Hydralazine Prevents Nitroglycerin Tolerance by Inhibiting Activation of a Membrane-bound NADH Oxidase
A New Action for an Old Drug
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Abstract
Hydralazine has been shown to reduce mortality in patients with congestive heart failure when given concomitantly with isosorbide dinitrate. Recently, we demonstrated that nitrate tolerance is in part due to enhanced vascular superoxide \( \cdot \text{O}_2^- \) production. We sought to determine mechanisms whereby hydralazine may prevent tolerance. Rabbits either received no treatment, nitroglycerin patches (1.5 \( \mu \text{g/kg/min} \times 3 \text{ d} \)), hydralazine alone (10 mg/kg/d in drinking water), or hydralazine and nitroglycerin. Aortic segments were studied in organ chambers and relative rates of vascular \( \cdot \text{O}_2^- \) production were determined using lucigenin-enhanced chemiluminescence. Nitroglycerin treatment markedly inhibited relaxations to nitroglycerin (maximum relaxations 67% in nitroglycerin-treated patients and increased vascular \( \cdot \text{O}_2^- \) production by over twofold (\( P < 0.05 \)). Treatment with hydralazine in rabbits not receiving nitroglycerin significantly decreased \( \cdot \text{O}_2^- \) production in intact rabbit aorta and increased sensitivity to nitroglycerin. When given concomitantly with nitroglycerin, hydralazine completely prevented the development of nitrate tolerance and normalized endogenous rates of vascular \( \cdot \text{O}_2^- \) production. Studies of vessel homogenates demonstrated that the major source of \( \cdot \text{O}_2^- \) was a NADH-dependent membrane-associated oxidase displaying activities of 67 \( \pm \) 12 nmo1 \( \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1} \) in nitroglycerin-treated vs. untreated aortic homogenates. In additional studies, we found that acute addition of hydralazine (10 \( \mu \text{M} \)) to nitroglycerin-tolerant vessels immediately inhibited \( \cdot \text{O}_2^- \) production and NADH oxidase activity in vascular homogenates. The chemiluminescence signal was inhibited by a recombinant heparin-binding superoxide dismutase (H-SOD) demonstrating the specificity of this assay for \( \cdot \text{O}_2^- \). These observations suggest that a specific membrane-associated oxidase is activated by chronic nitroglycerin treatment, and the activity of this oxidase is inhibited by hydralazine, providing a mechanism whereby hydralazine may prevent tolerance. The ability of hydralazine to inhibit vascular \( \cdot \text{O}_2^- \) anion production represents a novel mechanism of action for this drug. (J. Clin. Invest. 1996. 98:1465–1470.) Key words: superoxide • nitric oxide • nitrate tolerance • acetylcholine • lucigenin chemiluminescence

Introduction
The vasodilator capacity of organic nitrates is attenuated over the first 24 h of continuous treatment, due to the development of nitrate tolerance. The mechanisms underlying this phenomenon are likely multifactorial and may involve both neurohormonal adjustments and impairment of intracellular nitroglycerin metabolism (for review see reference 1). Recently, we found that prolonged treatment of rabbits with nitroglycerin increases endogenous rates of vascular \( \cdot \text{O}_2^- \) production, which inactivates the vasorelaxant actions of nitric oxide (NO) released from nitroglycerin, or released endogenously from the endothelium (2).

An important issue relevant to increased vascular \( \cdot \text{O}_2^- \) production is the enzymatic source involved. In our previous study, diphenylene iodonium attenuated nitroglycerin-induced increases in rates of \( \cdot \text{O}_2^- \) production, inferring a flavin-derived source of \( \cdot \text{O}_2^- \) (2). It has been demonstrated recently that a major source of oxygen radical production in vascular cells is a membrane-bound, flavin-containing NADH/NADPH-dependent oxidase (3, 4), which is regulated in vitro and in vivo by angiotensin II (5, 6). Since nitrate therapy is associated with activation of the renin-angiotensin system, we hypothesized that nitrate therapy might also be associated with activation of this oxidase system.

Hydralazine has been shown to prevent nitrate tolerance and to improve mortality when given in combination with isosorbide dinitrate in patients with congestive heart failure (7). Therefore, a second purpose of this study was to examine the effect of hydralazine on the function of vascular NADH/NADPH oxidases and vascular \( \cdot \text{O}_2^- \) production and to determine if such an action might account for the beneficial effect of this drug in nitrate tolerance.

Methods
Animal model, in vivo nitrate tolerance. New Zealand White rabbits of either sex, weighing 3–6 kg, were studied. Four groups were studied: an untreated group (no nitroglycerin or hydralazine), a hydralazine-treated group (10 mg/kg/d added to the drinking water for 3 d), a nitroglycerin-treated group (nitroglycerin patch, 1.5 \( \mu \text{g/kg/min} \) for 3 d), and a group treated with nitroglycerin and hydralazine. The nitroglycerin patch was applied to the skin on the dorsal aspect of the thorax. The treatment started between 8 and 10 a.m., and the nitroglycerin patch was changed each morning for the ensuing 2 d. Hydralazine hydrochloride (10 mg/kg/d) was added to the drinking wa-
ter for some animals. The volume of drinking water (400 ml) was adjusted so that the animals would consume the entire amount each day. On the morning of the third day, after initiation of nitroglycerin and/or hydralazine treatment, the animals were given an intravenous injection of 1,000 U of heparin and sufficient sodium pentobarbital to produce death. The chests was then rapidly opened and the descending aorta removed. The vessel was cleaned of adventitia. All studies were performed with the endothelium left intact.

**Vessel preparation and organ chamber experiments.** Eight 5-mm ring segments of thoracic aorta were suspended in individual organ chambers (25 ml) filled with Krebs buffer of the following composition (mM): NaCl, 118.3; KCl, 4.69; CaCl\(_2\), 1.87; MgSO\(_4\), 1.20 K-HPO\(_4\), 1.03; NaHCO\(_3\), 25.0; and glucose 11.1; pH 7.40. During the following hour, the resting tension was increased to optimize constriction to KCl. In preliminary experiments, this was found to be 5 grams for aortic rings from nitroglycerin-treated and untreated animals. To test vasodilator responses to endothelium-dependent and -independent vasodilators, such as acetylcholine, SIN-1, and nitroglycerin, vessels were preconstricted with phenylephrine to achieve 30–50% of maximal (KCl-induced) tone.

**Measurement of \(O_2^\cdot\) production in intact vessels.** \(O_2^\cdot\) production in intact vessels was measured using lucigenin-enhanced chemiluminescence as described previously (2, 8). Briefly, after preparation, 5-mm ring segments were placed in modified Krebs/Hepes buffer and allowed to equilibrate for 30 min at 37°C. Scintillation vials containing 1.5 ml Krebs/Hepes buffer with 500 μl lucigenin (250 μM) were placed into a scintillation counter switched to the out of coincidence mode. After 15 min, background counts were recorded and a vascular segment was added to the vial. Counts were then recorded for 15 min and the respective background was subtracted. Some vessels from untreated and nitroglycerin-treated animals were incubated for 30 min with hydralazine (10\(^{-4}\)) to determine any direct effects on vascular \(O_2^\cdot\) production. All vessels were then dried for 24 h in a 90°C oven for expressing results on a milligram vessel dry weight basis. Calibration of lucigenin chemiluminescence was accomplished using known rates of \(O_2^\cdot\) production from 0–5 mU/ml xanthine oxidase plus 100 μM xanthine, as determined by cytochrome c reduction (Emax 21 mM\(^{-1}\) cm\(^{-1}\)).

Lucigenin chemiluminescence, when used in physiological systems such as this, has been shown to be quite specific for \(O_2^\cdot\). In the present experiments, the levels of nitric oxide might be higher in vessels from nitroglycerin-treated animals than in controls. If nitric oxide could produce lucigenin chemiluminescence, this might have artificially increased the lucigenin signal. To rule this possibility out, we performed studies in which MAHMA NONOate (10 nM) was added to intact vessels. This might have artifi-
cially increased the lucigenin signal. To clarify, supernatants were subjected to a modified Tsuchihashi fractionation, as described earlier (10). The resulting suspension was dialyzed against 100 mM ZnSO\(_4\), 100 μM CuSO\(_4\), 50 mM Tris, pH 7.4, placed over an SP high flow column (Pharmacia Biotech Inc., Piscataway, NJ), and eluted against NaCl gradient. Samples with the highest SOD activity were pooled, concentrated, and dialyzed against 10 mM KPO\(_4\), pH 7.4, and chromatographed on heparin-Sepharose 6B (Pharmacia Biotech Inc.) using a linear NaCl gradient. Fractons displaying SOD activity were pooled, concentrated, and dialyzed against phosphate-buffered saline, pH 7.4, and stored at –80°C.

In experiments where HB-SOD was used, a 14-amino acid peptide, ERKKRRRESECKAA, corresponding to the heparin-binding sequence of the protein was used as a control.
measurement in a scintillation vial containing Hepes buffer, lucigenin, and the method of Bradford (11).

Protein content was measured in an aliquot of homogenate by the method of Bradford (11).

NADH or NADPH oxidase activity was measured by chemiluminescence in a scintillation vial containing Hepes buffer, lucigenin, and 100 μM NADH or 100 μM NADPH as the substrate. No activity could be measured in the absence of NADH and NADPH. Reactions were initiated by addition of 25 μl homogenate (25–50 μg protein).

In some experiments, membranes and cytosol were separated by centrifugation (50,000 g for 30 min). The supernatant (cytosolic fraction) was removed, and the pellet, containing both plasma and mitochondrial membranes, was resuspended in 200 μl of Hepes buffer. NADPH- and NADPH oxidase-dependent ·O2− production was then measured as before.

For experiments using either whole homogenates or membrane and cytosolic subfractions, lucigenin-enhanced chemiluminescence was measured after addition of NADH or NADPH for 7 min. Net chemiluminescence yields were integrated by calculating the area under the curve of chemiluminescence for the 7 min and converted to nmol ·O2− as before.

Materials. Nitroglycerin was supplied by Dupont (Wilmington, DE). SIN-1 was obtained from Casella (Frankfurt, Germany). All other chemicals were purchased from Sigma Immunochemicals (St. Louis, MO).

Statistical analysis. Results are expressed as mean ±SEM. The ED90 value for each experiment was obtained by logit transformation. To compare NADH-and NADPH-driven ·O2− production in normal and nitrate tolerant vessels, one-way ANOVA was used. Comparisons of vascular responses were performed using multivariate ANOVA with treatment status (with or without hydralazine and with or without nitroglycerin) as the independent variable, and percent relaxation and EC50 as dependent variables. A Scheffe post-hoc test was used to examine differences between groups when significance was indicated. P values < 0.05 were considered significant.

Results

Effects of in vivo hydralazine treatment on vasodilator responses. Nitroglycerin relaxed aortas from untreated rabbits in a dose-dependent fashion with an ED90 of −7.25±0.04. Treatment with nitroglycerin for 3 d markedly attenuated maximal relaxations in response to nitroglycerin (control 95±1 vs. tolerant 67±2.7%) and caused cross-tolerance to exogenous NO (SIN-1) and NO endogenously released by acetylcholine. Concomitant treatment with nitroglycerin and hydralazine prevented development of tolerance and cross-tolerance (see Fig. 1 and Table I). Interestingly, the relaxations caused by nitroglycerin and SIN-1 were enhanced in vessels from rabbits treated with hydralazine compared with untreated rabbit vessels.

Effects of in vivo hydralazine treatment on ·O2− levels in aortas from control and nitroglycerin-treated animals. Rates of ·O2− production, as estimated by lucigenin-enhanced chemiluminescence, were increased more than twofold in animals treated with nitroglycerin, as compared with vessels from untreated animals. Concomitant in vivo treatment with hydralazine significantly reduced rates of ·O2− production in vessels from untreated and nitroglycerin-treated animals to below baseline (untreated) rates (459±59 and 477±31 counts/mg/min, respectively, Table II).

Effects of in vitro hydralazine treatment on ·O2− levels in aortas from control and nitroglycerin-treated animals. Incubation of aortas with hydralazine (1 μM), from both untreated and nitroglycerin-treated rabbits, for 30 min, significantly reduced vascular ·O2− production (Table II). Because one proposed vasodilator mechanism of hydralazine-mediated vasodilation is membrane hyperpolarization (12), we performed additional experiments in which KCl (20 mM) was added concomitantly with hydralazine. The concomitant addition of KCl negated the effect of hydralazine (untreated counts = 1,410±218; hydralazine-treated counts = 781±101; hydralazine + KCl counts = 2,440±395 counts/mg/min). The fact that membrane potential might modulate vascular ·O2− production was further supported by the finding that the potassium channel opener, pinacidil (1 μM), also decreased lucigenin-enhanced chemiluminescence in aortas from both untreated and nitrate-treated animals (712±67 and 612±60 counts/mg/min, respectively).

Effect of nitroglycerin treatment on vascular NADH- and NADPH-dependent oxidase activity. To determine the ·O2− dependency of the lucigenin-enhanced chemiluminescence obtained from homogenates of rabbit aorta, we examined the ef-

Table I. Effects of Hydralazine Treatment on ED90 and Maximal Relaxations to Endogenous and Exogenous Nitrovasodilators in Aorta from Untreated and Nitroglycerin-treated Animals

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ED90 (−log M)</th>
<th>Maximal relaxation (%)</th>
</tr>
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<tbody>
<tr>
<td>Untreated</td>
<td>NTG 7.25±0.04, SIN-1 6.35±0.05, ACh 7.39±0.11</td>
<td>NTG 95±1, SIN-1 92±3, ACh 90±2</td>
</tr>
<tr>
<td>Hydralazine</td>
<td>NTG 7.61±0.11*, SIN-1 6.71±0.07*, ACh 7.45±0.14</td>
<td>NTG 98±1, SIN-1 100±0*, ACh 97±2*</td>
</tr>
<tr>
<td>NTG</td>
<td>NTG 6.99±0.07, SIN-1 5.76±0.11†, ACh 6.53±0.17†</td>
<td>NTG 67±2†, SIN-1 81±5†, ACh 68±6†</td>
</tr>
<tr>
<td>NTG + hydralazine</td>
<td>NTG 7.37±0.08*, SIN-1 6.54±0.11*, ACh 7.61±0.05*</td>
<td>NTG 93±3*, SIN-1 99±1*, ACh 85±2*</td>
</tr>
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Note: *P < 0.05 NTG-treated vs. untreated. †P < 0.05 vs. the same group (untreated or nitroglycerin-treated) without hydralazine.
of HB-SOD had no effect on chemiluminescence signals. In contrast, as little as 20 U/ml of HB-SOD almost inhibited the chemiluminescence signal. The peptide corresponding to the heparin-binding domain alone of HB-SOD had no effect on -O2 production. Each value is mean±SEM of two to five experiments.

![Figure 2](image)

**Figure 2.** Effects of Cu/Zn SOD and a recombinant HB-SOD on superoxide (-O2) production after stimulation of a vascular homogenate from a nitrate-tolerant aorta with NADH. Only very high concentrations (2,000 U/ml) of conventional SOD were capable of completely inhibiting chemiluminescence signals. In contrast, as little as 20 U/ml of HB-SOD almost inhibited the chemiluminescence signal. The peptide corresponding to the heparin-binding domain alone of HB-SOD had no effect on -O2 production. Each value is mean±SEM of two to five experiments.

Discussion

In previous studies, we found that nitroglycerin tolerance is associated with an increase in vascular -O2 production (2), which results in inhibition of the vasorelaxant action of NO derived from exogenously administered nitrovasodilators or stimulated endogenously by acetylcholine. In this study, we characterized the substrate dependency and cellular distribution of the oxidase likely involved in this process. We also demonstrated that a commonly used vasodilator, hydralazine, potently inhibits -O2 production, likely via inhibition of this oxidase. The present findings define a new mode of action for hydralazine and may explain why it has been found beneficial when given concomitantly with nitroglycerin.

![Figure 3](image)

**Figure 3.** Effects of in vivo nitroglycerin treatment on NADH and NADPH oxidase activity in aortas from rabbits with or without concomitant hydralazine treatment. In vivo treatment with nitroglycerin increased superoxide (-O2) production in response to NADH almost 2.5-fold, while having no effect on NADPH oxidase activity. Concomitant treatment with hydralazine decreased the activity of both NADH- and NADPH-driven superoxide in homogenates of vessels from animals with and without nitroglycerin treatment. Each value is mean±SEM of 4–12 experiments. *P < 0.01 untreated vs. nitroglycerin-treated, †P < 0.05 vs. without hydralazine treatment.
the membrane fractions of nitroglycerin-treated vessels was substantially increased compared with control membranes. The mechanism whereby nitroglycerin treatment increases activity of NADH/NADPH-dependent oxidases remains unclear. However, it may involve activation by neurohumoral stimuli, such as angiotensin II. Studies of cultured vascular smooth muscle cells have shown that angiotensin II can markedly increase NADH oxidase activity (13). Likewise, we have also found that hypertension caused by angiotensin II, but not norepinephrine, is associated with activation of vascular NADH activity (15). Preliminary studies have shown that the angiotensin II (type 1) receptor antagonist losartan prevents the increase in vascular \( \cdot O_2^- \) production and normalizes relaxations to nitroglycerin in nitrate-treated animals (16).

An interesting finding in this study is the relative effectiveness of HB-SOD in scavenging \( \cdot O_2^- \) generated by homogenates of vascular tissues. As in previous studies, we found that native \( \text{Cu}^{2+}/\text{Zn}^{2+} \) SOD was relatively ineffective at inhibiting the lucigenin chemiluminescence signal. In contrast, HB-SOD was \( \sim 100 \)-fold more potent. Because of its pKa, \( \text{Cu}^{2+}/\text{Zn}^{2+} \) SOD is both electrostatically and sterically repelled from the anionic cell surface and the interstitial matrix (17). Therefore, \( \cdot O_2^- \) produced in restricted environments may preferentially react with lucigenin, rather than being scavenged by conventional SOD. In contrast, the high-affinity binding of HB-SOD to membrane-associated glycosaminoglycans brings SOD scavenging activity in close proximity to sources of the vessel wall \( \cdot O_2^- \) production, thus potently inhibiting lucigenin-enhanced chemiluminescence.

Hydralazine has been shown to prevent the development of nitroglycerin tolerance in both experimental animals and in humans with congestive heart failure (18, 19). Hydralazine also has beneficial effects on mortality when administered concomitantly with long-acting nitrates to patients with congestive heart failure (20, 21). Hydralazine stimulates reflex increases in vasoconstrictor stimuli, including circulating catecholamines and plasma renin activity (reflecting increased circulating angiotensin II levels) (22). This would seem, upon first inspection, to worsen, rather than improve, tolerance by enhancing the neurohumoral counterregulatory adjustments to the nitrate (23, 24). Our current findings may explain this paradox. In both untreated and nitrate-treated animals who received concomitant hydralazine therapy, vascular \( \cdot O_2^- \) production was markedly reduced. Further, acute addition of hydralazine in clinically relevant concentrations (1 \( \mu \)M) to segments of aorta from control- and nitroglycerin-treated animals markedly reduced \( \cdot O_2^- \) production. In homogenates of vessels from hydralazine and nitroglycerin-treated animals, the NADH-driven oxidase activity was reduced compared with that observed in homogenates of aorta from animals treated with nitroglycerin alone.

The mechanisms by which hydralazine inhibits the NADH oxidase remains unclear. Hydralazine has been shown to inhibit other oxidases, including aldehyde oxidase and the mitochondrial \( \text{CoQ}_{10}^- \cdot \text{NADH} \) oxidase (25, 26). It is unlikely that inhibition of mitochondrial oxidases by hydralazine had any role in our present findings, as mitochondrial sources of \( \cdot O_2^- \) do not contribute significantly to lucigenin chemiluminescence by intact vessels or homogenates (2, 4). Hydralazine was only effective when administered in vivo or incubated with intact rings, but had no effect when added to the vascular homogenates. One explanation for this finding is that hydralazine prevents

![Figure 4](image-url)

**Figure 4.** Bar graphs showing the effect of 3 d of nitroglycerin treatment on the NADH-driven enzyme activity in cellular subfractions. Almost 100% of the NADH oxidase activity was present in the particulate (membrane) fraction. The activity of the membrane-associated enzyme was increased almost threefold in nitroglycerin-treated animals. Each value is mean \( \pm \) SEM of four to eight experiments. *\( P < 0.01 \) untreated vs. nitroglycerin-treated.

Recently, it has become more apparent that vascular tissues possess significant membrane-bound oxidase-specific activity using NADH and NADPH as cofactors for \( \cdot O_2^- \) production (4, 13). It is unclear whether this activity represents a single oxidase which uses NADH in preference to NADPH or if it represents multiple enzymes with different specific activities and substrate or cofactor specificities. These oxidases represent the major source of detectable \( \cdot O_2^- \) production by both the endothelium and vascular smooth muscle (4, 13). Unlike oxidases of phagocytic cells, vascular NADH/NADPH-dependent oxidases produce \( \cdot O_2^- \) at a constant rate, rather than in bursts. Like the neutrophil oxidases, these are inhibited by diphenylene iodonium, suggesting that flavins are critically involved in the electron transfer reactions (14).

Based on these emerging lines of evidence regarding the importance of the NADH and NADPH oxidases in nonendothelial cells of vascular tissues and our prior observation that DPI inhibited vascular \( \cdot O_2^- \) production in intact vessels, we hypothesized that these oxidases are likely the major oxidase remains unclear. Hydralazine has been shown to inhibit other oxidases, including aldehyde oxidase and the mitochondrial \( \text{CoQ}_{10}^- \cdot \text{NADH} \) oxidase (25, 26). It is unlikely that inhibition of mitochondrial oxidases by hydralazine had any role in our present findings, as mitochondrial sources of \( \cdot O_2^- \) do not contribute significantly to lucigenin chemiluminescence by intact vessels or homogenates (2, 4). Hydralazine was only effective when administered in vivo or incubated with intact rings, but had no effect when added to the vascular homogenates. One explanation for this finding is that hydralazine prevents...
assembly of the oxidase rather than directly inhibiting the enzyme. Another possibility is that the effect of hydralazine requires the intact cell to exert its effect, possibility via its hyperpolarizing properties. This possibility is strengthened by the fact that depolarizing concentrations of KCl could inhibit the effect of hydralazine and that another hyperpolarizing agent, pinacidil (27), also markedly inhibited vascular \( \cdot O_2 \) production.

In additional studies, we examined the functional consequences of hydralazine-mediated inhibition of vascular \( \cdot O_2 \) production by examining vascular relaxation responses in organ chambers.

Tolerance to nitroglycerin and cross-tolerance to SIN-1 and acetylcholine were completely normalized by hydralazine treatment. Of note, hydralazine also improved relaxations to nitroglycerin and SIN-1 in control vessels to a modest extent, suggesting that basal activity of the oxidase opposes relaxation to these vasodilators even in normal vessels.

The present findings suggest that tolerance might not be expected during treatment with drugs which are capable of releasing nitric oxide and also producing hyperpolarization. Indeed, it has been shown recently that nicorandil, a drug which combines both features, can be administered for prolonged periods without the development of tolerance (28–30).

In summary, in this study, we demonstrate that an enhanced rate of vascular \( \cdot O_2 \) production is a key underlying mechanism of nitrate tolerance and we have characterized the oxidase involved in this process. Additionally, we have identified a novel mechanism of action of an old drug, hydralazine, in vascular tissue. It is possible that inhibition of \( \cdot O_2 \) production contributes to the antihypertensive properties of this agent and may explain why its vasodilator actions are in part endothelium dependent.

Acknowledgments

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