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# Influence of the endothelium on ex vivo tolerance and metabolism of glyceryl trinitrate in rat aorta

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**Abstract**

The influence of the endothelium on glyceryl trinitrate metabolism and relaxation and the relationship to tolerance induced by transdermal glyceryl trinitrate was explored in rat aorta. Metabolism was assessed in artery segments incubated with glyceryl trinitrate (1.0 μM) for 2 min and the contents of 1,2- and 1,3-glyceryl dinitrate measured by gas chromatography. In non-tolerant arteries mean contents of glyceryl trinitrate, 1,2-glyceryl dinitrate and 1,3-glyceryl dinitrate were 3.2, 0.23 and 0.10 nmol/g, respectively; in tolerant arteries the content of 1,2-glyceryl dinitrate was reduced by approximately 60%. Endothelium removal or nitric oxide synthase (NOS) inhibition did not affect metabolite contents but increased the relaxant response to glyceryl trinitrate in the tolerant artery to an extent that tolerance was significantly attenuated. It is concluded that (i) tolerance is associated with depression of glyceryl trinitrate metabolism by an endothelium-independent mechanism and (ii) the endothelium contributes to tolerance by a mechanism which is independent of metabolism and may be linked with endothelial NOS.


*Keywords:* Aorta, rat; Endothelium; Glyceryl trinitrate metabolism; Glyceryl dinitrate; Artery relaxation; Tolerance, ex vivo; NO (nitric oxide) synthase inhibition

## 1. Introduction

The hypothesis that tolerance to glyceryl trinitrate is a consequence of diminished bioconversion to an active metabolite (nitric oxide; NO or congener) in the vascular smooth muscle is supported by evidence of diminished metabolism of glyceryl trinitrate in isolated vessels when tolerance is induced either in vitro (Brien et al., 1986; Slack et al., 1989) or measured ex vivo after sustained in vivo glyceryl trinitrate administration (Fung and Poliszczuk, 1986; Ratz et al., 2000a,b; Sage et al., 2000). This metabolic evidence is reinforced by the recent identification of the principal glyceryl trinitrate-bioconverting enzyme in rabbits as aldehyde dehydrogenase and findings that the activity of this enzyme is depressed in the rabbit aorta by in vitro tolerance (Chen et al., 2002). The evidence for impaired biotransformation is generally consistent with the minimal nature of cross tolerance to non-nitrate sources of NO accompanying in vitro tolerance (Henry et al., 1989) and ex vivo tolerance (De la Lande et al., 1999; Ratz et al., 2000a; Sage et al., 2000).

While it is generally assumed that the smooth muscle is the major site of glyceryl trinitrate bioconversion, it has been reported in rabbit and rat that ex vivo vascular tolerance is attenuated after removing the endothelium (Munzel et al., 1995a, 2000). While this attenuation was not significant in two other studies on rat aorta (De la Lande et al., 1999; Ratz et al., 2000a), this draws attention to the possibility that the endothelium might contribute to the impairment of glyceryl trinitrate metabolism in the tolerant vessel. Several studies have addressed the role of endothelium in the non-tolerant artery and concluded that it does not influence conversion of glyceryl trinitrate to its glyceryl dinitrate metabolites or to NO (McGuire et al., 1994; Feelisch and Kelm, 1991). However, the relevant question is whether glyceryl trinitrate metabolism is influenced by the endothelium in the tolerant aorta. To answer this question, we have examined the comparative effects of removing endothelium and inhibiting its nitric oxide synthase (eNOS) activity, on the metabolism and relaxant effect of glyceryl

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trinitrate in non-tolerant and tolerant rat aortae. Tolerance was induced by transdermal glyceryl trinitrate as described earlier (De la Lande et al., 1999), using conversion to 1,2-glyceryl dinitrate as a surrogate for active metabolite formation (Bennett et al., 1994). Vasorelaxant responses to glyceryl trinitrate were measured to enable the functional significance of the metabolic findings to be assessed.

## 2. Methods

Sprague–Dawley rats were exposed to transdermal glyceryl trinitrate (37 mg/kg/day) or to placebo patches for 2 days, as described previously (De la Lande et al., 1999). Twenty-four hours after the last application, the rat was sacrificed under halothane anaesthesia and the aorta excised, placed in Krebs solution at 37 °C and cut into segments. The experimental protocol was approved by the North Western Adelaide Health Service animal ethics committee and conforms to the Australian National Health and Medical Research Council guidelines of animal usage for experimentation.

### 2.1. Metabolic studies

Metabolism of glyceryl trinitrate was examined in 3 to 4 mm segments with and without endothelium that were either relaxed or contracted at the time of adding the GTN (the latter to mimic conditions used for studying relaxant activity). The relaxed segment ( ~ 5 mm) was incubated in 8 ml of Krebs solution gassed with 95% $O_2$, 5% $CO_2$ at 37 °C for 30 min after which it was rapidly blotted on moist filter paper prior to weighing and returned to fresh medium for a further 60 min. The segment was then incubated in 1.0 ml of glyceryl trinitrate (1.0 μM) for 2 min, washed in ice-cold Krebs solution for 5 s and immediately placed in liquid nitrogen. Both the frozen segment and glyceryl trinitrate incubating medium were stored at −80 °C. A second segment was incubated in glyceryl trinitrate-free medium but otherwise treated identically. In all experiments, a third segment was tested for endothelium integrity, using the relaxant effect of 1.0 μM acetylcholine as the index.

In some experiments on endothelium-intact vessels only, the effects of the NOS inhibitor (*N*-ω-nitro-L-arginine methyl ester; L-NAME 300 μM) were examined. The L-NAME was present 30 min prior to and during the 2 min incubation with (or without) glyceryl trinitrate. Experiments where the artery was contracted prior to incubation with glyceryl trinitrate were confined to those on effects of endothelium removal. The segment was first equilibrated for 60 min under 2 g tension in an organ bath containing 15 ml of Krebs solution after which phenylephrine was applied cumulatively until the contractile response was in the upper region of the concentration–response curve. The relaxant response to acetylcholine (1.0 μM) was then determined in order to assess endothelium function. Thirty minutes after bath washout of acetylcholine, the segment was contracted with phenylephrine 0.1 μM. When the response to phenylephrine had plateaued, the segment was exposed to glyceryl trinitrate 1.0 μM for 2 min after which it was rinsed in ice-cold Krebs solution for 5 s and frozen in liquid nitrogen. A second segment was treated identically but not exposed to glyceryl trinitrate.

### 2.2. Pharmacological studies

When relaxant activities alone were determined, the segment ( ~ 3 mm) was contracted with phenylephrine as described above after which glyceryl trinitrate was applied cumulatively (0.001–1.0 μM). Thirty to forty minutes after glyceryl trinitrate washout, the segment was again contracted with phenylephrine to enable relaxation to acetylcholine to be measured. Finally, a full concentration–response curve to phenylephrine was elicited to establish its maximal contractile effect. In order to minimise the varying influence of functional antagonism by the contractile agent on the relaxant response to glyceryl trinitrate, only responses to glyceryl trinitrate where the steady state levels of contraction to phenylephrine were in a defined range (60–85%) of the phenylephrine maximum were accepted. The actual steady state levels for control and tolerant arteries ($n = 14–15$) with endothelium were 72 ± 2% and 71 ± 2% and without endothelium were 75 ± 2% and 76 ± 2%, respectively. The effects of L-NAME were examined in a separate series of experiments, the L-NAME being added 30 min prior to the glyceryl trinitrate and was present for the remainder of the experiment. Mean steady state contraction levels for control and tolerant arteries ($n = 14$) with L-NAME absent were 68 ± 3 and 74 ± 2%, respectively, and with L-NAME present were 72 ± 2 and 78 ± 2%, respectively.

### 2.3. Endothelium removal

This was achieved by gently drawing dental floss held in forceps through the lumen. The loss of endothelium cells was confirmed by scanning electron microscopy in preliminary experiments while pharmacological testing in all experiments indicated that in most there was complete loss of the relaxant effect of acetylcholine 1.0 μM. Segments were rejected when relaxation to acetylcholine exceeded 5%. Conversely, the routine criterion for endothelium integrity was relaxation exceeding 50%. Evidence that the weighing procedure in the metabolic experiments did not result in significant endothelium damage was provided by the finding that neither $EC_{50}$'s nor $E_{max}$ values for acetylcholine-induced relaxation differed significantly between weighed and unweighed segments from seven control rats (data not shown).

### 2.4. Glyceryl trinitrate metabolite assay

Glyceryl trinitrate and its dinitrate metabolites were extracted from the segments of rat aorta using a modifica-

tion of the method described in an earlier study (Sage et al., 2000). Briefly, glyceryl trinitrate and its metabolites in the frozen segments, with added internal standard (1,4-dinitroxy-butan-2-ol) were extracted with 5 ml methyl tert-butyl ether which was then dried with anhydrous sodium sulphate, concentrated to approximately 200 μl under a stream of nitrogen and stored at −20 °C. Glyceryl trinitrate and the dinitrates were separated using gas chromatography with electron capture detection as previously described (Sage et al., 2000) and their peak height ratios to the internal standard were used for quantitation. Standard curves were prepared by spiking 1 ml Krebs with glyceryl trinitrate (1–50 pmol), 1,2-glyceryl dinitrate (0.1–5 pmol) and 1,3-glyceryl dinitrate (0.05–2.5 pmol) and extracted as above. The inter-assay coefficient of variation measured at 5, 1 and 0.25 pmol glyceryl trinitrate, 1,2-glyceryl dinitrate and 1,3-glyceryl dinitrate, respectively, was $<10\%$ ($n=5$). Tissue contents were expressed as nmol/g wet weight. Artefactual levels of 1,2-glyceryl dinitrate due to thermal degradation of glyceryl trinitrate on the column were estimated in each assay and kept to a level of approximately 1.0% or less by routine column maintenance (overnight baking and removal of 20 to 30 cm from the injector end).

### 2.5. Measurement of tolerance to glyceryl trinitrate

Responses to glyceryl trinitrate were compared in tolerant and non-tolerant arteries. The responses were quanitified in terms of $EC_{50}$ and $E_{max}$ values, obtained from the concentration–response curve by non-linear curve fitting using the Prism 3 GraphPad program. When sensitivity to glyceryl trinitrate is depressed in the rat aorta, as in tolerance, the concentration–response curve usually assumes a biphasic shape with the maximum of the first phase in the 0.3–1.0 μM range (Malta, 1989; De la Lande et al., 1999). In the present study, concentrations of glyceryl trinitrate were confined to the first phase. In addition, responsiveness to glyceryl trinitrate was quantified in terms of the area under the concentration–response curve (AUC) to 1.0 μM (since 1.0 μM is at the maximum in the non-tolerant artery and at the first phase maximum in the tolerant artery). The advantage of the AUC is that it takes into account the tendency for both the potency and maximum effect of glyceryl trinitrate to be depressed in the tolerant artery. It was estimated from the actual (not curve fitted) data, with percentage reduction in contraction as ordinate and log concentration at half log intervals as abscissa.

### 2.6. Data analysis

Results are expressed as mean ± S.E.M. or as mean (95% confidence intervals) for $EC_{50}$ values. Effects of endothelium removal were assessed by two-tailed paired *t*-test. The effect of the glyceryl trinitrate pre-treatment on relaxation was assessed by unpaired *t*-test. In addition, removal of endothelium or NOS inhibition by L-NAME was assessed by two-way analysis of variance (ANOVA) with repeated measures (Statistica Version, Statsoft). Glyceryl trinitrate/placebo pre-treatment was one factor and endothelium present/absent the repeated measures factor. Three-way ANOVA was used for the metabolic data with glyceryl trinitrate/placebo pre-treatment as one factor and endothelium present/absent and artery relaxed/contracted as repeated measures. The Duncan's Multiple Range test was used for post hoc analysis. Probability levels less than 0.05 were considered significant.

### 2.7. Materials

Acetylcholine chloride, L-phenylephrine hydrochloride and *N*-ω-nitro-L-arginine, methyl ester hydrochloride were purchased from Sigma (St Louis, MO, USA). Glyceryl trinitrate was purchased from David Bull Laboratories (Mulgrave, Vic, Australia) and 1,2-glyceryl dinitrate and 1,3-glyceryl dinitrate were purchased from Radian International (Austin, TX, USA). Methyl tert-butyl ether Omnisolv grade was purchased from EM Science (Gibbstown, NJ, USA). The internal standard 1,4-dinitroxy-butan-2-ol was prepared from 1,4-dibromo-butan-2-ol (Aldrich, Milwaukee, WI, USA) by displacement of the bromo groups using silver nitrate and subsequent purification by silica-gel column chromatography. The Krebs was gassed with carbogen (95% $O_2$, 5% $CO_2$) and was of the following composition (mM) Krebs: NaCl (118), KCl (3.89), $KH_2PO4$ (1.18), $NaHCO_3$ (25), $MgCl_2$ (1.05), $CaCl_2$ (2.34), EDTA (0.01), glucose (5.56), pH 7.4.

## 3. Results

### 3.1. Metabolic studies

After 2 min incubation with glyceryl trinitrate (1.0 μM), control artery segments with intact endothelium from rats pre-treated with glyceryl trinitrate-vehicle contained 1,2-glyceryl dinitrate, 1,3-glyceryl dinitrate and glyceryl trinitrate in amounts of 0.23, 0.10 and 3.2 nmol/g, respectively. The content of the 1,2- but not the 1,3-glyceryl dinitrate was significantly lower in tolerant segments from glyceryl trinitrate pre-treated rats so that the mean ratio of the two (termed the metabolite ratio) was reduced from 2.3 to 0.8 (Table 1). When the data were assessed by three-way ANOVA the effect of glyceryl trinitrate pre-treatment on 1,2-glyceryl dinitrate content was highly significant ($P<0.0001$) but effects of endothelium removal, of contraction and the combination of these factors were not significant (each $P>0.1$). The experiments with L-NAME, although fewer in number ($n=4–5$), showed similar trends to those with endothelium removal in that contents of 1,2-glyceryl dinitrate in control and tolerant arteries, namely 0.17 ± 0.05 and 0.07 ± 0.01 nmol/g, respectively, did not differ signifi-

Table 1
Effect of endothelium removal and tolerance induction on contents of glyceryl trinitrate and metabolites in relaxed and contracted rat aorta

| | Endothelium | Content (nmol/g) | | | |
|---|---|---|---|---|---|
| | | Relaxed aorta ($n=7$) | | Contracted aorta ($n=7$) | |
| | | Control | Tolerant | Control | Tolerant |
| 1,2-GDN | E+ | 0.23 ± 0.02 | 0.08 ± 0.01[a] | 0.24 ± 0.03 | 0.08 ± 0.01[a] |
| | E− | 0.18 ± 0.02 | 0.07 ± 0.02[a] | 0.23 ± 0.02 | 0.10 ± 0.04[a] |
| 1,3-GDN | E+ | 0.10 ± 0.01 | 0.10 ± 0.01 | 0.09 ± 0.01 | 0.09 ± 0.02 |
| | E− | 0.06 ± 0.01 | 0.09 ± 0.01 | 0.10 ± 0.01 | 0.14 ± 0.06 |
| GTN | E+ | 3.24 ± 0.37 | 3.56 ± 0.56 | 2.10 ± 0.20 | 2.91 ± 0.68 |
| | E− | 1.72 ± 0.18[b] | 2.50 ± 0.35 | 1.48 ± 0.17 | 1.58 ± 0.21 |
| *Log ratio* | | | | | |
| 1,2/1,3-GDN | E+ | 0.37 ± 0.05 | −0.15 ± 0.10[a] | 0.44 ± 0.05 | −0.07 ± 0.07[a] |
| | E− | 0.37 ± 0.09 | −0.16 ± 0.06[a] | 0.43 ± 0.07 | −0.08 ± 0.09[a] |

Values are mean ± S.E.M. E+ = endothelium present, E− = endothelium absent. GTN, glyceryl trinitrate; 1,2-GDN, 1,2-glyceryl dinitrate; 1,3-GDN, 1,3-glyceryl dinitrate. Tolerance was induced by transdermal glyceryl trinitrate application (15 mg/day for 2 days).
[a] Difference between control and tolerant aorta significant, $P<0.05$, unpaired *t*-test.
[b] Effect of endothelium removal significant, $P<0.05$, paired *t*-test.

cantly from those treated with L-NAME (0.15 ± 0.03 and 0.07 ± 0.01 nmol/g, respectively). The corresponding contents of 1,3-glyceryl dinitrate in all groups were in the 0.04–0.05 nmol/g range.

Residual glyceryl trinitrate content varied considerably, ranging from 0.9 to 3.6 nmol/g in the different artery groups. This compares with an estimated distribution in the extracellular compartment alone of about 0.6 nmol/g (from the data on rabbit aorta of Sweet and Levin, 1983). There was a tendency for endothelium removal to reduce the glyceryl trinitrate content, which reached significance in the control artery. This was not evident after L-NAME where contents in nmol/g in the absence and presence of L-NAME were in control arteries 0.87 ± 0.28 and 1.22 ± 0.34, respectively, and in tolerant arteries were 1.69 ±0.79 and 0.92 ± 0.07 ($n=4$), respectively. However, data on glyceryl trinitrate contents should be treated with caution, as these will include glyceryl trinitrate, which still adheres to the tissue after the 5 s wash period. The short wash period was used in order to minimise efflux of metabolites from the tissue.

### 3.2. Relaxation studies

#### 3.2.1. Effect of endothelium removal and L-NAME

In the vessels with intact endothelium and NOS, tolerance to glyceryl trinitrate's relaxant action was indicated by decreased potency (2–3 fold increase in $EC_{50}$) and efficacy (a 20% decrease in first phase maximum). (Fig. 1, Table 2). Note—the first phase maximum was within the concentration range 0.3–3.0 μM and effects of higher concentrations (De la Lande et al., 1999) were not tested in the present study; in the non-tolerant vessel, the first phase maximum was too close to 100% to be distinguished from the true maximum. Effects of endothelium removal were significant in the tolerant vessel only and comprised a reduction in $EC_{50}$, amounting to about doubling of potency and an increase in $E_{max}$ (efficacy) from 76% to 92%. The increase in $E_{max}$ meant that the shape of concentration–response curve was now similar to the curves in the non-tolerant vessels. When assessed by two-way ANOVA, the interaction between

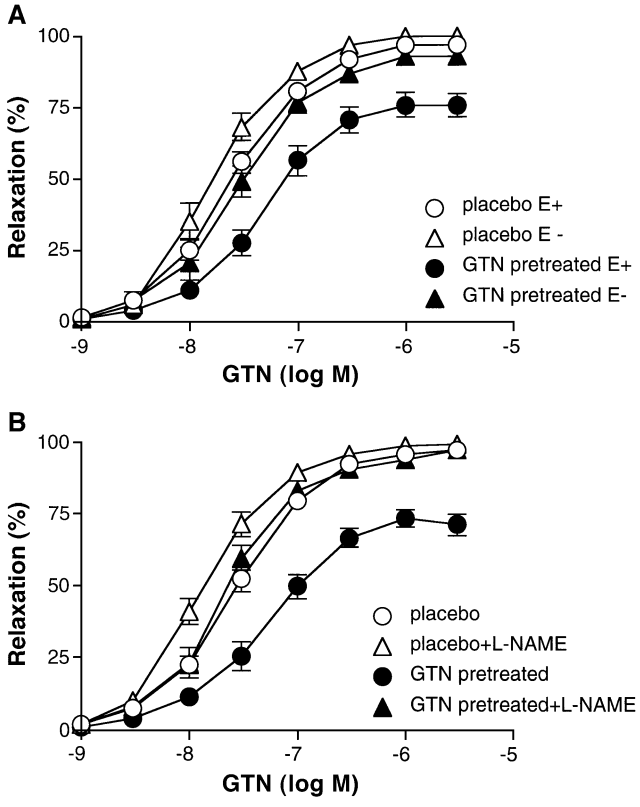


Fig. 1. The effect of endothelium removal (A, $n=14–15$) and the effect of NOS inhibition using L-NAME 300 μM (B, $n=14$) on relaxant responses to glyceryl trinitrate (GTN) in arteries from control and tolerant (GTN pretreated) rats. E+ = endothelium present and E− = endothelium absent.

Table 2
Effects of endothelium removal, NOS inhibition and tolerance induction on relaxant responses to glyceryl trinitrate

| | Endothelium removal | | | |
|---|---|---|---|---|
| | Endothelium present | | Endothelium absent | |
| | Control | Tolerant | Control | Tolerant |
| $EC_{50}$ (nM) | 23 (17–32) | 47 (33–66)[a] | 16 (11–22) | 25 (16–39)[b] |
| $E_{max}$ (%) | 97 ± 1 | 76 ± 4[a] | 98 ± 1 | 92 ± 2[a,b] |
| AUC[c] | 156 ± 5 | 105 ± 9[a] | 173 ± 7 | 145 ± 9[a,b] |
| | NOS inhibition | | | |
| | L-NAME absent | | L-NAME present | |
| | Control | Tolerant | Control | Tolerant |
| $EC_{50}$ (nM) | 26 (16–42) | 58 (43–77)[a] | 13 (9–18)[b] | 18 (12–25)[b] |
| $E_{max}$ (%) | 98 ± 1 | 75 ± 3[a] | 99 ± 1 | 93 ± 1[a,b] |
| AUC[c] | 156 ± 8 | 97 ± 7[a] | 185 ± 8[b] | 158 ± 7[a,b] |

Values are mean ± S.E.M or for $EC_{50}$ mean (95% confidence intervals).
[a] Effect of tolerance significant, $P<0.05$, unpaired $t$-test.
[b] Effect of endothelium removal/NOS inhibition significant, $P<0.05$, paired $t$-test.
[c] AUC, Area under log concentration–response curve to 1 μM glyceryl trinitrate, expressed in arbitrary units.

tolerance and endothelium integrity was not significant when based on $EC_{50}$ values ($P=0.52$) but was highly significant ($P<0.001$) when based on $E_{max}$ values.

L-NAME (300 μM) increased the potency of glyceryl trinitrate in the control vessel and both potency and efficacy in the tolerant artery (Table 2). The interaction between tolerance and L-NAME was marginally significant ($P=0.07$) when based on $EC_{50}$ values but was highly significant ($P<0.0001$) when based on $E_{max}$ values. The reduction in AUC (control/tolerant) was used as a crude measure of the magnitude of tolerance to take into account changes in both $EC_{50}$ and $E_{max}$. By this measure, tolerance in endothelium intact vessels (a 33% reduction) was about halved in the endothelium denuded vessels (a 16% reduction; from the data in Table 2). These reductions were similar to those in the absence and presence of L-NAME, namely 38% and 15%, respectively. The AUC values in Table 2 show that tolerance, after endothelium removal or L-NAME treatment, although reduced, was still significant ($P<0.01$).

### 3.2.2. Contractile responses to phenylephrine

Both tolerance and endothelium removal increased contractile responses to phenylephrine, in agreement with earlier findings (Molina et al., 1987; Munzel et al., 1995b; De la Lande et al., 1999). The present results show that L-NAME also increased contractile activity; the $EC_{50}$ 's for phenylephrine (in nM, $n=13–14$) being decreased from 144 (96, 216) to 50 (28, 89) in control arteries and from 80 (56, 113) to 32 (24, 43) in tolerant arteries. $E_{max}$ values were increased in control arteries, from 2.5 ± 0.1 to 3.2 ± 0.1 g ($P<0.01$, unpaired $t$-test), but in the tolerant arteries values with and without L-NAME did not differ significantly, namely 2.8 ± 0.1 and 2.7 ± 0.1 g, respectively.

## 4. Discussion

The pattern of metabolite contents in the control artery, comprising a predominance of 1,2- over 1,3-glyceryl dinitrate and selective reduction of the 1,2-glyceryl dinitrate content in the tolerant artery, agrees with previous results on rat aorta (Fung and Poliszczuk, 1986; Ratz et al., 2000b) and human saphenous vein (Sage et al., 2000). There is evidence (summarised by Bennett et al., 1994) that 1,2-glyceryl dinitrate is selectively formed in the artery during brief exposure to low concentrations of glyceryl trinitrate and has a rapid time course of generation consistent with a close link with vascular relaxation; a slow time course and low affinity characteristics argue against a similar link for 1,3-glyceryl dinitrate formation. These kinetic considerations have led to the use of the metabolite ratio (1,2-/1,3-glyceryl dinitrate) as a measure of the functionally relevant component of GTN metabolism. In the present study, the reduction in the 1,2-glyceryl dinitrate content following the transdermal glyceryl trinitrate pre-treatment was sufficient to reduce the metabolite ratio from 2.3 to 0.8. A similar result was obtained by Ratz et al. (2000b) in a rat model where tolerance was induced by sub-dermal glyceryl trinitrate but the level of tolerance (about a 6 fold rightward shift of the glyceryl trinitrate concentration–response curve) was greater than the 2–3 fold shift achieved in our model. Thus our data provide evidence that glyceryl trinitrate metabolism is depressed at the low level of ex vivo tolerance induced in the rat by the transdermal dosage regime.

Endothelium removal was without effect on metabolite contents or their ratio in the non-tolerant artery, in agreement with findings of McGuire et al. (1994). However, the present results show that it was also without effect in the tolerant artery, i.e. it did not modulate the depression of metabolism.

The failure to affect glyceryl trinitrate metabolism in either tolerant or non-tolerant vessels appears at variance with evidence that endothelial cells and smooth muscle cells metabolise glyceryl trinitrate to 1,2-glyceryl dinitrate at comparable rates (Bennett et al., 1989). The explanation may lie in the morphology of large conducting arteries like the aorta, where the population of endothelial cells is small compared with that of the smooth muscle cells, so that the contribution of the former is hard to detect. A potential for endothelium-derived NO to inhibit bioconversion of glyceryl trinitrate (Kojda et al., 1994, 1998) can be excluded as a factor since L-NAME, by eliminating this inhibition, would be expected to increase glyceryl trinitrate metabolism but there was no indication of this.

The level of contractile tone is an important determinant of the response to vasorelaxants and for this reason its effect on glyceryl trinitrate metabolism was examined. The effect

does not appear to have been examined previously, the closest being metabolic studies carried out under organ bath conditions (Brien et al., 1986) or in which metabolism was measured with contractile agent in the incubating medium (Bennett et al., 1994). However, the present experiments show that glyceryl trinitrate metabolite contents did not differ between vessels which were relaxed or which were contracted at the time of adding glyceryl trinitrate. The result implies that vascular tone does not affect the metabolism of glyceryl trinitrate.

Glyceryl trinitrate's relaxant action was measured to assess the functional significance of the metabolic data. The magnitude of tolerance to this action was comparable with that documented in an earlier study (De la Lande et al., 1999). However, in that study removal of endothelium, although potentiating the response to glyceryl trinitrate, did not reduce tolerance to a significant degree. A trend towards an increase in efficacy was more pronounced in the present study and resulted in significant attenuation of tolerance. This result agrees with that of Munzel et al. (2000) but is in apparent contrast with that of Ratz et al. (2000a), who observed no effect of endothelium removal on glyceryl trinitrate responses in tolerant arteries. Both studies and ours are in agreement that tolerance estimated in terms of changes in glyceryl trinitrate potency is not affected by endothelium removal. However, it appears that the endothelium has a varying potential to contribute to tolerance by an inhibitory affect on the first phase maximum of the glyceryl trinitrate response. Further analysis of the endothelium effect was confined to the role of eNOS. That the role was important was suggested by the similar effects of the eNOS inhibitor, L-NAME, to those of endothelium removal. The similarity extended to (i) increased efficacy in the tolerant vessel leading to a reduction in tolerance and (ii) the magnitudes of the reduction (about two-thirds) based on decreases in AUC (Table 2). The only difference was minor in that L-NAME increased the potency of glyceryl trinitrate in both non-tolerant and tolerant arteries, whereas a similar tendency on the part of endothelium removal was significant only in the tolerant vessel. The similarity also extended to the potentiation of contractile activity. Overall, the agreement is sufficiently striking to leave little doubt that loss of eNOS is primarily responsible for the effects of endothelium removal.

The mechanism of the contribution of endothelium (or eNOS) to tolerance has been studied only to the extent of showing that it is not accompanied by changes in glyceryl trinitrate metabolite contents and hence is unlikely to be mediated by a reduction in glyceryl trinitrate biotransformation within the smooth muscle. We cannot rule out the possibility that a reduction within the endothelium plays a role, particularly in view of the evidence that generation of superoxide by endothelium and by eNOS is increased in the tolerant artery (Munzel et al., 1995a, 2000). However, a major role for superoxide seems unlikely in view of the small magnitude of cross tolerance to NO derived from non-nitrate sources, relative to tolerance (Molina et al., 1987; De la Lande et al., 1999; Ratz et al., 2000a). Furthermore, the present evidence that eNOS activity contributes to tolerance is complicated by findings that (a) chronic loss of eNOS activity does not affect ex vivo tolerance in the mouse aorta (Wang et al., 2002) and (b) that, in a haemodynamic study in the rat, L-NAME potentiated glyceryl trinitrate's hypotensive action but this effect was abolished after tolerance induction (Ratz et al., 2002) (rather than enhanced as would be anticipated from the organ bath data). These anomalies draw attention to the possibility that the role of the endothelium in tolerance is influenced both by species and by vessel type, i.e. conduit in our study and resistance vessels in the haemodynamic study.

There are several limitations to this study. One is that for technical reasons we have measured vascular contents rather than rates of formation, of metabolites. However, errors due to metabolite efflux from the tissue have been minimised by using a short 2 min incubation period and a short 5 s wash period. Another is that 1,2-glyceryl dinitrate formation is only a surrogate measure of formation of NO (or congener) and, although unlikely, we cannot exclude the possibility that endothelium derived factors may influence the latter but not the former process. Unfortunately, there do not appear to be any convincing demonstrations that direct measurement of NO from glyceryl trinitrate is feasible in isolated large vessel segments except at high and unphysiological concentrations (Bennett et al., 1994). However, the possibility of a new approach, that of measuring the activity of the glyceryl trinitrate metabolising enzyme directly, is raised by a recent study identifying the enzyme as aldehyde dehydrogenase and showing that its activity is depressed in tolerance induced in vitro (Chen et al., 2002).

In summary, endothelium removal and inhibition of NOS have proved useful techniques for defining the role of glyceryl trinitrate metabolism in ex vivo tolerance. The results in the intact vessel show that impaired metabolism is a sensitive index of ex vivo tolerance while the effects of endothelium removal suggest that endothelium-derived factors which are independent of glyceryl trinitrate metabolism also contribute to tolerance. By providing a metabolic basis for distinguishing between endothelium-independent and -dependent tolerance, the results complement the earlier distinction of Munzel et al. (1995a) based on the association of the endothelium with superoxide-generation. Finally, it is pointed out the characteristics of ex vivo tolerance in rat aorta resemble those in human vessels (internal mammary artery and saphenous vein) where ex vivo tolerance produced by a 24-h infusion of glyceryl trinitrate is associated with depressed 1,2-glyceryl dinitrate formation and with absence of cross tolerance to non-nitrate NO donors (Sage et al., 2000). Whether there are both endothelium-dependent and -independent components to the tolerance in the human vessels is not known but it is an important question since its

resolution has the potential to provide new insights into the determinants of glyceryl trinitrate tolerance in the clinical setting.

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## References

Bennett, B.M., Leitman, D.C., Schroder, H., Kawamoto, J.H., Nakatsu, K., Murad, F., 1989. Relationship between biotransformation of glyceryl trinitrate and cyclic GMP accumulation in various cultured cell lines. J. Pharmacol. Exp. Ther. 250 (1), 316–323.

Bennett, B.M., McDonald, B.J., Nigam, R., Simon, W.C., 1994. Biotransformation of organic nitrates and vascular smooth muscle cell function. Trends Pharmacol. Sci. 15 (7), 245–249.

Brien, J.F., McLaughlin, B.E., Breedon, T.H., Bennett, 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.

Chen, Z., Zhang, J., Stamler, J.S., 2002. Identification of the enzymatic mechanism of nitroglycerin bioactivation. Proc. Natl. Acad. Sci. 99, 8306–8311.

De la Lande, I.S., Stafford, I., Horowitz, J.D., 1999. Tolerance induction by transdermal glyceryl trinitrate in rats. Eur. J. Pharmacol. 374, 71–75.

Feelisch, M., Kelm, M., 1991. Biotransformation of organic nitrates to nitric oxide by vascular smooth muscle and endothelial cells. Biochem. Biophys. Res. Commun. 180 (1), 286–293.

Fung, H.L., Poliszczuk, R., 1986. Nitrosothiol and nitrate tolerance. Z. Kardiol. 75 (Suppl 3), 25–27.

Henry, P.J., Horowitz, J.D., Louis, W.J., 1989. Nitroglycerin-induced tolerance affects multiple sites in the organic nitrate bioconversion cascade. J. Pharmacol. Exp. Ther. 248, 762–768.

Kojda, G., Beck, J.K., Meyer, W., Noack, E., 1994. Nitrovasodilator-induced relaxation and tolerance development in porcine vena cordis magna: dependence on intact endothelium. Br. J. Pharmacol. 112, 533–540.

Kojda, G., Patzner, M., Hacker, A., Noack, E., 1998. Nitric oxide inhibits vascular bioactivation of glyceryl trinitrate: a novel mechanism to explain preferential venodilation of organic nitrates. Mol. Pharmacol. 53, 547–554.

Malta, E., 1989. Biphasic relaxant curves to glyceryl trinitrate in rat aortic rings: evidence for two mechanisms of action. Arch. Pharm. 339, 236–243.

McGuire, J.J., Anderson, D.J., Bennett, B.M., 1994. Inhibition of the biotransformation and pharmacological actions of glyceryl trinitrate by the flavoprotein inhibitor, diphenyleneiodonium sulfate. J. Pharmacol. Exp. Ther. 271, 708–714.

Molina, C.R., Andresen, J.W., Rapoport, R.M., Waldman, S., Murad, F., 1987. Effect of in vivo nitroglycerin therapy on endothelium-dependent and independent vascular relaxation and cyclic GMP accumulation in rat aorta. J. Cardiovasc. Pharmacol. 10, 371–378.

Munzel, T., Sayegh, H., Freeman, B.A., Tarpey, M.M., Harrison, D.G., 1995a. Evidence for enhanced vascular superoxide anion production in nitrate tolerance. A novel mechanism underlying tolerance and cross-tolerance. J. Clin. Invest. 95 (1), 187–194.

Munzel, T., Giaid, A., Kurz, S., Stewart, D.J., Harrison, D.G., 1995b. Evidence for a role of endothelin 1 and protein kinase C in nitroglycerin tolerance. Proc. Natl. Acad. Sci. 92, 5244–5248.

Munzel, T., Li, H., Mollnau, H., Hink, U., Matheis, E., Hartmann, M., Oelze, M., Skatchkov, M., Warnholtz, A., Duncker, L., Meinertz, T., Forstermann, U., 2000. Effects of long-term nitroglycerin treatment on endothelial nitric oxide synthase (NOS III) gene expression, NOS III-mediated superoxide production, and vascular NO bioavailability. Circ. Res. 86 (1), E7–E12.

Ratz, J.D., McGuire, J.J., Anderson, D.J., Bennett, B.M., 2000a. Effects of the flavoprotein inhibitor, diphenyleneiodonium sulfate, on ex vivo organic nitrate tolerance in the rat. J. Pharmacol. Exp. Ther. 293 (2), 569–577.

Ratz, J.D., Fraser, A.B., Rees-Milton, K.J., Adams, M.A., Bennett, B.M., 2000b. Endothelin receptor antagonism does not prevent the development of in vivo glyceryl trinitrate tolerance in the rat. J. Pharmacol. Exp. Ther. 295 (2), 578–585.

Ratz, J.D., Adams, M.A., Bennett, B.M., 2002. Effect of in vivo nitrate tolerance on hypersensitivity to NO donors after NO-synthase blockade. Can. J. Physiol. Pharm. 80, 1106–1118.

Sage, P.R., de la Lande, I.S., Stafford, I., Bennett, C.L., Phillipov, G., Stubberfield, J., Horowitz, J.D., 2000. Nitroglycerin tolerance in human vessels. Evidence for impaired nitroglycerin bioconversion. Circulation 102, 2810–2815.

Slack, C.J., McLaughlin, B.E., Brien, J.F., Marks, G.S., Nakatsu, K., 1989. Biotransformation of glyceryl trinitrate and isosorbide dinitrate in vascular smooth muscle made tolerant to organic nitrates. Can. J. Physiol. Pharm. 67, 1381–1385.

Sweet, W.D., Levin, J.A., 1983. Two mechanisms of 3H-catecholamine accumulation in rabbit aorta media differentiated by sensitivity to temperature and corticosterone. Blood Vessels 20, 265–282.

Wang, E.Q., Lee, W.L., Fung, H.L., 2002. Lack of critical involvement of endothelial nitric oxide synthase in vascular nitrate tolerance in mice. Br. J. Pharmacol. 135, 299–302.