An Inhibitor of Macrophage Arginine Transport and Nitric Oxide Production (CNI-1493) Prevents Acute Inflammation and Endotoxin Lethality

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ABSTRACT

Background: Nitric oxide (NO), a small effector molecule produced enzymatically from L-arginine by nitric oxide synthase (NOS), is a mediator not only of important homeostatic mechanisms (e.g., blood vessel tone and tissue perfusion), but also of key aspects of local and systemic inflammatory responses. Previous efforts to develop inhibitors of NOS to protect against NO-mediated tissue damage in endotoxin shock have been unsuccessful, largely because such competitive NOS antagonists interfere with critical vasoregulatory NO production in blood vessels and decrease survival in endotoxemic animals. Accordingly, we sought to develop a pharmaceutical approach to selectively inhibit NO production in macrophages while sparing NO responses in blood vessels.

Materials and Methods: The processes of cytokine-inducible L-arginine transport and NO production were studied in the murine macrophage-like cell line (RAW 264.7). A series of multivalent guanylhydrazones were synthesized to inhibit cytokine-inducible L-arginine transport. One such compound (CNI-1493) was studied further in animal models of endothelial-derived relaxing factor (EDRF) activity, carrageenan inflammation, and lethal lipopolysaccharide (LPS) challenge.

Results: Upon activation with cytokines, macrophages increase transport of L-arginine to support the production of NO by NOS. Since endothelial cells do not require this additional arginine transport to produce NO, we reasoned that a competitive inhibitor of cytokine-inducible L-arginine transport would not inhibit EDRF activity in blood vessels, and thus might be effectively employed against endotoxic shock. CNI-1493, a tetravalent guanylhydrazone, proved to be a selective inhibitor of cytokine-inducible arginine transport and NO production, but did not inhibit EDRF activity. In mice, CNI-1493 prevented the development of carrageenan-induced footpad inflammation, and conferred protection against lethal LPS challenge.

Conclusions: A selective inhibitor of cytokine-inducible L-arginine transport that does not inhibit vascular EDRF responses is effective against endotoxin lethality and significantly reduces inflammatory responses.

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INTRODUCTION

Lethal tissue injury in septic shock syndrome, a frequent complication of critical illness, causes excessive mortality in association with any number of disease states, including trauma. Septic shock syndrome and organ damage are directly attributable to the action of endogenous mediators released by macrophages activated by pathogenic stimuli such as bacterial endotoxin (lipopolysaccharide [LPS]). These mediators include a variety of cytokines (especially tumor necrosis factor [TNF] and interleukin 1 [IL-1]) and nitric oxide (1–4). When produced in smaller physiological amounts, these factors are beneficial in host defense, but when overproduced in pathological quantities, their injurious and lethal effects predominate (5). Identification of the molecular mechanisms underlying the lethality of septic shock has led to the development of novel experimental strategies targeted against these endogenous mediators in the hope of limiting morbidity and mortality in this grave disease process.

Nitric oxide (NO) is a short-lived effector molecule that has been implicated in causing hypotension, vascular leakage syndrome, and tissue injury in septic shock (for review see Refs. 6 and 7). It is enzymatically produced from L-arginine by nitric oxide synthase (NOS), an enzyme present as various inducible and constitutive isoforms in smooth muscle, neurons, endothelial cells, macrophages, and other cell types. A number of agents that competitively inhibit NOS [(e.g., N^G-methyl-L-Arginine (L-NMA) and N^G-nitro-L-arginine methyl estes (L-NAME))] have been developed for use as anti-inflammatory agents. These have been shown to be effective in preventing, for instance, the inflammation associated with experimental adjuvant arthritis, and the development of immune complex glomerulonephritis in MRL-lpr/lpr mice (8,9). Unfortunately, these agents are deleterious when administered in experimental endotoxic shock, where they actually decrease survival relative to vehicle-treated controls (10–14). Recent evidence reveals that this increase in mortality is a side effect of the non-selective action of these compounds, which indiscriminately inhibit vital NOS activity in blood vessels (termed endothelium-derived relaxing factor [EDRF]), rather than the desired action of inhibiting macrophage-derived NO (13). As a result, local vasoconstriction cannot be relieved by EDRF, reducing blood flow to critical tissues, limiting oxygen delivery to tissues, and worsening ischemic organ damage already present during shock (13,15). Thus, the presently available NOS inhibitors further compromise organ perfusion during endotoxic shock and hasten rather than allay mortality in this condition.

Based in part on these observations, there is widespread interest in the identification of experimental agents to inhibit the production of NO in macrophages and other cells, but preserve the vasoregulatory NO responses in blood vessels. In the present study, we designed and developed a novel compound that limits L-arginine uptake by macrophages and inhibits their NO production. This agent (CNI-1493) selectively inhibited cytokine-inducible L-arginine uptake in macrophages, but did not inhibit EDRF activity in vivo. Moreover, it was effective in preventing carrageenan-induced inflammation and conferred protection against endotoxin lethality. We conclude that novel compounds that inhibit cytokine-inducible L-arginine transport activity may be effective therapeutic agents against inflammation and shock.

MATERIALS AND METHODS

Synthesis of CNI-1493

CNI-1493 (N,N′-bis[3,5-diacetylphenyl]decanediamide tetrakis[amidinohydrazone] tetrahydrochloride) was prepared by reacting 3,5-diacetylaniline (16) with sebacoyl chloride in dichloromethane containing pyridine. The resulting tetraketone was collected by filtration, and reacted with aminoguanidine hydrochloride in aqueous ethanol at reflux. The final product was purified from the reactants by crystallization, and subjected to elemental analysis, proton NMR, and melting point determinations to confirm structure and assess purity prior to use. These data indicated ≥98% purity, with the identity of the principal contaminant as the corresponding tris-aminohydrazone. The compound was not contaminated with detectable quantities of aminoguanidine. Material from a single synthesis was used in all the studies reported, but comparable results have been obtained from two separate preparations of the compound.

Measurement of NO Synthesis by Murine Macrophages

The murine macrophage-like cell line RAW 264.7 was obtained from ATCC, expanded, and subcultured in RPMI (1×10^6 cells/ml in 6-well plates) containing fetal bovine serum (FBS)

(10%). L-arginine was added to the medium in the concentrations shown for each experiment. Cells were allowed to adhere for 2 hr, then stimulated by incubation with LPS (100 ng/ml) and IFNγ (25 U/ml). Total nitrite concentration in conditioned media was determined at the time points indicated. Aliquots of conditioned media (200 μl) were assayed in triplicate for nitrite content by the Greiss method using dilutions of $NaNO_2$ as a standard (17). Standard curves obtained in the presence and absence of CNI-1493 were indistinguishable, indicating that CNI-1493 did not interfere with the Greiss method.

Determination of L-Arginine Uptake in RAW 264.7 Cells

L-arginine uptake was measured by the method of Bogle and others (18). Briefly, RAW 264.7 cells were plated (1×10^5 cells/well) in RPMI (with 10% FBS) in 96-well plates, allowed to adhere for 2 hr, then stimulated by the addition of rm-interferon-γ (rm-IFNγ) (25 U/ml; Genzyme) and LPS (*Escherichia coli* 0111:B4, 100 ng/ml; Sigma Chemical Co., St. Louis, MO, U.S.A.). At the times indicated after addition of stimulating agents, the cells were washed twice with HEPES-buffered Krebs solution containing NaCl (131 mM), KCl (5.5 mM), $MgCl_2$ (1 mM), $CaCl_2$ (2.5 mM), $NaHCO_3$ (25 mM), NaH_2PO_4 (1 mM), D-glucose (5.5 mM), and HEPES (20 mM), pH 7.4, 37°C. In separate experiments, CNI-1493 was supplied at the concentrations shown either 1 hr before the cells were stimulated (preactivation), or 8 hr after the cells were stimulated (postactivation). In both cases, CNI-1493 was added to the above buffer and cells incubated for 10 min at 37°C in the presence of L-arginine (100 μM); L-[2,3-^{3}H]-arginine (35 Ci/mmol) was then added to each well, and after 5 min the cells were washed three times with ice-cold PBS, solubilized in formic acid (100 μl), and the incorporated radioactivity determined by liquid scintillation counting. In other experiments and in agreement with previous reports, [^{3}H]-L-arginine transport in macrophages was observed to be linear for 10 min (18,19). In some experiments the efficiency of the washing step was verified by quantitative recovery of D-[^{14}C]mannitol included as an extracellular tracer (18). There was no significant efflux of transported label into the washes (not shown). In agreement with other studies in macrophages, 85% of L-arginine is metabolized via arginase to ornithine and urea, and the remaining 15% via NOS to NO and citrulline (17).

Determination of NOS Activity in Stimulated Macrophage Lysates

Activity of NOS present in activated macrophage lysates was measured by determining the conversion of radiolabeled L-arginine into citrulline by a modification of previously described methods (20). Briefly, macrophages were stimulated with IFNγ and LPS (as outlined above). After 18 hr, the cells were washed with PBS and lysed by three cycles of freeze/thawing in HEPES (20 mM) containing sucrose (0.32 M), EDTA (1 mM), DTT (1 mM), glycerol (5%), protease inhibitors (PMSF, leupeptin, aprotinin, and iodoacetamide), pH 7.2. The lysate was pelleted by centrifugation (100,000 × g) for 30 min. To measure NOS activity in the lysates, 300 μl of lysate was added to 60 μl of a reaction mixture containing NADPH (2 mM), $CaCl_2$ (0.45 mM), L-arginine (50 μM), calmodulin (10 μg/ml), [^{3}H]-L-arginine (1 μCi/ml), and Tween (20%). After 45 min at 37°C HEPES buffer (2 ml) was added and the labeled L-arginine and citrulline separated by chromatography (DOWEX AS 50W column). NOS activity is expressed as nanomole citrulline per minute per milligram total protein.

Determination of EDRF Activity In Vivo

Female Sprague-Dawley rats (225–250 g body wt) were anesthetized with nembutal (50 mg/kg, i.p.), a tracheostomy tube inserted, and the carotid artery and jugular vein cannulated by standard methods using polyethylene tubing (PE 50) (21). Blood pressure was recorded continuously with a pressure transducer and recorder (Model RS-3200; Gould, Inc.). In the experiment shown here, animals received a single sterile intra-arterial dose of either N^G-methyl-L-arginine (50 mg/kg; Sigma), CNI-1493 (10 mg/kg), or vehicle (0.4 ml). Acetylcholine diluted in LPS-free sterile water was administered via the jugular vein cannula at the doses indicated. The solutions were diluted to provide a constant injectable volume of 1 ml/kg body wt. The hypotensive (EDRF) response was measured as the decline in mean arterial blood pressure recorded 30 sec after administration of acetylcholine. The number of animals studied at each dose of acetylcholine was four to six for each experimental condition. The data are expressed as the mean ± standard error.

Carrageenan-Induced Footpad Inflammation

Paw edema was induced by injecting 1% Lambda-carrageenan (50 μl; Sigma) in HEPES 25 mM, pH 7.4, into the plantar surface of the left hindpaw of female C3H/HeN mice (20–25 g body wt) (22). The right hindpaw was injected with HEPES alone (50 μl). At 1.5 hr prior to paw injection, CNI-1493 was administered intraperitonealy at the doses indicated in a volume of 0.2 ml/animal. Three hours after paw injection, the thickness of the carrageenan- and saline-injected paws was measured using a caliper, and the data expressed as the difference between the diameters of the two paws. Data shown are the mean ± standard error of the differences between paws; four animals were studied per dose of CNI-1493. At the conclusion of the experimental period, animals were euthanized and the paw tissues fixed with 10% formalin, sectioned, and stained with hematoxylin and eosin for histological study.

Endotoxin Lethality Studies

These studies were performed by modification of a recently published protocol (23). Briefly, BALB/c mice (19–21 g) were housed in a climate-controlled, 12-hr light-dark cycled facility for at least 2 weeks after arrival. On the morning of the experiment, mice were given either CNI-1493 (1 mg/kg, i.p.), N^G-methyl-L-arginine (50 mg/kg, i.p.), or vehicle alone. LPS (*E. coli* 0111: B4; Sigma) in a dose of 13.75 mg/kg, i.p. (0.2 ml/mouse) was given 90 min after the experimental agents. Stock LPS solutions (10 mg/ml) were sonicated initially for 20 min, diluted in LPS-free water (1.375 mg/ml), then sonicated again for 10 min immediately prior to injection. All experiments consisted of 10 animals per group, and similar results were obtained at least twice for each condition.

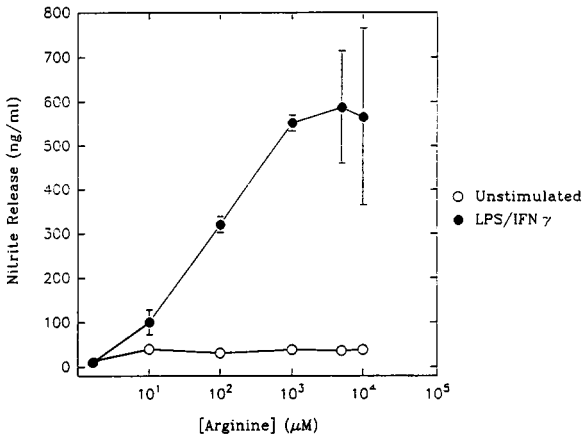

FIG. 1. Dependence of RAW cells on extracellular arginine to support NO production

RAW 264.7 cells were plated in RPMI devoid of L-arginine or in RPMI supplemented with L-arginine as indicated, then stimulated by the addition of LPS (100 ng/ml) and IFNγ (25 U/ml) at time zero. Total nitrite accumulated in the medium conditioned by 1×10^6 cells over the subsequent 18 hr is shown. Assays were performed in triplicate, and the experiment repeated at least three times. Data shown are mean ± SEM (n = 3 experiments).

RESULTS

Macrophages Rely on Extracellular L-Arginine as Substrate for NOS Synthase

It is widely known that murine macrophages produce copious quantities of NO after stimulation with LPS and IFNγ (17,24–28). In the present investigation, we used the murine macrophage-like cell line, RAW 264.7, stimulated by the addition of IFNγ (25 U/ml) and LPS (100 ng/ml) and measured the resultant cytokine-stimulated NO production by the accumulation of nitrite in the conditioned medium. In agreement with others (17), we first ascertained that extracellular L-arginine availability was rate limiting for macrophage NO production (Fig. 1). Cytokine-stimulated macrophage NO production was significantly attenuated when the culture medium was depleted of L-arginine, but was restored when L-arginine was present at physiologically relevant concentrations (50–150 μM) (Fig. 1). Although macrophages are known to possess the enzymatic machinery necessary to convert glutamine or citrulline into L-arginine for NO synthesis (29–36), the present data give evidence that macrophages rely principally on exogenous L-arginine to produce significant amounts of NO.

Increased L-Arginine Transport in Activated Macrophages Provides Substrate for NOS

In agreement with others (18,19,33,35), we showed that macrophages activated by LPS/IFNγ increase L-arginine uptake to provide substrate for NO production (Fig. 2). L-arginine transport in macrophages was increased shortly after mac-

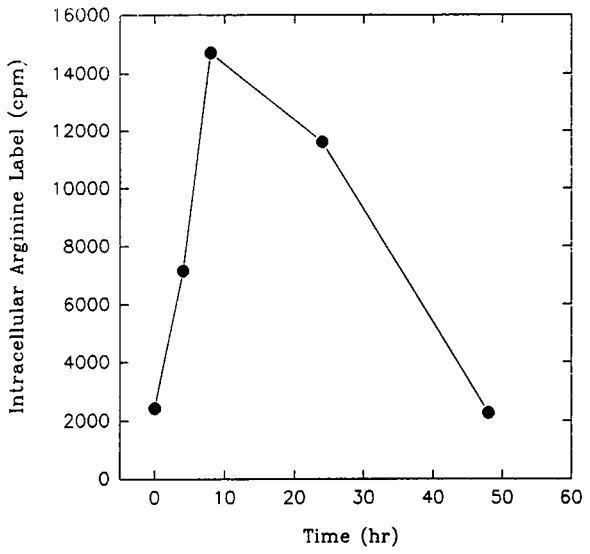

FIG. 2. Arginine transport is increased in activated RAW 264.7 cells

RAW cells were plated and activated with LPS and IFNγ at $t = 0$; and, at the times indicated, after addition of stimulating agents, the cells were washed, then incubated with L-[2,3-^{3}H]-arginine (1 μCi/ml, 69 Ci/mg, 0.025 μl/well) with carrier L-arginine (100 μM) at 37°C. Data shown are the intracellular accumulation of label during the first 5 min after addition of tracer.

rophages were stimulated and remained elevated for more than 24 hr (Fig. 2). Since exogenous L-arginine availability is rate limiting for macrophage NO production (above), and L-arginine transport is stimulated during macrophage activation, we reasoned that an inhibitor of L-arginine transport had the potential to inhibit macrophage NO production. Moreover, since endothelial cells are dependent upon neither extracellular L-arginine nor L-arginine transport to produce NO (29,30), this strategy could be expected to selectively inhibit NO production in macrophages, while preserving endothelial NO production in blood vessels, measured as EDRF activity.

Development of a Novel Compound (CNI-1493) That Inhibits Cytokine-Inducible L-Arginine Transport

Reasoning that a bulky, polyvalent guanylhydrazone compounds might interfere with the function of L-arginine transporters, we designed and synthesized a candidate inhibitor of cytokine-inducible L-arginine uptake that exploits the structural similarity between guanylhydrazone

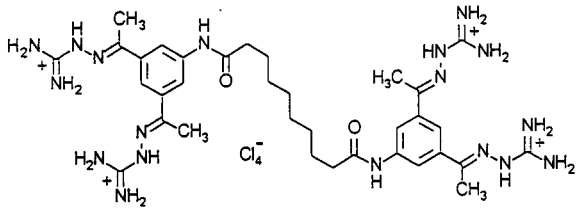

FIG. 3. Structure of CNI-1493

CNI-1493 (N,N'-bis[3,5-diacetylphenyl]decanediamide tetrakis[amidinohydrazone] tetrahydrochloride). Note the four guanidinium groups.

(more properly termed amidinohydrazone) functions and the guanidinium group of L-arginine (Fig. 3). This tetravalent guanylhydrazone compound, termed CNI-1493, inhibited cytokine-inducible L-arginine transport when added to the culture medium of macrophages that had been stimulated 8 hr previously with LPS/IFNγ, and thus were activated to transport L-arginine (Fig. 4). There was a CNI-1493 dose-dependent inhibition of L-arginine transport; the I.C.$_{50}$ of CNI-1493 against L-arginine transport in these postactivation experiments was 59 ± 15 μM, a

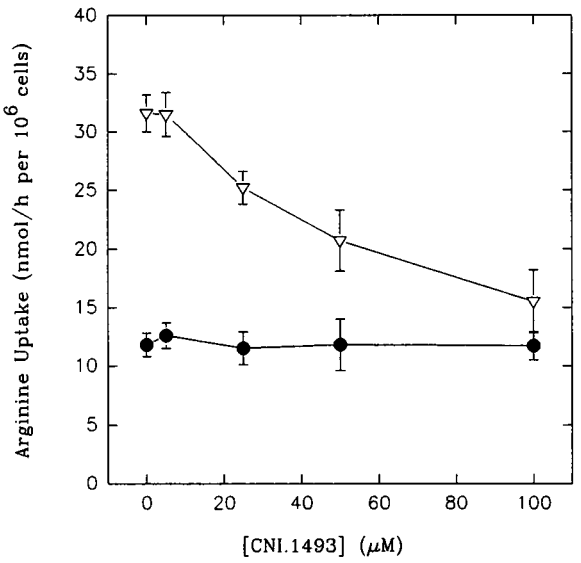

FIG. 4. CNI-1493 inhibits cytokine-inducible L-arginine transport in activated RAW cells

In this postactivation experiment, CNI-1493 was added to cells that had been stimulated 8 hr previously. The control, unstimulated macrophages have low levels of basal L-arginine uptake (filled circles), whereas activation with LPS and IFNγ stimulates L-arginine uptake (open triangles). CNI-1493 caused a dose-dependent inhibition of the cytokine-inducible component of L-arginine uptake. Data shown are L-arginine uptake expressed in nmol/hr/10^6 cells (mean ± SEM, $n = 3$ experiments).

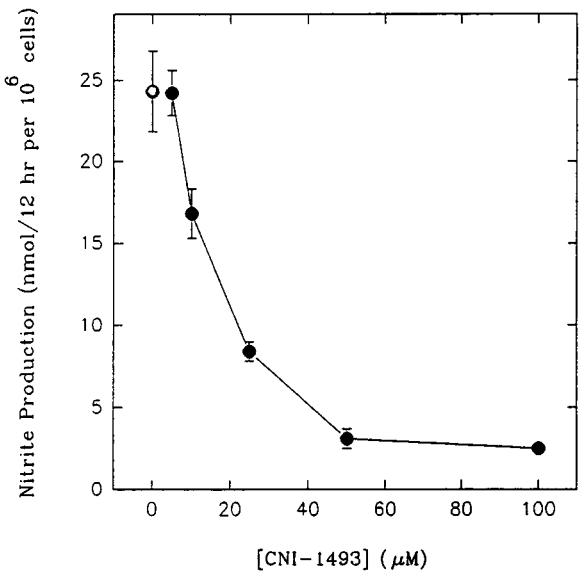

FIG. 5. CNI-1493 inhibits cytokine-inducible NO production in activated RAW cells

In this postactivation experiment, RAW 264.7 cells were stimulated for 8 hr with LPS and IFNγ as indicated, then the medium replaced, and CNI-1493 added to achieve the concentrations indicated for 4 hr. Nitrite production by these treated cells into fresh medium containing L-arginine (100 μM) and CNI-1493 was measured for the subsequent 12 hr period by assay for total nitrites accumulated in the culture medium. Data shown are nitrite production expressed in nmol/12 hr/10^6 cells (mean ± SEM, n = 3 experiments). Where the error bar is not visible, it lies within the extent of the symbol.

concentration at least 5-fold lower than other known transport inhibitors such as L-lysine and L-ornithine (each about 350 μM) (18). Moreover, CNI-1493 did not inhibit basal, unstimulated L-arginine transport in resting macrophages, but was an effective inhibitor of cytokine-inducible L-arginine transport (Fig. 4). This provides evidence that CNI-1493 defines a functionally distinct class of transporters for L-arginine uptake in cytokine-activated macrophages.

CNI-1493 Inhibits NO Production in Macrophages

CNI-1493 effectively suppressed NO production in macrophages that had been previously activated with LPS/IFNγ (Fig. 5). In the absence of CNI-1493, control cultures produced significant quantities of NO estimated by measuring nitrite accumulation during a 12-hr period (24.3 ± 2.5 nmol/12 hr/10^6 cells). Addition of CNI-1493 to the cultures suppressed the production of nitrites over the same period. The inhibitory effect of CNI-1493 on macrophage NO production was dose-dependent; the I.C.$_{50}$ for CNI-1493 as an inhibitor of NO production by previously activated macrophages in these postactivation experiments was 20 ± 2 μM. This concentration is comparable to the estimated I.C.$_{50}$ of L-NMA and L-NAME (15–25 μM) as inhibitors of macrophage NO production in our experiments (not shown) and previous reports (37,38).

CNI-1493 Prevents Macrophage Activation

During the course of a mammalian infection, individual macrophages are stimulated to become activated at different times, so at any instant there are functionally distinct macrophage populations that are either quiescent, primed, or already activated. To evaluate the effects of CNI-1493 in preventing induction of L-arginine transporters in macrophages that have not yet been stimulated, the inhibitor was added to quiescent macrophages 1 hr before the addition of LPS and IFNγ, and L-arginine uptake measured 8 hr later. In the presence of typical plasma concentrations of L-arginine (100 μM), CNI-1493 (7.5 μM) reduced L-arginine uptake to 53% of control values (19.4 ± 1.8 nmol/18 hr/10^6 cell versus 36.2 ± 1.9 nmol/18 hr/10^6 cell in controls). CNI-1493 also prevented cytokine-inducible NO production (measured as nitrite accumulation) in this preactivation model (Fig. 6). This inhibitory effect of CNI-1493 on NO production was partially competed by increasing concentrations of L-arginine in the medium. The I.C.$_{50}$ for CNI-1493 in preventing cytokine-inducible NO production by pretreating quiescent macrophages was 4 ± 1 μM (extracellular L-arginine concentration = 100 μM). This result indicates that CNI-1493 is significantly more effective in preventing the induction of cytokine-mediated NO production in quiescent macrophages than in reversing an ongoing NO response in macrophages that previously had been activated.

CNI-1493 Is Not a Direct NOS Inhibitor

The goal of these studies was to develop an inhibitor of macrophage NO production that would confer a survival advantage in endotoxic shock. To be beneficial, a candidate inhibitor of NO production should work effectively in macrophages, yet inhibit neither NOS activity in vitro (because this enzyme activity is required to gen-

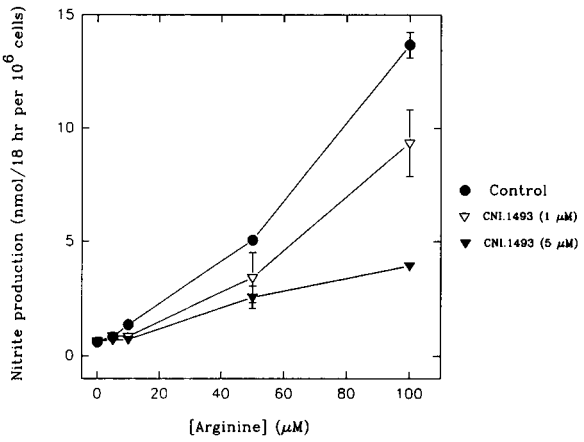

FIG. 6. Pretreatment with CNI-1493 prevents cytokine-inducible NO production in quiescent RAW cells

In this preactivation experiment, there was a dose-dependent inhibition of nitrite production by addition of CNI-1493 at either 1 μM (open triangles) or 5 μM (closed triangles). Note that the inhibitory activity of CNI-1493 competes with increasing L-arginine concentration in the medium.

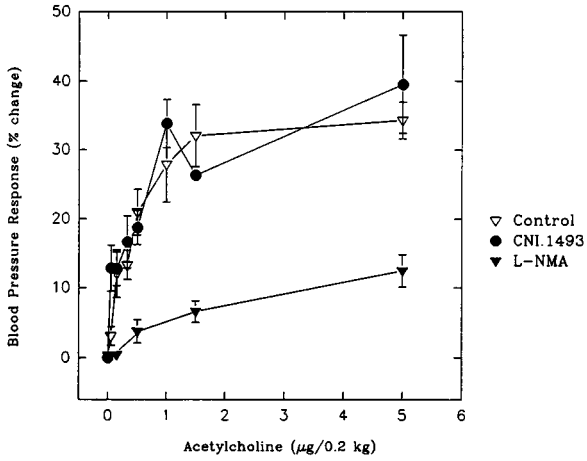

FIG. 7. CNI-1493 does not inhibit EDRF activity

Note that the normal response to increasing doses of the EDRF-dependent drug acetylcholine is an increasing blood pressure response (controls). In agreement with previously published work, pretreatment with L-NMA (positive controls) blunts EDRF activity. This stands in contrast to animals pretreated with CNI-1493, which manifest normal EDRF responses following acetylcholine challenge.

erate EDRF activity), nor EDRF responses measured in vivo. To examine the direct effect of CNI-1493 on NOS activity, we prepared lysates of RAW 264.7 cells that had been stimulated with LPS and IFNγ for 8 hr. As a positive control, the competitive substrate inhibitor of NOS, L-NMA (1 mM added to cell lysate preparations) was observed to completely suppress NOS activity measured by conversion of arginine into citrulline (L-NMA treated = 0.005 nmol/mg/min versus control = 0.058 nmol/mg/min). By contrast, CNI-1493 (50 μM) in macrophage lysates did not inhibit NOS (0.058 nmol/mg/min), suggesting that CNI-1493 does not function as a direct antagonist of NOS enzyme activity. When considered together, these data give strong evidence that CNI-1493 inhibits NO production in cytokine-activated macrophages by interfering with the activation-specific increase in uptake of L-arginine (which is normally required to support macrophage NO production), not by inhibiting NOS directly.

CNI-1493 Preserves Blood Vessel Endothelial-Derived Relaxing Factor Activity

The goal of these studies was to develop an agent that would effectively inhibit NO synthesis by activated macrophages but not inhibit the activity of endothelial-derived relaxing factor (EDRF) in vivo. We next examined the effect of CNI-1493 on EDRF responses in an in vivo animal model (Fig. 7). Blood pressure responses to acetylcholine (an EDRF-dependent vasodilator) were measured continuously in pentobarbital-anesthetized rats before, during, and after administration of CNI-1493, L-NMA, or vehicle only. As expected from previously published data (39), the EDRF response was inhibited by L-NMA, as evidenced by attenuated blood pressure responses in L-NMA-treated animals compared with vehicle-treated controls (Fig. 7). Moreover, in agreement with its known vasoconstrictive properties, we observed that L-NMA caused transient hypertension (data not shown) (13,39,40). In contrast, CNI-1493 did not suppress acetylcholine-induced blood pressure changes or cause hypertension (Fig. 7).

CNI-1493 Prevents Carrageenan-Induced Inflammation

The effectiveness of CNI-1493 in suppressing NO production while preserving EDRF activity prompted us to test its effects in murine models of inflammation. We first utilized a carrageenan-induced paw edema model that has been used

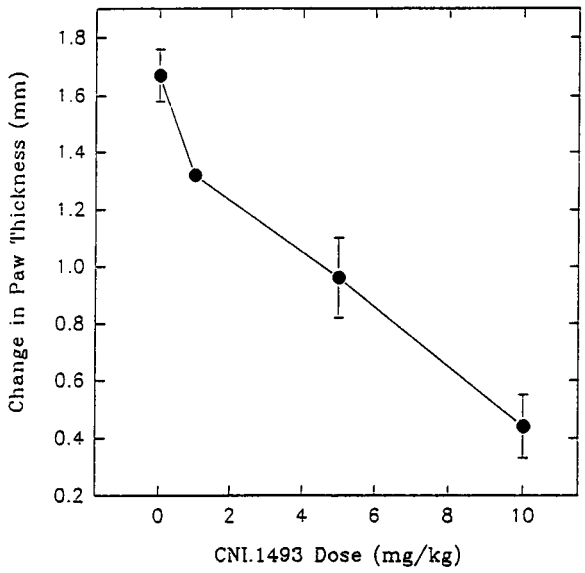

FIG. 8. CNI-1493 suppresses carrageenan-induced paw edema

CNI-1493 was administered intraperitoneally, and 90 min later carrageenan was injected into the footpad. The data shown are the changes in footpad thickness between the carrageenan-injected and the vehicle-injected footpad after 3 hr. The results shown are from a representative experiment; this experiment was repeated at least three times (n = 6 animals per dose).

for more than 30 years as a screening assay for the development of anti-inflammatory compounds (22). Mice received injections of carrageenan into the right hind paw and saline vehicle alone into the left paw. The inflammatory response to carrageenan caused significant paw swelling within 3 hr in controls as measured by an increase in the diameter of the carrageenan-injected footpad (Fig. 8). CNI-1493 administered intraperitoneally at various doses (Fig. 8) prevented the development of carrageenan-induced paw swelling. Histological examination of carrageenan-inflamed paw tissue revealed marked subcutaneous edema and moderate to severe neutrophil infiltration (Fig. 9 a and b). Polymorphonuclear leukocytes were observed to be marginated around dilated capillaries in the soft tissues. CNI-1493 conferred significant protection against the development of this subcutaneous edema and neutrophil recruitment (Fig. 9 c and d). These results give evidence that, in doses that are comparable to other widely used anti-inflammatory agents (e.g., the therapeutic doses of acetaminophen and ibuprofen in humans are between 10 and 15 mg/kg), CNI-1493 prevents inflammation.

Further evidence implicating NO as a mediator of edema in this model was obtained by administering S-nitro-N-acetyl-penicillamine (SNAP) directly into the footpad. SNAP is a chemical precursor of NO that decomposes to NO and N-acetyl-penicillamine. SNAP injected directly into foot pads (0.5 mg/50 μl/paw) caused significant subcutaneous edema within 3 hr (1.0 + 0.09 mm); but controls that received N-acetyl-penicillamine did not develop edema (0.03 $\pm$ 0.035 mm) as measured by paw swelling.

To test whether CNI-1493 inhibited macrophage NO production in vivo, the agent (0.4 mg/kg, i.p.) or vehicle alone was administered to BALB/c mice, and peritoneal macrophages isolated by lavage 2 hr later. Upon subsequent LPS/IFNγ treatment in vitro, NO production over 18 hr was attenuated 76% in macrophages obtained from CNI-1493-treated animals (NO production in controls = 28 $\pm$ 4 nmol/1 $\times$ 10^6 cells versus CNI-1493 treated = 7 $\pm$ 2 nmol/1 $\times$ 10^6 cells). Considered together, these results indicate that CNI-1493 is effective in preventing carrageenan-induced inflammation and NO production by macrophages in vivo.

CNI-1493 Confers Survival Advantage Against Endotoxin Lethality

The development and characterization of CNI-1493 in these experiments enabled us to next address the central hypothesis: an inhibitor of macrophage NO production that preserves EDRF responses can protect against the lethal toxicity of LPS in vivo. Accordingly, we utilized a murine model of endotoxemia to evaluate the effect of CNI-1493 in preventing lethal toxicity (Fig. 10). Administration of CNI-1493 (1 mg/kg) to BALB/c mice 1.5 hr before LPS challenge (at an $L.D._{50}$ dose) increased survival from 50% for vehicle-treated controls to 90% for the CNI-1493-treated group ($p < 0.05$). In agreement with previous observations, administration of L-NMA 1.5 hr before LPS challenge decreased survival to only 10% ($p < 0.05$). Controls receiving LPS and vehicle, and animals treated with LPS and L-NMA, were unkempt, showed decreased spontaneous movement about the cage, and huddled together; these visible signs of LPS toxicity were also markedly suppressed by administration of CNI-1493. Diarrhea occurred in all animals and was not suppressed by CNI-1493. These data indicate that an inhibitor of L-arginine transport that preserves EDRF activity is protective against LPS lethality.

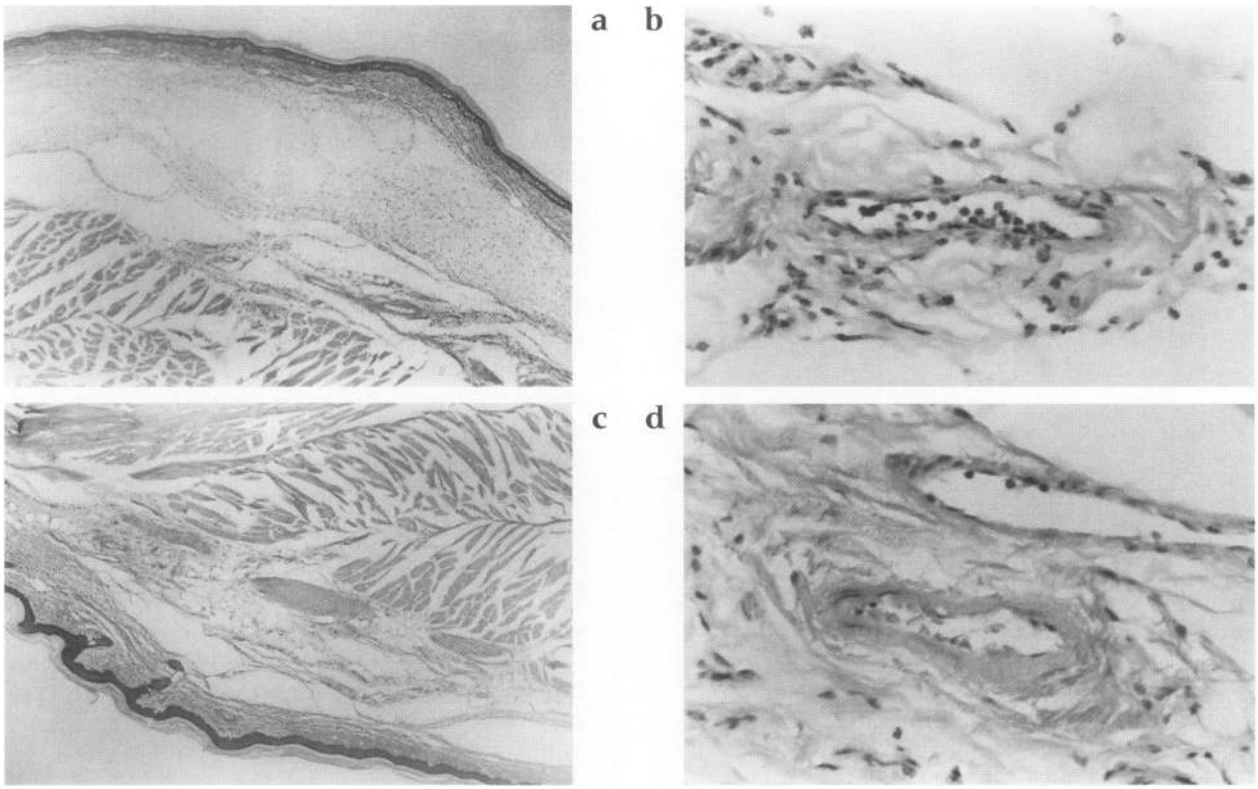

FIG. 9. Histological appearance of paw tissues obtained from the carrageenan-injected paw

Three hours after carrageenan administration, paw tissues were fixed with 10% formalin, and sections were stained with hematoxylin and eosin. (a and b) Sections taken from the carrageenan-treated footpad of a control animal not receiving CNI-1493. (a) A low power panoramic view; (b) a high power view of the same tissue showing neutrophil margination in a dilated capillary. (c and d) Comparable sections taken from the carrageenan-treated footpad of an animal treated with CNI-1493 (10 mg/kg, i.p.).

DISCUSSION

These studies give direct evidence that a novel compound (CNI-1493) that inhibits cytokine-inducible L-arginine transport in macrophages is effective in preventing NO production, carrageenan-induced inflammation and LPS lethality. To our knowledge, this is the first compound to target macrophage NO production but preserve EDRF activity in blood vessels, thereby offering protection against lethal endotoxic shock and organ damage.

CNI-1493 Prevents Macrophage Activation

An unexpected but important finding from these experiments is our observation that CNI-1493 is an extremely potent inhibitor of LPS-induced activation of macrophages. These results are somewhat analogous to the actions of glucocorticoids, which effectively prevent macrophage activation when administered before LPS (as measured by NO production), and prevent both carrageenan-induced inflammation and LPS lethality. While the present data give direct evidence that CNI-1493 is an inhibitor of cytokine-inducible L-arginine transport in macrophages, it is unclear whether its effect as a transport inhibitor accounts directly for its suppression of macrophage activation. The cytokine-inducible L-arginine transporter in macrophages has been identified as MCAT-2b (41), and it is plausible that CNI-1493 pretreatment may inhibit the earliest population of MCAT-2b transporters to be induced following cytokine treatment. This early interference might, in turn, interrupt any NO-dependent "feed forward" signals controlling further up-regulation of the cytokine-inducible component of arginine transport and thus potently inhibit total NO production. Another possibility is that CNI-1493 acts at another site in the signaling process of macrophage activation, perhaps by down-regulating cytokine production.

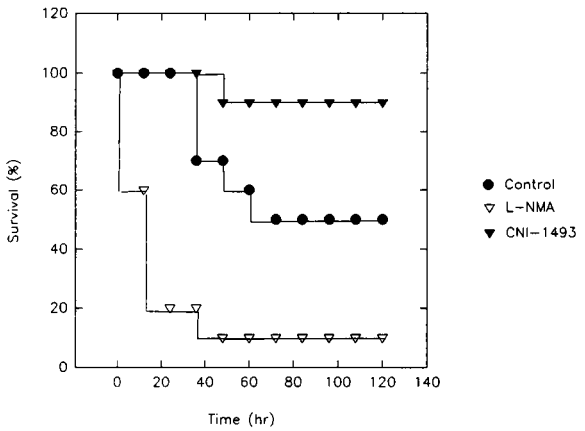

FIG. 10. CNI-1493 protects against LPS-induced lethality

All animals received an L.D.$_{50}$ dose of *E. coli* 0111:B4 LPS (13.75 mg/kg). Note that L-NMA significantly decreased survival, but CNI-1493 significantly improved survival. Each group consisted of 10 mice; differences between CNI-1493 and controls, and between L-NMA and controls, are statistically significant ($p < 0.05$ by the two-tailed Fisher's exact test).

This possibility is particularly intriguing, and probably unique, since other known inhibitors of L-arginine transport (e.g., lysine and ornithine) are ineffective in preventing macrophage activation as measured by NO production, with estimated I.C.$_{50}$ in the range of 10 mM (18). Albina and coworkers have previously reported that arginine availability directly influences macrophage function (42). The availability of a potent inhibitor of L-arginine uptake (CNI-1493) may now prove useful in delineating the functional role of cytokine-inducible L-arginine transporters (MCAT-2b) and L-arginine availability in the biology of macrophage activation.

Anti-Inflammatory Effects of CNI-1493

The macrophage occupies a paradoxical role in defense against invasion or infection because once unleashed, its armamentarium may indiscriminately injure tissues and kill the very host it evolved to protect (1,21,43,44). Novel therapeutic strategies designed to specifically prevent this "collateral damage" during inflammation are at all stages of preclinical testing and clinical use. Since CNI-1493 prevents carrageenan-induced inflammation, it seems likely that it acts to effectively interrupt the mediator cascade that normally recruits inflammatory cells, and also prevents the release of toxic products that injure tissues. Nonselective inhibitors of NOS (e.g., L-NMA and L-NAME) are protective against carrageenan-induced inflammation when co-injected directly in the paw (45), but are relatively ineffective in preventing acute edema formation when given systemically (46). When considered together with the present data, it is likely that inhibiting NO locally in the paw accounts (at least partially) for the beneficial activity of CNI-1493 in carrageenan-induced inflammation.

CNI-1493 in Endotoxic Shock

The development of a pharmacological inhibitor of macrophage arginine transport should prove useful in further delineating specific details of macrophage function in endotoxin and septic shock states. The unique actions of CNI-1493 in preventing both macrophage NO production and LPS lethality differ significantly from nonselective NOS inhibitors. For instance, evidence obtained with human immune cells indicates that NO participates in the regulation of the synthesis of TNF and other cytokines (47). The principal mediator of LPS toxicity is TNF, and, when coupled with the loss of organ perfusion resulting from the nonselective NOS inhibitors, the increased lethality resulting from administration of unspecific NOS inhibitors is not surprising (2,10–14,21,44,48). It will be of interest now to examine directly the influence of systemically administered CNI-1493 on the cytokine response in LPS-treated animals, and in human immunocytes.

CONCLUSION

We have developed a selective inhibitor of cytokine-inducible L-arginine transport (CNI-1493) that effectively inhibits NO production by activated macrophages. In contrast to previously available NO inhibitors, this agent does not inhibit NOS directly, and preserves EDRF activity in blood vessels. When administered in vivo, CNI-1493 is effective against endotoxin lethality and carrageenan inflammation. These studies indicate that it is feasible to develop novel therapeutics for inflammation and septic shock syndrome by selective inhibition of cytokine-inducible L-arginine transporter function in activated macrophages.

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