Nitroglycerin relaxes coronary artery of the pig with no change in glutathione content or glutathione S-transferase activity

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1 The role of glutathione content and glutathione S-transferase activity in vascular relaxant responses to nitroglycerin was evaluated in potassium (30 mM)-contracted coronary artery strips of the pig by measuring changes in tension, glutathione content and glutathione S-transferase activity.

2 Prior exposure of coronary artery strips to nitroglycerin (10^{-5} M or 10^{-4} M for 20 min) resulted in tachyphylaxis to subsequent relaxation to nitroglycerin (10^{-8} – 10^{-5} M).

3 The glutathione content and glutathione S-transferase activity of the arterial strips rendered tachyphylactic by prior exposure to nitroglycerin (10^{-5} M for 20 min or 10^{-3} M for 120 min) were not significantly different from those of control strips.

4 Treatment with diethyl maleate (10^{-4} M or 10^{-3} M for 60 min) markedly depleted arterial glutathione content in a concentration-dependent manner with no change in glutathione S-transferase activity.

5 The relaxant response of coronary artery strips to nitroglycerin (10^{-8} – 10^{-5} M) was completely unaffected following treatment with diethyl maleate (10^{-4} M or 10^{-3} M for 60 min).

6 The results suggest that vascular glutathione content does not play an important role in vascular relaxation or tolerance development to nitroglycerin, at least in pig isolated coronary artery.

Keywords: Nitroglycerin; glutathione; glutathione S-transferase; diethyl maleate; tolerance; vasorelaxation; porcine coronary artery.

Introduction

It has been proposed that organic nitrates produce vascular smooth muscle relaxation resulting from generation of S-nitrosothiols or nitric oxide and that these processes require the presence of glutathione and glutathione S-transferase. The nitric oxide or S-nitrosothiols thus formed then activate soluble guanylate cyclase producing guanosine 3':5'-cyclic monophosphate and vascular relaxation (Needleman *et al.*, 1973; Ignarro *et al.*, 1981; Ignarro & Kadowitz, 1985; Kukovetz & Holzmann, 1985). Moreover, it has been proposed that the development of tolerance following repetitive application of nitroglycerin is due to depletion of vascular glutathione, thus preventing enzymic conversion of the nitrovasodilator to the active species (Needleman & Johnson, 1973; Neeldman *et al.*, 1973). This speculation is supported by the finding that the sulphhydryl compound, *N*-acetylcysteine, reverses tolerance to nitrovasodilators in vascular smooth muscle by restoring glutathione content (Torresi *et al.*, 1985; Winniford *et al.*, 1986; Packer *et al.*, 1987; Svendsen *et al.*, 1989). Thus, glutathione consumption is considered to be needed for both vascular smooth muscle relaxation and tolerance development to nitrates. Despite this, however, only one study has been described in which measurement of vascular glutathione content has been performed in association with vascular relaxation and tolerance development (Gruetter & Lemke, 1985). The present work, therefore, was designed to clarify the relationship between vascular relaxation, tolerance development, and vascular glutathione content. In this study, relaxant responses to nitroglycerin and changes in glutathione content induced by nitroglycerin treatment were examined in isolated coronary arteries of the pig. In addition, the effects of diethyl maleate, which is known to deplete glutathione (Boyland & Chasseaud, 1967; 1968), were observed on relaxant responses and changes in glutathione content. Furthermore, glutathione S-transferase activity was also measured to clarify the role of this enzyme in vascular relaxation.

Methods

Preparation of coronary artery rings

After slaughter, hearts were removed from pigs of either sex weighing 70–100 kg and placed in ice-cold Krebs-Henseleit solution of the following composition (mM): NaCl 120, KCl 4.8, CaCl₂ 1.25, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25 and glucose 11. The proximal part of the left circumflex coronary artery was cut into ring preparations of 2–3 mm diameter and 1–1.5 mm wide. These arterial preparations were not denuded of endothelium, and thus the present study was carried out on the whole coronary artery with endothelium. The ring preparations were suspended in organ baths filled with Krebs-Henseleit solution maintained at 37°C and gassed with 95% O₂ and 5% CO₂. One end of the arterial rings was fixed at the base of the bath, and the other was connected to an isometric force-displacement transducer (Nihon Kohden, TB-611T, Tokyo, Japan) for measurement of changes in tension. The resting tension of the rings was adjusted to 1.0 g. Changes in tension were recorded on an ink-writing recorder (Nihon Kohden, RJG-4004).

Experimental protocol

After 90 min of equilibration time, KCl 30 mM was added to test the viability of the preparations and then washed out. Fifteen min later, nitroglycerin or Krebs-Henseleit solution (vehicle) was added and the rings were incubated for 20 min, before the bathing solution was exchanged for fresh Krebs-Henseleit solution. Twenty min after exchanging the bathing solution, KCl 30 mM was added to the baths again. After stabilization of the potassium-induced contracture, nitroglycerin was added cumulatively. Relaxant responses to nitroglycerin were represented as the percentage relaxation when the potassium (30 mM)-induced contracture was defined as 100% (100% = 1.8 ± 0.1 g in vehicle-incubated preparations as controls or 1.5 ± 0.2 g in nitroglycerin-incubated preparations, respectively).

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In another series of experiments, the preparations were preincubated with diethyl maleate for 60 min and washed with fresh Krebs-Henseleit solution. KCl 30 mM was added to test the viability of the rings, and then washed out. Fifteen min after washing the preparations, KCl 30 mM was applied again, and after stabilization of the induced contracture, nitroglycerin was added cumulatively. In this series of experiments, too, relaxant responses to nitroglycerin were represented as the percentage relaxation when the potassium (30 mM)-induced contracture was defined as 100% ($100\% = 2.1 \pm 0.1$ g in vehicle-incubated preparations as controls or 1.9 ± 0.2 g in diethyl maleate-incubated preparations, respectively).

Measurement of glutathione content and glutathione S-transferase activity

Small segments (5–10 mm) of coronary artery were collected from 5 pig hearts and equilibrated with Krebs-Henseleit solution for 90 min at 37°C. The arteries were then incubated with nitroglycerin 10^{-5} M for 20 min or with nitroglycerin 10^{-3} M for 120 min, or with diethyl maleate 10^{-4} M or 10^{-3} M for 60 min. The artery segments were washed 3 times with Krebs-Henseleit solution and homogenized in 5 volumes of 0.9% NaCl solution with a polytron homogenizer (Kinematch, Switzerland). After centrifugation at 9000 *g* for 30 min at 4°C, the supernatant was retained. The protein content was measured by the method of Lowry *et al.* (1951). Glutathione S-transferase activity was measured by the method of Habig *et al.* (1974) using 1-chloro-2,4-dinitrobenzene 1 mM and reduced glutathione 1 mM as substrates, and the glutathione content was measured by the method of Reed *et al.* (1980) and Gruetter & Lemke (1985) with a Toyo Soda high-performance liquid chromatograph (CCPM; Tokyo, Japan), a UV 8000 detector using iodoacetic acid (4 μ M), an excess of NaHCO₃ (dry powder) for neutralization and an alcoholic solution of 1-fluoro-2,4-dinitrobenzene (1.5 ml/98.5 ml absolute ethanol) as the reaction solvent.

Drugs

Drugs used in this study were nitroglycerin (Millisrol; Nihonkayaku, Tokyo, Japan), diethyl maleate (Sigma, St. Louis, U.S.A.), reduced glutathione (Sigma), 1-chloro-2,4-dinitrobenzene (Wako, Tokyo, Japan), iodoacetic acid (Sigma), NaHCO₃ (Wako) and 1-fluoro-2,4-dinitrobenzene (Wako). All other chemicals were of analytical reagent grade.

Statistics

Data were analyzed by Student's *t* test, and a significant difference was assumed when a *P* value was less than 5%.

Table 1 Effect of nitroglycerin (NG) on glutathione content (GSH) and glutathione S-transferase activity (GST) in pig coronary artery

Incubation	n	GSH (nmol mg ⁻¹ protein)	GST (nmol mg ⁻¹ protein min ⁻¹)	
Control	5	18.2 ± 5.5	45.9 ± 6.0	37°C, 20 min
NG 10 ⁻⁵ M	5	17.2 ± 4.8	44.4 ± 4.7	incubation
Control	5	14.7 ± 2.9	36.3 ± 7.1	37°C, 120 min
NG 10 ⁻³ M	5	14.4 ± 4.3	35.3 ± 7.1	incubation

Each value is the mean ± s.d.

Table 2 Effect of diethyl maleate (DEM) on glutathione content (GSH) and glutathione S-transferase activity (GST) in pig coronary artery

Incubation	n	GSH (nmol mg ⁻¹ protein)	GST (nmol mg ⁻¹ protein min ⁻¹)	
Control	5	14.3 ± 1.0	47.1 ± 1.7	37°C, 60 min
DEM 10 ⁻⁴ M	5	7.7 ± 2.3***	48.1 ± 2.7	incubation
DEM 10 ⁻³ M	5	0.9 ± 0.6***	49.3 ± 4.7	

Each value is the mean ± s.d.

*** *P* < 0.001 vs. the corresponding control values.

Results

Potassium chloride, 30 mM, contracted pig coronary artery strips to a level of 1.8 ± 0.1 g (*n* = 26). In 14 preparations which were previously incubated with Krebs-Henseleit solution as the control, nitroglycerin 10^{-8} – 10^{-5} M produced concentration-dependent relaxations as shown in Figure 1. In 7 preparations which were previously incubated with nitroglycerin 10^{-5} M for 20 min, the concentration-relaxation curve for nitroglycerin 10^{-8} – 10^{-5} M was significantly shifted to the right. Prior incubation with nitroglycerin 10^{-4} M for 20 min (*n* = 7) shifted the concentration-relaxation curve for nitroglycerin 10^{-8} – 10^{-5} M even further to the right (Figure 1). Thus, prior incubation with nitroglycerin resulted in the development of tachyphylaxis.

No change in arterial glutathione content or glutathione S-transferase activity was observed following incubation at 37°C with nitroglycerin 10^{-5} M for 20 min, or 10^{-3} M for 120 min (Table 1).

In order to evaluate further the relationship between vascular relaxation and glutathione content, the effects of the glutathione depleting agent, diethyl maleate, were examined.

Figure 1 The relaxant effects of nitroglycerin on pig isolated coronary artery rings following contraction with potassium chloride (30 mM). Controls (○); preparations previously incubated with nitroglycerin 10^{-5} M for 20 min (△); preparations previously incubated with nitroglycerin 10^{-4} M for 20 min (□). Each value represents the mean percentage relaxation (*n* = 7–14) of potassium-induced tone; vertical lines show s.e.mean.

* *P* < 0.05 and ** *P* < 0.01 indicate a difference from untreated tissues.

Figure 2 Effects of prior exposure to diethyl maleate on the relaxant response of potassium (30 mM)-contracted pig coronary artery rings to nitroglycerin. Controls (○); preparations previously incubated with diethyl maleate (●) at 10^{-4} M (a) and 10^{-3} M (b). Each value represents the mean percentage relaxation ($n = 6$) of potassium-induced tone; vertical lines show s.e.mean.

Diethyl maleate 10^{-4} M and 10^{-3} M significantly decreased the glutathione content to about 45% and 5%, respectively, of the control level after 60 min incubation, with no change in glutathione S-transferase activity (Table 2). The decrease in glutathione content with no change in glutathione S-transferase activity persisted for at least 120 min after the 60 min incubation with diethyl maleate 10^{-4} M or 10^{-3} M (unpublished data). As shown in Figure 2, concentration-relaxation curves for nitroglycerin 10^{-8} – 10^{-5} M obtained on arterial rings previously incubated with diethyl maleate 10^{-4} M or 10^{-3} M for 60 min were not significantly different from those from untreated controls.

Discussion

The present results show that the glutathione content of pig coronary arteries was not changed at all following treatment

with relatively large doses of nitroglycerin although tolerance (tachyphylaxis) did develop to the relaxant actions of this agent. As prior exposure to nitroglycerin resulted in the development of tolerance under conditions in which glutathione content was unaffected, it is unlikely that depletion of glutathione content is involved in the development of tolerance. It is also unlikely that consumption of glutathione in the vascular smooth muscle is needed for the relaxant response to nitroglycerin. Our results do not, therefore, agree with those of other investigators who proposed that depletion of arterial glutathione content is the cause of tolerance development (Needleman & Johnson, 1973; Needleman *et al.*, 1973). In addition, our findings do not agree with those of Yeates *et al.* (1989) who, using indirect techniques, suggested that glutathione S-transferase activity was inhibited in rabbit aortic strips by nitroglycerin: our direct measurement of glutathione S-transferase activity in pig coronary artery showed no inhibition even with high concentrations of nitroglycerin (10^{-3} M).

As our findings suggested that neither depletion of glutathione content nor loss of glutathione S-transferase activity was involved in the development of tolerance, further experiments were performed using diethyl maleate, a known glutathione depleting agent (Boyland & Chasseaud, 1967; 1968). As expected, diethyl maleate dose-dependently decreased the glutathione content of pig coronary artery, and at 10^{-3} M of diethyl maleate the arterial glutathione content was only just detectable. In contrast, the arterial glutathione S-transferase activity was not changed at all after treatment with diethyl maleate. Under these experimental conditions, nitroglycerin-induced relaxation of potassium-contracted pig coronary artery rings was completely unaffected. This finding supports the hypothesis that glutathione is not involved in the relaxant process or the development of tolerance to nitroglycerin in vascular smooth muscle. The present study, however, does not exclude the idea of Feelisch & Noack (1987) that not glutathione but cysteine may be involved in vascular relaxation and the development of tolerance to nitroglycerin.

In conclusion, prior exposure to high concentrations of nitroglycerin easily produced tachyphylaxis (tolerance) to relaxation in pig isolated coronary artery rings without any change in arterial glutathione content or glutathione S-transferase activity. Furthermore, although arterial glutathione content was almost completely depleted following treatment with diethyl maleate, the relaxant response to nitroglycerin 10^{-8} – 10^{-5} M was completely unaffected. Our results suggest that vascular smooth muscle relaxation and tolerance development to nitroglycerin do not involve changes in vascular glutathione content, at least in pig isolated coronary artery.

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