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Inhibition of Inducible Nitric Oxide Synthase Expression and Stimulation of the Endothelial Formation of Nitric Oxide Most Likely Accounts for the Protective Effect of 2-(Allylthio)pyrazine in a Murine Model of Endotoxemia

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The lipopolysaccharide (LPS)-induced expression of inducible nitric oxide synthase (iNOS) in the vascular wall accounts, at least in part, for the severe hypotension in endotoxemia. The present study investigated whether 2-(allylthio)pyrazine (2-AP), an antioxidant, affects the LPS-induced expression of iNOS in rat aortic rings and the LPS-induced mortality in mice. 2-AP prevented the LPS-induced attenuation of contractions to phenylephrine, formation of cyclic GMP, and expression of iNOS in aortic rings without endothelium and caused endothelium-dependent nitric oxide-mediated relaxations. The mortality of mice receiving a lethal bolus of LPS was decreased by 2-AP, and this effect was associated with a reduced serum nitrite and nitrate level. These findings suggest that agents which inhibit the expression of iNOS but stimulate the formation of endothelium-derived nitric oxide may be of therapeutical value for the treatment of endotoxemia.

Septic shock and endotoxemia are characterized by a severe hypotension, which reflects, in part, the expression of the inducible nitric oxide synthase (iNOS) in vascular smooth muscle cells as well as in many other cell types (1, 2). Once the protein is synthesized, the iNOS generates large amounts of nitric oxide (NO) over prolonged periods of time (2, 3). NO depresses the responsiveness of the vascular smooth muscle to contractile agonists by increasing the activity of soluble guanylyl cyclase which, in turn, stimulates the formation of cyclic GMP (1, 4). The activity of iNOS is regulated predominantly at the transcriptional level through the activation of several transcription factors including nuclear factor-κB (5). Although the signalling cascade mediating the expression of iNOS in response to LPS and pro-inflammatory cytokines remains poorly defined, oxidative stress is most likely involved since antioxidants such as pyrrolidine dithiocarbamate, prevented the expression of iNOS in cultured macrophages and rat aortic smooth muscle cells (6-8). Recently, we have shown that 2-(allylthio)pyrazine (2-AP) protected the liver against noxious agents (acetaminophen and carbon tetrachloride) by selectively inhibiting hepatic cytochrome P450 2E1 (9) as well as by directly scavenging oxygen species (10). The aim of the present study was to determine whether 2-AP affects the LPS-induced expression of iNOS in isolated blood vessels and also the mortality evoked by the administration of a lethal bolus of LPS to mice. In addition, the possibility that 2-AP affects the formation of nitric oxide by the calcium-calmodulin-dependent nitric oxide synthase in endothelial cells, which plays a pivotal role in the local control of vascular functions, was also assessed.

MATERIALS AND METHODS

Materials. 2-AP was synthesized at Bukwang Pharmaceutical Industrial Co. (Seoul, Korea) (9). The chemical structure of 2-AP is shown Fig. 1. Minimum Essential Medium (MEM) was purchased grom Gibco BRL (Grand Island, NY). N^G-nitro-L-arginine (NLA) was obtained from Aldrich chemical Co, (Milwaukee, WI, U.S.A.). Rabbit anti-macrophage iNOS antibody and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G were purchased from Transduction Laboratories (Lexington, KY, U.S.A.) and BioRad (Hercules, CA, U.S.A.), respectively. Phenylephrine hydrochloride (PE), indomethacine, LPS (Escherichia coli, 0127:B8) and all other chemicals were obtained from Sigma chemical Co. (St. Louis, MO, U.S.A.). cGMP radioimmunoassay kits were purchased from Amersham (Buckinghamshire, U.K.).

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Abbreviations used: 2-AP, 2-(allylthio)pyrazine; NO, nitric oxide; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; [NO_X], serum nitrite and nitrate; NLA, N^G-nitrio-L-arginine; MB, methylene blue.

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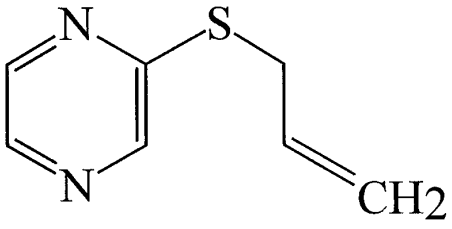

FIG. 1. Chemical structure of 2-AP.

Survival rates. The survival rate of male ICR mice (20-25 g) in response to LPS was assessed. LPS (LD_{80} value: 55 mg/kg, i.v.) was injected 1 hr after a single intraperitoneal injection of 10 and 100 mg/kg of 2-AP. 2-AP was dissolved in olive oil. Control group received olive oil as a vehicle. The survival was monitored for 72 hr.

Measurement of serum nitrite and nitrate ($[NO_X]$). LPS (20 mg/kg, i.p.) was injected 1 hr after treatment of mice with 2-AP at the doses of 10, 30 and 100 mg/kg, i.p. Control group received olive oil as a vehicle. After 20 hr, 500 μl of blood were collected from retroorbita. The blood samples were centrifuged at 5,000 rpm for 10 min to prepare serum. One (l of sample was injected into 20 ml of hot (90-95°C) vanadium (III) solution (0.1 M, dissolved in 2N HCl). NO released by reduction of nitrite and nitrate under these conditions was stripped from solution into the gas phase by a steady flow of helium. NO was determined by chemiluminescence method with ANTEK model 7020 (Houston, TX, U.S.A.) (11, 12).

Organ bath study. Male Sprague-Dawley rats (300-350 g) were used. Rings of rat thoracic aorta without endothelium were incubated in MEM in the presence of 20 μg/ml of LPS for 6hr at 37°C in the incubator supplying O_2 containing 5 % CO_2. 2-AP was added at the concentrations of 1, 10 and 100 μM to the incubation medium at the same time as LPS. The aortic rings were suspended in organ chamber containing Krebs solution (composition in mM : NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, Ca-EDTA 0.016 and glucose 11.1) bubbled with O_2 containing 5 % CO_2 and cumulative concentration-response curves to phenylephrine (10^{-8} - 10^{-5} M) were obtained. To examine the endothelium-dependent relaxation to 2-AP, rings of rat thoracic aorta with endothelium were prepared for the study in organ chambers (13). All experiments were carried out in the presence of indomethacin (10^{-5} M) to prevent the production of vasoactive prostanoids. In some experiments, NLA (10^{-5} M) or methylene blue (MB, 10^{-6} M) were added 30 min before the addition of phenylephrine. Cumulative concentration-relaxation effects to 2-AP (10^{-6} - 10^{-4} M) was assessed in aortic rings contracted with phenylephrine (10^{-6} M).

Measurement of tissue content of cyclic GMP. Rings of rat thoracic aorta without endothelium were incubated in MEM containing 20 μg/ml of LPS for 6 hr. After incubation, rings were incubated in fresh Krebs solution containing indomethacin (10^{-5} M) and isobutylmethyl xanthine (IBMX, 10^{-4} M) for 30 min. Aortic rings were frozen rapidly in liquid nitrogen after the incubation. Tissue cGMP content was measured by radioimmunoassay using cGMP assay kits (14).

Immunoblot analysis. The levels of iNOS protein in cytosolic fractions of homogenates of rat aortic rings without endothelium incubated in MEM containing LPS (20 μg/ml) with or without 2-AP (1, 10 and 100 μM) for 6 hr at 37°C were assessed by western blot analysis. 2-AP was added at the same time as LPS or 1, 3 and 5 hr after incubation with LPS. SDS-PAGE analysis was carried out according to Laemmli (15) using a BioRad Mini-Protean II apparatus. Proteins were separated by 7.5% SDS-PAGE and electrophoretically transferred to nitrocellulose paper followed by immunoblotting with rabbit anti-macrophage iNOS antibody (1 : 1000) (16). Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G was used as the secondary antibody, and color was developed using 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT).

Stastical analysis. Data were expressed as mean ± SEM. Statistical significance of differences between means were analyzed by Student's t-test for paired or unpaired observations, and p values less than 0.05 were considered significant.

RESULTS

Survival Rates

LPS-induced survival rates in mice were 18, 9 and 9% at 24, 48 and 72 hr, respectively. In contrast, LPS-injected into mice which had been pretreated with 2-AP at the doses of 10 and 100 mg/kg for 1 hr showed the survival rates of 70% and 90% at 24 hr, and 40% and 80% at 48 to 72hr. Animals treated with 2-AP alone showed no mortality (Fig. 2).

Serum Nitrites and Nitrates Concentrations ($[NO_X]$)

Administration of LPS to mice caused an increase in serum $[NO_X]$ by 32-fold, relative to control, during the first 6 hr (basal $[NO_X]$ were 13.6 ± 3.52 μM). When 2-AP was administered 1 hr before LPS, 2-AP inhibited the LPS-induced increase in serum $[NO_X]$ in a dose-dependent manner (Fig. 3). Pretreatment with 2-AP inhibited the LPS-induced increases in serum $[NO_X]$ by 47%, 55% and 68% at the dose of 10, 30 and 100 mg/kg, respectively. The ED_{50} value of 2-AP was 15.2 ± 1.1 mg/kg.

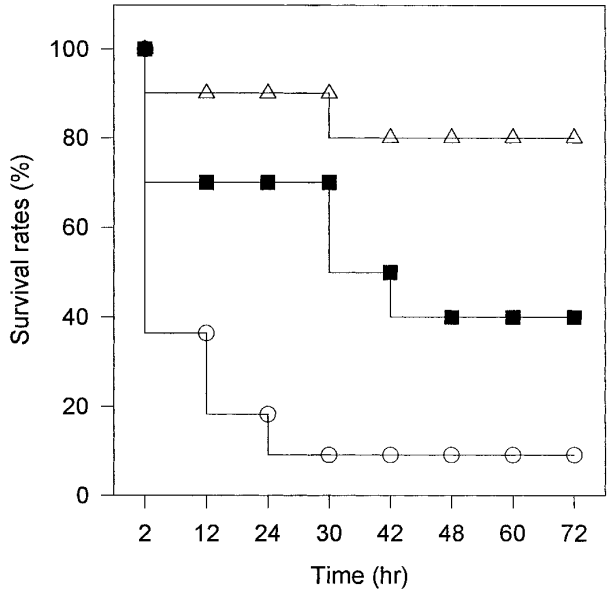

FIG. 2. Effect of 2-AP on survival rates in LPS-treated mice. LPS (55 mg/kg) was given i.v. 1 hr after a single intraperitoneal injection of mice with 2-AP (10 (n=10, ■) and 100 mg/kg (n=10, △), i.p.). The LPS control group received olive oil as vehicle (n=11, ○). The survival was monitored for 72 hr.

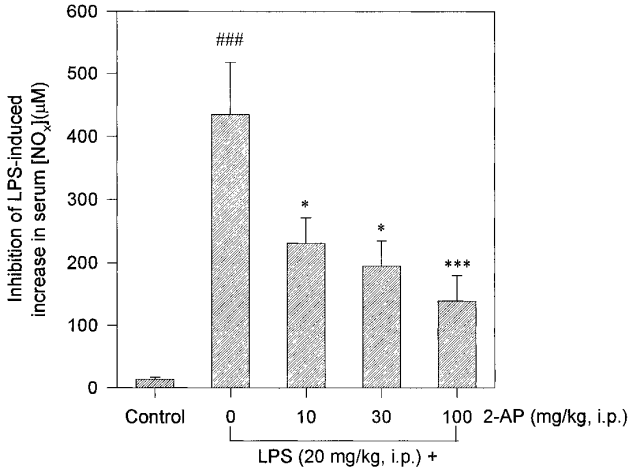

FIG. 3. Effect of 2-AP on the LPS-induced increase in serum [NO_x] in mice. LPS (20 mg/kg) was given i.p. 1 hr after treatment of mice with 2-AP (10, 30 and 100 mg/kg, i.p.) Animals were sacrificed 20 hr post-LPS treatment and serum [NO_x] were determined. Data are expressed as mean ± SEM, n=7-10.

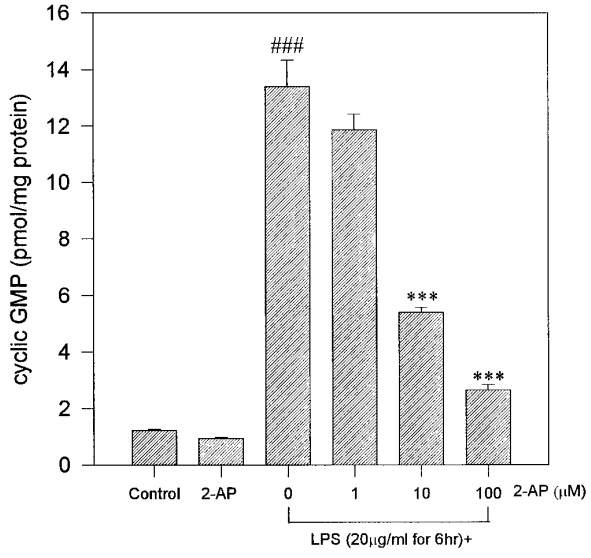

FIG. 5. Effect of 2-AP on the cyclic GMP content in rat aortic rings without endothelium incubated in the absence and presence of LPS (20 μg/ml) for 6 hr. All experiments were performed in the presence of indomethacin 10^{-5} M and IBMX 10^{-4} M. Results were expressed as the mean ± S.E.. ### : Significantly different from control (E−), $p<0.005$, *** : significantly different from LPS treated aorta, $p<0.005$, n=6-8.

Vascular Contractile Response

The incubation of aortic rings without endothelium in culture medium containing LPS (20 μg/ml) for 6hr significantly decreased the contractile response to phenylephrine (Fig. 4). The EC_{50} changed from −7.32 ± 0.005 to −6.54 ± 0.004 μM and the maximal contractions to phenylephrine (10^{-5} M) was reduced by 48.5 ± 4.6 %. 2-AP restored the concentration-contraction curve to phenylephrine in LPS-treated aortic rings in a concentration-dependent manner whereas the antioxidant treatment alone (100 μM) had no significant effect (data not shown).

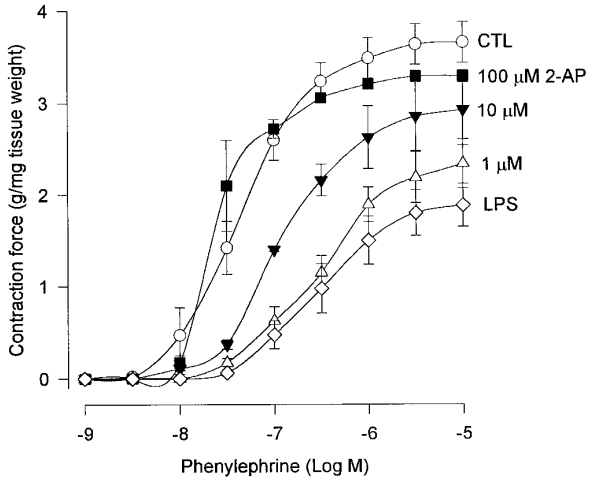

FIG. 4. Cumulative concentration-contraction curves to phenylephrine of control (○) and LPS-treated aortic rings without endothelium in the absence (◇) and presence of 2-AP (1 μM, △ ; 10 μM, ▼; 100 μM, ■). Results are expressed as the mean ± S.E., n=4.

Tissue Content of Cyclic GMP

The tissue content of cyclic GMP was 10-fold greater in the LPS (20 μg/ml for 6hr)-treated aortic rings without endothelium than in control rings (13.4 ± 0.9 compared to 1.2 ± 0.04 pmol/mg protein, respectively; Fig. 5). 2-AP prevented the LPS-induced formation of cyclic GMP in a concentration-dependent manner (Fig. 5). Exposure of aortic rings to 2-AP (100 μM) alone for 6hr did not affect significantly the basal level of cyclic GMP (Fig. 5). 2-AP did not affect the formation of cyclic GMP evoked by sodium nitroprusside in the aortic rings without endothelium (data not shown).

Immunoblot Analysis

A low level of iNOS protein of about 130 kDa was seen in cytosolic fractions from homogenates of rat aortic rings without endothelium incubated for 6hr in culture medium (Fig. 6). The abundance of iNOS protein was significantly increased, by about 3-fold, in aortic rings which had been exposed to LPS (20 μg/ml) for 6hr, and this effect of LPS was prevented in a concentration-dependent manner by 2-AP (Fig. 6). The inhibitory effect of 2-AP was most pronounced when the antioxidant was added to aortic rings simultaneously with LPS (Fig. 6). Treatment of aortic rings with 2-AP (100 μM for 6hr) alone did not induce iNOS expression (Fig. 6).

Endothelium-Dependent Relaxation

2-AP significantly relaxed rat aortic rings with endothelium contracted with phenylephrine in a concentra-

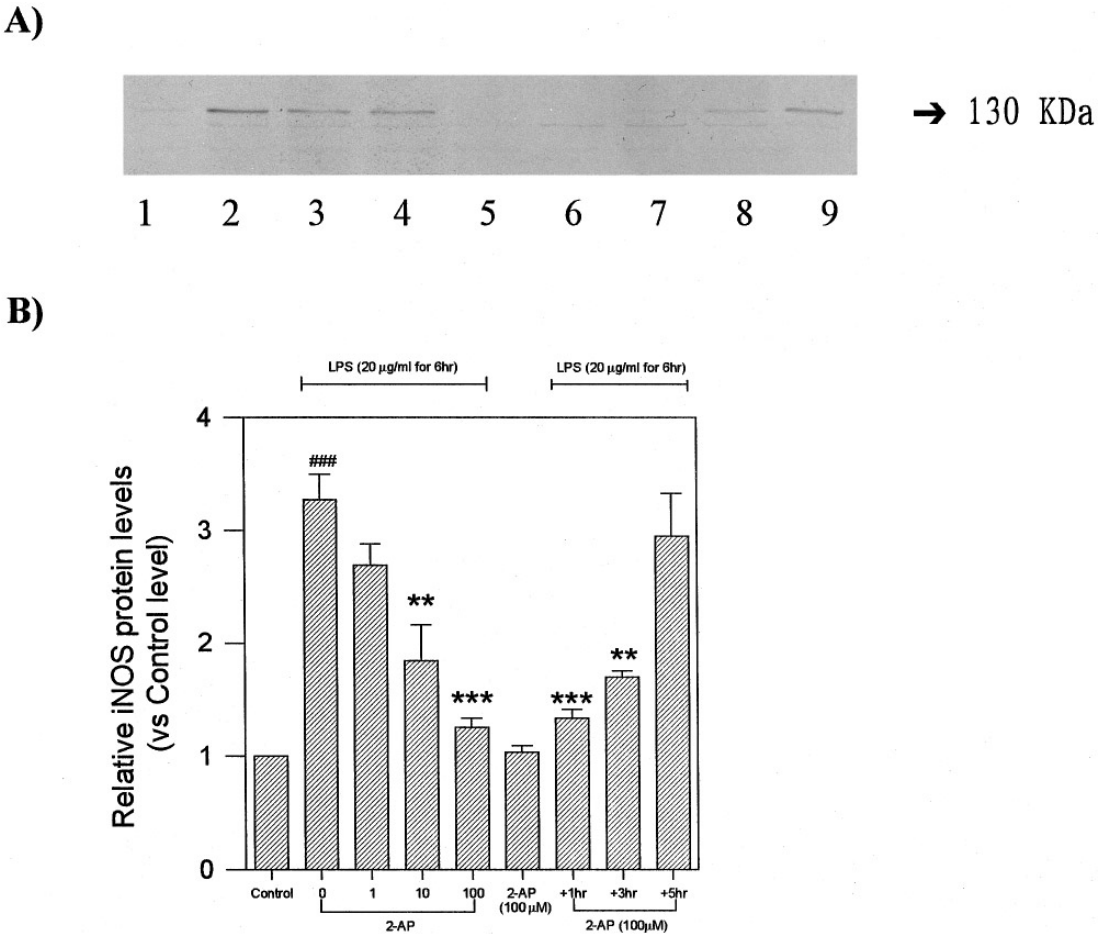

FIG. 6. A) Immunoblot analysis : Effect of 2-AP on the expression of iNOS protein in rat aortic rings without endothelium incubated with or without LPS (20 μg/ml). (lane 1, control; 2, LPS (20 μg/ml); 3, 2-AP 1 μM+LPS; 4, 2-AP 10 μM+LPS; 5, 2-AP 100 μM+LPS; 6, 2-AP 100 μM; 7, 2-AP 100 μM 1 hr post-LPS; 8, 2-AP 100 μM 3 hr post-LPS; 9, 2-AP 100 μM 5 hr post-LPS). B) Cumulative data obtained from 3 different experiments. The level of iNOS protein was quantified by densitometry, and expressed relative to the control level. Densitometric scanning of immunoblot analysis. Each bar represents mean ± S.E.. ### : Significantly different from control (E-), $p<0.005$, **, *** : significantly different from LPS alone treated aorta, $p<0.01$, $p<0.005$, n=3.

tion-dependent manner (from 10^{-6} to 10^{-4} M), but did not affect those without endothelium (Fig. 7). Preincubation of the rings with endothelium with either NLA (10^{-5}M) or MB (10^{-6}M) abolished relaxations to 2-AP (Fig. 7).

DISCUSSION

The present study demonstrates that the antioxidant 2-AP restored phenylephrine-evoked contractions in LPS-treated rat aortic rings without endothelium by preventing iNOS expression in the vascular wall. In addition to the prevention of iNOS expression, 2-AP increased the endothelial formation of nitric oxide as indicated by the endothelium-dependent relaxation of rat aortic rings. Both of these effects may contribute to explain the ability of 2-AP to increase the survival rate in LPS-treated mice.

Exposure of isolated arteries to LPS and/or cytokines such as interleukin-1β and tumor necrosis factor α for several hours leads to an attenuation of the contractile response to various stimuli (17 - 19). The impaired contraction reflects the expression of the iNOS in the vascular wall and the subsequent synthesis of substantial amounts of nitric oxide for prolonged periods of time which in turn increase the activity of soluble guanylyl cyclase. The present findings indicate that 2-AP fully restored contractions to phenylephrine and reduced the formation of cyclic GMP in LPS-treated arteries. Since 2-AP treatment alone did not significantly affect the basal content of cyclic GMP, the reduced LPS-induced formation of cyclic GMP is unlikely to be due to the inhibition of soluble guanylyl cyclase by 2-AP. Western blot analysis using a polyclonal antibody directed against iNOS indicate that the protective effect of 2-AP is rather due to the prevention of the expression of

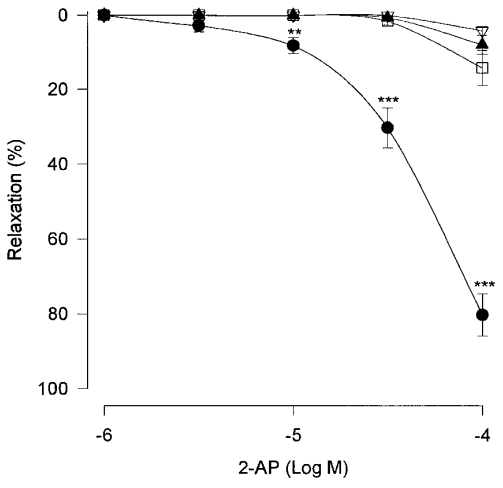

FIG. 7. Concentration-relaxation curves evoked by 2-AP in rat aortic rings with (●) or without (□) endothelium contracted with phenylephrine 10^{-6} M. The effect of methylene blue (MB, 10^{-6} M, ▽) and N^G-nitro-L-arginine (NLA, 10^{-5} M, ▲) on the endothelium-dependent relaxation to 2-AP are also shown. All experiments were performed in the presence of indomethacin (10^{-5} M). Results are shown as mean ± S.E. of 5 different experiments. ** : $p<0.01$, *** : $p<0.005$.

iNOS in the vascular wall. Indeed, the abundance of iNOS protein in the cytosolic fractions obtained from homogenates of LPS-treated aortas was significantly reduced by 2-AP. The activity of the iNOS is calcium independent and appears to be controlled predominantly at the transcriptional level through the activation of several transcriptional factors including nuclear factor-κB and interferon regulatory factor-1 (5). Consistent with a this regulation of iNOS expression at the transcriptional level, the inhibitory effect of 2-AP was most pronounced in aortic rings exposed simultaneously to the antioxidant and LPS. Whereas the addition of 2-AP several hours after LPS prevented only partially (3 hours after LPS) or not at all (5 hours after LPS) the expression of the iNOS. Besides 2-AP, other antioxidants such as pyrrolidine dithiocarbamate, have been shown to prevent the expression of iNOS in vascular smooth muscle cells as well as in other cell types presumably by inhibiting the activation of nuclear factor-κB (7, 8, 20). Hence, nuclear factor-κB activation is a likely target for the inhibitory effect of 2-AP which, however, remains to be determined.

Endotoxemia in rats and mice as well as in patients is associated with a marked increase in serum [NO_X] reflecting presumably an excessive expression of the iNOS (21 - 23). The present study shows that 2-AP prevented the increase in serum [NO_X] in a murine model of endotoxemia. These findings in conjunction with those obtained with cultured vascular smooth muscle cells suggest that 2-AP presumably also prevent the expression of iNOS in vivo. Moreover, the 2-AP-induced reduction of the serum [NO_X] levels were associated with an improvement of the survival rate of mice following administration of a lethal bolus of LPS. In contrast to 2-AP, administration of an inhibitor of nitric oxide synthase activity such as N^G-mono-methyl-L-arginine decreased rather than increased the rates of survival of endotoxemic mice. N^G-mono-methyl-L-arginine is a non-selective inhibitor of nitric oxide synthase that affects the activity of both the iNOS and the endothelial calcium-calmodulin-dependent nitric oxide synthase (3). Therefore, it is likely that a reduction of the protective role of endothelium-derived nitric oxide, which controls vascular tone (24) and the activation of both neutrophils and platelets (25, 26), may account at least in part for the deleterious effect of the non-selective inhibitor of nitric oxide synthase activity in endotoxemia. In contrast to N^G-mono-methyl-L-arginine, 2-AP evoked endothelium-dependent nitric oxide-mediated relaxations of rat aortic rings indicating that the antioxidant increased rather than inhibited the endothelial formation of nitric oxide. The dual effect of 2-AP to inhibit the pro-inflammatory mediator-induced formation of copious amounts of nitric oxide, and to increase endothelial formation of nitric oxide, might be of therapeutic value for the treatment of hyperdynamic circulatory states elicited by the induction of iNOS such as in sepsis and endotoxemia.

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