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Effect of a Nitric Oxide Synthase Inhibitor, S-Ethylisothiourea, on Cultured Cells and Cardiovascular Functions of Normal and Lipopolysaccharide-Treated Rabbits[1]

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Nitric oxide (NO) is synthesized from L-arginine by three isoforms of NO synthase (NOS). It is essential to suppress the function of the inducible isoform (macNOS) for amelioration of some inflammatory diseases in which the cytotoxic effect of NO is involved. *S*-Ethylisothiourea (*S*-EIU) was reported to be a potent and specific inhibitor of macNOS. We also confirmed that it rather specifically inhibited the activity of the purified macNOS and the formation of nitrite by RAW264.7 cells compared to N^G-monomethyl-L-arginine (L-NMA) and N^G-nitro-L-arginine (L-NNA), the other isoforms being less effective. *S*-EIU suppressed the release of nitrite and lactate dehydrogenase from rat vascular smooth muscle cells treated with interleukin-1β and forskolin more potently than L-NMA or L-NNA. *S*-EIU also slightly suppressed internucleosomal DNA cleavage in pancreatic β-cells induced by NO produced by macNOS. Intravenous administration of either *S*-EIU at 0.1 mg/kg/min or L-NMA at 1 mg/kg/min increased the blood pressure but decreased the heart rate in normal rabbits, while aminoguanidine at 1 mg/kg/min affected neither cardiovascular function. These inhibitors at these doses caused recovery of the blood pressure in lipopolysaccharide-treated rabbits that exhibited lowered blood pressure similar to that in the case of septic shock. Although *S*-EIU seemed not to be an adequate inhibitor for therapeutic use *in vivo* due to its side effects on cardiovascular functions, it is one of the most potent inhibitors of macNOS among reported inhibitors *in vitro*.

Key words: blood pressure, *S*-ethylisothiourea, nitric oxide, nitric oxide synthase, septic shock.

Many cells, such as endothelial cells, macrophages, hepatocytes, and neuronal cells, produce nitric oxide (NO) by oxidizing L-arginine (*1*). Three NO synthase (NOS) isoforms have been identified and cloned (*2*). Various stimulants, such as cytokines and lipopolysaccharide (LPS), induce Ca^{2+}-independent NOS activity in macrophages, liver, and smooth muscle. The brain exhibits Ca^{2+}-dependent activity of a constitutive enzyme (bNOS). Another form of NOS associated with the plasma membrane is expressed mainly in endothelial cells (ecNOS).

While NOS induced in macrophages contributes to the antiproliferative activity of interferon (IFN)-γ toward tumor cells and bacteria (*3*), a large amount of NO is toxic and mutagenic (*4*). We have shown that macrophage-type NOS (macNOS) induced by interleukin-1β is responsible for apoptotic death of pancreatic β cells (*5*). Inactivation of enzymes such as aconitase (*6*), glyceraldehyde-3-phosphate dehydrogenase (*7*), and glutathione peroxidase (*8*) may be the cause of this cytotoxic effect of NO. Endotoxin is an initiator of the septic syndrome, which is characterized by systemic vasodilatation, a diminished response to vasoconstrictors, and hypotension (*9*). Macrophages and smooth muscle cells are activated by endotoxin and release a large amount of NO, which plays a key role in the pathophysiologies of a variety of diseases, including sepsis, in which the blood pressure decreases to an abnormally low level. Thus, inhibition of macNOS may ameliorate these diseases (*10, 11*). Non-selective inhibition of NO formation, however, would cause side effects by inhibiting ecNOS or bNOS, and, therefore, selective and potent inhibitors are a prerequisite for therapeutic purposes.

Aminoguanidine (AG) is approximately seven times more potent than N^G-monomethyl-L-arginine (L-NMA) in inhibiting macNOS (*11, 12*), while it is 40 times less potent in increasing the mean arterial pressure in anaesthetized rats (*13*) and 30 times less potent in the contraction of isolated porcine splenic arteries. These observations suggest significant selectivity of aminoguanidine in inhibiting macNOS, rather than bNOS and ecNOS. However, EC_{50} of the compound for macNOS is 100 μM and therefore fairly large amounts are required for effective inhibition. More potent and specific inhibitors would be ideal compounds for

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Abbreviations: NO, nitric oxide; NOS, NO synthase; bNOS, brain NOS; macNOS, macrophage NOS; ecNOS, endothelial NOS; LPS, lipopolysaccharide; AG, aminoguanidine; L-NMA, N^G-monomethyl-L-arginine; L-NNA, N^G-nitro-L-arginine; *S*-EIU, *S*-ethylisothiourea; APMSF, (*p*-amidinophenyl)methanesulfonylfluoride; VSMC, vascular smooth muscle cells; LDH, lactate dehydrogenase.

therapy. Recently, a new type of selective and potent inhibitor, isothiourea derivatives, for macNOS compared to constitutive enzymes was reported (*14-17*). In this study, in addition to the *in vitro* effects with purified enzymes and cultured cells, we evaluated the *in vivo* effects of *S*-ethylisothiourea (*S*-EIU) on the blood pressure and heart rate in normal and septic shock rabbits induced by injection of LPS.

EXPERIMENTAL PROCEDURES

Materials—L-[*U*-^{14}C]Arginine monohydrochloride (319 mCi/mmol) and [α-^{32}P]dCTP were obtained from Amersham. Pepstatin, leupeptin and calmodulin were purchased from Sigma. NADPH and tetrahydrobiopterin were from Boehringer Mannheim and Research Biochemical, respectively. Soybean trypsin inhibitor, (*p*-amidinophenyl)methanesulfonylfluoride (APMSF), and FAD were obtained from Wako Pure Chemical Industries. Dowex 50WX-8 was obtained from Muromachi Kagaku Kogyo. Other reagents were of the highest grade available.

Purification of bNOS from Rat Brain—bNOS was purified from rat brain essentially as described (*18, 19*). Briefly, rat cerebellum were homogenized in 5 volumes of ice-cold buffer A (50 mM Tris, 1 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, 5 μM leupeptin, 2 μM pepstatin, 20 μM APMSF, 10 mg/liter soybean trypsin inhibitor, pH 7.4) containing 250 mM sucrose. The homogenate was centrifuged at 15,000$\times g$ for 30 min, and then solid ammonium sulfate (30% saturation) was added to the supernatant, followed by centrifugation for 15 min at 10,000$\times g$. The precipitate was resuspended in buffer A and then applied to a 2′,5′-ADP-Sepharose column. After washing with buffer A containing 0.5 M NaCl, the enzyme was eluted with 10 mM NADPH. This provided substantially pure bNOS.

Purification of macNOS from RAW264.7 Cells—RAW264.7 cells were stimulated with 100 ng/ml LPS for 24 h and then collected by centrifugation. After homogenization in buffer A and centrifugation for 15 min at 10,000$\times g$, the resultant supernatant was directly applied to a 2′,5′-ADP-Sepharose column. The enzyme fraction eluted with 10 mM NADPH was used for the NOS assay without further purification.

Purification of ecNOS from Baculovirus-Infected Insect Cells—Recombinant human ecNOS was produced in insect cells by infection of Sf21 insect cells with a baculovirus carrying human ecNOS cDNA at an infection multiplicity of 10, followed by incubation for 3 days (*20*). Hemin was added directly to the medium after viral infection. The ecNOS produced by the Sf21 cells was purified from 10^8 cells using 2′,5′-ADP-Sepharose and DEAE-cellulose. After extraction with 1% Triton X-100, chromatography on a 2′,5′-ADP-Sepharose column was performed as described for bNOS purification.

Assaying of NOS—Nitric oxide synthase activity was determined as the conversion of radiolabeled L-arginine to L-citrulline by the method described previously (*18*) with a minor modification (*19*). Briefly, 10 μl of a sample was incubated for 10 min at 37°C in a solution of 50 mM Hepes, 1 mM DTT, 1 mM $CaCl_2$, 0.1 mM tetrahydrobiopterin, 1 mM NADPH, 10 μg/ml calmodulin, 10 μM FAD, and 1.55 μM L-[*U*-^{14}C]arginine (pH 7.8), in a final volume of 100 μl. The reaction was terminated by the addition of 200 μl of buffer B (100 mM Hepes, 10 mM EDTA, pH 5.2). The whole reaction mixture was then applied to a 0.3 ml Dowex 50 WX column (Na^+ form, 200-400 mesh) that had been equilibrated with buffer B. Citrulline was eluted with 0.5 ml of buffer B and then its radioactivity was determined with a liquid scintillation counter. Enzyme activity was expressed as percent inhibition, the maximum activity being taken as 100%.

LPS-Induced Nitrite Formation by RAW264.7 Cells—RAW264.7 cells (a mouse macrophage line transformed with the Abelson leukemia virus, obtained from the American Type Culture Collection) were removed from growth dishes (100 mm in diameter) by trypsin/0.05% EDTA treatment. The cells were cultured in Dulbecco's modified minimum essential medium containing 4.5 g/liter glucose, 100 units of penicillin and 100 units of streptomycin supplemented with 10% heat-inactivated fetal bovine serum (GIBCO/BRL) at 37°C under an atmosphere of 95% air and 5% CO_2. 6.5×10^5 cells/well of 12-well plates were incubated for 22 h in medium supplemented with 10 ng/ml LPS and increasing concentrations of L-NMA, L-NNA or *S*-EIU. Following incubation, the supernatant was obtained by centrifugation and nitrite was quantitated in aliquots of the supernatant as described (*21*).

Effects of NOS Inhibitors on the Blood Pressure and Heart Rate of Rabbits—Male white rabbits (2.1-2.6 kg), cared for in accordance with NIH guidelines for laboratory animal welfare, were used without anesthesia. The right femoral artery was cannulated, and connected to a pressure transducer for measurement of the mean arterial blood pressure and heart rate. The left and right femoral veins were cannulated for the administration of drugs. To cause septic shock, animals received LPS (2 mg/kg, i.v.) as a slow injection. After 5 h, NOS inhibitors were injected continuously and then L-arginine was administered when the pressure was stable.

Assaying of Lactate Dehydrogenase (LDH) Activity—Vascular smooth muscle cells (VSMC) were isolated from rat aortae and cultured in Dulbecco's modified Eagle's medium containing 100 units of penicillin and 100 units of streptomycin supplemented with 20% heat-inactivated fetal bovine serum (GIBCO/BRL) at 37°C under an atmosphere of 95% air and 5% CO_2. Confluent VSMC cultured in 24-multiwell plates were incubated for 28 h with 5 ng/ml IL-1β and various concentrations of NOS inhibitors. After incubation, the medium was collected and stored at −30°C until the assay. LDH activity in 40-μl aliquots of the medium was measured spectrophotometrically at 340 nm in 1 ml of 1 mM Na-pyruvate, 0.1 mM NADH, and 100 mM K-phosphate, pH 7.4.

Assaying of NO-Induced DNA Cleavage—HIT cells, a pancreatic β-cell line, were used for evaluating the effects of NOS inhibitors on the internucleosomal DNA cleavage triggered by NO from macNOS induced by IL-1β as described (*5*). After stimulation of the cells with 10 ng/ml IL-1β in the presence of various concentrations of NOS inhibitors for 24 h, isolated DNA was subjected to 1% agarose gel electrophoresis followed by staining with ethidium bromide.

Statistical Analysis—The paired Student's *t* test was used to determine the significance of the differences between data.

RESULTS

Effects of S-EIU on L-Citrulline Formation by NOS and Nitrite Accumulation by RAW264.7 Cells—The dose response inhibition by a novel inhibitor, S-EIU, and widely used inhibitors, L-NMA and L-NNA, of partially purified rat bNOS, mouse macNOS and human ecNOS, as well as of LPS-induced nitrite formation by RAW264.7 cells is shown in Fig. 1. These compounds inhibited the partially purified NOS isozymes, with different specificities, in a dose-dependent manner. The inhibitory effect of S-EIU on macNOS was especially prominent, it being 400-fold and 600-fold more potent than L-NMA and L-NNA, respectively. The inhibitory effect of S-EIU on nitrite formation by LPS-stimulated murine macrophage RAW264.8 cells in which macNOS was induced was about three orders less than that on the activity of purified macNOS, but S-EIU was about 16- and 60-fold more potent than L-NMA and L-NNA, respectively. The potency of the inhibition by S-EIU of ecNOS and bNOS was less, and the difference from L-NMA and L-NNA was within one order. Thus, on *in vitro* assaying, S-EIU seemed to be a potent and rather selective inhibitor of mouse macNOS, and the data were consistent with those reported by Garvey *et al.* (*14*).

Effects of S-EIU on NO-Induced LDH Release from VSMC—Since a recent report (*22*) showed that NO produced in VSMC stimulated with IL-1β induced cellular damage, we examined the effects of S-EIU as well as L-NMA and L-NNA on the levels of nitrite and LDH activity in the conditioned medium of rat VSMC after stimulation with 5 ng/ml IL-1β and 10 μM forskolin. As shown in Fig. 2, S-EIU suppressed the release of nitrite more potently than L-NMA and L-NNA. S-EIU at lower than 10 μM also effectively suppressed the release of LDH activity from the cells, but increased it at 100 μM, suggesting a cytotoxic effect of this compound at higher concentrations.

Effects of S-EIU on Internucleosomal DNA Cleavage in HIT Cells—We then examined the effects of these NOS inhibitors on internucleosomal DNA cleavage in HIT cells in which apoptotic cell death occurred due to NO from macNOS induced by IL-1β (*5*). As shown in Fig. 3, S-EIU at 1 μM slightly suppressed IL-1β-induced internucleosomal DNA cleavage. However, it was not effective at higher concentrations, while L-NMA and L-NNA effectively suppressed the DNA cleavage at 100 μM.

Effects of S-EIU on Rabbit Cardiovascular Functions—

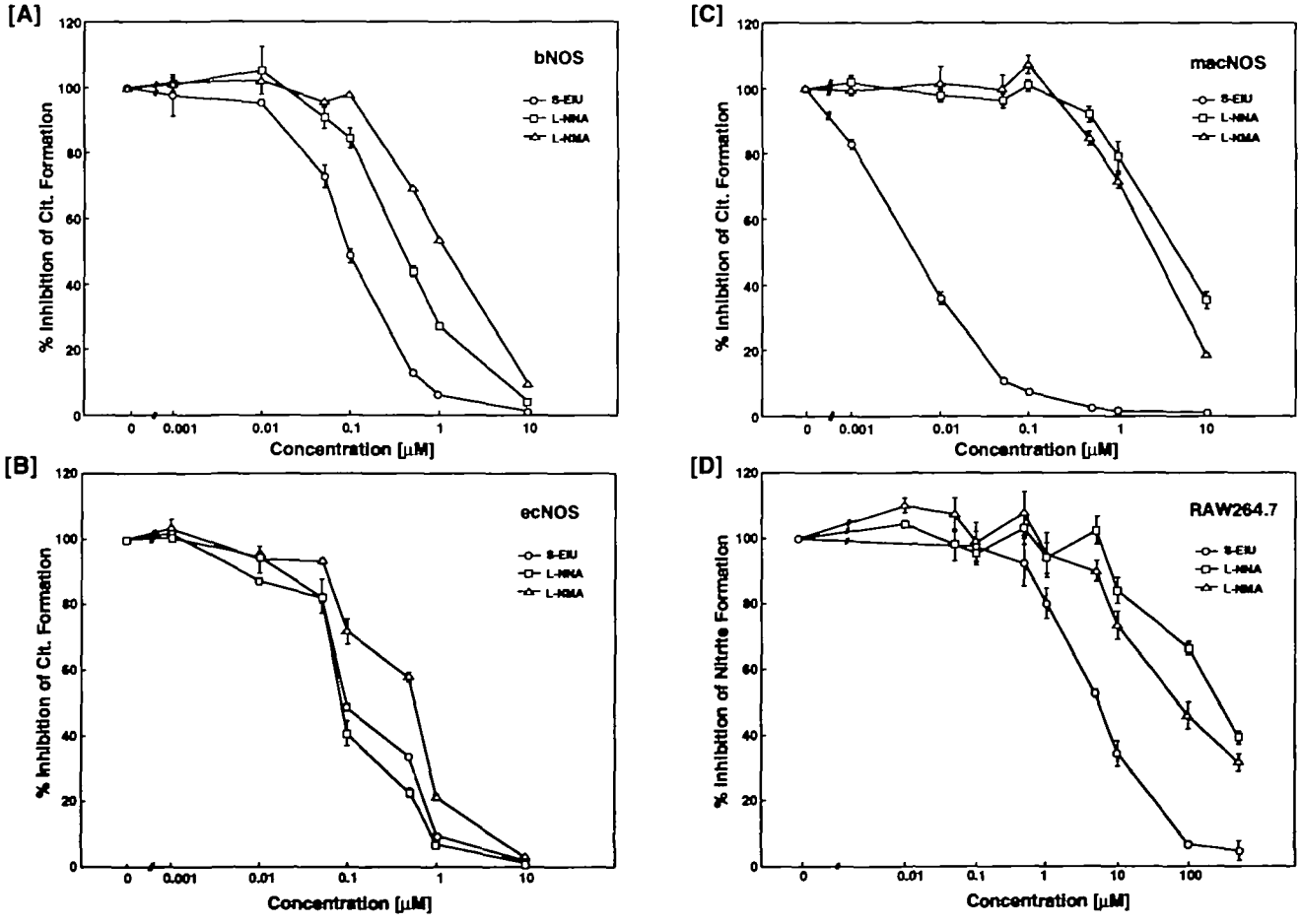

Fig. 1. **Effects of inhibitors on partially purified NOS isozymes and nitrite formation by RAW264.7 cells.** Dose-dependent inhibition by S-EIU, L-NNA, and L-NMA was examined for bNOS purified from rat cerebellum (A), human ecNOS from baculovirus-infected Sf21 cells (B), macNOS from RAW264.7 cells stimulated with 100 ng/ml of LPS (C), and LPS-induced nitrite formation by RAW264.7 cells (D). The means±SD for 3-4 experiments are shown.

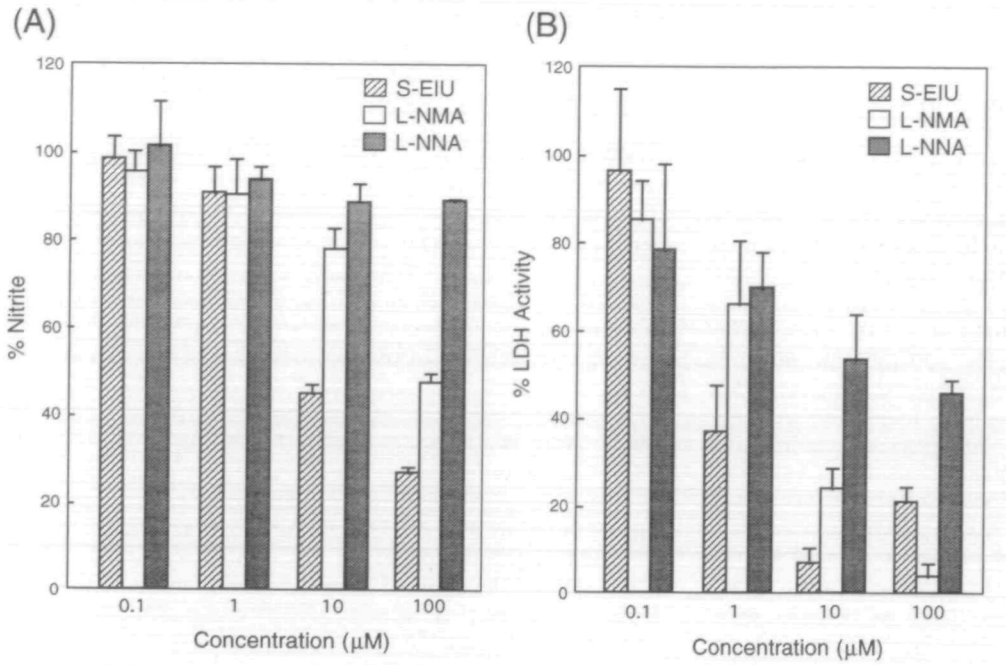

Fig 2 **Effects of NOS inhibitors on the release of nitrite and LDH from VSMC stimulated with IL-1β and forskolin.** VSMC were stimulated with 5 ng/ml IL-1β and 10 μM forskolin in the presence of various concentrations of NOS inhibitors for 24 h. Conditioned media from VSMC were assayed for nitrite (A) and LDH activity (B) The mean control value without inhibitors for nitrite was 43.8 μM. The means ± SD for triplicate experiments are shown.

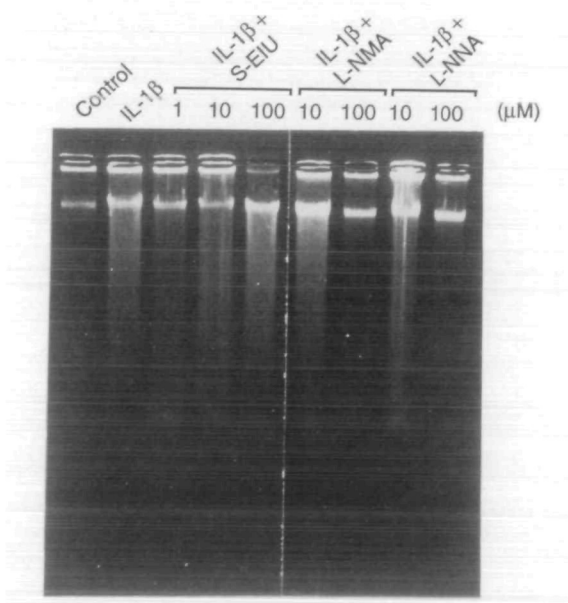

Fig. 3. **Effects of NOS inhibitors on the internucleosomal DNA cleavage in HIT cells stimulated with IL-1β.** HIT cells were stimulated with 10 ng/ml IL-1β in the presence of various concentrations of NOS inhibitors for 24 h. DNA was isolated and subjected to 1% agarose gel electrophoresis.

Since one side effect of an NOS inhibitor *in vivo* concerns the cardiovascular system, we administered *S*-EIU, L-NMA, and aminoguanidine, a selective inhibitor for macNOS, to rabbits intravenously, and then measured their blood pressure and heart rate (Fig. 4). *S*-EIU at 0.1 mg/kg/min or L-NMA at 1 mg/kg/min increased the blood pressure and concomitantly decreased the heart rate, while aminoguanidine at 1 mg/kg/min affected neither function. These findings suggested that *S*-EIU as well as L-NMA exerted a negative inotrophic effect on cardiac muscle, probably through inhibition of NO production by NOS constitutively expressed in endothelial cells and other tissues.

Effect of S-EIU on LPS-Induced Septic Shock Model Rabbits—To evaluate the usefulness of *S*-EIU in septic shock, we induced a rabbit septic shock model by injecting 2 mg/kg LPS intravenously (Fig. 5). After 5 h, the decrease in blood pressure was 21-27 mmHg, which was similar to that under the septic shock conditions (*9*). The administration of *S*-EIU (0.1 mg/kg/min), aminoguanidine (1 mg/kg/min), or L-NMA (1 mg/kg/min) caused recovery of the blood pressure to 5-7 mmHg less than the normal level. Again *S*-EIU significantly decreased the heart rate in this rat model. This inhibitory effect was totally suppressed by the administration of L-arginine, a natural substrate for NOS, at 100 mg/kg/min. This dose of L-arginine alone had no effect on blood pressure in normal rabbits (data not shown). No significant differences were observed for the three inhibitors or blood pressure, except that *S*-EIU was effective even at a one order less concentration.

DISCUSSION

We investigated the effects of a new inhibitor, *S*-EIU, as well as L-NNA and L-NMA on three types of NOS isozymes from different species, and found that *S*-EIU was a potent inhibitor of both purified mouse macNOS and nitrite formation by LPS-activated RAW264.7 cells, although the EC_{50} value for cultured cells was about three orders higher than that for purified macNOS (Fig. 1). This would be explained by a decrease in the effective concentration due to low membrane permeability or metabolic conversion to an inactive form within cells. A large amount of L-arginine in the medium may be another cause. The potency of *S*-EIU toward human ecNOS was indistinguishable from those of other inhibitors. We thus obtained essentially the same results as reported (*14, 16*), although our work was carried out independently and the NOS enzyme sources were

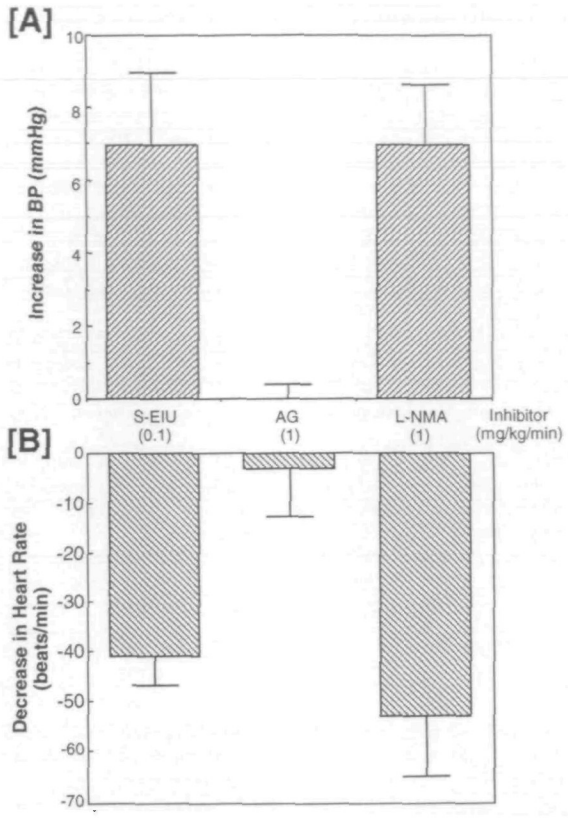

Fig 4. **Effects of NOS inhibitors on the blood pressure and heart rate of normal rabbits.** The changes in blood pressure (A) and heart rate (B) after the administration of each inhibitor are indicated, normal values being taken as zero The mean blood pressure and heart rate of control rabbits were 102 and 301 mmHg, respectively. The means ± SE for 3-4 experiments are shown

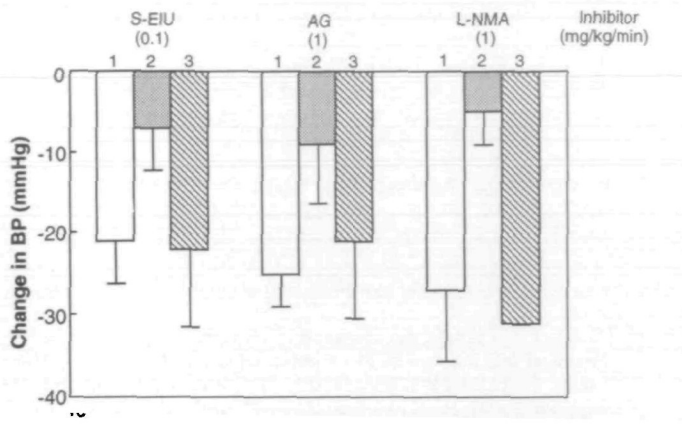

Fig 5. **Effects of NOS inhibitors on the blood pressure of LPS-treated rabbits.** The inhibitors were administered at 5 h after LPS-injection of rabbits. The blood pressures before (column 1) and after (column 2) injection of an inhibitor is indicated, normal values being taken as zero. Then L-arginine at 100 mg/kg/ml was administered (column 3). The mean blood pressure of control rabbits was 103 mmHg. The means ± SE for 3-4 experiments are shown

slightly different. *S*-EIU also potently suppressed NO formation and NO-induced cellular damage in rat VSMC at low concentrations, while it seemed to be cytotoxic at higher concentrations (Fig. 2). In terms of DNA cleavage triggered by NO in HIT cells, *S*-EIU was not more effective than L-NMA or L-NNA (Fig. 3). This would also be explained by the cytotoxicity of the compound.

We also investigated the effects of *S*-EIU on the blood pressure and heart rate of rabbits, and compared the results with those for a well known inhibitor, L-NMA, and a specific inhibitor for macNOS, aminoguanidine, to assess the effects of the compound on cardiovascular functions of rabbits *in vivo*. Although aminoguanidine is more specific to macNOS, its EC_{50} value was high and nearly corresponded with that of L-NMA. We therefore used 1 mg/kg/min aminoguanidine and L-NMA, and 0.1 mg/kg/min *S*-EIU. There was, however, an essential difference between *S*-EIU and aminoguanidine in the *in vivo* effect (Fig. 4). *S*-EIU affected both the blood pressure and the heart rate of untreated rabbits even with a one order less dose than that of aminoguanidine. It is not clear at present why *S*-EIU affected these cardiovascular functions in untreated rabbits. There may be another function of this compound. All these inhibitors, however, seemed to effectively ameliorate the blood pressure decrease in septic shock model rabbits (Fig. 5). The changes in the blood pressure and heart rate would be caused through inhibition of ecNOS in endothelial cells and bNOS in several tissues. This inhibition of the blood pressure change was totally suppressed by the administration of L-arginine, a natural substrate of NOS, consistent with the notion that *S*-EIU binds to a guanidino pocket in the substrate site of NOS and thereby competitively inhibits the enzyme activity (*14*). Although *S*-EIU is a selective and potential inhibitor in an *in vitro* system, it seems not to be an ideal inhibitor for *in vivo* use because it affects cardiovascular physiology, probably by inhibiting the production of NO *via* NOS isozymes other than macNOS. It is thus necessary to evaluate the usefulness of chemicals in both *in vitro* and *in vivo* assay systems at an early step of screening for therapeutic purposes.

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