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Aldehyde dehydrogenase, nitric oxide synthase and superoxide in ex vivo nitrate tolerance in rat aorta

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Abstract

The role of aldehyde dehydrogenase (ALDH) in ex vivo tolerance to transdermal glyceryl trinitrate was explored in rat aorta. ALDH activity, measured by aldehyde-induced NADH formation, was strongly depressed in the tolerant arteries. ALDH inhibitors, chloral hydrate (0.3 mM) and cyanamide (0.1–1 mM) inhibited relaxation to glyceryl trinitrate in non-tolerant and tolerant arteries. The inhibition differed from tolerance in that (a) the glyceryl trinitrate concentration–response curve was sigmoidal cf. biphasic in tolerance, (b) the potentiating effect of nitric oxide synthase (eNOS) inhibition was unchanged cf. increased in tolerance and (c) superoxide inhibited the response cf. no significant effect in tolerant or non-tolerant arteries. Hence, reduced ALDH activity does not account fully for ex vivo tolerance. The discrepancies are consistent with evidence that (a) organic nitrates, unlike chloral and cyanamide, irreversibly inactivate ALDH (hence reduced enzyme saturability can explain the biphasic curve) and (b) eNOS contributes to tolerance by a mechanism independent of glyceryl trinitrate metabolism.

Keywords: Glyceryl trinitrate; Tolerance, ex vivo; Aldehyde dehydrogenase; Superoxide; Nitric oxide synthase

1. Introduction

Recently aldehyde dehydrogenase (ALDH) has been identified as an enzyme mediating glyceryl trinitrate bioconversion in the artery wall. In the same study, it was shown that activity of the enzyme is depressed in rabbit aorta following nitrate tolerance induction by exposure to a high concentration of glyceryl trinitrate in vitro (Chen et al., 2002). We and others have shown that glyceryl trinitrate metabolism is diminished in isolated vessels when tolerance is induced in vivo (Fung and Poliszczuk, 1986; Bennett et al., 1994; Sage et al., 2000; de la Lande et al., 2004). Difabio et al. (2003) have confirmed the role of ALDH in glyceryl trinitrate metabolism in rat liver, but since ALDH inhibitors depressed glyceryl trinitrate relaxation about equally in tolerant and non-tolerant rat vessels, it was concluded that inhibition of the enzyme did not play a role in vascular tolerance. We have also examined whether inhibitors of ALDH induce changes identical to those occurring with nitrate tolerance induction. An answer was sought by examining (a) whether ALDH activity is depressed in the ex vivo tolerant rat aorta (as opposed to liver) and (b) whether effects of tolerance induction on the glyceryl trinitrate response are mimicked by the non-nitrate ester ALDH inhibitors, chloral hydrate and cyanamide. Tolerance is characterised by a biphasic glyceryl trinitrate concentration–response curve (de la Lande et al., 1999) and is attenuated when the endothelium is removed or endothelial nitric oxide synthase (eNOS) is inhibited (de la Lande et al., 2004). Hence, the present study includes analysis of the interaction between effects of eNOS and ALDH inhibition. Since there is evidence that the same active site on the ALDH enzyme complex mediates dehydrogenase activity and the esteratic activity responsible for metabolism of nitrate esters (Kitson, 1989; Mukerjee and Pietruszko, 1994), we have also examined whether the non-nitrate ALDH inhibitors affect the tolerant vessels in a fashion which is consistent with a common site of inhibition of the

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glyceryl trinitrate response. Finally, we have examined whether increased superoxide concentration, which has been postulated to play a major role in tolerance (Munzel et al., 1995a, 2000), influences effects of ALDH inhibitors.

2. Methods

Sprague–Dawley rats were exposed to transdermal glyceryl trinitrate (62 mg/kg/day) or to placebo patches (de la Lande et al., 1999). The patches were applied for 2 days. On the morning of the 3rd day, i.e., 24 h after the last application, rats were sacrificed under halothane anaesthesia and the thoracic aortae was 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 with Australian National Health and Medical Research Council guidelines of animal usage for experimentation.

2.1. ALDH assay

A modification of the method of Chen et al. (2002) for assay of ALDH activity in rabbit aorta was used. In brief, rat aorta was frozen in liquid nitrogen either immediately after its removal or after incubation in Krebs solution for 1 h. The frozen segment was ground in liquid nitrogen, homogenised and sonicated in N_2-deoxygenated 30 mM KPi buffer pH 7.5 (1: 5 w/v ground tissue weight/KPi ratio) and centrifuged at 20,000 × *g* at 4 °C for 20 min. The cell-free supernatant was incubated with 100 mM Tris–HCl (pH 8.5), 1 mM propionaldehyde, 1 mM 4-methylpyrazole, 2 mM NAD^+ and in some experiments, glyceryl trinitrate (0.01 and 0.1 μM). The formation of NADH at 340 nm during a 50 min period was used as the measure of ALDH activity. Two aortae were required per assay to provide sufficient supernatant for comparative analyses.

2.2. Organ bath studies

Glyceryl trinitrate relaxation in rat aorta rings was measured by the procedure described (de la Lande et al., 1999). In brief, rings were pre-contracted in Krebs solution with phenylephrine to 70–80% of maximum and glyceryl trinitrate was applied cumulatively (0.001–30 μM). In some experiments, glyceryl trinitrate was replaced by sodium nitroprusside (0.0001–1.0 μM) or acetylcholine (0.01–3.0 μM). Where the effects of drugs which modified relaxation were examined (*N*-ω-nitro-L-arginine, methyl ester (L-NAME); chloral hydrate; cyanamide), these were added 30 min prior to the relaxant agent.

2.3. Superoxide

Superoxide release in artery segments was measured by lucigenin (10 μM) luminescence after stimulation by diethyl dithiocarbamate (DETCA) plus NADPH. In the absence of either agent, luminescence was too low for reliable measurement but the combination of DETCA plus NADPH increased the luminescence at least 70-fold to easily measurable levels. The NADPH (100 μM) was present for 90 min whereas DETCA was present for the 30- to 60-min interval only. During the last 30 min, the Krebs was replaced by HEPES solution. Luminescence was measured in 0.3 ml of HEPES buffer containing 10 μM lucigenin, in a Wallac microbeta counter. That the luminescence (890 ± 170 luminescence units mg^{-1} s^{-1}, averaged over 12 min) was due mainly to superoxide was indicated by an 80 ± 3% reduction when the SOD mimetic, tiron 10 mM, was present ($n = 3$).

When the effect of superoxide on vasorelaxation was studied, the same procedure was used to generate the superoxide except that the vessels were in Krebs solution throughout. Relaxation responses to glyceryl trinitrate were measured, following pre-contraction with phenylephrine to 70–80% maximum, as in Section 2.2 above. The NADPH plus DETCA combination did not affect phenylephrine responses.

2.4. Measurement of tolerance to glyceryl trinitrate

Responses to glyceryl trinitrate were examined in tolerant and non-tolerant arteries. In the non-tolerant arteries, the concentration–response curves were sigmoidal in shape, enabling the responses to be quantified in terms of EC_{50} and E_{max} derived from curves fitted by non-linear regression (Prism 3 GraphPad). In tolerant arteries, the concentration–response curves were usually biphasic in shape with the first phase possessing a maximum of about 70% in the 0.3–1.0 μM range and the second phase achieving a maximum of about 100% at concentrations of 30 μM or greater (de la Lande et al., 1999). Previously, we had estimated an "EC_{50}" derived from the first phase maximum but sometimes this maximum was not clearly defined. For this reason, in the present study, when measuring effects in tolerant arteries, or comparison between non-tolerant and tolerant artery, we have preferred estimates of relaxant activity based on the uncorrected rather than curve-fitted data. The estimates refer to the concentration of glyceryl trinitrate producing a 50% reduction in the response to the contractile agent.

2.5. Data analysis

Results are expressed as mean ± S.E.M. with significance assessed by unpaired or paired *t*-test. Where there were two variables, e.g., L-NAME and cyanamide, the interaction was assessed by two-way analysis of variance (ANOVA) with repeated measures (Statistica Version, Statsoft). The Duncan's Multiple Range test was used for post hoc analysis. Probability levels less than 0.05 were considered significant.

2.6. Materials

Acetylcholine chloride, chloral hydrate, cyanamide, cysteine, diethyl dithiocarbamic acid sodium salt, 4,5-dihydroxy-1,3-benzene-disulfonic acid (tiron), bis-*N*-methyl acridinium nitrate (lucigenin), HEPES sodium salt, 4-methyl pyrazole hydrochloride, *N*-ω-nitro-L-arginine, methyl ester hydrochloride, L-phenylephrine hydrochloride, propionaldehyde and sodium nitroprusside were purchased from Sigma (St. Louis, MO, USA). Glyceryl trinitrate was purchased from David Bull Laboratories (Mulgrave, Vic, Australia) and NAD, NADPH were purchased from Roche Diagnostics (Mannheim, Germany). The Krebs 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), $MgCl_2$ (1.05), $CaCl_2$ (2.34), EDTA (0.01) and glucose (5.56), pH 7.4. Krebs–HEPES was gassed with carbogen (95% O_2, 5% CO_2) and was of the following composition NaCl (99), KCl (4.69), K_2HPO_4 (1.03), $NaHCO_3$ (25), $MgCl_2$ (1.20), $CaCl_2$ (1.87), Na–HEPES (20) and glucose (11.1), pH 7.4.

3. Results

3.1. ALDH activity

Mean ALDH activity in aorta which was frozen immediately after excision was 23 ± 3 nmol NADH mg protein^{-1} min^{-1}, $n = 5$. The activity was reduced by glyceryl trinitrate 0.01 and 0.1 μM in the assay medium by $62 \pm 7\%$ and $82 \pm 7\%$, respectively, ($n = 3$) (from 24 ± 0.6 to 9.3 ± 1.5 and 3.6 ± 1.6 NADH mg protein^{-1} min^{-1}). The activity was dramatically reduced in tolerant arteries, irrespective of whether they were immediately frozen or were incubated for 1 h in Krebs solution prior to freezing (Table 1). The latter conditions mimicked those of glyceryl trinitrate assay in a preceding study (de la Lande et al., 2004), enabling assay data from that study to provide an approximate estimate of residual glyceryl trinitrate retained in the tolerant artery. This was done by subtracting noise values for non-tolerant vessels from the values for the tolerant vessels (since only the latter would contain glyceryl trinitrate). The difference amounted to 0.02 nmol/g tissue ($n = 18–20$).

Table 1
ALDH activity in non-tolerant and tolerant rat aorta

		ALDH activity[a] (nmol NADH mg protein^{-1} min^{-1})	
Tissue treatment[b]	n	Non-tolerant	Tolerant
Frozen at 0 min	5	23 ± 2.9	4.6 ± 0.6[c]
Frozen at 60 min after 1 h at 37 °C	5	18 ± 0.6	0.8 ± 0.5[c]

[a] ALDH activity values are mean ± S.E.M.
[b] Rat aorta was either frozen in liquid nitrogen immediately following excision or incubated in Krebs at 37 °C for 1 h prior to freezing.
[c] Difference between non-tolerant and tolerant aorta significant, $P < 0.05$, unpaired *t*-test.

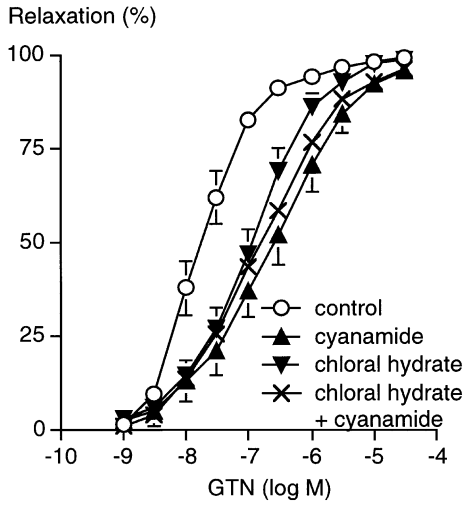

Fig. 1. The effect of pre-incubation with the ALDH inhibitors, chloral hydrate alone (0.3 mM), cyanamide alone (0.1 mM) or a combination of chloral hydrate (0.3 mM) and cyanamide (0.1 mM) (error bars omitted for clarity), on relaxant responses to glyceryl trinitrate (GTN) in control and ALDH inhibitor-treated arteries from non-tolerant rats ($n = 8$).

3.2. Organ bath studies

3.2.1. Effects of ALDH inhibition in non-tolerant arteries

Chloral hydrate (0.3 mM) and cyanamide (0.1 mM) inhibited the relaxant effect of glyceryl trinitrate (Fig. 1); the EC_{50} 's being shifted to the right approximately 7- and 14-fold, respectively. The difference in shifts was significant ($P < 0.05$; paired *t*-test, $n = 8$). Maximum relaxations (100%) were unchanged. The EC_{50} (log M) for the combination of the two agents (-6.83 ± 0.21) was intermediate between those of chloral (-6.93 ± 0.14) and cyanamide (-6.60 ± 0.21). Hence, there was no incremental EC_{50} increase in the EC_{50} for cyanamide when the two agents were combined (Fig. 1), consistent with a common site of action. The specificity of the interaction with glyceryl trinitrate was indicated by lack of effect of the inhibitors on relaxant responses to sodium nitroprusside (Fig. 2A, B). The only effect on acetylcholine-induced relaxation was a modest inhibition by cyanamide 1.0 mM, with a 0.32 ± 0.12 log unit shift in EC_{50} ($P = 0.042$, paired *t*-test $n = 6$; Fig. 2D). Neither chloral hydrate (0.3 mM) nor cyanamide (0.1–1.0 mM) affected the concentration–contractile response curve to phenylephrine when added 30 min prior to the phenylephrine. Nevertheless, both had a mild relaxant effect when added during the steady-state contraction to phenylephrine, as indicated by reductions in contraction by chloral hydrate of $26 \pm 6\%$ ($n = 3$) and by cyanamide of $27 \pm 7\%$ ($n = 5$).

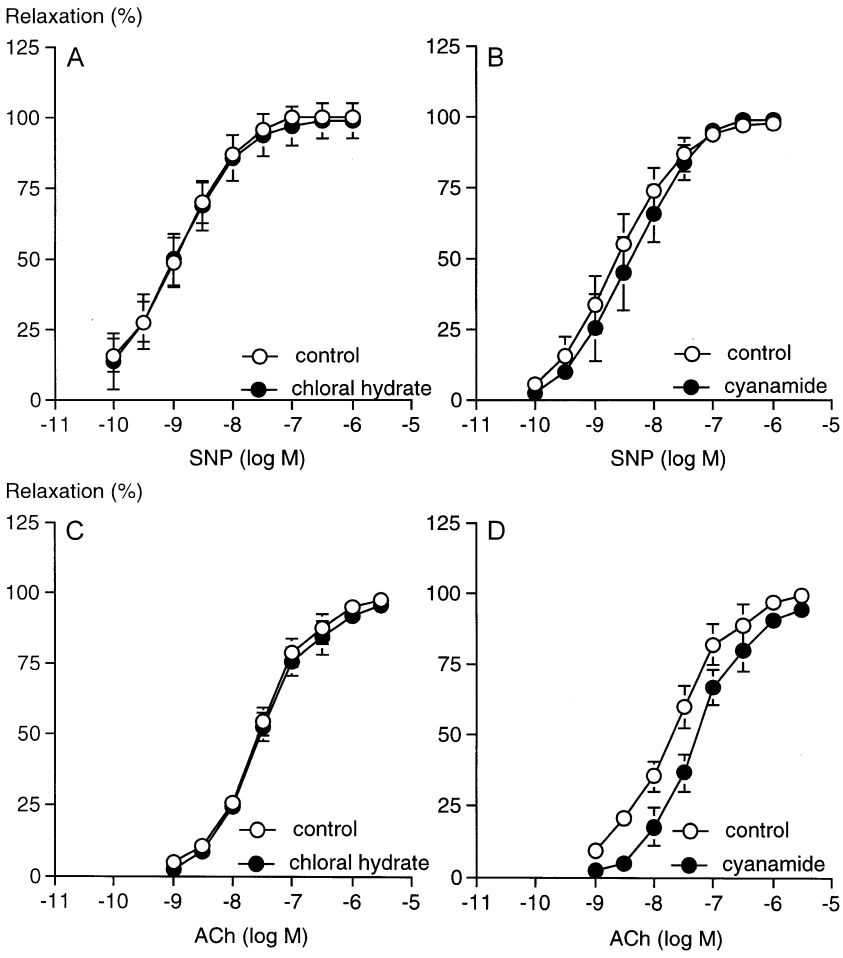

Fig. 2. The effect of pre-incubation with the ALDH inhibitors, chloral hydrate (0.3 mM) (A, $n=4$) or cyanamide (1.0 mM) (B, $n=4$) on relaxant responses to sodium nitroprusside (SNP) and the effect of chloral hydrate (0.3 mM) (C, $n=8$) or cyanamide (1.0 mM) (D, $n=6$) on relaxant responses to acetylcholine (ACh), in control and ALDH inhibitor-treated arteries from non-tolerant rats.

3.2.2. ALDH inhibition in tolerant arteries

In agreement with earlier findings (de la Lande et al., 1999), induction of tolerance was characterised by the appearance of a biphasic concentration–response curve to glyceryl trinitrate. The shape of the curve reflected a tendency to plateau at about 70% relaxation in the region of 1.0 μM; the plateau is termed as first phase maximum. Both chloral hydrate and cyanamide caused marked inhibition of glyceryl trinitrate responsiveness in the range of glyceryl trinitrate concentrations between threshold (approximately 0.01 μM) and first phase maximum (1.0–3.0 μM). In this range, the inhibitions by both agents were comparable with those seen in the non-tolerant artery (Fig. 3A, B). Thus, the concentration (in log M) of glyceryl trinitrate which reduced the maximum contractile action of phenylephrine by 50% was increased by chloral from -8.0 ± 0.07 to -7.2 ± 0.09 ($P<0.05$, $n=10$) in the non-tolerant artery and from -7.33 ± 0.13 to -6.36 ± 0.25 ($P<0.05$, $n=10$) in the tolerant artery. Cyanamide's inhibitory effect also was comparable in non-tolerant and tolerant arteries, irrespective of whether 0.1 mM cyanamide or 1.0 mM cyanamide was used. The effect of cyanamide on glyceryl trinitrate relaxation responses in the non-tolerant and tolerant artery at a concentration of 0.1 mM is shown in Fig. 3B. At 1.0 mM cyanamide, the concentration (in log M) of glyceryl trinitrate which reduced the maximum contractile action of phenylephrine by 50% was increased by 1.0 mM cyanamide from -7.84 ± 0.17 to -6.22 ± 0.17 ($P<0.05$, $n=5$) in the non-tolerant artery and from -7.30 ± 0.14 to -5.67 ± 0.19 ($P<0.05$, $n=9$) in the tolerant artery. These results suggest that tolerance induction and the ALDH inhibitors exert additive inhibitory effects on glyceryl trinitrate responses with the possible exception of responses at the lower and upper extremes of the glyceryl trinitrate curve. Here, the effects of both ALDH inhibitors appeared to be considerably diminished suggesting less-than additive interactions with tolerance.

3.2.3. L-NAME interactions

L-NAME (300 μM) increased the potency of glyceryl trinitrate 2- to 4-fold in control vessels, in accord with previous observations (de la Lande et al., 2004). When assessed by two-way ANOVA, the interaction between L-NAME and either chloral hydrate or cyanamide was not

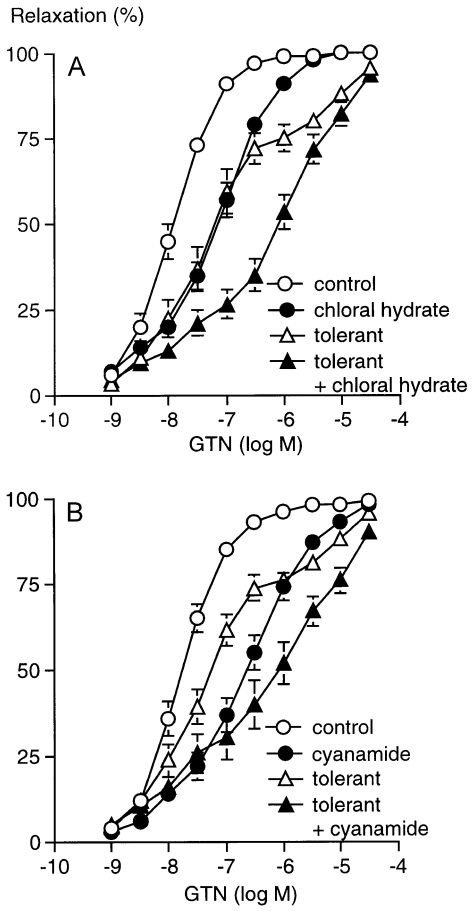

Fig. 3. The effect of ALDH inhibitor pre-incubation on relaxant responses to glyceryl trinitrate (GTN) in arteries from non-tolerant and tolerant rats. A represents the effect of chloral hydrate (0.3 mM) in $n=10$ arteries from non-tolerant and $n=9$ arteries from tolerant rats. B represents the effect of cyanamide (0.1 mM) in $n=14$ arteries from non-tolerant and $n=10$ arteries from tolerant rats.

significant ($P>0.05$), indicating that L-NAME's potentiating effect was unchanged in the presence of ALDH inhibitors (Fig. 4A, B).

3.3. Superoxide

We were unable to demonstrate differences between control and tolerant arteries in 10 μM lucigenin-induced luminescence which had been stimulated with DETCA-NADPH treatment and measured over the first 12 min (control = 722 ± 143; tolerant = 685 ± 132 luminescence units mg^{-1} s^{-1}, $n=6$, $P>0.05$, unpaired t-test). The treatment was without significant effect on the glyceryl trinitrate response (Fig. 5A, B). However, it strongly inhibited the acetylcholine response (Fig. 6), the EC_{50} (log M) being reduced from -7.73 ± 0.08 to -7.13 ± 0.11 while the E_{max} was depressed from 99 ± 2% to 61 ± 6%. These differences were highly significant ($P<0.01$, $n=5$). There was significant further depression of the glyceryl trinitrate responses when these were already depressed by ALDH inhibitors (Fig. 5A, B), but a tendency for DETCA-NADPH treatment to depress the glyceryl trinitrate response in the tolerant artery (Fig. 7) proved to be not significant when the data were analysed by two-way ANOVA using concentrations reducing phenylephrine responses by 50% ($P=0.27$). Tiron did not affect the glyceryl trinitrate response in the tolerant artery (Fig. 7).

4. Discussion

The inhibition of ALDH activity by glyceryl trinitrate was of the order of 60–80% between 0.01 and 0.1 μM and hence was well within the range of glyceryl trinitrate's vasorelaxant potency. Similarly, the marked reduction in activity in the tolerant artery is consistent with evidence that ex vivo tolerance is associated with depressed glyceryl trinitrate metabolism. Previously, Difabio et al. (2003) showed that ALDH activity is inhibited in nitrate-tolerant

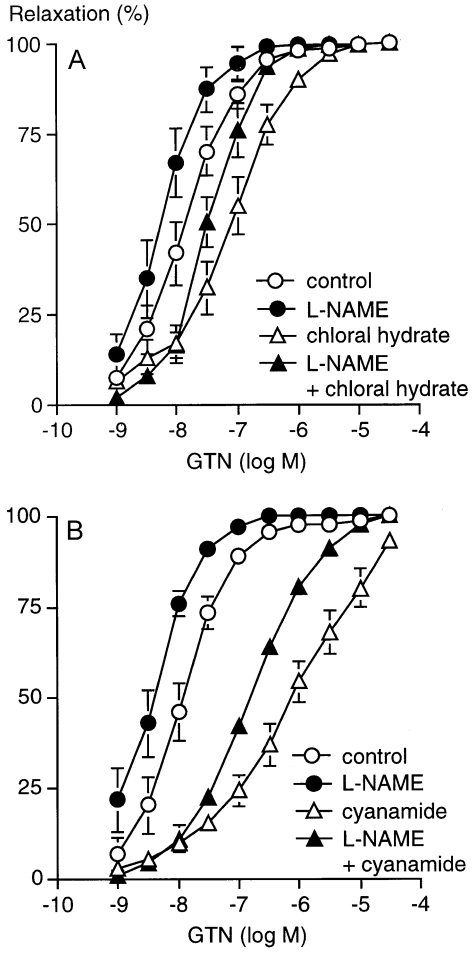

Fig. 4. The effect of L-NAME (300 μM) on relaxant responses to glyceryl trinitrate (GTN) in control and ALDH inhibitor pre-incubated arteries from non-tolerant rats. A represents the effect of L-NAME on control and chloral hydrate (0.3 mM) pre-treated arteries ($n=6$). B represents the effect of L-NAME on control and cyanamide (1.0 mM) pre-treated arteries ($n=6$).

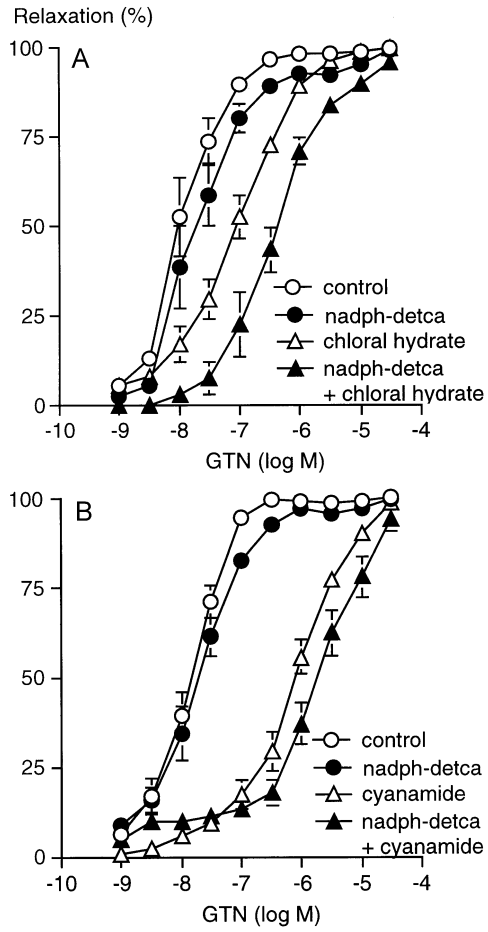

Fig. 5. The effect of NADPH (100 μM) plus DETCA (10 mM) on relaxant responses to glyceryl trinitrate (GTN) in control and ALDH inhibitor pre-incubated arteries from non-tolerant rats. A represents the effect of NADPH plus DETCA on control and chloral hydrate (0.3 mM) pre-treated arteries ($n=6$). B represents the effect of NADPH plus DETCA on control and cyanamide (1.0 mM) pre-treated arteries ($n=4$). Effects of NADPH-DETCA are not significant in the control arteries but significant in the chloral hydrate and cyanamide-treated arteries ($P<0.05$, paired t-tests).

rat liver mitochondria. However, the present finding is more directly relevant to vascular tolerance since it shows that the inhibition extends to the enzyme in the blood vessel wall.

The above result raises several questions. One is whether loss of ALDH activity is due to the presence of residual glyceryl trinitrate. That this is possible is suggested by our crude estimate that the residue could be as high as 0.02 nmol/g tissue, i.e., a concentration reducing ALDH activity by 60–80%. However, the magnitude of the loss in activity (to almost unmeasurable levels) suggests that there is in addition true loss of enzyme (discussed later) and at the same time implies that the activity is an extremely sensitive index of tolerance. Another question is that the extent of the ALDH reduction seems disproportionately large compared with the modest degree of vascular tolerance induced by the transdermal procedure. It is conceivable that the relationship between the two includes a large reserve of ALDH to be depleted before glyceryl trinitrate metabolism is depressed.

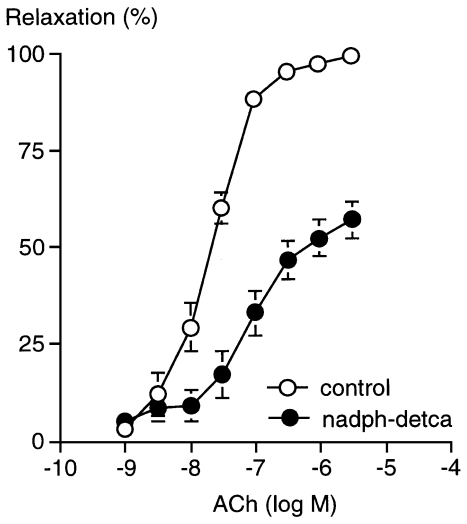

Fig. 6. The inhibitory effect of NADPH (100 μM) plus DETCA (10 mM) on relaxant responses to acetylcholine (ACh) ($n=5$) in non-tolerant rat aorta.

At the residual low ALDH levels, glyceryl trinitrate may then be metabolised by less efficacious mechanisms such as glutathione-*S*-transferase (Nigam et al., 1996; Lee and Fung, 2003) and cytochrome P450 (McDonald and Bennett, 1993). However, the influence of ALDH inhibition on their activities is not known.

The pharmacological studies in the non-tolerant arteries revealed inhibitory effects of chloral hydrate and cyanamide which were highly specific for organic nitrate-induced relaxation in rat aorta, (as indicated by minimal effect on relaxant responses to sodium nitroprusside and acetylcholine) in agreement with the results of Chen et al. (2002) in rabbit aorta and Difabio et al. (2003) in rat aorta. A qualification is that in rat aorta, cyanamide was considerably

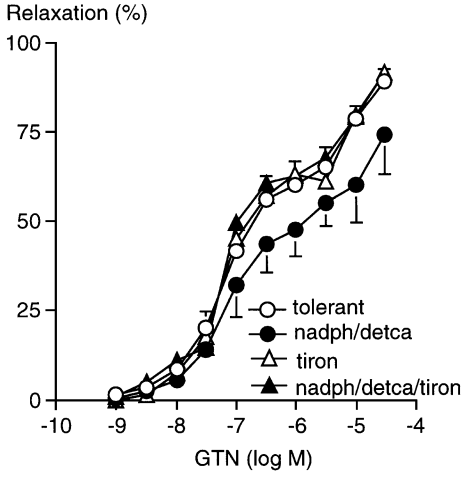

Fig. 7. The effect of NADPH (100 μM) plus DETCA (10 mM), with and without tiron (10 mM), on relaxant responses to glyceryl trinitrate in tolerant artery ($n=8$). For ease of presentation, the error bars are not shown for the tiron-treated vessels.

more potent, presumably due to greater availability of catalase which is required for conversion of cyanamide to its bioactive intermediate, (believed to be nitroxyl ion, DeMaster et al., 1985, 1998). Importantly, the inhibitory effects of cyanamide and chloral hydrate were non-additive, which accords with evidence of a common site of action, namely reactive cysteines at the catalytic site of the enzyme (DeMaster et al., 1998). In accord with the findings of Shahidullah et al. (2002), cyanamide displayed modest relaxant activity when added during the steady-state contractile response to phenylephrine and a similar effect was observed with chloral hydrate in the present study. It is unlikely that these relaxant effects influenced inhibition of the glyceryl trinitrate response to an important degree since neither agent influenced the response to the contractile agent when added 30 min prior to the latter, which was the procedure routinely used for assessing their effects on relaxant responses to glyceryl trinitrate. These findings emphasise the highly specific nature of the interactions between ALDH and glyceryl trinitrate and hence reinforce the evidence of Chen et al. (2002) that ALDH plays an important role in the biotransformation and relaxant effect of glyceryl trinitrate.

Since the metabolism of glyceryl trinitrate is depressed in ex vivo tolerance (see Introduction), we expected to find that the effects of ALDH inhibition in the non-tolerant vessel mimicked those of ex vivo tolerance. However, the ALDH-inhibited vessel did not exhibit two characteristics of tolerance, namely, the biphasic concentration–response curve to glyceryl trinitrate (de la Lande et al., 1999; also Fig. 3 in the present study), nor was the degree of inhibition of the glyceryl trinitrate response reduced in the presence of L-NAME (de la Lande et al., 2004). It should be noted that the biphasic curve is not confined to the tolerant artery, the first detailed description (by Malta, 1989) being in non-tolerant endothelium-denuded rat aorta. In contrast, there is no evidence of this curve in most studies on non-tolerant rat aorta (including the present) and it may be important that the vessels in these studies are generally more sensitive to glyceryl trinitrate than those in Malta's study. However, the failure of the ALDH inhibitors to elicit a biphasic curve is an indication that the shape of the curve is not simply a consequence of depressed glyceryl trinitrate sensitivity. Malta interpreted the biphasic curve in terms of two pathways of glyceryl trinitrate biotransformation. One had a high affinity for glyceryl trinitrate and was saturated at a submaximal concentration of about 1.0 μM, accounting for the appearance of the first phase maximum in the concentration–response curve. The other was considered to possess low affinity for glyceryl trinitrate and high saturability to account for responses to concentrations above the first phase maximum. When he induced tolerance in vitro, only the high affinity pathway was depressed. His interpretation is consistent with metabolic evidence of high and low affinity pathways mediating bioconversion to 1,2- and 1,3-glyceryl dinitrate, respectively, with only the high affinity one being depressed in tolerance both in vitro and ex vivo (Bennett et al., 1994). It follows from these considerations that the differing shapes of the glyceryl trinitrate concentration–response curves in tolerant and ALDH-inhibited arteries can be explained in terms of a reduced saturability of high-affinity biotransformation in the tolerant but not in the ALDH-inhibited artery.

The additive nature of the interaction between ALDH inhibition and tolerance was also examined in the expectation a common mechanism should result in combined inhibition which was less than additive (as seen with the interaction between chloral hydrate and cyanamide). This expectation was fulfilled only insofar as inhibition by chloral hydrate was insignificant at glyceryl trinitrate concentrations close to threshold ($\leq$10 nM) and above the first phase maximum. If lack of significance at the threshold level is discounted in view of the variable nature of threshold responses, it would appear that only the low-affinity glyceryl trinitrate response is inhibited by a mechanism common to tolerance and the ALDH inhibitors. In the physiologically important range encompassing the high affinity response (0.03–1.0 μM), the additive nature of the inhibitions implies that the mechanisms of inhibition differ. This may be because the sites of inhibition differ, as assumed by Difabio et al. (2003), but it could also reflect different kinetics of inhibition at the same catalytic site. The latter possibility arises because there is evidence that bioconversion of nitrate esters involves inactivation of ALDH (termed mechanism-based) which is irreversible and that chloral hydrate, a reversible inhibitor, is able to prevent this process (and hence glyceryl trinitrate relaxation) by competing with the ester for the active site on the enzyme (Mukerjee and Pietruszko, 1994; Pietruszko et al., 1995). If tolerance is attributed to the irreversible loss of enzyme, it follows that chloral hydrate added after tolerance is established will not be able to restore enzyme activity but will still prevent further loss if glyceryl trinitrate is added. Hence, in theory, chloral hydrate will continue to exert its inhibitory effect on the relaxant response to glyceryl trinitrate in the tolerant artery, explaining the additive nature of the interaction with tolerance. Irreversible loss of enzyme would also account for the reduced saturability of high affinity bioconversion in the tolerant artery. Presumably, this is not evident when loss of enzyme activity is surmountable as in the chloral hydrate-inhibited artery.

One aspect of a recent report by Sydow et al. (2004) is in apparent conflict with one of our findings. Namely they find that chloral hydrate is no longer inhibitory to the response of glyceryl trinitrate in the tolerant artery. However, a difference, apart from the strain of rat used is that the method of chronic glyceryl trinitrate infusion used to produce ex vivo tolerance in their study, resulted in a 43-fold increase in the EC_{50} without a change in the E_{max}. This level of tolerance is about 10 times greater than in our study and in that of Difabio et al. (2003). Surprisingly, in an earlier paper from

the same group (Munzel et al., 2000), the ex vivo tolerance produced by apparently the same chronic administration regimen resulted in a small increase in EC_{50} (about 3-fold) and a reduction in E_{max} which is not evident in their present communication. Our level of tolerance agrees with that obtained by Difabio et al. (2003), who also showed that the inhibitory actions of the ALDH inhibitors (cyanamide and propionaldehyde) were unchanged in the tolerant artery (i.e., the same degree of shift was seen in both non-tolerant and tolerant artery preparations). We suggest therefore that the differing effects of ALDH inhibitors are a consequence of the different biochemical effects of severe tolerance (in the study of Sydow et al., 2004) versus mild to moderate tolerance in our study and that of Difabio et al. (2003).

As discussed already, it is possible that the metabolism responsible for glyceryl trinitrate relaxation in the tolerant artery differs from the ALDH-mediated relaxation in the non-tolerant artery. It is conceivable that this is the low affinity component of metabolism where the effect of glyceryl trinitrate is reversible based on the tendency for the ALDH inhibitors to display less than additive interaction with tolerance. This explanation has focussed on chloral hydrate rather than cyanamide since the former is a reversible inhibitor of ALDH whereas the latter, apart from being indirect, is a mixed inhibitor of the purified enzyme, being reversible above pH 8.5 but becoming irreversible below pH 7. 5 (DeMaster et al., 1998). Hence, the interaction between tolerance and cyanamide in the intact tissue is likely to be more complex if both mechanisms can operate close to the physiological pH range.

While it is possible to explain additive inhibition in the above way, the possibility that the sites of inhibition are different still needs to be considered since ALDH activity is located mainly in smooth muscle (Chen et al., 2002) whereas there is evidence that the endothelium contributes to tolerance (Munzel et al., 1995a,b, 2000) via its eNOS activity (de la Lande et al., 2004). The smooth muscle location is consistent with the present finding that eNOS inhibition although potentiating the response to glyceryl trinitrate does not affect the inhibition of the glyceryl trinitrate response by chloral hydrate or cyanamide. It is also consistent with our earlier evidence that attenuation of tolerance by endothelium removal or by eNOS inhibition is independent of glyceryl trinitrate metabolism (de la Lande et al., 2004). It appears from these considerations that the endothelium qualifies as a site of inhibition of the glyceryl trinitrate response, which need not involve ALDH.

Since there is evidence to suggest that superoxide derived from endothelium contributes to tolerance (Munzel et al., 1995a, 2000), we sought to compare effects of superoxide (generated by DETCA plus NADPH), with those of the ALDH inhibitors. However, this approach was vitiated by the minimal effect of superoxide on the glyceryl trinitrate response. We had previously noted a lack of effect of superoxide generated by DETCA treatment alone on glyceryl trinitrate relaxation in segments of rat aorta and of human internal mammary artery (de la Lande et al., 2000; Sage et al., 2000). The present results show that the lack of effect persists despite the addition of NADPH to DETCA to dramatically increase superoxide production. The resistance of glyceryl trinitrate responses to superoxide was recently analysed by Hanspal et al. (2002) and shown to be selective when compared with sodium nitroprusside. They speculated that it might have been due to failure of superoxide to gain entry to the intracellular site of nitric oxide (NO) generation from glyceryl trinitrate or to loss of tissue thiols required for glyceryl trinitrate bioconversion. We tested a different explanation, based on the fact that NO and superoxide are chemical antagonists, namely that a reduced generation of NO from glyceryl trinitrate will favour the effect of superoxide. This explanation was supported by an inhibitory effect of superoxide in the non-tolerant artery when this was already inhibited by chloral or cyanamide. However, the relevance to superoxide resistance is obscured by our findings that the inhibition was not significant in the tolerant vessel. There remains the possibility that the insensitivity of glyceryl trinitrate response to superoxide is an indication that the glyceryl trinitrate response may not be mediated by the NO radical. In this respect, the superoxide-resistance adds to recent evidence arguing against NO as the bioactive mediator (Kleschyov et al., 2003).

In summary, a reduction in ALDH activity in the artery wall appears to be an extremely sensitive index of ex vivo tolerance. The magnitude of the reduction raises questions with the respect to the ability of ALDH to maintain the levels of metabolism and reactivity of glyceryl trinitrate in the tolerant artery and it is suggested that these levels may be maintained by other metabolic mechanisms of lower affinity. The pharmacological interactions between non-nitrate ALDH inhibitors and tolerance are consistent with roles for both ALDH and non-ALDH mechanisms in tolerance. A role for ALDH stems from evidence that the metabolism of nitrate esters by this enzyme is irreversible with resultant loss of ALDH. A non-ALDH mechanism is based on earlier evidence that the endothelium via its eNOS activity contributes to tolerance by a mechanism not involving depression of glyceryl trinitrate metabolism (de la Lande et al., 2004) while the currently demonstrated dissociation between eNOS and ALDH inhibition makes it unlikely that the contribution of eNOS involves ALDH. The possibility that the effects of eNOS relate to its capacity to generate superoxide and that this in turn depresses ALDH activity seems excluded by our failure to demonstrate effects of superoxide on the glyceryl trinitrate response in the non-tolerant artery. Similarly, the effects of superoxide in the tolerant artery do not seem of sufficient magnitude to suggest that superoxide becomes important when there is impaired generation of glyceryl trinitrate's bioactive product.

In conclusion, the results support a major role for ALDH in the response to glyceryl trinitrate in the non-tolerant

artery and point to its importance as a sensitive index of tolerance. However, it may be only one of several mechanisms contributing to the reduced response to glyceryl trinitrate in the ex vivo tolerant artery. The present evidence suggests that these include eNOS but not superoxide.

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