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RECOVERY OF VASCULAR SMOOTH MUSCLE RELAXATION FROM NITROGLYCERIN-INDUCED TOLERANCE FOLLOWING A DRUG-FREE INTERVAL

A TIME–COURSE *IN VITRO* STUDY

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Abstract—Hemodynamic tolerance occurs upon continuous exposure of vascular tissues to nitrogelycerin (NTG). This phenomenon is believed to be due to the depletion of the tissue sulfhydryl (SH) group, which is essential for NTG-induced increase in tissue cyclic GMP and vasorelaxation. To determine the effect of an NTG-free interval on recovery of tissue cyclic GMP accumulation and vasorelaxation following development of NTG tolerance, isolated rat aortic rings were kept in Krebs physiologic buffer at 37°, precontracted with epinephrine, and exposed to NTG. The mean concentration of NTG, which relaxed the rings by 50% (EC_{50}) upon first exposure, was 1.1×10^{-7} M (N = 20), and vascular cyclic GMP levels after NTG increased from 21 to 46 fmol/mg ($P < 0.02$). A second exposure to NTG 15 min later increased the EC_{50} to 1.3×10^{-4} M and cyclic GMP levels did not change ($P < 0.001$ vs first NTG exposure), indicating tolerance to NTG. However, acetylcholine-mediated relaxation of aortic rings was preserved even in NTG-tolerant rings. A second exposure of tissues to NTG separated by 30, 60, and 120 min from the first exposure progressively decreased the EC_{50}, such that at 120 min the EC_{50} of NTG was 0.4×10^{-7} M (P = NS vs first NTG exposure). Tissue cyclic GMP levels increased from 14 to 71 fmol/mg (P = NS vs first NTG exposure). These data confirm development of tolerance to the vasorelaxant effects of NTG following initial exposure. An interval of 2 hr between multiple exposures of tissues to NTG results in preservation of the smooth muscle relaxation and an increase in tissue cyclic GMP in response to NTG.

Tolerance to the hemodynamic effects of nitroglycerin (NTG) upon its continuous or frequent use is a well established phenomenon [see review in Ref. 1] Needleman and Johnson [2] first hypothesized that organic nitrates interact with a specific sulfhydryl (SH) group in the "organic nitrate receptor" followed by denitration and oxidation of the SH group to the disulfide form. It is now quite evident that NTG undergoes a series of biochemical events, involving organic nitrate denitration, inorganic nitrite liberation, nitric oxide formation, guanylate cyclase activation, and finally cyclic GMP accumulation [2, 3] which causes smooth muscle relaxation. This biotransformation of NTG is a prerequisite for its activation of guanylate cyclase. Previous studies indicated that a decrease in the biotransformation of NTG [4] as well as a reduction in activation of soluble guanylate cyclase [5] are responsible for the development of NTG tolerance. However, tissue cyclic GMP accumulation in response to acetylcholine or sodium nitroprusside or SIN-1, which do not undergo denitration and do not require an SH group for delivery of nitric oxide, is preserved or minimally impaired in NTG-tolerant tissues [6–8]. These observations imply that a deficiency in the metabolic activation of NTG is the major determinant for development of NTG tolerance.

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It is generally believed that a relative unavailability of the reduced intracellular thiol groups is responsible for the desensitization of tissues to NTG upon repeated exposure. This became evident in clinical and laboratory studies demonstrating reversal or prevention of tolerance to nitroso vasolidators upon delivery of the SH donors *N*-acetylcysteine and captopril [9–11]; however, other studies have not shown a similar benefit of SH donors, particularly when tolerance of vascular tissues develops after long-term use of large amounts of NTG or the related compound isosorbide dinitrate [12, 13].

Many studies have demonstrated that infrequent dosing of NTG or a period of NTG "holiday" may avoid the phenomenon of NTG tolerance [see review in Ref. 14]. The potential basis for the beneficial effect of NTG "holiday" is recovery of a reduced thiol group in the tissues, whereby biotransformation of NTG can occur. The major purpose of this study was to examine the effect of different periods of NTG-free interval on the recovery of the relaxant effect of NTG on vascular smooth muscle.

MATERIALS AND METHODS

Preparation of vessels. Sprague–Dawley rats (average weight 175 g) were killed after being lightly anesthesized with halothane. The thoracic aortae were quickly removed and placed into aerated (95% O_2, 5% CO_2) Krebs–Ringer buffer of the following composition (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5;

KH_2PO_4, 1.2; $MgCl_2$, 1.2; $NaHCO_3$, 12.5; sodium-EDTA, 0.01; and dextrose, 11.1. The vessels were carefully cleaned of fat and loose connective tissue and were cut into 4-mm rings. The rings were washed and mounted onto wire stirrups and suspended in tissue baths filled with 3 mL of aerated Krebs buffer at 37° and were subsequently connected to myograph transducers (Kistler-Morse, Redmond, WA) to record changes in isometric force [15–17]. The aortic rings were then stretched to a preload force of 5 g and allowed to equilibrate for approximately 2 h until they stabilized. During this period, the buffer in the tissue bath was replaced every 15 min. Following equilibration, vascular rings were tested for viability by addition of 20 mM KCl. Endothelial integrity was documented in all experiments by the presence of characteristic 60–80% relaxation in response to acetycholine (ACh, 10^{-6} M).

Study protocols. Aortic rings were stimulated with *l*-epinephrine to achieve approximately 3–5 g (80% of maximal) of stable contraction above the baseline. Once stable contraction was achieved, NTG in various concentrations (10^{-9} to 10^{-5} M) was added to the organ bath; the interval between the addition of the next higher concentration of NTG was 2–4 min depending on stabilization of relaxation in response to the previous addition. Following exposure of the aortic rings to 10^{-5} M, the highest concentration of NTG used, the rings were washed and allowed to re-equilibrate for various periods, i.e. 15, 30, 60 and 120 min (referred to as the drug-free period). The buffer in the organ bath was exchanged every 15 min during this period.

Following the drug-free period, the aortic rings were contracted with *l*-epinephrine to achieve the same degree of contraction as before the first exposure to NTG. Upon stable contraction, aortic rings were exposed to the same concentrations of NTG as before (10^{-9} to 10^{-5} M).

NTG tolerant rings (see Results) as well as some rings in parallel with first exposure to NTG were also exposed to different concentrations of ACh.

Measurement of cyclic GMP. In parallel experiments, aortic rings were prepared as described above and allowed to equilibrate for approximately 2 h in aerated Krebs buffer at 37°. Following precontraction with *l*-epinephrine and subsequent addition of NTG or ACh, the aortic rings were flash-frozen at −70°. The tissue cyclic GMP levels were assayed according to previously described methods [18, 19]. Briefly, the frozen rings were homogenized in 10% trichloroacetic acid, acetylated, and extracted with water-saturated ethyl ether. The resulting samples were then evaporated to dryness under a stream of air and frozen for later cyclic GMP analysis by radioimmunoassay (RIA). The extraction efficiency for our assay was 85 ± 4%.

Materials. *l*-Epinephrine was purchased from Elkin-Sinns, Cherry Hills, NJ. All other reagents were obtained from the Sigma Chemical Co., St. Louis, MO. Supplies for cyclic GMP RIA were obtained from New England Nuclear, Boston, MA. All buffers and reagents were prepared fresh daily and kept on ice at all times.

Statistical analysis. Aortic ring tension in grams was divided by the wet weight of the rings to obtain the isometric tension in g/mg tissue. The EC_{50} values for NTG and ACh are defined as the concentration required for 50% relaxation of peak *l*-epinephrine-induced contraction. The cyclic GMP value was divided by the wet weight of the vascular rings to obtain cyclic GMP levels in fmol/mg tissue. All data are expressed as means ± SEM and are based on a minimum of six experiments. Statistical analysis was performed using Student's *t*-test for paired and unpaired observations, as appropriate. A P value of less than 0.05 was considered significant.

RESULTS

Effect of NTG on vascular smooth muscle tone. As shown in Figs. 1 and 2, NTG upon first exposure caused a prompt relaxation of epinephrine-contracted rat aortic rings. The degree of relaxation was dependent on NTG concentration, and the mean EC_{50} of NTG was 1.1×10^{-7} M.

When the same vascular rings were re-exposed to NTG following a 15-min washout and re-equilibration period, the NTG-induced vasorelaxation was significantly ($P < 0.01$) attenuated with a marked shift in the concentration–response curve (Figs. 1 and 2). The mean EC_{50} of NTG (1.3×10^{-4} M) was almost 300-fold greater ($P < 0.001$) than upon first exposure of the rings to NTG.

In contrast, when vascular rings were exposed to identical NTG concentrations following a 30-, 60- or 120-min drug-free interval, the degree of NTG-induced relaxation was similar to that observed during first exposure. The mean EC_{50} values were 0.5×10^{-7}, 0.3×10^{-7} and 0.4×10^{-7} M at 30, 60 and 120 min of drug-free period (P = NS vs EC_{50} value after first exposure) (Figs. 1 and 2).

Effect of ACh on NTG-tolerant aortic rings. ACh caused a prompt and concentration-dependent relaxation of all aortic rings whether or not they had been pre-exposed to NTG. In control rings not exposed to NTG, the mean EC_{50} of ACh was 1.1×10^{-7} M. In aortic rings tolerant to the relaxant effects of NTG, the mean EC_{50} of ACh was slightly higher at 1.9×10^{-7} M (P = NS vs EC_{50} value in control rings).

Effects of NTG and ACh on cyclic GMP accumulation. First exposure to NTG was associated with a marked increase in tissue cyclic GMP levels (Table 1). However, second exposure to NTG after 15 min of washout and re-equilibration failed to cause any increase in cyclic GMP. In contrast, longer drug-free periods resulted in a significant increase in cyclic GMP, in conjunction with the appearance of a vasorelaxant effect. It is noteworthy that ACh-induced smooth muscle relaxation was associated with a substantial increase in cyclic GMP even in NTG-tolerant rings.

DISCUSSION

This study confirms the phenomenon of marked resistance to the vasorelaxant effects of NTG when the isolated rat aortic rings have been exposed previously to 10 μM NTG. This study also shows that if the interval between the two exposures is

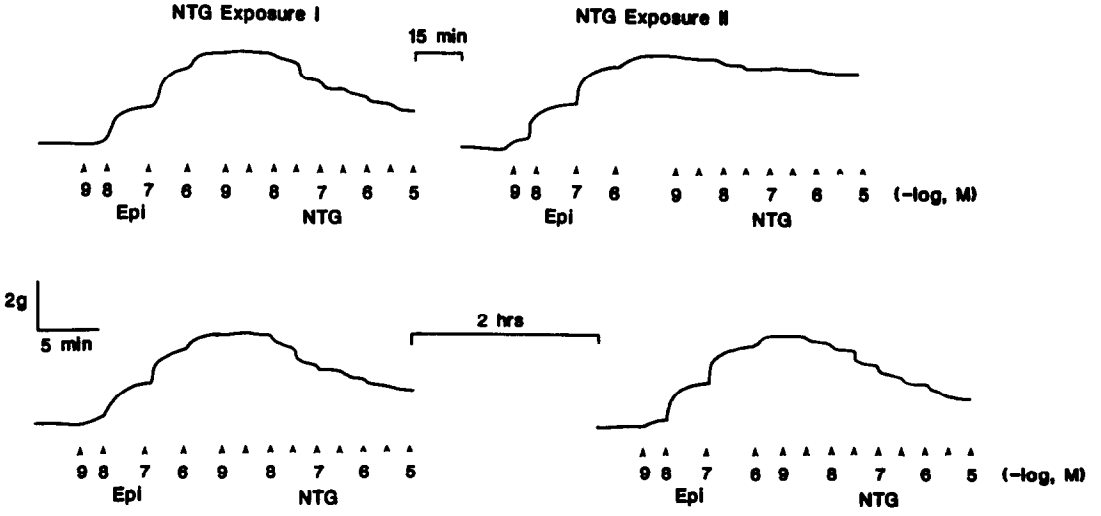

Fig. 1. A representative experiment showing minimal relaxation of vascular ring upon second exposure of rat aortic ring to nitroglycerin (NTG exposure II) 15 min following first exposure to NTG I (top panel). When the interval between exposures I and II was increased to 2 hr, the rat aortic ring relaxed normally in response to NTG.

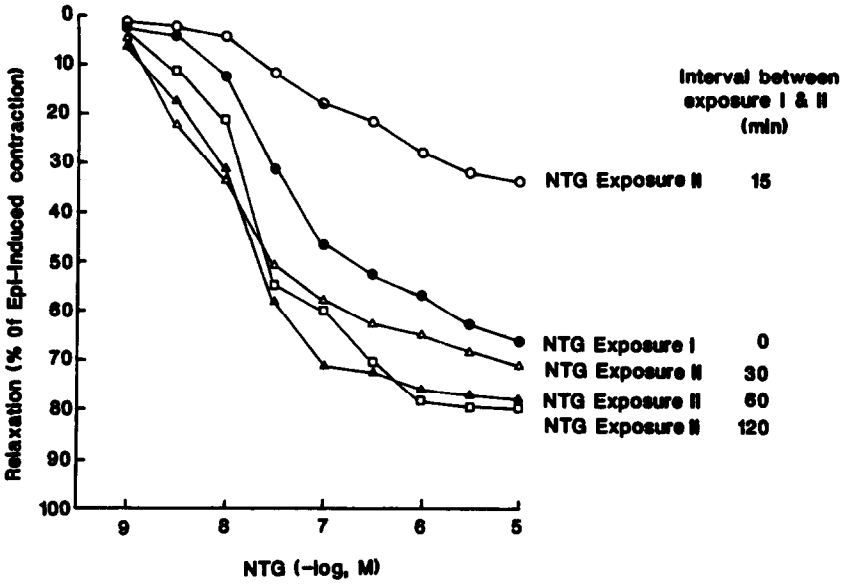

Fig. 2. Summary of the relaxant effects of NTG on rat aortic rings. Each data point is based on 4–8 experiments. Note tolerance to the vasorelaxant effects of NTG when the second exposure was 15 min after the first exposure. The relaxation was "normalized" when the washout and re-equilibration period was 30, 60 or 120 min.

prolonged from 30 to 120 min, there is a marked recovery of the vasorelaxant potential of NTG.

In association with the development of tolerance to the relaxant effect of NTG, tissue cyclic GMP levels did not increase. By itself, this observation cannot dissociate between the absence of biotransformation of NTG and failure to activate guanylate cyclase. However, ACh which releases a smooth muscle relaxing factor (most likely nitric oxide) from the endothelium promptly relaxed the NTG-resistant vessels and increased cyclic GMP concentrations. Importantly, endogenous nitric oxide does not require an SH group for its biotransformation. Since endothelium-derived nitric oxide causes vasorelaxation by stimulating cyclic GMP, our observations suggest that the tissue guanylate cyclase activity is preserved or only minimally decreased in NTG-tolerant rings [6, 7]. Several other investigators

Table 1. Cyclic GMP accumulation in rat aortic rings exposed to NTG

	Cyclic GMP (fmol/mg)
Epinephrine alone	21 ± 10
Epinephrine + NTG I	46 ± 12*
Epinephrine + ACh	111 ± 13*
Epinephrine + NTG II, 15 min	14 ± 14†
Epinephrine + ACh (after NTG II, 15 min)	110 ± 20*
Epinephrine + NTG II, 60 min	41 ± 21*
Epinephrine + NTG II, 120 min	71 ± 41*

Data (mean ± SEM) are from 3–6 experiments. NTG I and NTG II refer to first and second exposure; 15, 60 and 120 min refer to the interval between exposures I and II.

* $P < 0.01$ vs epinephrine alone.

† $P < 0.02$ vs NTG I exposure.

[8, 20, 21] showed persistence of response to SIN-1, which does not require biotransformation, in blood vessels rendered tolerant to NTG both *in vitro* and *in vivo*. In studies by Henry *et al.* [22], NTG-induced tolerance was not associated with attenuation of the effects of the endothelium-dependent vasodilator ionophore A23187. These and our studies support the observation of Bennett *et al.* [23], who showed that about 25% of guanylate cyclase activity is preserved in NTG-tolerant rat aortic rings. These observations are somewhat different from those of Rapoport *et al.* [24], who showed a modest tolerance to nitroprusside relative to its vasorelaxant effect and cyclic GMP accumulation in NTG-tolerant rings. SIN-1-induced guanylate cyclase activation in cell homogenates was attenuated after pretreatment of intact cells with NTG in studies by Mulsch *et al.* [21]. Ljusegren *et al.* [25] showed that response to ACh and the ionophore A23187 was blunted in NTG-pretreated bovine mesenteric arteries. Thus, there appear to be at least two different mechanisms of NTG tolerance: depletion of the SH group and desensitization of guanylate cyclase to activation *per se*. The present study suggests that desensitization of guanylate cyclase to activation is not very important in NTG tolerance in intact rat aortic rings when tissues are exposed to NTG for a short time.

In other ongoing studies in our laboratory, we observed that exposure of rat aortic rings to large amounts of NTG transiently rendered them resistant to the relaxant effects of ACh. Likewise, their exposure to ACh rendered them transiently resistant to the relaxant effects of NTG. This resistance was not reversed by addition of an SH group in the form of *N*-acetylcysteine, but could be rapidly overcome by treatment of rings with agents which restored sensitivity of guanylate cyclase. In light of this observation, it appears that following maximal and prolonged vasorelaxation by ACh or NTG, evolution of tolerance to NTG may reflect not only SH group depletion but also desensitization of guanylate cyclase to activation. As such, results of different studies [6–8, 20–25] need to be interpreted with caution with attention to study design.

Our observations on the recovery of relaxant effects of NTG on rat aortic rings after a drug-free interval of 30–120 min suggest that the vascular tissue recovers rapidly to allow biotransformation of NTG as well as cyclic GMP accumulation. This recovery of effects of NTG is similar to that reported recently by Henry *et al.* [26] and Kowaluk *et al.* [27]. Interestingly, recovery of NTG-induced cyclic GMP accumulation after initial tolerance has also been observed to occur in cultured rat lung fibroblasts after a prolonged washout period [28].

It appears from our studies that the recovery of tissues from NTG tolerance occurs after a drug-free period of ≥30 min when the rat aortic rings are exposed to small amounts (10 μM) of NTG for a brief period (approximately 10–15 min). It is likely that as the period of NTG exposure is increased, a longer drug-free period may be necessary to reverse the tolerance. Indeed, when bovine coronary artery rings were exposed to 1 or 100 μM NTG for 2–60 min, the extent of tolerance correlated with the NTG concentration as well as time of exposure [26]. However, NTG tolerance reversed with drug-free interval irrespective of the duration of NTG exposure and the degree of NTG tolerance induced [26]. Intermittent exposure compared to continuous exposure of rat aortic rings to the same amount of NTG also resulted in reduced NTG tolerance in studies by Kowaluk *et al.* [27], and the washout time necessary for reversal of tolerance was independent of the exposure time particularly when tolerance was complete.

These observations may be the basis of clinical observations of the requirement of a prolonged NTG-free interval when patients with congestive heart failure have been exposed to NTG for a long time [9]. Parker *et al.* [29] clearly demonstrated that tolerance to the clinical effects of organic nitrites as manifested by diminished exercise tolerance occurred when the drug was given four times a day but did not occur when it was given two or three times a day. As such, use of intermittent administration is becoming a mainstay of clinical therapy with nitrates.

In summary, our studies show that a marked tolerance to the vasorelaxant effects of NTG occurs after exposure of rat aortic rings to 10 μM NTG for

10–15 min. NTG tolerance is associated with inability to increase vascular cyclic GMP; however, endothelium-dependent vasodilator ACh can still cause relaxation and cyclic GMP accumulation in NTG-tolerant rings. The precise role of guanylate cyclase activity cannot be determined from these studies. Nonetheless, the tissue SH group and guanylate cyclase sensitivity can recover within 120 min in an *in vitro* system which presumably allows biotransformation of NTG and re-establishment of the relaxant effects of NTG.

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REFERENCES

1. Abrams J, Tolerance to organic nitrates. *Circulation* **74**: 1181–1185, 1987.
2. Needleman P and Johnson EM Jr, Mechanism of tolerance development to organic nitrates. *J Pharmacol Exp Ther* **184**: 309–315, 1973.
3. Ignarro LJ, Lippton H, Edwards JC, Baricos WH, Hyman AL, Kadowitz PJ and Gruetter GA, Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide. Evidence for the involvement of S-nitrosothiols as active intermediates. *J Pharmacol Exp Ther* **218**: 739–749, 1981.
4. Brien JF, McLaughlin BE, Breedon TH, Bennett BM, Nakatsu K and Marks GS, Biotransformation of glyceryl trinitrate occurs concurrently with relaxation of rabbit aorta. *J Pharmacol Exp Ther* **237**: I608–614, 1986.
5. Waldman SA, Rapoport RM, Ginsberg R and Murad F, Desensitization to nitroglycerin in vascular smooth muscle from rat and human. *Biochem Pharmacol* **35**: 3525–3531, 1986.
6. Keith RA, Burkman AM, Sokolski TD and Fertel RH, Vascular tolerance to nitroglycerin and cyclic GMP generation in rat aortic smooth muscle. *J Pharmacol Exp Ther* **221**: 525–531, 1982.
7. Molina CR, Andersen JW, Rapoport RM, Waldman SA and Murad F, The effect of *in vitro* nitroglycerin therapy on endothelium-dependent and independent vascular relaxation and cyclic GMP accumulation in rat aorta. *J Cardiovasc Pharmacol* **10**: 371–378, 1987.
8. Berkenboom G, Fontaine J and Degre S, Persistence of the response to SIN_1 on isolated coronary arteries rendered tolerant to nitroglycerin *in vitro* or *in vivo*. *J Cardiovasc Pharmacol* **12**: 345–349, 1988.
9. Packer M, Lee WH, Kessler PD, Gottlieb SS, Medina N and Yushak M, Prevention and reversal of nitrate tolerance in patients with congestive heart failure. *N Engl J Med* **317**: 799–804, 1987.
10. May DC, Popma JJ, Black WH, Schaffer S, Lee HR, Levine BD and Hillis LD, *In vivo* induction and reversal of nitroglycerin tolerance in human coronary arteries. *N Engl J Med* **317**: 805–809, 1987.
11. van Gilst WH, de Graeff PA, Scholtens FZ, de Langer J and Wesseling H, Potentiation of isosorbide dinitrate-induced coronary dilatation by captopril. *J Cardiovasc Pharmacol* **9**: 254–255, 1987.
12. Parker JO, Farrell B, Lahey KA and Rose BF, Nitrate tolerance: The lack of effect of *N*-acetylcysteine. *Circulation* **76**: 572–576, 1987.
13. Munzel T, Jurgen H, Mulsch A, Stewart DJ and Bassenge E, Nitrate tolerance in epicardial arteries or in the venous system is not reversed by *N*-acetylcysteine *in vivo*, but tolerance-independent interactions exist. *Circulation* **79**: 188–197, 1989.
14. Abrams J, Interval therapy to avoid nitroglycerin tolerance. Paradise regained? *Am J Cardiol* **64**: 931–934, 1989.
15. Lawson DL, Smith C, Mehta JL, Mehta P and Nichols WW, Leukotriene D_4 potentiates the contractile effects of epinephrine and norepinephrine on rat aortic rings. *J Pharmacol Exp Ther* **247**: 953–957, 1988.
16. Mehta JL, Lawson DL, Nichols WW and Mehta P, Modulation of vascular tone by neutrophils. Dependence on endothelial integrity. *Am J Physiol* **257**: H1315–H1320, 1989.
17. Mehta JL, Lawson DL and Nichols WW, Attenuated coronary relaxation after reperfusion. Effects of superoxide dismutase and T_xA_2 inhibitor U 63557A. *Am J Physiol* **257**: H1240–H1246, 1989.
18. Steiner AL, Parker CW and Kipris DM, Radioimmunoassay for cyclic nucleotides. *J Biol Chem* **247**: 1106–1111, 1972.
19. Nicolini FA, Wilson AC, Mehta P and Mehta JL, Comparative platelet inhibitory effects of human neutrophils and lymphocytes. *J Lab Clin Med* **116**: 147–152, 1990.
20. Stewart DJ, Holtz J and Bassenge E, Long-term nitroglycerin treatment: Effect on direct and endothelium-mediated large coronary artery dilation in dogs. *Circulation* **75**: 847–856, 1987.
21. Mülsch A, Busse R, Winter I and Bassenge E, Endothelium- and sydnonimine-induced responses of native and cultured aortic smooth muscle cells are not impaired by nitroglycerin tolerance. *Nauyn Schmiedebergs Arch Pharmacol* **339**: 568–574, 1989.
22. Henry PJ, Horowitz JD and Louis WJ, Nitroglycerin-induced tolerance affects multiple sites in the organic nitrate bioconversion cascade. *J Pharmacol Exp Ther* **248**: 762–768, 1989.
23. Bennett BM, Schroder H, Hayward LD, Waldman SA and Murad F, Effect of *in vitro* organic nitrate tolerance on relaxation, cyclic GMP accumulation, and guanylate cyclase activation by glyceryl trinitrate and the enantiomers of isotidide dinitrate. *Circ Res* **63**: 693–701, 1988.
24. Rapoport RM, Waldman SA, Ginsburg R, Molina CR and Murad F, Effects of glyceryl trinitrate on endothelium-dependent or endothelium-independent relaxation and cyclic GMP levels in rat aorta and human coronary artery. *J Cardiovasc Pharmacol* **10**: 82–89, 1987.
25. Ljusegren ME, Ahlner J and Axelsson KL, Studies on vascular smooth muscle tolerance to different cGMP-mediated vasolidators and cross-tolerance to glyceryl trinitrate. *Pharmacol Toxicol* **62**: 302–307, 1988.
26. Henry PJ, Horowitz JD and Louis WJ, Determinants of *in vitro* nitroglycerin tolerance induction and reversal: Influence of dose-regimen, nitrate-free period, and sulfhydryl supplementation. *J Cardiovasc Pharmacol* **14**: 31–37, 1989.
27. Kowaluk E, Hough K and Fung H-L, Effect of intermittent exposure and drug-free intervals on the *in vitro* vascular tolerance to nitroglycerin. *Life Sci* **44**: 1157–1163, 1989.
28. Schroder H, Leitman DC, Bennett BM, Waldman SA and Murad F, Glyceryl trinitrate-induced desensitization of guanylate cyclase in cultured rat lung fibroblasts. *J Pharmacol Exp Ther* **245**: 413–418, 1988.
29. Parker JD, Farrell B, Lahey KA and Moe G, Effect of intervals between doses on the development of tolerance to isosorbide dinitrate. *N Engl J Med* **316**: 1440–1444, 1987.