Release of nitric oxide from glyceryl trinitrate by captopril but not enalaprilat: *in vitro* and *in vivo* studies

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1 The hypotensive effects of glyceryl trinitrate (GTN, 0.5 mg kg⁻¹) but not of 3-morpholino-sydnonimine (SIN-1, 0.125 mg kg⁻¹) in anaesthetized rats were attenuated following a seven day (using a q.i.d. dosing schedule) oral treatment with isosorbide-5-mononitrate (IS-5-MN; 5 mg kg⁻¹) indicative of the induction of tolerance to GTN but not to SIN-1. The hypotensive effects of GTN did not decline when the sulphhydryl (SH) containing angiotensin converting enzyme inhibitor (ACE-I), captopril (CPT, 5 mg kg⁻¹) or the structurally unrelated SH-containing, N-acetylcysteine (NAC, 10 mg kg⁻¹) but not the non-SH-containing ACE-I, enalaprilat (ENA, 5 mg kg⁻¹) were given together with IS-5-MN for the seven days treatment.

2 The attenuated hypotensive effects of GTN (0.5 mg kg⁻¹) in rats treated with IS-5-MN were also restored when CPT (1 mg kg⁻¹) or NAC (2.5 mg kg⁻¹) but not ENA (1 mg kg⁻¹) was administered intraperitoneally (i.p.) 30 min before GTN. Furthermore, in control rats, CPT or NAC but not ENA given i.p. 30 min before GTN, potentiated its haemodynamic effects. These effects were blocked by methylene blue (10 mg kg⁻¹). At the same doses, CPT or NAC did not affect the hypotensive effects of SIN-1.

3 The reduced ability of cultured tolerant smooth muscle cells (SMC, 24 × 10³ cells) or endothelial cells (EC, 40 × 10³ cells) to potentiate the anti-platelet effects of GTN (44 µM) was restored by CPT or NAC but not by ENA or glutathione (all at 0.5 mM). Potentiation of the anti-platelet effects of tolerant SMC or EC by CPT or NAC was abolished by co-incubation with oxyhaemoglobin (Oxy-Hb, 10 µM) indicative of nitric oxide (NO) formation.

4 When GTN (150–2400 µM) was incubated with CPT, NAC or glutathione but not ENA (all at 0.1 mM) for 30 min in Krebs buffer at 37°C a concentration-dependent increase in nitrite (NO₂⁻) formation was observed.

5 The antiplatelet effects of GTN (5.5–352 µM) were potentiated by co-incubation with CPT or NAC but not with ENA or glutathione (all at 0.5 mM). The concentration of GTN required to inhibit platelet aggregation by 50% (IC₅₀) was 110 ± 2 µM for GTN alone, 14 ± 2 µM for GTN in the presence of NAC and 30 ± 2 µM for GTN in the presence of CPT. The potentiation of the effects of GTN by CPT or NAC was inhibited by co-incubation with Oxy-Hb (10 µM). By themselves, CPT or NAC did not inhibit platelet aggregation.

6 The ability of CPT to restore (a) the haemodynamic effects of GTN in tolerant rats and (b) the reduced capacity of tolerant SMC or EC to potentiate the anti-platelet effects of GTN is not related to its ACE inhibitory activity.

7 CPT also potentiated the hypotensive effects of GTN in non-tolerant rats, and *in vitro* CPT released NO from GTN in the absence of a GTN to NO converting cell, so that it is unlikely that reversal of tolerance by CPT is due to the replenishment of intracellular thiols. Rather it can be explained by the ability of CPT to release NO from GTN in the extracellular space. This extracellular formation of NO from GTN by CPT would then compensate for the impaired enzymic biotransformation of GTN to NO that develops during tolerance as was originally proposed for NAC.

Keywords: Glyceryl trinitrate; nitric oxide; tolerance; smooth muscle cells; endothelial cells; captopril; enalaprilat

Introduction

Glyceryl trinitrate (GTN) requires bioconversion to nitric oxide (NO) by various cells including smooth muscle cells (SMC) and endothelial cells (EC) in order to elicit vasodilatation (Ignarro *et al.*, 1981; Murad, 1986) and inhibition of platelet aggregation (Benjamin *et al.*, 1991; Salvemini *et al.*, 1992). This bioconversion is mediated by an enzyme not yet characterized (Chung & Fung, 1990; Feelisch & Kelm, 1991; Salvemini *et al.*, 1992). Enzymes such as the glutathione-S-transferase or cytochrome P₄₅₀ which metabolize GTN in the liver or kidney (Needleman & Johnson, 1973; Yeates *et al.*,

1989; Servent *et al.*, 1989; Schröder & Schrör, 1992) are not involved in the biotransformation of GTN to NO by SMC or EC (Gruetter & Lemke, 1985; Sakanashi *et al.*, 1991; Salvemini *et al.*, 1993). One hypothesis explains the action of GTN by denitration followed by interaction in vascular smooth muscle cells with reduced sulphhydryl (SH) groups (Needleman *et al.*, 1973) to form NO or a S-nitrosothiol (Ignarro *et al.*, 1981). The mechanism by which SH-containing molecules form NO from GTN is not known but may involve nucleophilic attack by thiolate anions on the nitrogen atom of the ester group of GTN (Feelisch, 1991).

The development of tolerance to nitrates in patients after continuous treatment for either chronic stable angina (Parker & Fung, 1984; Parker *et al.*, 1987) or congestive heart failure (Jordan *et al.*, 1985) is not completely understood. Desensitization of soluble guanylate cyclase or increased phosphodiesterase activity are not involved (see Ahlner *et al.*,

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1991; Elkayam, 1991 for reviews). Elevation of catecholamines and plasma renin activity have been observed in GTN-tolerant patients (Packer *et al.*, 1981; 1987) suggesting that neurohormonal compensation may play a role. However, the most widely-held hypothesis to explain nitrate tolerance is based on the work of Needleman and coworkers who demonstrated that the induction of GTN tolerance *in vivo* and *in vitro* was associated with a reduction of intracellular thiols thereby limiting the bioconversion of GTN. This explanation is supported by the finding that thiols, such as N-acetylcysteine (NAC), prevent or reverse tolerance to GTN both in *in vivo* and *in vitro* (Needleman & Johnson, 1973; Packer *et al.*, 1987). In addition, thiol-containing angiotensin converting enzyme inhibitors (ACE-I), such as captopril (CPT), prevent the development of nitrate tolerance and potentiate GTN-mediated vasorelaxations (Halon *et al.*, 1988; de Graeff *et al.*, 1989; Lawson *et al.*, 1991). Although these experiments favour the concept that tolerance to GTN is due to loss of intracellular thiols, this concept is not universally accepted (see Ahler *et al.*, 1991; Elkayam, 1991 for reviews).

Clearly, at least in cultured SMC and EC, tolerance is the result of impaired enzymic bioconversion of GTN to NO (Brien *et al.*, 1986; Feelisch & Kelm, 1991; Salvemini *et al.*, 1993). In addition we have proposed that reversal of tolerance to GTN by thiols such as NAC could not be explained solely by replenishment of intracellular thiols, for NAC was able to form NO from GTN in Krebs buffer and in the absence of metabolizing cells. Thus reversal of tolerance to GTN by thiols such as NAC may simply be due to extracellular formation of NO from GTN which would in turn compensate for the reduced metabolism by the enzymatic pathway.

Long-term administration of isosorbide-5-mononitrate (IS-5-MN) *in vitro* and *in vivo* induces tolerance to GTN (this is the so-called cross-tolerance; see Ahlner *et al.*, 1991; Elkayam, 1991, for reviews). Here we have studied the effects of CPT, NAC or ENA on the *in vivo* responses to GTN after long-term treatment of rats with IS-5-MN. In addition, we have compared the effects of CPT, ENA or glutathione to those of NAC on the anti-platelet effects of GTN in the absence or presence of tolerant cultured SMC or EC and on NO₂⁻ production from GTN in Krebs buffer.

Methods

Preparation of washed platelets

Human washed platelets were prepared as described by Radomski & Moncada (1983). Indomethacin (10 µM) was added to the final platelet suspension to prevent the formation of cyclo-oxygenase products. The platelet count was adjusted to approximately $1.5-2 \times 10^8 \text{ ml}^{-1}$.

Preparation of endothelial cells or smooth muscle cells

Bovine aortic EC were harvested and grown on cytodex 3 microcarrier beads (Pharmacia/LKB, Ltd.) in Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories) supplemented with 4 mM L-glutamine and 10% (v/v) foetal calf serum (Gibco) as described previously (de Nucci *et al.*, 1988). The approximate cell number was $3.5 \times 10^7 \text{ ml}^{-1}$ of beads. Bovine aortic SMC were characterized by the presence of specific alpha actin using a Sigma kit and were prepared as previously described (Mollace *et al.*, 1991a). Indomethacin (10 µM) was added to all final cell suspensions. In some experiments, GTN (600 µM) was incubated for 18 h with SMC or EC in culture (referred to as 'tolerant' cells; Salvemini *et al.*, 1993). The cell supernatant was removed and cells washed at least 3 times with warm Krebs buffer containing indomethacin (10 µM) before exposure to lower concentrations of GTN.

Platelet aggregation

A suspension of washed platelets was incubated at 37°C for 4 min in a Payton dual channel aggregometer (Born & Cross, 1963) with continuous stirring at 1,000 r.p.m. and then stimulated with thrombin (40 µM ml⁻¹) to give a submaximal aggregation (80–90%). The decrease in optical density was recorded for 5 min. After a 3 min incubation period with platelets, the inhibitory effects of GTN on platelet aggregation induced by thrombin were measured either alone or in the presence of captopril, NAC, glutathione, ENA or tolerant SMC or EC. When using cells of oxyhaemoglobin, the calibrations were performed in the presence of these agents to compensate for possible changes in light transmission (Salvemini *et al.*, 1989). Inhibition of platelet aggregation was calculated as described previously (Salvemini *et al.*, 1989).

Nitrite analysis

Nitrite (NO₂⁻) was measured by the Griess reaction. GTN (150–2400 µM) was incubated in Krebs buffer containing SOD (100 u ml⁻¹) and CPT, NAC, glutathione or ENA (all at 0.1–1 mM). The samples were incubated in a shaking water bath for 30 min at 37°C and then allowed to react with the Griess reagent (1% sulphanilamide/0.1% naphthylethylenediamine dihydrochloride/2.5% H₃PO₄) to form a chromophore absorbing at 546 nm. Nitrite concentration was determined with sodium nitrite as a standard. Results are expressed as nmol NO₂⁻ ml⁻¹ and each experiment was performed in triplicate.

In vivo measurements

Adult male Wistar Morini (S. Polo d'Enza, Reggio Emilia, Italy) normotensive rats (approx weight 200–300 g) were used throughout. The animals were divided into 2 groups: the first group received IS-5-MN (5 mg kg⁻¹) orally for 7 days using a q.i.d. dosing schedule treatment (08 h 00 min, 11 h 00 min, 14 h 00 min and 17 h 00 min) and the second group received saline at the same time periods. CPT (5 mg kg⁻¹), NAC (10 mg kg⁻¹) or ENA (5 mg kg⁻¹) were administered orally once daily (08 h 00 min) to a subgroup of IS-5-MN- or saline-treated rats. After 7 days, the animals were anaesthetized with sodium pentobarbitone (55 mg kg⁻¹) before surgical preparation. A catheter (PE50), filled with heparinized saline (200 u ml⁻¹) was inserted into the left common carotid artery to record blood pressure (BP) and heart rate (HR). The jugular vein was cannulated for i.v. injections of drugs. The animals were allowed to stabilize for at least 20 min after surgery. BP and HR were monitored on a Gemini polygraph via Bentley transducers (Ugo Basile, Comerio Vares, Italy). Each rat was used for only one dose of either GTN or SIN-1; GTN was given as a short 30 s infusion of 100 µl at a dose of 0.5 mg kg⁻¹ (i.e. 1 mg kg⁻¹ min⁻¹) and SIN-1 at 0.125 mg kg⁻¹ (i.e. 0.25 mg kg⁻¹ min⁻¹). In some experiments, CPT (1 mg kg⁻¹), NAC (2.5 mg kg⁻¹) or ENA (1 mg kg⁻¹) were given intraperitoneally to the IS-5-MN or saline-treated rats 30 min before the intravenous administration of GTN. When required methylene blue (MeB, 10 mg kg⁻¹, i.v.) was given 30 min before GTN or SIN-1. The mean arterial pressure (MAP) was calculated as the diastolic blood pressure plus one third of the pulse pressure. Results are expressed as changes in mean arterial pressure taken at the peak fall of the response (MAP changes, mmHg).

Materials

The composition of the modified (Sneddon & Vane, 1988) Krebs bicarbonate buffer was (mM): NaCl 137, KCl 2.7, NaHCO₃ 11.9, NaH₂PO₄ 0.3, MgSO₄ 0.8, glucose 5.6, and CaCl₂ 1. Human thrombin, haemoglobin (from bovine

blood), N-acetylcysteine (NAC), reduced glutathione, superoxide dismutase (SOD, from bovine erythrocytes), indomethacin, sulfanilic acid, N-1-naphthylethylenediamine dihydrochloride, Na_2CO_3 , sodium nitrite and kits for characterizing alpha smooth muscle actin (procedure number SIH903) were obtained from Sigma (Poole, Dorset). Phosphoric acid was obtained from BDH Analar (Essex). Oxyhaemoglobin (Oxy-Hb) was prepared by reduction of bovine haemoglobin with sodium hydrosulphite as described previously (Salvemini *et al.*, 1989). Glyceryl trinitrate (Nitronal) was obtained from Lipha Pharmaceuticals Ltd. (West Drayton, Middlesex). Prostacyclin was a gift from the Wellcome Research Laboratories (Beckenham, Kent), captopril a gift from Squibb (U.S.A.), enalaprilat (ENA) a gift from Sigma-Tau (Rome, Italy) and 3-morpholinosydnonimine (SIN-1) a gift from Hoeschst. For the *in vivo* experiments, pentobarbitone and N-acetyl-cysteine were obtained from Sigma (Milan, Italy), glyceryl trinitrate (Venitron) from Simes (Milan, Italy) and isosorbide-5-mononitrate (Nitrosorbide) from Lusofarmaco (Milan, Italy).

Statistics

Results are expressed as mean $\pm$ s.e.mean for (*n*) experiments. The results were analysed by Student's unpaired *t* test to determine the significance of differences between means, or by a two-way analysis of variance (ANOVA) followed by a least significance procedure to determine the nature of this response. A *P* value of <0.05 was taken as significant.

Results

Haemodynamic responses to glyceryl trinitrate in non-treated rats or in rats treated with isosorbide-5-mononitrate

Baseline blood pressure and heart rate in rats receiving IS-5-MN (5 mg kg^{-1}) were not different from those receiving saline (102.0 ± 2.0 or $104.0 \pm 3.2 \text{ mmHg}$ for MAP and 380.0 ± 4.0 or $374 \pm 5.2 \text{ b.p.m.}$ for HR in animals receiving saline or IS-5-MN, $n = 10$). Intravenous administration of GTN (0.5 mg kg^{-1}) produced a fall in MAP in control rats ($-31 \pm 2.8 \text{ mmHg}$, $n = 10$) which was larger than that seen in rats pretreated with IS-5-MN ($-12 \pm 2.2 \text{ mmHg}$, $P < 0.05$, $n = 5$; Figure 1a and b). Thus, the IS-5-MN treatment induced tolerance to the hypotensive action of GTN. CPT (5 mg kg^{-1}) or NAC (10 mg kg^{-1}) when given together with IS-5-MN for 7 days prevented the blunted hypotensive response to GTN ($-30.2 \pm 3.2 \text{ mmHg}$ or $-33.0 \pm 3.6 \text{ mmHg}$ for CPT- or NAC-treated rats, $n = 5$ for each group). However, ENA (5 mg kg^{-1}) did not prevent tolerance to GTN ($-18.0 \pm 3.2 \text{ mmHg}$, $n = 4$). Chronic (7 days) administration of CPT, NAC or ENA did not affect resting blood pressure and heart rate ($n = 4$, data not shown). In control rats (non-treated with IS-5-MN), CPT, NAC or ENA treatment did not modify the fall in blood pressure elicited by verapamil (2 mg kg^{-1} , i.v.; from -35 ± 4 to -30 ± 2.5 , -36 ± 3 or $-38 \pm 4 \text{ mmHg}$ respectively, $n = 4$), phentolamine (0.5 mg kg^{-1} , i.v.; from -42 ± 16 to -38 ± 2.6 , -38 ± 3.1 or $-40 \pm 3.2 \text{ mmHg}$ respectively, $n = 4$) or SIN-1 (0.125 mg kg^{-1} ; from -32 ± 3 to -35 ± 2 , -31 ± 3 or $-36 \pm 3 \text{ mmHg}$ respectively, $n = 4$). The haemodynamic changes obtained with verapamil, phentolamine or SIN-1 in rats treated with IS-5-MN were identical to those obtained in non-treated rats ($n = 4$, data not shown). CPT (1 mg kg^{-1}) or NAC (2.5 mg kg^{-1}), but not ENA (1 mg kg^{-1}) when given intraperitoneally 30 min before GTN restored the haemodynamic responses to GTN (0.5 mg kg^{-1}) in the IS-5-MN-treated rats (Figure 1b). The ability of CPT or NAC to restore the reduced responses to GTN in rats treated with IS-5-MN was abrogated by MeB (10 mg kg^{-1} , i.v.) (from $-34 \pm 3 \text{ mmHg}$ for GTN + CPT to $-6 \pm 1.2 \text{ mmHg}$ for

Figure 1 (a) The hypotensive effects of glyceryl trinitrate (GTN, 0.5 mg kg^{-1}) were reduced in rats rendered tolerant by treatment for seven days with isosorbide-5-mononitrate (IS-5-MN, 5 mg kg^{-1}). This effect was restored when captopril (CPT, 1 mg kg^{-1}) or N-acetylcysteine (NAC, 2.5 mg kg^{-1}) but not enalaprilat (ENA, 1 mg kg^{-1}) were given 30 min before the administration of GTN. (b) When given 30 min before GTN (0.5 mg kg^{-1}), CPT (1 mg kg^{-1}) or NAC (2.5 mg kg^{-1}) but not ENA (1 mg kg^{-1}) potentiated the hypotensive effects of GTN in control non-tolerant rats. The results are expressed as mean arterial pressure (MAP) changes (mmHg). Each point is the mean $\pm$ s.e.mean for the changes in five rats. * $P < 0.05$ when compared to the values obtained in the absence of CPT, NAC or ENA. † $P < 0.05$ when compared to the value obtained in control non-tolerant rats.

GTN + CPT + MeB, $n = 4$, $P < 0.05$ and from $-30 \pm 2.5 \text{ mmHg}$ for GTN + NAC to $-5 \pm 2.1 \text{ mmHg}$ for GTN + NAC + MeB, $n = 4$, $P < 0.05$). In control, saline-treated rats, CPT (1 mg kg^{-1}) or NAC (2.5 mg kg^{-1}) but not ENA (1 mg kg^{-1}) when given i.p. 30 min before GTN (0.5 mg kg^{-1}) potentiated the haemodynamic responses to GTN (Figure 1b). This potentiating effect was abrogated by MeB (from $-45 \pm 3 \text{ mmHg}$ for GTN + CPT to $-2 \pm 1 \text{ mmHg}$ for GTN + CPT + MeB, $n = 4$, $P < 0.05$ and from $-40 \pm 4 \text{ mmHg}$ for GTN + NAC to $-3 \pm 1 \text{ mmHg}$ for GTN + NAC + MeB, $n = 4$, $P < 0.05$). MeB also attenuated the fall

in blood pressure elicited by GTN (0.5 mg kg^{-1} ; from $-31 \pm 2.8 \text{ mmHg}$ to $-12 \pm 2 \text{ mmHg}$, $n = 5$, $P < 0.05$). At the doses used, CPT, NAC, ENA or MeB did not modify basal MAP ($102 \pm 2 \text{ mmHg}$ for control rats, $102 \pm 4 \text{ mmHg}$ in the presence of CPT, $100 \pm 4 \text{ mmHg}$ in the presence of NAC, $106 \pm 4 \text{ mmHg}$ in the presence of ENA and $107 \pm 3 \text{ mmHg}$ in the presence of MeB).

Effects of captopril, N-acetylcysteine, glutathione or enalaprilat on nitrite production from glyceryl trinitrate

Exposure of GTN ($150\text{--}2400 \mu\text{M}$) to CPT, NAC or glutathione (0.1 mM) for 30 min led to a concentration-dependent increase in the formation of NO_2^- (Figure 2). Glutathione was more potent than CPT or NAC whereas CPT and NAC produced similar amounts of NO_2^- from GTN (Figure 2). Enalaprilat (1 mM) failed to release NO_2^- from GTN ($n = 4$, not shown). GTN, CPT, NAC or glutathione by themselves did not produce NO_2^- .

Comparison of the effects of captopril, glutathione or enalaprilat to those of N-acetylcysteine on the antiplatelet effect of glyceryl trinitrate in the absence or presence of tolerant SMC or EC

Salvemini *et al.* (1993) found that NAC (0.5 mM) when incubated for 3 min in the presence of platelets and GTN increased the anti-platelet effects of GTN ($44 \mu\text{M}$). Here, we compared the effects of NAC to those of CPT, ENA or glutathione and found that CPT (0.5 mM) potentiated, but to a lesser extent than NAC, the anti-platelet effects of GTN ($5.5\text{--}352 \mu\text{M}$) (Figure 3). The concentration of GTN required to inhibit platelet aggregation by 50% (IC_{50}) was $110 \pm 2 \mu\text{M}$ for GTN alone, $30 \pm 2 \mu\text{M}$ for GTN in the presence of CPT and $14 \pm 2 \mu\text{M}$ for GTN in the presence of NAC ($n = 4$). Therefore, NAC enhanced 8 fold and CPT 4 fold the anti-platelet effect of GTN making NAC at least 2 fold more potent than CPT. Potentiation of the antiplatelet effects of GTN by CPT or NAC was abolished by co-incubation with OxyHb (10 M ; Figure 3). By itself NAC or CPT (both at 0.5 mM) did not modify platelet aggregation induced by thrombin. Enalaprilat or glutathione at a dose up to 2 mM failed to modify the inhibition of platelet aggregation by GTN ($5.5\text{--}352 \mu\text{M}$; $n = 4$, not shown). SMC or EC treated with GTN ($600 \mu\text{M}$) for 18 h became tolerant to GTN and lost their ability to potentiate the anti-platelet effects of GTN; this loss was restored by NAC (Salvemini *et al.*, 1993). In this study, loss of the potentiation of the anti-platelet effects of GTN ($44 \mu\text{M}$) by tolerant SMC (24×10^3 cells) or EC (40×10^3 cells) was restored by CPT but not by ENA or glutathione (all at 0.5 mM ; Figure 4a and b). The effect of CPT was abolished by co-incubation with OxyHb ($10 \mu\text{M}$; Figure 4a and b).

Figure 2 A 30 min exposure of glyceryl trinitrate (GTN, $150\text{--}2400 \mu\text{M}$) in Krebs buffer at 37°C to 0.1 mM captopril (CPT, $\bullet$), N-acetylcysteine (NAC, $\blacksquare$) or glutathione ($\blacktriangle$) produced a concentration-dependent increase in the formation of NO_2^- . Results are expressed in $\text{nmol NO}_2^- \text{ ml}^{-1}$. GTN, CPT, NAC or glutathione alone did not produce NO_2^- . Each point is the mean $\pm$ s.e.mean of 3 experiments each performed in triplicate.

Figure 3 The antiplatelet effects of glyceryl trinitrate (GTN, $5.5\text{--}352 \mu\text{M}$, $\bullet$) were potentiated by a 3 min co-incubation with captopril (CPT, $\blacktriangle$, 0.5 mM) or N-acetylcysteine (NAC, $\blacksquare$, 0.5 mM). Oxyhaemoglobin (OxyHb, $10 \mu\text{M}$) abolished the potentiation of the antiplatelet effects of GTN by CPT (Δ) or NAC ($\square$). Points represent the mean $\pm$ s.e.mean of 4 experiments. Results are expressed as % inhibition of platelet aggregation. $*P < 0.001$ for GTN + NAC vs GTN alone or for GTN + CPT vs GTN alone and $\dagger P < 0.001$ for GTN + NAC + OxyHb vs GTN + NAC or GTN + CPT + OxyHb vs GTN + CPT. Statistical analysis was performed using the ANOVA test followed by a least significant test.

Figure 4 (a) Tolerant smooth muscle cells (TSMC, 24×10^3 cells) or (b) tolerant endothelial cells (TEC, 40×10^3 cells) did not potentiate the anti-platelet effects of glyceryl trinitrate (GTN, $44 \mu\text{M}$). These effects were however potentiated when captopril (CPT) but not enalaprilat (ENA) or glutathione (Glu) (all at 0.5 mM) was co-incubated for 3 min with (a) TSMC and GTN or (b) TEC and GTN. This potentiation is abolished by oxyhaemoglobin (OxyHb, $10 \mu\text{M}$). Platelet aggregation was initiated by thrombin (40 mu ml^{-1}). Each point is the mean $\pm$ s.e.mean of 4 experiments. $*P < 0.005$ when compared to the value obtained in the presence of GTN and non-tolerant SMC or EC if the effect was an additive one; $\dagger P < 0.005$ when compared to the value obtained with GTN in the presence of non-tolerant SMC or EC and $*P < 0.005$ when compared to the value obtained with GTN in the presence of tolerant SMC or EC.

Discussion

It has been recognized for many years that the continuous administration of GTN or other organic nitrates such as IS-5-MN leads to the development of tolerance as evidenced by reduced vasodilator and anti-platelet effects of GTN, and that cross tolerance between GTN and IS-5-MN exists (Zelis & Mason, 1975; Thadani *et al.*, 1980). NAC partially reverses nitrate tolerance *in vitro* (Torresi *et al.*, 1985; Winniford *et al.*, 1986; Fung *et al.*, 1988; Lawson *et al.*, 1991) and *in vivo* as demonstrated by animal (Fung *et al.*, 1988; Boesgard *et al.*, 1991; Bauer & Fung, 1991) and human (Horowitz *et al.*, 1983) studies. In addition NAC potentiates the haemodynamic responses to GTN (Lawson *et al.*, 1991), restores the decreased anti-platelet responses to GTN during tolerance (Loscalzo, 1985; Folts *et al.*, 1989; Salvemini *et al.*, 1993) and increases the anti-platelet effects of GTN (Loscalzo, 1985; Salvemini *et al.*, 1993).

In the present study we have demonstrated that the development of tolerance to GTN in IS-5-MN treated rats can be restored by CPT or NAC when given together with the IS-5-MN during the seven day dosing period. The non-SH-containing ACE-I, ENA, failed to alter the development of tolerance. This supports previous studies which demonstrated that the beneficial effects of CPT were related to the presence of the SH-moiety and not to inhibition of ACE (van Gilst *et al.*, 1987; 1988; Cooke *et al.*, 1989; Lawson *et al.*, 1991). *In vitro*, the decreased ability of tolerant SMC or EC to potentiate the anti-platelet effects of GTN was restored by co-incubation with CPT or NAC but not by ENA. In addition, CPT or NAC but not ENA prevented the reduced haemodynamic effects of GTN in tolerant rats when given 30 min before GTN and they augmented the haemodynamic effects of GTN in saline-treated rats. SIN-1 releases NO spontaneously without metabolic activation (Feelisch, 1991). In the present study, the findings that neither NAC or CPT enhanced the hypotensive response of SIN-1 indicates that their *in vivo* effects on GTN are not likely to be due to increased survival of NO.

Incubation of cultured SMC or EC with high concentrations of GTN for 18 h leads to the development of tolerance as shown by reduced pharmacological and biochemical responses upon subsequent exposure to GTN (Salvemini *et al.*, 1993). Tolerance was found to be the result of impaired enzymic biotransformation of GTN to NO, and NAC by directly forming NO from GTN, compensating for this failing mechanism (Salvemini *et al.*, 1993). In the present study, the decreased ability of tolerant SMC or EC to potentiate the anti-platelet effects of GTN was restored by co-incubation with CPT but not ENA. The possibility that this reversal is due to replenishment of intracellular SH groups is unlikely, for CPT also increased the levels of NO_2^- . The potentiation by CPT in the absence or presence of tolerant SMC or EC was reversed by OxyHb indicating that an extracellular interaction between GTN and CPT took place with subsequent NO formation. Although the release of NO_2^- from GTN by CPT was similar to that by NAC, CPT was less effective in potentiating the anti-platelet effects of GTN. This indicates that CPT and NAC release the same amounts of NO_2^- but NAC releases more NO. Since in tolerant SMC or EC, the soluble guanylate cyclase (GC) activity is not affected (Kukowetz & Holzman, 1986; Mulsch *et al.*, 1988; Salvemini *et al.*, 1993), NO release from GTN by thiols would stimulate the soluble GC, increase the levels of cyclic GMP and restore both vasodilator and anti-platelet effects of

GTN. This effect would compensate for the failing enzymatic mechanism that converts GTN to NO (Salvemini *et al.*, 1993).

Glutathione interacts with GTN to form NO_2^- which lacks biological activity (Feelisch, 1991). In our experiments we have demonstrated that although glutathione is more potent than CPT or NAC in releasing NO_2^- , it fails to potentiate the anti-platelet effects of GTN, indicating that glutathione does not form NO from GTN. In our study, the effects of various thiols on NO release from GTN indicate that a non-enzymic pathway exists for the bioconversion of GTN to NO and NO_2^- . This strengthens and supports earlier work by Feelisch and co-workers who demonstrated that, depending on the structure of the thiol used, two pathways independent of each other are involved in the formation of either NO or NO_2^- . Thus, although 54 different thiols were capable of releasing NO_2^- from GTN in aqueous solution only 3 (namely thiosalicylic acid, NAC and cysteine) were able to release NO as well (Feelisch & Noack, 1987; Feelisch *et al.*, 1988). From our study, CPT fits into the category of a non-enzymic releaser of NO from GTN. Our experiments also favour the concept that the higher the catalytic capacity of the thiol to form NO from GTN, the better their ability to counteract tolerance (Chung & Fung, 1991; Salvemini *et al.*, 1993).

The experiments presented in this study reinforce the concept that *in vitro* reversal of tolerance by thiols is due to a direct interaction between GTN and the thiol and that subsequent NO formation is required in order to prevent or reverse tolerance.

MeB an inhibitor of the soluble guanylate cyclase prevented the potentiating effects of NAC or CPT in control rats and prevented the ability of these thiol donors to restore the responses to GTN in tolerant rats. MeB does not prevent the hypotensive effects of verapamil or phenolamine which act in a NO-independent way (Salvemini *et al.*, 1993). This suggests that the effects of NAC or CPT on GTN responses *in vivo* involve release of NO from GTN which subsequently stimulates the soluble guanylate cyclase. Such possible mechanism of action is supported by the *in vitro* results. However, whether this is the exact mechanism by which NAC or CPT potentiate the effects of GTN or reverse GTN tolerance *in vivo* remains to be evaluated further.

The presence of an SH group on CPT is responsible for the superoxide anion scavenging properties attributed to CPT (Goldschmidt & Tallarida, 1991). In this way, CPT preserves or enhances the biological activity of NO and prostacyclin released from the endothelium (Mollace *et al.*, 1991b), the actions of which can be affected by superoxide anions (see Salvemini & Botting, 1990 for review) generated during reperfusion of ischaemic tissues. This, together with the fact that CPT releases NO directly from the molecule of GTN and thus enhances the haemodynamic and anti-platelet effects of GTN, indicates that CPT may confer particular therapeutic benefits on patients with ischaemic heart disease or congestive heart failure who are undergoing concomitant therapy with GTN. It should, however, be kept in mind that since plasma levels of the ACE-I used in this study were not measured, extrapolation from *in vitro* to *in vivo* should be made with some caution.

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References

- AHLNER, J., ANDERSSON, R.G.G., TORFGARD, K. & AXELSSON, K.L. (1991). Organic nitrate esters: clinical use and mechanisms of actions. *Pharmacol. Rev.*, **43**, 351–423.
- BAUER, J.A. & FUNG, H.L. (1991). Differential hemodynamic effects and tolerance properties of nitroglycerin and S-nitrosothiol in experimental heart failure. *J. Pharmacol. Exp. Ther.*, **256**, 249–254.
- BENJAMIN, N., DUTTON, J.A.E. & RITTER, J.M. (1991). Human vascular smooth muscle cells inhibit platelet aggregation when incubated with glyceryl trinitrate: evidence for generation of nitric oxide. *Br. J. Pharmacol.*, **102**, 847–851.

- BOESGAARD, S., PETERSEN, J.S., ALDERSHILE, J., POULSEN, H.E. & FLACHS, H. (1991). Nitrate tolerance: effect of thiol supplementation during prolonged nitroglycerin infusion in an in vivo rat model. *J. Pharmacol. Exp. Ther.*, **258**, 851–856.
- BORN, G.V.R. & CROSS, M.J. (1963). The aggregation of blood platelets. *J. Physiol.*, **168**, 178–195.
- BRIEN, J.F., MCLAUGHLIN, B.E., BREEDON, T.H., BENNET, B.M., NAKATSU, K. & MARKS, G.S. (1986). Biotransformation of glyceryl trinitrate occurs concurrently with relaxation of rabbit aorta. *J. Pharmacol. Exp. Ther.*, **237**, 608–614.
- CHUNG, S.J. & FUNG, H.L. (1990). Identification of the subcellular site for nitroglycerin metabolism to nitric oxide in bovine coronary smooth muscle cells. *J. Pharmacol. Exp. Ther.*, **253**, 614–619.
- COOKE, J.P., ANDON, N. & LOSCALZO, J. (1989). S-nitrosocaptopril II: effects on vascular reactivity. *J. Pharmacol. Exp. Ther.*, **249**, 730–734.
- DE GRAEFF, P.A., DE LEEUW, M.J., VAN GILST, W.H. & WESSELING, H. (1989). Antianginal effects of captopril during chronic nitrate treatment. *Circulation*, **80**, II11–II14.
- DENUCCI, G., GRYGLEWSKI, R.J., WARNER, T.D. & VANE, J.R. (1988). Receptor-mediated release of endothelium-derived relaxing factor and prostacyclin from bovine aortic endothelial cells is coupled. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 2334–2338.
- ELKAYAM, U. (1991). Tolerance to organic nitrates: evidence, mechanisms, clinical relevance and strategies for prevention. *Ann. Intern. Med.*, **114**, 667–677.
- FEELISCH, M. (1991). The biochemical pathways of nitric oxide formation from vasodilators: appropriate choice of exogenous NO donors and aspects of preparation and handling of aqueous NO solutions. *J. Cardiovasc. Pharmacol.*, **17**, S25–S33.
- FEELISCH, M. & KELM, M. (1991). Biotransformation of organic nitrates to nitric oxide by vascular smooth muscle and endothelial cells. *Biochem. Biophys. Res. Commun.*, **180**, 286–293.
- FEELISCH, M. & NOACK, E.A. (1987). Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur. J. Pharmacol.*, **139**, 19–30.
- FEELISCH, M., NOACK, E. & SCHRODER, H. (1988). Explanation of the discrepancy between the degree of organic nitrate decomposition, nitrite formation and guanylate cyclase stimulation. *Eur. Heart J.*, **9**, 57–62.
- FOLTS, J.D., STAMLER, J. & LOSCALZO, J. (1989). N-acetylcysteine potentiates intravenous nitroglycerin in inhibiting periodic platelet thrombus formation in stenosed dog coronary arteries. *J. Am. Coll. Cardiol.*, **13**, 145A.
- FUNG, H.L., CHONG, S., KOWALUK, E., HOUGH, K. & KAKEMI, M. (1988). Mechanisms for the pharmacologic interaction of organic nitrates with thiols. Existence of an extracellular pathway for the reversal of nitrate vascular tolerance by N-acetylcysteine. *J. Pharmacol. Exp. Ther.*, **245**, 524–530.
- GOLDSCHMIDT, J.E. & TALLARIDA, R.J. (1991). Pharmacological evidence that captopril possesses an endothelium-mediated component of vasodilatation: effect of sulfhydryl groups on endothelium-derived relaxing factor. *J. Pharmacol. Exp. Ther.*, **257**, 1136–1145.
- GRUETTER, C.A. & LEMKE, S.M. (1985). Dissociation of cysteine and glutathione levels from nitroglycerin-induced relaxation. *Eur. J. Pharmacol.*, **111**, 85–95.
- HALON, D.A., ROSENFELD, T., HARDOFF, R. & LEWIS, B.S. (1988). Advantage of combined therapy with captopril and nitrates in severe congestive heart failure. *Isr. J. Med. Sci.*, **24**, 664–670.
- HOROWITZ, J.D., ANTMAN, E.M., LORELL, B.H., BARRY, W.H. & SMITH, T.W. (1983). Potentiation of the cardiovascular effects of nitroglycerin by N-acetylcysteine. *Circulation*, **68**, 1247–1253.
- IGNARRO, L.J., LIPPTON, H., EDWARDS, J.C., BARICOS, W.H., HYMAN, A.L., KADOWITZ, P.J. & GRUETTER, C.A. (1981). Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for involvement of S-nitrosothiols as active intermediates. *J. Pharmacol. Exp. Ther.*, **218**, 739–749.
- JORDAN, R.A., SETH, L., HENRY, D.A., WILEN, M.M. & FRANCIOSA, J.A. (1985). Dose requirements and hemodynamic effects of transdermal nitroglycerin compared with placebo in patients with congestive heart failure. *Circulation*, **71**, 980–986.
- KUKOVETZ, W.R. & HOLZMANN, S. (1986). Mode of action of nitrates with regard to vasodilatation and tolerance. *Z. Kardiol.*, **75** Suppl. 3, 8–11.
- LAWSON, D.L., NICHOLS, W.W., MEHTA, P. & MEHTA, J.L. (1991). Captopril-induced reversal of nitroglycerin tolerance: role of sulfhydryl group vs ACE-inhibitory activity. *J. Cardiovasc. Pharmacol.*, **17**, 411–418.
- LOSCALZO, J. (1985). N-acetylcysteine potentiates inhibition of platelet aggregation by nitroglycerin. *J. Clin. Invest.*, **76**, 703–708.
- MOLLACE, V., SALVEMINI, D., ANGGARD, E. & VANE, J. (1991a). Nitric oxide from vascular smooth muscle cells: regulation of platelet reactivity and smooth muscle cells guanylate cyclase. *Br. J. Pharmacol.*, **104**, 633–638.
- MOLLACE, V., SALVEMINI, D., SESSA, W.C. & VANE, J.R. (1991b). Inhibition of human platelet aggregation by endothelium-derived relaxing factor, sodium nitroprusside or iloprost is potentiated by captopril and reduced thiols. *J. Pharmacol. Exp. Ther.*, **258**, 820–823.
- MULSCH, A., BUSSE, R. & BASSENGE, E. (1988). Desensitization of guanylate cyclase in nitrate tolerance does not impair endothelium-dependent responses. *Eur. J. Pharmacol.*, **158**, 191–198.
- MURAD, F. (1986). Cyclic guanosine monophosphate as a mediator of vasodilation. *J. Clin. Invest.*, **78**, 1–5.
- NEEDLEMAN, P. & JOHNSON, E.M. JR. (1973). Mechanism of tolerance development to organic nitrates. *J. Pharmacol. Exp. Ther.*, **84**, 709–715.
- NEEDLEMAN, P., JAKSCHIK, B. & JOHNSON, E.M. JR. (1973). Sulphydryl requirement for relaxation of vascular smooth muscle. *J. Pharmacol. Exp. Ther.*, **187**, 324–331.
- PACKER, M., LEE, W.H., KESSLER, P.D., GOTTLIEB, S.S., MEDINA, N. & YUSHAK, M. (1987). Prevention and reversal of nitrate tolerance in patients with congestive heart failure. *N. Engl. J. Med.*, **317**, 799–804.
- PACKER, M., MELLER, J., MEDINA, N., YUSHAK, M. & GORLIN, R. (1981). Determinants of drug response in severe chronic heart failure. 1. Activation of vasoconstrictor forced during vasodilator therapy. *Circulation*, **64**, 506–514.
- PARKER, J.O. & FUNG, H.L. (1984). Transdermal nitroglycerin in angina pectoris. *Am. J. Cardiol.*, **54**, 471–476.
- PARKER, J.O., FARRELL, B., LAHEY, K.A. & ROSE, B.F. (1987). Nitrate tolerance: the lack of effect of N-acetylcysteine. *Circulation*, **76**, 572–576.
- PURI, R.N. & MEISTER, A. (1983). Transport of glutathione, as gamma-glutamylcysteinylglycyl ester into liver and kidney. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 5258–5260.
- RADOMSKI, M.W. & MONCADA, S. (1983). An improved method for the washing of human platelets with prostacyclin. *Thromb. Res.*, **30**, 383–389.
- SAKANASHI, M., MATSUZAKI, T. & ANIYA, Y. (1991). Nitroglycerin relaxes coronary artery of the pig with no change in glutathione content of glutathione S-transferase activity. *Br. J. Pharmacol.*, **103**, 1905–1908.
- SALVEMINI, D. & BOTTING, R. (1990). The effect of free radical scavengers on platelet adhesion and aggregation. In *Drug News and Perspectives*. ed. Prous, J.R. Science Publishers, 3, 202–212.
- SALVEMINI, D., DE NUCCI, G., GRYGLEWSKI, R.J. & VANE, J.R. (1989). Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 6328–6332.
- SALVEMINI, D., MOLLACE, V., PISTELLI, A., ANGGARD, E. & VANE, J. (1992). Metabolism of glyceryl trinitrate to nitric oxide by endothelial cells and smooth muscle cells and its induction by E. coli lipopolysaccharide. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 982–986.
- SALVEMINI, D., PISTELLI, A. & VANE, J. (1993). Conversion of glyceryl trinitrate to nitric oxide in tolerant and non-tolerant smooth muscle and endothelial cells. *Br. J. Pharmacol.*, **108**, 162–169.
- SCHRÖDER, H. & SCHRÖR, K. (1992). Cytochrome P₄₅₀ mediates bioactivation of glyceryl trinitrate in cultured cells. In *'Biology of Nitric Oxide'* ed. Moncada, S., Marletta, M., Hibbs, H. Jr. & Higgs, E.A. London: Portland Press.
- SERVENT, D., DELAFORGE, M., DUCROCQ, C., MANSUY, D. & LENFANT, M. (1989). Nitric oxide formation during microsomal hepatic degradation of glyceryl trinitrate: involvement of cytochrome P₄₅₀. *Biochem. Biophys. Res. Commun.*, **163**, 1210–1216.
- SNEDDON, J.M. & VANE, J.R. (1988). Endothelium-derived relaxing factor reduces platelet adhesion to bovine endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 2800–2804.
- THADANI, U., MANYARI, D., PARKER, J.O. & FUNG, H.L. (1980). Tolerance to the circulatory effects of oral isosorbide dinitrate. Rate of development and cross tolerance to glyceryl trinitrate. *Circulation*, **61**, 526–535.
- TORRESI, J., HOROWITZ, J.D. & DUSTING, G.J. (1985). Prevention and reversal of tolerance to nitroglycerine with N-acetylcysteine. *J. Cardiovasc. Pharmacol.*, **7**, 777–783.

- VAN GILST, W.H., DE GRAEFF, P.A., SCHOLTENS, E., DE LANGEN, C.D.J. & WESSELING, H. (1987). Potentiation of isosorbide dinitrate-induced coronary dilatation by captopril. *J. Cardiovasc. Pharmacol.*, **9**, 254–255.
- VAN GILST, W.H., SCHOLTENS, E., DE GRAEFF, P.A., DE LANGEN, C.D.J. & WESSELING, H. (1988). Differential influences of angiotensin converting-enzyme inhibitors on the coronary circulation. *Circulation*, **77**, 124–29.
- WINNIFORD, M.D., KENNEDY, P.L., WELLS, P.J. & HILLIS, L.D. (1986). Potentiation of nitroglycerin-induced coronary dilatation by N-acetylcysteine. *Circulation*, **73**, 138–142.
- YEATES, R.A., SCHMID, M. & LEITOLD, M. (1989). Antagonism of glyceryl trinitrate activity by an inhibitor of glutathione S-transferase. *Biochem. Pharmacol.*, **38**, 1749–1753.
- ZELIS, R. & MASON, D.T. (1975). Isosorbide dinitrate. Effect on the vasodilator response to nitroglycerin. *J. Am. Med. Assoc.*, **234**, 166–170.

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