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European Journal of Pharmacology 374 (1999) 71–75

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Short communication

# Tolerance induction by transdermal glyceryl trinitrate in rats

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Received 28 January 1999; received in revised form 23 April 1999; accepted 27 April 1999

**Abstract**

Mechanisms of mild glyceryl trinitrate tolerance in rats (transdermal application; 15 mg/day/2 days) were examined in isolated aortic rings contracted with phenylephrine. Tolerance to glyceryl trinitrate was comparable in both endothelium-intact and -denuded vessels; the maximum relaxation decreased to 70–80% and the $EC_{50}$ increased 3–4-fold. There was minimal cross-tolerance to acetylcholine (1.7-fold increase in $EC_{50}$) and none to sodium nitroprusside. The results suggest that mild tolerance to glyceryl trinitrate in rats is mediated by mechanisms which are predominantly endothelium-independent and which produce little activation of the cellular mechanism responsible for cross-tolerance. 

*Keywords:* Aorta, rat; Tolerance induction; Vascular relaxation; Glyceryl trinitrate; Acetylcholine; Sodium nitroprusside

## 1. Introduction

Hypotheses of the mechanisms of tolerance to organic nitrates have centred around (i) impaired biotransformation to nitric oxide (Brien et al., 1986; Henry et al., 1989), (ii) desensitisation of one or more of the cellular processes initiated by nitric oxide e.g., activation of guanylyl cyclase (Waldman et al., 1986; Kukovetz and Holzmann, 1990) and (iii) increased generation of superoxide by vascular endothelium, resulting in chemical inactivation of the nitric oxide (Munzel et al., 1995). Mechanisms (i) and/or (ii) have been evoked for vascular tolerance induced in vitro, and mechanism (iii) for tolerance induced in vivo. An important part of the evidence has been the extent of cross-tolerance to nitric oxide generators which are not organic nitrates, the absence of cross-tolerance favouring mechanism (i) and the presence favouring mechanisms (ii) or (iii). In the present study, we have explored the extent of cross-tolerance and endothelium dependence of tolerance in isolated aorta from a rat model where a moderate degree of tolerance was induced by transdermal application of glyceryl trinitrate. Transdermal application was used previously in the studies on rabbits (Du et al., 1991; Munzel et al., 1995); the results of Munzel et al. (1995) formed part of the evidence for mechanism (iii) above. Transdermal application to rats does not appear to have been documented, other studies having used repeated bolus (Molina et al., 1987; Ferdinandy et al., 1995) or sustained parenteral administration (Laursen et al., 1996) to induce tolerance. In one of these (Molina et al., 1987), the aorta exhibited a high level of cross-tolerance to the endothelium-dependent nitric oxide generator, acetylcholine, but this was in association with a high level of tolerance.

## 2. Materials and methods

### 2.1. Materials

Acetylcholine chloride, L-phenylephrine hydrochloride and sodium nitroprusside were purchased from Sigma, St. Louis, MO, USA. Glyceryl trinitrate was purchased from Fisons, Australia. Stock solutions were made up in either ethanol (glyceryl trinitrate) or distilled water (acetylcholine, L-phenylephrine) and stored at −20°C. Sodium nitroprusside was made up fresh (in distilled water) on day of use and protected from light. Dilutions of the stock solutions were made fresh in distilled water and maintained on ice.

The Krebs solution was gassed with carbogen (95% $O_2$, 5% $CO_2$) and was of the following composition (mM): NaCl (118), KCl (3.89), $KH_2PO_4$ (1.18), $NaHCO_3$ (25),

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0014-2999/99/$ - see front matter 
PII: S0014-2999(99)00319-2

$MgCl_2$ (1.05), $CaCl_2$ (2.34), EDTA (0.01) and glucose (5.56), pH 7.4.

### 2.2. Methods

Male Sprague–Dawley rats (380–420 g) were lightly anaesthetised with nitrous oxide/halothane, hair on the dorsal surface removed with wax strips and one and a half glyceryl trinitrate patches (Minitran 10: 3M Pharmaceuticals, Thornleigh, NSW, Australia) were applied and covered with transparent dressing (Tegaderm). The theoretical delivery of glyceryl trinitrate was 15 mg/24 h. Placebo patches (Minitran) were applied under identical conditions to control rats. The patches were replaced after 24 h and at 48 h, the rats were sacrificed under halothane anaesthesia. The aorta was divided into 3 mm rings and mounted in organ baths (15 ml) under a tension of 2 g. In some experiments the endothelium was removed by means of dental floss wrapped around a capillary tube.

### 2.3. Experimental procedure

Baseline tension usually stabilised within 60 min of mounting, during which time there were four replacements of bathing solution. The segment was then contracted with phenylephrine added incrementally from 0.01 μM until the response was considered to be in the upper region of its concentration–response curve. The concentrations of phenylephrine commonly used were 1.0 μM (placebo patches), 0.3 μM (glyceryl trinitrate patches) and 0.1 μM for endothelium-denuded vessels. When the response to phenylephrine was steady, the vasorelaxant (one of glyceryl trinitrate, acetylcholine or sodium nitroprusside) was added cumulatively until relaxation was maximal. The concentration–response curves for these agents were commenced at 97 ± 2 min ($n = 39$) after sacrifice of rats. Two further procedures which were carried out, commencing approximately 30 min after the preceding bath washout and following recontraction with phenylephrine, comprised (a) measuring the relaxant effect of acetylcholine (0.03–1.0 μM) to assess endothelium function and (b) eliciting the maximum contractile response to phenylephrine (5 or 10 μM); in the majority of experiments, the phenylephrine was added cumulatively commencing with 0.01 μM. The steady state levels of phenylephrine contraction at which vasorelaxation of the various compounds was examined was estimated from (b). The values (expressed as percentage maxima) were comparable in control and tolerant preparations for each of the relaxants, namely for glyceryl trinitrate in vessels with intact endothelium (64 ± 2%; $n = 12$ vs. 69 ± 3%; $n = 14$), acetylcholine (68 ± 3%; $n = 9$ vs. 71 ± 3%; $n = 13$), sodium nitroprusside (67 ± 4%; $n = 7$ vs. 67 ± 3%; $n = 10$) and glyceryl trinitrate in endothelium-denuded vessels (78 ± 3%; $n = 9$ vs. 78 ± 3%; $n = 13$). These levels did not include vessels where responses were less than 55% or greater than 90% of maximal; these were excluded from the results.

This protocol was approved by the North Western Adelaide Health Service animal ethics committee and conforms with Australian National Health and Medical Research Council guidelines of animal usage for experimentation.

### 2.4. Data analysis

$E_{max}$ and $EC_{50}$ values were calculated from sigmoid concentration–response curves which were fitted using the non-linear regression program, Graphpad Prism 2.01. In tolerant preparations, the concentration–response curve for glyceryl trinitrate was biphasic in shape, and the $E_{max}$ and $EC_{50}$ values for this agent refer only to the first phase maximum (which occurred in the 0.3–1.0 μM range). Tolerance was quantitated in terms of (i) increase in $EC_{50}$ and (ii) decrease in percent maximum. To take into account the changes in both (i) and (ii), the tolerance was also quantitated in terms of (iii) decrease in area under log concentration–response curve (in arbitrary units) using GraphPad Prism 2.01. The upper limit of concentrations used in calculating the areas under the log concentration–response curves was 1.0 μM in all cases.

All results are expressed as either mean ± S.E.M. or for $EC_{50}$, mean with 95% confidence intervals in brackets. Significance of differences ($P < 0.05$) was determined by unpaired *t*-tests; *n* refers to the number of animals.

## 3. Results

### 3.1. Relaxant responses

Aorta with intact endothelium, from rats receiving glyceryl trinitrate patches (termed tolerant aorta), exhibited biphasic concentration–response curves to glyceryl trinitrate with a first phase maximum in the 0.3–1.0 μM range. The biphasic shape was not evident in aortas from rats receiving the placebo patches (termed control aorta) presumably because the vessels were fully relaxed within this concentration range. Tolerance to glyceryl trinitrate was indicated by a marked decrease in the first phase maximum, an approximately 3-fold increase in the $EC_{50}$ derived from this maximum and a decrease in the area under the log concentration–response curves (Fig. 1A, Table 1). Tolerance was also evident in the endothelium-denuded vessels where the tolerant aorta also exhibited a biphasic concentration–response curve and the changes in first phase maximum and $EC_{50}$ were comparable with those in the vessels with intact endothelium (Fig. 1B).

Cross-tolerance to acetylcholine was slight although significant (Fig. 1C), while cross-tolerance to sodium nitroprusside was insignificant (Fig. 1D, Table 1); these

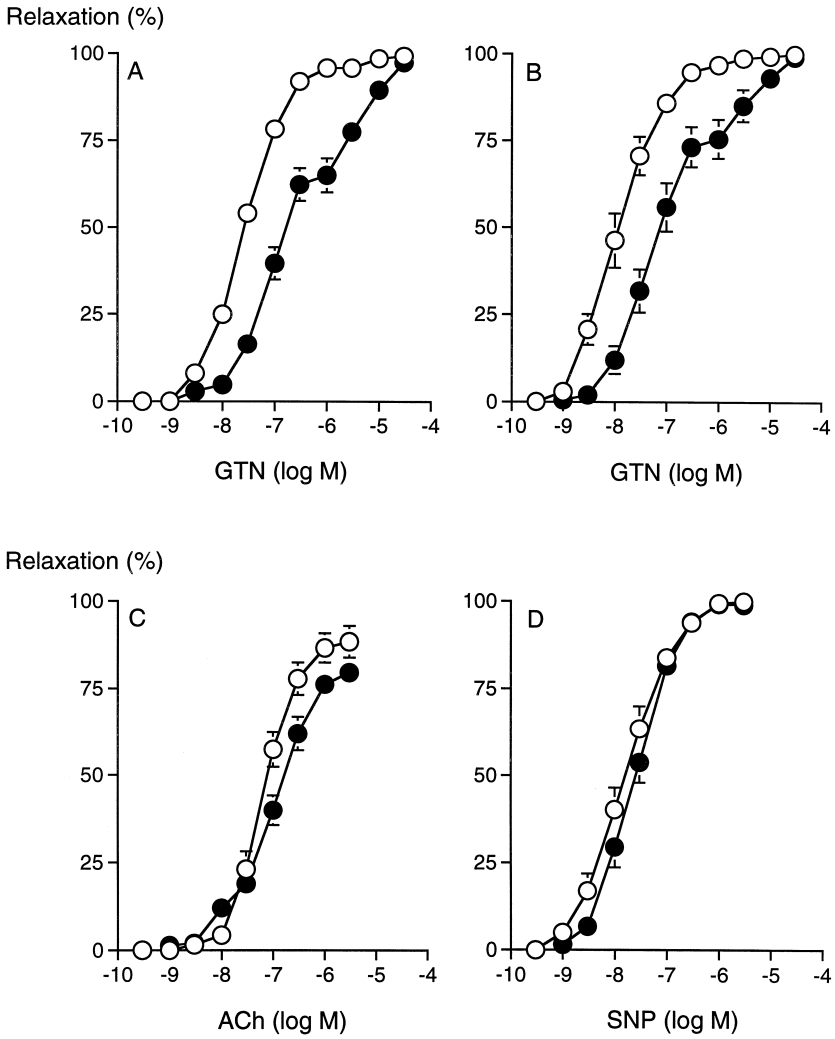


Fig. 1. Effect of transdermal glyceryl trinitrate (GTN) in arteries from control (○) and tolerant (●) rats, on relaxant responses to GTN (A, $n = 12–14$), GTN (B, $n = 9–13$, endothelium absent), acetylcholine (ACh; C, $n = 9–13$) and sodium nitroprusside (SNP; D, $n = 7–10$).

agents elicited sigmoidal concentration–response curves, both in the non-tolerant and tolerant vessels.

### 3.2. Contractile responses to phenylephrine

The aortae from tolerant rats were more responsive to phenylephrine, as indicated in tolerant ($n = 18$) and control ($n = 17$) rats by $EC_{50}$ values of 96 (77,121) nM and 279 (179,435) nM, respectively and maximum tensions of $2.36 \pm 0.1$ g and $1.97 \pm 0.1$ g, respectively. These differences were less evident and not significant in endothelium-denuded arteries, due primarily to a marked increase in sensitivity to phenylephrine in the control aorta;

Table 1
Effect of tolerance induction by transdermal glyceryl trinitrate

| Relaxant | $n$ | | $EC_{50}$ (nM) | | $E_{max}$ (%) | | AUC[a] | |
|---|---|---|---|---|---|---|---|---|
| | Control | Tolerant | Control | Tolerant | Control | Tolerant | Control | Tolerant |
| GTN | 12 | 14 | 26 (21,32) | 77 (64,94)[b] | $99 \pm 1$ | $70 \pm 5$[b] | $152 \pm 5$ | $79 \pm 7$[b] |
| GTN E − | 9 | 13 | 11 (6,19) | 42 (25,71)[b] | $97 \pm 1$ | $79 \pm 6$[b] | $184 \pm 11$ | $107 \pm 12$[b] |
| ACh | 9 | 13 | 63 (47,85) | 108 (75,155)[b] | $88 \pm 4$ | $81 \pm 3$ | $104 \pm 9$ | $77 \pm 6$[b] |
| SNP | 7 | 10 | 15 (7,30) | 24 (15,39) | $102 \pm 1$ | $101 \pm 1$ | $176 \pm 11$ | $154 \pm 9$ |

[a]AUC: area under log concentration–response curve to 1.0 μM relaxant (arbitrary units).
[b]Effect of transdermal GTN significant, $P < 0.05$, unpaired $t$-test.
Abbreviations: GTN, glyceryl trinitrate; SNP, sodium nitroprusside; ACh, acetylcholine; E − , endothelium absent.

$EC_{50} = 47$ (34,65) nM, $n = 9$. The $EC_{50}$ in the tolerant aorta was 34 (23,50) nM, $n = 15$.

## 4. Discussion

The results show that a 2-day patch application of glyceryl trinitrate to the rat, produces a measurable degree of tolerance in the isolated aorta and that the tolerance is endothelium-independent. The association of tolerance with a biphasic concentration–response curve accords with the appearance of this curve in other vessels under conditions where sensitivity is depressed (eg bovine coronary artery; De la Lande et al., 1999). This biphasic curve in rat aorta was first documented by Malta (1989). The present results also show that tolerance is associated with minimal and no cross-tolerance to two other nitric oxide generators (acetylcholine and sodium nitroprusside, respectively). An implication is that the cross-tolerance to acetylcholine, amounting to a reduction in $E_{max}$ of 40%, (and the lesser cross-tolerance to sodium nitroprusside; a 3-fold increase in $EC_{50}$) observed by Molina et al. (1987) in a comparable rat study, was related to the higher dosage of glyceryl trinitrate (approximately 50 mg/day over 4–7 days) and a resultant high level of tolerance (amounting to an increase in $EC_{50}$ of approximately 3 log units compared with 0.5 log units in our study). Other rat studies have employed sustained subcutaneous infusion for 3 days and in one of these (Laursen et al., 1996) there is the seeming discrepancy that tolerance in the aorta was abolished following endothelium removal. However a contributing factor may have been the use of potassium to precontract the aorta, since these conditions may have diminished the contribution of $K_{Ca}$ channel activation (Li et al., 1998) to nitric oxide induced vasodilatation. Consistent with our findings is the report of Ferdinandy et al. (1995) that the endothelium-denuded aorta from tolerant rats exhibited considerable tolerance to glyceryl trinitrate as indicated by an increase in the $EC_{50}$ of about 1.5 log units. In the latter study, the aorta was precontracted with an adrenoceptor agonist (noradrenaline) as was the case in our experiments (phenylephrine).

There are also anomalies with respect to comparable experiments in rabbits. When glyceryl trinitrate was applied transdermally for 3 days, Munzel et al. (1995) observed considerable cross-tolerance in the aorta to acetylcholine and to 3-morpholino-sydnonimine, as well as a large reduction in tolerance following endothelium removal. The results form part of the evidence for an important role of endothelium-generated superoxide (believed to arise from enhanced activity of the renin-angiotensin system) in the induction of tolerance to glyceryl trinitrate. Nevertheless, Du et al. (1991) using transdermal glyceryl trinitrate application for a shorter period (2 days, as in our study) observed that tolerance was not accompanied by cross-tolerance to endothelium-dependent (A23187) and -independent (sodium nitroprusside) nitric oxide generators. Whether the different results of the two studies reflected the slightly different conditions under which the arteries were examined, the different duration of glyceryl trinitrate exposure or the use of different nitric oxide generators, is not known. One of the difficulties in interpreting the differences is that none of the studies on rats or rabbits specify precisely the time which has elapsed after removing the artery and bathing it in the organ bath medium prior to establishing the in vitro sensitivity to the glyceryl trinitrate. Since tolerance is reversible, the possibility arises that factors responsible for cross-tolerance are washed out at different rates to those responsible for tolerance.

In our studies, as in the various studies on rabbits and rats outlined above, the tolerant vessels displayed increased sensitivity to the contractile agent. However a causal relationship to tolerance induction seems unlikely in view of the failure of the endothelium-denuded vessels to exhibit a similar increase despite the persistence of tolerance.

The results on rats alone emphasise the probability that cellular tolerance to glyceryl trinitrate is multifactorial in nature, a primary mechanism operating at an early stage of induction or at low levels of tolerance, and a secondary or contributory mechanism, responsible for cross-tolerance, becoming increasingly important as the duration or dosage of glyceryl trinitrate is increased. The minimal nature of cross tolerance in our study favours ‘impaired biotransformation’ as the primary mechanism, although our data does not exclude the possibility that desensitization and/or superoxide formation constitute secondary mechanisms. However, there still remains a major difficulty to the ‘impaired biotransformation’ hypothesis that in a study in the tolerant rat where nitric oxide was measured in vivo by spin trapping, there was no evidence of decreased formation of nitric oxide in response to glyceryl trinitrate (Laursen et al., 1996). Nevertheless, the present results show that the rat, in which tolerance is induced by a 2-day transdermal application, offers a useful model for future studies of the nature of the postulated primary mechanism. Furthermore, the characteristics of this model are consistent with comparable studies in human tissue. Ex vivo data of nitroglycerin tolerance at the platelet level in patients with angina pectoris indicated a similar degree of tolerance to that seen in our rat model and no cross-tolerance to sodium nitroprusside (Chirkov et al., 1997). Lack of cross-tolerance to sodium nitroprusside and acetylcholine was also seen in studies on human internal mammary arteries isolated from patients receiving glyceryl trinitrate prior to coronary artery bypass (Du et al., 1992). In vivo human data in forearm resistance vessels and dorsal hand veins of healthy subjects indicated lack of cross-tolerance to sodium nitroprusside or 3-morpholino-sydnonimine when nitrate tolerance was induced by 1 week of medium-

dose patch application (Sutsch et al., 1997) and was comparable with the tolerance seen in our experiments.

## Acknowledgements

This study was supported by a grant from the National Health and Medical Research Council of Australia.

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