

Renal D-amino acid oxidase mediates chiral inversion of N^G-nitro-D-arginine

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- d) **ABBREVIATIONS:** NO, nitric oxide; NOS, nitro oxide synthase; L-NNA, N^G-nitro-L-arginine; D-NNA, N^G-nitro-D-arginine; MAP, mean arterial pressure; DAAO, D-amino acid oxidase; CEC, capillary electrochromatography; HPLC, High Performance Liquid Chromatography
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ABSTRACT

N^G -nitro-D-arginine (D-NNA), i.v. injected into rats, produced a pressor response, and was presumed to act via chiral inversion into N^G -nitro-L-arginine (L-NNA), an inhibitor of nitric oxide (NO) synthase. We examined the possible role of renal D-amino acid oxidase (DAAO) in the chiral inversion of D-NNA to L-NNA. In pentobarbital-anesthetized rats, L-NNA was detected via capillary electrochromatography (CEC) in the blood immediately after i.v. injection of D-NNA. The time course of appearance of L-NNA paralleled the increase in blood pressure elicited by D-NNA. Unilateral renal ligation partially, and bilateral ligation completely, blocked the pressor response as well as the conversion of D-NNA to L-NNA. Furthermore, injection into conscious rats of sodium benzoate, a selective D-amino acid oxidase (DAAO) inhibitor, completely blocked the pressor response to naïve D-NNA, but not pressor response to D-NNA pre-incubated with homogenates of the kidney. Homogenates of the kidneys, liver (lesser degree) and brain (much lesser degree) converted D-NNA to L-NNA and the chiral inversion was blocked by the addition of benzoate. Moreover, D-NNA chiral inversion correlates with the activity of DAAO. Our results reveal a novel pathway of chiral inversion of D-amino acids where the renal DAAO plays an essential role that accounts for the biological activity of D-NNA.

Nitric oxide (NO), a second messenger, is synthesized by NO synthase (NOS) that catalyzes the oxidation of L-arginine to form NO and L-citrulline. Certain analogs of N^G-substituted arginine such as N^G-monomethyl-L-arginine, N^G-nitro-L-arginine (L-NNA) and N^G-nitro-L-arginine methyl ester have been shown to inhibit NO synthesis and endothelium-dependent relaxation of isolated arteries and to induce sustained pressor responses in whole animals (see review by Moncada *et al.*, 1991; Wang *et al.*, 1993). It has been widely reported that the N^G-substituted arginine analogs exhibit a high degree of stereospecificity such that the L but not D enantiomers are capable of inhibiting NOS and increasing blood pressure (Moncada *et al.*, 1991; Wang *et al.*, 1993).

A number of unexpected observations have challenged this dogma. We and others have shown that N^G-nitro-D-arginine (D-NNA) is as efficacious as L-NNA in increasing blood pressure in anesthetized rats (Wang and Pang, 1990; Raszkievicz *et al.*, 1992) and conscious rats (Wang *et al.*, 1991; Wang *et al.* 1993), and in inhibiting endothelium-dependent relaxation *ex vivo* (Wang *et al.*, 1993; Cheng *et al.*, 1997). There are observations that support the notion that D-NNA is converted into an active compound *in vivo*. Pressor response to D-NNA is slower in onset than L-NNA (Wang *et al.* 1991). In addition, the potency of pressor response to D-NNA is half that of L-NNA, whereas the potency of D-NNA is only 3% that of L-NNA *in vitro* (Wang *et al.* 1993). Furthermore, pressor response to D-NNA, similar of that to L-NNA, is blocked by L-arginine but not D-arginine (Wang *et al.* 1991). These results lead to the hypothesis that D-NNA is converted *in vivo* to L-NNA (Wang *et al.*, 1993). This

postulation of *in vivo* conversion from D-NNA into L-NNA was later verified in another laboratory through the use of HPLC (Wang *et al.*, 1999). The enzymatic process and the organs responsible for the conversion remain unclear. There is, however, support that the kidney may play an important role in the chiral inversion of D-NNA into L-NNA. The *in vitro* inhibitory action of D-NNA on endothelium-dependent relaxation is negligible, but is markedly increased following the incubation of D-NNA with homogenate of the kidney (Cheng *et al.*, 1997). Furthermore, ligation of the kidney blocks the action D-NNA on blood pressure (Wang *et al.* 1999).

The aim of this study was to examine the mechanism by which D-NNA is converted into L-NNA. The chiral separation of D-NNA and L-NNA was achieved via capillary electrochromatography (CEC) that is capable of separating D- and L-amino acids with high resolution and efficiency. We examined through bioassay and chemical analysis whether kidney ligation inhibited both pressor response to D-NNA and chiral inversion. Since D-amino acid oxidase (DAAO) has been reported to catalyze D-amino acid chiral inversion in mammals (Hasegawa *et al.*, 2004), we also examined whether inhibition of the action of renal DAAO through administration of sodium benzoate would abolish chiral conversion and pressor response to naïve D-NNA and D-NNA that was previously incubated with homogenate of the kidneys. Finally, we examined whether pure DAAO and tissue homogenates from various organs including the kidneys eliminated D-NNA as well as converted it into L-NNA.

Materials and Methods

Drugs and reagents. D-NNA was obtained from Bachem Bioscience (PA, USA) and L-NNA and aspartame were from Acros Co. (Geel, Belgium). DAAO (EC 1.4.3.3 from porcine kidney) was obtained from Sigma Co. (MO, USA). All other reagents were purchased from Shanghai Chemical Reagents Co. (Shanghai, China). D-NNA and L-NNA were dissolved in 5% glucose solution, and the dissolution required 20 min ultrasonication.

Animal preparation. Male, Sprague-Dawley rats (250 - 350 g; Fudan University Medical Animal Center, Shanghai, China) were anesthetized with sodium pentobarbital (65 mg/kg, i.p.). Polyethylene cannulae (PE50, Becton Dickinson Co., USA) were inserted into the left femoral artery for the measurement of mean arterial pressure (MAP) by a pressure transducer (model YPJ01, Chengdu Instruments, Sichuan, China) and both femoral veins for the injection of drugs and the collection of blood samples. The body temperature was maintained at 37° C with a heating device connected to an electrical heated thermostat. In the kidney ligation studies, the anesthetized rats were given laparotomy followed by unilateral or bilateral ligation of the renal arteries and veins, or sham-operation. In studies involving the use of conscious rats, the vascular cannulae were tunneled subcutaneously and exteriorized at the back of the neck. The rats were given >12 h recovery from anesthesia and surgery before use.

Measurements of D-NNA and L-NNA. D-NNA and L-NNA were measured

by CEC in the chiral ligand exchange mode (Wang *et al.* 1999). Plasma samples (100 μ l) or incubation samples (100 μ l) of D-NNA or L-NNA were deproteinated through mixing for 20 min with methanol/acetone (1000 μ l; v/v = 1:1). After centrifugation at 10,000 rpm for 20 min, the organic layer was volatilized at -20° C, and the residue was dissolved in acetate buffer (pH 5.0, plasma in 100 μ l and incubation samples in 5 ml). After mixing for 1 min, an aliquot (10 nl) of the sample was injected into the CEC system (Unimicro Tech. Co., CA, USA). Cupric acetate (1 mM) and aspartame (2 mM) were dissolved in the methanol/acetate buffer (pH 5.0, v/v = 1:20) in order to allow the formation of diastereomeric pairs with D-NNA or L-NNA. The diastereomeric pairs were separated on a reverse phase C18 column (75 μ m \times 20 cm; Unimicro Tech. Ltd, Shanghai, China) at detection wavelength of 280 nm. The retention times were 17 and 19 min for L-NNA and D-NNA, respectively. The samples were quantified against standard curves that ranged from 0.025 to 0.75 mM ($r^2 > 0.98$). The between-day (n = 5) precision values measured by the time and area under the curve were 4.3% and 4.8% for L-NNA and L-NNA, respectively. The within-day precision of the quality control samples was 2% for both L-NNA and D-NNA.

Preparation of tissue homogenates. Male Sprague-Dawley rats were killed by a blow at the back of the neck. The kidneys, liver, lungs, heart, brain and skeletal muscles were removed and washed with Tris-HCl buffer (4° C, 0.1 M, pH 8.2). Tissue samples (1 g each) were homogenized in Tris-HCl buffer (3ml, pH 8.2) at 4° C with a homogenizer (Fluko Equipment Co., Shanghai, China). The homogenates

were centrifuged at 1,500 rpm for 10 min, and the supernatants were used for the determination of the activities of DAAO and conversions of D-NNA.

Tissue homogenates incubation with D-NNA. The effects of various homogenates from tissue (kidneys, liver, heart, lungs, brain and skeletal muscle, n = 5 rats each group) on the conversion of D-NNA into L-NNA were determined. The supernatants (200 μ l) of the tissue homogenate were incubated with D-NNA (200 μ l, 50 mM) dissolved in Tris-HCl buffer plus additional Tris-HCl buffer (150 μ l) in a shaking bath (700 rpm) at 37° C for 30 min. In some experiments, the homogenate was replaced with DAAO (200 μ l of 13.5 units/ml, dissolved in Tris-HCl buffer, pH 8.2). The control samples were treated similarly, except that the buffer (100 μ l) was replaced by sodium benzoate (100 μ l of 100 mg/ml), and in some cases, supernatants from the homogenate were denatured through heating (10 min at 100° C). The blank control contained the same components but with saline in place of the D-NNA solution. The incubation mixtures were centrifuged at 10,000 rpm for 15 min and filtered through Millex-GP (0.22 μ m \times 33 mm, Nihon Millipore Ltd, Japan) before injection into rats. The incubation supernatants were used for *in vivo* functional studies or *in vitro* and *ex vivo* determination of D-NNA and L-NNA by CEC.

Determination of the activity of DAAO. The activity of DAAO was determined according to the 'keto acid method' (D'Aniello *et al.*, 1996; Sarower *et al.*, 2003). The supernatants (200 μ l) of the homogenates were incubated with D-alanine (200 μ l of 0.1 M dissolved in the above Tris-HCl buffer for 30 min in a shaking bath (700 rpm) at 37° C. Afterwards, trichloroacetic acid (200 μ l of 25%) was added to

the incubated mixture, mixed again, and centrifuged at 12,000 rpm for 10 min. The supernatant (400 μ l) was mixed with 2,4-dinitrophenylhydrazine (400 μ l of 1 mM in 1 M HCl) and incubated at 37° C for 10 min. Subsequently, NaOH (800 μ l of 1.5 M) was added and mixed. The mixture was kept at room temperature for 10 min and the absorbance was read at 445 nm against a blank sample consisting of the same mixture without D-alanine. The activity of DAAO in the homogenates was quantified against the standard DAAO curves (from 3 to 54 units/ml, $r^2 > 0.97$).

Data Analyses. All results were expressed as means \pm S.E.M. and analyzed by the analysis of variance followed by Duncan's Multiple Range test. A probability of error ($P < 0.05$) was selected as the criterion of statistical significance.

Results

Effects of kidney ligation on pressor response to DNNA and chiral inversion of D-NNA in anesthetized rats. The anesthetized rats were divided into four groups ($n = 6$ in each group), namely, one with unilateral ligation and one with bilateral ligation of the renal artery and vein, and two groups with sham-operation. At 10 min after surgery, one of the sham-operated groups and both kidney-ligated (unilateral and bilateral) groups were given i.v. bolus injection of D-NNA (32 mg/kg), and the remaining sham-operated group was given the vehicle (5% glucose, 4 ml/kg). Arterial pressure was monitored for 1 hour after drug administration. Blood samples (0.2 ml) were taken at 0, 5, 10, 20, 30, 40, and 60 min after injection of the vehicle or D-NNA while MAP was monitored continuously. Plasma samples were stored at $-20\text{ }^{\circ}\text{C}$ for later analysis by CEC.

Baseline MAP was similar among the four groups of rats (92 ± 5 , 92 ± 4 , 92 ± 5 and 90 ± 4 mm Hg). In pentobarbital-anesthetized, sham-operated rats, the vehicle did not alter MAP whereas D-NNA (32 mg/kg) gradually increased MAP to a sustained level of 20 mm Hg above baseline (Fig. 1A). D-NNA caused markedly less increase in MAP in the rats with unilateral renal ligation relative to the sham-operated rats, but MAP gradually increased to the same level as that attained in the sham-operated rats. Bilateral kidney ligation completely abolished the pressor response to D-NNA.

Neither D-NNA nor L-NNA was detectable in the plasma prior to injection of

D-NNA. I.V. bolus injection of D-NNA was followed by a biphasic disposition curve in the sham-operated rats (Fig. 1B). Unilateral kidney-ligation had negligible effects on the disposition of D-NNA. Bilateral kidney ligation nearly completely blocked the beta phase of D-NNA disposition without affecting the alpha phase. These results suggest that the kidneys are the primary organs responsible for the disposition of D-NNA.

Upon injection of D-NNA into the sham-operated rats, L-NNA was detected immediately, and peak concentration (≈ 30 $\mu\text{g/ml}$) was attained at 20 min and it slowly declined to a plateau (≈ 13 $\mu\text{g/ml}$) at 40 min after injection (Fig. 1C). Unilateral kidney ligation significantly reduced the conversion of D-NNA into L-NNA; however, a similar plateau concentration of L-NNA (≈ 13 $\mu\text{g/ml}$) was reached at 40 min after injection. Bilateral kidney ligation, on the other hand, nearly completely abolished the conversion of D-NNA into L-NNA, with approximately only 3 $\mu\text{g/ml}$ of the peak plasma L-NNA level over the experiment period.

In a separate study, i.v. bolus injection of L-NNA (16 mg/kg) in the group of sham-operated, pentobarbital-anesthetized rats also elicited a pressor response (Fig. 2A). In contrast to D-NNA, pressor response to L-NNA was slightly potentiated (rather than abolished) in rats subjected to bilateral kidney ligation. Plasma L-NNA and D-NNA were measured and no trace of D-NNA was detected after injection of L-NNA at any time point (results not shown). Plasma L-NNA exhibited a similar biphasic pattern of disposition in the sham-operated rats and rats with bilateral kidney ligation (Fig. 2B). These results show that L-NNA is metabolized through a

pathway independent of the kidneys.

The area under the curve (AUC) was estimated by the trapezoidal rule. The chiral inversion rate of D-NNA into L-NNA was calculated with the formula of Pang and Kwan (1993) where $\text{Inversion rate} = [\text{AUC}_{(\text{L-NNA}) \text{ after (D-NNA)}} \times \text{dose}_{(\text{L-NNA})}] / [\text{AUC}_{(\text{L-NNA}) \text{ after (L-NNA)}} \times \text{Dose}_{(\text{D-NNA})}]$. The calculated chiral inversion rate of D-NNA into L-NNA was $50 \pm 3\%$ in the sham-operated rats and $10 \pm 1\%$ in the bilateral kidney-ligated rats over the observation period. These results suggest that kidneys account for $\approx 80\%$ of chiral inversion D-NNA *in vivo*.

Effects of sodium benzoate (the DAAO inhibitor) on the pressor response to D-NNA in conscious rats. The conscious rats were divided into four groups (3 – 6 in each group) under two treatments. One group was pretreated with i.v. bolus injection of the vehicle (5% glucose, 4 ml/kg), one group was given a low dose of sodium benzoate (80 mg/kg), and the remaining two groups were given a high dose of sodium benzoate (400 mg/kg). Sodium benzoate is a selective inhibitor of DAAO and was reported to effectively block DAAO activity at these doses *in vivo* (Moses *et al.*, 1996). At 20 min later, the vehicle-pretreated group was i.v. bolus injected with D-NNA (32 mg/kg). The low dose benzoate-pretreated group was given D-NNA (32 mg/kg), and the two high dose benzoate-pretreated groups were given the vehicle (5% glucose, 4 ml/kg) or D-NNA (32 mg/kg). Arterial pressure was monitored for 1 hour.

I.V. bolus injection of benzoate caused a very small increase in MAP that returned to the baseline 10 minutes after administration (data not shown). As shown

in Fig. 3A, i.v. bolus injection of D-NNA produced a pressor response in the vehicle-pretreated rats, with the slow onset (approximately 80% maximum effect at 30 minutes after injection). Pretreatment with benzoate (80 and 400 mg/kg) prevented the pressor response to D-NNA in a dose-dependent manner where 400 mg/kg benzoate completely abolished the pressor response.

The effect of benzoate on the pressor response to L-NNA was also tested in two groups of conscious (N = 3 in each group). As shown in Fig. 3B, i.v. bolus injection of L-NNA (16 mg/kg) in the vehicle-pretreated rats caused an increase in MAP that reached a similar maximum as did D-NNA, but with a faster onset (\approx 80% maximum after 10 min). I.V. bolus injection of sodium benzoate (400 mg/kg) did not alter the pressor response to L-NNA (16 mg/kg).

To further confirm that endogenous renal DAAO plays an essential role in the pressor responses elicited by D-NNA, the effect of kidney homogenates-incubated D-NNA on MAP was studied in rats pretreated with benzoate. Two groups of conscious rats (n = 3 - 5 each group) received i.v. bolus injection of sodium benzoate (400 mg/kg). After 20 min, the rats received i.v. bolus injection of the supernatants (4 ml/kg) from kidney homogenates incubated for 30 minutes with either the vehicle or D-NNA (equivalent to 32 mg/kg). In benzoate-pretreated rats, injection of supernatants from kidney homogenate incubated with D-NNA produced a marked and sustained pressor response of similar magnitude (Fig. 4) as that in conscious rats without benzoate pretreatment (Fig. 3A). I.V. bolus injection of supernatants from kidney homogenate incubated with the vehicle produced a much smaller increase in

MAP that may be mediated by unknown vasoactive substances contained in the homogenates (Fig. 4).

Correlation between D-NNA chiral inversion and DAAO activity. The inhibitory effect of DAAO on D-NNA conversion was first tested *in vitro* without tissue homogenates. Following 30 and 60 min incubation with DAAO (4.9 units/ml), the concentration of D-NNA (18 mM) was reduced by $\approx 46\%$ and 73% , respectively; however, no L-NNA was produced ($n = 3$ each treatment). The DAAO-mediated disappearance of D-NNA was blocked by the addition of sodium benzoate (0.13 M) (data not shown).

D-NNA conversion was measured in the homogenates of the kidneys, liver, heart, lungs, brain and muscle ($n = 5$ in each group). Incubation of D-NNA (18.2 mM) for 30 min with homogenates of the kidneys, liver, and brain resulted in the disappearance of D-NNA by 50%, 31%, and 14%, respectively (Fig. 5A). In contrast, incubation of D-NNA with homogenates of the heart, lungs and muscle did not significantly reduce the concentration of D-NNA. The reductions of the concentration of D-NNA concur with the increase in the concentration of L-NNA (Fig. 5B), and the two parameters were highly correlated ($r = 0.92$, $P < 0.05$; Fig. 5D).

Neither D-NNA reduction nor L-NNA production was detected in benzoate-treated (1.3×10^{-5} M) or heat-denatured kidney homogenates (data not shown). The activity of DAAO was highly expressed in the kidneys followed by the liver and brain, with minimal activities detected in the heart, lungs and muscle (Fig. 5C). High positive correlations were obtained between the activity of DAAO and

the disappearance of D-NNA (Fig. 5E, $r = 0.92$, $P < 0.05$), as well as between DAAO activity and L-NNA production (Fig. 5F, $r = 0.96$, $P < 0.05$).

Discussion

With advent of the chiral separation technology of CEC, it was possible to demonstrate that plasma L-NNA was produced less than 5 min (first sampling) following i.v. bolus injection of D-NNA. This chiral inversion is unidirectional as D-NNA was not produced from L-NNA. Moreover, pressor response to i.v. injection of D-NNA corresponds generally to the plasma concentration of L-NNA, but not that of D-NNA. Approximately 50% inversion ratio of D-NNA to L-NNA was obtained, which was similar to that previously estimated by blood pressure measurement (Wang *et al.*, 1991) and by HPLC (Wang *et al.*, 1999). The present results and previous findings (Wang and Pang, 1990; Wang *et al.*, 1991, 1993; Wang *et al.* 1999), indicate that D-NNA undergoes chiral inversion *in vivo* to form the active enantiomer L-NNA. Together with other reports of the conversion of D-phenylalanine ($\approx 33\%$; Lehmann *et al.*, 1983) and D-leucine ($\approx 30\%$; Hasegawa *et al.*, 2000) to the corresponding L-enantiomers, our results further suggest that chiral conversion of D-amino acids into L-amino acids is a common metabolic pathway in mammals.

However, pressor responses to L-NNA either i.v. bolus injected in a naive form or converted from D-NNA were not exactly parallel to plasma concentrations of L-NNA. For example, after bilateral kidney ligation, blood pressure did not increase in response to a small rise in plasma L-NNA levels ($\approx 5\mu\text{g/ml}$) (Fig. 1) while blood pressure remained elevated after naive L-NNA administration in rats with plasma L-NNA levels dropped to below $5\mu\text{g/ml}$ (Fig. 2). This discrepancy may be due to

that L-NNA 's pressor responses correlate with its cellular levels in the target cells/tissues rather than its plasma concentrations. Nitric oxide synthase is located in the endothelial cells and L-NNA is a positive charged amino acid derivative and does not readily pass the cell membrane. It would be reasonable to postulate that L-NNA slowly enters endothelial cells and stays in the cells for a relatively long period leading to sustained pressor responses with slow onset. The hypothesis is supported by: (1) peak time for L-NNA-induced pressor response was obtained 30 minutes after L-NNA injection although plasma concentration of L-NNA ($\approx 10\mu\text{g/ml}$) was much below that at injection time ($>300\mu\text{g/ml}$) (Fig. 2); (2) pressor response to converted L-NNA was sustained while plasma level of L-NNA dropped from $\approx 30\mu\text{g/ml}$ to $\approx 13\mu\text{g/ml}$ (Fig. 1). Hence, production of small amount of L-NNA (3-5 $\mu\text{g/ml}$) from D-NNA in bilateral kidney ligated rats would lead to trace of L-NNA entering endothelial cells and little effect in blood pressure (Fig. 1). On the other hand, once high dose of L-NNA (16 mg/kg) was administered in both sham control and bilateral kidney ligated rats, a large amount of L-NNA would presumably enter endothelial cells and subsequently produce long-lasting pressor responses when plasma level of L-NNA even dropped to as low as $\approx 3\mu\text{g/ml}$ (Fig. 2).

The occurrence of D-amino acids and DAAO in mammals has been reported in recent years although their physiological function is unclear (Pilone, 2000). It has been proposed that D-amino acids are converted to L-amino acids in two-steps that involve oxidation of the D-amino acids to the corresponding alpha-keto acids by DAAO followed by amination to L-amino acids by the action of yet unidentified

transaminase(s) (Hasegawa *et al.*, 2000). The existence of DAAO in microorganisms, mainly yeasts and bacterium, is related to the ability of yeasts to use D-amino acids for growth and bacterium to use them as constituents of the cell wall (Pilone, 2000). In this study, we have found that pure DAAO catalyzed the oxidation of D-NNA *in vitro*, leading to the elimination of D-NNA by 46% and 73% over 30 and 60 min, respectively, but not to production of L-NNA. The stereospecificity of DAAO is absolute, and is restricted to the D-enantiomer even when both D- and L-amino acids are present. DAAO displays broad substrate specificity *in vitro*, and is capable of deaminating neutral and basic D-amino acids; however, its most efficient substrates are amino acids with hydrophobic side chains (Pilone, 2000). Our results show that D-NNA is an excellent substrate for DAAO *in vitro*. Equally important is the fact that the selective inhibitor of DAAO sodium benzoate was effective in blocking the *in vitro* chiral inversion of D-NNA by homogenates of the kidneys, as well as completely blocked the pressor response of D-NNA in intact conscious rats. Moreover, there was a high correlation between the activity of DAAO and D-NNA conversion in tissues. Our results of the effectiveness of DAAO in chiral inversion of D-NNA is in agreement with the recent report of the conversion of D-Leucine to L-leucine in DAAO-intact mice (ddY/DAAO+) but not in mutant mice that lack DAAO activity (ddY/DAAO-) (Hiroshi *et al.*, 2004).

The importance of the kidneys on chiral conversion of D-NNA was shown by following observations: (1) Bilateral kidney ligation caused greater inhibition of

pressor response to D-NNA than unilateral ligation of the kidneys, as well as less disappearance of D-NNA, and greater appearance of L-NNA. These findings provide the first chemical evidence for the importance of the kidneys in chiral inversion of D-NNA. (2) Sodium benzoate abolished pressor response to naïve D-NNA but not to D-NNA preincubated with kidney homogenates thereby showing the involvement of renal DAAO in the conversion of D-NNA. (3) Incubations of D-NNA with homogenates of the kidneys caused markedly greater disposition of D-NNA and production of L-NNA than homogenates of the liver, which in turn, has greater activity than homogenates of the brain, heart, lungs and skeletal muscle. (4) The activity of DAAO is highest in the kidneys followed by the liver and brain, in accordance with reports of greater expression of DAAO in the kidneys (Konno *et al.*, 1997; Pilone, 2000; Hasegawa *et al.*, 2004). Taken together, it is concluded that the renal DAAO plays an essential role in the chiral inversion of D-amino acids and accounts for approximately 80% D-NNA conversion in the body. The kidney has also been shown to be the principle organ for the chiral inversion of D-leucine (Hasegawa *et al.*, 2004).

With the recent advent of chromatographic chiral separation and detection, it has become clear that many chiral pharmaceuticals undergo chiral inversion in the body. Such drugs include 2-arylpropionic acid analogs (Drummond *et al.*, 1990), the new quinoxaline topoisomerase poison 2-[4-(7-chloro-2-quinoxalinyloxy)phenoxy] propionic acid (Zheng *et al.*, 2002), and thalidomide (Eriksson *et al.*, 1998). The metabolic chiral inversion of 2-arylpropionic acids such as ibuprofen and ketoprofen

(non-steroidal anti-inflammatory agents) has been well characterized. Chiral inversion is normally unidirectional in various species including humans, e.g., from R to S inversion for profens, whereas S to R inversion is rare (Jamali *et al.* 1997). The long-chain acyl-coenzyme A (CoA) synthetase and 2-arylpropionyl-CoA epimerase have been identified as key enzymes involved in the chiral inversion of 2-arylpropionic acids (Reichel *et al.*, 1995, 1997; Brugger *et al.*, 1996). Epimerase mRNA and protein are strongly expressed in the rat liver and kidneys, and weakly expressed in heart and brain (Reichel *et al.*, 1995, 1997). Thus, the chiral inversion process for D-amino acids to L-enantiomers is distinctly different from that for 2-arylpropionic acids.

In conclusion, L-NNA was detected and pressor response is observed soon after injection of D-NNA. Chiral inversion and pressor responses to D-NNA were blocked by kidney ligation ($\approx 80\%$) and following injection of the selective DAAO blocker benzoate (completely). However, benzoate did not block pressor response to D-NNA that had been pre-incubated with homogenate of the kidneys. The pure DAAO disposed D-NNA but did not produce L-NNA, while homogenates of the kidneys, liver (lesser degree) and brain (much lesser degree) disposed of D-NNA while producing L-NNA at the same time. Moreover, D-NNA chiral inversion correlates with the activity of DAAO. Thus, renal DAAO plays an essential but not sufficient role in chiral inversion of D-amino acids.

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Footnotes

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Legends for figures

Fig. 1. Mean arterial pressure (MAP, A) and plasma concentrations of N^G-nitro-D-arginine (D-NNA, B) and N^G-nitro-L-arginine (L-NNA, C) after i.v. bolus injection of the vehicle (5% glucose, 4 ml/kg) or D-NNA (32 mg/kg) in four groups of pentobarbital-anesthetized rats (N = 6 per group). D-NNA and L-NNA were measured by capillary electrochromatography at the chiral ligand exchange mode. All readings are means ± S.E.M. △, vehicle response in sham-operated rats; ▲, D-NNA in sham-operated rats; □, D-NNA in unilateral kidney-ligated rats; ■, D-NNA in bilateral kidney-ligated rats. *significantly different (P < 0.05) from respective values in the sham-operated group given D-NNA.

Fig. 2. Mean arterial pressure (MAP) (A) and plasma concentration of N^G-nitro-L-arginine (L-NNA, B) after i.v. bolus injection of L-NNA (16 mg/kg) in pentobarbital-anesthetized rats (n = 3 each group). L-NNA and N^G-nitro-D-arginine (D-NNA) were measured by capillary electrochromatography at the chiral ligand exchange mode. D-NNA was not detected over the observation period after injection of L-NNA. ○, L-NNA in sham-operated rats; ●, L-NNA in bilateral kidney-ligated rats. *significantly different (P < 0.05) from respective values in the sham-operated group given L-NNA.

Fig. 3. Effects of sodium benzoate (inhibitor of D-amino acid oxidase) on mean arterial pressure (MAP; means ± S.E.M) response to i.v. bolus injection of

N^G -nitro-D-arginine (D-NNA, A) or N^G -nitro-L-arginine (L-NNA, B) in six groups of conscious rats. The rats were i.v. injected with the vehicle (5% glucose, 4 ml/kg) or sodium benzoate (80 or 400 mg/kg) followed 20 min later by i.v. injection the vehicle (5% glucose, 4 ml/kg), D-NNA (32 mg/kg) or L-NNA (16 mg/kg). □, benzoate + vehicle (n =3); ■, vehicle + D-NNA (n =3); △, benzoate (80 mg/kg) + D-NNA (n =4); ▲, benzoate (400 mg/kg) + D-NNA (n =6); ○, vehicle + L-NNA (n =3); ●, benzoate (400 mg/kg) + L-NNA (n =3). *significantly different ($P < 0.05$) from respective values in the group given vehicle + D-NNA.

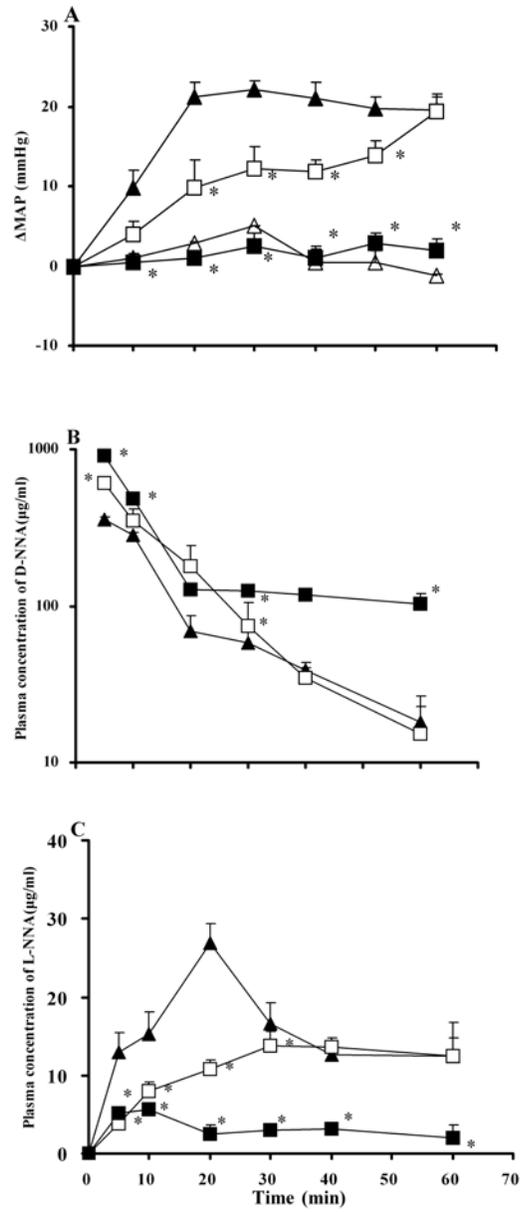
Fig. 4. Mean arterial pressure (MAP, means \pm S.E.M.) response to i.v. bolus injection of supernatants from kidney homogenates incubated with either the vehicle (5% glucose, △) or N^G -nitro-D-arginine (D-NNA, equivalent to 32 mg/kg, ▲) in two groups of conscious rats pretreated for 20 min with sodium benzoate (400 mg/kg, inhibitor of D-amino acid oxidase, n = 3 each group). *Significantly different ($P < 0.05$) from respective values in the group given supernatant from vehicle-incubated kidney homogenate.

Fig. 5. Decrease of N^G -nitro-D-arginine (D-NNA, A), Increase of N^G -nitro-L-arginine (L-NNA, B) and the activity of D-amino acid oxidase (DAAO, C) at 30 min after incubation of D-NNA (18.2 mM) with homogenates of the heart, liver, lungs, kidneys, brain and skeletal muscle (n = 5 rats per tissue). All readings are means \pm S.E.M. Increase of L-NNA was expressed as percentage of D-NNA where

1 unit of L-NNA was considered to be derived from 1 unit of D-NNA. Correlations between the rate of disappearance of D-NNA and the rate of appearance of L-NNA (D), the rate of disappearance of D-NNA and the activity of DAAO (E), and the rate of appearance of L-NNA and activity of DAAO (F).

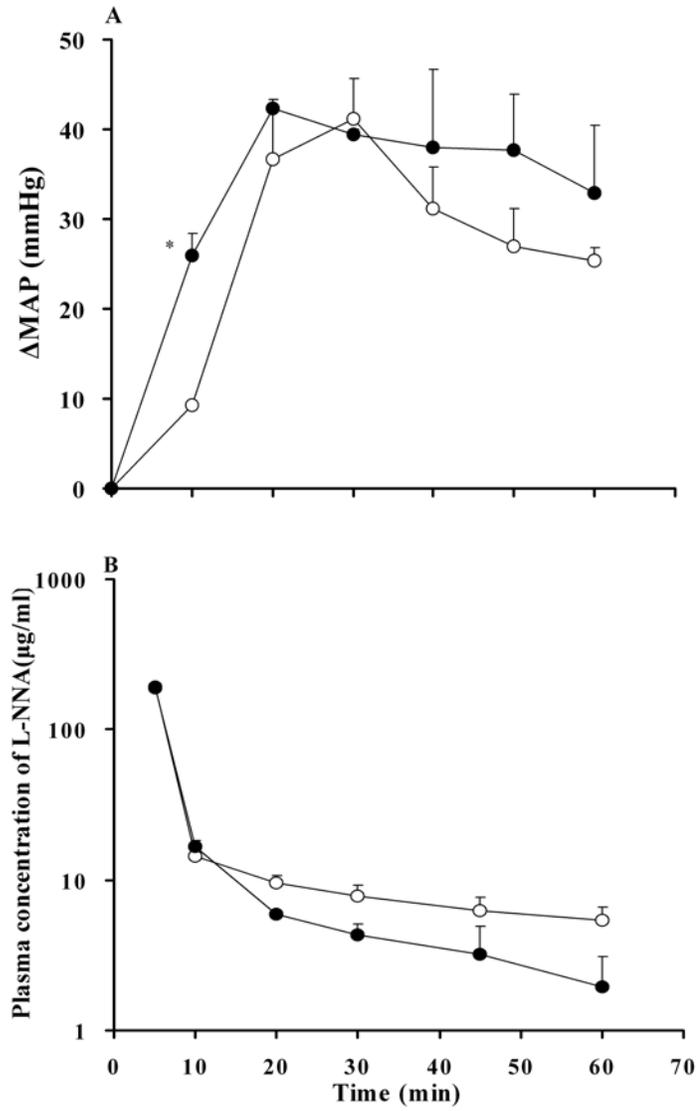
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Fig 1



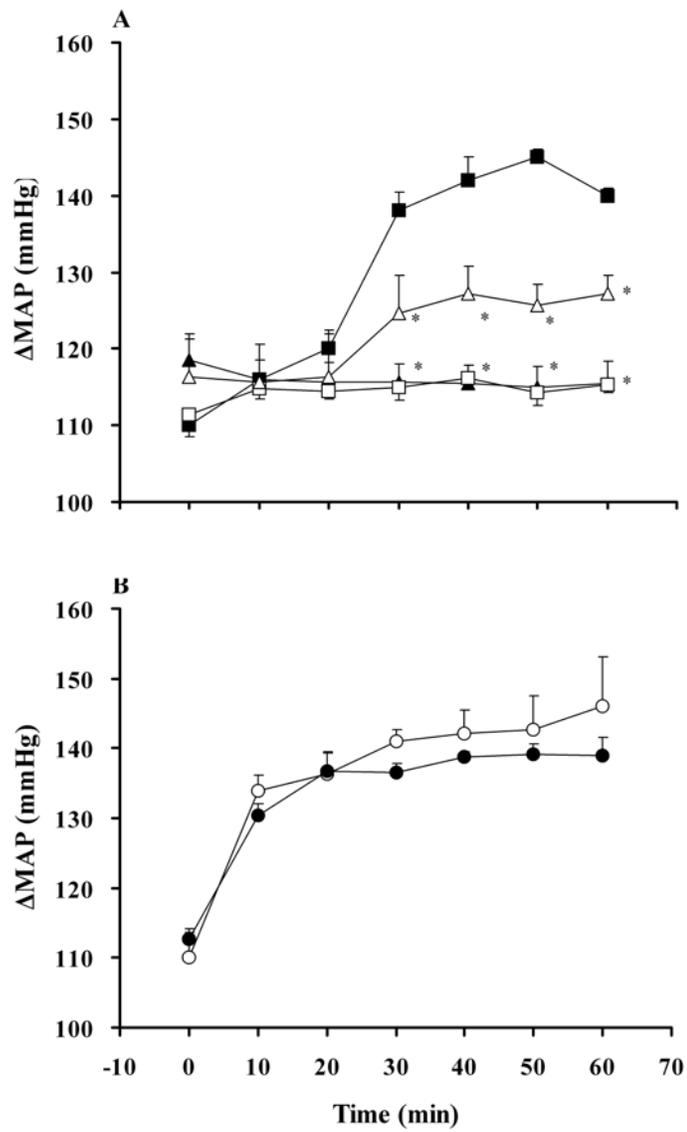
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Fig 2



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Fig 3



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Fig 4

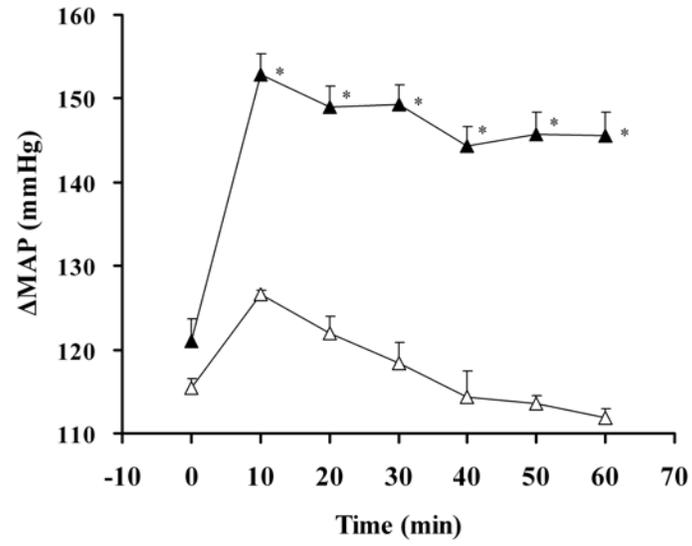


Fig 5

