Aldehyde Dehydrogenase 2 Plays a Role in the Bioactivation of Nitroglycerin in Humans


Objective—Nitrates are used widely in clinical practice. However, the mechanism underlying the bioactivation of nitrates to release NO remains unclear. Recent animal data suggest that mitochondrial aldehyde dehydrogenase (ALDH2) plays a central role in nitrate bioactivation, but its role in humans is not known. We investigated the role of ALDH2 in the vascular effects of nitroglycerin (NTG) in humans in vivo.

Methods and Results—Forearm blood flow (FBF) responses to intra-arterial infusions of NTG, sodium nitroprusside (SNP), and verapamil were measured in 12 healthy volunteers before and after ALDH2 inhibition by disulfiram. All drugs caused a dose-dependent vasodilatation. However, only the response to NTG was significantly reduced after disulfiram therapy (33% reduction in area under the curve [AUC]; \(P = 0.002\)). Separately, 11 subjects of East Asian origin, with the loss-of-function glu504lys mutation in the ALDH2 gene, received intra-arterial NTG, SNP, and verapamil. Only the FBF response to NTG was lower in the volunteers with the glu504lys mutation compared with East Asian and non-Asian wild-type control subjects (40% reduction in AUC; \(P = 0.02\)).

Conclusions—The findings suggest that ALDH2 is involved in the bioactivation of NTG in humans in vivo but accounts for less than half of the total bioactivation. This may be of clinical importance in patients with mutations in the ALDH2 gene and in those taking drugs that inhibit ALDH2. (Arterioscler Thromb Vasc Biol. 2005;25:0-0.)

Key Words: nitroglycerin • nitric oxide • nitrates • blood flow • vasodilation

Nitrates have been used for >100 years in the treatment of angina, heart failure, and hypertension. Several different forms are in clinical use, including nitroglycerin (NTG), isosorbide mononitrate, and isosorbide dinitrate. Nevertheless, they all induce vasodilatation via NO release and display tolerance with continued use. The exact mechanism of bioactivation of nitrates is unclear, but several candidate enzymes have been suggested, including glutathione-S-transferase, 1 cytochrome p450 reductase, 2 cytochrome p450, 3 old yellow enzyme, 4 and xanthine oxidoreductase. 5

Recent work by Chen et al identified mitochondrial aldehyde dehydrogenase (ALDH2) as an important enzyme involved in the bioactivation of nitrates in animals and suggested that nitrate tolerance may be caused by ALDH2 inhibition. 6 Although the role of ALDH2 in the bioactivation of nitrates has been confirmed by subsequent in vitro and in vivo animal studies, 7-9 its role in humans remains unclear. Interestingly, a common glu504lys point mutation in the ALDH2 gene is present among people of East Asian ethnic origin and causes a low-activity form of the enzyme, with heterozygotes having \(\approx 6.25\%\) of normal ALDH2 enzyme activity 10 and 0% homozygotes. The mutation is associated with flushing after alcohol ingestion, but its effect on the bioactivation of nitrates has not been investigated.

To investigate the hypothesis that ALDH2 is involved in the bioactivation of nitrates in humans, we designed 2 clinical studies. In the first study, the ALDH2 inhibitor disulfiram was administered to healthy volunteers, and the vasodilatory response to NTG was measured before and after inhibition of the enzyme. In the second study, the response to NTG in a group of healthy volunteers heterozygous or homozygous for the ALDH2 mutation was compared with that in East Asian and non-Asian wild-type control subjects. Venous occlusion plethysmography was used to measure forearm blood flow (FBF) responses to intra-arterial infusions of NTG in both studies. The responses to 2 other control vasodilators that do not undergo the same mechanism of bioactivation as NTG, sodium nitroprusside (SNP), which acts via direct release of NO, and verapamil, a calcium channel blocker, acting via an NO-independent mechanism, were also measured.

Methods

Subjects
Subjects were drawn from a community-based volunteer register, and all were free from cardiovascular disease and were nonsmokers. Individuals with hypertension, diabetes mellitus, hypercholesterolemia, or those receiving medication were excluded. All subjects were genotyped for the glu504lys ALDH2 mutation. Twelve healthy

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volunteers participated in study 1, which investigated the effect of disulfiram on FBF response to NTG. Sixteen subjects participated in study 2, which investigated the influence of ALDH2 genotype on NTG response; these subjects did not receive disulfiram. All subjects abstained from alcohol for the duration of the study (and for 10 more days after the end of study 1). Subjects were included in order of enrollment. No further selection of subjects occurred. The protocol was approved by the local research ethics committee and was performed according to Declaration of Helsinki principles. All subjects gave written informed consent before inclusion.

Forearm Blood Flow

FBF was assessed by venous occlusion plethysmography as described previously. A 27-gauge needle (Cooper’s Needle Works) attached to a 16-gauge epidural catheter (Portex) was inserted into the left brachial artery under local anesthesia (1% lidocaine; Phoenix Pharma), and saline or drugs were infused at a rate of 1 mL/min. After 30 minutes of 0.9% saline infusion, SNP was infused at incremental doses, followed by NTG and then verapamil. There was a 30-minute washout period between drugs. Measurements of FBF were made for the last 3 minutes of each infusion period. Blood pressure and heart rate were recorded at regular intervals throughout.

Drugs

Infused drugs were freshly prepared aseptically, using 0.9% saline as diluent. SNP (Faulding Pharmaceuticals) was infused at 0.2, 0.4, 0.8, and 3.0 µg/min, an alcohol-free preparation of NTG (Nitronal; Lipha Pharmaceuticals Ltd) at 0.25, 0.5, 1.0, and 4.0 µg/min, and verapamil (Securen; Abbot Laboratories Ltd) at 10 and 30 µg/min. Each dose was given for 6 minutes in a stepwise fashion.

Disulfiram (600 mg; Dume-X Alpharma A/S) was taken orally for 2 days before the second visit of study 1. The dose of disulfiram given was considered to be adequate to produce enzyme inhibition, guided by the effects on inhibition of alcohol metabolism, which start within 12 hours of the first dose and persist for up to 10 days after the final dose.

Genotyping

Blood samples were taken for DNA analysis for ALDH2 genotype. Genotyping was performed for the exon 12 glu504lys single-nucleotide polymorphism using the restriction enzyme Eco57I (Fermentas; Helena Biosciences Europe) as described previously.

Protocol

Study 1: Effects of ALDH2 Inhibition

Twelve subjects made 2 visits separated by 1 week. The study was of single-blinded crossover design. At 48 and 24 hours before each visit, subjects took either placebo (before visit 1) or disulfiram (before visit 2). All subjects then received intra-arterial SNP and NTG, and FBF was measured. In addition, 6 subjects also received verapamil. The order of administration of placebo and disulfiram was not randomized because of the unknown period of carryover attributable to the irreversible effect of disulfiram on the ALDH enzyme (which requires new synthesis to recover function). Compliance with disulfiram therapy was ensured by witnessing the first dose.

Study 2: Effects of ALDH2 Genotype

Eleven subjects with the glu504lys ALDH2 mutation (9 heterozygotes and 2 homozygotes), all of whom were of East Asian origin and 5 of whom were East Asian control subjects homozygous for the wild-type allele, made a single visit and underwent a FBF study with intra-arterial infusions of SNP, NTG, and verapamil, as described above. All subjects received SNP and NTG; 10 of the subjects with the ALDH2 mutation and 4 of the wild-type controls also received verapamil. Genotyping of subjects was performed after the clinical study to avoid bias.

Statistics

Results are expressed as the percentage change in ratio of FBF (infused arm: control arm) from baseline and also as change in absolute blood flow in the infused forearm from baseline (mL/100 g/min). Post hoc tests were performed using the Bonferroni method. A P value of <0.05 was considered significant. Data are presented as means±SEM unless otherwise stated.

Results

Study 1: Effects of ALDH2 Inhibition

The mean age of the subjects was 30±7 years (7 females), and all were normotensive (mean BP 117±10/79±5 mm Hg; Table 1). Genotyping confirmed that all 12 subjects were homozygous for the wild-type ALDH2 allele.

There was a dose-dependent increase in FBF in response to intra-arterial infusions of NTG, SNP, and verapamil at both visits (Table 2). The FBF response to intra-arterial NTG was significantly reduced after disulfiram therapy compared with placebo (P=0.002; ANOVA; Figure, B), but there was no significant change in the response to either SNP (P=0.8; Figure, A) or verapamil (P=0.5). Overall, disulfiram reduced AUC significantly from 798±91 to 532±90 AU for NTG (P=0.004) but not for SNP (P=0.7) or verapamil (P=0.2).
Baseline blood pressure (112±6/72±3 versus 113±4/77±2 mm Hg), heart rate (58±3 versus 63±4 bpm), and FBF (2.7±1.4 versus 3.0±1.3 mL/100 mL per minute) were not significantly different between visits, and there was no change in FBF in the noninfused arm, blood pressure, or heart rate during either study.

Study 2: Effects of ALDH2 Genotype

Eleven East Asian subjects with the glu504lys mutation in the ALDH2 gene were studied, and the results were compared with those obtained in 5 East Asian subjects homozygous for the wild-type allele, and also to those obtained on the placebo visit for the 12 non-Asian wild-type homozygotes who participated in study 1. The baseline characteristics of the volunteers are summarized in Table 1.

Again, there was a dose-dependent increase in FBF in response to intra-arterial infusions of NTG, SNP, and verapamil in all subjects (Table 3). There was a significantly blunted response to NTG in the East Asian subjects with the glu504lys mutation compared with the East Asian wild-type and also to those obtained on the placebo visit for the 12 non-Asian wild-type homozygotes who participated in study 1. The baseline characteristics of the volunteers are summarized in Table 1.

In addition, the response to NTG was comparable between the East Asian subjects with and without the glu504lys ALDH2 mutation and the (non-Asian) wild-type controls from study 1. Overall, there was a significant difference in the response to NTG between the 3 groups (P<0.001). Post hoc analysis revealed that the response to NTG was significantly blunted in the subjects with the ALDH2 mutation (P=0.005 versus East Asian wild-type controls; P<0.001 versus non-Asian wild-type controls) but was not significantly different between the 2 wild-type control groups (P>0.9). The presence of the glu504lys mutation did not influence the response to SNP or verapamil.

### Discussion

The main findings of this study are that inhibition of ALDH2 by disulfiram reduces the vasodilatory response to NTG, and that inherited reduction in ALDH2 activity is associated with a similarly reduced response to NTG. In contrast, the response to control vasodilators was unaffected by pharmacological or inherited reduction in ALDH2 activity. The reduction in response was 33% in the case of pharmacological inhibition of ALDH2 using disulfiram and 40% in the case of genetic reduction in ALDH2 activity on the basis of AUC calculations. These data suggest that ALDH2 is involved in the bioactivation of NTG to NO in vivo in humans; but because there was incomplete inhibition of the NTG response in both cases, it is likely that other enzymes or pathways are also involved.

Our results in humans are consistent with those reported in previous animal studies. Chen et al demonstrated a concentration-dependent rightward shift in the NTG relaxation curve in isolated rabbit aortic rings after treatment with ALDH2 inhibitors. They also demonstrated that in vivo blood pressure responses to NTG could be attenuated by the administration of the ALDH2 inhibitors chloral hydrate and cyanamide to anesthetized rabbits and rats. DiFabio et al and de la Lande et al confirmed that inhibition of ALDH2 with cyanamide caused a rightward shift in NTG relaxation curves in isolated rat aorta. Sydow et al also demonstrated a reduction in NTG response in vessels treated with the ALDH2 inhibitors cyanamide and acetaldehyde. Most recently, cyanamide was shown to attenuate the increase in coronary blood flow and decline in blood pressure and left

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**TABLE 2. Change in Absolute FBF From Baseline in the Infused Arm During Study 1**

<table>
<thead>
<tr>
<th></th>
<th>SNP (µg/min)</th>
<th>NTG (µg/min)</th>
<th>Verapamil (µg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2</td>
<td>0.4</td>
<td>0.8</td>
</tr>
<tr>
<td>ΔFBF, mL/100 mL/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Visit 1: placebo</td>
<td>1.4±0.3</td>
<td>2.0±0.4</td>
<td>3.8±0.5</td>
</tr>
<tr>
<td>Visit 2: disulfiram</td>
<td>1.7±0.3</td>
<td>2.5±0.4</td>
<td>3.9±0.6</td>
</tr>
</tbody>
</table>

ΔFBF indicates change in absolute FBF from baseline in the infused arm. Values are means±SEM; n=12 (SNP, NTG); n=6 (verapamil).

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**Figure**

Percentage change from baseline in FBF ratio (infused arm:control arm) in response to intra-arterial SNP (A) and NTG (B) before (△) and after (▲) inhibition of ALDH2 by disulfiram (n=12) in study 1. Percentage change from baseline in FBF ratio (infused arm:control arm) in response to intra-arterial SNP (C) and NTG (D) in East Asians with glu504lys ALDH2 mutation (▲; n=11) and East Asian wild-type controls (△; n=5) in study 2. Values are means±SEM; *P<0.002; **P<0.02.
ALDH2 inhibition is the mechanism responsible for nitrate tolerance in animal studies. Our current study was not designed to determine whether this is attributable to ALDH2 inactivity is not currently known.

Although we demonstrated a significantly blunted response to NTG in our studies, the effect was only partial. However, this is in keeping with existing data suggesting that several different enzymes are involved in the bioactivation of NTG. Moreover, the relative importance of ALDH2 compared with other enzyme systems in the clinical response to nitrates is not known, and it is possible that there may be compensatory upregulation of other systems when ALDH2 activity is reduced, either because of subacute pharmacological inhibition or genetically determined low activity. As expected, SNP and verapamil responses were unaffected by reduced ALDH2 activity, supporting the view that they act via different vasodilatory mechanisms and do not undergo the same type of bioactivation as NTG.

The ALDH2 mutation is a guanine to adenine base change at residue 1951 within exon 12 of the ALDH2 gene on chromosome 12. This single-nucleotide polymorphism results in a glutamic acid to lysine substitution in the enzyme and is dominant with homozygotes and heterozygotes for the mutation having a low-activity form of ALDH2. Subjects with the mutation typically experience facial flushing and a “disulfiram-like” reaction after ingestion of alcohol. The dominant-negative behavior of the allele is attributed to tetramer formation by the enzyme, and the activity of the enzyme in heterozygotes is estimated to be 6.25% of normal. As expected from the fact that the mutation is dominant, there was no apparent difference between the drug responses in the heterozygotes and the homozygotes in the study, although the number of homozygotes in the study was too small to perform any separate statistical analysis. It might be the case that other mechanisms of bioactivation of NTG can be recruited when ALDH2 activity is decreased for whatever reason, and this could be particularly important in the case of homozygotes for the glu504lys mutation. The mutation is rare in white populations but relatively common in people of East Asian ethnic origin, including the Japanese (up to 50% heterozygotes) and Chinese (up to 17% heterozygotes). We chose to study volunteers with the mutation as a natural example of reduced activity of ALDH2.

One study found that Japanese men homozygous for the glu504lys mutation had an increased risk of myocardial infarction but concluded that this may be attributable to influences of a low alcohol intake in these men on high-density lipoprotein cholesterol levels. However, 2 studies looking at the relationship between ALDH2 genotype and hypertension in Japanese populations have identified a negative association between hypertension and the glu504lys mutation in men and no association in women. The negative association was again thought to be secondary to reduced alcohol intake because of alcohol intolerance in the group with the glu504lys mutation.

Limitations of the current study include the fact that we only measured local vascular effects of NTG and assessed the effect of subacute, not chronic, inhibition of ALDH2. Also, we did not measure plasma levels of NTG or its metabolites because this was a local infusion study using very low doses of NTG. Disulfiram has been suggested to have some direct vascular effects, including increasing blood pressure in some patients. However, in our study, blood pressure and baseline FBF were not significantly different between the 2 visits, before or after disulfiram was given. Moreover, in the current study, there was no difference in the response to the control vasodilators SNP and verapamil after disulfiram therapy.

Disulfiram was chosen as the ALDH2 inhibitor used in this study because it has been used widely in humans in clinical settings and has been administered previously to healthy volunteers without ill effect. We did not test whether complete inhibition of ALDH2 was achieved by the dosages of disulfiram given in this study. However, the effects of...
disulfiram on alcohol metabolism start within 12 hours of the first dose, and disulfiram causes irreversible inhibition of ALDH2, and resynthesis of the enzyme is required to reverse the effect, which takes several days. Therefore, we assumed that the dose of 600 mg of disulfiram daily for 2 days was sufficient to achieve a high degree of inhibition of ALDH2.

In summary, we have shown that ALDH2 plays a role in the bioactivation of NTG in humans in vivo. However, because more than half of the response to NTG was maintained after pharmacological inhibition of ALDH2 and in people with genetically determined low activity of ALDH2, this suggests that ALDH2 is unlikely to be the only enzyme involved in the bioactivation of nitrates. The findings of this study may be of clinical importance in patients with mutations in the ALDH2 gene and may also have implications for patients taking drugs that inhibit ALDH2 who require nitrate therapy. Another important question that requires investigation in the future is whether ALDH2 is involved in the development of nitrate tolerance in humans.

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