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Investigation of role for oxidant stress in vascular tolerance development to glyceryl trinitrate *in vitro*

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1 The role of reactive oxygen species (ROS) during the development of vascular cellular tolerance to glyceryl trinitrate (GTN), was studied in the rat isolated aorta.

2 Nitrate tolerance induced by a 30 min incubation with GTN (30 or 100 μM) *in vitro*, was not affected by pretreatment with the intracellular superoxide anion scavenger, tiron (10 mM), or the intracellular scavenger of peroxynitrite anion and hydroxyl radical, dimethylsulphoxide (DMSO, 0.2% v v^{-1}). In contrast, pretreatment with the intracellular sulphydryl donor, N-acetyl-L-cysteine (NAC, 1 mM), significantly attenuated GTN-induced tolerance.

3 Pretreatment with a putative inhibitor of oxidant stress-mediated, transcription factor NF-κ B activation, pyrrolidine dithiocarbamate (PDTC, 50 μM), an inhibitor of gene activation by NF-κB, dexamethasone (1 μM) or an inhibitor of protein synthesis, cycloheximide (10 μM), failed to affect tolerance development to GTN.

4 Pretreatment with DMSO (0.2% v v^{-1}) or PDTC (50 μM) depressed non-tolerant vasorelaxation to GTN (1 nM – 1 μM) *per se*.

5 Tiron (10 mM) abolished the reduction of ferricytochrome c by a superoxide anion generating system, assessed photometrically *in vitro*. In contrast, DMSO (0.2% v v^{-1}), NAC (1 mM) and PDTC (50 μM) were without effect.

6 Our data suggests that neither oxidant stress nor nuclear activation, is important in the development of cellular tolerance to GTN in rat isolated aortic smooth muscle.

Keywords: Nitrate tolerance; nitric oxide; reactive oxygen species; rat aorta

Introduction

Among nitrovasodilators (for review, see Harrison & Bates, 1993), the organic nitrates offer a special therapeutic advantage in the treatment of ischaemic heart disease and congestive heart failure (for review, see Ahlner *et al*., 1991). In addition, they have afforded a new approach to portal hypertension (Bosch *et al*, 1993). However, the clinically important haemodynamic and anti-ischaemic effects of organic nitrates are limited due to the development of tolerance (Bogaert, 1991; Elkayam, 1991). Nitrate tolerance is clinically managed by interrupting dosing so as to avoid continuous drug exposure (Reicher, 1989; Amsterdam, 1992; Rutherford, 1995); but this practice is not without clinical problems (Thadani & Vane, 1992; Ferratini, 1994; Rutherford, 1995).

Nitrate tolerance *in vivo* probably has several components, including haemodynamic and pharmacokinetic counter-regulation (Bogaert, 1988; Parker *et al*., 1991; Bassenge & Zanzinger, 1992; Elkayam *et al*., 1992; Fung, 1993) and cellular resistance at the level of the vasculature (cellular tolerance) (Needleman & Johnson, 1973; Feelisch & Noack, 1987; Chong & Fung, 1990; Bennett *et al*., 1994). Cellular tolerance to organic nitrates such as glyceryl trinitrate (GTN), extensively studied *in vitro*, has widely been attributed to reduced biotransformation to NO (Needleman & Johnson, 1973; Noack, 1990; Forster *et al*., 1991; Fung *et al*., 1992; Feelisch, 1993; Bennett *et al*., 1994). Thus, a diminution in the activity of a putative organic nitrate converting enzyme(s) (ONCE) (see Chong & Fung, 1990a; Kowaluk & Fung, 1991; Bennett *et al*., 1994) and/or a deficit in intracellular thiols concerned with non-enzymatic biotransformation or perhaps intermediate nitrosothiol formation (see Axelsson *et al*., 1982; Hütter *et al*., 1988; Chong & Fong, 1990b), has been held to be a key event in tolerance.

Despite the suggestion that NO may impair nitrate biotransformation (Kojda *et al*., 1994), evidence that NO *per se* is a major mediator of cellular nitrate tolerance *in vitro* is lacking, since exogenous NO is unable to mimic fully nitrate self-tolerance (Yeates & Schmid, 1992). Recently, several studies have described the ability of antioxidants to attenuate or even abrogate, tolerance to organic nitrates assessed both *in vitro* (Yeates & Schmid, 1992; Münzel *et al*., 1995; Laight & Anggård, 1995; Marfella *et al*., 1995) and *in vivo* (Bassenge *et al*., 1995; Bassenge & Fink, 1995; Skatchkov *et al*., 1995; Utepbergenov *et al*., 1996). This provides a new insight into the mechanism(s) of tolerance, delineating the involvement of oxidant stress. In particular, Münzel *et al*. (1995, 1996) and Skatchkov *et al*. (1995) have outlined roles for the superoxide and peroxynitrite anion.

Given the probable participation of reactive oxygen species (ROS) in intracellular signalling and transcriptional activation (Johnson & McKnight, 1989; Schreck *et al*., 1992; Baeuerle & Henkel, 1994; Grimm & Baeuerle, 1993), including the regulation of thiol levels (see DeLeve & Kaplowitz, 1991), it is possible that superoxide anions generated during exposure to an organic nitrate (see Kappus & Sies, 1981; Biaglow *et al*., 1986; Hill *et al*., 1989; Yeates & Schmid, 1992) may promote a tolerant state where nitrate biotransformation is impaired. Of particular interest is the oxidative stress-response transcription factor nuclear factor (NF)-κB (for reviews, see Schreck *et al*., 1992; Baueuerle & Henkel, 1994), which is involved in the rapid activation of multiple genes concerned in the early defence reactions of higher organisms, the expression of which may be impaired by glucocorticoids such as dexamethasone (Ray & Prefontaine, 1994; Wilckens, 1995) and putative NF-κB inhibitors such as pyrrolidine dithiocarbamate (PDTC) (Schreck *et al*., 1992).

The aim of the present study was therefore to investigate the role of the superoxide and peroxynitrite anion together with the effects of putative inhibitors of stress-mediated nuclear activation, including *de novo* protein synthesis, in tolerance induction to GTN in rat isolated aortic smooth muscle *in vitro*

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Methods

General

Male Wistar rats (250–300 g) were anaesthetized with pentobarbitone sodium (60 mg kg^{-1}, i.p.) and the thoracic aorta carefully excised after a thorectomy. Endothelium-intact aortic rings approximately 2 mm in length, were mounted under a resting tension of 2 g in organ baths in physiological salt solution (PSS) gassed with carbogen and warmed at 37°C. The PSS had the following composition (in mM): NaCl 133, KCl 4.7, NaH_2PO_4 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, (+)-glucose 7.8 and $CaCl_2$ 2.52. Stabilization was allowed for 1 h, during which time the PSS was changed every 15 min.

Experimental protocol

Tolerance was induced by incubating with GTN (30 or 100 μM) for 30 min followed by a thorough washout over 30 min, i.e. a GTN-free interval. Rings were then precontracted with a concentration of noradrenaline (NA, 100 nM) which elicited approximately 90% of the maximal response and cumulative vasorelaxation to GTN (1 nM–1 μM) assessed. In studies in which the ability of intracellular ROS scavengers and thiol supplementation to prevent tolerance development was investigated tiron (10 mM), DMSO (0.2% v v^{-1}) or NAC (1 mM) was added 10 min before incubation with GTN (30 or 100 μM) and then washed out during the GTN-free interval. In studies in which the role of nuclear activation and *de novo* protein synthesis was addressed, PDTC (50 μM), dexamethasone (1 μM) or cycloheximide (10 μM) was present 1 h before the incubation with GTN (30 or 100 μM) and then throughout the experiment.

Assessment of superoxide anion scavenging activity

Superoxide anion scavenging activity was assessed photometrically by a modification of the method described by McCord & Fridovich (1969). Briefly, the reduction of ferricy-

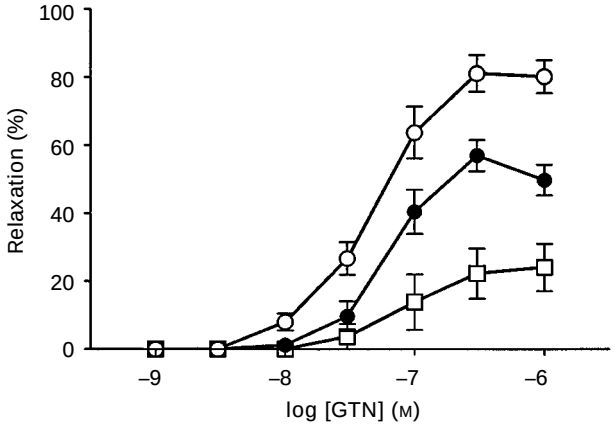

Figure 1 Vasorelaxation to glyceryl trinitrate (GTN) in the rat isolated aorta, either non-tolerant (○) or made tolerant by a 30 min incubation with GTN 30 μM (●) or 100 μM (□). Data shown are means ($n=8$); vertical lines indicate s.e.mean.

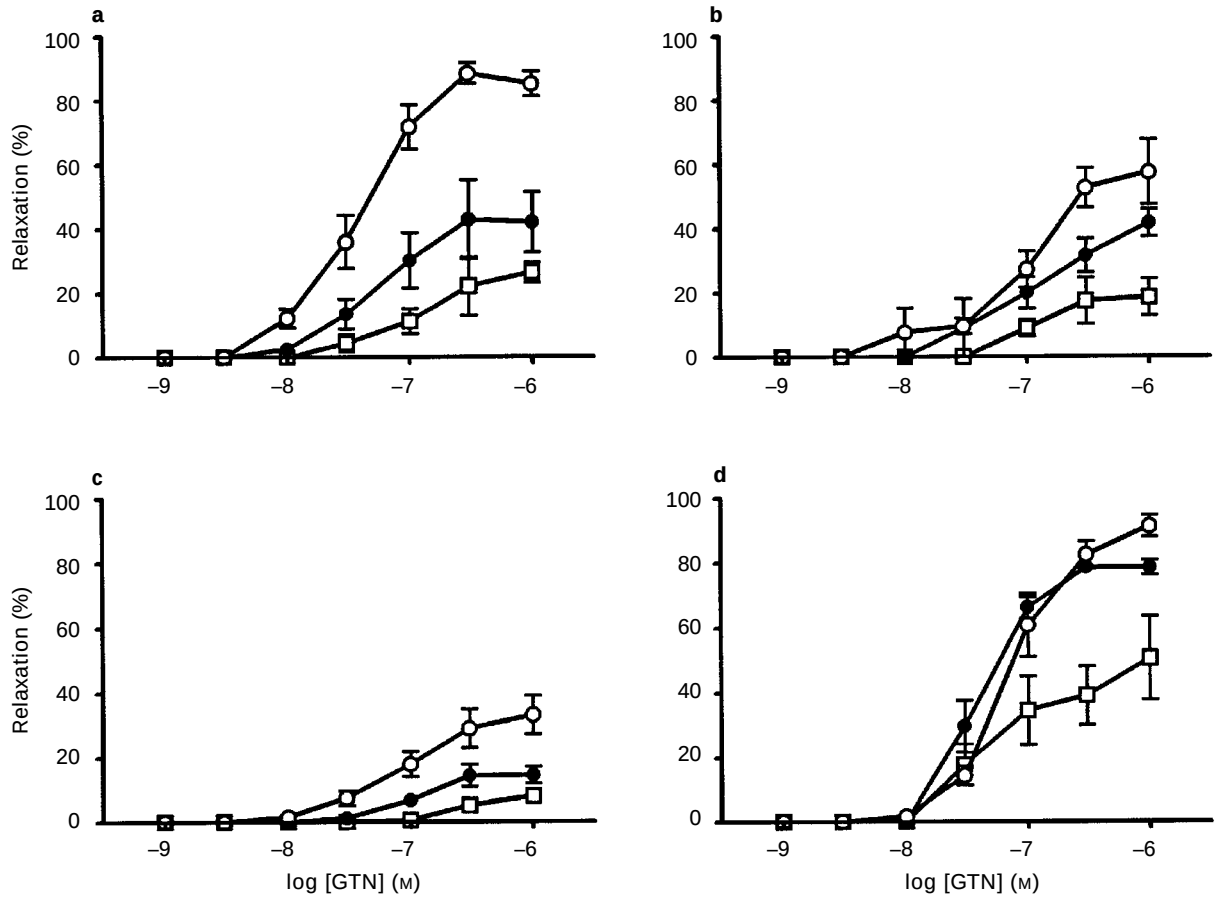

Figure 2 Vasorelaxation to glyceryl trinitrate (GTN) in the rat isolated aorta, either non-tolerant (○) or made tolerant by a 30 min incubation with GTN 30 μM (●) or 100 μM (□): effect of (a) tiron (10 mM, $n=5$); (b) dimethylsulphoxide (0.2%, $n=7$); (c) pyrrolidine dithiocarbamate (50 μM, $n=5$); or (d) N-acetyl-L-cysteine (1 mM, $n=4$). Vertical lines show s.e.mean.

tochrome c (100 μM) by superoxide anions generated by a xanthine oxidase (20 mu ml^{-1})/hypoxanthine (100 μM) system was monitored at 550 nm at room temperature in a kinetic platereader, as previously described (Laight *et al*., 1996). Superoxide anion scavenging activity was investigated for tiron (10 mM), dimethylsulphoxide (DMSO, 0.2% v v^{-1}), N-acetyl-L-cysteine (1 mM) and PDTC (50 μM). Superoxide dismutase (SOD, 200 u ml^{-1}) was included in the study as a reference agent.

Drugs

Pyrrolidine dithiocarbamate, cycloheximide, N-acetyl-L-cysteine, 1,2-dihydroxybenzene-3,5-disulphonate (tiron), dimethylsulphoxide, dexamethasone phosphate, (−)-noradrenaline bitartrate (NA), xanthine oxidase (xanthine: oxygen oxidoreductase; EC 1.1.3.22) derived from buttermilk (Grade 1) and bovine superoxide dismutase (superoxide: superoxide oxidoreductase, EC 1.15.1.1) were obtained from Sigma Chemical Co. (Poole, Dorset). Glyceryl trinitrate (Nitronal) was obtained from Lipha Pharmaceuticals Ltd. (West Drayton, Middlesex, U.K.).

Statistics

Data are expressed as mean ± s.e.mean. The differences between two means were evaluated by Student's unpaired *t* test. A multicomparison of means was conducted by 1W ANOVA followed by Dunnett's test. Tolerance was assessed by the area under the concentration-response curve (AUC) for vasorelaxation to GTN (1 nM – 1 μM) and expressed as a percentage of non-tolerant AUC (=100%). Statistical significance was accepted at the 5% level.

Results

Cellular tolerance to GTN

NA (100 nM) elicited non-tolerant precontraction of 13.7 ± 1.0 mN (n=8) in the control group which was not significantly affected after treatment with tiron (10 mM) (10.8 ± 1.0 mN, n=5), DMSO (0.2%) (10.8 ± 1.0 mN, n=7) or N-acetyl-L-cysteine (1 mM) (13.7 ± 2.0 mN, n=4) or in the presence of PDTC (50 μM) (13.7 ± 1.0 mN, n=5), dexamethasone (1 μM) (12.7 ± 1.0 mN, n=6) or cycloheximide (10 μM) (13.7 ± 1.0 mN, n=6). Furthermore, a previous 30 min exposure to GTN (30 or 100 μM) did not affect precontraction to NA (100 nM) in any of the groups (data not shown).

GTN (1 nM – 1 μM) elicited vasorelaxation which was depressed in a graded manner by a previous 30 min exposure to GTN (30 or 100 μM) (Figure 1 and Table 1). Co-incubation of tiron (10 mM) (Figure 2a) or DMSO (0.2% v v^{-1}) (Figure 2b) with GTN (30 or 100 μM) during tolerance induction or the continuous presence of PDTC (50 μM) (Figure 2c) did not affect tolerance development (Table 1). However, co-incubation with N-acetyl-L-cysteine (1 mM) (Figure 2d), abolished tolerance development to 30 μM GTN (P<0.05) and attenuated the effect of 100 μM GTN, although statistical significance was not attained (Table 1). Furthermore, the continuous presence of dexamethasone (1 μM) (Figure 3a) or cycloheximide (10 μM) (Figure 3b) had no effect. In addition, the AUC for non-tolerant vasorelaxation to GTN (1 nM – 1 μM) was markedly depressed following both co-incubation with DMSO (0.2% v v^{-1}) and in the presence of PDTC (50 μM) (Table 2).

Assessment of superoxide anion scavenging activity

The initial rate of reduction of ferricytochrome c (100 μM) by a xanthine oxidase (20 mu ml^{-1})/hypoxanthine (100 μM) system was abolished by tiron (10 mM) and almost completely depressed by SOD (200 u ml^{-1}) (Table 3). In contrast, DMSO

Table 1 Vasorelaxation to glyceryl trinitrate (GTN, 1 nM – 1 μM) in the rat isolated aorta made tolerant by a 30 min incubation with GTN (30 or 100 μM): effects of treatments

Treatment	n	*AUC (%) 30 μM GTN*	*AUC (%) 100 μM GTN*
Control	8	63.2 ± 6.7†	23.8 ± 9.0†
Tiron (10 mM)	5	47.4 ± 13.6†	22.2 ± 7.2†
DMSO (0.2%)	7	54.8 ± 2.4†	17.7 ± 6.5†
NAC (1 mM)	4	105.7 ± 6.6*	55.7 ± 13.4†
PDTC (50 μM)	5	59.2 ± 13.8†	11.4 ± 6.1†
CHX (10 μM)	6	48.7 ± 11.6†	24.3 ± 8.5†
Dex (1 μM)	6	68.0 ± 4.1†	22.0 ± 5.9†

Tolerant area under the concentration-response curve (AUC) for vasorelaxation to GTN after exposure to GTN (30 or 100 μM), is expressed as a percentage of non-tolerant AUC (=100%). Dimethysulphoxide, DMSO; N-acetyl-L-cysteine, NAC; pyrrolidine dithiocarbamate, PDTC; cycloheximide, CHX; dexamethasone, Dex. DMSO and NAC were washed out 30 min before vasorelaxation to GTN was assessed while PDTC, CHX and Dex were present throughout. †P<0.05 with respect to corresponding non-tolerant AUC. *P<0.05 with respect to corresponding control group value.

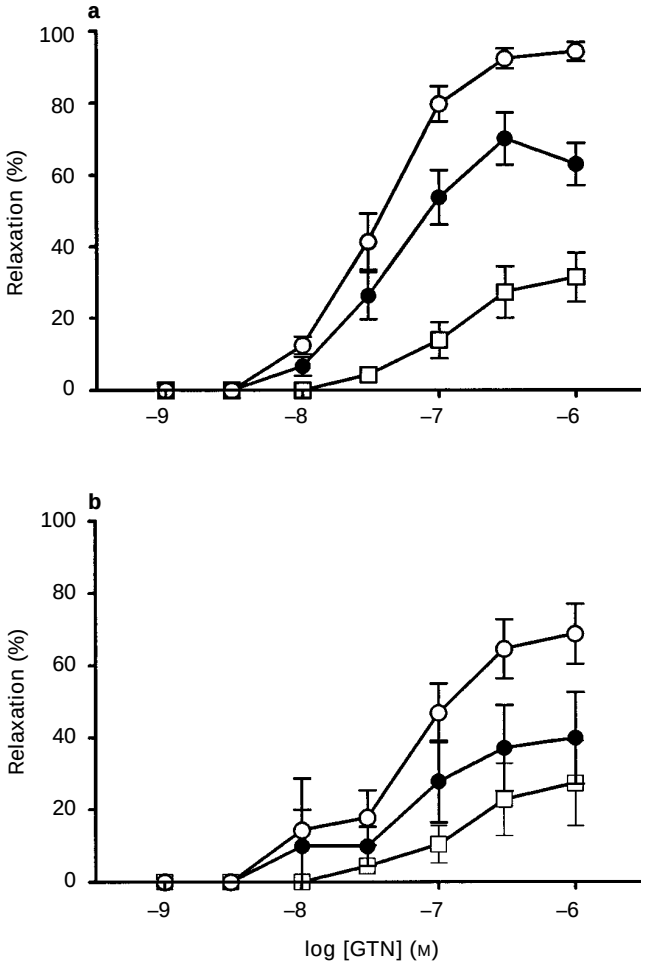

Figure 3 Vasorelaxation to glyceryl trinitrate (GTN) in the rat isolated aorta, either non-tolerant (○) or made tolerant by a 30 min incubation with GTN 30 μM (●) or 100 μM (□): effect of (a) dexamethasone (1 μM, n=6) or (b) cycloheximide (10 μM, n=6). Vertical lines show s.e.mean.

Table 2 Vasorelaxation to glyceryl trinitrate (GTN, 1 nM – 1 μM) in the non-tolerant, rat isolated aorta: effect of treatments

Treatment	n	*GTN* pD_2	E_{max} (%)	*AUC* (units)
Control	8	7.35±0.02	80.1±4.8	110.0±10.3
Tiron (10 mM)	5	7.45±0.03	85.1±3.9	126.2±10.2
DMSO (0.2%)	7	7.02±0.03*	57.5±10.1	60.3±9.0*
NAC (1 mM)	4	7.14±0.11*	91.3±3.4	103.3±8.1
PDTC (50 μM)	5	7.10±0.03*	33.2±5.9*	37.5±8.2*
CHX (10 μM)	6	7.23±0.01	68.9±8.3	83.8±14.2
Dex (1 μM)	6	7.46±0.01	94.3±2.6	137.6±7.7

Area under the concentration-response curve, AUC; dimethylsulphoxide, DMSO; N-acetyl-L-cysteine, NAC; pyrrolidine dithiocarbamate, PDTC; cycloheximide, CHX; dexamethasone, Dex. DMSO and NAC were washed out 30 min before vasorelaxation to GTN was assessed while PDTC, CHX and Dex were present throughout. *$P<0.05$ with respect to corresponding control group value.

Table 3 Initial rate of reduction of ferricytochrome c by a xanthine oxidase/hypoxanthine system *in vitro*: effect of treatments

Treatments	n	*Initial rate* (mOD min^{-1})
Control	3	25.9±0.8
SOD (200 u ml^{-1})	3	1.23±0.2**
Tiron (10 mM)	3	0**
DMSO (0.2%)	3	26.2±1.0
NAC (1 mM)	3	27.8±1.5
PDTC (50 μM)	3	29.3±3.2

Superoxide dismutase, SOD; dimethylsulphoxide, DMSO; N-acetyl-L-cysteine, NAC; pyrrolidine dithiocarbamate, PDTC. OD= optical density at 550 nm. **$P<0.01$ with respect to control group value.

(0.2% v v^{-1}), N-acetyl-L-cysteine (1 mM) and PDTC (50 μM) were without effect.

Discussion

It was hypothesized that vascular cellular nitrate tolerance could represent an adaptive response to the generation of the superoxide anion during the biotransformation of organic nitrates. In this 'nitrate stress' scenario, the postulated generation of ROS would rapidly activate stress-response transcription factors, such as NF-κB (Schreck *et al*., 1992), to induce a tolerant biochemical phenotype in which further biotransformation was impaired. However, the inability of PDTC or dexamethasone, which have been shown to inhibit NF-κB activation effectively in a number of cell types (Sherman *et al*., 1993; Eberhardt *et al*., 1994; Ray & Prefontaine, 1994; Wilckens, 1995), together with the failure of the protein synthesis inhibitor cycloheximide, to affect tolerance development to GTN, does not support a role for nuclear activation *in vitro*.

Similarly, the inefficacy of pretreatment with the intracellular antioxidants tiron and DMSO argues against roles for the superoxide anion, peroxynitrite anion and the hydroxyl radical during the *in vitro* development of cellular nitrate tolerance. The concentration of tiron employed (10 mM) was probably adequate, as it was shown to abolish the reduction of ferricytochrome c by a superoxide anion generating system (see also Laight *et al*., 1996) and has been previously applied to ameliorate oxidative damage *in vitro* (Mohazzab *et al*., 1994). Furthermore, other ROS generated from the superoxide anion, such as hydrogen peroxide (see Fridovich, 1986; Halliwell & Gutteridge, 1986) may be tentatively excluded. In contrast, Yeates & Schmid (1992), using a number of structurally diverse, poorly defined antioxidants, showed a total prevention of *in vitro* tolerance development to both isosorbide mononitrate and GTN in the rabbit isolated aorta. This disparity in observations could reflect species differences in the aetiology of cellular nitrate tolerance; or conceivably, non-specific effects of those antioxidants employed by Yeates & Schmid (1992). Interestingly, the results of attempts to implicate a specific role for the superoxide anion in nitrate tolerance were not supportive of such a role (Yeates & Schmid, 1992).

Since our study was designed, in principle as described by Yeates & Schmid (1992), to investigate the prevention of the development of cellular nitrate tolerance by antioxidants, the protocol does not address the possibility that superoxide anion may contribute to a pseudo-tolerance by attenuating vasorelaxation to GTN directly via the inactivation of NO (Gryglewski, 1986; Tsao & Lefer, 1990; Pagano *et al*., 1993; Butler *et al*., 1995). Indeed, Münzel *et al*. (1995) have shown that tiron reverses not only self-tolerance to GTN but also cross-tolerance to the endothelium-dependent vasodilator, acetylcholine and the NO donor, 3-morpholino-sydnominine, in the aorta isolated from rabbits made tolerant to GTN *in vitro*. However, preliminary studies designed to address this eventuality, have so far been unsuccessful since tiron (10 mM) was determined to abrogate NA-induced vasoconstrictor tone in the rat isolated aorta; while an alternative intracellular superoxide anion scavenger, tempo (Samuni *et al*., 1990; Laight *et al*., 1996), was found to depress markedly vasorelaxation to GTN *per se* (personal observations).

In contrast to the other antioxidants tested, the sulphydryl donor N-acetyl-L-cysteine was able to abolish tolerance development to the lowest level of GTN, while clearly tending to attenuate tolerance to the higher level. While a number of previous studies have failed to find improvement with N-acetyl-L-cysteine in tolerant animals (Abdollah *et al*., 1987; Hütter *et al*., 1988; Münzel *et al*., 1989; Holtz *et al*., 1989) or man (Hogan *et al*., 1989), the present observations are in agreement with the results of Lawson *et al*. (1991) and Newman *et al*. (1990) that N-acetyl-L-cysteine mitigates nitrate tolerance both *in vitro* and *in vivo*. Furthermore, given that N-acetyl-L-cysteine was washed out before vasorelaxation to GTN was studied, the beneficial action of N-acetyl-L-cysteine in the rat isolated aorta is clearly distinguishable from a simple reversal of tolerance due to the extracellular metabolism of GTN (see Fung *et al*., 1988; Levy *et al*., 1988; Hütter *et al*., 1988; Boesgaard *et al*., 1991). Our results would therefore support the sulphydryl depletion hypothesis of nitrate tolerance (Needleman & Johnson, 1973), although the role of intracellular thiol depletion in nitrate tolerance has been recently challenged (Kojda *et al*., 1993; Boesgaard *et al*., 1994). The present study would further suggest that N-acetyl-L-cysteine-attenuated cellular tolerance is not mediated by the scavenging of superoxide anion.

In conclusion, the data provide no evidence for the involvement of the superoxide anion or derived ROS in the development of cellular tolerance to GTN in rat aortic smooth muscle *in vitro*. Additionally, the use of PDTC as an inhibitor of NF-κB activation does not support a primary role for stress-mediated nuclear activation in this model of nitrate tolerance.

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