Evidence for a Causal Role of the Renin-Angiotensin System in Nitrate Tolerance

Sabine Kurz, MD; Ulrich Hink, MD; Georg Nickenig, MD; Alain B. Borthayre, BS; David G. Harrison, MD; Thomas Münzel, MD

Background—We have previously shown that nitroglycerin (NTG) therapy increases vascular expression of endothelin 1 (ET-1) and stimulates vascular superoxide (O$_2^-$) production via activation of NADH/NADPH oxidases. Both phenomena are stimulated by angiotensin II in vitro, and the renin-angiotensin system is activated during early nitrate therapy. We hypothesized that either angiotensin II or ET-1 may increase vascular O$_2^-$ production during nitrate therapy.

Methods and Results—In New Zealand White rabbits, 3 days of treatment with NTG patches increased plasma renin activity for the entire treatment period. After 24 hours of NTG treatment, angiotensin II type 1 (AT$_1$) receptor expression and vascular ACE activity were significantly decreased. At this time, constrictions to angiotensin I and II were depressed, but there was no loss of NTG vasodilator potency. Within 3 days of continuous NTG treatment, relaxations to NTG were markedly blunted. This was associated with an increase in AT$_1$ receptor mRNA expression, a return of ACE activity back to baseline, and a marked increase in constrictions to angiotensin I and II despite continuously increased plasma renin activity. Tolerance was associated with a 2-fold increase in vascular O$_2^-$, as estimated by lucigenin-enhanced chemiluminescence. Concomitant treatment with the AT$_1$ receptor antagonist losartan (5 to 25 mg · kg$^{-1}$ · d$^{-1}$) dose-dependently normalized vascular O$_2^-$ and prevented tolerance to NTG and cross-tolerance to endogenous nitric oxide released by acetylcholine. The nonselective ET-1 receptor blocker bosentan (100 mg · kg$^{-1}$ · d$^{-1}$) had similar but less pronounced effects.

Conclusions—The positive effects of AT$_1$ and ET-1 receptor blockade on tolerance and O$_2^-$ production imply a pathophysiological role for angiotensin II and to some extent for ET-1 in the development of nitrate tolerance.

Key Words: angiotensin II receptors • losartan • bosentan • chemiluminescence • nitric oxide

The usefulness of organic nitrates is limited by tolerance, which develops shortly after onset of treatment. The mechanisms underlying nitrate tolerance remain poorly defined but are most likely multifactorial. An early adaptation to long-term nitrate therapy involves neurohumoral adjustments, including activation of the renin-angiotensin system, retention of sodium and water, and volume redistribution. These impair the vasodilator and preload-reducing effects of the organic nitrates. Recently, we found that 3 days of nitrate treatment doubled vascular superoxide (O$_2^-$) production. This increased vascular O$_2^-$ decreases the vasodilation produced by nitric oxide released from nitroglycerin (NTG), other nitrovasodilators, and endogenously by acetylcholine, because treatment with cell-permeant forms of superoxide dismutase corrected these defects. Subsequent studies of vascular homogenates suggested that the source of O$_2^-$ in these vessels is a membrane-bound NADH oxidase. Increased vascular O$_2^-$ production was observed only in vessels removed from rabbits treated with NTG, but not in vessels treated in vitro with even high concentrations of NTG. This observation suggests that in vivo adjustments to nitrate therapy may be important in modulating vascular O$_2^-$ production. One prominent response to the hypotension and chronic vasodilation caused by NTG is stimulation of the plasma renin-angiotensin system, which could in turn activate the NADH oxidase in vascular cells. Furthermore, recent experimental and clinical studies suggest that concomitant treatment with high-dose angiotensin I–converting enzyme (ACE) inhibitors prevent nitrate tolerance.

Taken together, these lines of evidence imply that the renin-angiotensin system may contribute to nitrate tolerance. In the present study, we sought to characterize changes in the circulating and local renin-angiotensin system that might participate in vascular adaptations to long-term NTG therapy.

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From the Department of Medicine, Emory University School of Medicine, and Veterans Administration Hospital (S.K., A.B.B., D.G.H.), Atlanta, Ga; and the Cardiology Division, Albert Ludwig University, Freiburg (S.K.); Eppendorf University, Hamburg (U.H., T.M.); and University of Cologne, Division of Cardiology (G.N.), Germany.

Correspondence to Thomas Münzel, MD, Abteilung für Kardiologie, Universität-Es-Krankenhaus Eppendorf, Martinistraße 52, D-20246 Hamburg, Germany. E-mail muenzel@uke.uni-hamburg.de

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Second, we tested the hypothesis that concomitant treatment with the angiotensin II type 1 (AT$_1$) receptor antagonist losartan could prevent NTG-induced increases in vascular O$_2^-$ production and nitrate tolerance. Because a portion of the effect of angiotensin II is mediated by increasing vascular endothelin (ET-1) secretion and expression, we also examined the effect of ET-1 receptor blockade on nitrate tolerance and vascular O$_2^-$ production.

**Methods**

**Animal Model**

New Zealand White rabbits of either sex (weight, 3 to 6 kg) were studied. Rabbits were treated with NTG patches as described recently and were studied either 24 or 72 hours later. On the morning of the study day, an intravenous injection of 1000 U heparin was administered, followed by a lethal dose of pentobarbital. The chest was then rapidly opened and the descending thoracic aorta removed.

Three protocols were followed. In one, we examined the time course of nitrate tolerance and the effects of NTG on various components of the renin-angiotensin system. Separate groups of rabbits were studied for vascular responsiveness (n=5 each for 0, 24, and 72 hours, respectively), AT$_1$ receptor mRNA expression (n=8, 7, and 8 for 0, 24, and 72 hours, respectively), plasma renin activity (6 controls and 6 NTG-treated), and vascular ACE activity (5, 4, and 4 for 0, 24, and 72 hours, respectively).

In a second protocol, we examined the effect of the AT1 receptor antagonist losartan on vascular responsiveness and O$_2^-$ production. Rabbits were treated with an NTG patch and losartan (DuPont/Merck Inc), 5, 10, or 25 mg · kg$^{-1}$ · d$^{-1}$, added to their drinking water.

In a previous study, we found that ET-1 expression was markedly increased in aortas of rabbits with NTG tolerance. In a third protocol, therefore, we examined the effects of the nonselective ET1 receptor antagonist bosentan (Hoffmann La Roche) on nitrate tolerance and vascular O$_2^-$ production. Four groups (n=5 to 6 each) were studied: control, control + bosentan, NTG-treated, and NTG-treated + bosentan. Bosentan 100 mg · kg$^{-1}$ · d$^{-1}$ in the drinking water was given 2 days before and throughout NTG treatment (total, 5 days).

**Determination of Plasma Renin Activity**

Rabbits were briefly anesthetized with xylazine and ketamine (0.2 and 30 mg/kg, respectively). A 22-gauge Teflon catheter (Angiocath, Becton Dickinson) was inserted percutaneously into the central ear artery and secured into place with tape. The rabbits recovered overnight, and NTG therapy was started the following morning. Before onset of NTG therapy and on days 1 and 3, the rabbits were placed in a Lucite restraining device and allowed to acclimate for 30 minutes. Blood (3 ml) was drawn from the indwelling catheter and placed in tubes containing EDTA. Plasma renin activity was measured as previously described.

**Vessel Preparation and Organ Chamber Experiments**

In organ chambers, relaxations to either NTG 1 nmol/L to 3 μmol/L or acetylcholine 1 nmol/L to 3 μmol/L were examined after the vessels had been submaximally constricted with phenylephrine, as previously described. Constrictions to angiotensin II were also examined and expressed as a percentage of contractions to 80 mmol/L KCl.

**Estimation of Vascular O$_2^-$ Production**

O$_2^-$ production in vascular tissue was estimated by use of lucigenin-enhanced chemiluminescence, as published previously.

**Table 1. Plasma Renin Activity in Control Animals and Animals Treated with NTG for 24 and 72 Hours, Respectively**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NTG-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>1.9±0.6</td>
<td>2.8±0.9</td>
</tr>
<tr>
<td>24 hours</td>
<td>1.1±0.2</td>
<td>4.2±1.6</td>
</tr>
<tr>
<td>72 hours</td>
<td>2.5±1.2</td>
<td>5.5±1.4*</td>
</tr>
</tbody>
</table>

Data from 6 experiments are presented as mean±SEM. *P<0.05 vs control.

**Northern Analysis**

After vessel excision and removal of the adventitial tissue, the aortas were quickly frozen in liquid nitrogen. RNA was extracted with TRI reagent according to the manufacturer’s protocol to obtain total cellular RNA. RNA was quantified spectrophotometrically by measurement of absorbance values at 260 and 280 nm. Aliquots (10 μg) were electrophoresed through 1.2% agarose/0.67% formaldehyde gels and stained with ethidium bromide to verify the quantity and quality of the RNA. After transfer to Hybond N membranes, Northern analysis of AT$_1$ receptor mRNA was performed as previously described.

**Determination of Vascular ACE Activity**

Rabbit aortas were homogenized with a glass–glass homogenizer in ACE buffer (containing 0.05 mol/L HEPES, pH 7.5, 0.1 mol/L NaCl [vol/vol], and Triton X-100). After short-term low-speed centrifugation (3000 g, 2 minutes), ACE activity was measured with an assay based on the hydrolysis of L-histidyl l-phenylalanyl-pro (Ventrex Laboratories) by the protocol previously described. Samples were assayed for total cellular protein by the method of Bradford, and identical protein concentrations were adjusted to yield results in the linear range of substrate utilization (3 to 30 μg protein per assay). Activity calculations were based on Michaelis-Menten first-order kinetics. ACE activity was expressed as hydrolytic activity/μg protein that could be inhibited by 1 μmol/L ramiprilat (Upjohn Inc), in arbitrary enzymatic units. These units represent percent utilization of substrate hydrolyzed per microgram protein. Because the calculation used for ACE activity is logarithmic, a value of 3.0 U/μg is equivalent to 0.5 pmol substrate hydrolyzed/μg protein. Each assay was done in triplicate.

**Statistical Analysis**

Results are expressed as mean±SEM. The ED$_{50}$ value for each experiment was obtained by logit transformation. Comparisons of vascular responses (ED$_{50}$ and maximal percent relaxation), O$_2^-$ production, and angiotensin II production, and angiotensin II receptor mRNA levels were performed by use of ANOVA. When significance was indicated, a Student-Newman-Keuls post hoc analysis was used to indicate between-group differences. Plasma renin activity was compared by paired t tests. Vessel ACE activity and vascular AT$_1$ receptor expression were compared among groups by unpaired t tests with Bonferroni corrections. Significance was assumed at a value of P<0.05.

**Results**

**Effects of NTG Treatment on Plasma Renin Activity**

In control rabbits, plasma renin activity remained constant for the 3 days of observation. In contrast, plasma renin activity was increased by nitrate therapy and remained elevated throughout the treatment period (Table 1).

**Effects of NTG Treatment on Vascular ACE Activity**

By examining ACE activity in vessels with and without endothelium, it was possible to determine the relative contributions of...
the endothelium and vascular smooth muscle to total ACE activity. The activity of nonendothelial ACE was not changed by nitrate therapy. In contrast, endothelial ACE activity was decreased by 50% at 24 hours and returned to baseline 72 hours after nitrate treatment was begun (Figure 1).

Effects of NTG Treatment on Vascular AT₁ Receptor Expression
Twenty-four hours of NTG treatment significantly decreased aortic AT₁ receptor mRNA expression (P<0.01, Figure 2). Three days after onset of NTG treatment, AT₁ receptor mRNA expression had increased 2-fold compared with the values at 24 hours (P<0.01, Figure 2a and 2b). GAPDH mRNA expression was not altered by NTG treatment. Thus, despite persistently elevated plasma renin activity, vascular ACE activity and AT₁ receptor mRNA levels returned to values equal to or above control values after 3 days of nitrate therapy.

Effects of NTG Treatment on Vascular Reactivity
Twenty-four hours after initiation of NTG therapy, contractions induced by both angiotensin I and angiotensin II were shifted rightward, whereas relaxations to NTG were unaltered (Figure 3). In marked contrast, 3 days after onset of NTG treatment, relaxations to NTG were markedly depressed, indicating the presence of nitrate tolerance. At this time, responses to angiotensin I and II had changed dramatically from those observed 1 day after onset of treatment and were now substantially enhanced compared with baseline responses before the onset of nitrate therapy (Figure 3, Table 2).

Effects of Losartan Treatment on Relaxations to NTG and Acetylcholine
As previously demonstrated, treatment for 3 days with NTG markedly impaired relaxations to NTG (Figure 4, left). Treatment with 5 mg · kg⁻¹ · d⁻¹ of losartan improved relaxations to NTG, whereas treatment with either 10 or 25 mg · kg⁻¹ · d⁻¹ of losartan completely normalized peak relaxations to NTG. There were no differences in the ED₅₀ to NTG between any of the groups studied (Table 3).

Percent peak relaxations and sensitivity (as reflected by the ED₅₀) to acetylcholine were also impaired in nitrate-treated vessels (P<0.01, Figure 4, right). Treatment with either 5, 10, or 25 mg · kg⁻¹ · d⁻¹ completely normalized relaxations to acetylcholine in rabbits receiving NTG.

In rabbits not receiving NTG, treatment with even the highest dose of losartan (25 mg · kg⁻¹ · d⁻¹) had no effect on relaxations to acetylcholine. Relaxations to NTG were slightly but not significantly enhanced by losartan treatment in these vessels.

Effects of Losartan on Vascular O₂⁻ Production
As demonstrated previously, O₂⁻ production was increased 2-fold in tolerant aortic segments with intact endothelium (P<0.05, Figure 5). Losartan treatment decreased O₂⁻ production modestly in vessels from rabbits not receiving NTG. Importantly, in NTG-treated rabbits, losartan dose-dependently reduced vascular O₂⁻ production. Losartan 10 mg · kg⁻¹ · d⁻¹ completely normalized vascular O₂⁻ levels observed in untreated rabbits, and 25 mg · kg⁻¹ · d⁻¹ reduced vascular O₂⁻ production below levels attained by untreated
rabbits. In vitro, incubation of tolerant tissue with losartan (10^{-2} \text{ mol/L for 30 minutes, n=4}) did not significantly alter the lucigenin-enhanced chemiluminescence signal (1245 ± 152 versus 1148 ± 122 cpm/mg).

**Effect of Bosentan Treatment on Vascular Relaxations to NTG and O}_2^- Production**

Cotreatment with bosentan slightly but significantly increased sensitivity to NTG in vessels from NTG-tolerant animals (ED50), whereas it had no effect on relaxations to NTG in rings from animals not pretreated with NTG patches (Figure 6A). Maximal responses to NTG were similar in all 4 treatment groups.

Treatment of control rabbits with bosentan did not affect vascular O}_2^- production (1145 ± 63 versus 854 ± 115 cpm/mg, Figure 6B). In the setting of tolerance, lucigenin-enhanced chemiluminescence signal was increased 2-fold (2126 ± 546 cpm/mg). Concomitant treatment with bosentan significantly decreased O}_2^- production to 1547 ± 154 cpm/mg dry weight (P<0.05, Figure 6B). This value remained significantly higher than that observed in controls, both treated and untreated with bosentan.

**Discussion**

The therapeutic efficacy of organic nitrates is rapidly blunted during continuous treatment with these agents.\(^{3,15}\) It is likely that this phenomenon is multifactorial and involves both direct effects on the vasculature and systemic adaptations that counteract NTG-induced vasodilation. These systemic responses include increases in circulating plasma renin activity, increased vasopressin levels, enhanced catecholamine release rates, and increased intravascular volume\(^{2,4,15}\) and have been referred to collectively as pseudotolerance.

The time courses for development of tolerance and pseudotolerance are disparate. Many aspects of pseudotolerance begin immediately after initiation of nitrate treatment and are fully manifest 24 hours later.\(^{15}\) In contrast, the loss of the direct vascular effect of NTG (true tolerance) requires at least 3 days of treatment.\(^{15,18}\) Largely because of these time differences, true vascular tolerance and pseudotolerance have been thought to represent unrelated phenomena. The present studies provide a mechanism whereby one component of pseudotolerance, activation of the renin-angiotensin system, could contribute to true vascular tolerance.

Previously, we found that increased vascular O}_2^- production contributes to tolerance by inactivating nitric oxide released from NTG. Treatment with a liposomal superoxide dismutase preparation almost completely restored nitrate sensitivity in tolerant rabbit aorta.\(^5\) In subsequent studies, we found that the source of O}_2^- in these vessels is an NADH/NAD(P)H-dependent membrane oxidase, which is probably involved in the development of tolerance.
the predominant source of \( O_2^- \) in vascular cells. The fact that the AT\(_1\) receptor antagonist losartan prevented nitrate tolerance is compatible with previous studies showing that the activity of this oxidase is modulated by angiotensin II both in tissue culture and in intact animals. It is of interest that even in control animals, losartan therapy also decreased vascular \( O_2^- \) production. This suggests that ambient levels of angiotensin II, present even in normal conditions, probably modulate vascular \( O_2^- \) production. In contrast to the in vivo effect of losartan, in vitro incubation of tolerant tissue with losartan did not alter vascular \( O_2^- \), as estimated by lucigenin-enhanced chemiluminescence. Thus, unlike hydralazine, which is effective in vitro, losartan does not modify \( O_2^- \) production by a direct effect on the NADH oxidase but rather inhibits \( O_2^- \) production by preventing AT\(_1\) receptor stimulation in vivo.

To examine a potential role of the renin-angiotensin system in nitrate tolerance, we related vascular responsiveness to various components of the renin-angiotensin system during the 3 days of nitrate treatment. In agreement with previous observations, we found that NTG therapy in rabbits caused a persistent increase in plasma renin activity throughout the entire treatment period. Twenty-four hours after initiation of NTG, we found no shift in the dose-response relationship for NTG, an inhibition of vascular ACE activity, and a decrease in vascular AT\(_1\) receptor mRNA expression. Not surprisingly, these phenomena were associated with a blunted vascular constriction to both angiotensin I and II. Seventy-two hours after onset of NTG therapy, ACE activity and AT\(_1\) receptor mRNA levels had returned to control values and significantly above control levels, respectively, and constrictions to both angiotensin I and II had increased to values above those observed in control vessels. It has previously been reported that increased levels of angiotensin II inhibit vascular ACE activity in vivo and decrease expression of the AT\(_1\) receptor in vitro. The explanation for why AT\(_1\) receptor mRNA expression and vascular ACE activity are not depressed at a time when plasma renin activity and presumably the level of angiotensin II remain elevated is unclear. These differences may reflect variations between the in vivo and in vitro conditions and other direct effects of NTG on the vasculature.

Recently, we demonstrated that nitrate tolerance is associated with increased expression of ET-1 in the vascular media. In this previous study, we showed that this phenomenon increased responses to a variety of vasoconstrictors, most likely via activation of protein kinase C. Because several of the effects of angiotensin II may be mediated by ET-1, we examined the effect of ET-1 receptor blockade on nitrate tolerance and vascular \( O_2^- \) production in additional studies. Administration of a high dose of the nonselective ET-1 receptor antagonist bosentan modestly improved NTG-induced relaxations and partially corrected the increase in vascular \( O_2^- \) production in nitrate-tolerant rabbit aorta. These observations suggest that ET-1 may contribute to a portion of nitrate tolerance and the associated increase in \( O_2^- \) production. Of note, bosentan therapy was not as effective as treatment with losartan, suggesting that angiotensin II has effects independent of ET-1 in nitrate tolerance.

A causal role of the renin-angiotensin system in NTG tolerance has been supported by observations that ACE inhibition, in some but not all instances, can reverse or prevent nitrate tolerance. It is important to note that lower doses of ACE inhibitors failed to show a beneficial effect, whereas high doses have been shown to prevent nitrate tolerance. In the present study, we found that the higher doses

![Figure 5](http://circ.ahajournals.org/)

**Figure 5.** Effect of losartan (Los) treatment on vascular \( O_2^- \) production as estimated with lucigenin-enhanced chemiluminescence in NTG-treated animals. Data are mean±SEM of 4 to 6 experiments. *P<0.001 untreated vs NTG-treated, †P<0.05 vs without losartan.

![Table 3](http://circ.ahajournals.org/)

**Table 3.** Effect of Losartan (5, 10, or 25 mg · kg\(^{-1} \cdot \text{d}^{-1}\)) on ED\(_{50}\) and Maximal Relaxation to Various Vasodilators in Aortic Segments From NTG-Treated Animals

<table>
<thead>
<tr>
<th></th>
<th>NTG</th>
<th>ACh</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ED(_{50}) log mol/L</td>
<td>Max Rel, %</td>
</tr>
<tr>
<td>Control</td>
<td>7.24±0.10</td>
<td>90±1</td>
</tr>
<tr>
<td>Losartan 25</td>
<td>7.51±0.13</td>
<td>97±2</td>
</tr>
<tr>
<td>NTG</td>
<td>7.11±0.10</td>
<td>55±8†</td>
</tr>
<tr>
<td>NTG+Losartan 5</td>
<td>7.30±0.22</td>
<td>86±6</td>
</tr>
<tr>
<td>NTG+Losartan 10</td>
<td>7.27±0.33</td>
<td>90±4</td>
</tr>
<tr>
<td>NTG+Losartan 25</td>
<td>7.41±0.11</td>
<td>97±2</td>
</tr>
</tbody>
</table>

Potencies of NTG and acetylcholine (ACh) are expressed as ED\(_{50}\), the concentration that produces 50% of the maximal response to each drug. Each value is the mean±SEM of 3 to 7 experiments.

*P<0.05 vs control for ED\(_{50}\), †P<0.05 vs control for maximal relaxation.
of losartan (10 to 25 mg/kg) were most effective in preventing nitrate tolerance. Taken together, these findings suggest that complete inhibition of the renin-angiotensin system with either high-dose ACE inhibition or AT₁ receptor blockade is necessary to prevent nitrate tolerance. Another important caveat regarding the effect of ACE inhibitors or AT₁ receptor antagonists is that these may not prevent the pseudotolerance observed in the intact animal. Thus, although these may improve vascular reactivity, many of the manifestations of nitrate tolerance in vivo, such as redistribution of volume and loss of preload reduction, may not be prevented by antagonists of the renin-angiotensin system.

In summary, these studies demonstrate a rather dynamic regulation of the circulating plasma renin activity, expression of the AT₁ receptor mRNA, and modulation of vascular ACE activity during the development of nitrate tolerance. The time course of these events and the beneficial effects of losartan suggest that components of the renin-angiotensin system are quite important in the development of true vascular tolerance. Likewise, the effect of bosentan suggests that ET-1 probably also contributes to nitrate tolerance. The improvements in vascular reactivity caused by losartan were accompanied by a normalization of vascular O₂⁻⁻ production. These experiments, together with previous studies, indicate a causative role for O₂⁻⁻ in nitrate tolerance and support the concept that chronic nitrate therapy may exert an oxidant stress on the vasculature that is dependent on the renin-angiotensin system. Although studies like these must be extended to humans, these observations provide a basis for specific interventions to prevent some of the untoward effects of chronic nitrate therapy.

Acknowledgments

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