The Nitric Oxide Donor SIN-1 Is Free of Tolerance and Maintains Its Cyclic GMP Stimulatory Potency in Nitrate-Tolerant LLC-PK$_1$ Cells

Burkhard Hinz[1] and Henning Schröder[1,2]

Received December 22, 1998; accepted February 5, 1999

Purpose. Using an established cell culture model, the present study investigates whether linsidomine (SIN-1), a spontaneous donor of nitric oxide and active metabolite of the antianginal drug molsidomine, induces tolerance to its own cyclic GMP stimulatory action or shows a diminished response after tolerance induction with glyceryl trinitrate.
Methods. Incubations with nitric oxide donors were carried out in LLC-PK$_1$ kidney epithelial cells. Intracellular levels of cyclic GMP, the vasodilatory second messenger of nitric oxide, were determined by radioimmunoassay.
Results. A 5-h preincubation with glyceryl trinitrate (0.01–100 μM) led to complete inhibition of a subsequent cyclic GMP stimulation by glyceryl trinitrate but left the cyclic GMP response to SIN-1 unaltered. Similarly, cyclic GMP elevations by the spontaneous nitric oxide donors sodium nitroprusside and spermine NONOate were not affected after pretreatment with glyceryl trinitrate. Moreover, pretreatment with SIN-1 (1–1000 μM) had no significant effect on SIN-1-dependent cyclic GMP stimulation.
Conclusions. Our results show that in LLC-PK$_1$ cells, SIN-1 is free of tolerance induction and not cross-tolerant to glyceryl trinitrate. This may be due to the spontaneous nitric oxide release from SIN-1, which in contrast to nitric acid esters does not require enzymatic bioactivation and may therefore be unaffected by nitrate tolerance.

KEY WORDS: nitrate tolerance; nitric oxide; cyclic GMP; SIN-1; molsidomine; glyceryl trinitrate.

INTRODUCTION

Organic nitrates have been used for the therapy of myocardial ischemia and its principal symptom angina pectoris for more than one hundred years. The cellular mechanism underlying the antianginal effect of organic nitrates involves the conversion of these compounds into the nitric oxide free radical. Upon stimulation of the soluble guanylyl cyclase by nitric oxide the vasodilatory intracellular second messenger cyclic GMP is generated (1). However, the efficacy of organic nitrates in chronic situations is limited by the development of tolerance to the cardiovascular effects of these drugs in humans and experimental animals (2). Different mechanisms have been proposed to account for nitrate tolerance. On the cellular level, down-regulation of enzymes that participate in the bioactivation of nitric acid esters may lead to a diminished nitric oxide generation from these compounds (3–5). Sustained or irreversible inhibition of the nitric oxide-sensitive soluble guanylyl cyclase has also been demonstrated under conditions of nitrate tolerance (6).

The sydnonimine derivative molsidomine is frequently used to provide prophylaxis against anginal episodes, and has been shown to exhibit hemodynamic actions similar to nitric acid esters (7). Molsidomine is enzymatically metabolized in the liver to linsidomine (SIN-1), the latter releasing nitric oxide spontaneously without requiring further bioactivating steps (8,9). Recently, SIN-1, administered intravenously, has become available for the treatment of unstable angina pectoris (10).

However, conflicting experimental results have been published as to whether beneficial antianginal and hemodynamic effects of molsidomine or SIN-1 are altered in nitrate-tolerant blood vessels (11–15). Furthermore, previous and recent studies leave open the question whether SIN-1 may cause tolerance to its own vasodilatory effect (12,16).

Using a cultured kidney epithelial cell line (LLC-PK$_1$) the present study investigates whether SIN-1-induced intracellular cyclic GMP accumulation is altered by prolonged preexposure of cells to SIN-1 or tolerance-inducing glyceryl trinitrate concentrations. LLC-PK$_1$ cells have been established as a model for studying molecular mechanisms and pathways involved in organic nitrate-induced activation and desensitization of the guanylyl cyclase/cyclic GMP system. In particular, the cyclic GMP response in LLC-PK$_1$ cells to different nitric oxide donors very closely reflects the structure activity relationship and potency order of these compounds as to vascular relaxation in vivo or in isolated blood vessels (3,17).

MATERIALS AND METHODS

Materials

LLC-PK$_1$ cells (ATCC CL 101) were obtained from the American Type Culture Collection (Rockville, MD, USA). Fetal calf serum, Ham's F-12 medium, penicillin, streptomycin and L-glutamine were purchased from Life Technologies (Eggenstein, Germany). SIN-1 was a gift of Hoechst AG (Frankfurt/Main). Glyceryl trinitrate was a gift from Schwarz Pharma AG (Monheim, Germany). Sodium nitroprusside was obtained from Sigma (Deisenhofen, Germany). Spermine NONOate was bought from Alexis Deutschland GmbH (Grünberg, Germany). Culture dishes were from Falcon/Becton Dickinson GmbH (Heidelberg, Germany).

Cell Culture

LLC-PK$_1$ cells were maintained and subcultured in Ham's F-12 medium, supplemented with 15% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. The cells were grown in a humidified incubator at 37°C and 5% CO_2.

Incubation Procedure and Cyclic GMP Determination

Cells grown to confluence in 35 mm culture dishes were washed twice with phosphate buffered saline. Cells were preincubated for 5 h with a balanced salt solution (composition [mM]: NaCl: 130, KCl: 5.4, $CaCl_2$: 1.8, $MgCl_2$: 0.8, glucose: 5.5, and N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic

[1] Department of Pharmacology and Toxicology, School of Pharmacy, Martin Luther University Halle-Wittenberg, Wolfgang-Langenbeck-Str. 4, 06099 Halle (Saale), Germany.
[2] To whom correspondence should be addressed. (e-mail: schroeder@pharmazie.uni-halle.de)

ABBREVIATIONS: SIN-1, linsidomine (3-morpholinosydnonimine).

0724-8741/99/0500-0633$16.00/0 © 1999 Plenum Publishing Corporation

acid]-NaOH [HEPES-NaOH]: 20, buffered to pH 7.3) in the presence or absence of SIN-1 or glyceryl trinitrate (final incubation volume: 1 ml). After the preincubation period, cells were washed twice with 2 ml phosphate-buffered saline. Cells were preincubated with balanced salt solution containing isobutylmethylxanthine (final concentration: 0.5 mM). After 10 min, SIN-1, glyceryl trinitrate or balanced salt solution was added and the incubation was continued for another 10 min. The final assay volume was 1 ml. Supernatants were aspirated and cyclic GMP levels were determined by radioimmunoassay after addition of ethanol to the cells and subsequent evaporation as described previously (18).

RESULTS

Preincubation of cells with glyceryl trinitrate left SIN-1-induced cyclic GMP stimulation unaltered (Fig. 1, Panel A), whereas under the same conditions a substantial desensitization of the intracellular cyclic GMP response to a subsequent stimulation with glyceryl trinitrate was found (Fig. 1, Panel B). Likewise, glyceryl trinitrate-tolerant cells remained fully responsive to cyclic GMP accumulation induced by sodium nitroprusside and spermine NONOate (Fig. 2). As shown in Fig. 3, a 5-h pretreatment of the cells with SIN-1 at concentrations up to 1 mM did not reduce cyclic GMP elevation upon a subsequent exposure to SIN-1.

DISCUSSION

The present study demonstrates that the nitric oxide donor and molsidomine metabolite SIN-1 is devoid of tolerance development in a cultured cell line. Furthermore, we have shown that

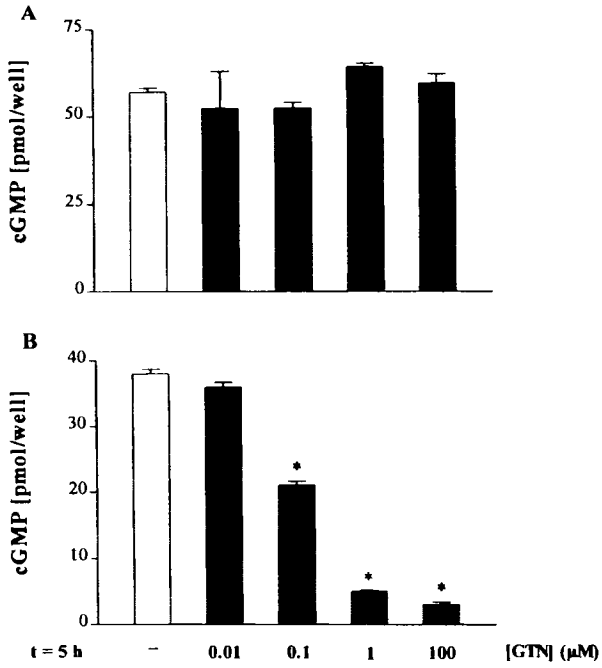

Fig. 1. Effect of a 5-h pretreatment with glyceryl trinitrate (GTN) at various concentrations on subsequent cyclic GMP accumulation by SIN-1 (50 μM for 10 min; Panel A) or glyceryl trinitrate (1 μM for 10 min; panel B) in LLC-PK$_1$ cells (10^6 cells/well). Values are means ± S.E.M. of n = 6 observations. *P < 0.05 (Ordinary one-way ANOVA plus Bonferroni test).

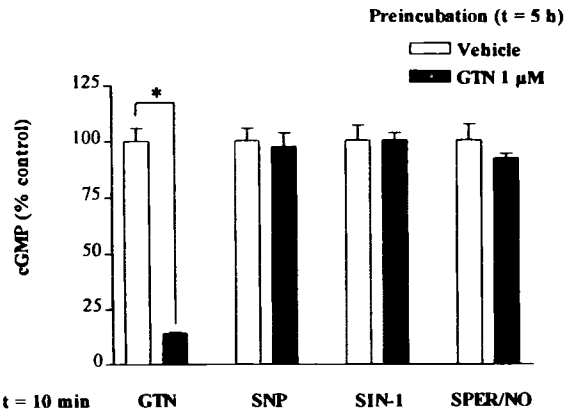

Fig. 2. Effect of a 5-h pretreatment with glyceryl trinitrate (GTN; 1 μM) on subsequent cyclic GMP accumulation by GTN (1 μM for 10 min), SIN-1 (10 μM for 10 min), sodium nitroprusside (SNP; 1 μM for 10 min) and spermine NONOate (SPER/NO; 1 μM for 10 min) in LLC-PK$_1$ cells. Values are means ± S.E.M. of n = 6 observations. *P < 0.05 (Student's two-tailed t-test).

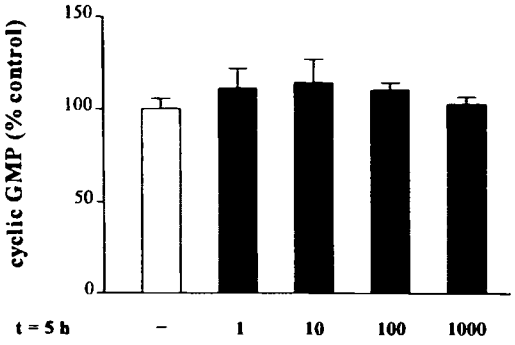

Fig. 3. Effect of a 5-h pretreatment with SIN-1 at various concentrations on subsequent cyclic GMP stimulation by SIN-1 (10 μM for 10 min) in LLC-PK$_1$ cells. Values are means ± S.E.M. of n = 6 observations.

cells made tolerant by glyceryl trinitrate remain fully sensitive to SIN-1-induced cyclic GMP accumulation. Likewise, tolerance induction by glyceryl trinitrate left cyclic GMP stimulation by the spontaneous nitric oxide donors sodium nitroprusside and spermine NONOate unaltered suggesting that tolerance to glyceryl trinitrate specifically affects nitric oxide release from nitric acid esters whereas the sensitivity of guanylyl cyclase remains unimpaired. Thus, our data support the view that nitrate tolerance is caused, at least in part, by a decrease in biotransformation of organic nitrates (3) and subsequent nitric oxide formation (4). That tolerance is specific for nitric acid esters such as glyceryl trinitrate can be explained by their enzymatic bioactivation via cytochrome P-450 (17–20) or as yet unidentified organic nitrate-bioactivating enzymes. In contrast, spontaneous nitric oxide donors such as sodium nitroprusside or spermine NONOate do not require metabolic activation prior to their pharmacological action but release nitric oxide spontaneously in aqueous solution (8,9). Downregulation or irreversible inactivation of organic nitrate-metabolizing enzymes during tolerance is also supported by a previous study showing that the reversal of nitrate tolerance in intact cells requires de novo synthesis of proteins (1). On the other hand, the spontaneous nitric oxide donor SIN-1 does not require enzymatic transformation into

nitric oxide (8,9). Accordingly, SIN-1 has been shown to activate the soluble guanylyl cyclase/cyclic GMP system in various organs as well as in cell-free systems under omission of any additional co-factors (8,21).

Conflicting results have been published as to whether SIN-1 causes tolerance to its own response. From the data published by Kuhn and Förstermann (13) it appears that prolonged exposure of human coronary arteries to SIN-1 does not modify relaxations to various vasodilators. Similarly, in a recent study vascular tolerance was not observed in rabbits treated chronically with SIN-1 (11), whereas a slight tolerance was documented in SIN-1-treated rats (16). On the other hand, Henry et al. (12) using isolated bovine coronary artery rings demonstrated that preexposure to SIN-1 attenuated subsequent SIN-1-induced relaxations.

The reason why some authors have observed tolerance after prolonged treatment with SIN-1, particularly during organ bath experiments, could be that SIN-1 in aqueous solutions and in the presence of unphysiologically high oxygen concentrations functions as a donor of superoxide and cytotoxic peroxynitrite rather than nitric oxide (8,22) and may thus inflict oxidant damage to proteins involved in cyclic GMP formation and vascular relaxation, possibly via tyrosine modification (23). However, in vivo and in the presence of electron acceptors other than oxygen, SIN-1 predominantly releases nitric oxide (24,25), an observation that might explain why tolerance induction by SIN-1 has mainly been reported under artificial experimental conditions.

Whereas numerous studies document a failure of glyceryl trinitrate to attenuate vascular effects of SIN-1 (4,11,13,14), there are, on the other hand, a few investigations reporting cross-tolerance between glyceryl trinitrate and SIN-1 (12,15). A marked attenuation of the responsiveness to SIN-1 in artery rings made tolerant by pretreatment with glyceryl trinitrate was noted by Henry et al. (12).

However, no plausible explanation has been suggested as yet to explain the conflicting data of these studies. A possible mechanism by which glyceryl trinitrate under some experimental conditions may induce cross-tolerance to other nitric oxide donors such as SIN-1 is a direct desensitization of soluble guanylyl cyclase. Thus, in previous studies inhibitory effects on guanylyl cyclase have been detected following long-term exposure to organic nitrates in vascular and nonvascular tissues (1,6). From those investigations desensitization of guanylyl cyclase appears to be a function of glyceryl trinitrate preexposure concentration and to occur only at rather high concentrations in the millimolar range. Our data presented here clearly demonstrate that at lower, therapeutically more relevant concentrations, nitrate tolerance is confined to glyceryl trinitrate and does not affect cyclic GMP stimulation by SIN-1 or other spontaneous nitric oxide donors thus precluding down-regulation of guanylyl cyclase as basic mechanism of nitrate tolerance. As indicated above, our data rather suggest that tolerance induction occurs at a site upstream of nitric oxide-dependent guanylyl cyclase activation which is the enzymatic bioconversion of glyceryl trinitrate to nitric oxide. Therefore, spontaneous nitric oxide donors such as SIN-1, sodium nitroprusside or spermine NONOate, which by-pass this bioactivation step, are unaffected by nitrate tolerance and can trigger cyclic GMP accumulation despite blockade of glyceryl trinitrate metabolizing enzymes. Under certain clinical conditions, neurohormonal counterregulation and intravascular volume expansion, also referred to as pseudo-tolerance, have been observed during therapy with vasodilators in general, i.e. not exclusively with glyceryl trinitrate, and do therefore not meet the criteria of nitrate tolerance. Since nitrate tolerance in its onset, duration and intensity is a substance-specific phenomenon, pseudo-tolerance or unspecific neurohormonal counterregulation does not appear to be its underlying cause in vivo. Accordingly, Unger et al. (26) observed activation of endogenous vasoconstrictor mechanisms during therapy with molsidomine/SIN-1, and showed that this type of neurohormonal activation is not necessarily followed by tolerance. Moreover and in agreement with the findings of the present study, the majority of clinical reports clearly document that the vascular effects of SIN-1 are not diminished by tolerance and that nitrate-tolerant patients are still responsive to molsidomine or SIN-1 (27–29).

Recently, it has been demonstrated that nitrate tolerance is associated with an enhanced vascular superoxide production which may cause tolerance by quenching the nitric oxide free radical and forming peroxynitrite (15). The increase in vascular superoxide generation under conditions of nitrate tolerance is thought to result from angiotensin II-dependent activation of endothelial NAD(P)H oxidases (15). Also, superoxide originating during the redox process of nitrate bioactivation might inactivate organic nitrate converting enzymes (15,30). However, if superoxide was responsible for tolerance induction in our cellular model, all other nitric oxide-dependent pathways should be expected to show cross-tolerance, i.e. to be desensitized, after glyceryl trinitrate or SIN-1 pretreatment. Since SIN-1-dependent cyclic GMP stimulation remained unaffected by prolonged preexposure to both glyceryl trinitrate and SIN-1, our findings as well as many clinical observations documenting lack of cross tolerance between glyceryl trinitrate and SIN-1 (27–29) do not support oxidative stress as a crucial mechanism for nitrate tolerance. Moreover in a recent study from our group (17), various oxygen radical scavengers and a specific scavenger of nitric oxide left cellular tolerance induction unaltered providing additional evidence that reactive oxygen species or nitric oxide do not contribute to the development of nitrate tolerance.

In summary, our results show that the antianginal compound SIN-1 does not induce tolerance to its own actions at the cellular level and together with other spontaneous donors of nitric oxide maintains its capacity to stimulate the vasodilatory second messenger cyclic GMP under conditions of nitrate tolerance. Our results support and confirm clinical observations showing a lack of cross-tolerance between glyceryl trinitrate and SIN-1. Thus, nitrate tolerance appears to be a substance-specific phenomenon that is confined to nitric acid esters such as glyceryl trinitrate.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (Schr 298/8-2).

REFERENCES

1. H. Schröder, D. C. Leitman, B. M. Bennett, S. A. Waldman, and F. Murad. Glyceryl trinitrate-induced desensitization of guanylate cyclase in cultured rat lung fibroblasts, *J. Pharmacol. Exp. Ther.* **245**:413–418 (1988).
2. J. D. Parker and J. O. Parker. Nitrate therapy for stable angina pectoris, *N. Engl. J. Med.* **338**:520–531 (1998).
3. B. M. Bennett, D. C. Leitman, H. Schröder, J. H. Kawamoto,

K. Nakatsu, and F. Murad. Relationship between biotransformation of glyceryl trinitrate and cyclic GMP accumulation in various cultured cell lines, *J. Pharmacol. Exp. Ther.* **250**:316–323 (1989).
4. S. Förster, I. Woditsch, H. Schröder, and K. Schrör. Reduced nitric oxide release causes nitrate tolerance in the intact coronary circulation, *J. Cardiovasc. Pharmacol.* **17**:867–872 (1991).
5. H. L. Fung, S. J. Chung, J. A. Bauer, S. Chong, and E. A. Kowaluk. Biochemical mechanism of organic nitrate action, *Am. J. Cardiol.* **70**:4B–10B (1992).
6. S. A. Waldman, R. M. Rapoport, R. Ginsburg, and F. Murad. Desensitization to nitroglycerin in vascular smooth muscle from rat and human, *Biochem. Pharmacol.* **35**:3525–3531 (1986).
7. W. Rudolph and J. Dirschinger. Clinical comparison of nitrates and sydnonimines, *Eur. Heart J.* **12** (Suppl E):33–41 (1991).
8. M. Feelisch and J. S. Stamler. Donors of nitrogen oxides. In. M. Feelisch and J. S. Stamler (eds) Methods in Nitric Oxide Research, Wiley, Chichester, New York, 1996, pp. 77–115.
9. T. Ullrich, S. Oberle, A. Abate, and H. Schröder. Photoactivation of the nitric oxide donor SIN-1, *FEBS Lett.* **406**:66–68 (1997).
10. J. Delonca, T. Giraud, P. Beaufils, B. Dupuis, R. Haiat, and C. Thery. Comparative efficacy of the intravenous administration of linsidomine, a direct nitric oxide donor, and isosorbide dinitrate in severe unstable angina. A French multicentre study. French Group of Investigators, *Eur. Heart J.* **18**:1300–1306 (1997).
11. C. Govantes, M. A. Rodriguez-Martinez, and J. Marin. Vasodilator effect and tolerance induced by the nitrocompound SIN-1 in rabbit femoral artery, *Methods Find. Exp. Clin. Pharmacol.* **18**:387–395 (1996).
12. P. J. Henry, J. D. Horowitz, and W. J. Louis. Nitroglycerin-induced tolerance affects multiple sites in the organic nitrate bioconversion cascade, *J. Pharmacol. Exp. Ther.* **248**:762–768 (1989).
13. M. Kuhn and U. Förstermann. Endothelium-dependent vasodilatation in human epicardial coronary arteries: effect of prolonged exposure to glyceryl trinitrate or SIN-1, *J. Cardiovasc. Pharmacol.* **14** (Suppl 11):S47–S54 (1989).
14. A. Mülsch, R. Busse, I. Winter, and E. Bassenge. Endothelium- and sydnonimine-induced responses of native and cultured aortic smooth muscle cells are not impaired by nitroglycerin tolerance, *Naunyn Schmiedeberg's Arch. Pharmacol.* **339**:568–574 (1989).
15. T. Münzel and D. G. Harrison. Evidence for a role of oxygen-derived free radicals and protein kinase C in nitrate tolerance, *J. Mol. Med.* **75**:891–900 (1997).
16. G. Berkenboom, Z. Y. Fang, P. Unger, and J. Fontaine. Effects of in vivo SIN-1 treatment on nitrovasodilator relaxation and on EDRF-mediated responses in rat aorta, *J. Cardiovasc. Pharmacol.* **16**:636–640 (1990).
17. B. Hinz and H. Schröder. Vitamin C attenuates nitrate tolerance independently of its antioxidant effect, *FEBS Lett.* **428**:97–99 (1998).
18. H. Schröder. Cytochrome P-450 mediates bioactivation of organic nitrates, *J. Pharmacol. Exp. Ther.* **262**:298–302 (1992).
19. B. M. Bennett, B. J. McDonald, R. Nigam, P. G. Long, and W. C. Simon. Inhibition of nitrovasodilator- and acetylcholine-induced relaxation and cyclic GMP accumulation by the cytochrome P-450 substrate, 7-ethoxyresorufin, *Can. J. Physiol. Pharmacol.* **70**:1297–1303 (1992).
20. R. Yuan, M. Sumi, and L. Z. Benet. Investigation of aortic CYP3A bioactivation of nitroglycerin in vivo, *J. Pharmacol. Exp. Ther.* **281**:1499–1505 (1997).
21. E. Böhme, G. Grossmann, J. Herz, A. Mülsch, C. Spies, and G. Schultz. Regulation of cyclic GMP formation by soluble guanylate cyclase: stimulation by NO-containing compounds, *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **17**:259–266 (1984).
22. A. Schrammel, S. Pfeiffer, K. Schmidt, D. Koesling, and B. Mayer. Activation of soluble guanylyl cyclase by the nitrovasodilator 3-morpholinosydnonimine involves formation of S-nitrosoglutathione. *Mol. Pharmacol.* **54**:207–212 (1998).
23. A. Van der Vliet, J. P. Eiserich, C. A. O'Neill, B. Halliwell, and C. E. Cross. Tyrosine modification by reactive nitrogen species: a closer look, *Arch. Biochem. Biophys.* **319**:341–349 (1995).
24. J. S. Beckman and W. H. Koppenol. Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and the ugly, *Am. J. Physiol.* **271**:C1424–C1437 (1996).
25. T. Polte, S. Oberle, and H. Schröder. The nitric oxide donor SIN-1 protects endothelial cells from tumor necrosis factor-α-mediated cytotoxicity: possible role for cyclic GMP and heme oxygenase, *J. Mol. Cell. Cardiol.* **29**:3305–3310 (1997).
26. P. Unger, A. Leone, M. Staroukine, S. Degre, and G. Berkenboom. Hemodynamic response to molsidomine in patients with ischemic cardiomyopathy tolerant to isosorbide dinitrate, *J. Cardiovasc. Pharmacol.* **18**:888–894 (1991).
27. T. Störk, M. Möckel, S. Störk, G. Piske, O. Danne, T. Bodemann, R. Müller, H. Eichstädt, and H. Hochrein. Hemodynamic effect of molsidomine in coronary patients with heart failure with clinically manifest nitrate tolerance, *Z. Kardiol.* **82**:293–301 (1993).
28. G. Sutsch, J. H. Kim, C. Bracht, and W. Kiowski. Lack of cross-tolerance to short-term linsidomine in forearm resistance vessels and dorsal hand veins in subjects with nitroglycerin tolerance, *Clin. Pharmacol. Ther.* **62**:538–545 (1997).
29. P. Unger, J. L. Vachiery, D. de Canniere, M. Staroukine, and G. Berkenboom. Comparison of the hemodynamic responses to molsidomine and isosorbide dinitrate in congestive heart failure, *Am. Heart J.* **128**:557–563 (1994).
30. E. Bassenge and B. Fink. Tolerance to nitrates and simultaneous upregulation of platelet activity prevented by enhancing antioxidant state, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **353**:363–367 (1996).