Effects of Cyanamide, an Aldehyde Dehydrogenase Type 2 Inhibitor, on Glyceryl Trinitrate- and Isosorbide Dinitrate-Induced Vasodilation in Rabbit Excised Aorta and in Anesthetized Whole Animal

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The contribution of aldehyde dehydrogenase type 2 (ALDH2) to bioactivation of glyceryl trinitrate (GTN) and isosorbide dinitrate (ISDN) was systematically examined in excised rabbit aorta and anesthetized whole animal with cyanamide, an ALDH2 inhibitor. In excised aortic preparation, the degree of inhibition by cyanamide in GTN-induced vasorelaxation (concentration ratio, calculated as EC_{50} in the presence of cyanamide/EC_{50} in the absence of cyanamide; 5.61) was twice that in ISDN-induced relaxation (2.78). However, the degree of inhibition by cyanamide, as assessed by the dose ratio (as described above, but calculated with doses) in anesthetized rabbits was 2.29 in GTN-induced hypotension (assessed by area under the curve (*AUC*) of 50 mmHg·min) and 7.68 in ISDN-induced hypotension. Thus, the inhibitor was 3 times more potent in ISDN-induced hypotension, a finding in conflict with to that obtained in excised aortic preparation. The rate of increase in plasma nitrite (NO_2^-) concentration at certain hypotensive effect (50 mmHg·min of *AUC*) in the presence and absence of cyanamide (ΔNO_2^- ratio) was larger in ISDN-induced hypotension (15.01) than in GTN-induced hypotension (3.28). These results indicate that the bioactivation pathway(s) of GTN is ALDH2-dependent in aortic smooth muscle, while ADLH2-independent mechanism(s) largely take place in the whole body. In contrast, the activation mechanism(s) of ISDN is largely ALDH2-dependent in both aortic smooth muscle and whole body. Plasma NO_2^- may be derived from pathways other than the cyanamide-sensitive metabolic route.

Key words glyceryl trinitrate; isosorbide dinitrate; cyanamide; rabbit; nitrite

Glyceryl trinitrate (GTN) has been widely used in the treatment of ischemic heart disease for more than a century[1] and the main mechanism of its vascular action is the activation of the intracellular nitric oxide (NO) receptor enzyme, soluble guanylate cyclase, leading to increased bioavailability of guanosine 5′-cyclic monophosphate (cGMP) and activation of cGMP-dependent protein kinases and/or cyclic nucleotide-gated ion channels.[2] However, the mechanism of bioactivation of the agent, upstream of intracellular signal transduction, was not elucidated until recently.

Another new concept argues that the high potency nitrate, GTN, is mainly bioactivated by mitochondrial aldehyde dehydrogenase type 2 (ALDH2) when used at low, clinically relevant concentrations ($<1\,\mu$M, high affinity pathway), and this notion is supported by evidence from various laboratories.[3–5] On the other hand, the low potency nitrate, isosorbide dinitrate (ISDN), is suggested to be mainly metabolized by P450 in the endoplasmic reticulum, directly yielding nitric oxide.[5–7] Although the above scenarios are based on many molecular, biochemical, mechanical (excised vascular preparation) and whole animal studies, there are no systematic studies that include both excised vascular and whole animal preparations to elucidate the difference between GTN and ISDN in one species.

The present study was designed to compare the role of ALDH2 in bioactivation of GTN and ISDN using an ALDH2 inhibitor, cyanamide, in rabbit excised aorta and in anesthetized whole animal. In addition, this study includes a special reference to changes in arterial plasma NO_2^- concentration in whole animal experiment, because it is considered to reflect NO status in whole body[8,9] and that measurement of these changes in concentration are feasible by our sophisticated method with high sensitivity and reliability.[10] The rationale for the study was that if bioactivation status or pathways of GTN and ISDN are differently affected by ALDH2 inhibitor, changes in plasma NO_2^- concentration should reflect changes in the active product (NO) or its oxidized product (NO_2^-).

MATERIALS AND METHODS

The Ethics Committee of Kanazawa Medical University approved all animal procedures, and animals were dealt with in a humane way in accordance with "Guiding Principles for the Care and Use of Laboratory Animals" set by the Japanese Pharmacological Society.

Excised Aortic Preparation Japanese white rabbits weighing 2.5–3.5 kg were anesthetized with intravenous sodium pentobarbital (30 mg/kg) and bled. The thoracic aorta was immediately excised, placed in a chamber filled with cold Krebs–Henseleit solution and dissected free of excess fat and connective tissue. The aorta was cut into rings about 3 mm wide and the endothelium was removed by gently rubbing the intimal surface with a stainless steel rod. Each ring was suspended between two wire hooks in a 10-mL organ bath containing Krebs–Henseleit solution kept at 37°C and was treated as described elsewhere.[11] In brief, isometric tension of

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the ring preparation was measured under resting tension of 1 g and the ring was contracted by phenylephrine (3 μM) several times to confirm steady contraction. The complete removal of the endothelium was confirmed by the lack of relaxation following the addition of 1 μM of acetylcholine. After about 30 min of the final contraction by phenylephrine, cyanamide (30, 300 μM in final concentration) or vehicle (distilled water) was added and another 30 min elapsed for new stabilization. Vasodilators were added in a cumulative manner and relaxations were expressed as % changes, where 100% was defined as a tension range between just before application of the first concentration of vasorelaxant and resting tension (just before the final phenylephrine). The ionic composition of the Krebs–Henseleit bicarbonate solution was as follows (in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4. 1.2; $NaHCO_3$, 25; and glucose, 11.1. The solution was aerated with 95% O_2–5% CO_2 gas mixture (pH 7.4 at 37°C).

Anesthetized Rabbit Preparation Japanese white rabbits weighing 2.8–3.1 kg were fastened overnight and anesthetized with intravenous sodium pentobarbital (30 mg/kg). Cannulae were inserted into the jugular vein (for drug administration), the carotid artery (to monitor blood pressure), and the femoral artery (for blood sampling). A tachometer to measure pulse rate (PR) was triggered by the pulse waves of arterial pressure. The first control parameters and arterial blood sample were obtained after a stabilization period of 20–30 min and the latter was treated with special attention to NO_x contamination as described previously.[10,12] The exact time period from blood sampling to plasma separation was measured for each sampling and was used for correction of plasma NO_2^- concentration *in vivo* (as described below). After intragastric administration of cyanamide (1 mL/kg of 100 mg/mL solution, *i.e.*, 100 mg/kg) or vehicle (1 mL/kg of distilled water), another 60 min was allowed with additional pentobarbital for a steady-state followed by measurement of the second control parameters and sampling of the control blood specimen. Then, experimental procedures were performed after a short stabilization period. Glyceryl trinitrate or ISDN was injected through the jugular vein for 10 s followed by flush with 1 mL of saline for 10 s. Arterial blood sample was obtained after 3 min of the administration. Two other doses of the agent were administered cumulatively and the same sampling procedure was repeated.

Measurement of Plasma NO_2^- Plasma NO_2^- was measured by the highly sensitive HPLC-Griess method. The sensitivity and discrimination ability was 1 nM[13] with extensive care to avoid NO_x contamination as described elsewhere in detail.[12] In this study, only arterial plasma NO_2^- concentration was measured to estimate NO_2^- concentration *in vivo*. Because the disappearance rate of NO_2^- *ex vivo* (after sampling) is slower in arterial blood than in venous blood, error could be smaller in arterial blood when the measured NO_2^- concentration is corrected for time (from sampling to plasma separation) and the disappearance rate for estimating plasma NO_2^- concentration *in vivo*.[10] Furthermore, arterial plasma NO_2^- concentrations are considered to preferentially reflect changes (increases) in plasma NO_2^- concentration even by derangement with exogenous NO_2^- *in vivo*.[10]

Agents Cyanamide was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Injection solutions of glyceryl trinitrate (GTN, Millisrol; Nihon Kayaku Co., Tokyo, Japan), and isosorbide dinitrate (ISDN, Nitrol; Daiichi Co., Tokyo, Japan) were used. These solutions were diluted to desired concentrations with saline. All other chemicals were of analytical grade or the highest commercially available grade.

Statistical Analysis All data are expressed as mean± S.E.M. Within-group comparisons were made using the paired Student's *t*-test, and between-group comparisons were performed with the unpaired Student's *t*-test. For repeated measures, or for more than three groups, comparisons were made by one- or two-way analysis of variance (ANOVA) followed by Scheffe's method. Differences were considered statistically significant at $p<0.05$.

RESULTS

***Ex Vivo* Experiment Using Excised Aortic Ring Preparation** Excised ring preparations were divided into two major groups (GTN and ISDN groups), and each group was subdivided into three subgroups according to pretreatment (control, cyanamide 30 or 300 μM). Phenylephrine evoked tension of 2.3–4.7 g in the preparations, which was similar in the six different subgroups. Although pretreatment with cyanamide (30, 300 μM) tended to reduce phenylephrine-induced tension, there was no significant difference among the treatments (control, cyanamide 30, 300 μM, by one-way ANOVA) in both GTN and ISDN groups. As shown in Fig. 1 (left upper panel), the concentration-dependent vasorelaxation by GTN tended to be attenuated only in the higher range by 30 μM of cyanamide. Significant inhibition was observed with 300 μM of cyanamide ($p<0.05$, by two-way ANOVA) and relaxation by higher concentrations of GTN tended to be largely attenuated. The concentrations of GTN estimated to evoke 50% of relaxation (EC_{50}) in the control group (30.9±2.5 nM) and in the lower cyanamide (30 μM) group (47.6±10.7 nM) were significantly ($p<0.05$, by one-way ANOVA) smaller than that in the higher cyanamide (300 μM) group (173.2±24.4 nM). Thus, the concentration ratio due to inhibition with 300 μM of cyanamide, as evaluated by EC_{50}s in the presence and absence of the inhibitor, was 5.61 for GTN-induced vasorelaxation (Fig. 1, right panel).

Cyanamide at 300 μM caused rightward parallel shift in ISDN-induced concentration-dependent relaxation, which was significantly ($p<0.05$, by two-way ANOVA) different from the control and lower cyanamide (30 μM) groups (Fig. 1, left lower panel). The concentrations of ISDN that evoked 50% of relaxation (EC_{50}) in the control group (532±92 nM) and in the lower cyanamide (30 μM) group (323±49 nM) were significantly ($p<0.05$, by one-way ANOVA) smaller than that in the higher cyanamide (300 μM) group (1480±221 nM). The concentration ratio of inhibition by 300 μM of cyanamide, as evaluated by EC_{50}s in the presence and absence of the inhibitor, was 2.78 for ISDN-induced vasorelaxation (Fig. 1, right panel).

***In Vivo* Experiment in Anesthetized Rabbits** Rabbits were randomly divided into four groups according to pretreatments; distilled water (DW) or cyanamide (CYM) and vasodilators (GTN and ISDN). Figure 2 shows an overview of the experiment in real values. There were no significant differences in hemodynamic parameters and arterial NO_2^- concentrations among the four groups (DW+GTN, DW+ISDN, CYM+GTN and CYM+ISDN groups) before pretreatment. Pretreat-

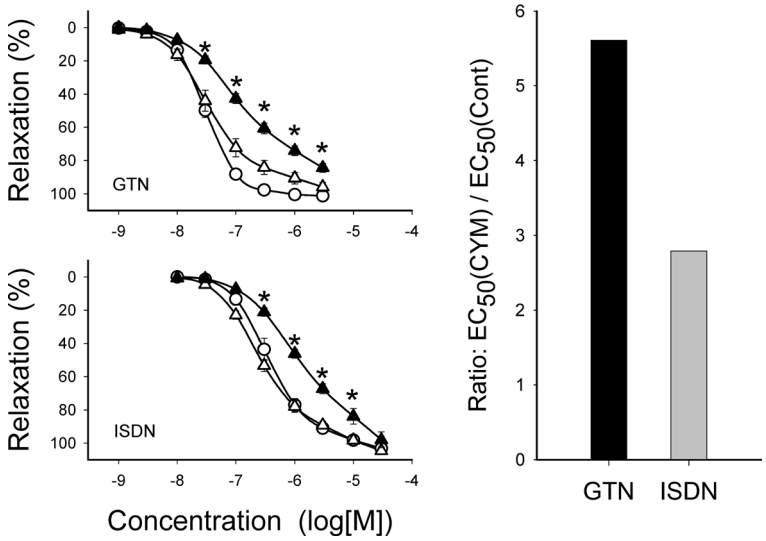

Fig. 1. Inhibitory Effects of Cyanamide on Vasorelaxation Induced by GTN and ISDN

Glyceryl trinitrate (left upper panel) or ISDN (left lower panel) was administered in cumulative manner in the absence (open circles, $n=6$) or presence of cyanamide (CYM) (open triangles: 30 μM, $n=6$, closed triangles: 300 μM, $n=6$). Only 300 μM of CYM significantly inhibited both GTN- and ISDN-induced vasorelaxation ($p<0.05$ by two-way ANOVA). The concentrations that elicited 50% relaxation (EC_{50}) were calculated and the concentration ratios of the mean value in the presence of 300 μM of CYM to the control by GTN and ISDN are also shown (right panel). *Significantly ($p<0.05$, by one-way ANOVA) different from both control (pretreatment by distilled water) and pretreatment by 30 μM of CYM.

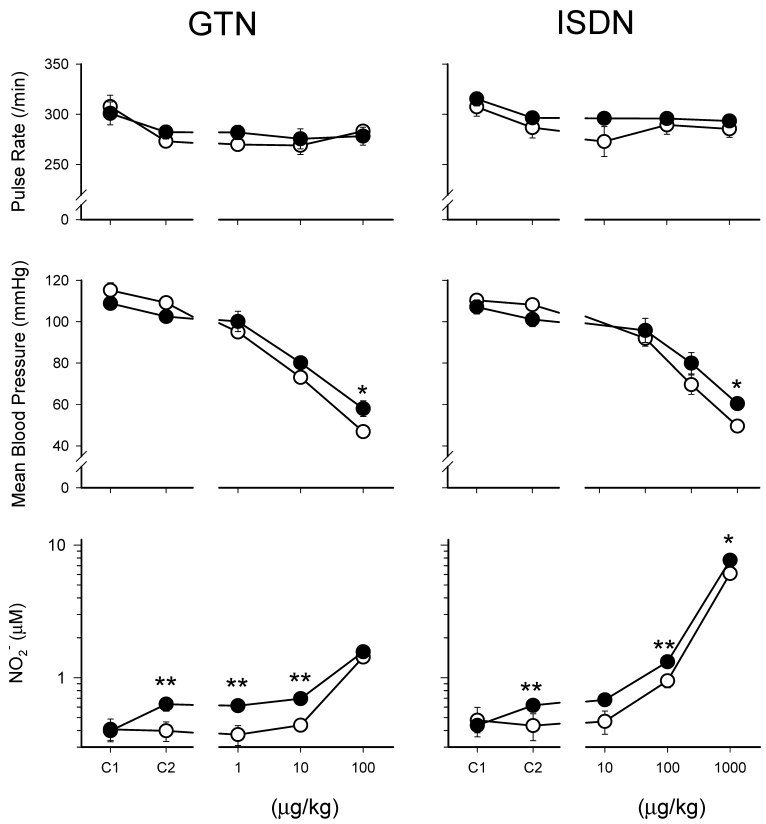

Fig. 2. Control Values before GTN or ISDN and Changes by These Agents in Anesthetized Rabbits

Control parameters obtained before pretreatment (C1: 1 mL/kg of distilled water or 1 mL/kg of 100 mg/mL CYM solution) and after 60 min of the pretreatment, just before GTN or ISDN (C2) were shown in real values in the left part. A significant differences in arterial NO_2^- concentrations between before and after pretreatment by CYM was indicated (two-way ANOVA). Real values of the minimum mean blood pressure and the corresponding pulse rate and arterial NO_2^- concentrations after each dose of GTN or ISDN were depicted in respective right parts. A significant difference by pretreatment was also indicated except for changes in pulse rate by GTN or ISDN (by two-way ANOVA). Significant changes were further analyzed by one-way ANOVA and the results were indicated in the figure. Open circles show pretreatment with vehicle (distilled water) and closed circles with cyanamide. ** and *Significantly ($p<0.01$ and 0.05, respectively) different from respective control groups. Data are mean±S.E.M. of 6 experiments in all groups.

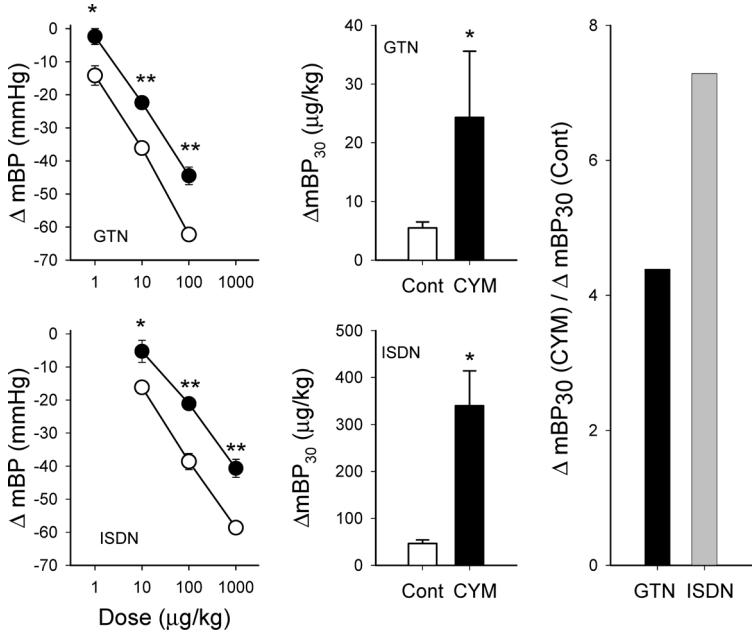

Fig. 3. Inhibitory Effects of Cyanamide on Maximum Reduction of Mean Blood Pressure by GTN and ISDN

Glyceryl trinitrate or ISDN was administered in a cumulative manner and the maximum changes in mean blood pressure (mBP) from control blood pressure was shown (left column). Cyanamide significantly inhibited both GTN- and ISDN-induced hypotension ($p<0.05$ by two-way ANOVA). These changes were further analyzed by one-way ANOVA and the results were indicated in the figure. Doses that elicit mBP reduction of 30 mmHg (ΔmBP_{30}) were calculated (middle column) and the dose ratio of the mean value in the presence of cyanamide, ΔmBP_{30} (CYM), to the vehicle control, ΔmBP_{30} (Cont), by GTN and ISDN are also shown (right column). Data are mean±S.E.M. of 6 experiments in all groups. * and ** Significantly ($p<0.01$ and 0.05, respectively) different from respective vehicle control groups (open circle or Cont).

ment with CYM did not affect PR and mean blood pressure (mBP), but increased arterial NO_2^- concentrations significantly ($p<0.05$ by two-way ANOVA). Cumulative administration of GTN and ISDN decreased blood pressure dose-dependently, but reached stable levels within 3 min. Both agents significantly ($p<0.05$ by ANOVA) decreased mBP and increased arterial NO_2^- concentrations dose-dependently, but did not affect PR.

Above significant changes were further analyzed as changes from control values. As is shown in Fig. 3, the maximum changes in mBP by both vasodilators were statistically significant ($p<0.05$ by one-way ANOVA). Pretreatment with cyanamide significantly ($p<0.05$ by two-way ANOVA) attenuated these vasodilatory effects (Fig. 3 left). Since the maximum reduction in mBP by the vasodilators was about 60 mmHg, the doses for the 30 mmHg reduction (about half of the maximum) were estimated. The GTN dose required to induce 30 mmHg reduction in the cyanamide pretreatment group was significantly ($p<0.05$ by non-paired *t*-test) greater than in the vehicle control group (Fig. 3, middle). Similarly, the dose of ISDN required for 30 mmHg reduction in the presence of cyanamide was significantly ($p<0.05$ by non-paired *t*-test) larger than in the absence of the inhibitor (Fig. 3, middle). Thus, the inhibitory ratio of cyanamide on GTN-induced vasodilation was 4.42 and that on ISDN-induced vasodilation was 7.29 (Fig. 3, right).

Because the time course of vasodilation by GTN and ISDN was not identical, the hypotensive responses were also analyzed based on the area under the curve (*AUC*) of the mBP for 3 min. Figure 4 shows significant inhibitory effects of cyanamide ($p<0.05$ by two-way ANOVA) on the hypotensive effects of both GTN and ISDN (Fig. 4, left). Since the maximum *AUC* by GTN was about −100 mmHg·min, the doses for *AUC* of −50 mmHg·min were estimated for comparison. The dose of GTN required for −50 mmHg·min in the cyanamide pretreatment group was significantly ($p<0.05$ by non-paired *t*-test) larger than in the vehicle control group (Fig. 4, middle). Similarly, the dose of ISDN required for *AUC* of −50 mmHg·min in the presence of cyanamide was significantly ($p<0.05$ by non-paired *t*-test) larger than in the absence of the inhibitor (Fig. 4, middle). Thus, the inhibitory potency of cyanamide on GTN-induced vasodilation as assessed by the dose ratio with *AUC* was 2.29 and that on ISDN-induced vasodilation was 7.68 (Fig. 4, right).

With regard to NO_2^- concentration, pretreatment with cyanamide significantly increased plasma NO_2^- concentration, while that by distilled water (vehicle) did not (Fig. 2, $p<0.05$ by two-way ANOVA). To determine the cause of the difference, total NO_2^- exogenously administered before blood sampling was estimated. The concentrations of NO_2^- in solutions were listed in Table 1 and the amounts of NO_2^- by intragastric administration (DW+GTN group: 57±7, CYM+GTN group: 53±2, DW+ISDN group: 48±1, CYM+ISDN group: 48±1 nmol/kg: NS by one-way ANOVA) were calculated. The estimated increases in NO_2^- concentration in the circulating blood (when whole blood is assumed to be 8% of body weight) after pretreatment with saline or cyanamide solution were less than 1 nM in all groups and were significantly different ($p<0.05$ by non-paired *t*-test) from the observed changes shown in the Fig. 2.

Cumulative administration of GTN and ISDN increased arterial plasma NO_2^- concentration in a dose-dependent manner (Fig. 2). Although augmentation by cyanamide was significant in both GTN- and ISDN-induced increases as assessed by real values (by two-way ANOVA), changes were further evaluated as differences from respective control values (C2 in Fig. 2) just before GTN or ISDN. After this transformation, the increases by GTN after cyanamide (6.2±18.7, 87.7±18.4 and 961.0±37.4 nM after 1, 10 and 100 μg/kg, respectively;

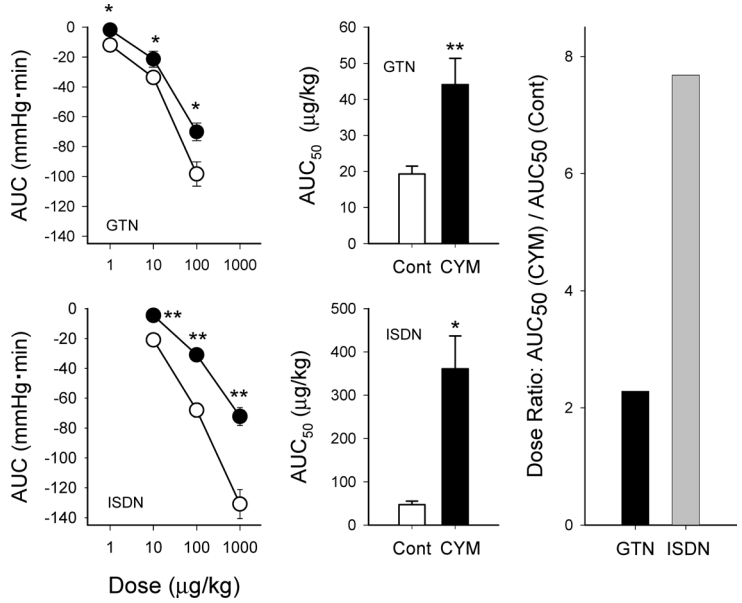

Fig. 4. Inhibitory Effects of Cyanamide on Hypotension (Evaluated as *AUC*) Induced by GTN and ISDN

Glyceryl trinitrate or ISDN was administered in a cumulative manner and the area under curve (*AUC*) below respective control blood pressure was calculated for the initial 3 min. The extent of the hypotensive effect (*AUC*) against the dose of GTN and ISDN are shown. Results after vehicle control (open circles, $n=6$) and after cyanamide (100 mg/kg; closed circles, $n=6$) are depicted. Cyanamide significantly inhibited both GTN- and ISDN-induced hypotension (left column, $p<0.05$ by two-way ANOVA). Doses that elicit -50 mmHg·min of *AUC* (AUC_{50}) were calculated (middle column) and the dose ratio of the mean value in the presence of cyanamide, AUC_{50} (CYM), to the vehicle control, AUC_{50} (Cont), by GTN and ISDN are also shown (right column). Data are mean±S.E.M. of 6 experiments in all groups. * and ** Significantly ($p<0.01$ and 0.05, respectively) different from respective vehicle control groups (open circle or Cont).

Table 1. Concentrations of NO_2^- in Injection Solutions

Groups	NO_2^- (nM)		
	Saline	DW	CYM solution
DW+GTN	58±2	38±7	
CYM+GTN	55±5		34±1
DW+ISDN	49±2	31±1	
CYM+ISDN	50±2		30±1

Nitrite concentrations of injection solutions in each group were listed. DW: distilled water, GTN: glyceryl trinitrate, CYM: cyanamide, ISDN: isosorbide dinitrate. Solutions of 1 mL/kg of saline, DW or 1 mL/kg of 100 mg/mL CYM were injected. Data are mean±S.E.M. of 6 measurements at each group.

$n=6$) were not significantly different from those after vehicle treatment (3.9±8.2, 69.4±24.2 and 1064.1±45.0 nM after 1, 10 and 100 μg/kg, respectively; $n=6$). In contrast, the increases in arterial plasma NO_2^- concentrations by ISDN after cyanamide (33.5±12.0, 511.1±18.1 and 5665.1±272.2 nM after 10, 100 and 1000 μg/kg, respectively; $n=6$) were significantly larger ($p<0.05$ by two-way ANOVA) than those after vehicle treatment (62.4±19.4, 699.0±19.9 and 7069.1±488.6 nM after 10, 100 and 1000 μg/kg, respectively; $n=6$).

The relationships between changes in arterial plasma NO_2^- concentrations and the hypotensive effect of GTN and ISDN were further analyzed. As is shown in Fig. 5, the degrees of the hypotensive effects of GTN and ISDN correlated with changes in arterial plasma NO_2^- concentrations, and cyanamide shifted this relation to the right, although the magnitudes of the shifts were different between the two hypotensive agents. The increase in arterial plasma NO_2^- concentration at the hypotensive effect of -50 mmHg·min *AUC* by GTN was significantly smaller ($p<0.05$ by unpaired *t*-test) than in the presence of cyanamide (Fig. 5, middle). Similarly, the increase in arterial plasma NO_2^- concentration at the hypotensive effect of -50 mmHg·min *AUC* by ISDN in the presence of cyanamide was significantly larger ($p<0.05$ by unpaired *t*-test) than in the absence of cyanamide (Fig. 5, middle). The calculated ΔNO_2^- ratio was 15.01 for ISDN and 3.28 for GTN (Fig. 5, right).

The results of the whole animal experiment can be summed up as follows: Pretreatment with cyanamide or vehicle (distilled water) did not affect hemodynamic parameters, but cyanamide increased plasma NO_2^- concentration. Furthermore, cyanamide attenuated the hypotensive effect of both GTN and ISDN and its inhibitory potency against these agonists as assessed by the dose ratio was 4.42 and 7.29, respectively. Cyanamide significantly enhanced a dose-dependent increase in NO_2^- concentration by ISDN, but not by GTN. However, at certain hypotensive potency, the extent of the increase in NO_2^- in the presence of cyanamide was significant for both GTN and ISDN, and was greater by ISDN (ΔNO_2^- ratio, 15.01) than that by GTN (3.28).

DISCUSSION

The major finding of the present study was that a larger inhibition of ALDH2 by cyanamide was recognized in GTN-induced vasorelaxation than in ISDN-induced vasorelaxation in excised aortic preparation, while the inverse relationship was observed in the whole animal experiment. The former is consistent with the notion presented by Münzel *et al.*[5] that GTN, a high potency nitrate, is mainly metabolized and activated by mitochondrial ALDH2 (high affinity pathway) except for its high doses (low affinity pathway), while ISDN, a low potency nitrate, is by the P-450 system in the smooth muscle endoplasmic reticulum. With regard to the *in vitro*-preparation experiment, the dominant role of ALDH2 in bioactivation of GTN, compared with ISDN, has been suggested in rat[6] and

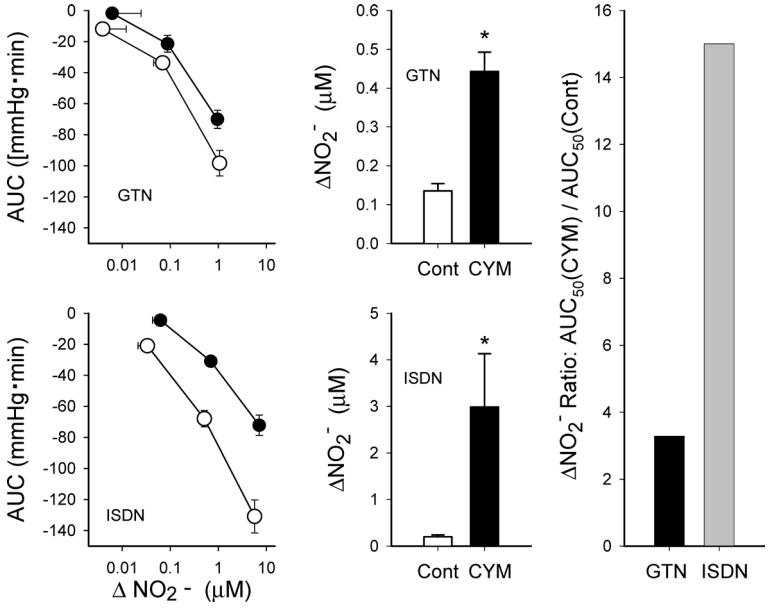

Fig. 5. Relationship between Changes in NO_2^- Concentrations and Hypotensive Effects of GTN and ISDN in the Presence or Absence of Cyanamide

The hypotensive effects (*AUC*) against changes in arterial plasma NO_2^- concentration by GTN and by ISDN are shown. Results after vehicle control (open circles, $n=6$) and after cyanamide (100 mg/kg; closed circles, $n=6$) are depicted in the left colum. Increases in arterial plasma NO_2^- concentration corresponding to -50 mmHg·min of *AUC* (AUC_{50}) were calculated (middle column) and the dose ratios of the mean values (ΔNO_2^- ratio) in the presence of cyanamide, AUC_{50} (CYM), to the vehicle control, AUC_{50} (Cont), by GTN and ISDN are also shown (right column). Data are mean±S.E.M. of 6 experiments in all groups. *Significantly ($p<0.05$) different from respective vehicle control groups (Cont).

mouse aorta.[14] And the results of our *ex vivo* study are in general consistent with the above data. However, the difference of inhibition by cyanamide between GTN- and ISDN-induced vasorelaxation was not very large (Fig. 1). It is noteworthy that human ALDH2 metabolizes ISDN to form isosorbide-2-mononitrate and the process is sensitive to ALDH2 inhibitors.[15] We should be also aware that species differences in sensitivity to both inhibitors and vasodilators (including possible difference in bioactivation) might contribute to the differences as we have previously experienced in examination of guanylate cyclase activation system in rabbit, guinea-pig and rat aorta with several types of activators and inhibitors.[16]

A conflicting result to that in excised aortic preparations was noted in anesthetized whole animal: the inhibitory effect of cyanamide on ISDN-induced hypotension was remarkable, while that by GTN was small (Figs. 3, 4). If bioactivation of GTN is largely dependent on ALDH2 in our whole animal preparation, the inhibitory effect of cyanamide could be larger in GTN than in ISDN, as observed in the excised preparation. To our knowledge, there are not so many whole animal experiments that have demonstrated different contribution of ALDH2 to bioactivation among organic nitrates. In mice, ALDH2 gene deletion is reported to largely attenuate GTN-induced hypotension, but not SNP-induced one.[14] The inhibitory effect of cyanamide has been shown against the hypotensive effect of GTN in anesthetized canine preparation, but the inhibitor was ineffective in those of sodium nitroprusside (SNP) and adenosine.[17] Similarly, it is reported that the vasodilatory effects of GTN and NO_2^- on pulmonary and systemic vascular beds of anesthetized rats were attenuated by cyanamide, while the inhibitor had no effect on SNP-induced vasodilation.[18] The doses of GTN used in the above studies correspond to the middle dose (about 10 μg/kg) that used in the present *in vivo* study. However, no direct comparison between GTN and ISDN *in vivo* with an ALDH2 inhibitor has been reported previously, although cyanamide has been used clinically for many years.[19]

Interestingly, there is a difference in human polymorphisms at codon 487 (*1, GAA=glu: *2, AAA=Lys) of ALDH2 genotypes with high activity (*1/*1) and low activity (*2/*2). The pioneering work of Mackenzie *et al.*[20] demonstrated attenuation of GTN-induced increase in human forearm blood flow in *1/*2 and *2/*2 populations and also in healthy volunteers (*1/*1) after disulfiram. It is also reported that the clinical efficacy of sublingual GTN in relieving anginal pain in *1/*1 is superior to that in *1/*2 and *2/*2 populations.[21] However, a recent sophisticated study showed that changes in arterial diameter by GTN and ISDN are not essentially different between *1/*1 and *2/*2,[22] suggesting that not only ALDH2, but also other enzymes are probably involved in GTN bioactivation.

In fact, organic nitrates have been shown to be bioactivated by several members of the vascular ALDH family.[23] Aldehyde dehydrogenase 2, ALDH3[23] and cytosolic ALDH2 (alternative splicing variant of mitochondrial ALDH2),[24] but not mitochondrial ALDH2, may be responsible for bioactivation of GTN. Isosorbide dinitrate may be bioactivated by ALDH1 (cytosolic isoform), ALDH2 (mitochondrial isoform)[15,25] and ALDH3, including ALDH3D.[23] These evidences indicate that possible different sensitivities of these enzymes to vasodilators[23,25] and inhibitors,[19] and possible different tissue contribution,[26,27] including intracellular compartments,[24] may account for our present results. Indeed, different sensitivities to different ALDH2 inhibitors have been reported: GTN bioactivation in rat liver was sensitive to chloral hydrate but not to daidzin.[28] As discussed above, our hemodynamic results are not consistent with other reports,[5] but the data presented here offer valuable information regarding systematic examination both *ex vivo* and *in vivo* in one species with the vasodilatory effect as an index.

In the present whole animal experiment, a dose-dependent decrease in blood pressure by both GTN and ISDN accompanied the dose-dependent increases in plasma NO_2^-. Although the details of GTN metabolism are not fully understood, possible pathways yielding 1,2-glyceryl dinitrate and NO_2^- have been suggested[3] and those yielding 1,2-glyceryl dinitrate and NO, part of the latter may be the source of NO_2^-, have also been reported.[4] Increases in NO and NO_2^- by ISDN have been reported not only *in vitro*,[7,15,25,29,30] but also *in vivo*,[29,30] although the possible metabolic pathways, including whether NO_2^- is a direct or indirect product, are not fully elucidated. Nevertheless, the dose-dependent increase in plasma NO_2^- with concomitant decrease in blood pressure by ISDN is in good agreement with previous studies.

The hypotensive effect of GTN was significantly inhibited by pretreatment with cyanamide, but the degree of the inhibition was significantly smaller than that by ISDN. The weak inhibitory effect of cyanamide on GTN-induced hypotension with concurrent small increase in plasma NO_2^- indicate that, in whole animal, cyanamide-insensitive bioactivation mechanism(s) could be largely operative in organs other than vascular smooth muscle. Indeed, it is reported that the formation of NO by GTN is quite larger in liver, lung and kidney than vascular tissues in rabbits[31,30] and that high doses of GTN are metabolized by several other enzymes (low affinity pathway), including glutathione-*S*-transferases, xanthine oxidoreductase, and cytochrome P450.[5] These possible mechanisms might release at least small amounts of NO_2^- or NO into the plasma where the latter is easily affected by oxidation to yield NO_2^-. When a cyanamide-sensitive bioactivation mechanism yields NO_2^- as a byproduct or NO as an active metabolite, plasma NO_2^- concentrations might not be different at certain hypotensive potency in the absence or presence of cyanamide ("ΔNO_2^- ratio" in Fig. 5 could be around 1). Again, our middle dose GTN (10 μg/kg) is similar to that at which cyanamide inhibited the hypotensive effect of GTN in a canine model,[17] which is considered within the range of high affinity pathway.

Isosorbide dinitrate may be largely bioactivated by cyanamide-sensitive mechanisms in both vascular smooth muscle and other organs. Liver, kidney and lung are considered the major organs responsible for NO production by ISDN in rabbits.[30] As was already discussed above, the ΔNO_2^- ratio (15.01 in Fig. 5) is far above 1.0, plasma NO_2^- may be derived from other pathways insensitive to cyanamide and its contribution to hypotension remains unclear.

In our study, intragastric administration of cyanamide itself increased plasma NO_2^- concentration. Cyanamide is a prodrug bioactivated by catalase and H_2O_2 to form nitrosyl cyanamide, an intermediate, which is further metabolized to cyanide and nitroxyl, the latter serving as an inhibitor of ALDH2.[19] In addition, it has been reported that NO_2^- and CO_2 are also metabolites of nitrosyl cyanamide.[32] This could be a source of the elevated plasma NO_2^- in our experiments using cyanamide. Pentobarbital is known to decrease blood pressure due to both venodilation and reduced cardiac output, evoking reflex increase in heart rate.[33,34] These hemodynamic effects may indirectly affect basal plasma NO_2^- concentration. In addition, a possible direct effect of the anesthetic agent on NO or NO_2^- metabolism may be taken into account. However, the four groups were treated in a same procedure and total doses of pentobarbital were not remarkably different. Therefore, in our experimental condition, effect of pentobarbital may be largely offset to enable interpretation of the results.

One limitation of this study might relate to the selection of rabbits as preparation and the possibility remains that the results observed here are specific to rabbits. Indeed, we have demonstrated that the rabbit aorta, compared with rat and guinea pig, is quite insensitive to guanylate cyclase inhibitors, LY83583 and methylene blue, in SNP-, but not GTN-induced vasorelaxation and cGMP accumulation.[16]

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