment, with urine output and water intake measured during the final 24 hours. An aliquot of the urine sample was retained and frozen until analysis for indices of oxidative stress.

**In Vitro Blood-Perfused Juxtamedullary Nephron Technique**

Under pentobarbital anesthesia, the left kidney was removed and flash frozen, and the right kidney was perfused and harvested for study of afferent arteriolar function using the rat in vitro blood-perfused juxtamedullary nephron technique, as detailed previously.10,11 Perfusion pressure at the cannula tip in the renal artery was set and maintained at 110 mm Hg. The tissue surface was bathed in Tyrode solution containing 10 g/L of BSA, approximating the composition of renal interstitial fluid. Videometric techniques were used to measure arteriolar lumen diameter, as described previously,8 during completion of the following protocols.

For protocol 1, after a stabilization period, during which a single arteriole was selected for study, images of the arteriole were recorded during a 10-minute baseline period (exposure to Tyrode bath alone). Subsequently, the impact of Kir channels on afferent arteriolar diameter was evaluated based on the effects of sequential exposure to Tyrode bath containing 10, 30, and 100 μmol/L of BaCl2 (5 minutes each), followed by a 10-minute recovery period (Tyrode bath alone). This procedure was typically followed by an assessment of the tonic impact of Kir1.1/Kir3.x channels, based on the effects of sequential 5 minutes exposure to 1, 10, and 100 nmol/L of tertiapin-Q (TPQ).12

For protocol 2, in other kidneys (not exposed to Ba2+ or TPQ), the baseline period was followed by an evaluation of the impact of KATP channels on afferent arteriolar function based on responses to sequential exposure to 30, 100, and 300 μmol/L of glibenclamide (5 minutes each). The efficacy and specificity of the concentrations of Ba2+, TPQ, and glibenclamide used in this study have been addressed previously.7,8

**Indices of Oxidative Stress**

Dihydroethidium (DHE) staining was used to detect O2− in kidneys from each group of rats, according to the method described by Zalba et al.13 Flash-frozen kidneys were sectioned, and images of the renal cortex were obtained by laser confocal microscopy. DHE staining intensity was quantified from these images using Adobe Photoshop CS4 Extended.

Plasma and urine samples from each group of rats were assayed for thiobarbituric acid reactive substances (TBARS) using the OxiSelect TBARS Assay kit (Cell Biolabs). The Amplex Red Hydrogen Peroxide/Peroxidase Assay kit (Molecular Probes) was used to quantify H2O2 levels. Both assays were performed according to the manufacturer instructions.

**Preglomerular Microvascular Smooth Muscle Cell Culture**

Preglomerular microvascular smooth muscle cells (PVSMCs) from normal rat kidney were cultured by the explant method, as described previously.14 PVSMCs at passage 3 were maintained in 5 or 20 mmol/L of glucose for 2 to 3 weeks and then growth arrested by exposure to serum-free conditions for 1 day before harvesting.

**RNA Extraction and Real-Time RT PCR**

RNA was extracted from cultured PVSMCs using the Absolutely RNA Miniprep kit (Stratagene). Total RNA was measured in a 2-μL sample using a Nanodrop (Thermo Scientific). Samples were stored at −80°C until real-time RT-PCR was performed using the Rotor Gene Real-Time Detection System, following the QuantiTect SYBR Green RT protocol (Qiagen).

**Cortical Microvessel Isolation**

Vessels were isolated from rat kidney by sieving, according to the following method. Rats were anesthetized with pentobarbital sodium, and the kidney was perfused with cold Ca2+- and Mg2+-free Hank balanced salt solution containing 5 or 20 mmol/L of glucose (for sham and STZ rats, respectively). The kidney was removed, sliced longitudinally, and demedullated. The remaining cortex pieces were sequentially sieved over 250- and 150-μm nylon mesh, rinsed by dental water jet, placed in a cold collagenase solution, and incubated at 37°C while shaking for 20 minutes. After incubation, the vessels were gently sieved again over the 150-μm nylon mesh to wash away the collagenase and placed in cell lysis buffer for Western blotting.

**Western Blot**

Proteins in cortical microvessel lysates were separated by SDS-PAGE (4% to 15% gradient gel), transferred onto polyvinylidene fluoride membranes, dried, blocked, and incubated overnight with one of the following primary antibodies: rabbit anti-Kir1.1 (Chemicon, AB5196; 1:200 dilution), rabbit anti-Kir2.1 (Alomone, APC-026; 1:100), rabbit anti-Kir6.1 (Santa Cruz, sc20808; 1:200), goat anti-SUR2B (Santa Cruz, sc5793; 1:200), or mouse anti-β-actin (Abcam, ab627c; 1:200). The membranes were then washed 5 times before 1-hour incubation with secondary antibody conjugated to an infrared dye. Quantification was achieved using the Odyssey Infrared Imaging System.

**Statistical Analyses**

Simple between-group comparisons were made by ANOVA. Effects of pharmacological agents on arteriolar lumen diameter were evaluated based on the average diameter measured during the final 2 minutes of each treatment period, with statistical comparisons made by 2-way repeated-measures ANOVA and, when appropriate, the Holm-Sidak multiple comparisons test. P values <0.05 were considered statistically significant. All of the data are presented as mean±SEM.

**Results**

During the 3 to 4 weeks after injection of STZ/vehicle, blood glucose concentration was measured twice weekly and found to average 23.5±0.7 mmol/L (n=33 rats) in STZ rats, a value that was elevated significantly compared with sham rats.
Tempol: 155 ± 3 mL/d; STZ+Tempol: 35 ± 3 mL/d. Tempol rats drank more water than anticipated to a greater extent of the osmotic diuresis was also magnified. As a result, the degree of hyperglycemia achieved in the rats with T1D was somewhat greater than evident in our preliminary studies, the extent of the osmotic diuresis was also magnified. As a result, the STZ+Tempol rats drank more water than anticipated (STZ+Tempol: 155 ± 10 mL/d; sham+Tempol: 35 ± 3 mL/d; P < 0.05). Consequently, the dose of Tempol received by STZ+Tempol rats drinking 0.4 mmol/L of Tempol (62 ± 4 μmol/d) was greater than that received by sham+Tempol rats drinking 1 mmol/L of Tempol (35 ± 3 μmol/d; P < 0.05).

Plasma levels and urinary excretion of TBARS were elevated significantly after 3 to 4 weeks of hyperglycemia in STZ and STZ+Tempol rats (Table). Urinary H₂O₂ excretion was similar in sham and sham+Tempol rats but was significantly increased in STZ rats when compared with sham (P < 0.001). The increased H₂O₂ excretion evident in STZ rats was blunted significantly in STZ+Tempol rats. Thus, Tempol reduced the hyperglycemia-induced increase in H₂O₂ excretion but had no effect on plasma H₂O₂ levels.

DHE staining was used to assess the effect of Tempol on production of ROS in the kidneys of sham and STZ rats. Figure 1A provides representative DHE fluorescence images of renal cortical sections from each treatment group. The quantitative analysis shown in Figure 1B reveals that renal cortical vessel DHE fluorescence in STZ rats was significantly greater than that evident in sham rats. Chronic Tempol treatment of STZ rats prevented the elevation in vascular DHE fluorescence. Although the tendency for increased DHE fluorescence intensity in renal cortical tubules of STZ rats did not achieve statistical significance (P = 0.06 vs Sham), tubular DHE staining in STZ+Tempol rats was significantly lower than in untreated STZ rats. Tempol treatment of sham rats did not alter DHE fluorescence intensity in vessels or tubules. These data are in agreement with Tempol acting as an antioxidant in the renal cortex, especially in vascular structures.

Figure 2 shows afferent arteriolar lumen diameter under baseline conditions in the 4 animal groups. Baseline afferent arteriolar diameter in STZ rats was 25% greater than in sham rats (P < 0.05). In contrast, baseline lumen diameter did not

### Table. Indices of Oxidative Stress

<table>
<thead>
<tr>
<th>Groups</th>
<th>Plasma TBARS, μmol/L</th>
<th>TBARS Excretion, μmol/d</th>
<th>Plasma H₂O₂, nmol/L</th>
<th>H₂O₂ Excretion, nmol/d</th>
</tr>
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<tbody>
<tr>
<td>Sham</td>
<td>7.8 ±1.1 (n = 5)</td>
<td>0.31 ±0.03 (n = 12)</td>
<td>1295 ±54 (n = 5)</td>
<td>33.2 ±4 (n = 14)</td>
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<tr>
<td>Sham+Tempol</td>
<td>6.6 ±0.4 (n = 6)</td>
<td>0.36 ±0.04 (n = 10)</td>
<td>1041 ±109 (n = 6)</td>
<td>19.9 ±3 (n = 12)</td>
</tr>
<tr>
<td>STZ</td>
<td>19.0 ±2.3 (n = 5)*</td>
<td>17.9 ±2.5 (n = 12)*</td>
<td>1792 ±172 (n = 6)</td>
<td>403 ±89 (n = 13)*</td>
</tr>
<tr>
<td>STZ+Tempol</td>
<td>18.3 ±1.5 (n = 6)*</td>
<td>19.2 ±2.5 (n = 12)*</td>
<td>1663 ±181 (n = 6)</td>
<td>190 ±57 (n = 12)*†</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. TBARS indicates thiobarbituric acid reactive substances (malondialdehyde equivalents); STZ, streptozotocin. Excretory data were derived from 24-h urine collection in metabolic cages during the 24 h before the terminal experiment. Plasma samples were obtained under anesthesia before harvesting the kidney for study of arteriolar function.

*P < 0.05 vs sham.
†P < 0.05 vs STZ.

(5.2 ± 0.1 mmol/L; n = 31 rats). Chronic Tempol treatment had no effect on blood glucose levels in either group (sham + Tempol: 5.2 ± 0.1 mmol/L; n = 34 rats; STZ + Tempol: 23.3 ± 0.9 mmol/L; n = 37 rats). The rats in each group gained weight during this period; however, STZ and STZ+Tempol rats gained less weight than sham and sham+Tempol rats.
Effects of type 1 diabetes mellitus (T1D) and chronic Tempol treatment on afferent arteriolar baseline diameter. n = 20 to 23 arterioles per group. †P < 0.05 vs sham.

differ among STZ + Tempol rats, sham rats, and sham + Tempol rats. Thus, chronic Tempol treatment prevented the afferent arteriolar dilation typically evident in the STZ model of T1D while not altering baseline afferent diameter in nondiabetic rats.

Figure 3A illustrates the afferent arteriolar lumen diameter responses to increasing concentrations of Ba²⁺ in each group of rats. In sham rats, Ba²⁺ evoked a vasoconstrictor response (11 ± 3% decrease in diameter; P < 0.05) only at the highest concentration used (100 µmol/L). In STZ rats, significant constrictor responses were evident during exposure to 30 and 100 µmol/L of Ba²⁺, and these responses were exaggerated compared with arterioles from sham rats (30 µmol/L: Δ diameter = −15 ± 2%; 100 µmol/L: Δ diameter = −35 ± 6%; both P < 0.05 versus sham). Afferent arteriolar responses to Ba²⁺ did not differ between sham and sham + Tempol rats; however, the exaggerated afferent arteriolar Ba²⁺ responsiveness evident in STZ rats was not manifest in STZ + Tempol rats. Indeed, the response to 100 µmol/L of Ba²⁺ was reduced in STZ + Tempol rats compared with STZ rats (P < 0.05). As a result, afferent arteriolar diameter responses to Ba²⁺ did not differ significantly among STZ + Tempol, sham, and sham + Tempol groups. Thus, chronic Tempol treatment prevented the exaggerated afferent arteriolar contractile response to Ba²⁺ otherwise evident in STZ rats, suggesting that oxidative stress contributes to the augmented dilator impact of Kᵦ₆ channels on afferent arteriolar tone during T1D.

Figure 3B summarizes the effects of T1D and Tempol on afferent arteriolar responses to TPQ. Afferent arteriolar luminal diameter was unaffected by TPQ in kidneys from sham and sham + Tempol rats. In contrast, 100 nmol/L TPQ evoked a significant decrease in afferent arteriolar diameter in STZ rats (Δ diameter = −11 ± 3%; P < 0.05) but not in STZ + Tempol rats. Thus, chronic Tempol treatment prevented the afferent arteriolar constrictor response to TPQ that otherwise occurs in STZ rats, suggesting that tonic activation of afferent arteriolar Kir1.1/Kir3.x channels arises via an oxidative stress-dependent mechanism during T1D.

The results of experiments performed to evaluate the effects of T1D and Tempol on Kₐ₆ channel-dependent regulation of afferent arteriole tone are illustrated in Figure 3C. Glibenclamide (30–300 µmol/L) had no effect on afferent arteriolar lumen diameter in kidneys from sham or sham + Tempol rats. However, significant contractile responses to glibenclamide were evident in kidneys from STZ rats, with afferent arteriolar diameter decreasing 11 ± 2% and 21 ± 4% during exposure to 100 and 300 µmol/L of glibenclamide, respectively. Moreover, across the entire glibenclamide concentration range used in this study, afferent arteriolar diameter responses in STZ rats significantly exceeded those observed in sham rats. Compared with the responses observed in STZ rats, afferent diameter responses to 100 and 300 µmol/L of glibenclamide were significantly attenuated in kidneys from STZ + Tempol rats. In fact, glibenclamide did not significantly alter afferent arteriolar diameter in
STZ+Tempol rats, yielding responses that did not differ from those observed in sham and sham+Tempol rats. The ability of chronic Tempol treatment to prevent the afferent arteriolar contractile response to glibenclamide that is typically evident in STZ rats suggests that oxidative stress contributes to the emergence of a tonic dilator impact of K<sub>ATP</sub> channels on the afferent arteriole during T1D.

Real-time RT-PCR was used to measure mRNA expression of the pore-forming subunits of TPQ-sensitive channels (Kir1.1, Kir3.1, and Kir3.4), Ba<sup>2+</sup>-sensitive K<sub>IR</sub> channels (Kir2.1), and K<sub>ATP</sub> channels (Kir6.1, Kir6.2, and Kir6.3; characteristically expressed in the vasculature, pancreas, and zebrafish, respectively). Figure 4 summarizes the real-time RT-PCR data for the 7 channels after maintenance of PVSMCs for 2 to 3 weeks in culture medium containing either normal (5 mmol/L) or high (20 mmol/L) concentrations of glucose. Kir1.1, Kir2.1, and Kir6.1 mRNA were detected in cultured PVSMCs, whereas no mRNA was detected for Kir3.1, Kir3.4, or the K<sub>ATP</sub> negative controls (Kir6.2 and Kir6.3). The threshold cycle did not differ between normal and high glucose-treated PVSMCs for any of the channels studied, indicating that the T1D-induced changes in afferent arteriolar lumen diameter responses to K<sup>+</sup> channel blockers were not likely attributed to a glucose-induced increase in Kir gene transcription in PVSMCs.

Figure 5 summarizes the Kir channel protein levels in renal cortical microvessels obtained from each group of rats. Western blotting confirmed the presence of Kir1.1, Kir2.1, Kir6.1, and SUR2B at the protein level in renal cortical microvessels. The Kir1.1 antibody also detected a strong signal in renal medullary thick ascending limb homogenate (positive control; data not shown). Kir1.1 and Kir2.1 protein expressions did not differ among vessels from the sham, sham+Tempol, STZ, and STZ+Tempol groups. Thus, neither T1D nor chronic antioxidant therapy altered Kir1.1 or Kir2.1 expression in renal cortical microvessels. Additionally, we probed for pore-forming and regulatory subunits composed of vascular K<sub>ATP</sub> channels Kir6.1 and SUR2B, respectively; however, there was no significant difference in protein levels of either subunit among sham, sham+Tempol, STZ, and STZ+Tempol groups. Thus, neither T1D nor chronic antioxidant therapy altered Kir6.1 or SUR2B expression in renal cortical microvessels. These Western blot data demonstrate that the effects of T1D and chronic Tempol treatment on Kir regulation of arteriolar function are not attributed to change in channel protein level but, rather, likely reflect posttranslational alterations in channel activity.

**Discussion**

Accumulating evidence suggests that oxidative stress plays a role in a variety of vascular and renal complications of T1D. The present study explored the possibility that oxidative stress contributes to the dysregulation of K<sup>+</sup> channel-dependent control of afferent arteriolar tone that occurs during the early stage of T1D in the rat. Animals were studied 3 weeks after onset of STZ-induced T1D, during which partial insulin replacement yielded a condition of moderate hyperglycemia. The STZ rats demonstrated the polydipsia and polyuria characteristic of this model, as well as an
increase in basal afferent arteriolar lumen diameter that is
generally considered to contribute to diabetic hyperfiltration.5,15,16 We found that chronic Tempol treatment prevented the T1D-induced increase in basal afferent arteriolar diameter but did not affect this parameter in kidneys from nondiabetic rats. In addition, the effect of T1D to exaggerate afferent arteriolar constrictor responses to blockade of various Kir channel family members was prevented by Tempol treatment. Tempol also prevented increased DHE staining in renal cortical vessels from kidneys of STZ rats. To the extent that the effects of Tempol represent its antioxidant actions, these observations implicate oxidative stress as a significant factor underlying the renal microvascular dysfunction that arises during the early stage of T1D in the rat, acting at least in part through effects on K+ channel regulation of arteriolar tone.

In normal rat kidney, voltage-gated K+ channels ( Kv channels), large-conductance Ca2+-activated K+ channels (BKCa channels), and Ba2+-sensitive Kir channels contribute a tonic dilator influence on afferent arteriolar tone.6 In kidneys from STZ rats, members of the Kir channel family contribute to afferent arteriolar tone, and pharmacological agents that block various Kir channels reduce afferent arteriolar diameter to normal values.7,8 The multiple members of the Kir channel family differ with regard to electrophysiological and pharmacological properties.17 Using real-time RT-PCR, we detected mRNA encoding Kir2.1 (the most prominent vascular Kir channel), Kir1.1 (the ROMK channel), and Kir6.1 (porforming subunit of the vascular KATP channel) in PVSMCs. Notably, we were unable to detect in PVSMCs mRNA encoding Kir3.1 (GIRK1) or Kir3.4 (GIRK4), both of which can be inhibited by TPQ. Western blotting confirmed the presence of Kir2.1, Kir1.1, Kir6.1, and SUR2B at the protein level in cortical microvessels. Thus, it is likely that the afferent arteriolar effects of Ba2+ reflect inhibition of Kir2.1 (and, perhaps, other Kir family members, which exhibit varying Ba2+-sensitivities), those of TPQ reflect inhibition of Kir1.1, and those of glibenclamide reflect inhibition of the vascular KATP channel (Kir6.1/SUR2B) in PVSMCs. Similar to our previous observations,7,8 exaggerated afferent arteriolar contractile responses to Ba2+, TPQ, and glibenclamide were observed in kidneys from STZ rats compared with sham rats, thereby implicating Kir2.1, Kir1.1, and KATP channels in the afferent arteriolar dilation accompanying T1D. Importantly, chronic Tempol treatment prevented this phenomenon, normalizing arteriolar diameter and contractile responsiveness to Ba2+, TPQ, and glibenclamide. Evaluation of mRNA expression and K+ channel protein levels did not reveal any differences between Tempol and vehicle treatments in either rat group. Therefore, the presented evidence indicates that neither T1D nor high levels of glucose, per se, alters channel expression in the preglomerular microvasculature. Rather, the altered K+ channel regulation of afferent arteriolar tone evident in these experiments more likely reflects posttranslational modulation of channel function.

It is well documented that Tempol can act as a O2− scavenger, as well as exerting other antioxidant effects by functioning as a superoxide dismutase mimic, increasing endogenous superoxide dismutase activity, exerting a catalase-like action, and reducing NADPH-dependent O2− generation.18 Tempol is generally administered to rats as 1 to 2 mmol/L in drinking water, resulting in a dose of &sim;30 to 60 μmol/d.19 In our attempt to choose a concentration of Tempol to provide for STZ+Tempol rats, we underestimated the extent of the polydipsia that these animals would experience; however, the resulting doses administered in sham+Tempol and STZ+Tempol groups fall within the range (30–180 μmol/d) at which Tempol is maximally effective in exerting its antioxidant effects without apparent dose dependency.18 Within this dosage range, the effects of Tempol are almost exclusively attributed to its antioxidant actions,18,19 although a few studies have found that Tempol has effects that are not mimicked by other antioxidants, such as tiron.20,21 Tempol has also been reported to exert beneficial effects without altering systemic indices of oxidative stress, such as plasma F2-isoprostane levels.22 Similarly, we found no effect of Tempol on the T1D-induced increase in plasma TBARS or TBARS excretion, indices of systemic and renal lipid peroxidation. Some component of the sustained elevation in TBARS evident in STZ+Tempol rats may reflect the ability of H2O2 to promote lipid peroxidation via the Fenton reaction, because Tempol did not decrease plasma H2O2 levels and only blunted (but did not completely prevent) the increase in H2O2 excretion accompanying T1D. Indeed, Tempol exerts complex renal effects in STZ rats, decreasing renal cortical NADPH oxidase activity23,4 and reducing glomerular O2− and H2O2 levels while not altering the increased tubular H2O2 or the elevated renal HOCl levels.5 Importantly, in the present study that focused on renal vascular function, Tempol prevented the increase in vascular DHE staining (an indicator of O2− generation in situ) in kidneys from STZ rats. Thus, although we cannot rule out any off-target effects of Tempol, the beneficial impact of this agent on K+ channel control of afferent arteriolar tone likely reflects its antioxidant action exerted in the T1D-induced state of renal cortical oxidative stress.5,23,24

The mechanism through which the pro-oxidant milieu accompanying T1D alters function of these Kir channel family members is not known. Among the 3 channels targeted by this study, the literature provides the strongest evidence for ROS modulation of KATP channel function. H2O2 and peroxynitrite dilate cerebral arterioles by activating KATP channels,25 and peroxynitrite hyperpolarizes and relaxes smooth muscle in rabbit internal carotid artery (but not in the common carotid artery) through KATP channel activation.26 O2− has been reported to variably inhibit or activate KATP channels, depending on the vascular bed studied.9 ROS could influence KATP channel function by altering ATP levels in the cell or by acting directly on either the Kir6.1 or the SUR2B subunits of the channel. Recent studies of stably expressed Kir6.1 in HEK-293 cells (in the absence of any SUR subunit) revealed that coapplication of hypoxanthine and xanthine oxidase produced a significant increase in Kir6.1 currents that could be abolished by Tempol.27 Thus, O2− or other ROS might act directly on Kir6.1 to increase the contribution of KATP channels to afferent arteriolar tone during T1D. Such a scenario might also contribute to the exaggerated impact of
Ba²⁺-sensitive Kir2.1 channels on afferent arteriolar tone during diabetes mellitus. These channels are generally activated by moderate increases in extracellular K⁺ concentration and, indeed, are responsible for K⁺-mediated vasodilation. Although we are unaware of any reports that ROS directly modulate function of this channel, an ROS-induced increase in K<sub>ATP</sub> channel activity may create a microenvironment of high extracellular [K⁺] sufficient to provoke Kir2.1 activation. This scenario might explain our previous observation that exposure to 20 mmol/L of K⁺ does not further dilate afferent arterioles from STZ rats. Thus, ROS-stimulated K<sub>ATP</sub> channel activity and subsequent Kir6.1 activation could contribute to vascular smooth muscle membrane depolarization, reduced Ca²⁺ influx, and vasodilation. Obviously, more studies are needed to investigate the validity of this scenario.

The mechanism through which ROS might stimulate a Kir1.1-dependent influence on afferent arteriolar tone during T1D also remains speculative. On one hand, O₂⁻ reduces Kir1.1 activity in renal epithelial cells via an Src kinase-dependent mechanism that results in channel endocytosis. This scenario is directionally opposite of that necessary to underlie our observations. On the other hand, in pulmonary artery smooth muscle cells, H₂O₂ increases expression of the stress-responsive serum and glucocorticoid-inducible kinase Sgk1, which is a well-documented activator of Kir1.1 in renal epithelial cells. H₂O₂ excretion is markedly increased during T1D; hence, an Sgk1-mediated process may contribute to the ROS-dependent increase in the Kir1.1 contribution to afferent arteriolar dilation during T1D. However, there is no information available regarding whether regulation of Kir1.1 channels in afferent arteriolar vascular smooth muscle and renal epithelial cells occurs via similar mechanisms.

Finally, it seems prudent to note the potential interplay between the phenomena unveiled by the present study (ie, that oxidative stress promotes afferent arteriolar dilation during T1D via effects on K⁺ channels) and evidence that oxidative stress during T1D diminishes afferent arteriolar NO bioavailability, reducing agonist-induced NO-dependent dilation, as well as the tonic dilator impact of endogenous NO (which should promote afferent arteriolar contraction). Undoubtedly, control of afferent arteriolar tone involves both K⁺ channel-dependent regulation of membrane potential, as well as the tonic influence of NO (which does not generally evoke hyperpolarization), with the net effect of these processes likely determining dilator tone. Thus, the decline in NO bioavailability during T1D may actually blunt the dilator impact of increased K<sub>R</sub> channel activation during this state of oxidative stress. Further study is required to unravel the interplay between these processes in control of afferent arteriolar function during T1D.

**Perspectives**

Diabetes mellitus is the leading cause of end-stage renal disease, making it increasingly important to unravel the complex mechanisms through which T1D impairs renal function. Evidence from this study and others suggests that the antioxidant Tempol may be useful for preventing the renal damage thought to contribute to the progression of diabetic nephropathy. However, the effects of Tempol are complex and not uniformly exerted within the kidney, or systemically, and it has not yet been possible to discern the identity of the offending oxidant and its specific impact on various K<sub>R</sub> channel family members in the renal microvasculature. Continued investigation in this field may unveil targeted antioxidant approaches for preventing the renal complications of T1D.

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**Disclosures**

None.

**References**


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Tempol Prevents Altered K⁺ Channel Regulation of Afferent Arteriolar Tone in Diabetic Rat Kidney

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Running Title: Diabetes and afferent arteriolar K⁺ channels

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Expanded Methods

Animals
Animals in this study were treated according to the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* using procedures approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. Male Sprague-Dawley rats \(n=135\); Harlan) weighing 275-300 g were anesthetized \(50 \text{mg/kg IP methohexital sodium}\) to facilitate injection streptozotocin \(65 \text{mg/kg IV; STZ rats}\) or vehicle (Sham rats). The following day, blood glucose levels were measured (Accu-Chek® Advantage® kit; Roche Diagnostics) and the rats were anesthetized again to allow SC insertion via a 16-gauge needle of a 2.3×2.0 mm sustained-release insulin or vehicle implant (Linplant®; Linshin Canada). The animals were housed individually in a temperature-controlled room with a 12:12 hr light-dark cycle, and provided ad libitum access to standard laboratory chow and water. Half of the rats in each group received chronic antioxidant therapy, achieved by addition of tempol \(4\)-hydroxy-TEMPO; Aldrich) to the drinking water. Sham+Tempol rats were provided 1 mmol/L tempol, while STZ+Tempol rats were provided 0.4 mmol/L tempol (to compensate for their polydipsic behavior). Blood glucose levels were measured twice weekly until terminal experiments were performed 26±1 days after STZ (or vehicle) injection.

In Vitro Blood-Perfused Juxtamedullary Nephron Technique
Afferent arteriolar function was monitored using the rat in vitro blood-perfused juxtamedullary nephron technique, according to previously described methods.\(^1\,^2\) Briefly, under sodium pentobarbital anesthesia \(50 \text{mg/kg IP}\), the right kidney was perfused with Tyrode's solution containing 52 g/L bovine serum albumin, a mixture of L-amino acids,\(^3\) and either 5.5 or 20 mmol/L D-glucose (for tissue from sham and STZ, rats, respectively). The left kidney was removed from the rat and flash-frozen. The right kidney was removed from the rat during continued Tyrode’s perfusion and a dissection procedure was performed to expose the tubules and microvasculature of juxtamedullary nephrons. Blood collected from the rat was processed and filtered as described previously,\(^1\,^2\) after which the Tyrode’s perfusate was replaced with the reconstituted blood. Perfusion pressure at the cannula tip in the renal artery was maintained at 110 mmHg throughout the experiment. The tissue was warmed to 37°C, and its surface was continuously bathed with Tyrode's solution containing 10 g/L bovine serum albumin, approximating the composition of renal interstitial fluid. Stock solutions containing vasoactive agents were stored at \(-20^\circ\text{C}\) until the day of the experiment, at which time they were diluted with Tyrode's bath to the appropriate final concentrations. Videometric techniques were used to measure arteriolar lumen diameter as previously described.\(^4\) Afferent arteriolar lumen diameter was monitored at a single measurement site (>100 µm upstream from the glomerulus and >80 µm downstream from the interlobular artery) under several experimental conditions, according to the protocols detailed below.

Protocol 1: Afferent arteriolar function was assessed in kidneys from Sham, Sham+Tempol, STZ, and STZ+Tempol rats. After a stabilization period, during which a single arteriole was selected for study, images of the arteriole were recorded during a 10 min baseline period (exposure to Tyrode’s bath alone). Subsequently, in some kidneys, the impact of \(K_{IR}\) channels on afferent arteriolar diameter was evaluated based on the effects of sequential exposure to Tyrode’s bath containing 10, 30 and 100 µmol/L \(\text{BaCl}_2\) (5 min each), followed by a 10 min recovery.
period (Tyrode’s bath alone). This procedure was typically followed by an assessment of the
tonic impact of Kir1.1/Kir3.x channels, based on the effects of sequential 5 min exposure to 1, 10
and 100 nmol/L Tertiapin-Q (TPQ; Tocris Bioscience).5

Protocol 2: In other kidneys (not exposed to Ba2+ or TPQ), the baseline period was followed by
an evaluation of the impact of KATP channels on afferent arteriolar function based on responses to
sequential exposure to 30, 100 and 300 μmol/L glibenclamide (5 min each).

For each protocol, the data were discarded if lumen diameter failed to return to within 10%
of baseline values during the 10 min recovery period following exposure to a K channel blocker.
The efficacy and specificity of the concentrations of Ba2+, TPQ and glibenclamide used in this
study have been addressed previously.4,6

Indices of Oxidative Stress
Dihydroethidium (DHE) staining was utilized to detect O2•− in kidneys from untreated and
tempol-treated rats, according to the method described by Zalba et al.7 Briefly, flash frozen
kidneys were cut into 14-μm thick sections, placed on glass slides and exposed to 10 μmol/L
DHE in the dark at 37°C for 30 min. A laser confocal imaging system (Zeiss LSM-510 Meta)
with a 585-nm long-pass filter was used to obtain DHE images of the sections. The images were
not manipulated except for cropping and superimposition of a scale bar. Average DHE staining
intensity in selected renal cortical structures was quantified using the image analysis capabilities
of Adobe® Photoshop® CS4 Extended (version 11.0.2).

Lipid peroxidation is a marker of cellular damage due to oxidative stress, and thiobarbituric
acid reactive substances (TBARS) indicate the presence of oxidized lipids. Therefore, TBARS
were quantified in urine and plasma samples using the OxiSelect™ TBARS Assay Kit (Cell
Biolabs, San Diego, CA) following the manufacturer’s directions. The protocol uses the reaction
between TBARS and malondialdehyde (MDA) compounds to form a MDA-RBA adduct which
can be measured colorimetrically. The samples and standards were processed in triplicate and
read at the absorbance of 532 nm. TBARS levels were determined from an MDA equivalence
standard, with the data expressed as μmol/L in plasma and μmol/day for urinary excretion.

The Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Eugene,
OR) was used to quantify H2O2 levels. Plasma and urine samples were prepared according to the
manufacturer’s instructions. Samples were measured in microtiter plates and fluorescence
intensity was measured (excitation=528 nm; emission=590 nm). Background-subtracted
fluorescence signals were compared with a standard curve and expressed as nmol/L in plasma
and nmol/day for urinary excretion.

Preglomerular Microvascular Smooth Muscle Cell Culture
Preglomerular microvascular smooth muscle cells (PVSMCs) from normal rat kidney were
cultured by the explant method as previously described.8 Briefly, rats were anesthetized with
pentobarbital sodium (50 mg/kg IP) and the abdominal aorta was cannulated to allow perfusion
of the kidneys with physiological saline solution (PSS, containing 100 U/mL penicillin and 100
mg/mL streptomycin), followed by 400 U/mL collagenase in Trypsin-EDTA (0.05%; Gibco),
then 2.5% Fe3O4 in PSS. The kidneys were removed, then the cortex was minced and transferred
to 1100 U/mL collagenase and 400 U/mL hyaluronidase in PSS and incubated (37°C, gentle
shaking, 30 min). The iron oxide-containing tissue was isolated from the suspension using a magnet, washed 4-5 times with ice cold PSS and incubated in 270 U/mL collagenase (37°C, gentle shaking, 10 min). The iron-laden tissue was again collected using the magnet, washed with cold PSS and inspected under the microscope to confirm that it consisted of only microvessels devoid of glomeruli. The microvessels were then cultured in DMEM containing 20% fetal calf serum (FCS), 100 U/mL penicillin and 100 U/mL streptomycin, as described by Endlich et al. Cultures were maintained at a temperature of 37°C with 85% humidity and at a CO₂ concentration of 5% in air, with the medium changed three times per week. PVSMCs at passage 3 were maintained in 5 or 20 mmol/L glucose for 2-3 weeks, then growth-arrested by exposure to serum-free conditions for 1 day prior to harvesting. The cells obtained by this method have been characterized as VSM based on morphological and immunostaining criteria.

RNA Extraction and Real-Time RT PCR
RNA was extracted from PVSMCs cultured in two 100 mm dishes, according to the manufacturer’s directions (Absolutely RNA Miniprep Kit, Stratagene). Briefly, cells were harvested at passage 3 using Trypsin-EDTA (0.05%; Gibco), followed by cell lysis. Next, the sample was pre-filtered in a spin cup to remove large contaminants and reduce the total amount of DNA. The filtrate was transferred to a second spin cup where the RNA was allowed to bind to the silica-based fiber matrix of the cup. This sample was subjected to a low-salt wash and digestion with DNase to remove any remaining DNA, followed by a second series of washes to remove the DNase and other protein contaminants. Finally, RNA was eluted from the fiber matrix with 30 μL of low-ionic-strength elution buffer. Total RNA was measured in a 2 μL sample using a Nanodrop (Thermo Scientific). Samples were stored at –80°C until real-time RT-PCR was performed.

The mRNA encoding various K⁺ channels was detected using real-time RT-PCR. These measurements were made using the Rotor Gene Real-Time Detection System with output to a computer-based acquisition system (Rotor Gene software). We employed a one-step protocol in which both reverse transcription (RT) of RNA and PCR take place in a single tube. The protocol (QuantiTect® SYBR® Green RT PCR handbook, Qiagen) consisted of reverse transcription (50°C for 30 min), denaturation (95°C for 15 sec), amplification and quantification repeated 45 times (95°C for 25 sec, 58°C for 25 sec, 72°C for 25 sec). For each reaction the mixture consisted of 2x QuantiTect SYBR Green RT-PCR master mix, 20 μmol/L sense primer, and 20 μmol/L antisense primer in RNase-free H₂O. Primers were designed using NCBI sequence database and using the published sequences via the Primer 3 web site (www.broad.mit.edu/cgi-bin/primer/primer3.cgi). Primers were designed to have the target sequences for quantification of copy number nested within the standard sequences at a length of 125–128 base pairs. The primer sets were as follows:

Kir1.1 >GCGAGACCATCATTTTGGAT <CATGTGGAAGAAAGGGCTGT
Kir2.1 >AACGTGGGAGAGAAAGGACA <CCAAAGAACAGCCAGGAGAG
Kir3.1 >CTGACCAGCTTCACATAGCAA <TTGCCAGACTGGGATAGACC
Kir3.4 >GAAGTAGCCTCAAGGGGTTC <TTGGGGATCTTTTGTGGTCT
Cortical Microvessel Isolation

Vessels were isolated from rat kidney by sieving, according to the following method. Rats were anesthetized with pentobarbital sodium (50 mg/kg IP) and the kidney was perfused with an experiment-specific rinse buffer to flush out blood. For western blotting, the buffer was cold Ca\(^{2+}\)- and Mg\(^{2+}\)-free HBSS containing 5 or 20 mmol/L glucose, for Sham and STZ rats, respectively. The kidney was removed, sliced longitudinally, and demedullated. The remaining cortex pieces were sieved over 250 \(\mu\)m nylon mesh and then again over 150 \(\mu\)m nylon mesh. The vessels were then rinsed by dental water jet and placed into cold collagenase (~1 mg/mL) and incubated at 37\(^\circ\)C while shaking for 20 min. After incubation the vessels were gently sieved again over the 150 \(\mu\)m nylon mesh to wash away the collagenase. Samples were then placed in microcentrifuge tubes containing cell lysis buffer for western blotting.

Western Blot

Protein concentrations in cortical microvessel lysates were measured using the BCA Protein assay (Pierce) using BSA as the standard. The samples were diluted into SDS loading buffer and heated to 95\(^\circ\)C for 5 min, vortexed, and then loaded onto a 4-15% gradient polyacrylamide gel (20 \(\mu\)g (Kir6.1), 40 \(\mu\)g (all other proteins) per well). Proteins were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore) at 300 mA for 90 min. Membranes were then dried and blocked for 1 hr with Odyssey™ blocking buffer (LI-COR Biosciences), and incubated overnight at 4\(^\circ\)C with primary antibody in Odyssey™ blocker with 0.1% Tween-20. The following primary antibodies were used: rabbit anti-Kir1.1 (Chemicon, AB5196; 1:200 dilution), rabbit anti-Kir2.1 (Alomone, APC-026; 1:100), rabbit anti-Kir6.1 (Santa Cruz, sc20808; 1:200), goat anti-SUR2B (Santa Cruz, sc5793; 1:200), mouse anti-\(\beta\)-actin (Abcam, ab627c; 1:200). The membranes were then washed 5-times in PBS + 0.1% Tween-20 at room temperature prior to incubation with the secondary antibody for 1 hr at room temperature. The use of secondary antibodies conjugated to an infrared dye allowed direct quantification using the OdysseyTM Infrared Imaging System (LI-COR), which can simultaneously detect two antigens on the same blot using secondary antibodies tagged with IRDye® 800 (800 nm wavelength) and AlexaFluor® 680 (680 nm wavelength). Molecular weights were estimated using the Odyssey Molecular Weight Marker. Negative controls included pre-adsorption of the primary antibody with a 5-fold excess of the appropriate blocking peptide or using commercially available fusion protein.

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