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L-N^6-(1-Iminoethyl)-lysine potently inhibits inducible nitric oxide synthase and is superior to N^G-monomethyl-arginine in vitro and in vivo

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Abstract

L-N^6-(1-Iminoethyl)-lysine is a novel inhibitor of nitric oxide (NO) synthase, which similar to aminoguanidine but unlike N^G-monomethyl-L-arginine is 30-fold more selective for the inducible than for the constitutive isoform of the enzyme. Here, we characterized this inhibitor for the first time in intact cells and during infection of mice with a NO-sensitive parasite (*Leishmania major*). L-N^6-(1-Iminoethyl)-lysine potently inhibited the activity of inducible NO-synthase in primary macrophages. After stimulation by interferon-γ the IC_{50} of L-N^6-(1-iminoethyl)-lysine was 0.4 ± 0.1 μM and 10- or 30-fold lower than that of N^G-monomethyl-L-arginine or aminoguanidine, respectively. In vivo, L-N^6-(1-imino-ethyl)-lysine (0.4–9 mM in the drinking water) suppressed inducible NO-synthase activity and caused a dramatic exacerbation of leishmaniasis, despite a counterregulatory increase of inducible NO-synthase protein in the tissue. In contrast, considerably higher concentrations of N^G-monomethyl-L-arginine (20–50 mM) were required in order to achieve comparable effects. N^G-Monomethyl-L-arginine, but not L-N^6-(1-imino-ethyl)-lysine led to weight loss, reduced water and food consumption. We conclude that L-N^6-(1-iminoethyl)-lysine should be used instead of N^G-monomethyl-L-arginine for potent suppression of inducible NO-synthase in vitro and in vivo.

Keywords: Aminoguanidine; Nitric oxide (NO) synthase, inducible; Leishmaniasis; L-N^6-(1-Iminoethyl)-lysine; Nitric oxide (NO); N^G-Monomethyl-L-arginine

1. Introduction

During the past 10 years nitric oxide (NO) has emerged as an important effector molecule which is involved in a wide spectrum of physiological and pathophysiological processes in eukaryotic organisms (reviewed in: Nathan, 1992; Schmidt and Walter, 1994). One of the first functions ascribed to NO when generated from L-arginine by the inducible isoform of NO synthase of activated murine macrophages was the killing of both intra- and extracellular microbes (Green and Nacy, 1993). Although not all microorganisms appear to be susceptible to NO (Doi et al., 1993; O'Brien et al., 1994; Michaliszyn et al., 1995; Nunoshiba et al., 1995), various experimental approaches have demonstrated direct or indirect antimicrobial activity of NO. In vitro, authentic NO-gas or NO-donors were shown to be toxic to protozoa, bacteria and viruses (Liew et al., 1990; Flesch and Kaufmann, 1991; Croen, 1993; Doi et al., 1993; Karupiah et al., 1993). Furthermore, expression of inducible NO-synthase by macrophages correlated with the elimination of a variety of microbial organisms not only in vitro, but also in vivo. Conversely, abrogation of cytokine-induced NO production by arginine-analogues or deletion of the gene for the inducible isoform of NO-synthase supported parasite survival and dissemination (Liew et al., 1990; Beckerman et al., 1993; Evans et al., 1993; Green et al., 1993; Nüssler et al., 1993; Boockvar et al., 1994; Petray et al., 1994; Seguin et al., 1994; Stenger et al., 1994; Chan et al., 1995; MacMicking et al., 1995; Wei et al., 1995). The majority of studies, which aimed to demonstrate that the elimination of microbes is dependent on the activity of *inducible* NO-synthase, relied on the use of N^G-monomethyl-L-arginine. This compound, however, is equally effective as a competitive inhibitor of the

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inducible *and* the constitutive (neuronal or endothelial) isoforms of NO-synthase (Hibbs et al., 1987; Knowles et al., 1989; Palmer and Moncada, 1989). The small amounts of NO generated by constitutive NO-synthases are believed to fulfill homeostatic functions such as the regulation of blood pressure, glomerular and coronary perfusion and gastrointestinal motility (reviewed in Nathan, 1992; Schmidt and Walter, 1994). Furthermore, in at least two studies N^G-monomethyl-L-arginine has been reported to exert significant side effects (weight loss and alopecia) in infected mice (Granger et al., 1991; Evans et al., 1993), which might be due to its lack of selectivity and could have contributed to the clinical deterioriation of the infection following treatment.

In order to delineate the function of NO generated by the inducible as opposed to the constitutive isoform of NO-synthase, more potent and selective inhibitors are required both for basic research and future therapeutic applications. Aminoguanidine was the first NO-synthase inhibitor which was found to be relatively selective for the inducible isoform (Corbett et al., 1992). A new and promising compound is L-N^6-(1-iminoethyl)-lysine. When the inhibitory effects of L-N^6-(1-iminoethyl)-lysine and N^G-monomethyl-L-arginine on inducible NO-synthase partially purified from the murine RAW264.7 cell-line and on constitutive NO-synthase from rat cerebellum were compared, L-N^6-(1-iminoethyl)-lysine exhibited a much higher degree of selectivity for inducible NO-synthase than N^G-monomethyl-L-arginine (factor 30 vs. 0.5) (Moore et al., 1994). So far, however, the inhibitory effect of L-N^6-(1-iminoethyl)-lysine on the NO production by intact cells has not been analysed. L-N^6-(1-Iminoethyl)-lysine was found to suppress NO production and inflammation in rats with adjuvant arthritis (Connor et al., 1995), but no other in vivo studies have been published to date. We therefore decided to compare this novel inducible NO-synthase inhibitor (i) with N^G-monomethyl-L-arginine and aminoguanidine in primary murine macrophages and (ii) with N^G-monomethyl-L-arginine during the course of an infectious disease. As a model we chose the infection of resistant mice with the protozoan parasite *Leishmania major*, the resolution of which requires NO production (Liew et al., 1990; Evans et al., 1993) and is paralleled by the expression of inducible NO-synthase in the infected tissue (Stenger et al., 1994).

2. Materials and methods

2.1. Reagents

Recombinant mouse interferon-γ (batch M3RD48; protein concentration 1.0 mg/ml; specific activity 5.2 $\times 10^6$ U/mg; lipopolysaccharide content < 10 pg/ml) was a kind gift of Dr. G. Adolf (Ernst Boehringer-Institut für Arzneimittelforschung, Vienna, Austria). NADPH, FAD, L-arginine hydrochloride, aminoguanidine hemisulfate, pepstatin A, chymostatin, phenylmethyl-sulfonylfluoride, and lipopolysaccharide from *E. coli* O111:B4 were obtained from Sigma Chemie (Deisenhofen, Germany). (6*R*)-2-Amino-4-hydroxy-6-(L-erythro-1,2-dihdroxypropyl)-5,6,7,8-tetrahydropteridine (tetrahydrobiopterin, BH_4) was purchased from Dr. Schircks Co. (Jona, Switzerland), the L- and D-analogues of N^G-monomethyl-arginine and of N^G-nitro-arginine-methyl-ester were from Alexis Co. (Läufelfingen, Switzerland). The L- and D-analogue of N^6-(1-iminoethyl)-lysine were synthesized and kindly provided by Drs. W. M. Moore and M.G. Currie (G.D. Searle Research and Development, Monsanto Co., St. Louis, MO).

2.2. Animals, parasites, in vivo infection and treatment

Female C57BL/6 and CD1 mice, weighing 16–18 g, were purchased from Charles River Breeding Laboratories (Sulzfeld, Germany), housed in our own facilities and used at 6–8 weeks of age. The mice were kept on a normal rodent diet and were allowed to drink freely from dispensers containing acidified drinking water. Origin, in vivo passage and in vitro propagation of the *Leishmania major* isolate (MHOM/IL/81/FE/BNI) were reported in detail elsewhere (Solbach et al., 1986). Mice were inoculated bilaterally into the hind footpads with 3×10^6 stationary-phase *Leishmania major* promastigotes (after two to four in vitro subcultures) each so that the tissue from one animal could be used for both immunohistology and analysis of parasite burden. At regular intervals after infection the footpad swelling was measured with a metric caliper and the mean (±S.D.) percentage increase of footpad thickness was calculated (Solbach et al., 1986). Food and water uptake as well as body weight were recorded throughout the course of infection. The number of parasites in the tissue of infected mice was determined by a limiting dilution analysis applying Poisson statistics and the χ^2-minimization method as published earlier (Solbach et al., 1986). The NO-synthase inhibitors were added to the drinking water (adjusted to pH 2.7 in order to prevent microbial growth) and provided freshly every second day. L-N^6-(1-Iminoethyl)-lysine concentrations in the serum were measured by electrospray-tandem mass spectrometry (kindly performed by Dr. Kevin Duffin, Searle, St. Louis).

2.3. Macrophage cultures

4 days after intraperitoneal injection with 4% Brewer's thioglycollate broth (Difco, Detroit, MI) peri-

toneal cells were harvested with phosphate-buffered saline solution (PBS) from female CD1 or C57BL/6 mice and enriched for macrophages by 1–2 h adherence step as described (Vodovotz et al., 1993). The cells were cultured with RPMI 1640 medium supplemented with 2 mM glutamine, 10 mM Hepes, 13 mM $NaHCO_3$, 100 μg/ml penicillin, and 160 μg/ml gentamicin (all reagents from Seromed Biochrom, Berlin, Germany) plus 5% selected fetal bovine serum (Sigma) (final lipopolysaccharide-content: < 10 pg/ml) in 24-well plates (10^6 macrophages/well in a volume of 500 μl) or 78 cm^2 culture dishes (14×10^6/dish; 500 μl medium per 10^6 macrophages) (Nunc, Wiesbanden, Germany) at 37°C in 5% CO_2-95% humidified air. Subsequently, macrophages were stimulated with interferon-γ (20 ng/ml) ± lipopolysaccharide (20 ng/ml) in the absence or presence of NO-synthase inhibitors for 24–72 h as detailed in the text.

2.4. Determination of nitrite accumulation

Nitrite (NO_2^-) accumulation in the supernatant of cultured cells was used as an indicator of NO production and was determined by the Griess-reaction with sodium nitrite as a standard as described (Vodovotz et al., 1993). 100 μl of each supernatant (corresponding to 2×10^5 macrophages) were assayed in triplicate and absorbance measured at 550 nm using a MR5000 plate reader (Dynatech, Denkendorf, Germany).

2.5. Preparation of cell lysates

For analysis of inducible NO-synthase by enzyme activity assays and Western blot, macrophage monolayers were washed 3 times with PBS (37°C), scraped in 1 ml of Tris-buffer (40 mM, pH 8) containing 5 μg/ml pepstatin A, 1 μg/ml chymostatin, 5 μg/ml aprotinin and 100 μM phenylmethyl-sulfonylfluoride, and lysed by sonication with a Sonifier B-12 (Branson Ultrasonics Co., Fa. Heinemann, Schwäbisch-Gmünd, Germany) (2 × 10 s at intensity 2). Aliquots of the total cell lysates were used for Bradford protein assay (Bio-Rad, München, Germany). Popliteal lymph nodes isolated from *Leishmania major*-infected mice were also disrupted in lysis buffer by sonication (2 × 30 s at intensity 2).

2.6. Inducible NO-synthase enzyme assay

Inducible NO-synthase enzyme activity was measured in a microplate assay as described (Stuehr et al., 1991; Vodovotz et al., 1993). Briefly, 4–13 μg of macrophage lysate or 40–60 μg of lymph node lysate were incubated for 120–150 min at 37°C in 20 mM Tris-HCl (pH 7.9) containing 4 μM BH_4, 4 μM FAD, 3 mM dithiothreitol, 2 mM NADPH and 2 mM L-arginine in a final volume of 100 μl. In some experiments NO-synthase inhibitors were added before the enzyme reaction was initiated by L-arginine and NADPH. The reaction was stopped by the addition of lactate dehydrogenase (20 U/ml) to oxidize residual NADPH. Product NO_2^- was measured by the Griess reaction. Non-specific absorbance due to lysate turbidity was measured in wells containing all reagents except for L-arginine and NADPH, and was substracted.

2.7. Sodium dodecyl sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE) and Western blot analysis

15–20 μg of macrophage lysate were separated on 7.5% reducing SDS-PAGE, transferred to reinforced nitrocellulose membranes (Schleicher and Schüll, Dassel, Germany), and immunoblotted with a rabbit anti-mouse inducible NO-synthase immunoglobulin G raised against an octapeptide derived from the C-terminus of inducible NO-synthase (kindly provided by Drs. Q.-w. Xie and C. Nathan, New York) exactly as described previously (Vodovotz et al., 1993).

2.8. In situ detection of inducible NO-synthase and Leishmania major

Cryostat sections (5 μM) of footpad lesions and lymph nodes from *Leishmania major*-infected C57BL/6 mice were analysed for the presence of inducible NO-synthase protein and *Leishmania major* parasites by immunoenzymatic staining techniques as published recently (Stenger et al., 1994). A rabbit-anti-*Leishmania* major antiserum was generously supplied by Dr. H. Moll (Würzburg, Germany).

3. Results

3.1. Effect of L-N^6-(1-iminoethyl)-lysine, N^G-monomethyl-L-arginine and aminoguanidine on NO_2^- accumulation, inducible NO-synthase enzyme activity and inducible NO-synthase protein expression of cytokine-activated intact mouse macrophages

In a previous in vitro study, the inducible NO-synthase-inhibitory activity of L-N^6-(1-iminoethyl)-lysine was evaluated with partially purified enzyme preparations, but not by using cytokine-activated intact cells (Moore et al., 1994). We therefore investigated whether L-N^6-(1-iminoethyl)-lysine is able to suppress NO production by interferon-γ- or interferon-γ/lipopolysaccharide-stimulated mouse peritoneal macrophages. The data in Fig. 1 and Table 1 demonstrate that L-N^6-(1-iminoethyl)-lysine, but not its D-isomer is highly active when added to macrophage cultures: it is 10- or 30-fold more potent in suppressing NO_2^- accumulation

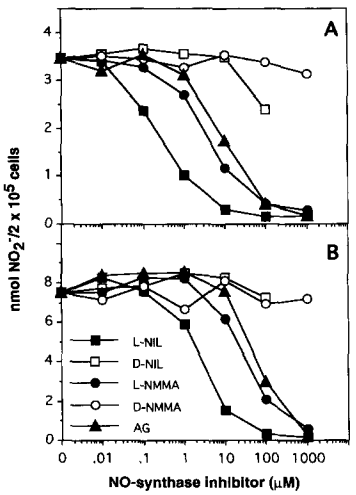

Fig. 1. Effect of various inhibitors on the NO production of macrophages stimulated with interferon-γ (panel A) or interferon-γ plus lipopolysaccharide (panel B) for 48 h. Active L- or inactive D-analogues of the inhibitors were added from the beginning of stimulation. NO_2^- accumulation in unstimulated cultures was 0.15 (panel A) or 0.19 nmol/2×10^5 macrophages (panel B). One of four experiments. L-, D-NIL: L-, D-N^6-(1-iminoethyl)-lysine; L-, D-NMMA: L-, D-N^G-monomethyl-arginine; AG: aminoguanidine.

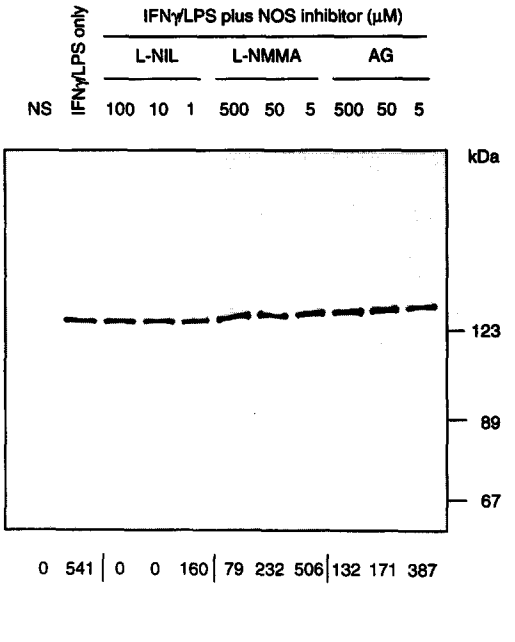

Fig. 2. Western blot analysis (20 μg lysate/lane) of the expression of inducible NO-synthase protein (ca. 130 kD) in macrophages activated by interferon-γ plus lipopolysaccharide for 24 h in the presence or absence of NO-synthase inhibitors (L-NIL: L-N^6-(1-iminoethyl)-lysine; L-NMMA: L-N^G-monomethyl-arginine; AG: aminoguanidine). The inducible NO synthase (iNOS) activities of the lysates are listed below the respective lanes. NS = no stimulus. One of three similar experiments.

than N^G-monomethyl-L-arginine or aminoguanidine, respectively. The slight inhibition observed with the D-N^6-(1-iminoethyl)-lysine in interferon-γ-activated macrophages (Fig. 1A) presumably results from a 1% contamination by the L-isomer (Moore et al., 1994). Western blot analysis and enzyme activity assays revealed that the reduced accumulation of NO_2^- is due to inhibition of inducible NO-synthase enzyme activity. Even at high concentrations neither L-N^6-(1-iminoethyl)-lysine nor N^G-monomethyl-L-arginine or aminoguanidine reduced the expression of inducible NO-synthase protein (Fig. 2). No suppression of NO release was seen with L-N^G-nitro-arginine-methyl-ester (0.01–1000 μM), an inhibitor which mainly acts on constitutive NO-synthase (Gross et al., 1990), in this experimental setting (data not shown).

Table 1
Effect of L-N^6-(1-iminoethyl)-lysine (L-NIL), N^G-monomethyl-L-arginine (L-NMMA) and aminoguanidine (AG) on interferon-γ- or interferon-γ/lipopolysaccharide-induced accumulation of nitrite by macrophages

Stimulus [a]	NO-synthase inhibitor	IC_{50} [b]
Interferon-γ	L-NIL	0.46± 0.1 (6)
	L-NMMA	5.05± 1.5 (5)
	AG	12.6 ± 4.2 (3)
Interferon-γ + lipopolysaccharide	L-NIL	2.13± 0.5 (7)
	L-NMMA	26.0 ± 6.5 (8)
	AG	66.8 ±15.8 (6)

[a] Macrophages were stimulated with 20 ng/ml interferon-γ ±20 ng/ml lipopolysaccharide for 48 h. [b] Numbers represent the mean (±S.E.M.) 50% inhibitory concentrations for each inhibitor (in μM; number of experiments in parentheses). NO_2^- accumulation in untreated control cultures was 3.3±0.3 and 6.7±0.2 nmol/2×10^5 macrophages after activation by interferon-γ or interferon-γ/lipopolysaccharide, respectively (means±S.E.M., n = 11 or 8).

3.2. *Inhibition of total cellular inducible NO-synthase by L-N^6-(1-iminoethyl)-lysine, N^G-monomethyl-L-arginine and aminoguanidine*

In order to rule out that the stronger suppression of inducible NO-synthase activity by L-N^6-(1-iminoethyl)-lysine in intact macrophages is due to a better uptake of this compound into the cells during the in vitro culture period, we compared the ability of L-N^6-(1-iminoethyl)-lysine, N^G-monomethyl-L-arginine and aminoguanidine to suppress inducible NO-synthase activity in total lysates prepared from activated peritoneal macrophages (Fig. 3). Under these experimental conditions L-N^6-(1-iminoethyl)-lysine was again much more potent than N^G-monomethyl-L-arginine and aminoguanidine (Fig. 3). The IC_{50} of the three compounds were 1.2 ± 0.2, 42 ± 3.0 and 23.5 ± 1.5 μM, respectively, when lysates from interferon-γ-activated macrophages were used, and 1.7 ± 0.3, 75 ± 16 and 36.9 ± 7.2 μM, respectively, when the inhibitors were added to lysates from interferon-γ/lipopolysaccharide-activated

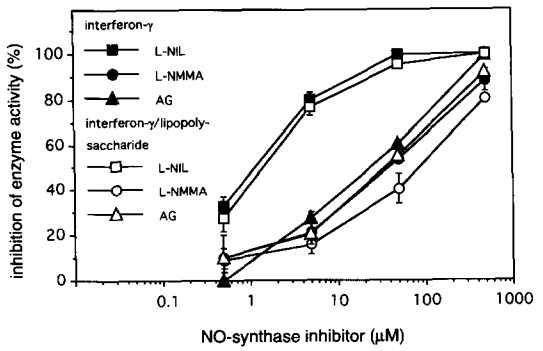

Fig. 3. Effect of NO-synthase inhibitors (L-NIL: L-N^6-(1-iminoethyl)-lysine; L-NMMA: L-N^G-monomethyl-arginine; AG: aminoguanidine) on inducible NO-synthase activity of lysates prepared from macrophages activated with interferon-γ (IFN-γ; closed symbols) or interferon-γ/lipopolysaccharide (LPS; open symbols) for 24 h. Inhibitors and all cofactors were added to the lysates and the enzyme reaction was initiated by the addition of NADPH (2 mM) and L-arginine (2 mM) within 1 min. Numbers represent the mean (±S.E.M.) percentage inhibition of enzyme activity in three to six experiments as compared with control (the enzyme activities in the absence of inhibitors were 198±5.3 and 907±96 pmol/mg protein per min for lysates of interferon-γ or interferon-γ/lipopolysaccharide-activated macrophages, respectively).

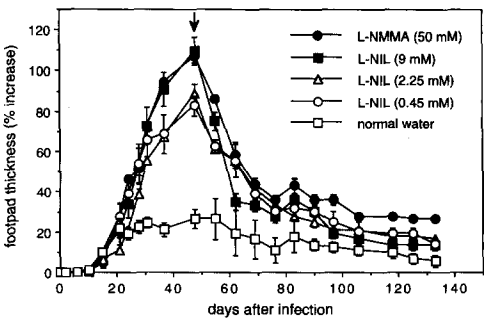

Fig. 4. L-N^6-(1-Iminoethyl)-lysine or N^G-monomethyl-L-arginine aggravate the course of *L. major* infection in C57BL/6 mice. At day 0 groups of three mice were infected with *L. major* and placed on drinking water without additives or with N^G-monomethyl-L-arginine (50 mM) or L-N^6-(1-iminoethyl)-lysine (0.45, 2.25 or 9 mM). (↓) Indicates the end of treatment at day 48 of infection when all groups were switched to normal drinking water. Lesion development (mean increase of footpad thickness ± S.D.) was monitored throughout the course of infection. Where error bars are not visible, they fall within the symbols denoting the mean. One of three similar experiments.

macrophages (means ± S.E.M. of three to six experiments). Interestingly, aminoguanidine was a stronger inhibitor than N^G-monomethyl-L-arginine when the inhibitors were added to cell lysates (Fig. 3) rather than to intact cells (Table 1). This might be due to differences in the uptake by cells or stability of these compounds under culture conditions.

3.3. L-N^6-(1-Iminoethyl)-lysine aggravates murine Leishmaniasis in the absence of overt side effects

Based on the reported relative selectivity of L-N^6-(1-iminoethyl)-lysine for the inducible isoform of NO-synthase (Moore et al., 1994) and intrigued by its potency in vitro, we decided to compare the activity of L-N^6-(1-iminoethyl)-lysine and N^G-monomethyl-L-arginine in *Leishmania major*-infected C57BL/6 mice. We did not include aminoguanidine in this study as it was consistently inferior to L-N^6-(1-iminoethyl)-lysine in vitro (Figs. 1–3; Table 1). In control mice, which received normal drinking water without inhibitors, the footpad lesions after cutaneous infection with *Leishmania major* were maximal at day 30–50 after infection and healed thereafter (Fig. 4) as expected from previous studies (for review: Bogdan et al., 1993). When the drinking water was supplemented with L-N^6-(1-iminoethyl)-lysine (0.45, 2.2 or 9 mM), the lesions enlarged dramatically and finally exulcerated. For N^G-monomethyl-L-arginine, considerably higher concentra-

Table 2
Effect of L-N^6-(1-iminoethyl)lysine (L-NIL) and N^G-monomethyl-L-arginine (L-NMMA) on the parasite burden in the lymph nodes of *Leishmania major*-infected C57BL/6 mice (day 41) as determined by limiting dilution analysis [a]

Additive to the drinking water	*Leishmania* per 10^3 lymph node cells [c]	*Leishmania* per total lymph node [e]
none	1.1 (0.64–3.76)	4.4×10^3
L-NMMA (20 mM) [b]	5.1×10^4 (2.9×10^4–12.5×10^4) [d]	2.3×10^9
L-NMMA (5 mM)	1.5×10^2 (1.1×10^2–2×10^2) [d]	2.7×10^6
L-NIL (2.25 mM)	1.0×10^5 (0.5×10^5–1.6×10^5) [d]	1.6×10^9
L-NIL (0.45 mM)	1.2×10^4 (0.1×10^4–2.2×10^4) [d]	4.0×10^8
L-NIL (0.09 mM)	1.8×10 (1.2×10–2.3×10) [d]	5.0×10^5

[a] Groups of three mice were infected with 3×10^6 parasites into both hind footpads. [b] Treatment of mice with 50 mM N^G-monomethyl-L-arginine (L-NMMA) was discontinued after 25 days because of severe side effects (wasting syndrome). [c] 95% confidence interval is given in parentheses. [d] Significant difference when compared with the corresponding control group (significance was assumed when the 95% confidence limits did not overlap). [e] Calculated from the total number of cells per lymph node.

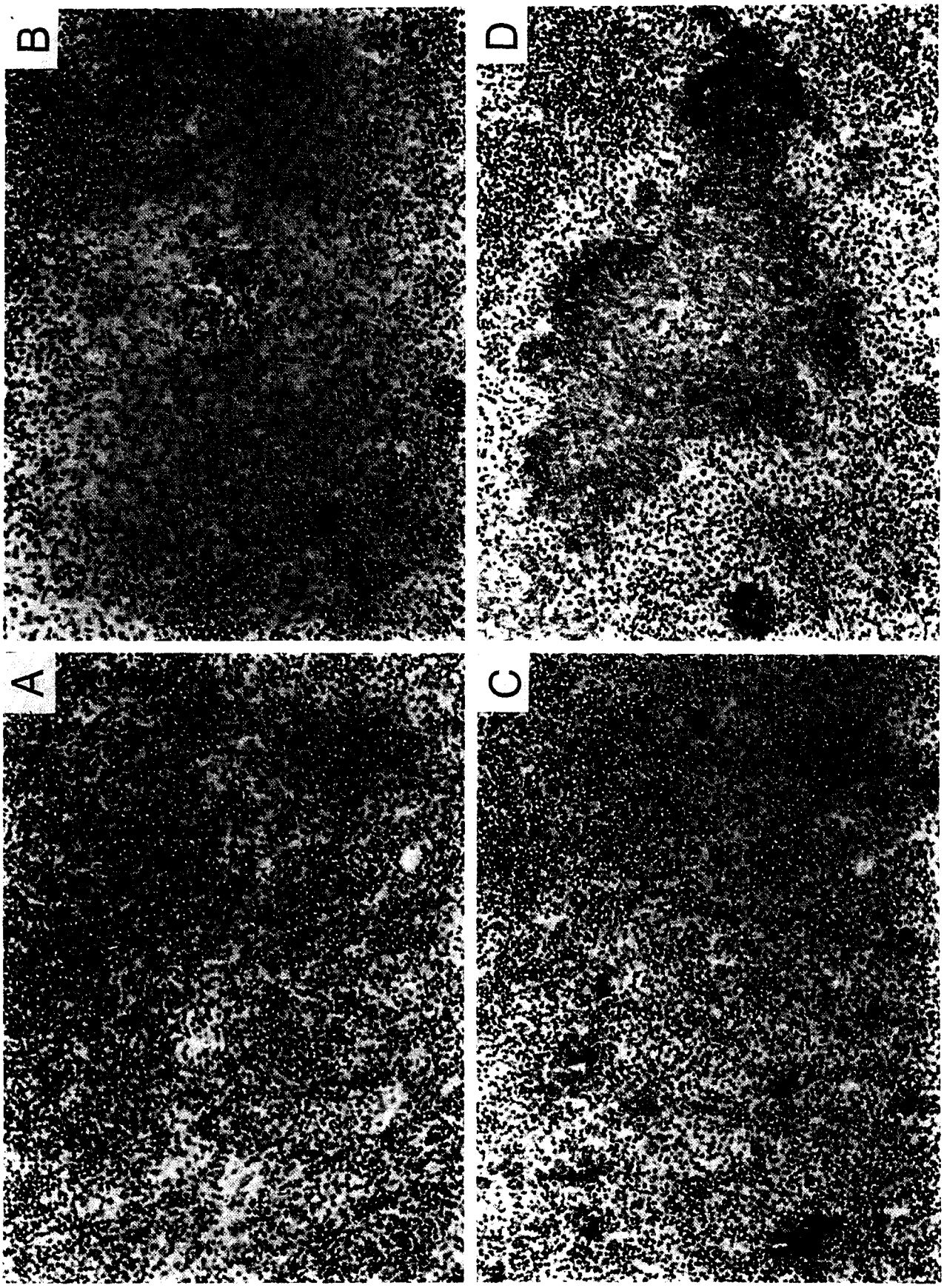
B
D
A
C

Table 3
Suppression of inducible NO-synthase activity by L-N^6-(1-iminoethyl)-lysine (L-NIL) in lymph nodes of *Leishmania major*-infected mice [a]

Additive to the drinking water [b]	Specific activity [c]
None	101.8 ± 19 (3)
4.5 mM L-NIL	10.1 ± 4 (5)

[a] Popliteal lymph nodes were removed from C57BL/6 mice at day 25 or 26 after infection. [b] L-N^6-(1-Iminoethyl)-lysine (L-NIL) was added to the drinking water from the day of infection. [c] pmol NO_2^-/min per mg protein in a 150 min assay. Numbers represent the mean (±S.E.M.) activities of inducible NO-synthase in the tissue (number of lymph nodes in parentheses). Activities in lymph nodes from uninfected mice were 1.06 ± 0.31 and 0.72 ± 0.6 pmol/mg per min ($n = 3$) after a 1 week treatment with normal water or water supplemented with 4.5 mM L-NIL, respectively.

tions (20–50 mM) were required in order to achieve comparable clinical effects (Fig. 4). As little as 0.09 mM L-N^6-(1-iminoethyl)-lysine or 5 mM N^G-monomethyl-L-arginine in the drinking water led to a detectable increase of the footpad swelling (not shown). Importantly, as soon as the application of L-N^6-(1-iminoethyl)-lysine or N^G-monomethyl-L-arginine was discontinued, even sloughed lesions started to clear and finally healed completely (Fig. 4).

In all cases progressive lesion growth following treatment with L-N^6-(1-iminoethyl)-lysine (or N^G-monomethyl-L-arginine) was paralleled by strikingly increased numbers of parasites in the footpad (not shown) and the draining lymph node as quantified by limiting dilution analysis (Table 2) and visualized by immunohistochemical staining (Fig. 5, panel A vs. B).

Previously, we showed that inducible NO-synthase is undetectable in the skin and lymph nodes of naive mice, but induced and strongly upregulated in the resistant C57BL/6 strain during the course of infection with *Leishmania major* (Stenger et al., 1994). In the lymphoid tissue of infected C57BL/6 mice treated with L-N^6-(1-iminoethyl)-lysine, inducible NO-synthase protein was present in large, confluent areas and even more abundant than in the infected control animals which received water without L-N^6-(1-iminoethyl)-lysine (Fig. 5, panel C vs. D). However, the enzymatic activity of inducible NO-synthase was almost completely blocked following treatment with L-N^6-(1-iminoethyl)-lysine (Table 3). Considering the assay conditions (2 mM L-arginine) and the mean (±S.E.M.) concentration of L-N^6-(1-iminoethyl)-lysine in the serum (62.7 ± 19 μM; $n = 3$), this result demonstrates potent and presumably irreversible inhibition of inducible NO-synthase in vivo. The lack of inducible NO-synthase activity was also indirectly documented by immunohistochemical staining of serial lymph node sections. In mice treated with L-N^6-(1-iminoethyl)-lysine expression of inducible NO-synthase coincided with the presence of large amounts of *Leishmania major* parasites, whereas in control animals only few parasites were

Table 4
Effect of N^G-monomethyl-L-arginine (L-NMMA) and L-N^6-(1-iminoethyl)-lysine (L-NIL) on the water and food consumption and on the body weight of *L. major*-infected or naive C57BL/6 mice [a]

Additive to the drinking water	Water consumption (ml/day per mouse) [b]	Food consumption (ml/day per mouse) [b]	Weight gain (%) [b,c]
Infected mice			
None	3.6 ± 0.4	3.2 ± 0.1	6.3 ± 0.4
L-NMMA (50 mM) [d]	1.5 ± 0.4 [f]	2.4 ± 0.4 [g]	−20.1 ± 1.8 [e]
L-NMMA (20 mM)	2.2 ± 0.2 [g]	2.9 ± 0.3	−6.2 ± 0.8 [e]
L-NMMA (5 mM)	3.3 ± 0.1	3.1 ± 0.1	1.1 ± 0.5 [e]
L-NIL (2.25 mM)	3.8 ± 0.2	3.0 ± 0.1	5.3 ± 0.9
L-NIL (0.45 mM)	3.8 ± 0.5	3.5 ± 0.1	7.8 ± 1.2
L-NIL (0.09 mM)	4.0 ± 0.4	3.6 ± 0.1	6.5 ± 1.5
Naive mice			
None	3.6 ± 0.6	3.7 ± 0.4	10 ± 0.1
L-NMMA (50 mM)	1.8 ± 0.2 [f]	3.7 ± 0.6	−11 ± 1.1 [e]

[a] Groups of three mice each were infected with *L. major*. Food and water consumption as well as body weight were monitored from day 0 to 41 after infection, when the mice were killed for limiting dilution analysis (see Table 2). [b] Values denote means ± S.D. [c] Percentage gain of weight was calculated by the formula [(weight at day 42 − weight at day 0):(weight at day 0)] × 100%. Negative numbers denote loss of weight. [d] Treatment of mice with 50 mM N^G-monomethyl-L-arginine (L-NMMA) had to be discontinued after 25 days of infection because of severe side effects (wasting syndrome). [e,f,g] Significant decrease as compared to control group (Student's *t*-test for unpaired samples; [e] $P < 0.001$, [f] $P < 0.01$; [g] $P < 0.05$).

Fig. 5. Treatment with L-N^6-(1-iminoethyl)-lysine leads to upregulation of inducible NO-synthase and increased numbers of parasites in *L. major*-infected mice. Immunoperoxidase staining of *L. major* parasites (panel A and B) or inducible NO-synthase protein (panel C and D) in consecutive sections of lymph nodes from C57BL/6 mice (day 41 of *L. major* infection) which were kept on normal drinking water (panel A and C) or on water supplemented with 2.25 mM L-N^6-(1-iminoethyl)-lysine (panel B and D) from the beginning of infection. Magnification × 200.

detectable in inducible NO-synthase-positive areas (Fig. 5 panel A vs. C and B vs. D). In both groups of mice inducible NO-synthase colocalized with NADPH diaphorase-activity in the tissue (not shown).

Treatment of *Leishmania major*-infected mice with L-N^6-(1-iminoethyl)-lysine (0.09, 0.45, 2.25 or 9 mM in the drinking water) never led to reduced water or food uptake or impaired weight gain (Table 4). The behavior of the mice was indistinguishable from the control groups. In contrast, *Leishmania major*-infected mice which were kept on water with 50 mM N^G-monomethyl-L-arginine (which is the concentration used in most in vivo studies), had a stongly reduced fluid intake, a lower food consumption, lost weight (Table 4) and showed much less agility during handling. 5 of a total 17 mice treated with 50 mM N^G-monomethyl-L-arginine had to be taken out of the experiment (e.g. Table 2) because of a severe 'wasting syndrome'. At a concentration of 20 mM, the side effects of N^G-monomethyl-L-arginine were less prominent, but still significant. Weight loss and reduced fluid intake were also observed in uninfected mice treated with N^G-monomethyl-L-arginine (50 mM) (Table 4). Weight development as well as food and water consumption of mice treated with the D-analogue of N^G-monomethyl-arginine or ammonium acetate as a taste and salt control were indistinguishable from the control group kept on normal water (not shown).

4. Discussion

The data presented here demonstrate that L-N^6-(1-iminoethyl)-lysine (i) is highly active in intact primary macrophages, where it completely inhibits interferon-γ- or interferon-γ/lipopolysaccharide-induced NO production, and (ii) leads to a dramatic exacerbation of *Leishmania major*-infection in C57BL/6 mice, the control of which is known to be dependent on the production of NO (Liew et al., 1990; Evans et al., 1993) and correlates with enhanced tissue expression of inducible NO-synthase (Stenger et al., 1994). Our study provides several novel aspects as to the activity of L-N^6-(1-iminoethyl)-lysine in vitro and in vivo. First, L-N^6-(1-iminoethyl)-lysine is considerably more potent than N^G-monomethyl-L-arginine in suppressing NO_2^- accumulation by intact macrophages (Fig. 1) or the enzyme activity of inducible NO-synthase when added to lysates from interferon-γ ($\pm$ lipopolysaccharide)-activated cells (Fig. 3) (the respective molar IC_{50} values of L-N^6-(1-iminoethyl)-lysine were roughly 10- or 44-fold lower than those of N^G-monomethyl-L-arginine). Second, in vivo 0.45 mM L-N^6-(1-iminoethyl)-lysine in the drinking water were sufficient to cause dramatic aggravation of disease, whereas 20–50 mM N^G-monomethyl-L-arginine were required to obtain a comparable effect (Fig. 4). Third, even at the highest concentration tested (9 mM in the drinking water) L-N^6-(1-iminoethyl)-lysine did not cause adverse clinical effects. In particular, L-N^6-(1-iminoethyl)-lysine did not result in weight loss, reduced water uptake or impaired food consumption, side effects we regularly observed in infected mice treated with 20–50 mM N^G-monomethyl-L-arginine in the drinking water (Table 4). Evans and Hibbs also reported a reduction of weight in *Leishmania major*-infected, resistant C3H mice treated with N^G-monomethyl-L-arginine, but in contrast to our findings the food uptake was only transiently reduced and the water intake not significantly altered in their study (Evans et al., 1993). As hypertrophy of the pyloric sphincter develops in mice with a disrupted neuronal NO-synthase gene (Huang et al., 1993), it is tempting to speculate that inhibition of constitutive NO-synthase by N^G-monomethyl-L-arginine causes subtle impairment of gastric motility and emptying, which subsequently leads to decreased water and food consumption and weight loss. This hypothesis is supported by our observation that administration of L-N^G-nitro-arginine-methyl-ester (50 mM in the drinking water), a strong inhibitor of constitutive NO-synthase, was also followed by reduced fluid uptake and body weight (data not shown). The alopecia observed by Hibbs and colleagues in N^G-monomethyl-L-arginine-treated *Leishmania major*-infected BALB/c mice (Evans et al., 1993) might be a deficiency symptom caused by the reduced intake of food.

When we performed enzyme activity assays with lysates prepared from macrophages, which were activated with interferon-γ/lipopolysaccharide in the presence of L-N^6-(1-iminoethyl)-lysine, aminoguanidine or N^G-monomethyl-L-arginine during a 48 h culture period, we noticed sustained suppression of inducible NO-synthase activity, although no exogenous NO-synthase inhibitor was added to the assay mixture (see inducible NO-synthase activity values in Fig. 2). This was particularly striking in the case of L-N^6-(1-iminoethyl)-lysine, where inducible NO-synthase activity remained suppressed even in the presence of a 200- to 2000-fold excess of L-arginine (2 mM). These findings indicate that L-N^6-(1-iminoethyl)-lysine is a stable compound which might block the activity of inducible NO-synthase in an irreversible manner as recently suggested (Stuehr and Griffith, 1992).

Upon addition to *intact* macrophages, the IC_{50} values of L-N^6-(1-iminoethyl)-lysine, N^G-monomethyl-L-arginine and aminoguanidine were approximately 5-fold higher after stimulation with interferon-γ plus lipopolysacccharide as compared to interferon-γ alone (see Table 1). No such difference was found when the inhibitors were tested on *lysates* from interferon-γ- versus interferon-γ/lipopolysaccharide-activated macrophages (Fig. 3). It is possible that stimulation of

macrophages in the presence of lipopolysaccharide leads to reduced uptake or enhanced metabolism of the inhibitors. Alternatively, lipopolysaccharide has been shown to strongly induce the transport of arginine (Sato et al., 1992), which might increase the intracellular substrate/inhibitor ratio and thereby the IC_{50} of the inhibitors.

Similarly to L-N^6-(1-iminoethyl)-lysine, aminoguanidine is 26–40 times more selective for the inducible than for the constitutive isoform of NO-synthase (Corbett et al., 1992; Connor et al., 1995). Aminoguanidine increased mortality in *Mycobacterium tuberculosis*- or *Listeria monocytogenes*-infected mice (Beckerman et al., 1993; Chan et al., 1995), counteracted NO-dependent protection against *Plasmodium* sporozoites in the liver of immunized mice (Seguin et al., 1994) and partially inhibited adjuvant arthritis in rats (Connor et al., 1995). However, as shown in this study, aminoguanidine is 20–30-fold less effective than L-N^6-(1-iminoethyl)-lysine in suppressing inducible NO-synthase in vitro (Table 1, Fig. 3). Furthermore, in the rat model its oral bioavailability and/or potency in vivo was inferior to L-N^6-(1-iminoethyl)-lysine (Connor et al., 1995).

More recently, isothioureas, non-amino acid analogs of L-arginine, were identified as novel inhibitors of NO-synthase. *S*-Methylisothiourea reversed hypotension and improved survival in rats or mice with endotoxic shock. Although this compound is 10 times more potent than N^G-monomethyl-L-arginine in inhibiting macrophage NO-synthase, it suppresses equally well the inducible (macrophage) and constitutive (endothelial) isoform of NO-synthase (Szabó et al., 1994). *S*,*S*′-(1,3-Phenylenebis(1,2-ethanediyl))bisisothiourea, in contrast, was 190-fold more selective for the inducible NO-synthase, but this inhibitor was poorly active on intact cells and turned out to be acutely toxic in mice and rats at doses as low as 10 mg/kg (Garvey et al., 1994). Thus, the isothioureas evaluated so far appear to be inferior to L-N^6-(1-iminoethyl)-lysine.

Previous studies which aimed to inhibit inducible NO-synthase in murine *Leishmania major* infections utilized N^G-monomethyl-L-arginine (Liew et al., 1990; Evans et al., 1993), leaving the possibility that a simultaneous suppression of constitutive NO-synthase is required for the aggravation of leishmaniasis. This appears now unlikely, as the same degree of clinical exacerbation was achieved with 100-fold lower concentrations of L-N^6-(1-iminoethyl)-lysine (0.45 mM in the drinking water; Fig. 4) which, for example, did not affect the constitutive NO-synthase-regulated arterial blood pressure in rats (Connor et al., 1995).

Continuous administration of L-N^6-(1-iminoethyl)-lysine or N^G-monomethyl-L-arginine from the beginning of infection caused inexorable lesions in *Leishmania major*-infected resistant mice. Even when exulceration had already occurred and the cutaneous and lymph node tissue was densely packed with parasites, the lesions regressed and healed completely as soon as the administration of the inhibitors was stopped (Fig. 4). This indicates that the presence of high amounts of parasites per se does not lead to a non-protective (type 2 T-helper cell) bias in the T cell immune response as has been hypothesized based on in vitro data (Janeway et al., 1988). In fact, the upregulation of inducible NO-synthase seen in mice treated with L-N^6-(1-iminoethyl)-lysine or N^G-monomethyl-L-arginine (Fig. 5) presumably results from an enhanced interferon-γ response of the host organism, which attempts to overcome the action of the inhibitor and to resolve the lesions by generating more inducible NO-synthase protein.

In conclusion, the experiments presented here identified L-N^6-(1-iminoethyl)-lysine as a potent inhibitor of inducible NO-synthase in primary mouse macrophages and in *Leishmania major*-infected mice, which is superior to aminoguanidine and to the widely used substrate analogue N^G-monomethyl-L-arginine. N^G-Monomethyl-L-arginine turned out to be much more toxic than previously appreciated. Considering the relative isoform-selectivity of L-N^6-(1-iminoethyl)-lysine for inducible NO-synthase, its potency and, as far as tested, its apparent safety in vivo following oral administration, L-N^6-(1-iminoethyl)-lysine is currently the inhibitor of choice whenever suppression of inducible NO-synthase is to be achieved.

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