Role of Mitochondrial Aldehyde Dehydrogenase in Nitroglycerin-Induced Vasodilation of Coronary and Systemic Vessels
An Intact Canine Model

Jian Zhang, MD; Zhiqiang Chen, PhD; Frederick R. Cobb, MD; Jonathan S. Stamler, MD

Background—It has recently been shown that mitochondrial aldehyde dehydrogenase 2 (mtALDH) catalyzes the formation of 1,2-glyceryl dinitrate and nitrite from nitroglycerin (glyceryl trinitrate [GTN]) within mitochondria, leading to production of cGMP and vasorelaxation. However, whether this mechanism operates in the systemic and coronary beds that subserve the antianginal action of GTN is not known. In this study, we address this question in an intact canine model.

Methods and Results—Fourteen healthy mongrel dogs (weight, 20 to 25 kg) were studied. Coronary blood flow and hemodynamics were continuously monitored by a pulse Doppler flow probe implanted around the left circumflex coronary artery and with catheters in left ventricle and aorta, respectively. Each dog was given a 1-mL bolus injection of GTN, sodium nitroprusside (SNP), or adenosine through a catheter in the left atrium before and 30 minutes after infusion of cyanamide (17 mg/kg), an inhibitor of mtALDH. Cyanamide significantly inhibited both the classic dehydrogenase and GTN reductase activities of mtALDH in situ and attenuated the coronary blood flow increase and declines in blood pressure and left ventricular end-diastolic pressure produced by GTN in vivo. In contrast, mtALDH inhibition had no effect on the coronary and systemic effects of SNP and adenosine.

Conclusions—Our data suggest that mtALDH contributes to GTN biotransformation in vivo and thus at least partly underlies the antianginal mechanism of drug action. Our findings also highlight the differences in biometabolism of clinically relevant nitrosovasodilators. (Circulation. 2004;110:750-755.)

Key Words: nitroglycerin | nitric oxide | angina | blood flow

Nitroglycerin (glyceryl trinitrate [GTN]) has been used for the treatment of angina and heart failure for >130 years.1,2 It is thought that the effects of GTN are mediated by nitric oxide (NO) or a closely related molecule; however, the evidence remains indirect.3–6 Although it is likely that NO derived from GTN contributes to the activation of smooth muscle guanylate cyclase, which mediates vasodilation, recent studies have suggested that GTN activity may be dissociated from NO production both in vitro and in vivo.5,6 In addition, GTN can exert effects that are independent of guanylate cyclase/cGMP.7 Unlike other vasodilators (eg, sodium nitroprusside [SNP]) and agonists (eg, adenosine) that operate at least partly through the NO/cGMP pathway, the therapeutic effects of GTN are compromised by development of vascular tolerance.8–12 Furthermore, continued use of GTN is associated with endothelial dysfunction, a predictor of adverse cardiac events.11,13,14 Thus, the molecular basis of neither GTN bioactivation nor tolerance is well understood.

We recently identified the mitochondrial aldehyde dehydrogenase (mtALDH) with the enzymatic biotransformation of GTN to 1,2-glyceryl dinitrate and nitrite15 and showed that this GTN reductase activity plays a central role in GTN vasorelaxation in vitro and hypotension in vivo. We also demonstrated that SNP-mediated vasodilation was unaffected by mtALDH. In rats administered GTN continuously,16 the evolution of tolerance coincided with the loss of mtALDH activity, whereas responsiveness to SNP was preserved. It is not known, however, if the same molecular mechanisms operate in the coronary and venous beds of large mammals to account for the antianginal properties of GTN. In the present study, we tested the effects of GTN in an intact canine model that is highly predictive of human responsiveness to nitrosovasodilators.

Methods

General Preparation
Fourteen healthy mongrel dogs (weight, 20 to 25 kg) were studied. Animals were anesthetized with 2% isoflurane. A thoracotomy was

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From the Department of Medicine, Division of Cardiology (J.Z., F.R.C., J.S.S.) and Howard Hughes Medical Institute (Z.C., J.S.S.), Duke University Medical Center, Durham, NC.

Correspondence to Dr Jonathan S. Stamler, Department of Medicine, Box 2612, Duke University Medical Center, Durham, NC 27710. E-mail staml001@mc.duke.edu

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Experimental Protocols

The levels of oxygen and carbon dioxide in blood (P$_{O_2}$ and P$_{CO_2}$) were monitored continuously, as were aortic pressure, left ventricular end-diastolic pressure (LVEDP), dP/dt, and coronary blood flow. Each dog was given a 1-mL bolus injection of GTN, SNP, or adenosine via the left atrial catheter before and 30 minutes after infusion of cyanamide (17 mg/kg). Between injections, coronary blood flow and hemodynamics were allowed to return to preinjection levels (minimum, 10 minutes). At the end of the study, animals were euthanized, and the coronary artery and aorta were removed and stored at $-80^\circ$C for measurements of the activities of ALDH and GTN reductase.

GTN Biotransformation

The formation of 1,2-glyceryl dinitrate and 1,3-glyceryl dinitrate were measured by TLC and liquid scintillation spectrometry, as described in previous studies. Briefly, the artery rings were blotted and weighed and then left for 1 hour in Krebs solution. GTN (1 $\mu$mol/L) was added (total volume, 1 mL) at 37°C for 10 to 30 minutes. The reaction was stopped (dry ice or 4°C), GTN and its metabolites were extracted with 3×4 mL ether and pooled, and the solvent was evaporated by a stream of nitrogen. The final volume was kept to <100 $\mu$L in ethanol for subsequent TLC separation and scintillation counting. Buffer control (Kreb's buffer plus GTN) and nonspecific biotransformation (heat-inactivated rings plus GTN) activities were also measured, and the results were corrected accordingly.

<table>
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<th>Agents, mol/L</th>
<th>Aortic Pressure, $\Delta$%</th>
<th>Heart Rate, $\Delta$%</th>
<th>LVEDP, $\Delta$%</th>
<th>dP/dt, $\Delta$%</th>
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<td>0*</td>
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*P<0.05 from before cyanamide.

Preparation of Drugs

GTN was synthesized according to Dean and Baun, purified by thin-layer chromatography (TLC), and diluted to serial concentrations before use in 0.9% NaCl that had been deoxygenated with nitrogen for >30 minutes. Cyanamide, SNP, and adenosine were obtained from Sigma Chemical Co. GTN, SNP, and adenosine were diluted to 10$^{-8}$, 10$^{-7}$, 10$^{-6}$, and 10$^{-5}$ mol/L before use. Cyanamide was prepared in 0.9% NaCl before intracoronary infusion.

Experimental Protocols

The levels of oxygen and carbon dioxide in blood (P$_{O_2}$ and P$_{CO_2}$) were monitored continuously, as were aortic pressure, left ventricular
ALDH Assay
The artery ring was homogenized in 30 mmol/L phosphate buffer (deoxygenated with nitrogen gas), pH 7.5, and the homogenate was then sonicated and centrifuged at 10,000×g for 10 minutes. ALDH activity in the supernatant was monitored at room temperature by following NADH formation at 340 nm as described.15

Data and Statistical Analyses
Enzyme activities, coronary blood flow, heart rate, aortic pressure, and LVEDP were compared before and after each injection of GTN, SNP, and adenosine. The means of the peak responses of coronary blood flow were plotted as a function of increasing doses of GTN, SNP, and adenosine. The response of coronary blood flow to GTN, SNP, and adenosine followed a characteristic sigmoid dose-response curve that is described by the following equation20:

\[
\text{Effect} = \frac{\text{Maximal Effect} \times \text{Dose}}{K_d + \text{Dose}}
\]

The \(K_d\) has been described as the dissociation constant of drug-receptor complex and is the dose at which 50% of the maximum response (EC\(_{50}\)) has been achieved. The values for EC\(_{50}\) and maximum effect were calculated in each animal by performing a nonlinear least-squares regression (\(r^2 > 0.90\)) on the dose-response data. Time course was measured as the time for the increase in coronary blood flow and decrease in blood pressure to return to baseline level. All data are presented as mean±SEM. The changes of coronary blood flow and hemodynamics were analyzed with paired \(t\) tests. The responses were considered significant at \(P<0.05\).

Results

Effects of Cyanamide on Coronary Blood Flow, Heart Rate, and Systemic Hemodynamics
Intracoronary infusion of cyanamide (17 mg/kg) did not significantly affect \(P_O\) and \(P_CO_2\). It caused transient increases in coronary blood flow (×2), heart rate, and dP/dt and decreases in aortic blood pressure and LVEDP. All parameters returned to basal levels within approximately 5 minutes.

Effects of GTN, SNP, and Adenosine on Heart Rate, LVEDP, and dP/dt With and Without Cyanamide
The Table shows the hemodynamic responses of 14 dogs to bolus injections of GTN, SNP, and adenosine before and after cyanamide. These agents caused a transient dose-related decrease in aortic blood pressure and LVEDP and an increase in heart rate and dP/dt. Cyanamide did not significantly affect the responses to SNP or adenosine but blocked the effects of GTN. The systemic response to GTN was seen within 10 seconds, and the maximal responses occurred 25 seconds after bolus injection. Figure 1 illustrates the selective blockade by cyanamide on GTN (versus SNP and adenosine).

Effects of GTN, SNP, and Adenosine on Coronary Blood Flow With and Without Cyanamide
Figure 2 shows a direct recording of coronary blood flow and hemodynamic responses to bolus injection of GTN (via the left atrium) before and after cyanamide. Both the duration and amplitude of coronary blood flow and systemic responses to GTN were significantly attenuated after the administration of cyanamide. Inhibition of coronary blood flow was more marked than the systemic effect of GTN, likely reflecting the route of administration. The peak coronary blood flow response to GTN was observed 10 seconds before the systemic effect, indicating a direct effect of GTN on coronary resistance vessels rather than a secondary reflex response. Figure 3 shows the coronary blood flow response to GTN, SNP, and adenosine. Cyanamide did not alter the effects of SNP and adenosine; however, the responses of GTN (10\(^{-9}\) to 10\(^{-6}\) mol/L) were decreased significantly (before: 21%, 89%, and 129%; after: 0%, 12%, and 60%). The EC\(_{50}\) values of GTN were 5×10\(^{-8}\) and 3×10\(^{-7}\) mol/L before and after cyanamide, respectively (\(P<0.05\)).

Duration of Action of GTN, SNP, and Adenosine: Coronary Blood Flow and Aortic Blood Pressure
Figure 4 shows the effect of cyanamide on the duration of coronary (blood flow increase) and systemic (decrease in
blood pressure) vasodilation by GTN. At the highest dose of GTN, values for the duration of action of GTN on coronary blood flow and blood pressure were reduced from 60.0/1100615.0 to 25.0/110063.5 seconds and from 136.7/1100621.9 to 65.0/110066.0 seconds, respectively (P<0.05). In contrast, cyanamide did not significantly change the duration of action of SNP and adenosine (data not shown).

Activities of mtALDH and GTN Reductase
Figure 5 shows the activities of GTN reductase and mtALDH from 8 control and 6 treated animals. The activity of GTN reductase was decreased by 50% (P<0.05) and that of mtALDH was decreased by 80% (P<0.05) compared with values before administration of cyanamide.

Discussion
In the present study, we demonstrate in an intact canine model that the vasodilator effect of GTN in both coronary and systemic vessels is mediated, at least in significant part, by GTN reductase (mtALDH). In contrast, mtALDH plays no significant role in the coronary or systemic actions of SNP or in the response to adenosine.

We recently reported that mtALDH has GTN reductase activity,15 which specifically catalyzes the formation of 1,2-glyceryl dinitrate within mitochondria,15,16 leading to the formation of cGMP and vascular smooth muscle relaxation. We further demonstrated that the inhibition of GTN reductase significantly decreases the formation of 1,2-glyceryl dinitrate and cGMP in vascular rings and blocks relaxation by GTN. In these studies, inhibition of mtALDH was accomplished with multiple classes of ALDH inhibitors21,22 (disulfiram, chloral hydrate, benomyl, daidzin, and cyanamide) as well as by the substrate acetaldehyde. Responses to cyanamide (at concentrations that do not affect SNP) were predictive and virtually identical to those of the other agents.15,16 (We caution against using this drug in tolerant vessels because of the confounding interactions with reactive oxygen species that may alter its potency.) Our choice of this agent was based mainly on our finding that its hemodynamic effects were modest by comparison with other agents (not shown) and that we could inhibit mtALDH in situ, without producing undesired systemic effects. We found that cyanamide significantly inhibited the responses of coronary blood flow and hemodynamics to GTN; the EC50 value after cyanamide was approximately 6-fold that before cyanamide. The duration of the systemic and coronary responses to GTN was also significantly reduced after the inhibition. Furthermore, the finding that responses to SNP were unaffected by cyanamide indicates that the NO/cGMP axis remained intact.

It is generally accepted that the bioactivity of GTN depends on production of NO or S-nitrosothiol, which activates soluble guanylate cyclase and in turn increases cGMP, leading to vascular smooth muscle relaxation.3,4,23–25 It should be noted, however, that GTN may exert a direct effect on guanylate cyclase6 as well as a cGMP-independent effect that contributes to vasodilation.26,27 We have suggested that mtALDH mediates the cGMP-dependent component of vasorelaxation,15,16 whereas the remaining activity may not require GTN biotransformation.6,23 Thus, the fact that some residual GTN activity is seen after cyanamide infusion is to be expected (further, the mtALDH enzyme was inhibited by only 50% to 80% in our studies).

Coronary blood flow can be influenced by many factors, including myocardial O2 consumption, pH, and sympathetic
drive. 28,29 GTN caused transient increases in heart rate and dP/dt (attributed to sympathetic-adrenergic reflex activation associated with a fall in blood pressure). However, the coronary blood flow response to GTN preceded the fall in blood pressure and increase in heart rate by $10^{-10}$ seconds.

Thus, we conclude that the effect of GTN on coronary flow is direct. GTN preferentially acts on large arteries and capacitance vessels, with lesser effects on coronary resistance vessels. 30,31 The mechanism underlying the differential responsivity to GTN is unknown. Kurz et al 32 and Sellke et al 33 suggested that the availability of sulfhydryl groups in different vascular beds may be responsible. However, in a previous study, we showed that GTN and S-nitroso-L-cysteine, which probably target different sulfhydryl groups, exhibited comparable potency in coronary conductance and resistance coronary vessels. 34 It has also been suggested that endogenous NO

Figure 3. Effects of inhibition of GTN reductase on coronary blood flow. Coronary blood flow response to GTN in the presence or absence of cyanamide is shown. Increases in the flow at $10^{-8}$, $10^{-7}$, and $10^{-6}$ mol/L were 21.1±5.4%, 89.0±13.0%, and 128.9±18.6%, respectively, in the absence of cyanamide and 0%, 11.8±4.3%, and 60.0±7.1%, respectively, ($P<0.05$) in the presence of cyanamide. The $EC_{50}$ values were $5\times10^{-8}$ mol/L in the absence of cyanamide inhibition and $3\times10^{-7}$ mol/L in the presence of cyanamide ($P<0.05$). Data are expressed as mean±SEM. B, Dose responses of coronary blood flow to SNP in the presence or absence of cyanamide. Cyanamide did not significantly alter coronary blood flow responses. C, Dose responses of coronary blood flow to adenosine (ADO) in the presence or absence of cyanamide. Cyanamide did not significantly alter coronary blood flow responses.

Figure 4. Duration of action of GTN ($10^{-6}$ mol/L) on coronary blood flow and aortic blood pressure. Coronary blood flow increases lasted 136.7±21.9 seconds and aortic blood pressure decreases lasted 91.7±23.5 seconds before mtALDH inhibition (cyanamide) and 60.0±15.0 seconds and 25.0±3.5 seconds, respectively, after cyanamide ($P<0.05$).

Figure 5. A, GTN reductase activity in vascular rings. GTN reductase activity was significantly decreased from 44.4±6.4 to 23.6±3.1 nmol/min per milligram in the absence or presence of cyanamide ($P<0.05$), respectively. Data are expressed as mean±SEM (n=6). B, mtALDH activity in vascular rings. mtALDH activity was significantly decreased from 2.6±0.4 to 0.6±0.2 nmol/min per milligram in the absence or presence of cyanamide ($P<0.05$). Data are expressed as mean±SEM (n=7).
production, which is higher in arteries than in veins, may inhibit GTN biotransformation, but we find that arterial mtALDH activity is robust. It remains to be seen whether differences in distribution or activity of mtALDH explain these findings.

In summary, inhibition of mtALDH attenuated the coronary and systemic hemodynamic responses of GTN and shortened the duration of its action. In contrast, responses to SNP and adenosine were unaffected by mtALDH inhibition. Our results thus provide new mechanistic insights into the profile of cardiovascular effects exerted by different classes of nitrovasodilators. Because the hemodynamic effects of GTN in dogs are very similar to those in humans, our findings may have clinical relevance.

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References

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