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Beneficial effects and improved survival in rodent models of septic shock with *S*-methylisothiourea sulfate, a potent and selective inhibitor of inducible nitric oxide synthase

(endotoxin/diabetes/inflammation/arthritis/cancer therapy)

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ABSTRACT **Enhanced formation of nitric oxide (NO) by both the constitutive and the inducible isoforms of NO synthase (NOS) has been implicated in the pathophysiology of a variety of diseases, including circulatory shock. Non-isoform-selective inhibition of NO formation, however, may lead to side effects by inhibiting the constitutive isoform of NOS and, thus, the various physiological actions of NO. *S*-Methylisothiourea sulfate (SMT) is at least 10- to 30-fold more potent as an inhibitor of inducible NOS (iNOS) in immunostimulated cultured macrophages (EC_{50}, 6 μM) and vascular smooth muscle cells (EC_{50}, 2 μM) than N^G-methyl-L-arginine (MeArg) or any other NOS inhibitor yet known. The effect of SMT on iNOS activity can be reversed by excess L-arginine in a concentration-dependent manner. SMT (up to 1 mM) does not inhibit the activity of xanthine oxidase, diaphorase, lactate dehydrogenase, monoamine oxidase, catalase, cytochrome P450, or superoxide dismutase. SMT is equipotent with MeArg in inhibiting the endothelial, constitutive isoform of NOS *in vitro* and causes increases in blood pressure similar to those produced by MeArg in normal rats. SMT, however, dose-dependently reverses (0.01–3 mg/kg) the hypotension and the vascular hyporeactivity to vasoconstrictor agents caused by endotoxin [bacterial lipopolysaccharide (LPS), 10 mg/kg, i.v.] in anesthetized rats. Moreover, therapeutic administration of SMT (5 mg/kg, i.p., given 2 hr after LPS, 10 mg/kg, i.p.) attenuates the rises in plasma alanine and aspartate aminotransferases, bilirubin, and creatinine and also prevents hypocalcaemia when measured 6 hr after administration of LPS. SMT (1 mg/kg, i.p.) improves 24-hr survival of mice treated with a high dose of LPS (60 mg/kg, i.p.). Thus, SMT is a potent and selective inhibitor of iNOS and exerts beneficial effects in rodent models of septic shock. SMT, therefore, may have considerable value in the therapy of circulatory shock of various etiologies and other pathophysiological conditions associated with induction of iNOS.**

Nitric oxide (NO) is produced by a group of isoenzymes collectively termed NO synthases (NOSs) (1, 2). NO derived from the constitutive isoform is a neurotransmitter in the central and peripheral nervous system (3). NO generated by the constitutive isoform of NOS in the vascular endothelium (eNOS) is involved in the regulation of blood pressure and organ blood flow distribution and inhibits the adhesion of platelets and polymorphonuclear granulocytes to the endothelial surface (1, 2, 4). Dysfunction of NO formation by the vascular endothelium is implicated in the pathogenesis of hypertension, hypercholesterolemia, diabetes mellitus, ischemia–reperfusion injury, angina pectoris, subarachnoid hemorrhage, and various forms of circulatory shock (5–11). NO derived from eNOS also plays a protective role in endotoxin-induced gastointestinal damage (12, 13).

Another isoform of NOS (inducible NOS, iNOS) can be induced in various cells, including macrophages, by a variety of agents such as endotoxin (bacterial lipopolysaccharide, LPS), interleukin 1, tumor necrosis factor, and γ-interferon (IFN-γ). The cytotoxicity of NO from activated macrophages plays a key role in their antimicrobial activity (1, 2). Enhanced formation of NO following the induction of iNOS contributes importantly to the circulatory failure (hypotension and vascular hyporeactivity to vasoconstrictor agents) in circulatory shock of various etiologies (14–19). Moreover, iNOS plays a role in the pathophysiology of a variety of other diseases, including diabetes and transplant rejection (20–28). However, non-isoform-selective inhibition of NO formation may lead to side effects by inhibiting the constitutive isoform of NOS and, thus, the various physiological actions of NO. Thus, selective inhibitors of iNOS may well be of great therapeutic potential. Some L-arginine analogues show a limited isoform selectivity in their inhibition of NOS. For instance, N^G-cyclopropyl-L-arginine, N^G-nitro-L-arginine (NO_2Arg), and its methyl ester (after hydrolysis) are more potent inhibitors of eNOS than of iNOS (29–32), whereas N^G-methyl-L-arginine (MeArg), N^G-amino-L-homoarginine, and N^G-amino-L-arginine are equipotent inhibitors of both iNOS and eNOS (29–32). MeArg is the preferred L-arginine-based NOS inhibitor for targeting iNOS *in vivo* or *in vitro*. Aminoguanidine and some related guanidines, however, are weaker inhibitors of eNOS than iNOS and, hence, are relatively selective as inhibitors of iNOS (33, 34). Here we report that *S*-methylisothiourea sulfate (SMT) is a potent NOS inhibitor which is relatively selective for iNOS activity and exerts beneficial effects in rodent models of septic shock.

METHODS

Cell Culture. The mouse macrophage cell line J774.2 and rat aortic smooth muscle cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 3.5 mM L-glutamine and 10% fetal bovine serum (35, 36). Cells were cultured in 96-well plates (200 μl of medium per well) until they reached confluence. To induce iNOS in macrophages, fresh culture medium containing *Escherichia coli* lipopolysaccharide (LPS, 1 μg/ml) was added and nitrite accumulation in the culture medium was measured 24 hr later. In vascular smooth muscle cells, iNOS was induced by LPS (1 μg/ml) and γ-interferon (IFN-γ, 10 units/ml), and nitrite was measured after 48 hr. To assess the effects of

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Abbreviations: IFN-γ, γ-interferon; LPS, bacterial lipopolysaccharide; MAP, mean arterial blood pressure; MeArg, N^G-methyl-L-arginine; NOS, nitric oxide synthase; eNOS, constitutive endothelial NOS; iNOS, inducible NOS; NO_2Arg, N^G-nitro-L-arginine; SMT, S-methylisothiourea sulfate.

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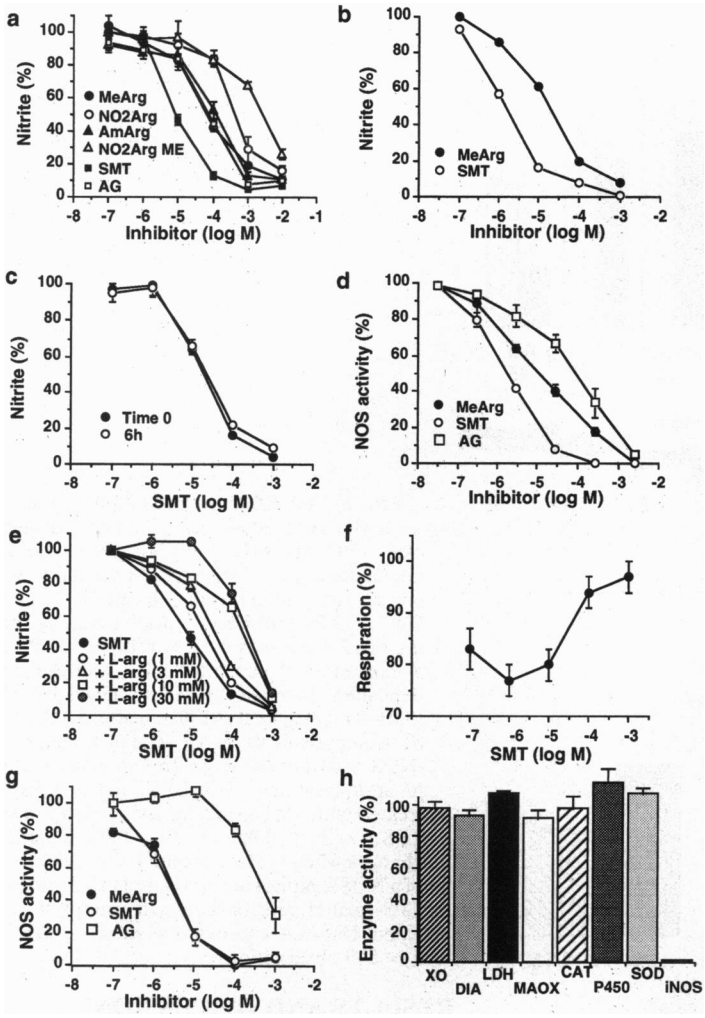

FIG. 1. (*a*) Effect of SMT, MeArg, N^G-amino-L-arginine (AmArg), aminoguanidine (AG), NO_2Arg, or its methyl ester (NO_2Arg ME) on the increase in nitrite concentration in the supernatant of cultured J774.2 macrophages activated with LPS for 24 hr. (*b*) Effect of SMT or MeArg on the increase in nitrite concentration in the supernatant of cultured rat aortic smooth muscle cells activated with LPS and IFN-γ for 48 hr. (*c*) Effect of SMT, given either together with LPS or 6 hr after LPS, on the increase in nitrite concentration in the supernatant of cultured J774.2 macrophages activated with LPS for 24 hr. (*d*) Effect of SMT, MeArg, or AG on citrulline formation (an indicator of iNOS activity) in lung homogenates obtained from rats treated with LPS (10 mg/kg, i.v.; 3 hr). (*e*) Effect of L-arginine (1–30 mM) on the inhibition of nitrite accumulation caused by SMT. (*f*) Mitochondrial respiration of cultured macrophages (24 hr after LPS) in the presence of various concentrations of SMT. (*g*) Effect of SMT, MeArg, or AG on citrulline formation (an indicator of eNOS activity) in endothelial cell homogenates. (*h*) Effect of SMT (1 mM) on the activity of xanthine oxidase (XO), diaphorase (DIA), lactate dehydrogenase (LDH), monoamine oxidase (MAO), catalase (CAT), cytochrome P450 (P450), superoxide dismutase (SOD), and iNOS. Data are expressed as percentage of nitrite concentration produced by cells activated with LPS alone (*a*, *c*, and *e*) or with LPS and IFN-γ (*b*), or as percentage of total enzymatic activity (*d*, *g*, *h*) in the absence of inhibitors, or as percentage of cellular respiration seen in cells not treated with LPS (*f*). Means ± SEM of n = 6–9 observations are shown. Nitrite concentration in the culture medium of J774.2 cells at 24 hr was 36 ± 1 μM; nitrite concentration in the culture medium of smooth muscle cells at 48 hr was 74 ± 3 μM. eNOS activity in the homogenates of endothelial cells was 4.12 ± 0.6 pmol/mg of protein per min; iNOS activity in the homogenates of lung was 6.2 ± 0.8 pmol/mg of protein per min. Formazan production (an indicator of cellular respiration) in J774.2 cells not treated with LPS amounted to 0.32 ± 0.07 μg per well per min.

various inhibitors on nitrite production, agents were added to the cells either 5 min before or 6 hr after LPS. Nitrite production, an indicator of NO synthesis, was measured by the Griess reaction (35, 36). Cell respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (35, 36). Formazan production is expressed as a percentage of that observed in cells not treated with LPS.

iNOS and eNOS Activities. Bovine aortas were obtained from the local slaughterhouse and the intimal surface (endothelium) was removed by scraping. Lungs were obtained from rats 3 hr after injection of LPS (10 mg/kg, i.v.). To measure eNOS activity in endothelial cell homogenates, production of L-[^{3}H]-citrulline from L-[^{3}H]arginine (10 μM, 5 kBq per tube) was measured in the presence of NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 μM), Ca^{2+} (2 mM), and vehicle, SMT, or MeArg (10^{-7}–10^{-3} M) for 20 min at 37°C in Hepes buffer (pH 7.5) (17, 18). To measure iNOS activity, lung homogenates were incubated with the above factors and EGTA (5 mM), but without Ca^{2+} (17, 18). Reactions were stopped by dilution with 1 ml of ice-cold Hepes buffer, pH 5.5/2 mM EGTA/2 mM EDTA. Reaction mixtures were applied to Dowex 50W (Na^+ form) columns; L-[^{3}H]citrulline was eluted and measured by scintillation counting.

Enzyme Activities. Xanthine oxidase (0.04 unit/ml) was measured by its reduction of nitroblue tetrazolium (600 μM) using xanthine (0.1 mM) as substrate in the presence of Ca^{2+} (5 mM) and Mg^{2+} (5 mM). Diaphorase activity was measured by a kinetic colorimetric assay based on its reduction of nitroblue tetrazolium (100 μM) using porcine heart diaphorase (0.05 unit/ml; Sigma) with NADPH (50 μM) as cofactor. Lactate dehydrogenase from rabbit muscle (60 μg/ml; 12 ± 0.2 units/ml) was measured by an end-point colorimetric assay (Sigma). Monoamine oxidase activity (bovine plasma, 0.09 unit/ml), was measured by the conversion of benzylamine (100 μM) to benzaldehyde and determined as ΔA_{250}. Catalase (10 units/ml, Sigma) was measured colorimetrically by using FOX 1 reagent and xylenol orange (37). Cytochrome P450 activity in rat liver microsomes was measured by the conversion of 7-pentoxyresorufin (5 μM) to resorufin in the presence of NADPH (250 μM) (38). (Control cytochrome P450 activity was 30 nmol of resorufin per min per mg of protein.) Superoxide dismutase (20 units/ml; Sigma) activity was measured by its effect on xanthine oxidase in the xanthine oxidase assay.

Hemodynamic Measurements. Male Wistar rats (260–320 g) were anesthetized with thiopentone sodium (120 mg/kg, i.p.). The trachea was cannulated to facilitate respiration and rectal temperature was maintained at 37°C with a homeothermic blanket. The right carotid artery was cannulated and connected to a pressure transducer (P23XL; Spectramed, Stratham, Oxnard, CA) for the measurement of phasic and mean arterial blood pressure (MAP) and heart rate, which were displayed on a polygraph recorder. Left and right femoral veins were cannulated for the administration of drugs. To cause shock, animals received LPS (10 mg/kg, i.v.) as a slow injection over 10 min (17, 18). After 180 min, SMT (0.01–3 mg/kg, i.v.) was injected. For comparison, the effect of MeArg, NO_2Arg methyl ester, and aminoguanidine were also investigated.

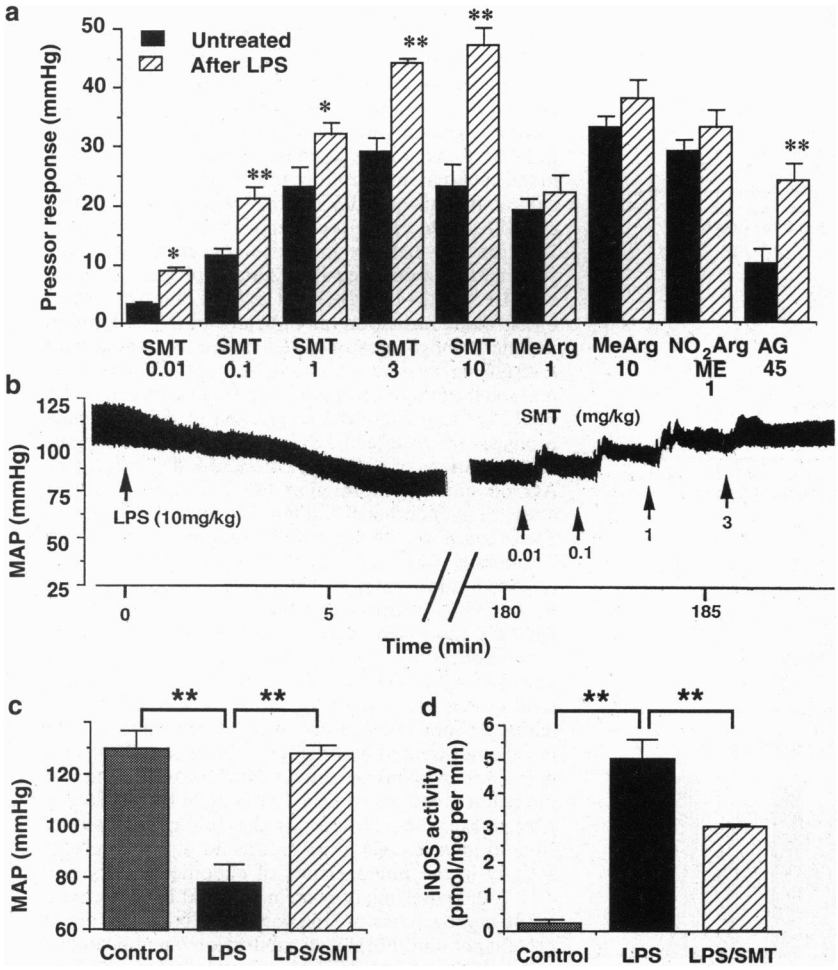

FIG. 2. (*a*) Effect of SMT (0.01–10 mg/kg, i.v.), MeArg (1–10 mg/kg, i.v.), NO_2Arg methyl ester (NO_2Arg ME, 1 mg/kg, i.v.), and aminoguanidine (AG, 45 mg/kg, i.v.) on MAP in control rats and in rats at 3 hr after LPS. (1 mm Hg = 133 Pa.) (*b*) Representative tracing showing MAP in an anesthetized rat before LPS and 5 min and 180 min after LPS, and the dose-dependent increase in MAP caused by SMT (0.01–3 mg/kg, i.v.) at 180 min after LPS. (*c* and *d*) Comparison of MAP values (*c*) and lung iNOS activities (*d*) in control animals, in rats 3 hr after treatment with LPS, and in LPS rats treated with SMT (3 mg/kg, i.v., at 180 min after LPS). *, $P < 0.05$; **, $P < 0.01$: significant changes when pressor responses to a given dose of a NOS inhibitor are compared in untreated vs. LPS-treated rats or between groups as indicated. Data are expressed as means ± SEM of $n = 5$–9 observations.

LPS-Induced Changes in Plasma Markers. Male Wistar rats (260–320 g) were injected with LPS (10 mg/kg, i.p.) or with vehicle. At 2 hr after LPS or vehicle, animals received SMT (5 mg/kg, i.p.). A separate group of rats was treated with SMT (50 mg/kg, i.p.) at 2 hr after injection of vehicle. Six hours after injection of LPS or vehicle, animals were anesthetized with halothane and blood was taken by cardiac puncture. Blood samples for assays of plasma alanine aminotransferase, aspartate aminotransferase, bilirubin, creatinine, and Ca^{2+}, as well as differential blood cell counts, were analyzed by Vetlab Services (Sussex, UK).

Survival Studies. Swiss albino mice (26–30 g) were injected with *E. coli* LPS (60 mg/kg, i.p.). Two and 6 hr after LPS, animals received SMT (0.1 or 1 mg/kg, i.v.); 8–12 mice were used in each group.

Materials. Bacterial LPS (*E. coli*, serotype 0127:B8), calmodulin, DMEM, L-glutamine, MTT, NADPH, NO_2Arg and its methyl ester, sodium nitrite, and Dowex 50W anion-exchange resin were from Sigma. MeArg monoacetate was from Calbiochem. Fetal bovine serum was obtained from Advanced Protein Products (West Midlands, U.K.). L-(2,3,4,5-^{3}H]Arginine hydrochloride was from Amersham. N^G-Amino-L-arginine was synthesized by O.W. Griffith (Medical College of Wisconsin, Milwaukee). SMT was purchased from Aldrich.

Statistical Evaluation. All values in the figures and text are expressed as mean ± SEM of *n* observations. For each drug, at least nine wells from three independent experiments were studied. Student's unpaired *t* test was used to compare means between groups. For comparisons in the survival rate, the χ^2 test was used. $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

SMT Is a Competitive Inhibitor of iNOS. Comparison of the inhibitory effects of SMT and MeArg on LPS-induced nitrite production in cultured J774.2 macrophages and rat aortic vascular smooth muscle cells showed that SMT was 10 times more potent in cultured macrophages ($EC_{50} \approx 6$ μM) and 30 times more potent in the rat vascular smooth muscle cells ($EC_{50} \approx 2$ μM) (Fig. 1 *a* and *b*). SMT is a more potent inhibitor of iNOS than any other known inhibitor of NOS, including N^G-amino-L-arginine, aminoguanidine, NO_2Arg, or its methyl ester, in both cell types studied (Fig. 1*a*). SMT is a direct inhibitor of the activity of iNOS and does not interfere with the induction of iNOS, for (*i*) the inhibitory effect of SMT on nitrite formation of LPS-activated macrophages remains unchanged when SMT is given after the stimulus of induction (Fig. 1*c*) and (*ii*) SMT also inhibits the formation of L-citrulline from L-arginine in homogenates of lungs obtained from rats treated with LPS in the absence of Ca^{2+} (Fig. 1*d*). This L-citrulline formation is due to iNOS activity (17). The effect of SMT is attenuated by excess L-arginine in the culture medium (Fig. 1*e*), suggesting that SMT is a competitive inhibitor of iNOS activity at the L-arginine site. SMT itself is not cytotoxic. Indeed, it prevents the NO-mediated cytotoxic effect of LPS in cultured macrophages (Fig. 1*f*) which is due to inhibition of mitochondrial respiratory-chain enzymes (39).

While SMT is more potent than MeArg on iNOS activity, SMT is equipotent with MeArg in inhibiting eNOS activity in endothelial cell homogenates (Fig. 1*g*). This suggests that SMT has a relative selectivity toward iNOS when compared with MeArg or other L-arginine-based NOS inhibitors (29–32). When compared with aminoguanidine, another non-

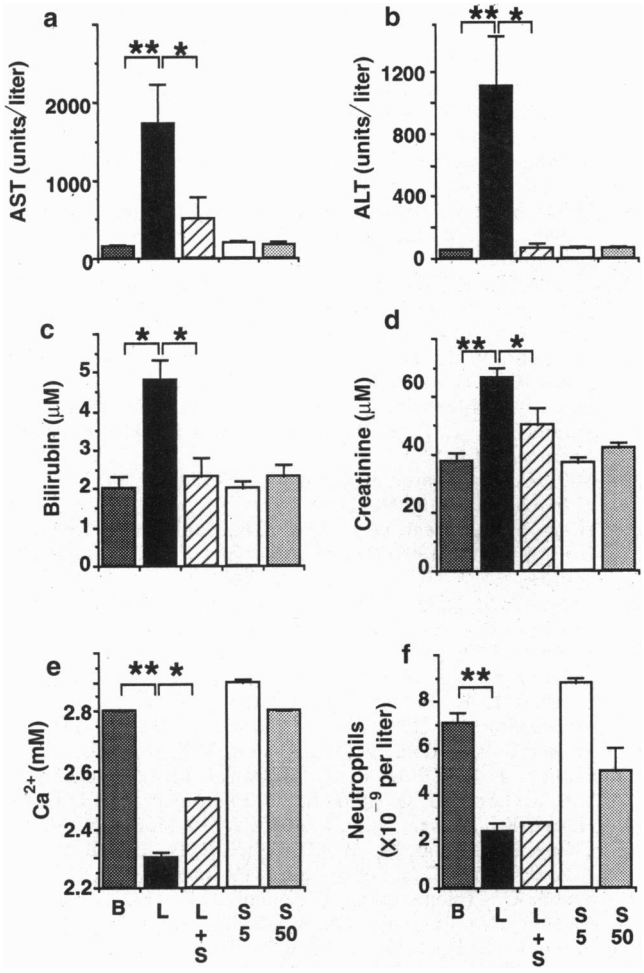

FIG. 3. Effect of SMT on LPS-induced increases in plasma concentrations of aspartate aminotransferase (AST) (*a*), alanine aminotransferase (ALT) (*b*), bilirubin (*c*), and creatinine (*d*) and on LPS-induced decreases in Ca^{2+} (*e*) and neutrophil count (*f*) at 6 hr after injection of LPS (10 mg/kg, i.p.). *, $P < 0.05$; **, $P < 0.01$: significant changes between groups as indicated. Student's unpaired *t* test was used to compare means between groups. Different groups of animals received vehicle (basal; B); LPS alone (L), LPS and SMT (5 mg/kg, i.p. 2 hr after LPS; L + S), or vehicle plus SMT (5 or 50 mg/kg, i.p., 2 hr after vehicle; S 5 and S 50, respectively). Data are expressed as means ± SEM of $n = 5$–8 observations.

arginine-based NOS inhibitor with selectivity toward iNOS (20, 21), SMT is 10–50 times more potent as an inhibitor of iNOS activity in LPS-treated J774.2 macrophages and in lung homogenates obtained from rats treated with LPS (Fig. 1 *a* and *d*). In addition, SMT is ≈100 times more potent than aminoguanidine on eNOS activity (Figs. 1*g* and 2*a*; see also refs. 20 and 21). This suggests that SMT is almost as selective as aminoguanidine as an inhibitor of iNOS activity, while it has a lower EC_{50} for iNOS activity *in vitro*.

The effect of SMT appears to be specific for NOS, since it does not inhibit (up to 1 mM) the activity of XO, diaphorase, lactate dehydrogenase, monoamine oxidase, catalase, cytochrome P450, or superoxide dismutase (Fig. 1*h*).

Hemodynamic Effects of SMT in the Rat. The pressor effects of moderate doses of SMT in control anesthetized rats are similar to those of MeArg and weaker than those of NO_2Arg methyl ester. This is in accord with the *in vitro* eNOS data. However, in contrast to the arginine analogues, the pressor responses afforded by SMT are much greater in rats treated with LPS than in control rats. Thus, SMT is a more potent pressor agent than MeArg in septic rats (Fig. 2 *a* and *b*), indicating that SMT is the more potent inhibitor of iNOS activity *in vivo*. Most notably, injection of SMT (3 mg/kg) into LPS-treated rats causes a prompt restoration of the blood pressure to pre-LPS levels (Fig. 2*c*). In addition, SMT inhibits iNOS activity measured in homogenates of lung from rats treated with LPS (Fig. 2*d*).

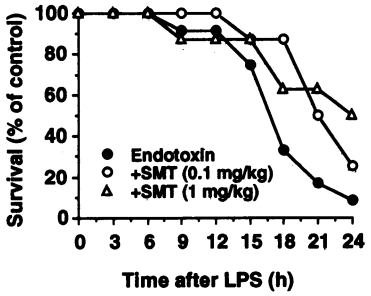

FIG. 4. (*a*) Effect of SMT (0.1 or 1 mg/kg, i.p.) on the survival of mice challenged with LPS (60 mg/kg, i.p.) ($n = 8$–10 animals were used in each group).

Aminoguanidine, in normal rats, did not markedly increase blood pressure even at a dose as high as 45 mg/kg. However, in rats treated with LPS, the same dose of aminoguanidine caused only a partial restoration of the blood pressure (Fig. 2*a*). This observation, together with the facts that (*i*) the vascular actions of aminoguanidine have a slow onset (40) and (*ii*) the drug inhibits a variety of other enzymes, suggests that SMT may have advantages over aminoguanidine in targeting iNOS activity *in vivo*.

LPS treatment for 6 hr increases the plasma levels of alanine and aspartate aminotransferases and of bilirubin (Fig. 3 *a*–*c*). Administration of SMT 2 hr after LPS prevents these changes, suggesting that SMT protects against the LPS-induced (and NO-mediated) liver damage (Fig. 3 *a*–*c*). These results do not support a previous proposal (41) that iNOS plays a protective role in hepatic dysfunction in sepsis. LPS also causes an impairment of renal function, as indicated by an increase in plasma creatinine (Fig. 3*d*). Inhibitors of eNOS are known to exaggregate this renal damage by causing further vasoconstriction and glomerular thrombosis (42), whereas SMT causes a significant improvement of this parameter of renal function (Fig. 3*d*), possibly by increasing MAP and, hence, perfusion pressure to the kidney. SMT also protects against the hypocalcemia associated with endotoxin shock (Fig. 3*e*). However, it does not enhance the neutropenia caused by LPS (Fig. 3*f*), suggesting either that SMT only slightly affects eNOS activity *in vivo* or that any potential inhibition of eNOS activity by SMT is not sufficient to cause an enhanced adhesion of neutrophils to the endothelium. When given to control rats, SMT (up to 50 mg/kg) has no effect on any of the above parameters, suggesting that acute administration of a high dose of SMT does not cause liver or kidney dysfunction or neutropenia (Fig. 3).

Effect of SMT on 24-hr Survival in Mice Treated with LPS. In mice injected with a high dose of LPS (60 mg/kg, i.p.), SMT (1 mg/kg) improves 24-hr survival from 8.3% to 50% ($P < 0.05$) (Fig. 4). We speculate that the selective inhibition by SMT on iNOS activity is responsible for this effect.

Conclusions. Here we demonstrate that SMT is a more potent inhibitor of iNOS activity than any of the yet described NOS inhibitors. SMT protects rats against the circulatory failure and organ dysfunction caused by endotoxin and improves survival in a murine model of septic shock. SMT, or other iNOS-selective inhibitors, are likely to have fewer side effects which are related to the inhibition of eNOS, such as excessive vasoconstriction and organ ischemia (1, 2, 4), increased platelet and neutrophil adhesion and accumulation (1, 2, 4, 43), and microvascular leakage (43). We speculate

that SMT will have advantages over L-arginine-based NOS inhibitors (e.g., MeArg) in the therapy of circulatory shock of various etiologies (14–19). These compounds may also be of therapeutic benefit in other diseases associated with iNOS induction, such as chronic arthritis (22, 23), chronic inflammation (24), diabetes (20, 21), transplant rejection (26), some forms of chronic inflammatory reactions of the central nervous system (25), and some forms of cancer (27, 28).

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