The In Vivo Unidirectional Conversion of Nitro-D-arginine to Nitro-L-arginine

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ABSTRACT
We recently reported that nitric oxide synthase in the brain can be inhibited not only by nitro-L-arginine (L-NA) but also by its D-enantiomer nitro-D-arginine (D-NA). In the present study, we found that D-NA, when tested in vitro, was 400 times less potent than L-NA. However, when D-NA was injected in vivo, its L-enantiomer, L-NA, was found to rapidly appear in plasma samples (~1 min), rose to a maximum concentration at 30 min (~40% conversion), and remained at this plateau for about 5 h. This was consistent with the changes in blood pressure. There was no conversion of L- to D-NA. The results suggested that D-NA has very weak biological actions by itself, but when administered in vivo, D-NA can be converted to L-NA.

Nitric oxide synthase (NOS), an enzyme widely expressed in mammals, produces nitric oxide (NO) by the oxidation of L-Arg to NO and citrulline (Palmer et al., 1988; Moncada et al., 1991). Nitro-L-arginine (L-NA) is one of a group of known inhibitors of NOS used experimentally to examine the role of NO (Mulsch et al., 1989; Moore et al., 1990; Hobbs and Gibson, 1990; Rees et al., 1990; Tucher et al., 1990). The enantiomer, nitro-D-arginine (D-NA), is assumed not to affect NOS activity and is often used as a placebo in studies involving L-NA (Moore et al., 1990; Hobbs and Gibson, 1990; Tucher et al., 1990). There is evidence, however, that following systemic administration, D-NA elicits pharmacological responses similar to those seen after L-NA treatment (Wang et al., 1991, 1993; Wang et al., 1994; Theard et al., 1995; Cheng et al., 1996). When administered in sufficiently high doses, D-NA has been shown to increase arterial pressure (Wang et al., 1991; Wang et al., 1994; Theard et al., 1995; Cheng et al., 1996) and decrease the bicuculline-induced seizure threshold in rats (Wang et al., 1994; Theard et al., 1995). Compared to L-NA, D-NA has a slower onset and weaker effect (Wang et al., 1991, 1993, 1994; Theard et al., 1995; Cheng et al., 1996). L-Arg, the natural substrate for NOS, reverses the in vivo effect of both L-NA and D-NA (Wang et al., 1991; Cheng et al., 1996). This reversal of action is not seen with the unnatural isomer, D-Arg (Wang et al., 1991; Cheng et al., 1996). Thus, it appears that both L-NA and D-NA act on NOS by a common mechanism. Because the activity was not due to contamination of D-NA with L-NA (Wang et al., 1991, 1994), we hypothesized that it resulted either from the intrinsic activity of D-NA or from the conversion of D-NA to a compound, such as L-NA, that is active against NOS.

Materials and Methods
All experiments were performed on male Sprague-Dawley rats (300–380 g, Charles River) according to Institutional Use of Animals in Research guidelines. To examine whether D-NA has intrinsic activity similar to L-NA, we measured the ability of L-NA or D-NA to inhibit the conversion of L-[14C]arginine to [14C]citrulline in rat brain homogenates (Bredt and Snyder, 1989). Fourteen rats were anesthetized with halothane and decapitated. Cerebral cortical tissue samples were obtained and frozen. After weighing, the individual frozen samples were homogenized for 30 s in 500 μl of 50 mM HEPES (pH 7.4) and 1 mM EDTA (pH 7.4). The homogenate was then centrifuged at 2000 to 3000 rpm for 5 min at 4°C and the supernatant (25 μl) was aliquoted into multiple vials. To each aliquot of brain homogenate we added 15 μl of either D-NA (concentration range 3 x 10⁻⁶–10⁻² M) or L-NA (concentration range 3 x 10⁻⁶–10⁻⁵ M) dissolved in reaction buffer. Control samples received reaction buffer only. Homogenates from seven rats each were used for D-NA or L-NA evaluations. Radioactive L-[14C]arginine (25 μl, 1 mCi/ml) and 85 μl of reaction buffer (50 mM HEPES, 1 mM EDTA, 1 mM reduced form of β-NADPH, and 1 mM Ca²⁺, pH 7.4) was added to each sample. The mixture was incubated at room temperature for 10 min and the reaction was stopped by the addition of a solution of 20 mM HEPES and 2 mM EDTA, pH 5.5. The reaction mixture was applied to a column containing Dowex AG50WX-8 (Na⁺ form; Bio-Rad, Richmond, CA). [14C]Citrulline was eluted with 2 ml of distilled water and determined by liquid scintillation spectrometry. Inhibition of NOS activity (conversion of L-[14C]arginine to [14C]citrulline) was determined by comparing NOS activity measured in L-NA- or D-NA-containing samples.

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ABBREVIATIONS: NO, nitric oxide; NOS, NO synthase; D-NA, nitro-d-arginine; L-NA, nitro-l-arginine.
treated samples to the activity of the untreated controls. Data were analyzed and EC$_{50}$ determined using the curve fitting program, Inplot (Graphpad Software, San Diego).

To evaluate the conversion of d-NA to l-NA in vivo, anesthetized rats were tracheotomized and mechanically ventilated with 1% halothane and 70% N$_2$O/30% O$_2$. Following insertion of bilateral femoral arterial and venous catheters, d- or l-NA (50 mg/kg dissolved in 3 ml of saline) was infused over 1 min and blood samples and blood pressure were obtained at 1, 5, 10, 20, 30, and 45 min and 1, 2, 3, 4, 5, and 24 h after completion of drug infusion, respectively. Concentrations of d- and l-NA in plasma were determined by high-performance liquid chromatography (HPLC). Samples were extracted by ultrafiltration. Rat plasma (100 µl) was placed into a Centrifree micropartition device (Amicon Inc., Beverly MA) and centrifuged at 1000g for 20 min. An aliquot (10 µl) of the collected extract was injected into the HPLC system and quantitated against standard curves ranging from 5 to 200 µg/ml d- or l-NA. d- and l-NA were measured by HPLC in the ligand exchange mode (Gilon et al., 1979). The mobile phase of cupric acetate (0.1 mM) and Asp-Phe methyl ester (aspartame, 0.2 mM), adjusted to pH 5.5 with acetate buffer, formed diastereomeric pairs with the d- and l-NA. The diastereomeric pairs were separated on a Nova-Pak C$_{18}$ column (3.9 × 150 mm, 4-µm particle size, stainless steel; Waters Associates, Milford, MA). The flow rate was 1 ml/min. The eluting analytes were detected by the UV absorbance of the nitro group at 280 nm. Retention times were immediately detectable in plasma (Figs. 2 and 3). D-NA was detectable immediately following 50-mg/kg i.v. injections. The parent compounds of d- and l-NA were measured by HPLC in the ligand exchange mode (Gilon et al., 1979). The mobile phase of cupric acetate (0.1 mM) and Asp-Phe methyl ester (aspartame, 0.2 mM), adjusted to pH 5.5 with acetate buffer, formed diastereomeric pairs with the d- and l-NA. The diastereomeric pairs were separated on a Nova-Pak C$_{18}$ column (3.9 × 150 mm, 4-µm particle size, stainless steel; Waters Associates, Milford, MA). The flow rate was 1 ml/min. The eluting analytes were detected by the UV absorbance of the nitro group at 280 nm. Retention times were immediately detectable in plasma (Figs. 2 and 3). D-NA and l-NA were measured by HPLC in the ligand exchange mode (Gilon et al., 1979). The mobile phase of cupric acetate (0.1 mM) and Asp-Phe methyl ester (aspartame, 0.2 mM), adjusted to pH 5.5 with acetate buffer, formed diastereomeric pairs with the d- and l-NA. The diastereomeric pairs were separated on a Nova-Pak C$_{18}$ column (3.9 × 150 mm, 4-µm particle size, stainless steel; Waters Associates, Milford, MA). The flow rate was 1 ml/min. The eluting analytes were detected by the UV absorbance of the nitro group at 280 nm. Retention times were immediately detectable in plasma (Figs. 2 and 3).

Pharmacokinetics was calculated from the determined plasma concentrations using compartmental and noncompartmental analysis. The L-NA and d-NA serum concentration-time data were fitted by nonlinear least-squares regression analysis (Seber, 1989) to a two-compartment i.v. bolus model for each rat. Data were weighted by the UV absorbance of the nitro group at 280 nm. Retention times were immediately detectable in plasma (Figs. 2 and 3). D-NA and l-NA were measured by HPLC in the ligand exchange mode (Gilon et al., 1979). The mobile phase of cupric acetate (0.1 mM) and Asp-Phe methyl ester (aspartame, 0.2 mM), adjusted to pH 5.5 with acetate buffer, formed diastereomeric pairs with the d- and l-NA. The diastereomeric pairs were separated on a Nova-Pak C$_{18}$ column (3.9 × 150 mm, 4-µm particle size, stainless steel; Waters Associates, Milford, MA). The flow rate was 1 ml/min. The eluting analytes were detected by the UV absorbance of the nitro group at 280 nm. Retention times were immediately detectable in plasma (Figs. 2 and 3). D-NA and l-NA were measured by HPLC in the ligand exchange mode (Gilon et al., 1979). The mobile phase of cupric acetate (0.1 mM) and Asp-Phe methyl ester (aspartame, 0.2 mM), adjusted to pH 5.5 with acetate buffer, formed diastereomeric pairs with the d- and l-NA. The diastereomeric pairs were separated on a Nova-Pak C$_{18}$ column (3.9 × 150 mm, 4-µm particle size, stainless steel; Waters Associates, Milford, MA). The flow rate was 1 ml/min. The eluting analytes were detected by the UV absorbance of the nitro group at 280 nm. Retention times were immediately detectable in plasma (Figs. 2 and 3). D-NA and l-NA were measured by HPLC in the ligand exchange mode (Gilon et al., 1979). The mobile phase of cupric acetate (0.1 mM) and Asp-Phe methyl ester (aspartame, 0.2 mM), adjusted to pH 5.5 with acetate buffer, formed diastereomeric pairs with the d- and l-NA. The diastereomeric pairs were separated on a Nova-Pak C$_{18}$ column (3.9 × 150 mm, 4-µm particle size, stainless steel; Waters Associates, Milford, MA). The flow rate was 1 ml/min. The eluting analytes were detected by the UV absorbance of the nitro group at 280 nm. Retention times were immediately detectable in plasma (Figs. 2 and 3). D-NA and l-NA were measured by HPLC in the ligand exchange mode (Gilon et al., 1979). The mobile phase of cupric acetate (0.1 mM) and Asp-Phe methyl ester (aspartame, 0.2 mM), adjusted to pH 5.5 with acetate buffer, formed diastereomeric pairs with the d- and l-NA. The diastereomeric pairs were separated on a Nova-Pak C$_{18}$ column (3.9 × 150 mm, 4-µm particle size, stainless steel; Waters Associates, Milford, MA). The flow rate was 1 ml/min. The eluting analytes were detected by the UV absorbance of the nitro group at 280 nm. Retention times were immediately detectable in plasma (Figs. 2 and 3).

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Results

The in vitro experiments demonstrated that both d-NA and l-NA cause dose-dependent inhibition of brain NOS activity (Fig. 1). The EC$_{50}$ of d-NA was $2.0 \times 10^{-4}$ M compared to $5.0 \times 10^{-7}$ M for l-NA. Thus, d-NA was 400 times less potent than l-NA in this in vitro test. Because d-NA shows much greater activity in vivo, when it is almost half as potent as l-NA (Wang et al., 1991), the pharmacological response to administered d-NA must result from something other than the intrinsic activity of d-NA on NOS.

We evaluated the pharmacokinetics of both l-NA and d-NA following 50-mg/kg i.v. injections. The parent compounds were immediately detectable in plasma (Figs. 2 and 3). d-NA was 400 times less potent than l-NA (calculated from EC$_{50}$).

![Fig. 1. In vitro dose-dependent inhibition of brain NOS activity by n-NA or L-NA. n-NA was 400 times less potent than L-NA (calculated from EC$_{50}$).](image-url)
had a significantly faster CL (0.58 ± 0.09 versus 0.08 ± 0.02 liters/h/kg) and shorter $T_{1/2}$ (1.0 ± 0.6 versus 13.4 ± 2.8 h) than L-NA. D-NA was almost undetectable in plasma by 5 h after dosing (Fig. 2), whereas L-NA was still present at 24 h (Fig. 3). In the rats that were administered D-NA (Fig. 2), L-NA quickly appeared in the plasma, having a mean lag time of 3.0 ± 1.8 min. L-NA reached a maximum concentration at approximately 1 h and remained at this plateau for at least 5 h (Fig. 2). Plasma concentrations of L-NA following D-NA administration were 12 ± 2 μg/ml at 1 h and 10 ± 2 μg/ml at 5 h. Following L-NA administration, corresponding values were 45 ± 5 and 40 ± 5 μg/ml. The half-life of L-NA during this phase was comparable following D-NA or L-NA administration (18.0 h after D-NA administration versus 19.6 h after L-NA). After 2 h following D-NA administration, plasma levels of L-NA began to exceed those of D-NA. By 5 h, D-NA was virtually undetectable, whereas significant levels of L-NA remained in both the L-NA- and D-NA-treated rats (Figs. 2 and 3). Estimates of the conversion of D-NA to L-NA (see Materials and Methods) yielded a value of approximately 40%. Administration of L-NA produced no measurable concentration of D-NA in the plasma.

The rise in blood pressure closely mirrored the measured concentrations of L-NA in the rat plasma. Following acute i.v. administration of L-NA, the rats showed a rapid increase in mean arterial blood pressure (MABP), reaching 30 to 40 mm Hg above baseline levels approximately 10 min after injection and remaining at that level during the experiment period (Fig. 3). After D-NA administration, the blood pressure rose more slowly. One hour after injection, MABP reached a level above baseline comparable to that seen in L-NA-treated rats with no further decrease over the 3-h experimental period (Fig. 2). Because the duration of effect parallels the pharmacokinetics of L-NA better than that of D-NA, it is quite apparent that the cardiovascular activity seen following D-NA administration is due to its conversion to L-NA.

In vitro experiments showed that D-NA is a substrate for D-AAO. After a 16-h incubation with D-AAO, the measured amount of D-NA had decreased by about two-thirds. No change in the D-NA concentration of the control sample was observed. D-NA is therefore a substrate for the first of two enzymes necessary for the conversion of D-NA to L-NA.

**Discussion**

It is generally accepted that NO is enzymatically synthesized from L-Arg, but not D-Arg, and that NOS can be inhibited by certain L but not D-Arg analogues including NA (Moore et al., 1990; Hobbs and Gibson, 1990; Tucher et al., 1990; Moncada et al., 1991). Those assumptions appeared to be contradicted by recent reports from our laboratory and others showing that systemic administration of D-NA elicited physiologic changes of a magnitude approaching that seen with comparable doses of L-NA (i.e., increased arterial pressure, reduced brain NOS activity [Wang et al., 1991, 1993, 1994; Theard et al., 1995; Cheng et al., 1996]). Of the three most likely explanations for such actions of D-NA—contamination of D-NA preparations by L-NA, direct pharmacological activity of D-NA, and conversion of D-NA to L-NA in vivo—only the third appears to have any merit. That is, the D-NA preparations used were devoid of any L-NA contamination (Wang et al., 1991, 1994), and D-NA itself, as demonstrated in the present study and others (Wang et al., 1993), has very weak biological actions. We found that it is 400 times less potent than L-NA in inhibiting brain NOS activity in vitro. Pharmacokinetic analysis indicated that about half of administered D-NA was converted to L-NA. Thus, it is apparent that the physiologic effects of D-NA we observed previously were due to the conversion of D- to L-NA in vivo.

Failure of previous studies to observe pharmacologic responses to D-NA can be attributed to the use of low equimolar doses of D- and L-NA (Rees et al., 1989; Moore et al., 1990; Hobbs and Gibson, 1990; Tucher et al., 1990). The L-NA derived from D-NA has a lower bioavailability and requires more time to achieve maximum plasma concentration compared to directly administered L-NA. Thus, at least 2-fold greater doses of this compound and longer observation times are required to observe responses similar to those seen with L-NA. Our bioavailability results of 40% agree well with previous findings showing that the ED$_{50}$ of D-NA for increasing blood pressure in rats was 2.5 times that of L-NA (Wang et al., 1991).

D-amino acids are present in plants and various classes of marine and terrestrial animals. Because mammals can only use L-amino acids, not D-amino acids, to synthesize proteins, the in vivo conversion of D-NA to L-NA is not unexpected. Furthermore, there is no barrier in animals against intestinal absorption of D-amino acids. If the ingested D-amino acids are not metabolized, they may accumulate in tissues and could interfere with normal protein synthesis. This includes the synthesis of essential enzymes, such as glutamate oxaloacetate transaminase, glutamic pyruvic transaminase, and lactate dehydrogenase (D'Aniello et al., 1993a). However, mammals do possess a capacity for conversion of D-amino acids.
acids to L-amino acids. The enzymes responsible for that conversion, the D-amino acid oxidases (Krebs, 1935), are widely distributed in mammalian tissues, especially in the liver. These enzymes are capable of oxidizing multiple D-amino acids, including D-Arg (D’Aniello et al., 1993b). D-amino acids are converted to D-enantiomers in mammals in a two-step process involving the oxidation of the D-amino acids to the corresponding α-keto acids by D-amino acid oxidase followed by amination to the L-amino acid by the action of a transaminase (Krebs, 1935; Meister, 1955). D- to L-amino acid conversions may also occur via racemases. These enzymes are not endogenously produced in mammals but may be present in contaminating bacteria. However, the unidirectional conversion of D-NA to L-NA seen in the present study is unlikely to be attributable to the action of a racemase, because racemases catalyze L. and D-enantiomer interconversions in both directions (Yorifuji and Ogata, 1971).

The question remains as to whether all experimentally used D-Arg analogs, as well as D-Arg itself, are influenced equally by D-amino acid oxidase actions. The literature shows that D-Arg is indeed a substrate for D-amino acid oxidase (D’Aniello et al., 1993b). That does not automatically permit one to assume that D-Arg analogues will be oxidized at the same rate, if at all. In fact, one might expect that the more natural substrate for D-amino acid oxidase, D-Arg, would be affected to a greater extent by oxidase action. In the present study, we found that D-NA is a substrate for D-amino acid oxidase in vitro. The rate of D-NA oxidation (67% reduction in D-NA levels over 16 h) was much slower than the rate of D-NA conversion to L-NA seen in the in vivo experiments. However, the present in vitro system clearly is not representative of conditions present in vivo. For example, one might consider the possibility that D-amino acid oxidase activity may be subjected to product (i.e., α-keto acid) inhibition. In the presence of the transaminase (the in vivo situation), the α-keto acid might be rapidly removed. In the experiments we conducted, the transaminase was absent, allowing the α-keto acid to accumulate. Whether this scenario (or others) is applicable in the present study is uncertain. Thus, until the appropriate experiments are performed, no adequate explanation for the differences in the rates of D-NA loss seen in the in vivo vs in vitro situation can be offered.

In conclusion, D-NA has very weak intrinsic pharmacological activity, but when given systemically in vivo, D-NA can be converted to L-NA. The assumption that D-Arg analogues do not affect NOS activity has provided the basis for their use as control agents in experiments using L-Arg analogs to inhibit NOS. However, based on present findings, that assumption does not appear to be always correct. Thus, caution must be exercised when interpreting data related to systemic administration of D-Arg analogs in vivo.

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References

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