Nitric Oxide May Prevent Hypertension Early in Diabetes by Counteracting Renal Actions of Superoxide

Michael W. Brands, Tracy D. Bell, Bradford Gibson

Abstract—The dependence of blood pressure on a balance between superoxide and nitric oxide may be amplified in diabetes. We have shown that the first occurrence of sustained hyperglycemia in type I diabetes causes hypertension when induced in rats that have had nitric oxide synthesis blocked chronically (L-NAME, 10 μg/kg per minute IV). This study used tempol (18 μmol/kg per hour IV) to test the hypothesis that superoxide mediates that hypertensive response. Induction of diabetes in untreated rats had no significant effect on mean arterial pressure (MAP, measured 18 h/d), and glomerular filtration rate (GFR) increased significantly during the 2 weeks of diabetes. Chronic infusion of L-NAME in a separate group of rats increased baseline MAP from ≈90 mm Hg to a stable level of ≈120 mm Hg after 6 days of infusion, and induction of diabetes (streptozotocin, 40 mg/kg IV) in those rats caused a rapid, progressive increase in MAP that averaged 156±5 mm Hg by day 14 of diabetes that was associated with a decrease in GFR and 4-fold increase in isoprostane excretion. Tempol infusion was begun on day 2 of diabetes in a subgroup of those rats, and the progressive hypertensive response was prevented, with MAP averaging 134±10 mm Hg by day 14. In addition, the normal renal hyperfiltration response was restored by tempol and the increase in isoprostane excretion did not occur. Thus, the hypertension and decrease in GFR caused by onset of diabetes in rats without a functioning nitric oxide system was prevented by chronic administration of the superoxide dismutase mimetic tempol. (Hypertension. 2004;43:57-63.)

Key Words: blood pressure • glomerular filtration rate • diabetes mellitus • nitric oxide • L-NAME

There is good evidence for opposing actions of superoxide and nitric oxide in the chronic control of arterial pressure under physiological and pathophysiologic states. Most of this evidence suggests that there is a balance between the blood pressure–lowering effects of nitric oxide and the hypertensive actions of superoxide that involves direct and indirect chemical interaction as well as physiological interaction at effector sites. Shifts in that balance can have wide-ranging effects, and there could be a more critical and tenuous balance between the two in the control of blood pressure in diabetes because of the neurohumoral and direct effects of hyperglycemia and the added stress imparted by renal fluid and electrolyte losses caused by poor glycemic control. Studying the interaction between nitric oxide and superoxide has been complicated, however, by evidence that production of both may be increased in diabetes but that nitric oxide synthesis becomes impaired over time.

We recently tested the hypothesis that nitric oxide was critical for preventing hypertension very early in diabetes. This was based on our previous work that suggested endothelium-dependent vasodilation was not impaired during the first week of type I diabetes and that angiotensin (Ang) II increased significantly during that same period. We found that blocking nitric oxide synthesis chronically with L-NAME caused induction of diabetes to increase mean arterial pressure (MAP) markedly. In addition, the increase in glomerular filtration rate (GFR) that occurred after onset of diabetes in untreated rats was prevented, and there was a progressive rather than transient increase in plasma renin activity (PRA). Because both diabetes and Ang II have been shown to stimulate superoxide and because superoxide may induce renal vasoconstriction, this study tested the hypothesis that superoxide mediates the hypertension and renal vasoconstriction caused by onset of diabetes in L-NAME–treated rats.

Methods

The experiments were conducted in male Sprague-Dawley rats (weight, 325 to 350 g, Harlan Sprague-Dawley), and the protocols were approved by the Institutional Animal Care and Use Committee. Anesthesia was induced with sodium pentobarbital (50 mg/kg IP), and atropine (40 μg IP per rat) was administered to ensure an unobstructed airway. Under aseptic conditions, an artery and vein catheter were implanted as described previously and were routed subcutaneously to the scapular region and exteriorized. After recovery, the rats were placed in individual metabolic cages and the catheters were connected to a dual-channel hydraulic swivel (Instech) mounted above the cage. The venous catheter was connected through the swivel to a syringe pump (Harvard Apparatus)
that ran continuously throughout the study. Sodium intake throughout the experiment was controlled by continuous intravenous infusion of 22 mL of sterile 0.9% saline per day, combined with sodium deficient rat chow (0.006 mmol sodium/g; Teklad). This enabled precise control of baseline sodium balance that enhanced the accuracy of cumulative sodium balance measurements. However, rats were allowed to increase sodium intake during diabetes by drinking tap water (3.7 μEq Na+/mL). The arterial catheter was filled with heparin solution (1000 USP U/mL) and connected, also through the swivel, to a pressure transducer (Cobe) mounted on the cage exterior at the level of the rat. Pulsatile arterial pressure signals were amplified and sampled continuously at 100 Hz, using PowerLab from noon to 6 AM (18 hours) every day.

Experimental Protocol

The rats were divided randomly into 4 diabetic groups (D) and one L-NAME–treated group (L), in which diabetes was not induced. After baseline measurements to ensure stable blood pressure and sodium balance, L-NAME (10 μg/kg per minute IV) was added to the infusion of the L rats and to two of the diabetic groups and maintained throughout the remainder of the study. Six days later, streptozotocin (40 mg/kg IV) was administered to the 4 diabetic groups, and after the first day of diabetes, the superoxide dismutase mimetic 4-hydroxy-tempo (tempol, 18 μmol/kg per hour IV) was added to the infusate of two of the diabetic groups. The diabetic groups, therefore, were divided into (1) diabetes only (D, n=8), (2) diabetes plus tempol (DT, n=5), (3) diabetes plus L-NAME (DL, n=12), and (4) diabetes plus L-NAME and tempol (DLT, n=9). On day 4 of the control period and once per week (days 5 to 6) during the 2-week diabetic period, 1 mL of arterial blood was collected from the arterial catheter for measurement of GFR, hematocrit, and plasma electrolyte and glucose concentrations. Samples were replaced with an equal volume of 0.9% saline.

Analytical Methods

GFR was measured after a 24-hour intravenous infusion of [125I]iothalamate (Glofil, ~20 μCi/kg per day). Because steady state is achieved during the 24-hour infusion, the isotope infusion rate was substituted for urinary isotope excretion rate to calculate clearance. Urinary sodium and potassium concentrations were determined with the use of ion-sensitive electrodes (Synchron El-ise, Beckman-Coulter). Urine from a subset of the rats was analyzed for 8-isoprostane (an index of superoxide) bicyclo-PGE2 (an index of PGE2) and 2,3-dinor thromboxane B2 (an index of thromboxane A2) concentrations, using EIA kits from Cayman Chemical. Data were analyzed by 2-factor ANOVA with repeated measures, using JMP 5 software (SAS Institute, Inc). Tukey honestly significant difference comparisons from the ANOVA were used for supplemental between-group comparisons, and the Dunnett t test was used for within-group changes over time. A value of P<0.05 was considered statistically significant, and data are presented as mean±SEM.

Results

Mean arterial pressure was not different among groups during the prediabetic control period, and chronic L-NAME treatment increased MAP similarly in the DL, DLT, and L groups (Figure 1). The onset of diabetes immediately began to increase MAP in the DL and DLT groups, increasing from (Figure 1). The onset of diabetes immediately began to increase MAP in the DL and DLT groups, increasing from 127±3 to 133±5 mm Hg on the first day of diabetes in the DL rats and from 121±4 to 127±5 mm Hg on the respective days in the DLT rats. MAP continued to increase throughout the 14-day diabetic period in the DL rats, becoming statistically greater than control by day 4 and averaging 156±5 mm Hg on day 14. The addition of tempol to the DLT rats on day 2 of diabetes, however, completely prevented this effect, and MAP in the DLT rats remained not different from MAP in the nondiabetic, L-NAME–treated rats (L) throughout the 2-week period. Figure 2 shows that this blood pressure effect was related closely to the changes in urinary 8-isoprostane excretion, which increased approximately 4-fold in the DL rats but did not change in the DLT rats. This is consistent with the effect of similar doses of tempol on isoprostane excretion reported by other laboratories. Thus, the progressive increases in MAP and urinary 8-isoprostane excretion caused by onset of diabetes in L-NAME–treated rats were prevented by chronic tempol administration. Tempol had no measurable effect, however, in the diabetic rats not treated with L-NAME (D versus DT).

Figure 3 shows the increases in GFR that occurred in the untreated diabetic rats (D) and the DT rats over the 2-week diabetic period. In addition, the figure shows that L-NAME–treated diabetic rats (DL) were not able to increase GFR during diabetes, which is consistent with our earlier observations. However, when those rats were treated with tempol (DLT), GFR increased significantly during the diabetic period.

The changes in cumulative sodium balance were consistent with these changes in GFR and MAP. Figure 4 shows that the L rats, which were not diabetic, had no significant changes in sodium balance during the study period, whereas sodium balance decreased significantly in the D, DT, and DLT diabetic groups. Urinary sodium excretion at baseline was not different among any groups, averaging 3.0±0.1, 2.9±0.1, 3.1±0.1, 3.2±0.1, and 3.2±0.1 mmol/d in the D, DT, DLT, DL, and L groups, respectively. The increase in sodium excretion caused by onset of diabetes is reflected in the progressive decrease in cumulative sodium balance shown in Figure 4, and a gradual increase in sodium intake (from the drinking water, Table 1) combined with some attenuation of...
the initial natriuretic surge brought the diabetic rats back into
daily balance, while remaining negative absolute. It is impor-
tant to note, however, that the decrease in cumulative sodium
balance was not statistically significant in the DL rats, which
was the diabetic group that did not have an increase in GFR
and that became hypertensive during the diabetic period. The
changes in hematocrit and plasma protein concentrations
tended to reflect the sodium balance, suggesting hemocon-
centration, but this was significant only for hematocrit in the
D and DT rats (Table 2).

Figure 5 shows the changes in bicyclo-PGE\(_2\) (PG) and
2–3,dinor thromboxane B\(_2\) (TX). The increases in PG and
TX excretion caused by onset of diabetes confirm our
earlier observations,\(^\text{14}\) but it is noteworthy that our previ-
ous study\(^\text{14}\) measured significant increases in 6-keto PGF\(_{1a}\)
and TXB\(_2\), indexes of PGI\(_2\) and renal TXA\(_2\) production,
respectively, whereas no differences were measured in any
group at any time point in the present study (data not
shown). The measurements were all made from the same
urine aliquot and analyzed on the same day; PGE\(_2\) and
2–3,dinor TXB\(_2\) were not measured previously,\(^\text{14}\) so the
explanation for this difference between studies is not clear.
Comparing the changes in the D versus DL rats suggests
that L-NAME did not affect the increase in PG caused by
diabetes but prevented the increase in TX. Interestingly,
tempol did not affect the diabetes-induced increase in TX
(DT versus D) but reversed the effect of L-NAME to
prevent the increase in TX excretion.

Discussion
The main finding from this study is that the hypertension
cau sed by onset of diabetes in rats without a functioning nitric
The role of nitric oxide in renal and cardiovascular control in diabetes is controversial. There is good evidence that diabetes impairs endothelial function, and the proposed mechanisms include inhibition of nitric oxide synthase and quenching of nitric oxide by superoxide. The latter mechanism fits with the evidence that nitric oxide production may be increased in our model, these findings support our hypothesis that there is increased dependence on nitric oxide for the maintenance of normal blood pressure at the onset of diabetes. The effect of tempol that we measured suggests, in turn, that an important action of nitric oxide in that regard is to oppose a hypertensive influence of superoxide.

The mechanism through which superoxide, or oxygen-derived free radicals in general, promotes hypertension is not known, but there is good evidence that direct interference with nitric oxide is an important mechanism. Consistent with that, we have reported that endothelium-mediated dilation is not impaired at the onset of type I diabetes. In addition, when the nitric oxide system is blocked chronically with L-NAME, onset of diabetes causes a marked, progressive increase in MAP, as shown by the DL rats in the present study and previously by our laboratory. Although we still need to determine whether nitric oxide production actually is increased in our model, these findings support our hypothesis that there is increased dependence on nitric oxide by superoxide. The latter mechanism fits with the evidence that nitric oxide production may be enhanced as well, and quenching of nitric oxide by superoxide.

### TABLE 1. Food, Water, and Sodium Intake During the Prediabetic Period and the 2 Weeks of Diabetes

<table>
<thead>
<tr>
<th>Variable</th>
<th>Prediabetic</th>
<th>Diabetes Week 1</th>
<th>Diabetes Week 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food intake, g/d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL</td>
<td>17 ± 1</td>
<td>17 ± 2</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>DLT</td>
<td>20 ± 1</td>
<td>20 ± 1</td>
<td>30 ± 2*</td>
</tr>
<tr>
<td>D</td>
<td>19 ± 1</td>
<td>19 ± 1</td>
<td>30 ± 3*</td>
</tr>
<tr>
<td>DT</td>
<td>19 ± 1</td>
<td>20 ± 2</td>
<td>35 ± 2*</td>
</tr>
<tr>
<td>L</td>
<td>20 ± 1</td>
<td>18 ± 1</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>Water intake, mL/d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL</td>
<td>14 ± 1</td>
<td>83 ± 15*</td>
<td>126 ± 6*</td>
</tr>
<tr>
<td>DLT</td>
<td>19 ± 1</td>
<td>87 ± 17*</td>
<td>194 ± 8*</td>
</tr>
<tr>
<td>D</td>
<td>15 ± 1</td>
<td>83 ± 15*</td>
<td>167 ± 6*</td>
</tr>
<tr>
<td>DT</td>
<td>12 ± 1</td>
<td>96 ± 25*</td>
<td>219 ± 18*</td>
</tr>
<tr>
<td>L</td>
<td>11 ± 1</td>
<td>11 ± 1</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>Sodium (Na+) intake, mmol/d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL</td>
<td>3.4 ± 0.0</td>
<td>3.7 ± 0.1*</td>
<td>3.8 ± 2.0*</td>
</tr>
<tr>
<td>DLT</td>
<td>3.6 ± 0.0</td>
<td>3.9 ± 0.1*</td>
<td>4.3 ± 0.0*</td>
</tr>
<tr>
<td>D</td>
<td>3.3 ± 0.0</td>
<td>3.5 ± 0.1*</td>
<td>3.9 ± 0.0*</td>
</tr>
<tr>
<td>DT</td>
<td>3.3 ± 0.0</td>
<td>3.6 ± 0.1*</td>
<td>4.1 ± 0.1*</td>
</tr>
<tr>
<td>L</td>
<td>3.6 ± 0.0</td>
<td>3.6 ± 0.0</td>
<td>3.6 ± 0.0</td>
</tr>
</tbody>
</table>

DL indicates diabetes plus L-NAME; DLT, diabetes plus L-NAME and tempol; D, diabetes only; DT, diabetes plus tempol; and L, L-NAME–treated group.

### TABLE 2. Hematocrit and Plasma Variables During the Prediabetic Period and the 2 Weeks of Diabetes

<table>
<thead>
<tr>
<th>Variable</th>
<th>Prediabetic</th>
<th>Diabetes Week 1</th>
<th>Diabetes Week 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL</td>
<td>43 ± 0</td>
<td>45 ± 1</td>
<td>46 ± 1</td>
</tr>
<tr>
<td>DLT</td>
<td>43 ± 1</td>
<td>45 ± 1</td>
<td>44 ± 1</td>
</tr>
<tr>
<td>D</td>
<td>43 ± 1</td>
<td>48 ± 1*</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>DT</td>
<td>41 ± 1</td>
<td>44 ± 1*</td>
<td>43 ± 1*</td>
</tr>
<tr>
<td>L</td>
<td>42 ± 0</td>
<td>43 ± 1</td>
<td>43 ± 1</td>
</tr>
<tr>
<td>Plasma protein, g/dL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL</td>
<td>6.8 ± 0.1</td>
<td>7.1 ± 0.1</td>
<td>7.3 ± 0.2</td>
</tr>
<tr>
<td>DLT</td>
<td>6.4 ± 0.1</td>
<td>6.7 ± 0.0</td>
<td>7.1 ± 0.2</td>
</tr>
<tr>
<td>D</td>
<td>6.3 ± 0.1</td>
<td>7.1 ± 0.3</td>
<td>6.9 ± 0.2</td>
</tr>
<tr>
<td>DT</td>
<td>6.4 ± 0.1</td>
<td>7.0 ± 0.3</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td>L</td>
<td>6.1 ± 0.1</td>
<td>6.3 ± 0.1</td>
<td>6.3 ± 0.1</td>
</tr>
<tr>
<td>Plasma [Na+], mmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL</td>
<td>144 ± 0.8</td>
<td>142 ± 0.9</td>
<td>143 ± 2.1</td>
</tr>
<tr>
<td>DLT</td>
<td>145 ± 1.7</td>
<td>148 ± 2.0</td>
<td>144 ± 2.1</td>
</tr>
<tr>
<td>D</td>
<td>143 ± 0.7</td>
<td>147 ± 0.9</td>
<td>138 ± 1.9</td>
</tr>
<tr>
<td>DT</td>
<td>147 ± 1.2</td>
<td>146 ± 1.7</td>
<td>138 ± 2.8</td>
</tr>
<tr>
<td>L</td>
<td>148 ± 0.5</td>
<td>148 ± 3.9</td>
<td>144 ± 1.1</td>
</tr>
<tr>
<td>Plasma glucose, mg/dL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL</td>
<td>107 ± 2</td>
<td>442 ± 27*</td>
<td>392 ± 52*</td>
</tr>
<tr>
<td>DLT</td>
<td>91 ± 2</td>
<td>354 ± 34*</td>
<td>357 ± 57*</td>
</tr>
<tr>
<td>D</td>
<td>114 ± 1</td>
<td>323 ± 47*</td>
<td>441 ± 46*</td>
</tr>
<tr>
<td>DT</td>
<td>107 ± 4</td>
<td>433 ± 28*</td>
<td>501 ± 10*</td>
</tr>
<tr>
<td>L</td>
<td>107 ± 4</td>
<td>100 ± 4</td>
<td>109 ± 5</td>
</tr>
</tbody>
</table>

*P < 0.05 vs prediabetic values.
Superoxide and Hypertension in Diabetes

Although the beneficial blood pressure effect of tempol in the L-NAME-treated rats suggests superoxide raised blood pressure by mechanisms other than through quenching of nitric oxide, the fact that we measured increased isoprostane excretion only in the DL rats suggests, on the other hand, that quenching of superoxide may be an important mechanism of action of nitric oxide early in diabetes. No significant change in the L rats (the L-NAME-treated rats in which diabetes was not induced) suggests that the increased isoprostane levels in the DL rats was not a consequence of NO synthase inhibition per se but was due to induction of diabetes under conditions in which NO was absent. Thus, the similar isoprostane excretions in the D versus DT rats may be evidence that increased nitric oxide production when diabetes is induced may quench superoxide. Majid et al. proposed that nitric oxide may play an important role to counteract superoxide mediated renal vasoconstriction, and we speculate similarly that an important action of nitric oxide early in diabetes is to counteract superoxide, but possibly by preventing increases in superoxide rather than, or perhaps in addition to, physiological antagonism of superoxide actions at effector sites.

We believe that one key effector site for superoxide early in diabetes may be the renal vasculature. We consistently report that GFR increases significantly during the first 3 weeks of diabetes, and MAP may or may not increase modestly in normal rats. However, in L-NAME-treated rats, not only is the hyperfiltration attenuated or prevented, but GFR actually tends to decrease during the diabetic period. The DL rats in the present study showed a similar GFR response, and we hypothesize this is because nitric oxide synthesis was blocked chronically with L-NAME; in other words, this suggests that superoxide may have increased blood pressure in the L-NAME-treated DL rats by a nitric oxide-independent mechanism. This is consistent with data from Majid et al. and Zou et al. showing significant superoxide-mediated renal vasoconstriction in the absence of nitric oxide. The mechanism for superoxide-mediated vasoconstriction per se is not known and was not the focus of this study, but there is evidence that superoxide can affect thromboxane A2/PGH2 receptor signaling and other cell-signaling pathways in vascular smooth muscle to cause vasoconstriction. In addition, Chen et al. reported that oxidative stress in the kidney increases production of adenosine, which is a powerful renal vasoconstrictor. The superoxide dismutase mimetic action of tempol also can lead to increased cyclooxygenase activity through metabolism of hydrogen peroxide, and this may explain the increase in PG excretion that we measured in the tempol-treated rats. Interestingly, although the increase in PG excretion in the tempol groups suggests it could have played a role in the restored renal vasodilation and attenuated hypertension in the DL rats, neither TX excretion nor the PG to TX ratio correlates with the blood pressure responses in the different groups. However, additional studies that include synthesis and/or receptor blockade will be needed to determine a more precise role for prostanoids.

Figure 5. Urinary bicyclo-PGE2 (top panel) and 2,3-dinor TXB2 (bottom panel) excretion in the 5 groups of rats during the pre-L-NAME (control) and prediabetic (L-NAME) periods and during the middle of each of the 2 weeks of diabetes. *P<0.05 vs prediabetic values within group.
edly, and our data suggest this is due, at least in part, to significant increases in superoxide. In addition, because GFR did not increase in the hypertensive rats with high urinary isoprostane excretion but did increase significantly in the rats in which tempol prevented the increase in blood pressure and isoprostane excretion, we hypothesize that GFR may play a significant role in blood pressure control early in diabetes through control of sodium balance. It is important to note that we study the period immediately after induction of diabetes in order to determine the cardiovascular and renal consequences of hyperglycemia before there has been time for significant renal injury to develop. We cannot rule out a contribution of renal injury to blood pressure in the later stages of the 2-week diabetic period, but the significant responses measured at the onset of diabetes in our model support the importance of functional effects of hyperglycemia and the important roles of nitric oxide and superoxide.

**Perspectives**

We and others have shown that PRA increases significantly during the first week of diabetes, and chronic L-NAME treatment amplifies and prolongs that response. We have a working hypothesis that an increase in GFR under these conditions actually is required to keep blood pressure from increasing. Thus, as long as nitric oxide synthesis is not impaired early in diabetes, the onset of hyperglycemia increases Ang II and GFR, and the balance between these factors maintains relatively normal blood pressure. Without nitric oxide, however, GFR does not increase, and the present results suggest that this is caused by superoxide. The hypertension also is Ang II–dependent, but the link between superoxide and the increase in Ang II that we measure remains to be established in our model. It should be noted that although this hypothesis linking GFR to blood pressure control at the onset of diabetes is consistent with our data, it remains largely speculative. It also is recognized that glomerular hyperfiltration contributes significantly to progressive renal injury in diabetes, and this may be a situation, therefore, in which a short-term physiological response becomes pathologic when activated chronically. Our main findings, however, do suggest that the control of GFR and arterial pressure at the onset of diabetes depends on nitric oxide synthesis and that the hypertension and renal vasoconstriction that ensue without nitric oxide are superoxide-dependent.

**Acknowledgments**

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**References**


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