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Research Article

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Evidence for Enhanced Vascular Superoxide Anion Production in Nitrate Tolerance

A Novel Mechanism Underlying Tolerance and Cross-Tolerance

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Abstract

We sought to examine mechanisms underlying nitroglycerin (NTG) tolerance and "cross-tolerance" to other nitrovasodilators. Rabbits were treated for 3 d with NTG patches (0.4 mg/h) and their aortic segments studied in organ chambers. Relaxations were examined after preconstriction with phenylephrine. In NTG tolerant rabbit aorta, relaxations to cGMP-dependent vasodilators such as NTG (45±6%), SIN-1 (69±7%), and acetylcholine (ACh, 64±5%) were attenuated vs. controls, (90±2, 94±3, and 89±2% respectively, $P < 0.05$ for all), while responses to the cAMP-dependent vasodilator forskolin remained unchanged. In tolerant aorta, endothelial removal markedly enhanced relaxations to NTG and SIN-1 (82±4 and 95±3%, respectively). Other studies were performed to determine how the endothelium enhances tolerance. Vascular steady state O_2^- levels (assessed by lucigenin chemiluminescence) was increased twofold in tolerant vs. control vessels with endothelium (0.31±0.01 vs. 0.61±0.01 nmol/mg per minute). This difference was less in vessels after denudation of the endothelium. Diphenylene iodonium, an inhibitor of flavoprotein containing oxidases, and Tiron a direct O_2^- scavenger normalized O_2^- levels. In contrast, oxypurinol (1 mM) an inhibitor of xanthine oxidase, rotenone (50 μM) an inhibitor of mitochondrial electron transport and N^G-nitro-L-arginine (100 μM) an inhibitor of nitric oxide synthase did not affect the chemiluminescence signals from NTG-tolerant aortas. Pretreatment of tolerant aorta with liposome-entrapped, pH sensitive superoxide dismutase (600 U/ml) significantly enhanced maximal relaxation in response to NTG, SIN-1, and ACh, and effectively reduced chemiluminescence signals. These studies show that continuous NTG treatment is associated with increased vascular O_2^--production and consequent inhibition of $NO^\cdot$ mediated vasorelaxation produced by both exogenous and endogenous nitrovasodilators. (*J. Clin. Invest.* 1995. 95:187–194.) Key words: SIN-1 • lucigenin diphenylene iodonium • liposomal entrapped superoxide dismutase

Introduction

A major therapeutic limitation inherent to organic nitrates is the development of tolerance which occurs during chronic treatment with these agents (for reviews see references 1 and 2). The mechanisms underlying nitrate tolerance remain poorly defined, and are likely multifactorial. One mechanism seems to be a diminished bioconversion of nitroglycerin to its active vasodilator metabolite (3). Other mechanisms likely include neurohumoral adaptations, e.g., increases in plasma volume (4), activation of the renin angiotensin system (5), and increases in plasma vasopressin and catecholamines (6). The extravascular effects serve to counteract the vasodilator and cardiac unloading actions of these agents.

A phenomenon related to nitroglycerin tolerance is cross-tolerance to other nitrovasodilators and endothelium-dependent vasodilators. This has been observed most commonly in situations where nitroglycerin was administered chronically in vivo (7, 8), and is usually not encountered in situations where nitroglycerin tolerance is produced by short-term exposure of vascular segments to nitroglycerin in vitro (9). The latter experimental situations may be criticized because they often use supra-pharmacologic concentrations of the drug for very short periods and may not have relevance to the in vivo situation. Cross-tolerance to other nitrovasodilators may be due to changes in the activity of the enzyme guanylate cyclase which is the target of the nitric oxide released from these drugs or perhaps increases in the activity of the phosphodiesterases degrading cGMP (7, 8).

In the present experiments, we have defined a new mechanism partially responsible for nitroglycerin tolerance and cross-tolerance to other nitrovasodilators. In these studies, we found that aortic segments from rabbits chronically treated with nitroglycerin demonstrate greater degrees of tolerance to nitroglycerin if the endothelium is present than if removed and is due to increased steady state concentrations of vascular O_2^-.

Methods

Materials. Bis-*N*-methyl acridinium nitrate (lucigenin), phenylephrine, acetylcholine, forskolin, oxypurinol, N^G-nitro-L-arginine (L-NNA),[1] rotenone, bovine Cu/Zn superoxide dismutase (SOD), and Tiron were all purchased from Sigma Chemical Co. (St. Louis, MO). 3-morpholino-sydnonimine (SIN-1) was obtained from Casella (Frankfurt, Germany). Nitroglycerin was supplied by Dupont and diphenylene iodonium was obtained from Toronto Research Chemicals (Downsview, Ontario).

Animal model. New Zealand White rabbits of either sex, weighing 3–6 kg were studied. A region either on the dorsal aspect of the thorax or between the scapulae was shaved and a nitroglycerin patch applied to the skin. This treatment period was started between 8 and 10 AM, and the nitroglycerin patch changed each morning for the ensuing 2 d. On the morning of the third day after initiation of nitroglycerin treat-

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1. *Abbreviations used in this paper:* ACh, acetylcholine; DPI, diphenylene iodonium; L-NNA, N^G-nitro-L-arginine; NTG, nitroglycerin; SIN-1, 3-morpholino-sydnonimine.

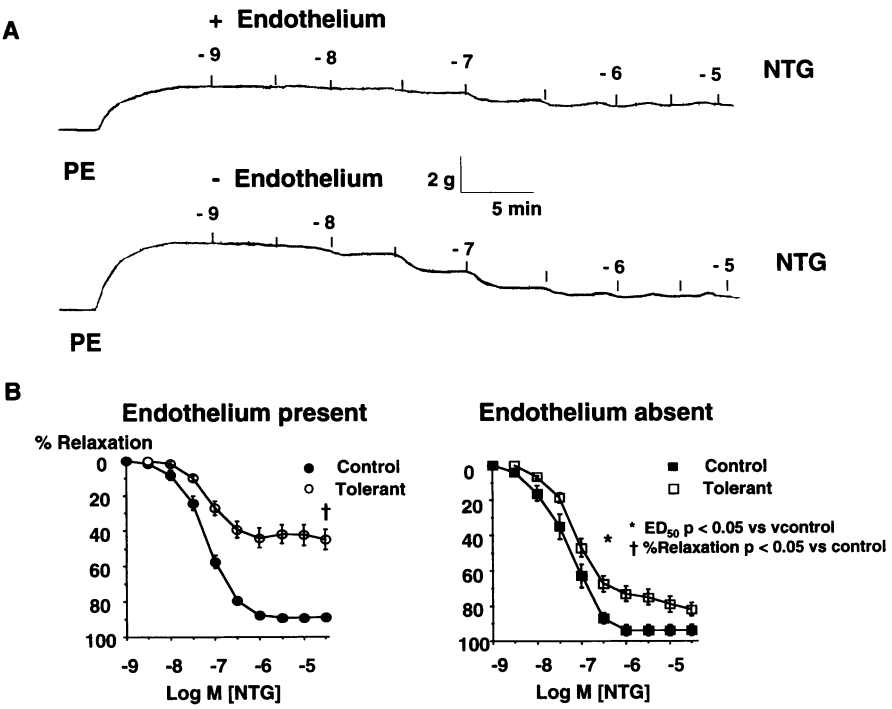

Figure 1. (*A*) Experimental record demonstrating the effect of endothelial removal on the relaxations to NTG (1 nM–30 μM) in tolerant rabbit aortic ring segments. Both segments were preconstricted with phenylephrine, and relaxations to cumulative concentrations of NTG examined. In the presence of the endothelium, the vessel relaxed maximally 37% and in the absence of the endothelium 78%. (*B*) Mean data demonstrating NTG induced relaxations in both control and NTG tolerant vessels with and without endothelium. Data are mean±SEM.

ment, the animals were given an intravenous injection of 1,000 U of heparin and sufficient sodium pentobarbital to produce death. The chest was then rapidly opened and the descending thoracic aorta removed. Rabbits of a similar size and sex distribution served as controls.

Vessel preparation. The aorta was placed in chilled Krebs buffer, cleaned of excessive adventitial tissue, and cut into 5-mm-ring segments. In some vessels, the endothelium was removed by inserting the closed tips of a metal hemostat into the ring segment and rolling it gently on moistened filter paper.

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_2HPO_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 constrictions to KCl. In preliminary experiments, this was found to be 5 g for both nitroglycerin tolerant and control aortic rings. Experiments were always performed on four sets of paired rings (with and without endothelium) in the presence of 10 μM indomethacin. The vessels were preconstricted with phenylephrine to achieve 30–50% of maximal tone. Rings were than exposed to increasing concentrations of either NTG, SIN-1, or ACh. After the addition of each concentration of drug, the subsequent dose was not added until the baseline had again stabilized.

Liposome entrapped SOD. Liposome-entrapped SOD was prepared as recently described by White et al. (10). Briefly, liposomes were composed of dioleoylphosphatidylethanolamine and dioleoylglycero-3-succinate (1:1). Lipids were dried under N_2 and hydrated 36 h in 210 mM sucrose/7 mM Hepes. During hydration, pH 8.5 was maintained with tetraethylammonium hydroxide. Lipids were added to SOD dissolved in sucrose Hepes buffer, vortexed and emulsions extruded through a 600 nM filter under N_2 pressure (Extruder; Lipex Biomembranes). The mean liposome diameter was 217 nm, determined by laser light scattering analysis.

To augment the vascular levels of superoxide dismutase, aortic rings from control and tolerant rabbits were incubated for 1 h at 37°C in a Hepes/Krebs buffer containing 600 U/ml of liposome entrapped SOD (final volume 1.5 ml). Thereafter, the aortic rings were removed from the liposomal SOD solution, washed, and placed in organ chambers as described above. All following experiments were performed in the absence of added native or liposomal SOD.

Determination of vascular SOD activity. The SOD activity of control aortic rings and aortic rings incubated with liposomal SOD (after extensive washing to remove residual nonincorporated liposomal SOD) was assayed in a 10% homogenate in 50 mM KPi, 0.1 mM EDTA, 0.1% CHAPS, pH 7.8. This was done for both untreated and nitroglycerin-tolerant vessels. After centrifugation at 10,000 *g* for 10 min, SOD was measured by inhibition of xanthine oxidase-mediated reduction of cytochrome c (11).

Estimation of vascular steady-state O_2^- levels. Vascular O_2^- levels were measured using lucigenin chemiluminescence. The details of this assay have been published previously (12). Briefly, after preparation, the vessels were placed in a modified Krebs/Hepes buffer and allowed to equilibrate for 30 min at 37°C. Scintillation vials containing 10 ml of Krebs/Hepes buffer with 250 mM lucigenin were placed into a scintillation counter switched to the out of coincidence mode. After 15 min, background counts were recorded and a vascular segment then added to the vial. Scintillation counts were then recorded 15 min later and the respective background counts subtracted. The vessels were then dried by placing them in a 90°C oven for 24 h. Lucigenin chemiluminescence counts were converted to net values of O_2^- by calibration of chemiluminescence yield with known quantities of xanthine and xanthine oxidase (12). The specificity of lucigenin chemiluminescence for O_2^- quantitation is supported by previous reports (13) and table 2 where chemiluminescence is inhibited by liposomal SOD and the SOD mimetic Tiron.

Statistics. Results are expressed as mean±SEM. The ED_{50} value for each experiment were obtained by logit transformation. Comparisons of vascular responsiveness were performed using a multivariate analysis of variance, with tolerance and endothelium as independent variables and the ED_{50} and percent maximal relaxations as dependent variables. Comparisons of steady state O_2^- levels were made using two factor analysis of variance. A Dunnett two tailed post hoc test was used to examine differences between groups when significance was indicated. P values < 0.05 were considered significant.

Results

Organ chamber studies. The maximal contractions to KCl averaged 6.17±0.51 and 6.00±0.4 in control and 6.85±0.72 and

6.33±0.54 in nitroglycerin tolerant aortic segments with and without endothelium respectively (P = NS). In concert with previous reports (7), the sensitivity to phenylephrine was significantly enhanced in nitroglycerin tolerant vessels. Thus, the amount of phenylephrine necessary to achieve 50% of maximal KCl contraction averaged 0.50 and 0.31 μM in the control vessels with and without endothelium and 0.21 and 0.10 μM in tolerant vessels with and without endothelium, respectively.

Responses to NTG. In normal vessels with and without endothelium, nitroglycerin produced maximal relaxations of 90±1 and 94±2%, respectively. In nitroglycerin tolerant vessels with endothelium, maximal relaxations to nitroglycerin were markedly less than that observed in normal rings (45±6%). Removal of the endothelium from nitroglycerin tolerant vessels substantially enhanced their maximal relaxations to nitroglycerin (81±4%) (Fig. 1, Table I).

Responses to SIN-1. In control vessels, removal of the endothelium slightly increased the sensitivity to SIN-1 (ED_{50}'s = −6.43±0.09 with and −6.80±0.07 without endothelium, respectively). After 3 d of NTG treatment, the sensitivity of vessels to SIN-1 was diminished, both in vessels with (ED_{50} = −5.75±0.10) and without endothelium (ED_{50} = −6.13±0.08). Maximal relaxations were attenuated to a greater extent in tolerant vessels with endothelium than in those without endothelium (Fig. 2, Table I).

Responses to acetylcholine. Acetylcholine produced dose-dependent relaxations of control vessels with an ED_{50} of -7.27±0.05. The maximal relaxation averaged 90±2%. After 3 d of NTG treatment, the sensitivity to ACh was diminished (Fig. 2, Table I).

Responses to forskolin. In control vessels, forskolin caused a dose dependent relaxation with an ED_{50} of -6.67±0.05. The maximal relaxation averaged 100%. 3 d of NTG treatment did not alter relaxations produced by forskolin (Table I).

Superoxide levels in normal and nitroglycerin tolerant vessels. In normal vessels with intact endothelium, steady state O_2^- levels, as estimated by lucigenin chemiluminescence, were 0.31±0.01 nmol/mg/min. Superoxide levels were twofold higher in aortic segments from nitrate tolerant vessels with intact endothelium (0.61±0.01 nmol/mg per minute). Endothelial removal minimally increased O_2^- levels in normal vessels

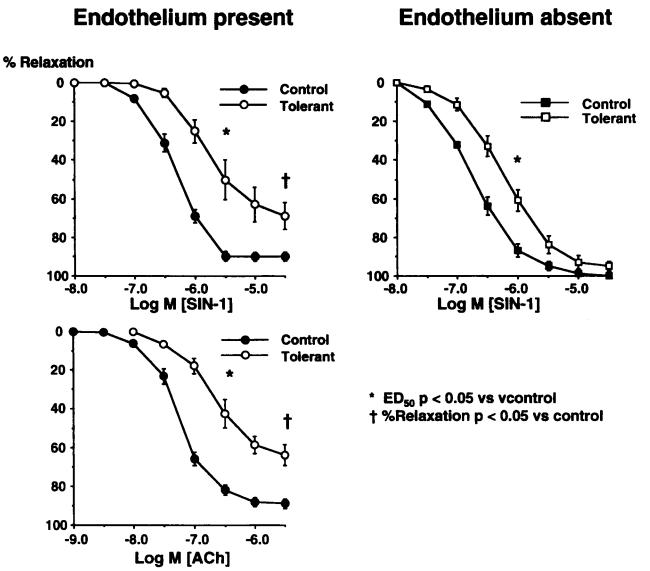

Figure 2. Effect of SIN-1 and ACh on control and tolerant aortic rings. After 3 d of NTG administration, relaxations to both agents were reduced. Removal of the endothelium markedly enhanced the relaxation of tolerant vessels to SIN-1. Data are mean±SEM.

(0.37±0.02 nmol/mg per minute) while paradoxically decreasing detectable O_2^- levels in nitrate tolerant vessels (0.50±0.02 nmol/mg per minute). After removal of the endothelium, the difference between O_2^- levels in normal and nitroglycerin tolerant vessels was diminished, but remained significantly different (Fig. 3).

Effects of pH sensitive liposomal SOD on NTG, SIN-1, ACh and forskolin induced vasorelaxation in control and tolerant rabbit aorta. Based on the above findings, we suspected that the enhanced levels of O_2^- in nitroglycerin tolerant vessels might contribute not only to nitroglycerin tolerance, but also to cross-tolerance to SIN-1 and endogenous NO production stimulated by acetylcholine. To test this hypothesis, we examined the effects of both native bovine CuZn SOD and bovine CuZn SOD

Table I. Effects of 3-d NTG Treatment and Liposomal-SOD on Sensitivity and Maximal Relaxations to NTG, SIN-1, ACh, and Forskolin

	NTG		SIN-1		ACh		Forskolin	
	$ED_{50(-log)}$	Max. Rel.	$ED_{50(-log)}$	Max. Rel.	$ED_{50(-log)}$	Max. Rel.	$ED_{50(-log)}$	Max. Rel.
		%		%		%		%
C+	7.17±0.03	90±1	6.43±0.09	91±2	7.27±0.05	90±2	6.73±0.05	100
SOD	7.68±0.09‡	94±4	6.67±0.07‡	100‡	7.57±0.05‡	93±3	6.84±0.07	100
C−	7.40±0.03	94±3	6.80±0.07	99±0.5	—	—	—	—
SOD	7.52±0.07	100	6.84±0.12	100				
Tol+	7.01±0.13	45±6*	5.75±0.10*	69±7*	6.67±0.12*	65±6*	6.70±0.05	100
SOD	7.18±0.08	81±4‡	6.40±0.17‡	76±6	6.97±0.10‡	87±6‡	6.69±0.08	100
Tol−	6.99±0.13*	82±4	6.13±0.08*	95±2	—	—	—	—
SOD	7.38±0.10	90±5	6.28±0.18	100				

The potencies of NTG, SIN-1, ACh, and forskolin are expressed as ED_{50} (concentration which produces 50% of the maximal response to each drug). +, Vessel with endothelium; −, vessel without endothelium; SOD, liposomal SOD pretreatment. Each value is the mean±SEM of 7 to 13 separate experiments except for liposomal SOD treatments which = 5–7. * $P < 0.05$ vs Control. ‡ $P < 0.05$ vs without liposomal SOD.

Table II. Effects of Rotenone, L-NNA, Oxypurinol, Tiron, and Liposomal Superoxide Dismutase on Vascular $O_2^{\cdot -}$ Production in Nitroglycerin Tolerant Vessels with Endothelium

	Tolerant	L-NNA (10 mM)	Rotenone (10 μM)	Oxypurinol (1 mM)	Lip. SOD (600 U/ml)	Tiron (10 mM)
nmol$O_2^{\cdot -}$/mg/min	0.71±0.05	0.74±0.14	0.63±0.07	0.64±0.11	0.42±0.04**	0.36±0.05**

** $P < 0.01$ vs tolerant vessel with endothelium, each value is the mean±SEM of 4 to 7 samples.

entrapped in pH sensitive liposomes. The latter was employed to specifically increase intracellular SOD specific activity.

Superoxide dismutase activity averaged 119±4 U/g tissue in controls and 97±10 U/g tissue in nitroglycerin tolerant vessels. These values were not statistically different. Incubation of control vessels ($n = 8$) with liposomal superoxide dismutase increased vascular superoxide dismutase activity to 144±3 U/g tissue ($P < 0.001$).

In control rabbit aortic segments with endothelium, pretreatment with liposomal SOD slightly enhanced the sensitivity to NTG, SIN-1, and ACh (Figs. 4–6, Table I). This effect was not observed in endothelium denuded vessels. In NTG tolerant aortic segments with endothelium, liposomal SOD markedly enhanced the relaxations evoked by NTG, SIN-1, and ACh. The effect of liposomal SOD pretreatment on maximal relaxation in response to NTG and SIN-1 was less pronounced in tolerant aortic segments in which the endothelium had been removed (Figs. 4 and 5, Table I).

In four separate experiments, we tested the effects of native nonliposomal CuZn SOD on NTG induced relaxation in tolerant rabbit aorta. In these studies, SOD (200 U/ml) was added to the organ chamber and was present during administration of nitroglycerin. In contrast to liposomal SOD, native SOD had no effect on relaxations to nitroglycerin (Fig. 4).

Liposomal SOD and a control liposomal preparation without SOD did not alter forskolin induced vasorelaxation in either control or tolerant rabbit aorta (Fig. 6).

Effects of diphenylene iodonium on superoxide production in normal and tolerant rabbit aorta. Pretreatment of tolerant vessels with diphenylene iodonium (an inhibitor of flavoprotein containing oxidoreductases, 100 μM) for 10 min markedly decreased lucigenin chemiluminescence to a value similar to control vessels (Fig. 7).

Since iodonium compounds may also inhibit several enzyme systems known to generate $O_2^{\cdot -}$ (14–17), we examined the effects of oxypurinol (an inhibitor of xanthine oxidase, 1 mM, $n = 4$) (18), rotenone (an inhibitor of the mitochondrial NADH dehydrogenase, 50 μM, $n = 4$) (19) and L-NNA (to inhibit $NO^{\cdot}$ synthases, 100 μM, $n = 5$) (20) on $O_2^{\cdot -}$ in tolerant rings with endothelium. None of these compounds altered lucigenin chemiluminescence signal in tolerant rings (Table II). In contrast, the $O_2^{\cdot -}$ scavenger Tiron (10 mM) (13) and pretreatment with liposomal SOD markedly decreased the $O_2^{\cdot -}$-dependent chemiluminescence in NTG-tolerant rabbit aorta.

Discussion

In the present experiments, we have defined a new mechanism partially responsible for nitroglycerin tolerance and cross-tolerance to other nitrovasodilators. In these studies, we found that aortic segments from rabbits chronically treated with nitroglycerin demonstrate greater degrees of tolerance to nitroglycerin if the endothelium is present than if it is removed. Our experimental data indicate that this is likely related to an increased steady-state concentration of vascular $O_2^{\cdot -}$.

In these studies, rabbits were treated with patches designed to release 0.4 mg of nitroglycerin per hour. Based on an average weight of 4 kg, and assuming uniform release of the drug, this would result in a constant delivery rate of 1.6 mg/kg per minute. This concentration is not uncommonly used in the treatment of patients with unstable angina pectoris or left heart decompensation and may therefore be achieved when nitrates are employed chronically. It is difficult to extrapolate the relevance of drug doses between different species, expecially when body surface areas are very different, however at the very least one can conclude that our present findings would have implications with respect to intravenous administration and to treatment with high doses of nitroglycerin employed in heart failure.

In the present experiments, we found that 3 d of nitroglycerin treatment produced not only tolerance to nitroglycerin, but also cross-tolerance to SIN-1 and acetylcholine (which released endothelium-derived nitric oxide). There has been substantial debate as to whether or not cross-tolerance to other nitrovasodilators occurs in the setting of nitroglycerin tolerance. One explanation for the discrepancy in previous studies has been differences in the method used to produce tolerance (7, 8, 21). Several studies have used short-term in vitro exposure of vascular segments to very high concentrations of nitroglycerin, and have generally not observed cross-tolerance (21). These studies may not have relevance to the in vivo situation because of the high concentration of nitroglycerin used and the lack of other physio-

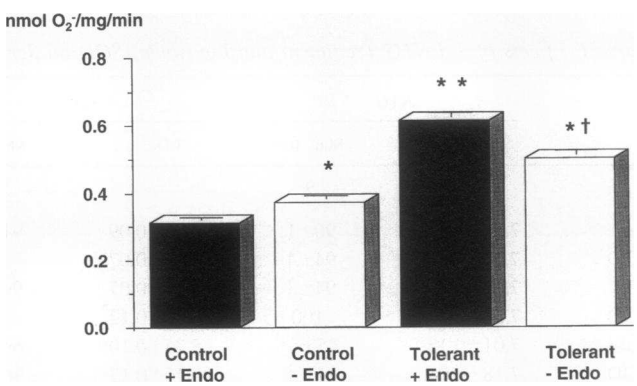

Figure 3. Superoxide levels in aortic segments from control and NTG treated rabbits. $O_2^{\cdot -}$ levels were estimated by lucigenin chemiluminescence in the presence and absence of the endothelium. Data are expressed as mean±SEM. * $P < 0.05$ vs. control with and without endothelium, ** $P < 0.001$ tolerant vs. control vessels with endothelium, † $P < 0.05$ tolerant vessel without vs. with endothelium.

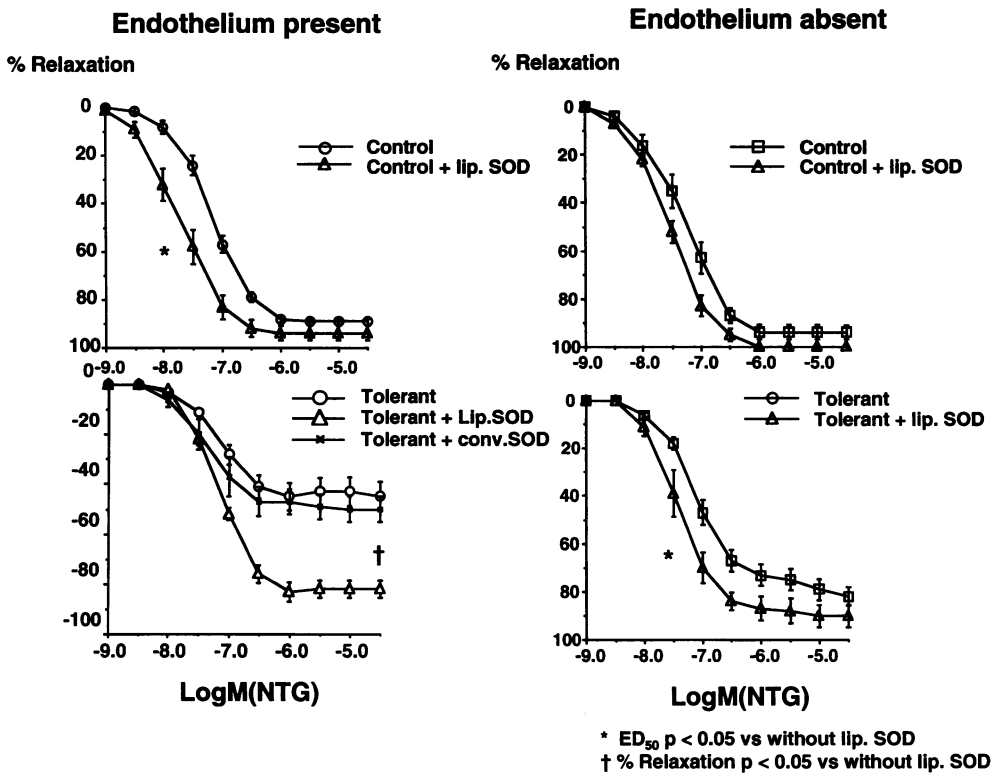

Figure 4. Effect of liposomal entrapped SOD on NTG-dose response in control NTG tolerant rabbit aorta. Control and tolerant aortic segments were incubated at 37°C in a Hepes/Krebs buffer for one hour containing 600 U/ml of SOD in this liposomal preparation. Segments were preconstricted with phenylephrine, and relaxations to cumulative concentrations of NTG were examined. In addition, in tolerant rabbit aorta, the effects of conventional SOD on NTG dose response was tested. Data are expressed as mean±SEM.

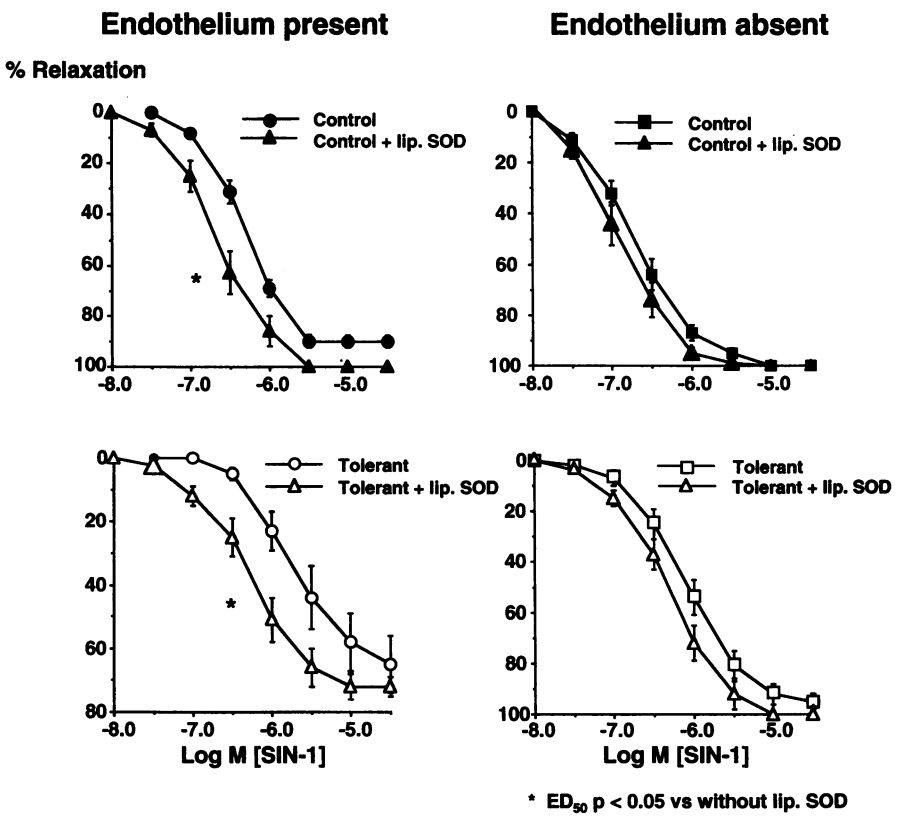

Figure 5. Effects of liposomal entrapped SOD on SIN-1 dose response in control and NTG tolerant rabbit aorta. Control and tolerant aortic segments were incubated at 37°C for 1 h in a Hepes/Krebs buffer containing 600 U/ml of SOD in this liposomal preparation. Segments were preconstricted with phenylephrine, and relaxations to cumulative concentrations of SIN-1 were examined. Data are expressed as mean±SEM.

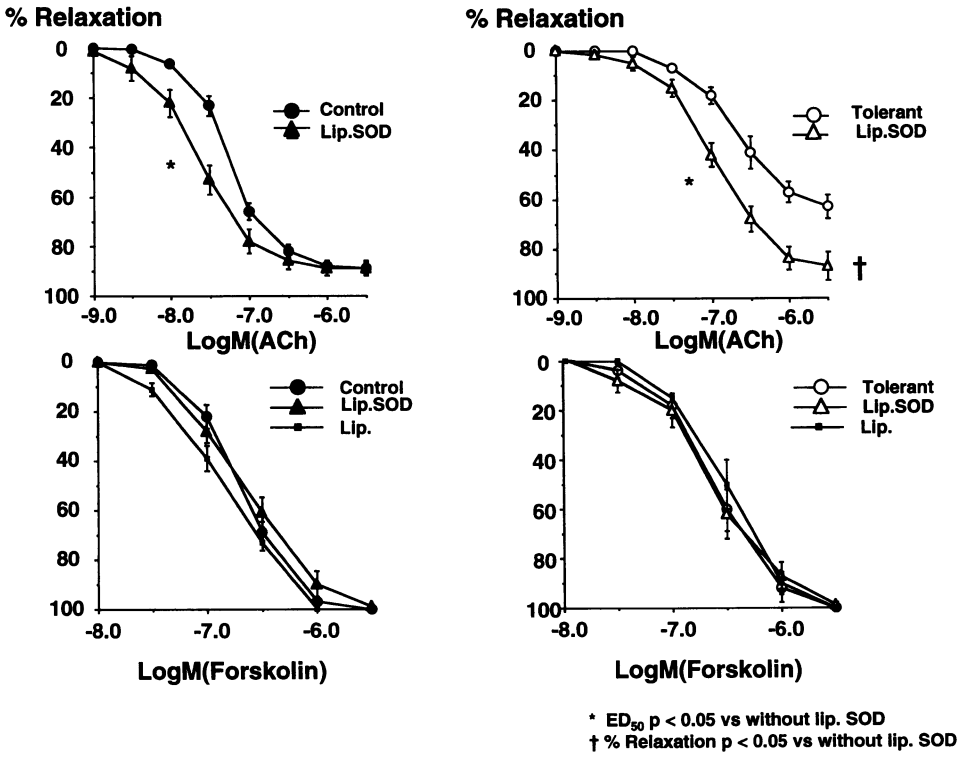

Figure 6. Effects of liposomal entrapped SOD and liposomes without SOD (*Lip.*) on ACh and Forskolin dose response in control and NTG tolerant rabbit aorta. Control and tolerant aortic segments were incubated at 37°C for 1 h in a Hepes/Krebs buffer containing 600 U/ml of SOD in this liposomal preparation. Segments were preconstricted with phenylephrine, and relaxations to cumulative concentrations of ACh and Forskolin were examined. Data are expressed as mean±SEM.

logical influences which may be important in-vivo (e.g., neurohormonal stimulation). Other studies, in which nitroglycerin was administered in-vivo have demonstrated cross-tolerance to endothelium-dependent vasodilators and other nitrovasodilators (7, 8).

In the present study, we made the interesting observation that in nitrate tolerance, the presence of the endothelium markedly impaired vasorelaxations evoked by NTG and SIN-1 in nitroglycerin tolerant vessels. Removal of the endothelium enhanced maximal relaxations by 37 and 27%, respectively. This lead us to hypothesize that the endothelium is either continuously releasing a vasoconstrictor or that nitric oxide released from these compounds might be chemically and physiologically inactivated before activating the vascular smooth muscle guanylate cyclase.

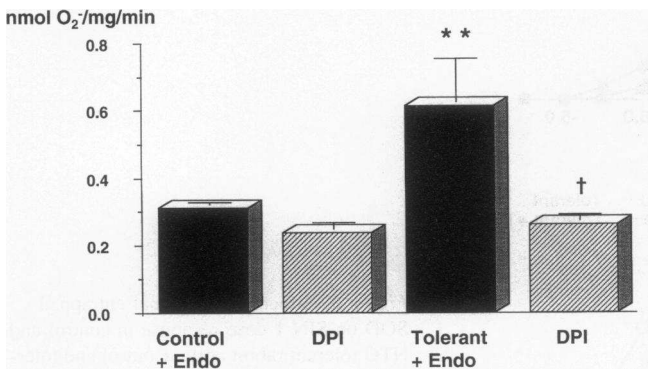

Figure 7. Effects of diphenylene iodonium (DPI) on O_2^- steady-state levels in control and tolerant rabbit aorta. Superoxide levels were determined using lucigenin chemiluminescence. Incubation of tolerant aortic segments for 10 min normalized O_2^- production in tolerant vessels, however, had no significant effect in control segments. $^{**}P < 0.001$ vs. control with endothelium, $^{\dagger}P < 0.05$ vs. without DPI pretreatment.

In support of the latter hypothesis, lucigenin-dependent chemiluminescence indicated that steady state levels of O_2^- in nitroglycerin tolerant vessels was approximately twice that of control vessels. In control aortic segments, endothelial denudation significantly increased detectable O_2^- consistent with previous studies (12). In the setting of nitroglycerin tolerance, however, removal of the endothelium had the opposite effect, and paradoxically decreased O_2^- levels. This finding strongly suggests that a major source of O_2^- in these vessels is either the endothelium or a cell type closely associated with the endothelium and was diminished, but remained significant following denudation. This finding indicates that nitroglycerin tolerance is associated with increases in both endothelial and vascular smooth muscle O_2^- concentrations.

Further support for a role of O_2^- in both nitroglycerin tolerance and cross-tolerance was obtained from experiments in which superoxide dismutase was administered. To increase intracellular SOD levels, we used pH sensitive liposomes containing CuZn SOD. The endosomal uptake of liposomes is rapidly followed by acidification of the endosome due to activation of proton pumps in the endosomal surface (10). The resultant destabilization of liposome membrane lipids favors fusion with the endosomal membrane and results in enhanced transfer of CuZn SOD into the cytoplasmic compartment.

Several lines of evidence indicate that incubation of control and tolerant aortic rings with liposomal preparations effectively increased intracellular SOD content. Treatment of control rings with liposomal SOD significantly increased vascular SOD activity. While the measured increase in activity might seem modest, it is important to note that only small increases in SOD concen-

trations will markedly decrease O_2^- half-life and concentrations (22), particularly when the levels of superoxide anion are low (as in these studies). Further, based on previous experiments, it is likely that the delivery of SOD by the liposomes was such that the concentration was increased in critical compartments even though the overall concentration might increase only modestly (10). Preincubation of control and tolerant rings with liposomal SOD significantly enhanced vasorelaxation to NTG, SIN-1, and ACh. Incubation with liposomal SOD decreased lucigenin chemiluminescence in a manner similar to the O_2^- scavenger Tiron. These effects were likely due to intracellular levels of SOD, because any exogenous liposomal SOD had been washed from the vessel prior to these assays. Moreover, native unentrapped CuZn SOD was ineffective, suggesting that the effect of the liposomal preparation was due to augmentation of intracellular superoxide dismutase. It is also unlikely that the action of the liposomal superoxide dismutase was due to nonspecific effects of the liposomes, because control liposomes preparations without superoxide dismutase did not increase relaxations. Further, liposomal superoxide dismutase had no effect on relaxations to forskolin.

The present experiments do not indicate that the only mechanism underlying nitroglycerin tolerance is increased O_2^- production. Liposomal SOD only partially improved relaxations to nitroglycerin. A modest impairment of relaxation persisted after liposomal-SOD treatment in both endothelium-intact and denuded vessels. It is likely that this remaining impairment is due to factors other than increased vascular O_2^- production such as decreased biotransformation of nitroglycerin to its active vasodilator metabolites (3).

The present studies also provide some insight into the potential sources of O_2^- production in nitroglycerin tolerant vessels. Diphenylene iodonium (DPI) completely normalized superoxide anion production these vessels. Iodonium salts are potent inhibitors of flavoprotein containing oxidoreductases. These include mitochondrial NADH dehydrogenase, nitric oxide synthase, xanthine oxidase, a plasmalemmal NADPH oxidase, and a cytosolic NADH oxidase (14–17, 23). The inhibitory effect of DPI could have been due to its action on any of these enzyme systems. In the present experiments, more specific inhibitors of the mitochondrial NADH dehydrogenase, NO• synthase, and xanthine oxidase had no effect on O_2^- production. Thus, it is unlikely that these enzyme systems were the source of O_2^- in nitroglycerin tolerant vessels. It has recently been suggested that the NADPH oxidase is a major source of cellular O_2^- release in rabbit aorta (24). In neutrophils, this enzyme complex consists of a membrane bound flavoprotein termed cytochrome b_{558} which consists of two subunits (25, 26). Two other components, p47 and p67, exist in the cytoplasm and are translocated to the membrane upon activation (for example by activation of protein kinase C). Iodonium compounds bind covalently to the flavin binding domains of b_{558} and result in inhibition of oxidase activity (14, 27). Whether or not this cascade of activation exists in vascular cells remains to be defined. It has recently been shown that angiotensin II can increase both NADH and NADPH oxidase activity in vascular smooth muscle, although the signaling process may be different than that known to exist in the neutrophil (28). It is not known if angiotensin II would produce a similar effect in the endothelium. Nevertheless, it is interesting to speculate that activation of the renin/angiotensin system which occurs in vivo during nitroglycerin therapy might contribute to this phenomenon.

The present findings do not exclude the possibility that other flavoprotein containing oxidoreductases are participating in superoxide anion generation in nitroglycerin tolerant vessels. In particular, it has been demonstrated that a major source of superoxide anion in bovine coronary endothelium is a cytosolic NADH oxidase which is inhibited by DPI (23). Other flavoprotein containing enzymes may also be involved. Finally, it is possible that defense mechanisms against O_2^- might be impaired.

In these studies, we also demonstrated that nitroglycerin tolerance did not alter relaxations to forskolin, which acts via adenylate cyclase. These findings are compatible with previous studies showing that other agents which act via adenylate cyclase are not affected by increased vascular superoxide anion production (29).

The present findings may also have implications regarding the beneficial effects of free radical scavengers such as *N*-acetylcysteine on systemic and coronary hemodynamics in the setting of NTG tolerance (5, 30). It is conceivable that a portion of the beneficial effect of such therapy occurs scavenging O_2^- by thiol groups present in higher concentrations (31).

Superoxide readily reacts with NO• to form peroxinitrite ($ONOO^-$) which, although capable of activating guanylate cyclase, has a half-life substantially shorter than NO• and is likely less potent (10). Furthermore, increased tissue O_2^- concentrations may serve as a source of not only $ONOO^-$ but other oxygen derived radicals which can mediate vascular injury, enhance vascular smooth muscle growth, and lipid oxidation therefore contributing to the atherosclerotic process.

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