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Nitroglycerin-induced desensitization of vascular smooth muscle may be mediated through cyclic GMP-disinhibition of phosphatidylinositol hydrolysis

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Summary. The purpose of this study was to investigate the hypothesis that nitroglycerin-induced desensitization of vascular smooth muscle is mediated through cyclic GMP-disinhibition of phosphatidylinositol hydrolysis. Norepinephrine-induced contraction and increased levels of inositol monophosphate, a measure of phosphatidylinositol hydrolysis, in rat aorta. Prior treatment with nitroglycerin inhibited both the norepinephrine-induced contraction and the elevated levels of inositol monophosphate to the same relative magnitude. The nitroglycerin-induced inhibition of contraction and inositol monophosphate formation were prevented in tissues desensitized with nitroglycerin. These results suggest that: 1) nitroglycerin may inhibit vascular smooth muscle contraction through cyclic GMP-inhibition of phosphatidylinositol hydrolysis and 2) desensitization to the relaxant effects of nitroglycerin may be due to disinhibition of the hydrolysis.
Key words. Vascular smooth muscle; nitroglycerin; cyclic GMP; relaxation; desensitization; phosphatidylinositol; inositol phosphates.

Tolerance to the hypotensive effects of nitroglycerin have been established clinically[1-3], and have been observed in animals treated with nitroglycerin[4-6]. The site of nitroglycerin-induced tolerance may be at the level of the vasculature, since relaxations to nitroglycerin were reduced in blood vessels removed from animals treated with nitroglycerin[4,6]. Nitroglycerin treatment of blood vessels in vitro has also been shown to inhibit their subsequent ability to relax in response to nitroglycerin[7-9]. Relaxation to the nitrovasodilators, including nitroglycerin, has been proposed to be mediated through the formation of cyclic GMP[10-13]. In support of this hypothesis, are the observations that the increased levels of cyclic GMP associated with nitroglycerin-induced relaxation are reduced in blood vessels removed from animals treated with nitroglycerin, or in vessels exposed to nitroglycerin in vitro[7,9,10,14-17]. However, the hypothesis that nitroglycerin-induced desensitization may be due to an inability to elevate cyclic GMP needs to be further evaluated, since the events which follow cyclic GMP elevation and result in relaxation, remain obscure.

There are a number of reports which have demonstrated that contraction of vascular smooth muscle is associated with increased phosphatidylinositol hydrolysis[18-20]. The increased hydrolysis of phosphatidylinositol is thought to elevate the levels of inositol trisphosphate and diacylglycerol, which may result in contraction[18,21]. Furthermore, it has been proposed that cyclic nucleotides may act as feedback inhibitors of contraction through inhibition of phosphatidylinositol hydrolysis[21]. Consistent with this hypothesis, we have recently shown that sodium nitroprusside inhibited the elevated levels of inositol monophosphate due to norepinephrine, and that this inhibitory effect was mimicked by 8-bromo cyclic GMP[22]. Thus, the purpose of the present study was to investigate whether nitroglycerin-induced tolerance in vascular smooth muscle was due to cyclic GMP-mediated disinhibition of phosphatidylinositol hydrolysis.

Materials and methods. Rats (Sprague-Dawley, male, 240–360 g) were decapitated, their thoracic aortae removed and cleaned of extraneous fatty tissue. Helical strips (approximately 2 mm × 1.5 cm) were prepared and the endothelium removed by rubbing with a scalpel[23]. Tissues were then incubated for 3 h with 8 μCi/ml ^{3}H-inositol (myo-[(2-^{3}H(N))]-inositol, 16.5 μCi/mmole, New England Nuclear) in 37 °C Krebs-Ringer bicarbonate solution which was gassed with 95% O_2–5% CO_2 and had the following composition (mM): NaCl, 118.5; KCl, 4.74; $MgSO_4$, 1.18; KH_2PO_4, 1.18; $CaCl_2$, 2.5; $NaHCO_3$, 24.9; glucose, 10.0. Strips were then mounted in organ baths and placed at 0.8 g-force resting tension which was maintained throughout the experiment. Tissues were allowed to equilibrate for 2 h prior to the addition of any drugs. Other strips were placed in flasks and desensitized by exposure for 1 h to 0.44 mM nitroglycerin (1:10 nitroglycerin:lactose from ICI Americas, Inc.) as we and others have previously described[9,15]. Control strips were exposed to 2.6 mM lactose. Lactose was added to strips unexposed to nitroglycerin since the nitroglycerin was added as a lactose powder. Tissues were then mounted in organ baths as above and washed every 15 min over the next 1 h. Strips were exposed to 10 or 100

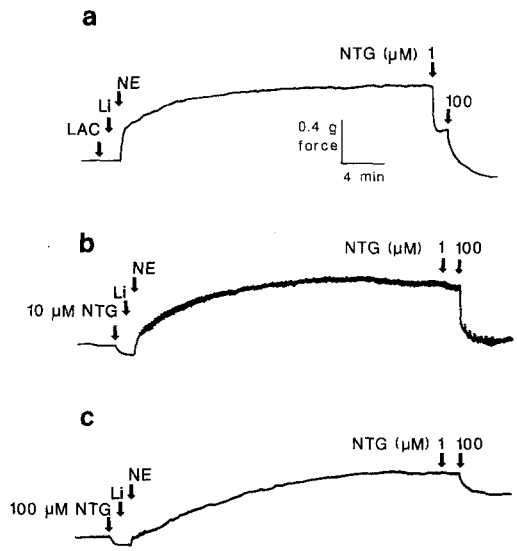

Figure 1. Effects of nitroglycerin on norepinephrine-induced contractions. Rat thoracic aortae without endothelium were exposed to 600 μM lactose (LAC; *a*), or 10 or 100 μM nitroglycerin (NTG; *b*, *c*), followed by 10 mM lithium chloride (Li) and then 0.3 μM norepinephrine (NE). Tissues were exposed to 1 and then 100 μM nitroglycerin 30 min after the addition of norepinephrine. Tracings of tension recordings are shown.

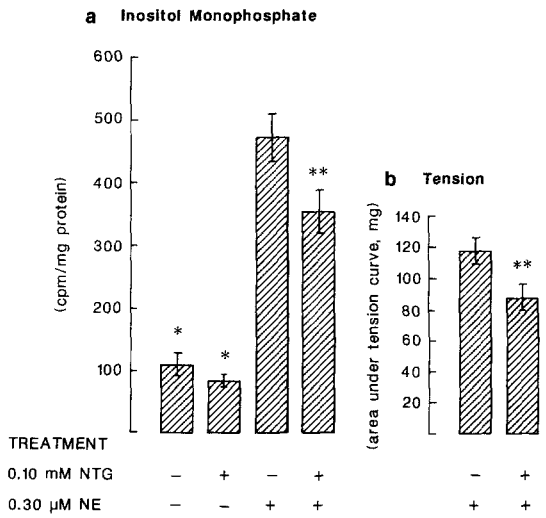

Figure 2. Effects of nitroglycerin on norepinephrine-induced tension and elevations of inositol monophosphate levels in rat thoracic aorta. *a* Segments of rat thoracic aorta without endothelium were exposed to 600 μM lactose or 0.10 mM nitroglycerin followed by 10 mM lithium chloride and then, in some cases, to 0.3 μM norepinephrine. Tissues were then frozen and assayed for inositol monophosphate as described in 'Methods'. Each of the four conditions within each experiment was tested on a quarter segment of aorta which was derived from a single vessel. In *b*, the area under the tension curve generated by tissues treated as above was quantitated as described in 'Results'. Shown are mean ± SE. N = 10 and 9 in experiments *a* and *b*, respectively. *Significantly less than tissues exposed to norepinephrine; **Significantly less than tissues exposed to norepinephrine in the absence of nitroglycerin.

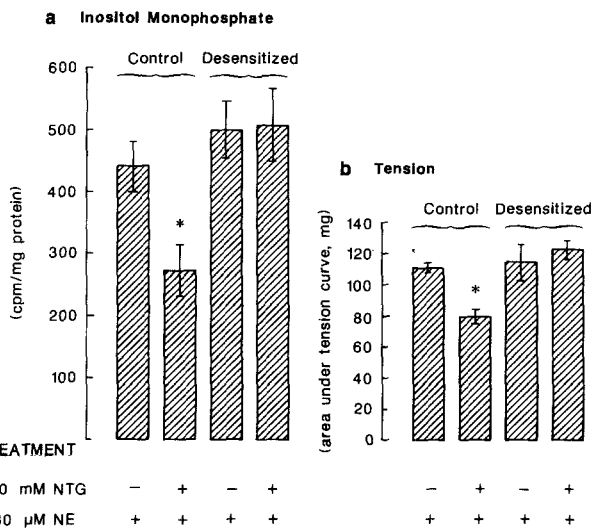

Figure 3. Effects of nitroglycerin on norepinephrine-induced tension and elevation of inositol monophosphate in desensitized rat thoracic aorta without endothelium. *a* Desensitization was induced by treatment of strips for 1 h with 0.44 mM nitroglycerin as described in 'Methods', while control aortae were treated for 1 h with 2.6 mM lactose. Tissues were then exposed to 600 μM lactose or 0.10 mM nitroglycerin followed by 10 mM lithium chloride and 0.3 μM norepinephrine. Tissues were then frozen and assayed for inositol monophosphate as described in 'Methods'. Each of the four conditions within each experiment was tested on a quarter segment of aorta which was derived from a single vessel. In *b*, the area under the tension curve generated by tissues treated as above was quantitated as described in 'Results'. Shown are mean ± SE. N = 5 and 4 in *a* and *b*, respectively. *Significantly less than other tissues.

μM nitroglycerin for 1 min followed by 10 mM LiCl for an additional 1 min, and then to 0.3 μM norepinephrine (l-norepinephrine, Sigma) for 30 min. LiCl was added to inhibit inositol monophosphate phosphatase[24]. Additional strips remained unexposed to nitroglycerin and/or norepinephrine. Inositol monophosphate levels in the tissues treated as above were assayed according to the procedure of Brown and Brown[25]. Briefly, aortae were frozen between clamps precooled in liquid nitrogen, homogenized in 10% trichloroacetic acid and centrifuged (1500 × g, 20 min). The supernatants were then extracted with water saturated ether (5 × 2 ml) and loaded onto anion exchange columns (175 mg; BioRad AG1-X8). The columns were then washed with 100 ml of water, and inositol monophosphate eluted with 8 ml of 0.2 mM ammonium formate in 100 mM formic acid. Scintillation cocktail (10 ml) was added to the eluate and the samples counted. The pellets were suspended in 1.0 N NaOH and their protein content assayed according to the procedure of Lowry et al.[26]. Statistical significance was accepted at the 0.05 level using Student's t-test.

Results. Nitroglycerin inhibited both the rate and maximum force of contraction due to norepinephrine (fig. 1). Therefore, the magnitude of inhibition was measured as the area under the norepinephrine-induced tension curve and was quantitated by weight. Treatment of rat aortae with 0.1 mM nitroglycerin inhibited contractions due to 0.3 μM norepinephrine by 25% (fig. 2b). Smaller magnitudes of nitroglycerin-induced inhibition of contraction were observed with lower concentrations of nitroglycerin (0.01 mM; fig. 1b, c). The relatively small magnitude of nitroglycerin-induced inhibition of contraction due to norepinephrine was probably due to desensitization to the relaxant effects of nitroglycerin that occurred during exposure to nitroglycerin, since nitroglycerin-induced relaxations were inhibited in tissues exposed to nitroglycerin (fig. 1). Oscillations of the contractile response due to norepinephrine were sometimes observed in tissues pretreated with nitroglycerin (fig. 1b). Basal tension was also decreased by exposure to nitroglycerin (fig. 1b, c).

Prior exposure to 0.1 mM nitroglycerin also decreased the elevated levels of inositol monophosphate due to norepinephrine (figs 2a and 3a). The magnitude of inhibition of inositol monophosphate accumulation (25%) was equivalent to the amount of decrease in the contractile response (figs 2b and 3b). Nitroglycerin also appeared to lower basal levels of inositol monophosphate, however, the decrease was not significant (fig. 2a). The inhibition of both the contractions and elevations of inositol monophosphate levels due to norepinephrine were abolished in tissues desensitized with nitroglycerin (fig. 3).

Discussion. Tolerance to nitroglycerin has been well established in clinical therapy[1]. Also, decreased relaxations to nitroglycerin has been observed in blood vessels obtained from animals treated with nitroglycerin in vivo, or in blood vessels exposed to nitroglycerin in vitro[6-9]. It appears that at least part of the underlying mechanism involved in the development of tolerance may be due to an inability to elevate cyclic GMP levels since the elevated cyclic GMP levels associated with nitroglycerin-induced relaxation were prevented in desensitized rat aorta and other vascular tissues[7,9,10,14-17]. The present results demonstrate that nitroglycerin prevented the accumulation of inositol monophosphate and contraction due to norepinephrine. Inositol monophosphate accumulation has been shown to be a measure of phosphatidylinositol hydrolysis[25] and is associated with agonist-induced contraction in rat aorta[19]. Nitroglycerin had no effect on inositol monophosphate accumulation in tissues desensitized with nitroglycerin. These results, and those which demonstrated that the elevated cyclic GMP levels induced by nitroglycerin were inhibited in tissues desensitized with nitroglycerin, suggest that nitroglycerin-induced desensitization is due to prevention of cyclic GMP-mediated inhibition of phosphatidylinositol hydrolysis.

The above hypothesis is consistent with that of Takai et al.[21],

who suggested that cyclic nucleotides may act as negative feedback inhibitors of agonist-induced hydrolysis of membrane phosphatidylinositols. In support of this hypothesis, sodium nitroprusside and 8-bromo cyclic GMP also inhibited contraction and inositol monophosphate accumulation due to norepinephrine in rat aorta[22]. In addition, it has recently been suggested that the endothelium inhibits vascular smooth muscle tone and contraction through the spontaneous release of a factor which elevates cyclic GMP levels, thereby inhibiting phosphatidylinositol hydrolysis[27].

Others have suggested that phosphatidylinositol hydrolysis increases the levels of 1) phosphatidic acid, which may act as a Ca^{2+} ionophore[28], 2) inositol trisphosphate, which induces Ca^{2+} release from the sarcoplasmic reticulum and thereby induces contraction[18], and 3) diacylglycerol, which activates protein kinase C[21]. The mechanism by which cyclic GMP may act to prevent the formation of any and/or all of these potential second messengers has not been established. Nitroglycerin tolerance may be due to an inability to activate cyclic GMP-dependent protein kinase, which would phosphorylate proteins involved in the regulation of phosphatidylinositol hydrolysis.

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Photochemical oxidation of actinidin, a thiol protease from *Actinidia chinensis*

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Summary. Actinidin was rapidly inactivated by methylene blue-catalyzed photooxidation at pH 7.9 and 20 °C. The rate of inactivation was pH-dependent and became slower at lower pH values, suggesting the involvement of a histidine residue in the inactivation.
Key words. Actinidine, photoinactivation; protease.

Dye-sensitized photochemical oxidation of an enzyme has often been used to obtain information on the amino acid residues essential for its catalytic activity. It is well known that the photooxidation of proteins in the presence of methylene blue causes a rapid destruction of histidine and tryptophan residues and a slower destruction of tyrosine, cysteine, and methionine residues[1-4]. Martinez-Carrion studied a histidine-specific oxidation with aspartic aminotransferase[5]. Actinidin (EC 3.4.22.14) isolated from the sarcocarp of the Chinese gooseberry or kiwi fruit, *Actinidia chinensis*, is a thiol protease[6,7].

This report shows that actinidin is sensitive to dye-sensitized photochemical oxidation, and suggests that destruction of histidine is responsible for inactivation.

Materials and methods. Actinidin was isolated from the Chinese gooseberry according to the procedure of McDowal[7]. The proteinase activity of actinidin was determined by a modified Kunitz method[8] using amidated casein instead of casein as a substrate. The assay was always performed in the presence of 1 mM cysteine and 1 mM EDTA. Reaction was carried out at pH 4.0 and stopped with 10% trichloroacetic acid. Amidated casein (pI 9–10) was prepared by amidation of casein with ethylenediamine.

The pH dependence of the rate of inactivation of actinidin by methylene blue-catalyzed photooxidation was tested as follows. The reaction mixture contained 120 μM actinidin and 180 μM methylene blue, respectively, in 0.2 M phosphate-citrate buffer containing 1 mM EDTA, in a total volume of 10 ml. The reaction mixture was irradiated from a distance of 15 cm with a 100 W incandescent lamp at 20 °C. Aliquots of 50 μl were withdrawn at appropriate time intervals and used for assay of the enzymatic activity, and at the same time aliquots of 1.0 ml were withdrawn for amino acid analyses.

Photooxidized protein was freed from the reagents by passage through a column (1.5 × 31 cm) of Sephadex G-25 equilibrated and eluted with 0.1 M formic acid. The protein fractions were pooled and lyophilized. Native and photooxidized actinidin