Nitric-oxide synthase (NOS) catalyzes the oxidation of L-arginine to citrulline and nitric oxide (NO). The enzyme is inhibited by a variety of N^6-monosubstituted L-arginine analogs, and some of these compounds are useful in reversing pathologies associated with the overproduction of NO (e.g., the hypotension of septic shock). We report here that L-thiocitrulline (γ-thioureido-L-norvaline) is a potent, stereospecific inhibitor of the constitutive brain and endothelial isoforms of NOS as well as the isoform induced in vascular smooth muscle cells by lipopolysaccharide and interferon-γ. Steady state kinetic studies show L-thiocitrulline inhibition is competitive with L-arginine (K_i 4–20% of K_m), indicating that initial binding is as a substrate/product analog. In contrast to L-arginine and N^6-methyl-L-arginine, the prototypic NOS inhibitor, L-thiocitrulline binding elicits a "Type II" difference spectrum, indicating a high spin to low spin transition of the iron in the heme cofactor. This finding suggests that L-thiocitrulline is contributing the sixth ligand to heme iron, probably through the thioureido sulfur. Such interaction with heme iron neither stimulates nor inhibits the direct flavin-mediated cytochrome c reduction activity of the enzyme, but it does inhibit heme-dependent superoxide formation. In vivo, L-thiocitrulline is a potent pressor agent in both normal and endotoxenic rats, the latter finding suggesting utility in treating the hypotension of septic shock.

Although there is only modest sequence homology among the three NOS isoforms (50–60%) (7, 8), all have a C-terminal domain that is somewhat homologous to cytochrome P-450 reductase (7, 9) and an N-terminal domain that is hypothesized to have cytochrome P-450-like activity (10). Consistent with this view, the C-terminal domain has sequences suggesting binding sites for NADPH and the required cofactors FAD and FMN; the N-terminal domain binds heme and arginine (9–11). Mechanistic studies suggest that catalysis by all isoforms involves two successive monoxygenase reactions, the first a ferryl heme-dependent hydroxylation of a guanidino nitrogen of arginine yielding NOH-Arg and the second a nucleophilic attack by peroxo heme on the guanidino carbon of NOH-Arg or on the one electron oxidation product of NOH-Arg (1, 2, 12–14). Both steps find precedent in cytochrome P-450 reductase/cytochrome P-450 biochemistry (1, 13–15). Electron flow is believed to follow the sequence NADPH → FAD → FMN → heme → O_2 (13, 14); in nNOS, the FMN → heme transfer is dependent on Ca^{2+}/calmodulin (16).

Reports over the past 7 years have implicated NO production by constitutive NOS isoforms in a wide variety of physiological processes including blood pressure homeostasis (17, 18), control of organ perfusion (19), inhibition of platelet adhesion and aggregation (20), and neurotransmission including control of gastrointestinal sphincters (21, 22). NOS may also have a role in subtle central nervous system effects such as long term potentiation and long term depression (7, 23). iNOS-mediated NO production, which can be quantitatively substantial, is implicated in the cytotoxic/cytostatic activity of macrophages (24, 25) and perhaps other cells (26). NOS-inhibitory N^6-monosubstituted L-arginine derivatives (e.g., N^6-methyl-L-arginine (N^6-MMA) (1, 24) and N^6-nitro-L-arginine (N^6-NTA) (1, 27)) have been extremely valuable in elucidating the NOS dependence of physiological phenomena studied in cells and whole organisms. It has also been found that NOS inhibitors are of potential therapeutic value in suppressing the overproduction of NO that accounts at least in part for the postischemic reperfusion injury of stroke (28), the hypotension of cytokine-induced and septic shock (29, 30), and the tissue damage associated with inflammatory conditions, including arthritis (31, 32).

The research and clinical utility of NOS inhibitors is dependent on both their specificity and their potency of inhibition. To date, NOS specificity has been best achieved with arginine analogs, some of which are tight-binding inhibitors (e.g., L-NNA, Refs. 33 and 34) and some of which are mechanism-based inhibitors (e.g., L-NMMA, Refs. 35 and 36). In the present studies, we have examined L-thiocitrulline (37), a novel amino acid that is recognized by the arginine/citrulline binding site of NOS and that appears to interact as an axial sixth ligand of the heme cofactor.
L-Thiocitrulline

EXPERIMENTAL PROCEDURES

Materials—Biochemical reagents and reagents for organic synthesis were obtained from Sigma and Aldrich, respectively: (6R,5S,7R)-Tetrahydrofuran (THF) was purchased from Fisher Scientific, B. Schirke, Jena, Germany, and N-[4-U-14C]arginine was from DuPont NEN. Male Sprague-Dawley rats (200–450 g) were from Harlan Labs, Madison, WI. New Zealand white rabbits were from New Franklin Rabbits, New Franklin, WI. L-NNA acetate salt was prepared for ornithine to be used in atm. and N,N-dimethyl-pseudoephedrine iodide as essentially as described (39, 40). L-Thiocitrulline, L-thiocitrulline (S-thiocitrulline, thiocitrulline or -lysinine) was mainly prepared from N'-benzoylcarbonyl-L-ornithine, N'-benzoylcarbonyl-lysine by modification of procedures described by Feldman for the synthesis of NOH-Arg (41); detailed synthetic procedures are reported elsewhere (37).

Rat nNOS was isolated from stably transfected kidney 293 cells (9) as described (10). Studies with nNOS were carried out with crude homogenate of cultured rat aortic smooth muscle cells grown in the presence of lipopolysaccharide and interferon-y to induce iNOS expression. The tissue was prepared and centrifuged and a 20-pl portion of the supernatant was derivatized with NADPH-dependent reduction of cytochrome c was determined spectrophotometrically in a final volume of 50 pl of 50 mM Na’ HEPES buffer, pH 7.5, 100 pM CaCl2, 2 mM dithiothreitol, 1 mg/ml bovine serum albumin, 2 mM CaCl2, 10 mg/ml calmodulin, 5 pM FAD, 5 pM FMN, 1 nM NADPH, 1 pM L-arginine, or 10 pM L-thiocitrulline or 10 pM L-NMA, and 18 mg of nNOS. At time intervals, a 20-pl aliquot was removed and the cuvette was flushed with 1 l of THB, 100 pl of the final volume of 500 pl of Na’ HEPES buffer, pH 7.5, 0.1 M EDTA, 100 mg/ml bovine serum albumin, 200 pl of THB, 10 pl of CaCl2, 10 pl of FMN, 50 pl of THB, 10 pl of L-thiocitrulline was added to the standard mixture. Nitric oxide-mediated oxidation of oxygenhemoglobin was monitored at 401 nm (e = 3.8 x 10^4/M); the reference cuvette contained a similar mixture without enzyme. Reported values are based on the maximum rate seen within 5 min (i.e. the "initial" rate after any lag period, see "Results").

NADPH-dependent reduction of cytochrome c was determined spectrophotometrically in a final volume of 50 pl of 50 mM Na’ HEPES buffer, pH 7.5, 0.1 M EDTA, 50 pM NADPH, 50 pM bovine heart cytochrome c, 0–1 mM L-thiocitrulline, and 2.4 pM of nNOS (specific activity = 200–220 nmol of cytochrome c reduced per min mg). Cytochrome c reduction was monitored at 550 nm (e = 21.1 x 10^3/M). In some studies, 20 pl of CaCl2, and 10 pl of FMN, 50 pl of THB, 100 pl of dithiothreitol, 100 pl of MgCl2 buffer, 50 pl of NADPH, and 100 pM potential substrate (L-thiocitrulline, L-arginine, or L-NMA). Reaction was initiated by the addition of 36 pM of nNOS, and the reaction mixtures were placed at 25 °C. At appropriate times, typically 3.5, 7, and 10.5 min, 25-pl portions of a stopping solution (0.2 M Na’ dithionite followed by gel filtration) were added to the sample cuvette and the mixture was flushed with 1 l of THB, 100 pl of CaCl2, and 10 pl of calmodulin were added to the standard mixture. Nitric oxide-mediated conversion of oxyhemoglobin to methemoglobin was determined (43). [14C]Citrulline was eluted with a 95% O2, 5% CO2 mixture. Isotonic tension was measured with force-generating tendons (Grass Instrument Co., Quincy, MA) and recorded on a polygraph interfaced with an analog-to-digital converter. Resting tension was adjusted to a length tension maximum of 2 g, and the rings were equilibrated for 1 h. After confirming responsibility to 40 mM KCl (3.7 ± 1.2 g of contraction), rings were washed repeatedly with Kreb’s buffer and precontracted with 1 uM norepinephrine. After stabilizing 5–10 min, a dose-response curve to methacholine (10^-10^-6 M) was performed, and percent relaxation to each dose was recorded. The rings were then rinsed with Kreb’s buffer and equilibrated with L-thiocitrulline or L-NMA (1–300 pM) for 10 min. The rings were then contracted with 1 uM norepinephrine, and the dose-response curve for methacholine was repeated. Each ring served as its own control; each concentration of inhibitor was examined three times.

Trituration of nNOS by optical difference spectrophotometry was performed from 9 ml of 50% Triton-HCl buffer, 10% glycerol, 0.1 mM EDTA, pH 7.5) were placed in the sample cuvette and the absorbance difference was adjusted to base line. All compounds used in the titrations were dissolved in 50 ml of Tri-HCl buffer, 10% glycerol, pH 7.5, and did not possess intrinsic absorbance at wavelengths used in the difference titrations. Titrations were conducted at 15 °C. Additions of perturbant to the sample cuvette were made using a Hamilton syringe, followed by mixing and recording of the resultant difference spectrum. The total change in sample volume was <2%. Spectra were normalized to zero absorbance as described in the figure legends.

Male Sprague-Dawley rats (200 and 450 g) were used for all in vivo studies. Anesthesia was induced with ketamine (50 mg/kg intramuscularly) and acepromazine (5 mg/kg intramuscularly) and maintained throughout the study by additional intramuscular injections of the mixture. Rats were placed on a heated table thermostatically controlled to 37 °C. Venous cannula were inserted either into the iliac or internal jugular vein for administration of inhibitors. A cannula in the iliac artery was connected to a pressure transducer and used to monitor blood pressure. For studies in septic rats, abdomens were opened under aseptic conditions and the cecum was isolated and ligated with a silk suture. A small (~4 mm) incision was made proximal to the ligation and fecal contents were expressed, allowing development of peritonitis (48).

RESULTS

Kinetic Characterization of nNOS and iNOS Inhibition by L-Thiocitrulline and Related Compounds—In preliminary studies, 10 or 100 pM L- or L-thiocitrulline was added to the standard nNOS and iNOS assay mixtures containing 20 M L-[4-14C]arginine, and the extent of inhibition was determined (Fig. 1). With both nNOS and iNOS, 100 pM L-thiocitrulline decreased L-[14C]citrulline formation by ≥96%, whereas the L-ornithinamide caused no inhibition (<1%). For comparison, 100 pM L-NMA inhibited about 90%. With both isoforms, product increased almost linearly with time, offering no suggestion of significant irreversible inhibition by the 10.0 M L-thiocitrulline (Fig. 1, 10-l M L-thiocitrulline). As reported by others (49), L-thiocitrulline (100 M) was not an inhibitor of either NOS isoform. To examine the possibility that small thioureas might inhibit, N-methylthiourea (100 M and 1 M) was also tested; it did not inhibit (not shown).

Initial-rate kinetic studies were carried out over a range of L-[14C]arginine and L-thiocitrulline concentrations to determine the nature of nNOS and iNOS inhibition by L-thiocitrulline (Fig. 2, a and b, respectively). As shown, inhibition was competitive.
with respect to L-arginine for both isoforms. Replots of $K_{n,app}$ versus L-thiocitrulline concentration (Fig. 2, a and b, insets) indicated that the $K_i$ for L-thiocitrulline is $0.06 \pm 0.01$ and $3.6 \pm 0.8 \mu M$ with nNOS and iNOS, respectively (mean ± S.D. for four independent determinations). In the same studies, $K_{n,app}$ values for L-arginine were $1.6 \pm 0.3$ and $18.8 \pm 3.0 \mu M$ with nNOS and iNOS, respectively. In parallel studies, initial inhibition by L-NMA was, as reported (35, 36), also competitive with L-arginine; $K_i$ values were $0.18$ and $6 \mu M$ for nNOS and iNOS, respectively (not shown). The $K_{n,app}$ and the $K_{NMA}$ values are consistent with those in the literature (33–36, 42).

Because L-homoarginine is an alternative substrate for both nNOS and iNOS (1), we also synthesized and tested L-homothiocitrulline. Although significantly less potent than L-thiocitrulline, L-homothiocitrulline inhibited nNOS and iNOS with $K_i$ values of 3 and $45 \mu M$, respectively (not shown). The relative potency of L-thiocitrulline and L-homothiocitrulline is reminiscent of our earlier observation that N'-methyl-L-homoarginine is a less effective inhibitor than L-NMA with both nNOS and iNOS.²

² M. A. Hayward and O. W. Griffith, unpublished observations.

Figure 1: Inhibition of nNOS and iNOS by L-thiocitrulline and related compounds. The effect of 100 μM L-thiocitrulline (A), L-thiocitrulline (V), L-NMA (●), L-citrulline (○), or 10 μM L-thiocitrulline (●) on the rate of [14C]citrulline formation from [14C]arginine was determined at the time points shown as described under "Methods." The rate without inhibitor (●) is also shown. Panel A shows the results with nNOS (2 μg); panel E shows the results with iNOS (10 μg).

Figure 2: Kinetic analysis of L-thiocitrulline inhibition. Reaction mixtures were as described under "Methods," except the total volume was 50 μl, and the concentration of L-[14C]arginine was varied over the range shown (3.0–10 μM for nNOS and 5–40 μM for iNOS). L-Thiocitrulline concentrations were 0 μM (●), 0.2 μM (●), 0.4 μM (▲), 0.6 μM (▼), and 0.8 μM (●) in the nNOS studies (panel A); they were 0 μM (●), 2 μM (●), 5 μM (▲), 10 μM (▼), and 15 μM (●) in the iNOS studies (panel B). For nNOS studies, each reaction mixture contained 0.5 μg of enzyme; for iNOS, each contained 6.5 μg of enzyme. Rates were calculated from a single time point at 5 min. The data points shown are averages of duplicate measurements; the experiments were carried out four times to obtain the kinetic constants reported in the text.

Reversibility of Inhibition by L-Thiocitrulline—Although initial inhibition of NOS by L-NMA is competitive with arginine and fully reversible, NOS undergoes mechanism-based irreversible inhibition by L-NMA with time (35, 36). Similar studies were carried out with L-thiocitrulline. As shown in Fig. 3, nNOS is moderately unstable when incubated with NADPH and cofactors, but the addition of L-thiocitrulline does not accelerate the rate of inactivation. In contrast, L-NMA substantially in-
L-Thiocitrulline-nNOS

Mcmillan and Masters (47) have previously shown that
min) as a function of time of preincubation with arginine or inhibitor. In
of the fractional residual activity
mined on the basis of NO-mediated conversion of oxyhemoglobin to
methemoglobin, monitored at 401 nm. The figure shows the natural log of the fractional residual activity (i.e., observed $\Delta A_{390}$/min initial $\Delta A_{390}$/min) as a function of time of preincubation with arginine or inhibitor. In separate studies, it was shown that the amount of inhibitor carried over did not affect the rate of oxyhemoglobin conversion to methemoglobin.

Increases the rate of inactivation, consistent with earlier reports (35, 36).

Inhibition of eNOS-dependent Aortic Ring Contraction by L-Thiocitrulline—nNOS and iNOS are soluble isozymes that are readily available via overexpression (nNOS) or cytokine-mediated induction (iNOS). In contrast, eNOS is membrane-bound and present in very low abundance in the COS expression system available to us (8); detailed kinetic studies were not possible. On the other hand, methacholine-induced relaxation of aortic rings precontracted with norepinephrine is an eNOS-dependent phenomenon that is readily quantitated. Studies were carried out using rabbit aortic rings exposed for 10 min to 1–300 pM L-thiocitrulline and then relaxed by cumulative addition of 0.003–10 pM methacholine (9 concentrations); percent relaxation was assessed after each addition. Similar studies were carried out with rings exposed to L-NMA. Percent relaxation was calculated based on results with the same rings prior to inhibitor treatment. At all concentrations tested, L-thiocitrulline and L-NMA were approximately equipotent in blocking relaxation; results for 10 pM methacholine are shown for each concentration of methacholine tested. The results for 10 pM methacholine are shown; qualitatively similar results were obtained at all other methacholine doses tested (i.e., L-NMA was a slightly less effective inhibitor than l-thiocitrulline). The data are shown as means ± S.D. for triplicate measurements.

Spectral Evidence for Interaction of L-Thiocitrulline with NOS—McMillan and Masters (47) have previously shown that the heme iron of nNOS as isolated is mainly high spin. However, titration of the enzyme with L-arginine, NOH-Arg, or L-NMA results in a Type I difference spectrum (a peak at ~380 nm, a trough at ~420 nm, and an isosbestic point at ~405 nm), suggesting that binding of these substrates (or inhibitor) results in a low spin to high spin heme iron transition in that subpopulation of the enzyme that was not initially high spin. Because low spin heme iron is associated with the hexacoordinated state, the transition was inferred to occur by displacement of an amino acid side chain or water molecule as the sixth axial ligand of heme iron (47, 50). Fig. 5 shows the results of a spectral titration of 7.7 μM nNOS with L-thiocitrulline (final concentrations = 0.4–9.0 μM). These difference spectra were normalized to zero absorbance at 400 nm for presentation. An isosbestic point of 427 nm for the L-thiocitrulline (200 μM)-perturbed difference spectrum was obtained by arithmetic subtraction of the absolute spectra using 8.8 μM nNOS (data not shown). The resulting difference spectra reflect a mixture of high spin and low spin species produced by this single compound. Since L-thiocitrulline, but not L-arginine or L-citrulline, produces this effect, the implication is that the thiol group interacts directly with the heme-iron, i.e., heme-ligation (see “Discussion”). As shown, L-thiocitrulline causes a perturbation in the absorbance spectrum of the heme Soret transition band characterized by a trough at ~392 nm, a shoulder at ~413 nm, and a peak at 435 nm. Spectral perturbation was stereospecific; D-thiocitrulline did not elicit heme spectral changes at concentrations up to 10 μM (data not shown). The inset of Fig. 5 shows a double reciprocal plot of the $\Delta A (A_{435} - A_{392})$ versus perturbant concentration that was used to estimate a spectral binding constant of 3 μM (uncorrected for bound ligand, which was <5% of total). In three separate determinations, the resulting spectral binding constants ranged from 1–3 μM.

For comparison, Fig. 6 shows the difference spectra obtained upon addition to 3.5 μM nNOS of final concentrations of 40 μM L-homothiocitrulline (curve A) or L-thiocitrulline (curve B).
which was used to estimate a spectral binding constant, line stock solutions were made as described under "Methods." The final absorbance differences (from this data set); the zero absorbance at 400 nm. The samples of L-thiocitrulline and L-homothiocitrulline.
imidazole were used as additional perturbants (Fig. 5).
ence spectra were obtained with 8.8 µM imidazole, which is a typical Type 1 interaction with a peak at 432 nm and a trough at 393 nm and, hence, the L-thiocitdline group, elicits a Type I difference spectrum, suggesting no direct sulfur-iron ligation. The difference spectrum of nNOS (Fig. 6) shows the difference spectrum of nNOS (3.5 µM) upon addition of 200 µM imidazole, which is a typical Type II difference spectrum characterized by a peak at 432 nm and a trough at 393 nm.

L-Homothiocitrulline, which contains an additional methylene group, elicits a Type I difference spectrum, suggesting no direct sulfur-iron ligation. The inset of Fig. 6 shows the difference spectrum obtained with the nitrogenous ligand, imidazole, which represents a typical Type II interaction with a peak at 432 nm and a trough at 393 nm and, hence, the L-thiocitrulline spectral perturbation is best described as a modified Type II.

To further elucidate L-thiocitrulline binding, L-arginine and imidazole were used as additional perturbants (Fig. 7). Difference spectra were obtained with 8.8 µM nNOS following the addition of L-thiocitrulline to a final concentration of 10 µM (Fig. 7A, curve A), followed by additions of L-arginine to 100 µM (curve B) and then L-thiocitrulline to 1010 µM (curve C). As shown, L-thiocitrulline produces a modified Type II difference spectrum with a shoulder at ~410 nm and a bathochromic-shifted peak at ~435 nm. Upon the addition of L-arginine, a typical Type I difference spectrum is obtained, while the final addition of L-thiocitrulline accentuates the 410-nm shoulder and restores the modified Type II difference spectrum. In the studies shown in Fig. 7B, nNOS was adjusted to a final concentration of 1000 µM imidazole (base line) and a titration with L-thiocitrulline (100-1100 µM) was conducted as described under "Methods." The final concentrations of L-thiocitrulline used for the difference spectra shown in the figure were as follows: A, 300 µM; B, 700 µM; and C, 1100 µM. A double-reciprocal plot of the absorbance difference (A₁₃₅ - A₄₃₅) against perturbant concentration showed Kₛ = 27 µM.

**Fig. 5. Spectral perturbation of nNOS by L-thiocitrulline.** Samples of nNOS (7.7 µM) were placed in cuvettes, the base line absorbance difference was recorded, and successive additions of L-thiocitrulline stock solutions were made as described under "Methods." The final concentrations of L-thiocitrulline were as follows: A, 0.4; B, 1.0; C, 3.0; D, 5.0; E, 7.0; and F, 9.0 µM. The difference spectra were normalized to zero absorbance at 400 nm. The inset is a double-reciprocal plot of the absorbance differences (A₁₃₅ - A₄₃₅) versus perturbant concentration, which was used to estimate a spectral binding constant, Kₛ = 3.1 µM (from this data set); the Kₛ ranged from 1-3 µM in three titration experiments.

**Fig. 6. Comparison of the spectral perturbation of nNOS by L-thiocitrulline and L-homothiocitrulline.** Difference spectra of nNOS (3.5 µM) were recorded after the addition of L-homothiocitrulline (A) or L-thiocitrulline (B) to a final concentration of 40 µM. All of the spectra were normalized to zero absorbance at 410 nm. The inset shows the difference spectrum of nNOS (3.5 µM) upon addition of 200 µM imidazole, which is a typical Type II difference spectrum characterized by a peak at 432 nm and a trough at 393 nm.

**Fig. 7. Effects of L-arginine and imidazole on L-thiocitrulline-nNOS heme interaction.** Panel A, the difference spectrum of nNOS (8.8 µM) following addition of L-thiocitrulline to 10 µM (A) was recorded. Subsequent additions of 100 µM L-arginine (B) and 1,000 µM more L-thiocitrulline (C) were made, and the resulting difference spectra were recorded. All spectra were normalized to zero absorbance at 400 nm. Panel B, NOS (8.8 µM) was adjusted to a final concentration of 1 mM imidazole (base line) and a titration with L-thiocitrulline (100-1100 µM) was conducted as described under "Methods." The final concentrations of L-thiocitrulline used for the difference spectra shown in the figure were as follows: A, 300 µM; B, 700 µM; and C, 1100 µM. A double-reciprocal plot of the absorbance difference (A₁₃₅ - A₄₃₅) against perturbant concentration showed Kₛ = 27 µM.

Effect of L-thiocitrulline on Cytochrome c Reduction by nNOS—It has been suggested that Ca²⁺/calmodulin serves to align the reductase and heme-containing oxidase domains of nNOS, facilitating transfer of electrons from flavins to heme (16). In the absence of Ca²⁺/calmodulin, heme is not reduced, and nNOS catalyzes the flavin-mediated, NADPH-dependent
administration of L-thiocitrulline to anesthetized rats caused a
conversion completely to citrulline, and 100 μM L-NMA yielded 90%
conversion to that elicited by L-NMA (Fig. 8A). At lower doses, L-thiocitrulline was also slightly more potent than L-NMA. The maximum pressor response to L-thiocitrulline (100 μM) inhibited cytochrome c reduction in the presence of Ca2+/calmodulin by 82 ± 3% (n = 4); β-thiocitrulline at the same concentration was without effect.

Possible Metabolism of L-Thiocitrulline—Because L-NMA has
been shown to be a poor substrate and mechanism-based inhibitor of NOS (35,36), we examined the possible conversion of L-thiocitrulline to citrulline or ornithine by NOS. Incubation of 36 μg of nNOS with 500 μM NADPH, 100 μM L-thiocitrulline, and necessary cofactors caused no diminution of the L-thiocitrulline present and no detectable product formation when the reaction mixtures were examined by HPLC or ion-exchange amino acid analysis. Formation of 1% citrulline or ornithine would have been easily detected. Under similar conditions, 100 μM L-arginine was converted completely to citrulline, and 100 μM L-NMA yielded 90 μM citrulline.3

In Vivo Activity of L-Thiocitrulline and Related Compounds—Administration of L-thiocitrulline to anesthetized rats caused a nearly immediate and profound increase in blood pressure (Fig. 8A). At a dose of 20 mg/kg, the pressor effect of L-thiocitrulline was about 20% greater in magnitude and comparable in duration to that elicited by L-NMA (Fig. 8B and C). Note that 20 mg/kg is a maximally effective dose for both L-thiocitrulline and L-NMA. At lower doses, L-thiocitrulline was also slightly more potent than L-NMA. The maximum pressor response to 5, 10, and 15 mg/kg of L-thiocitrulline was about 40, 50, and 85 mm Hg (systolic).

L-Thiocitrulline was also an effective pressor agent in hypertensive septic rats; a typical result is presented in Fig. 9. As shown, a rat with surgically induced peritonitis developed hypotension over a 6-h period. Infusion of normal saline caused only a small improvement in blood pressure, but L-thiocitrulline (20 mg/kg) returned pressure to normal levels for 20–30 min.

DISCUSSION

All available data indicate that each NOS monomer contains a single arginine/citrulline binding site that accommodates L-arginine, NOH-Arg (generated in situ or provided exogenously, Ref. 2) and a variety of N'-monosubstituted inhibitory L-arginine analogs. Initial binding of the L-arginine analogs invariably shows competitive kinetics versus L-arginine. The amino acid binding site is stereospecific for L-enantiomers and is thought to place one guanidino nitrogen of L-arginine in sufficiently close proximity to heme iron to allow two successive heme-mediated monooxygenation reactions to occur (formation of NOH-Arg from L-arginine and then oxidation of NOH-Arg to 3 Note that in preliminary studies we saw time- and NOS-dependent conversion of L-thiocitrulline to citrulline (57); this result has not been reproducible. Although L-thiocitrulline is stable in conventional buffers and is not affected by dithiothreitol, EDTA, Ca2+, or calmodulin, it does exhibit some nonenzymatic degradation to citrulline (and other products) with redox active cofactors (FAD, FMN). Such degradation requires several hours at 25 °C; however, and would seem to be too slow to account for our previous result. We are continuing to examine the stability of L-thiocitrulline.

Fig. 8. Effect of L-thiocitrulline and L-NMA on blood pressure of normal rats. Male Sprague-Dawley rats were anesthetized and instrumented as described under “Methods.” After blood pressure and heart rate had stabilized, NOS inhibitor was given by bolus intravenous injection over about 30 s. Blood pressure and heart rate were continuously monitored. The top panel for a typical rat shows the effect on systolic (S), mean (M), and diastolic (D) blood pressure upon giving L-thiocitrulline (20 mg/kg); the middle panel shows the results of a similar experiment with L-NMA (20 mg/kg). The lower panel compares the blood pressure response for six rats given L-thiocitrulline (20 mg/kg), seven given L-NMA (20 mg/kg), and six given L-NMA (30 mg/kg). The pressor effect of thiocitrulline is significantly greater than that of L-NMA at either dose (p < 0.05).
citrulline and NO). For N\textsuperscript{\textomega}-monosubstituted arginine substrates, it is the substituted guanidino nitrogen that is bound near heme; thus, the hydroxylated nitrogen of NOH-Arg is further oxidized to NO (2), and l-NMA is processed to N\textsuperscript{\textomega}-hydroxy-N\textsuperscript{\textomega}-methylarginine rather than to N\textsuperscript{\textomega}-hydroxy-N\textsuperscript{\textomega}-methylarginine (35).

Prior to the present work, several lines of evidence suggested that the NOS arginine/citrulline binding site had high affinity mainly for cationic arginine analogs. l-Arginine (33-36, 42, 47), l-NMA (35, 36), N\textsuperscript{\textomega}-amino-l-arginine (53, 54), and N\textsuperscript{\textomega}-iminoethyl-l-ornithine (55) are all tightly bound and contain highly basic side chains. In contrast, l-canavanine, which is isosteric with l-arginine but has a side chain pK\textsubscript{a} of 6, is a relatively weak competitive inhibitor (1, 55). Similarly, the NOS reaction presumably leaves l-citrulline transiently bound to the amino acid binding site, but when added exogenously, this neutral amino acid does not inhibit even when present at high concentration relative to arginine (49, present work). Nevertheless, our finding that l-thiocitrulline competes with l-arginine for its binding site and exhibits a K\textsubscript{i} substantially lower than the K\textsubscript{i} for arginine or the K\textsubscript{i} for l-NMA demonstrates that a neutral citrulline analog can be tightly bound and raises the question of what factors contribute to its binding affinity.

The observation that l-thiocitrulline elicits a Type II-like difference spectra indicates that its binding is accompanied by a shift of heme iron from a high spin to a low spin state consistent with a transition from pentacoordinate to hexacoordinate heme. Although our studies do not allow unambiguous assignment of the sixth heme ligand, the sulfur atom of thiocitrulline, present as either the thiono (-NH-CS-NH\textsubscript{2}) or the thiol (-NH-C(SH)=NH) tautomer, is an attractive candidate. The spectral results shown in Figs. 5-7 provide further understanding of the nature and extent of heme-ligand interaction. As shown in Fig. 5, l-thiocitrulline elicits an optical absorption difference spectrum with nNOS that resembles a Type II difference spectrum (see Fig. 6, iscet, for imidazole-induced spectrum for a typical Type II spectrum). The family of spectra shown in Fig. 5, however, reveals a distinct spectral signature that suggests additional interactions with the nNOS protein not previously observed with any other substrate or inhibitor of this enzyme. The difference spectra obtained with l-thiocitrulline after the addition of imidazole (Fig. 7B), resulting in the appearance of typical Type I spectrum and a >10-fold increase in \Delta absorbance, suggest that the citrulline-like structure of this inhibitor interacts in the l-arginine binding site producing a difference spectrum similar to that obtained with l-arginine.

On the other hand, the addition of l-thiocitrulline, followed by l-arginine, produces a Type I difference spectrum with l-arginine that, upon the subsequent addition of l-thiocitrulline, reverts to the modified Type II spectrum with an even more pronounced peak at \sim 408 nm. As discussed below, these results can be explained by the reversible binding of each of these substrate/inhibitors in its own individual orientation.

The use of the optical difference spectroscopy approach has the advantage of magnifying minor spectral changes observed in the absolute spectra because the absorbance background becomes the base line. However, in interpreting these spectra, it must be appreciated that only relative changes are measured, and the appearance of a peak may be the difference between two troughs in the absolute spectrum, which in the sample cuvette is less negative than in the reference cuvette. Nevertheless, all of the spectra obtained with l-thiocitrulline can be modeled by Gaussian curves for each of the spin states of nNOS. The anomalous spectra seen upon binding of l-thiocitrulline in the l-arginine binding site of native nNOS represent a shifting of mostly high spin native nNOS (47) to l-thiocitrulline-ligated nNOS (Scheme 1, reaction 1). The latter is an equilibrium mixture of low and high spin forms suggesting that the sulfur atom of l-thiocitrulline is not perfectly aligned with the open axial position of heme iron and achieves a statistical equilibrium, sometimes acting as a sixth ligand (low spin) and sometimes being perhaps near heme iron but not close enough to perturb the d electron orbitals of iron (high spin). In either the high or low spin state, l-thiocitrulline apparently prevents reduction and/or oxygen binding by heme since superoxide formation is reduced by 80-85%. Although our present data do not allow precise calculation of the NOS-l-thiocitrulline high spin/low spin equilibrium ratio, the fraction in the low spin form must be greater than the \sim 10% low spin form found in native nNOS since l-thiocitrulline binding is
accompanied by a Type II-like spectral shift (i.e. increasing low spin form). As shown previously, addition of L-arginine to nNOS greatly diminishes the fraction of low spin iron, perhaps to zero (47). The results shown in Fig. 7A can then be understood in terms of reactions 1, 2, and 3 (Scheme I) occurring in sequence as L-thiocitrulline was added to nNOS (modified Type II spectral shift), L-arginine was added to that complex (Type I spectral shift), and, finally, excess L-thiocitrulline was added to displace L-arginine and restore the modified Type II spectra.

The results from the study starting with the hexacoordinate, low spin NOS-imidazole complex (Fig. 7B) are accounted for by displacement of an imidazole ligand that interacts strongly with heme iron but relatively weakly with nNOS (IC50 = 240 μM Ref. 56; Kf = 160 μM, Ref. 47) by a L-thiocitrulline ligand that interacts less strongly with heme iron but strongly with nNOS (Kf = 0.06 μM) (Scheme I, reaction 4). Thus the displacement of the imidazole by L-thiocitrulline is accompanied by a Type I spectral shift attributable to formation of high spin NOS-L-thiocitrulline. Note that formation of low spin NOS-L-thiocitrulline also occurs when imidazole is displaced by L-thiocitrulline, but NOS is spectroscopically silent in these respects, inhibition by L-thiocitrulline is, however, not complete; reversibility of L-thiocitrulline binding is substantially slower (~30 min) than that with L-thiocitrulline, and L-NMA gives a Type I rather than Type II difference spectrum, suggesting it does not interact directly with the heme cofactor.

In vivo studies indicate that L-thiocitrulline is an effective pressor agent blocking formation of vasoactive NO by both eNOS (normotensive rat studies) and iNOS (hypotensive, septic rat studies). L-Thiocitrulline may thus be of therapeutic value in treating the hypotension of cytokine-induced and septic shock. Whether or not L-thiocitrulline is more effective than L-NMA for this purpose remains to be determined, but it has several potential advantages including a lower Kf with iNOS. Furthermore, in contrast to L-NMA, L-thiocitrulline is not metabolized to L-citrulline, a product that is converted to L-arginine.

Because NOS catalyzes the formation of NO and homocitrulline from L-homoarginine (24) and is inhibited by N'-methyl-L-lysine ("homo-NMA"), we anticipated that L-homothiocitrulline would inhibit. It does so, albeit with Kf values 12-50-fold higher than the Kf values for L-thiocitrulline. In our view, the higher Kf values are due only in part to the inherently lower affinity of NOS for L-homoarginine and its analogs; the relatively poor affinity also reflects the fact that this derivative apparently does not interact strongly with heme iron. Thus, L-thiocitrulline binding to native nNOS elicits a Type I rather than Type II difference spectrum indicating that the high spin/low spin statistical equilibrium for NOS-L-thiocitrulline is not significantly perturbed (Fig. 4). As shown previously, addition of L-arginine to nNOS results in a spectral perturbation similar to that of L-thiocitrulline (15147-15153).

Analogy between L-NNA and L-thiocitrulline is, however, not complete; reversibility of L-NNA binding is substantially slower than that of L-thiocitrulline, and L-NMA gives a Type I rather than Type II difference spectrum, suggesting it does not interact directly with the heme cofactor.

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