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Role of Mitochondrial Aldehyde Dehydrogenase in Nitrate Tolerance

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ABSTRACT

Glyceryl trinitrate (GTN) is used in the treatment of angina pectoris and cardiac failure, but the rapid onset of GTN tolerance limits its clinical utility. Research suggests that a principal cause of tolerance is inhibition of an enzyme responsible for the production of physiologically active concentrations of NO from GTN. This enzyme has not conclusively been identified. However, the mitochondrial aldehyde dehydrogenase (ALDH2) is inhibited in GTN-tolerant tissues and produces NO_2^- from GTN, which is proposed to be converted to NO within mitochondria. To investigate the role of this enzyme in GTN tolerance, cumulative GTN concentration-response curves were obtained for both GTN-tolerant and -nontolerant rat aortic rings treated with the ALDH inhibitor cyanamide or the ALDH substrate propionaldehyde. Tolerance to GTN was induced using both in vivo and in vitro protocols. The in vivo protocol resulted in almost complete inhibition of ALDH2 activity and GTN biotransformation in hepatic mitochondria, indicating that long-term GTN exposure results in inactivation of the enzyme. Treatment with cyanamide or propionaldehyde caused a dose-dependent increase in the EC_{50} value for GTN-induced relaxation of similar magnitude in both tolerant and nontolerant aorta, suggesting that although cyanamide and propionaldehyde inhibit GTN-induced vasodilation, these inhibitors do not affect the enzyme or system involved in tolerance development to GTN. Treatment with cyanamide or propionaldehyde did not significantly inhibit 1,1-diethyl-2-hydroxy-2-nitrosohydrazine-mediated vasodilation in tolerant or nontolerant aorta, indicating that these ALDH inhibitors do not affect the downstream effectors of NO-induced vasodilation. Immunoblot analysis indicated that the majority of vascular ALDH2 is present in the cytoplasm, suggesting that mitochondrial biotransformation of GTN by ALDH2 plays a minor role in the overall vascular biotransformation of GTN by this enzyme.

Most current hypotheses on the mechanism of action of organic nitrates consider that these compounds act as prodrugs, in that they undergo mechanism-based biotransformation in vascular smooth muscle cells to an activator of soluble guanylyl cyclase (sGC) (presumed to be NO or a related species) (Artz et al., 2001). The term clearance-based biotransformation has been coined to differentiate pathways that lead to nitrate metabolism *without* activation of sGC (Bennett et al., 1994). The biotransformation of glyceryl trinitrate (GTN, nitroglycerin) yields the dinitrate metabolites glyceryl-1,2-dinitrate (1,2-GDN) and glyceryl-1,3-dinitrate (1,3-GDN) as products, and in nitrate-tolerant tissues, the biotransformation of GTN is attenuated. Formation of NO using high concentrations of GTN has been demonstrated in intact and broken cell preparations, but an enzyme in vascular smooth muscle that catalyzes the three-electron reduction of the nitrate ester group of organic nitrates to NO has not been identified (Thatcher and Weldon, 1998). Several enzymes, however, have been identified that are capable of mediating the denitration of GTN, yielding GDN and inorganic nitrite ion (NO_2^-) as products, including the human and rat aortic glutathione *S*-transferases (Tsuchida et al., 1990; Nigam et al., 1996) and rat aortic cytochrome P450 (McDonald and Bennett, 1993). More recently, it was shown that mitochondrial aldehyde dehydrogenase (ALDH2) was capable of mediating denitration of GTN to GDN and NO_2^- (Chen et al., 2002). This important article illuminated the puzzle of nitrate biotransformation and nitrate tolerance: ALDH2 was proposed to be inactivated during nitrate tolerance and further, was proposed to be the enzyme responsible for the mechanism-based biotransformation of nitrates (Chen et al., 2002).

Several experimental approaches have been used to exam-

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ABBREVIATIONS: sGC, soluble guanylyl cyclase; GTN, glyceryl trinitrate; 1,2-GDN, glyceryl-1,2-dinitrate; 1,3-GDN, glyceryl-1,3-dinitrate; ALDH2, mitochondrial aldehyde dehydrogenase; DEA/NO, 1,1-diethyl-2-hydroxy-2-nitrosohydrazine; DPI, diphenyleneiodonium sulfate.

ine tolerance to GTN. In in vitro models, vascular tissue is incubated with high concentrations of an organic nitrate and then used for functional or biochemical measurements. Although this model can be used for assessing changes after short-term exposure to organic nitrates, because of the limited viability of in vitro vascular preparations, it cannot be used to investigate mechanisms of tolerance reversal, nor can it be used to examine the effects of counter-regulatory mechanisms or changes in gene expression that are associated with long-term exposure to nitrates. A second model has used in vivo administration of GTN, either by multiple subcutaneous dosing protocols or by administration of transdermal GTN patches (Münzel et al., 1995; De la Lande et al., 1999; Ratz et al., 2000a,b, 2002). The latter more closely resembles the clinical situation, where the rapid development of tolerance is observed during transdermal GTN therapy (Anonymous, 1991). We have focused much of our recent efforts in developing a subdermal GTN patch model of GTN tolerance in the rat. In our rat model of in vivo tolerance, we have found significant rightward shifts of the concentration-response curves for relaxation of aortae removed from these animals (Ratz et al., 2000a,b), decreased GTN-induced cyclic GMP accumulation and decreased GTN biotransformation (Ratz et al., 2000a). In addition, the blood pressure response to GTN is markedly reduced in the intact animal, and this is associated with an altered pattern of GTN metabolite formation (Ratz et al., 2002). These effects (with the exception of GTN-induced cyclic GMP accumulation) can be reversed completely by a 2-day washout period after removal of the patches.

In the study of Chen et al. (2002), the ALDH activity of rabbit aorta was decreased in aortae made tolerant to GTN by in vitro exposure to high GTN concentration, and GTN-induced relaxation of and cyclic GMP accumulation in nontolerant rabbit aorta was inhibited after exposure to several ALDH inhibitors. In addition, the ALDH inhibitors chloral hydrate and cyanamide inhibited GTN-induced decreases in mean arterial pressure in anesthetized rabbits and rats. These findings were taken as evidence for a role of ALDH2 in mechanism-based biotransformation of GTN and tolerance development. However, whether ALDH inhibitors affected GTN-induced relaxation in nitrate-tolerant tissues was not assessed. The current studies were undertaken primarily using an in vivo rather than an in vitro model of nitrate tolerance to further assess the potential role of ALDH2 in nitrate tolerance. If nitrate tolerance is caused by inactivation of ALDH2, then one would predict that the inhibitory effect on GTN-induced relaxation that occurs after exposure of nontolerant tissues to ALDH2 inhibitors would be attenuated in tissues from GTN-tolerant animals, because presumably the enzyme would already be inactivated. We also assessed the effect of long-term in vivo GTN exposure on hepatic mitochondrial GTN biotransformation and hepatic mitochondrial ALDH2 activity to estimate the extent to which ALDH2 was inhibited under the conditions in which in vivo GTN tolerance was induced.

Experimental Procedures

Drugs and Solutions. Transdermal GTN patches were purchased as Transderm-Nitro brand (0.2 mg/h) from CIBA Pharmaceuticals (Missisauga, ON, Canada). Drug-free (sham) patches were produced by soaking the patches for a minimum of 2 days in 95% ethanol (patches were allowed to air dry for 30 min before implantation). Removal of GTN from the patches by this procedure was confirmed by the absence of GTN or metabolites in the plasma of rats in which the sham patches were implanted. GTN was obtained as a solution (TRIDIL, 5 mg/ml) in ethanol, propylene glycol, and water (1:1:1.33) from DuPont Pharmaceuticals (Scarborough, ON, Canada). 1,2-GDN and 1,3-GDN were prepared by acid hydrolysis and purified by thin layer chromatography (Brien et al., 1986). Concentrations of GTN, 1,2-GDN, and 1,3-GDN in stock solutions were determined by a spectrophotometric method as described previously (Bennett et al., 1988). All other chemicals were of at least reagent grade and were obtained from a variety of sources.

Induction of GTN Tolerance in Vivo. All procedures for animal experimentation were undertaken in accordance with the principles and guidelines of the Canadian Council on Animal Care. GTN tolerance was induced by exposing rats to a continuous source of GTN via the subdermal implantation of two 0.2 mg/h transdermal GTN patches (tolerant) or drug-free patches (control) for 48 h as described previously (Ratz et al., 2000a). Briefly, a 1-cm transverse incision was made and the skin was separated from the underlying fascia by blunt dissection. Two transdermal patches were inserted back-to-back into the resulting subdermal space. The site was sutured closed and disinfected with 2.5% iodine. At 24 h, the site was reopened and both patches were replaced. Animals were sacrificed 24 h later and the livers or aortae removed.

Mitochondrial ALDH2 Activity. Livers were homogenized in 0.25 M sucrose, 5 mM Tris-HCl, and 0.5 mM EDTA, pH 7.2, centrifuged at 480*g*; the supernatant was centrifuged at 4,800*g* to obtain the mitochondrial fraction. Mitochondria were solubilized with deoxycholate (2.5 mg/mg protein), and ALDH activity was measured as the change in A_{340} during incubation with 1 mM NAD^+ in 50 mM sodium pyrophosphate, pH 8.8 containing 2 μM rotenone (to inhibit NADH consumption by complex I of the electron transfer chain), 1 mM 4-methylpyrazone (to inhibit alcohol dehydrogenase), and substrate (0.05 or 5 mM propionaldehyde) (Tottmar et al., 1973; Loomis and Brien, 1983). ALDH2 activity is reflected by changes in A_{340} at low substrate concentration.

GTN Biotransformation by Hepatic Mitochondria. The mitochondrial fraction was obtained as described above and resuspended to 0.5 mg/ml protein in 125 mM KCl and 10 mM Tris-HCl, pH 7.4. Samples (2 ml) were incubated with 200 nM GTN for 0 or 20 min at 37°C in the presence or absence of inhibitors. Samples were extracted with diethyl ether and the GTN metabolites 1,2-GDN and 1,3-GDN, quantitated by gas chromatography with electron capture detection as described previously (McDonald and Bennett, 1990)

Relaxation Studies in Isolated Rat Aorta. Isolated thoracic aortic rings were prepared from control and GTN-tolerant rats. Tissues were exposed to diluent (control), 1 mM cyanamide, or 1 mM propionaldehyde for 15 min and then contracted submaximally with 0.1 μM phenylephrine. Once the induced tone had stabilized, cumulative concentration-response curves for GTN (0.1 nM–0.1 mM) were obtained. After a 30-min washout period, treatments were repeated and cumulative concentration-response curves for 1,1-diethyl-2-hydroxy-2-nitrosohydrazine (DEA/NO) (0.1 nM–0.1 μM) were obtained. In other experiments, aortic rings prepared from naive animals were rendered tolerant by in vitro exposure to either 0.3 mM GTN for 30 min or 0.5 mM GTN for 60 min followed by a 60-min washout period. Tissues were then incubated with ALDH inhibitors and responses to GTN obtained as described above.

Immunoblot Analysis of ALDH2. Rat liver, rat aorta, and rabbit aorta were homogenized in 0.25 M sucrose, 50 mM Tris-HCl, 0.5 mM EDTA, pH 7.2, containing 0.1 mM phenylmethylsulfonyl fluoride, and protease inhibitors (Roche Diagnostics, Mannheim, Germany), centrifuged at 480*g*, and the supernatant centrifuged at 11,000*g*. Proteins in the mitochondrial and cytosolic fractions were separated on 10% gels by SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to polyvinylidene difluoride membranes.

Blots were then probed with a polyclonal antibody to human ALDH2, and immunoreactive bands were visualized by enhanced chemiluminescence. A purified recombinant ALDH2 protein was used as positive control (N. Lassen and V. Vasiliou, manuscript in preparation).

Data Analysis. In isolated blood vessel studies, the EC_{50} values for relaxation were determined from each concentration-response curve using a sigmoidal concentration-response curve-fitting algorithm. The -fold shifts in EC_{50} values caused by inhibitor treatment were calculated and compared using Student's t test for unpaired data. Other groups of data were compared using the appropriate statistical test, as indicated. The assumption of homogeneity of variance was tested in all cases using Bartlett's test. Because of inhomogeneity of variance, statistical analysis for the relaxation experiments was performed using logarithmically transformed data. A P value of 0.05 or less was considered statistically significant.

Results

Mitochondrial ALDH2 Activity. Because of the paucity of mitochondrial protein that could be obtained from aorta for assessment of enzyme activity, even after pooling of 10 rat aortae, we used hepatic mitochondrial ALDH2 activity as a surrogate marker to assess the effect of the in vivo GTN tolerance protocol on ALDH2 activity. ALDH2 has a low K_m value for substrate relative to the other ALDH isoforms, and therefore low (50 μM) and high (5 mM) substrate concentrations are used to differentiate ALDH2 from the other ALDH activities present in the mitochondrial preparation. In hepatic mitochondria from GTN-tolerant animals, ALDH activity at low substrate concentration was inhibited by more than 85% (Table 1), indicating that long-term exposure to GTN was highly effective for inhibition of ALDH2 activity. Total ALDH activity was also decreased in hepatic mitochondria from GTN-tolerant animals, albeit to a lesser extent (Table 1). We attempted to assess ALDH activity in homogenates of pooled rat aorta, but reliable rates of NADH formation could not be obtained.

GTN Biotransformation by Hepatic Mitochondria. GTN biotransformation by hepatic mitochondria in the absence of NAD^+ resulted in the highly selective formation of 1,2-GDN (1,2-GDN/1,3-GDN ratio ~6) and was characterized by an initial fast component (1,2-GDN was present in the 0-min samples) and then subsequent denitration over the 20-min period of incubation (Table 2). Both components of the denitration of GTN to 1,2-GDN were increased about 3-fold in the presence of NAD^+ and the 1,2/1,3 regioselectivity increased to >20. Pretreatment of samples with cyanamide or propionaldehyde resulted in a marked inhibition of both components of GTN biotransformation (Table 2). Mitochondrial NAD^+-dependent GTN denitration was also markedly inhibited in mitochondria from GTN-tolerant animals (Table 2), suggesting that long-term GTN exposure inhibits the GTN denitration activity of ALDH2 in addition to inhibition of the dehydrogenase activity of the enzyme. If it is assumed that vascular ALDH2 is equally susceptible to the inhibitory effects of long-term GTN exposure as the hepatic enzyme, then it can be concluded that the in vivo GTN tolerance protocol would have resulted in almost complete inhibition of the vascular enzyme.

TABLE 1

Low K_m ALDH activity is markedly inhibited in hepatic mitochondria from GTN-tolerant rats

ALDH activity was measured at low- and high-substrate concentration in hepatic mitochondria prepared from naïve and GTN-tolerant animals. Values represent the mean ± S.D. (n = 6).

Sample	[PRO]	Specific Activity
	mM	*nmol/min/mg of protein*
Control	0.05	11.5 ± 5.5
Control	5.0	33.0 ± 7.8
Tolerant	0.05	1.6 ± 0.3*
Tolerant	5.0	13.9 ± 1.5*

* $P < 0.05$ versus control, Student's t test for unpaired data.
PRO, propionaldehyde.

Effect of ALDH Inhibitors on Relaxation Responses to GTN and DEA/NO. A first series of experiments used the ALDH inhibitor cyanamide. Compared with control, nontolerant aortic rings, the mean EC_{50} value for GTN-induced relaxation in aortic rings from GTN-tolerant animals was shifted approximately 4.5-fold to the right (Fig. 1). Cyanamide treatment resulted in a 50-fold rightward shift in the GTN concentration-response curve relative to control tissues, whereas cyanamide treatment of GTN-tolerant tissues resulted in a 200-fold increase in the EC_{50} value relative to control. When the -fold shift in EC_{50} values caused by cyanamide was compared in tissues from each animal, the increase in EC_{50} value for GTN-induced relaxation was 51.9 ± 19.4-fold in aortae from control rats and 46.9 ± 16.4-fold in tissues from GTN-tolerant rats ($P > 0.05$, Student's t test for unpaired data). Similar results were obtained using the ALDH substrate propionaldehyde (Fig. 2). In this case, the increase in EC_{50} value for GTN-induced relaxation was 26.2 ± 13.6-fold in aortae from control rats and 29.4 ± 11.4-fold in tissues from GTN-tolerant rats ($P > 0.05$, Student's t test for unpaired data). Similar results were obtained using lower concentrations (100 μM) of cyanamide and propionaldehyde, whereas 10 μM concentrations of the inhibitors had no effect on the relaxation responses to GTN (data not shown). Thus, both cyanamide and propionaldehyde inhibited GTN-induced relaxation of both nontolerant and GTN-tolerant tissues to an equal extent. We also assessed the effect of cyanamide and propionaldehyde on relaxation by the authentic NO donor DEA/NO. Consistent with previous results of others using sodium nitroprusside (Chen et al., 2002), the two inhibitors did not alter relaxation-induced by DEA/NO (data not shown), indicating that the inhibitory effect of these compounds was unrelated to downstream signaling by NO.

In another series of experiments, we assessed the effect of ALDH inhibitors on aortic rings rendered tolerant to GTN by in vitro exposure to high GTN concentration (0.3 mM for 30 min or 0.5 mM for 60 min). This procedure causes a much more profound desensitization of the tissue to the vasodilator effect of GTN such that EC_{50} values are increased 50- to 100-fold rather than the 5-fold increases observed in aortae from GTN-tolerant animals. Similar to the results observed in aortae from GTN-tolerant animals, both cyanamide and propionaldehyde inhibited GTN-induced relaxation in tissues made tolerant to GTN in vitro (Figs. 3 and 4), although this inhibition was less that that observed in nontolerant tissues. This could be interpreted as evidence for a differential effect of the inhibitors in nitrate tolerant tissues. However, it should be noted that regardless of treatment regimen, all tissues eventually achieve 100% relaxation in response to GTN, and this limits the degree to which inhibitors can increase the EC_{50} value for GTN-induced relaxation.

Immunoblot Analysis of ALDH2. Because we were unable to obtain a reliable measure of ALDH2 activity in rat aorta, we wanted to ensure that the protein was at least present in this tissue. In subcellular fractions of rat liver, ALDH2 was localized in the mitochondrial fraction, with negligible immunoreactive protein present in the 11,000*g* supernatant fraction (Fig. 5). In marked contrast, there were immunoreactive protein bands in both fractions of rat and rabbit aorta, indicating that in these tissues, ALDH2 is distributed in both mitochondrial and extramitochondrial compartments. For the rat aorta samples, the intensity of staining was at least double in the 11,000*g* supernatant fraction sample than in the mitochondrial fraction. Equivalent amounts of protein were loaded on the gel, and because the amount of protein in the mitochondrial fraction makes up about 2% of the total protein, it would seem that at least 99% of vascular ALDH2 is present in the cytosol. For the rabbit aorta, although the intensity of staining was greater in the mitochondrial fraction than the 11,000*g* supernatant fraction, mitochondrial ALDH2 still only represents about 4 to 5% of the total immunoreactive protein in this tissue. We also observed another immunoreactive protein of about 25 kDa that was only present in fractions of vascular origin (Fig. 5). Whether this represents proteolytic degradation of vascular ALDH2 or a cross-reacting protein is uncertain.

Discussion

In vivo vasodilation by GTN is characterized by a rapid onset of action at nanomolar concentrations of drug and ensuing nitrate tolerance after long-term treatment. GTN undergoes rapid metabolism via both clearance-based biotransformation and mechanism-based biotransformation, the latter leading to sGC activation and vasodilation. There is evidence that a number of factors contribute to nitrate tolerance, including attenuation of one or more mechanism-based biotransformation pathways. However, it has recently been proposed that mitochondrial ALDH2 is responsible for mechanism-based biotransformation and is the underlying cause of nitrate tolerance (Chen et al., 2002). Unambiguous characterization of mechanism-based nitrate biotransformation is important because of the relevance to nitrate tolerance in current cardiovascular therapy and because of the prospective development of novel nitrate therapeutics (Reynolds et al., 2002; Wallace and del Soldato, 2003).

No purified enzyme system has been demonstrated to release significant quantities of NO from GTN, although it is widely held that the biologically active product of mechanism-based biotransformation is NO (Thatcher and Weldon, 1998). Nevertheless, several proteins have been shown to mediate the denitration of GTN to generate the biotransformation products 1,2-GDN, 1,3-GDN, and NO_2^-, including hemoglobin, myoglobin, xanthine oxidoreductase, old yellow enzyme, glutathione *S*-transferase, cytochromes P450, and cytochrome P450 reductase (Habig et al., 1975; Bennett et al., 1986; McDonald and Bennett, 1990; McGuire et al., 1998; Doel et al., 2000; Ratz et al., 2000b; Meah et al., 2001). Given the fundamental chemistry underlying these reactions, many other enzymes and proteins containing transition metals or cysteine groups may be involved in nitrate metabolism. The work described herein confirms previous reports that ALDH2, an enzyme that contains three cysteines at the active site, is capable of GTN metabolism. However, relative to hepatic microsomes, the rate of GTN denitration by hepatic mitochondria is very low. In the present study, there was about 15% metabolism of GTN over a 20-min period (Table 2), in contrast to a 30-s half-life for GTN during anaerobic incubation with rat hepatic microsomes, at the same GTN concentration and at a similar amount of protein (McDonald and Bennett, 1990).

The vascular biotransformation of GTN is regioselective for formation of the 1,2-GDN metabolite, as shown in isolated rabbit aorta (Brien et al., 1986), rat aorta (Fung and Poliszczuk, 1986; McGuire et al., 1994), human saphenous vein (Sage et al., 2000), and rat aortic microsomes (McDonald and Bennett, 1993; McGuire et al., 1998). Furthermore, it is exclusively 1,2-GDN formation that occurs during the initial exposure of tissues to GTN (Brien et al., 1988). However, in blood vessels or cultured cells made tolerant to GTN in vitro (Slack et al., 1989, Bennett et al., 1989) and in blood vessels from GTN-tolerant animals (Ratz et al., 2000a), this regioselective biotransformation of GTN is lost. These data might be taken to suggest that the mechanism-based biotransformation pathway is regioselective, but it is quite reasonable to expect clearance-based metabolism also to show regioselectivity and for both pathways to be attenuated in tolerant tissue. Regioselectivity is not therefore, by itself, a sufficient criterion for identification of mechanism-based biotransformation. The results from the present study on ALDH2 activity in hepatic mitochondria confirm that ALDH2 shows significant regioselectivity toward 1,2-GDN formation from GTN.

TABLE 2

Inhibition of GTN denitration in hepatic mitochondria by cyanamide and propionaldehyde, and in mitochondria from GTN-tolerant rats

Samples were incubated with 200 nM GTN in the presence or absence of NAD^+ for 0 or 20 min, and the GDN metabolites were quantitated. Values represent the mean ± S.D. (n = 3–6).

Sample	NAD^+	1,3-GDN		1,2-GDN	
		0 min	20 min	0 min	20 min
		nM			
Control					
Mitochondria	−	0.9 ± 0.9	1.7 ± 1.1	5.6 ± 4.4	9.1 ± 9.7
Mitochondria	+	0.7 ± 0.7	1.5 ± 1.0	18.8 ± 7.7*	29.8 ± 14.3*
Mitochondria + 1 mM cyanamide	+	0.3 ± 0.5	1.2 ± 1.6	3.3 ± 2.5	0.9 ± 1.0
Mitochondria + 1 mM propionaldehyde	+	0.3 ± 0.6	0.9 ± 0.8	0.0 ± 0.8	2.5 ± 2.0
Tolerant					
Mitochondria	−	1.4 ± 1.7	1.7 ± 2.8	1.1 ± 0.3	3.6 ± 2.4
Mitochondria	+	0.5 ± 0.8	1.1 ± 1.8	1.4 ± 0.7	3.7 ± 2.2

* $P < 0.05$ versus all other groups (one-way analysis of variance). All other comparisons were not significantly different.

The work of Chen et al. (2002) on ALDH2, following on from the observations of Mukerjee and Pietruszko (1994) on ALDH, and the original observations of Towell et al. (1995) on nitrate-ethanol interactions, stimulated us to examine the role of ALDH2 in ex vivo models of nitrate tolerance. The ex vivo model that uses in vivo administration of GTN by administration of transdermal GTN patches shows a blood pressure response to GTN that is markedly reduced in the intact animal (Münzel et al., 1995; De la Lande et al., 1999; Ratz et al., 2000a,b, 2002), which closely resembles the clinical situation observed during transdermal GTN therapy. In the study of Chen et al. (2002), total ALDH activity was reduced in GTN-tolerant rabbit aortae, and inhibitors of ALDH (1 mM chloral hydrate, cyanamide or acetaldehyde) inhibited GTN biotransformation, relaxation, and GTN-induced cyclic GMP accumulation in isolated blood vessels. It was therefore a surprise that the administration of the same ALDH inhibitor (cyanamide), and a similar ALDH substrate (propionaldehyde) used in the Chen study, caused a rightward shift in the concentration-response curve for GTN relaxation in aortic tissue from nitrate-tolerant animals, equivalent to that seen for naive animals (Figs. 1 and 2). However, these observations using propionaldehyde and cyanamide were similar to those made by us previously using the selective flavoprotein inhibitor diphenyleneiodonium sulfate (DPI). DPI inhibited the regioselectivity for formation of 1,2-GDN, inhibited cyclic GMP accumulation in blood vessels, and showed no inhibitory effect on tissue relaxation induced by authentic NO donors, but DPI also inhibited GTN-induced relaxation to the same extent in aortae from naive and GTN-tolerant animals (McGuire et al., 1994; Ratz et al., 2000b). Together, these data were taken as demonstrating that fla-

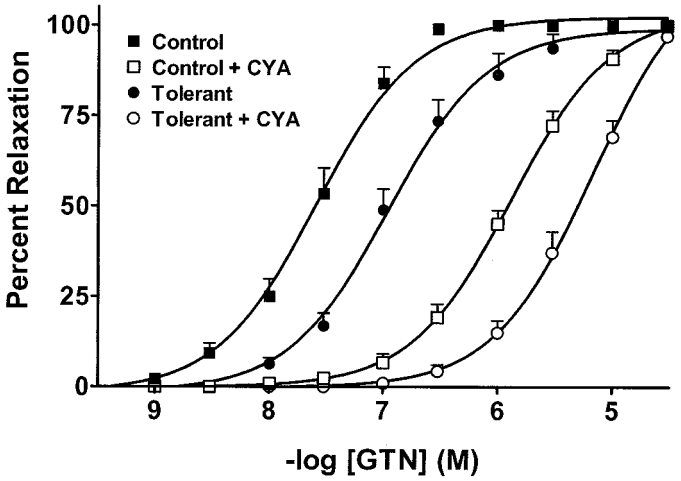

Fig. 1. Effect of in vivo GTN tolerance, 1.0 mM cyanamide (CYA), or both on GTN-induced relaxation of isolated rat aorta. The EC_{50} values for GTN-induced relaxation were control, 30 ± 17 nM; tolerant, 133 ± 86 nM; control + CYA, 1.4 ± 0.9 μM; and tolerant + CYA, 5.8 ± 3.2 μM. Each value represents the mean ± S.E.M. (n = 9). All EC_{50} values were significantly different to each other ($P < 0.001$, one-way analysis of variance).

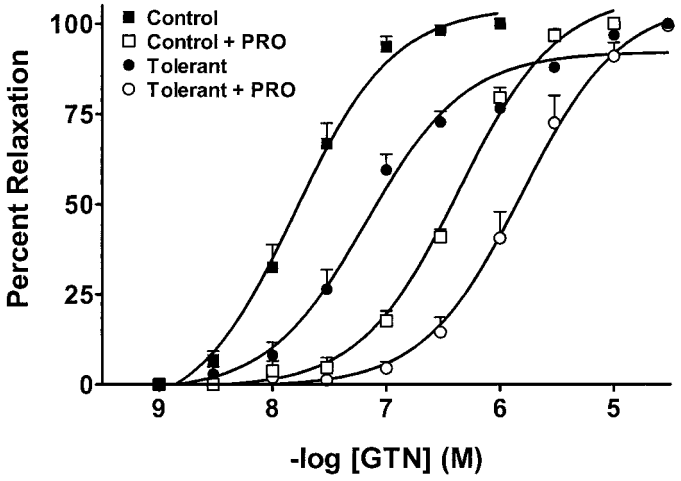

Fig. 2. Effect of in vivo GTN tolerance, 1.0 mM propionaldehyde (PRO), or both on GTN-induced relaxation of isolated rat aorta. The EC_{50} values for GTN-induced relaxation were control, 19.1 ± 8.3 nM; tolerant, 81.4 ± 57 nM; control + PRO, 0.42 ± 0.06 μM; and tolerant + PRO, 2.2 ± 1.5 μM. Each value represents the mean ± S.E.M. (n = 7–8). All EC_{50} values were significantly different from each other ($P < 0.001$, one-way analysis of variance).

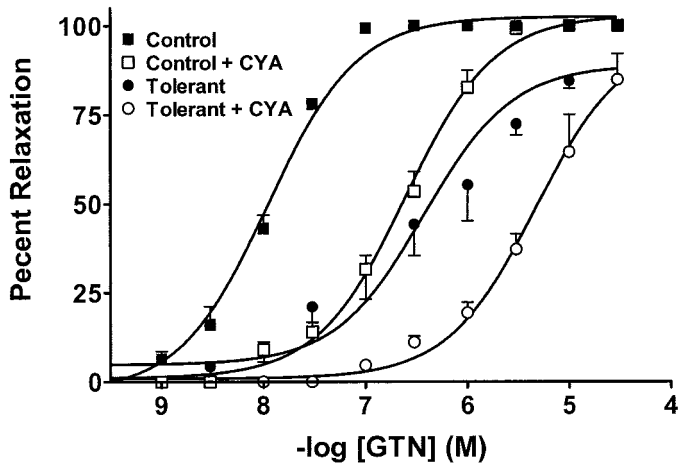

Fig. 3. Effect of in vitro GTN tolerance, 1.0 mM cyanamide (CYA), or both on GTN-induced relaxation of isolated rat aorta. The EC_{50} values for GTN-induced relaxation were control, 12 ± 3.7 nM; tolerant, 940 ± 580 nM; control + CYA, 350 ± 200 nM; and tolerant + CYA, 5.7 ± 3.0 μM. Each value represents the mean ± S.E.M. (n = 4–5). All EC_{50} values were significantly different to each other except for tolerant versus control + CYA ($P < 0.05$, one-way analysis of variance).

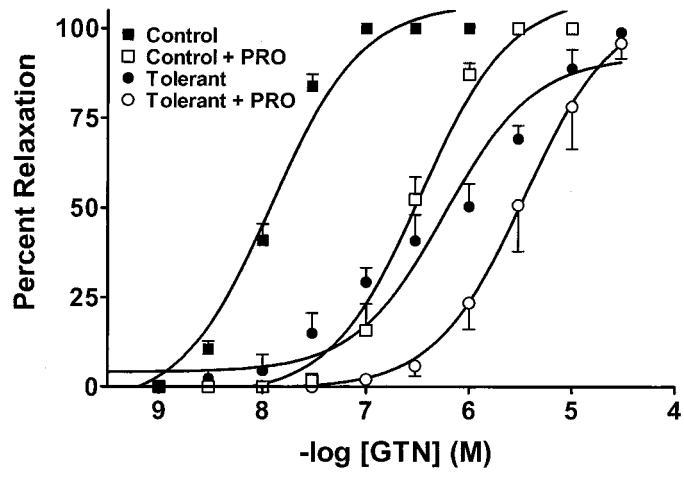

Fig. 4. Effect of in vitro GTN tolerance, 1.0 mM propionaldehyde (PRO), or both on GTN-induced relaxation of isolated rat aorta. The EC_{50} values for GTN-induced relaxation were control, 13 ± 2 nM; tolerant, 1.1 ± 0.9 μM; control + PRO, 320 ± 130 nM; and tolerant + PRO, 5.7 ± 6.6 μM. Each value represents the mean ± S.E.M. (n = 3–4). All EC_{50} values were significantly different to each other ($P < 0.05$ one-way analysis of variance).

voprotein pathways are involved in clearance-based and mechanism-based biotransformation of GTN and may be attenuated in nitrate-tolerant tissue but that the mechanism-based biotransformation pathways affected by nitrate tolerance were different from the pathways affected by DPI. Simply, flavoproteins are important for nitrate metabolism and may participate in biotransformation leading to vasodilation, but their alteration cannot be the basis for nitrate tolerance. The similar results obtained in the present work would require the same conclusions to be made about ALDH2.

Our results on ALDH2 activity clearly confirm that both the dehydrogenase and the denitration activities of ALDH2 (to the extent that mitochondrial NAD^+-dependent GTN biotransformation is mediated by ALDH2) are markedly inhibited in nitrate-tolerant animals and that cyanamide and propionaldehyde are effective inhibitors of hepatic mitochondrial GTN biotransformation activity. Assuming that there is equal susceptibility of hepatic and vascular ALDH2 to the effects of long-term GTN exposure, we can conclude that the in vivo GTN tolerance protocol used in the current study would result in almost complete inactivation of ALDH2 present in vascular smooth muscle. It then follows that any action of cyanamide or propionaldehyde to inhibit GTN-induced relaxation in GTN-tolerant aortae would be unrelated to inhibition of ALDH2 by these compounds and more likely reflects a nonspecific inhibitory action on GTN-induced relaxation. As discussed above, long-term in vivo protocols have replaced short-term in vitro protocols as a more relevant model of nitrate tolerance. Nevertheless we also used an in vitro tolerance model to assess the effects of ALDH inhibitors and reached the same conclusions: the ALDH inhibitors exert nonspecific inhibitory actions on GTN relaxation.

ALDH catalyzes NAD^+-dependent dehydrogenation of a wide range of aldehyde substrates, and an essential active site cysteine residue also provides esterase activity. Nitrates, including isosorbide dinitrate, have been shown to be substrates and inhibitors of ALDH, undergoing denitration mediated by the active site cysteine (Mukerjee and Pietruszko, 1994; Pietruszko et al., 1995). The mechanism of denitration and ALDH inhibition has not been fully defined, but initial formation of an enzyme thionitrate ester (E-SNO_2), followed by reaction with a second active site cysteine residue to yield a disulfide and NO_2^-, is consistent with the original "thiol receptor model" of Needleman and Johnson first proposed 30 years ago (Needleman and Johnson, 1973). GTN denitration simply requires binding of GTN adjacent to an active site cysteine to generate a thionitrate ester, because thionitrates react rapidly with thiols to generate NO_2^- and disulfide (Artz et al., 1996). Although this may account for inhibition of ALDH2 by GTN, the formation of disulfides, mixed disulfides, and other covalent modifications of active site cysteines have been reported for various ALDH inhibitors, consistent with the mixed inhibition observed for ALDH, including reversible, irreversible, fast, and slow components (Lipsky et al., 2001). In this work, we observe that GTN biotransformation by hepatic mitochondria in the presence of NAD^+ was characterized by an initial fast component (1,2-GDN in the 0-min samples) and then subsequent denitration over the 20-min period of incubation, in simile with observations on incubation of isosorbide dinitrate with purified human ALDH (Mukerjee and Pietruszko, 1994). Both components of GTN biotransformation were inhibited by treatment of samples with cyanamide or propionaldehyde or in mitochondria from GTN-tolerant animals.

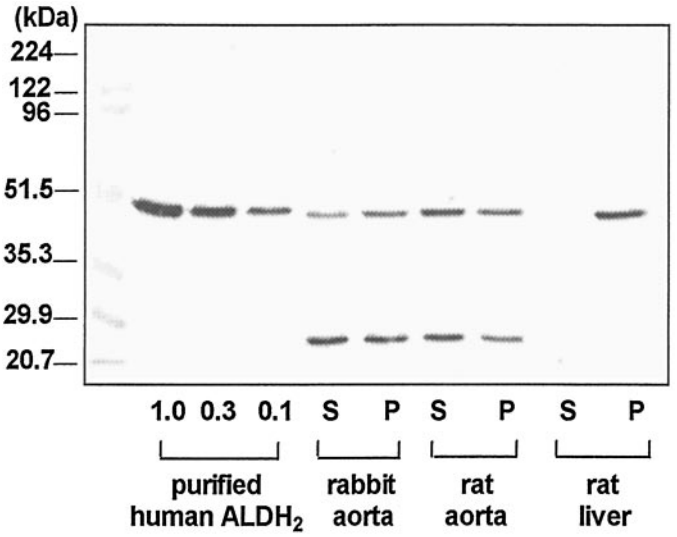

Fig. 5. Immunoblot analysis of the subcellular distribution of ALDH2 in rat liver and rat and rabbit aorta. The 11,000*g* supernatant (S) and pellet (P) fractions from liver and aorta were prepared, and samples were resolved on a 10% SDS-polyacrylamide gel electrophoresis gel under reducing conditions (10 μg protein/lane). Samples containing purified human ALDH2 (0.1, 0.3, and 1.0 μg of protein) were run for comparison.

It was proposed by Chen et al. (2002) that in ALDH2-mediated mechanism-based biotransformation, NO_2^- is the obligate intermediate in the formation of NO from GTN and that this further biotransformation occurs in mitochondria. We have argued that although NO_2^- is the predominant N,O-containing species formed during the vascular biotransformation of GTN, because of its low vasodilator potency and high endogenous levels of this species, it is unlikely that NO_2^- is the pharmacological activator of sGC or an intermediate in the formation of an activator of sGC (Bennett and Marks, 1984). The NO_2^- concentration in rat aorta has recently been reported to be 10 μM (Rodriguez et al., 2003), and pharmacokinetic studies have demonstrated that NO_2^- is distributed evenly between plasma and erythrocytes and between the intravascular and extravascular compartments (Parks et al., 1981). Because plasma and intracellular concentrations of NO_2^- are in the micromolar range and a pharmacologically effective concentration of GTN for vascular smooth muscle relaxation is in the nanomolar range, the NO_2^- derived from GTN would only incrementally increase the intracellular NO_2^- pool and thus would not be expected to exert pharmacological actions.

If, on the other hand, there was compartmentalized conversion of NO_2^- to NO in mitochondria subsequent to denitration of the organic nitrate by ALDH2, and then facile translocation of NO to its site of action, NO_2^- could be argued to be a plausible intermediate in the formation of bioactive NO from GTN. However, even the proposition of a mitochondrial source of NO_2^- conversion to NO is problematic. Two mechanisms of mitochondrial NO formation from NO_2^- have been suggested: reduction of NO_2^- to NO by components of the mitochondrial electron transport chain and disproportionation of nitrous acid (HNO_2) in the mitochondrial intermembrane space (Chen et al., 2002). The reduction of NO_2^- to

NO by the bc_1 complex of the respiratory chain (Kozlov et al., 1999), and by cytochrome oxidase (Brudvig et al., 1980), has been reported. However, this only occurs under anaerobic conditions and not under conditions of physiological oxygen tensions. Moreover, the reduction of oxygen to superoxide by the bc_1 complex predicts that under aerobic conditions, there would be competition between oxygen and NO_2^- for available electrons and that if NO formation were to occur, there might be quenching of NO by concomitant superoxide formation. NO binds to and inhibits cytochrome oxidase, and under aerobic conditions, the bound NO either will dissociate unchanged from the heme iron of the protein or will be oxidized to NO_2^-, depending on the rate of electron flux through the respiratory chain (Sarti et al., 2003). The NO_2^--cytochrome oxidase adduct only occurs with the fully oxidized form of the protein, and in contrast to the results of Brudvig et al. (1980), Giuffre et al. (2000) found that upon reduction of the iron-copper center of the protein, NO_2^- is released as such and that reduction of NO_2^- to NO does not occur. Under normal physiological conditions, cytochrome oxidase should be considered a pathway for NO clearance (by oxidation to NO_2^-) rather than NO formation. The second pathway via disproportionation of nitrous acid (HNO_2) formed from NO_2^- in the proton-rich environment of the mitochondrial intermembrane space, is controlled by kinetic and thermodynamic considerations, dictating that even at pH 4, the rate of NO formation is only about 0.01% per second (Samouilov et al., 1998). Thus, nanomolar concentrations of NO_2^- derived from GTN could only generate subpicomolar concentrations of NO in a relevant time frame for GTN-induced vasodilation (seconds), and this would be in the relatively small volume of the intermembrane space. In contrast, the EC_{50} values for relaxation of rat aorta by the spontaneous NO donor, DEA/NO, and by *S*-nitrosoglutathione are 9.2 and 26 nM, respectively (Ratz et al., 2000).

Of further relevance to mitochondrial biotransformation is our observation that in rat and rabbit aorta, the majority of ALDH2 is present in the cytosol rather than in mitochondria (Fig. 5). This is in contrast to rat liver, in which only a very small portion of cellular ALDH2 is localized to the cytosol. Extramitochondrial localization of ALDH2 is not without precedence; in human liver, ALDH2 is also distributed mainly in the cytosolic fraction (Tsutsumi et al., 1988; Takase et al., 1989). Therefore, to the extent that ALDH2 contributes to the vascular biotransformation of GTN, the majority of this biotransformation would presumably occur in the cytosol rather than in mitochondria. Assessment of mitochondrial GTN biotransformation by ALDH is further complicated by the fact that the respiratory chain can also mediate the denitration of GTN. Thus, at least two biotransformation pathways are present in mitochondria, and these may be linked via NAD^+-NADH reduction and oxidation.

To accommodate the observations contained herein with those in previous work, it could be suggested that ALDH2 mediates mechanism-based nitrate biotransformation, but that nitrate tolerance is not caused by attenuation of this biotransformation pathway. Alternatively, one concludes that ALDH2 is able to contribute to nitrate biotransformation but that there is no clear evidence to suggest that ALDH2 provides the primary pathway of mechanism-based biotransformation (or bioactivation), nor does attenuation of ALDH2 represent a primary cause of nitrate tolerance. It may be possible to glean the clinical relevance of ALDH2 in nitrate tolerance from the well know polymorphism of ALDH2 in Asian persons, which results in perturbed cofactor binding and a lack of ALDH2 activity (Vasiliou and Pappa, 2000). However, a search of the literature failed to find reference to altered therapeutic effects of nitrates in segments of the Asian population.

The actions of cyanamide and aldehydes on blood pressure and tissue relaxation must therefore reflect a nonspecific inhibitory action on GTN-induced relaxation. An obstacle to the identification of the enzyme responsible for nitrate biotransactivation and tolerance has been interpretation of data on enzyme inhibitors that are selective but not specific (Bennett et al., 1994). Disulfiram [$(Et_2NC(S)S)_2$], cyanamide (H_2NCN), aldehydes, and their hydrates (Cl_3CCHO, CH_3CHO, and EtCHO) cannot be considered, in a physiological setting, to be specific ALDH2 inhibitors, because most are extensively and rapidly metabolized in vivo; some, including cyanamide, are not ALDH2 inhibitors in vitro. Many are argued themselves to require biotransformation to inhibit ALDH2, and most have been shown to inhibit other sulfhydryl-dependent enzymes, cytochromes P450, and other metalloenzymes (DeMaster et al., 1986, 1998; Fleming et al., 1990; Jin et al., 1994; Berndt et al., 1996; Lipsky et al., 2001; Schindler, 2001). Finally, because biomimetic systems have been reported that demonstrate the feasibility of sulfhydryl-dependent and transition metal-dependent reduction of nitrates to NO (Artz et al., 1996; Murray et al., 2001; Zavorin et al., 2001), the search should continue for a physiological organic nitrate reduction system that is capable of generating NO and that does not function in nitrate-tolerant tissue.

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