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Arrest of endotoxin-induced hypotension by transforming growth factor β1

(septic shock/inducible nitric oxide synthase/selective inhibition/vascular smooth muscle cells)

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ABSTRACT **Septic shock is a cytokine-mediated process typically caused by a severe underlying infection. Toxins generated by the infecting organism trigger a cascade of events leading to hypotension, to multiple organ system failure, and frequently to death. Beyond supportive care, no effective therapy is available for the treatment of septic shock. Nitric oxide (NO) is a potent vasodilator generated late in the sepsis pathway leading to hypotension; therefore, NO represents a potential target for therapy. We have previously demonstrated that transforming growth factor (TGF) β1 inhibits inducible NO synthase (iNOS) mRNA and NO production in vascular smooth muscle cells after its induction by cytokines critical in the sepsis cascade. Thus, we hypothesized that TGF-β1 may inhibit iNOS gene expression *in vivo* and be beneficial in the treatment of septic shock. In a conscious rat model of septic shock produced by *Salmonella typhosa* lipopolysaccharide (LPS), TGF-β1 markedly reduced iNOS mRNA and protein levels in several organs. In contrast, TGF-β1 did not decrease endothelium-derived constitutive NOS mRNA in organs of rats receiving LPS. We also performed studies in anesthetized rats to evaluate the effect of TGF-β1 on the hemodynamic compromise of septic shock; after an initial 25% decrease in mean arterial pressure, TGF-β1 arrested LPS-induced hypotension and decreased mortality. A decrease in iNOS mRNA and protein levels in vascular smooth muscle cells was demonstrated by *in situ* hybridization and NADPH diaphorase staining in rats treated with TGF-β1. Thus these studies suggest that TGF-β1 inhibits iNOS *in vivo* and that TGF-β1 may be of future benefit in the therapy of septic shock.**

In septic shock, a severe underlying infection triggers a cascade of events leading to intractable hypotension, multiple organ system failure, and high mortality rates (1, 2). The infecting organism stimulates the release of cytokines and vasoactive mediators that ultimately results in vascular smooth muscle relaxation and consequent hypotension. Because several bacterial toxins and a redundant series of receptors and cytokines participate in this process, attempts at therapeutic intervention aimed at a single toxin or mediator have failed (2). Nitric oxide (NO), a potent vasodilator synthesized by nitric oxide synthase (NOS) (3–5), is a key mediator generated late in the sepsis pathway leading to hypotension (6–10). Therefore, NO represents a potentially important target for therapy.

In two recent studies, MacMicking *et al.* (11) and Wei *et al.* (12) generated mice carrying a disrupted inducible NOS (iNOS) gene. The resistance of these mice to endotoxin-induced death underscores the importance of NO in septic shock. Since the overabundance of NO produced during septic shock is generated through the inducible NO pathway, it would be beneficial to selectively inhibit this pathway. In fact, investigators have suggested that nonselective inhibition of both the inducible and constitutive (endothelial-cell derived) NO pathways in the treatment of septic shock may be detrimental (9, 13).

Transforming growth factor (TGF) β1, which is involved in a number of physiologic processes (14, 15), antagonizes the effects of interleukin 1β and tumor necrosis factor α (16–18), two cytokines produced early in the septic shock cascade. TGF-β1 inhibits iNOS gene expression in cultured macrophages (by reducing mRNA stability and translation) (19, 20) and in cultured vascular smooth muscle cells (by decreasing the rate of transcription) (21). Because of the critical role of vascular smooth muscle cells in the regulation of vascular tone and the ability of TGF-β1 to inhibit iNOS after its induction by cytokines *in vitro* (21), we hypothesized (*i*) that TGF-β1 could inhibit iNOS gene expression and protein production after its induction *in vivo* and (*ii*) that TGF-β1 could block the hypotension of septic shock. We found that TGF-β1 inhibited iNOS production and arrested hypotension, even after its initiation, in a rat model of septic shock produced by endotoxin. TGF-β1 did not increase mean arterial pressure in control rats (not receiving endotoxin), and it did not decrease endothelium-derived constitutive NOS (ecNOS) mRNA in rats receiving endotoxin. These studies suggest that TGF-β1 is an inhibitor of iNOS but not ecNOS during endotoxic shock *in vivo*. They also suggest that TGF-β1 may be of benefit in the therapy of septic shock.

MATERIALS AND METHODS

RNA Blot Analysis. Conscious male Sprague–Dawley rats (200–250 g) were injected with *Salmonella typhosa* lipopolysaccharide (LPS, 4 mg/kg i.p.). Immediately after LPS, the rats received an i.p. injection of vehicle (1% serum albumin, $n = 4$) or TGF-β1 (20 μg/kg, $n = 4$). The rats were killed 10 hr after LPS administration, and total RNA was extracted from the heart, kidney, liver, lung, and spleen as described (22). After electrophoresis (10 μg of total RNA per lane), the RNA was transferred to nitrocellulose filters, which were hybridized at 68° for 2 hr with ^{32}P-labeled smooth muscle cell iNOS and 18S rRNA probes in QuikHyb solution (Stratagene). Northern blots were also prepared by using total RNA from the heart, kidney, and lung of the same rats, and these blots were hybridized with ^{32}P-labeled ecNOS and 18S rRNA probes. The hybridized filters were washed in 30 mM NaCl/3 mM sodium citrate/0.1% SDS at 55°C and stored on phosphor screens or

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Abbreviations: NO, nitric oxide; TGF, transforming growth factor; NOS, NO synthase; iNOS, inducible NOS; ecNOS, endothelium-derived constitutive NOS.

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autoradiographed with Kodak XAR film at −80°C (the optimal iNOS exposure times were 8 hr for the lung and spleen and 24 hr for the heart, kidney, and liver; the ecNOS exposure time was 48 hr for all organs). Radioactivity was measured on a PhosphorImager running the IMAGEQUANT software (Molecular Dynamics).

Western Blot Analysis for iNOS. Tissue from rats injected with LPS followed by vehicle or LPS followed by TGF-β1 (as described above) was homogenized [in 50 mM Tris·HCl/10% (vol/vol) glycerol/5 mM magnesium acetate/0.2 mM ethylenediamine tetraacetate/0.5 mM dithiothreitol/1.0 mM phenylmethylsulfonyl fluoride], and an equal amount of soluble protein (200 μg) from each organ was fractionated by Tris/glycine/SDS-polyacrylamide gel electrophoresis. An identical gel was stained with Coomassie blue to assure equal loading. Protein on the gel was then transferred to a poly(vinylidene difluoride) membrane (Immobilon-P, Millipore). The membrane was incubated with 5% (vol/vol) skim milk in Tris-buffered saline for 1 hr at room temperature to block nonspecific adsorption. iNOS protein was detected by incubating the membrane with a rabbit anti-iNOS antibody (1:2500 dilution) (23) for 12 hr at 4°C and then with an alkaline phosphatase-conjugated goat anti-rabbit IgG (1:5000 dilution) (Kirkegaard & Perry Laboratories) for 1 hr at room temperature. The colorimetric reaction was completed by incubating the membrane in nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrate (Sigma).

Hemodynamic Experiment. Rats received an i.p. injection of thiobutabarbital (sodium salt, 100 mg/kg), which kept them anesthetized throughout the experiment. The trachea was cannulated with a tubing adapter. Then the right carotid artery was cannulated with PE-50 tubing to measure arterial pressure, and the left jugular vein was cannulated to infuse LPS, TGF-β1, or vehicle (24). Phosphate-buffered saline (PBS, vehicle 1) was used as a control for LPS and 1% serum albumin (vehicle 2) was used as a control for TGF-β1. The control group (n = 8) received only anesthesia and vehicles 1 and 2 in place of LPS and TGF-β1, respectively. The LPS group (n = 8) received LPS (4 mg/kg i.v.) and vehicle 2 in place of TGF-β1. The LPS/TGF-β1 group (n = 8) received LPS (4 mg/kg i.v.) followed by TGF-β1 (an i.v. bolus at 20 μg/kg followed by an infusion at 200 ng per kg per min). The TGF-β1 group (n = 6) received TGF-β1 (bolus at 20 μg/kg followed by an infusion at 200 ng per kg per min) and vehicle 1 in place of LPS. This TGF-β1 dosing regimen was chosen after pilot dose–response and route-of-administration experiments, which we performed because of the limited availability of TGF-β1 and its short circulating half-life (25, 26). After an initial 25% decrease in mean arterial pressure (corresponding to time 0) in the rats receiving LPS (LPS group and LPS/TGF-β1 group), TGF-β1 (or vehicle 2) was administered and mean arterial pressure was monitored over the next 150 min. Preliminary experiments revealed that all rats responded to LPS with time; however, if a rat's mean arterial pressure did not decrease within 90 min, it was excluded from the study. Thus, only the rats most sensitive to LPS were used and it was not necessary to prolong their time under anesthesia. In addition, our preliminary experiments revealed that iNOS mRNA levels increased by 90 min in rats responding to LPS (data not shown).

***In Situ* Hybridization and NADPH Diaphorase Staining.** At the completion of the hemodynamic experiments, a subset of rats was perfused with 4% (wt/vol) paraformaldehyde and the kidneys were removed and sectioned (27). Probe preparation and *in situ* hybridization were conducted by methods as described (27, 28). iNOS mRNA was detected with a ^{35}S-labeled UTP-labeled antisense RNA probe synthesized by using SP6 RNA polymerase from *Not* I-linearized rat iNOS cDNA in PCR II. As a control, a sense RNA probe was synthesized by using T7 RNA polymerase from *Hin*dIII-linearized rat iNOS cDNA in PCR II. The RNA probes were hydrolyzed for 20 min at 60°C to generate probes of ≈100 nt.

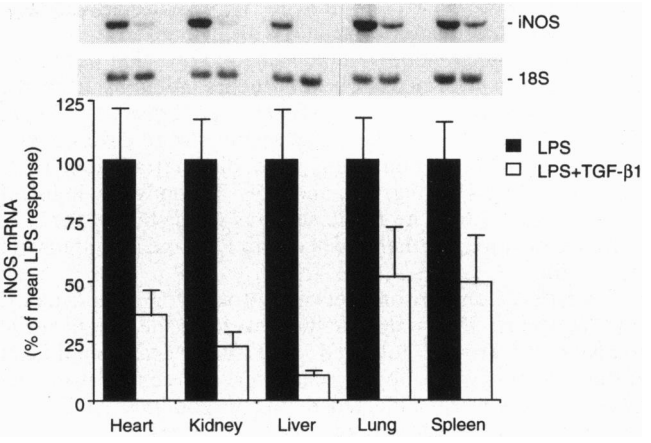

FIG. 1. Effect of TGF-β1 on LPS-induced iNOS mRNA *in vivo*. Male Sprague–Dawley rats (200–250 g) were injected with *S. typhosa* LPS (4 mg/kg i.p.). Immediately after the LPS, the rats received an i.p. injection of vehicle (1% serum albumin) or TGF-β1 (20 μg/kg). The rats were killed 10 hr after LPS administration, total RNA was extracted from the organs, and Northern blots were prepared. (*Upper*) iNOS and 18S transcripts from indicated organs of a representative rat in each group. (*Lower*) The signal intensity of each RNA sample hybridized to the iNOS probe was divided by that hybridized to the 18S probe. The normalized intensities were then plotted as a percentage of the mean LPS response (mean ± SEM, n = 4 rats in each group) in the organs of all rats studied. The mean LPS response was defined as the mean signal intensity of iNOS mRNA from the rats receiving LPS, and this signal intensity was given the value of 100%. Bars: solid, LPS followed by vehicle; open, LPS followed by TGF-β1.

Each tissue section was hybridized with 20 million cpm of probe at 50°C overnight. After the hybridization procedure, sections were washed at 50°C under stringent conditions and dried. The tissue sections were subsequently dipped into

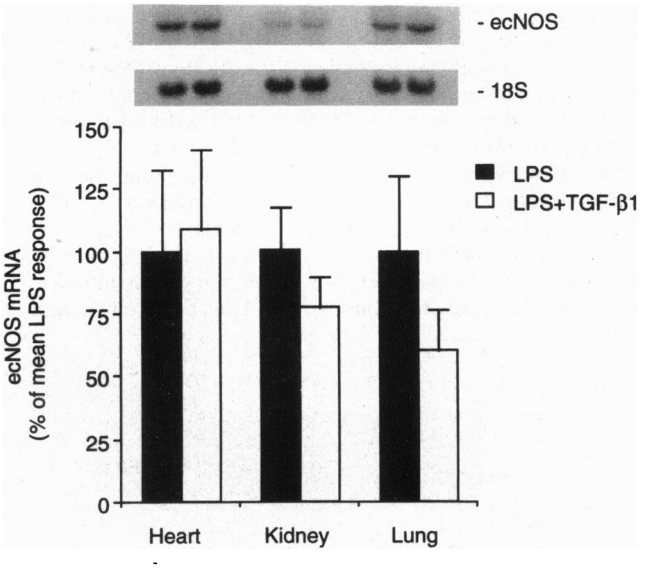

FIG. 2. Effect of TGF-β1 on ecNOS mRNA after administration of LPS *in vivo*. Total RNA was extracted from the organs of rats described in Fig. 1, and Northern blots were prepared. (*Upper*) ecNOS and 18S transcripts from indicated organs of a representative rat in each group. (*Lower*) The signal intensity of each RNA sample hybridized to the ecNOS probe was divided by that hybridized to the 18S probe. The normalized intensities were then plotted as a percentage of mean LPS response (mean ± SEM, n = 4 rats in each group) in the organs of all rats studied. Mean LPS response was defined as the mean signal intensity of ecNOS mRNA from the rats receiving LPS, and this signal intensity was given the value of 100%. Bars: solid, LPS followed by vehicle; open, LPS followed by TGF-β1.

emulsion (Kodak NTB2) solution and exposed for 48 hr at 4°C. The sections were counterstained with hematoxylin/eosin.

Tissue was sectioned from the same kidneys that were used for the *in situ* hybridization experiments. Sections from the kidneys were stained for NADPH diaphorase activity as described (23). Briefly, the tissue sections were covered with a reaction mixture containing nitro blue tetrazolium (0.25 mg/ml), NADPH (1 mg/ml), and 0.5% Triton X-100 in 0.1 M Tris·HCl (pH 7.6). The tissue sections were stained for 1 hr with this mixture and then washed with PBS and mounted with cover slips.

Statistics. Comparisons between groups for hemodynamic and Northern blot data analyses were made by factorial analysis of variance followed by Fisher's least significant difference test when appropriate. Survival comparisons between groups were subjected to the χ^2 goodness of fit test. Statistical significance was accepted at $P < 0.05$.

RESULTS AND DISCUSSION

TGF-β1 Inhibited Tissue iNOS mRNA and Protein but Not Tissue ecNOS. To determine whether TGF-β1 affected iNOS mRNA levels in an animal model of septic shock, we injected rats with *S. typhosa* LPS (29) followed immediately by vehicle or TGF-β1 (Fig. 1). Since septic shock involves multiple organ systems, we extracted total RNA from the heart, kidney, liver, lung, and spleen (22). iNOS mRNA was barely detectable in the organs of control rats that did not receive LPS (data not shown). LPS followed by vehicle induced an increase in iNOS mRNA levels in all five organs. In contrast, LPS followed by TGF-β1 promoted a significant decrease ($P < 0.05$) in LPS-induced iNOS mRNA levels in the heart, kidney, and liver (Fig. 1). LPS-induced iNOS mRNA levels also decreased in the lung and spleen of rats receiving TGF-β1, but less dramatically. Although iNOS mRNA levels in the five organs were analyzed after 10 hr of LPS stimulation, tissue iNOS mRNA was detectable as early as 90 min after i.p. administration of LPS (data not shown). Tissue mRNA from the heart, kidney, and lung (organs rich in endothelial cells) was also analyzed for ecNOS in the two groups of rats (Fig. 2). TGF-β1 promoted no significant decrease in the level of ecNOS mRNA from any of the organs studied. These data demonstrate the ability of TGF-β1 to decrease tissue iNOS mRNA without decreasing ecNOS mRNA during endotoxic shock *in vivo*.

We then extracted total protein from the lung and spleen (LPS stimulation of iNOS mRNA and protein was greatest in these organs) of the representative animals described in Fig. 1 to determine whether TGF-β1 had an effect on LPS-induced iNOS protein expression. TGF-β1 abolished expression of LPS-induced iNOS protein in both organs (Fig. 3), even though its effect on iNOS mRNA levels in the lung and spleen was modest (Fig. 1). These studies suggest that TGF-β1 decreased expression of iNOS at both the mRNA and protein levels *in vivo*, as described *in vitro* (19, 21).

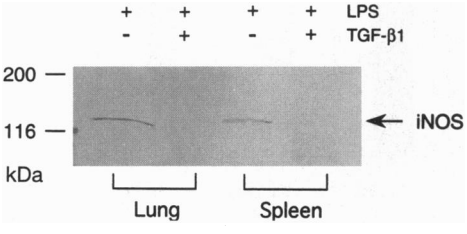

FIG. 3. Effect of TGF-β1 on LPS-induced iNOS protein expression in the lung and spleen. Tissue from rats injected with LPS followed by vehicle or LPS followed by TGF-β1 (as described in Fig. 1) was homogenized and an equal amount of soluble protein (200 μg) from each organ was fractionated by Tris/glycine/SDS polyacrylamide gel electrophoresis (22). Protein in the gel was then transferred to a poly(vinylidene difluoride) membrane. iNOS protein was detected by immunoblot analysis with a rabbit anti-iNOS antibody (1:2500 dilution) (23). Shown are the presence and absence of iNOS protein (130 kDa) in the lung and spleen from a representative rat in each treatment group.

TGF-β1 Arrested Hypotension Produced by Endotoxin. After realizing that TGF-β1 downregulated iNOS mRNA and NO production in vascular smooth muscle cells (21) and that TGF-β1 inhibited LPS-induced expression of iNOS in several organs affected by septic shock, we wanted to know whether TGF-β1 would have a beneficial effect on the hemodynamic compromise associated with septic shock. LPS was administered i.v. (4 mg/kg) to Sprague–Dawley rats (LPS group, Fig. 4*A*) to produce a consistent and profound decrease in mean arterial pressure. We then gave the same dose of LPS to another group of rats, followed by administration of TGF-β1 after an initial 25% decrease in mean arterial pressure (LPS/TGF-β1 group, Fig. 4*A*). The average time required for LPS to induce this decrease in arterial pressure was 68 ± 8 min in

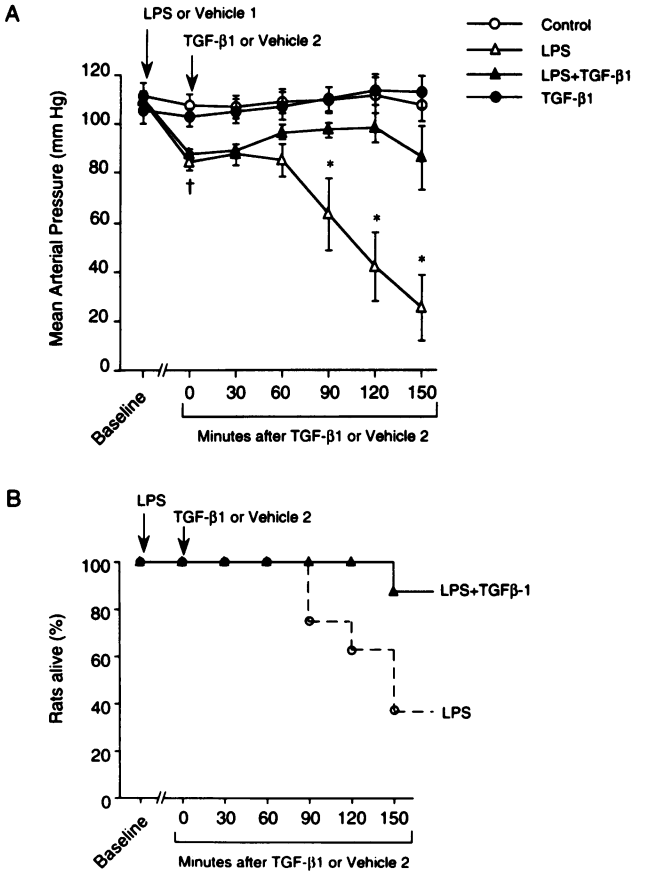

FIG. 4. Effect of TGF-β1 on LPS-induced hypotension and mortality in male Sprague–Dawley rats. (*A*) Control group ($n = 8$) received only anesthesia and vehicles in the place of LPS and TGF-β1, respectively. LPS group ($n = 8$) received LPS (4 mg/kg i.v.) and vehicle in place of TGF-β1. The LPS/TGF-β1 group ($n = 8$) received LPS (4 mg/kg i.v.) followed by TGF-β1 (bolus at 20 μg/kg followed by an infusion at 200 ng per kg per min). The TGF-β1 group ($n = 6$) received TGF-β1 (an i.v. bolus at 20 μg/kg followed by an infusion at 200 ng per kg per min) and vehicle in place of LPS. After an initial 25% decrease in mean arterial pressure (corresponding to time 0) in the rats receiving LPS (LPS group and LPS/TGF-β1 group), TGF-β1 (or vehicle 2) was administered and mean arterial pressure was monitored over the next 150 min. (*B*) Mortality rates for the LPS group and the LPS/TGF-β1 group described in *A*. There were no deaths in the control and TGF-β1 groups.

*Significant decrease in LPS group vs. all other groups.

†Significant decrease in mean arterial pressure in LPS and LPS/TGF-β1 groups vs. their own base lines and vs. control and TGF-β1 groups.

the LPS group and 55 ± 8 min in the LPS/TGF-β1 group ($P = 0.26$). (The rapid decrease in mean arterial pressure can be attributed to the intravenous route of LPS administration in this anesthetized rat model of septic shock.) TGF-β1 given after the onset of septic shock arrested a further fall in blood pressure and produced a significantly higher mean arterial pressure after 90, 120, and 150 min (LPS group vs. LPS/TGF-β1 group, Fig. 4*A*). Furthermore, after 60 min of TGF-β1 treatment, the mean arterial pressure in the LPS/TGF-β1 group was not statistically different from that in the control group or the group treated with TGF-β1 but not LPS.

The effect of NO, which is synthesized from L-arginine by NOS, depends on activation of soluble guanylate cyclase (6–10). Inhibitors of soluble guanylate cyclase (30) have been used to reverse the hypotension of LPS- and cytokine-induced septic shock in experimental animals. These agents by themselves produce an increase in arterial pressure when administered to normal animals, suggesting that they act systemically to inhibit vascular guanylate cyclase. Analogs of L-arginine that inhibit NOS (8, 9) have also been studied as possible agents in the treatment of septic shock. L-Arginine analogs block both the constitutive and inducible pathways of NO production and they increase blood pressure in normal animals. Unfortunately, this removal of the two pathways of NO-dependent vasodilation may lead to a decrease in the perfusion of vital organs and ultimately to an increase in end organ damage (9, 13). Inhibitors more selective for iNOS than for ecNOS, such as *S*-methylisothiourea and aminoguanidine, have also been considered as candidates for therapeutic use in septic shock (31–33). Unfortunately, even though these agents are more selective for iNOS *in vitro*, they appear to inhibit ecNOS *in vivo* because they increase arterial pressure in normal animals. TGF-β1—in contrast with L-arginine analogs, *S*-methylisothiourea, and aminoguanidine—does not increase mean arterial pressure when given alone to control rats (TGF-β1 group, Fig. 4*A*). Yet TGF-β1 has the unique ability to arrest the hypotension of septic shock. This characteristic may be related to the ability of TGF-β1 to selectively inhibit iNOS (21) without inhibiting ecNOS (Fig. 2) (34).

To determine whether TGF-β1 affected an important cytokine produced early in the septic shock cascade, we measured tumor necrosis factor α activity by using a cytotoxic assay as described (35). TGF-β1 did not decrease plasma tumor necrosis factor α activity in the LPS/TGF-β1 group (396 ± 66 units/ml, $n = 8$) in comparison with the LPS groups not receiving TGF-β1 (264 ± 45 units/ml, $n = 7$; $P = 0.141$). These data suggest that TGF-β1 affected iNOS expression directly as opposed to indirectly through the inhibition of inflammatory cytokines. However, we cannot exclude the possibility that TGF-β1 may decrease the activity of other cytokines such as interleukin 1β.

We also looked at mortality rates in these experiments (Fig. 4*B*). By 2.5 hr after the initial decrease in mean arterial pressure, five of the eight rats had died in the LPS group, whereas only one of the eight rats had died in the LPS/TGF-β1 group ($P < 0.05$).

TGF-β1 Inhibited Smooth Muscle Cell iNOS mRNA and Protein. To determine whether the beneficial effect of TGF-β1 on septic shock in rats was associated with a decrease in vascular iNOS, we performed *in situ* hybridization for iNOS and staining for NADPH diaphorase activity, a marker for NOS (23, 36), on sections of renal tissue from rats described in Fig. 4. We analyzed vessels with a diameter of <300 μm (arterioles) in the kidney because they have a dominant effect on vascular resistance and tone. Renal tissue hybridized with the antisense iNOS probe in rats receiving LPS showed intense concentration of autoradiographic grains in the wall of the arteriole (Fig. 5*A*). There were fewer grains in arterioles from rats receiving LPS/TGF-β1 (Fig. 5*B*). No autoradiographic grains specific to the arterial wall were visible in renal arterioles from rats receiving LPS or LPS/TGF-β1 when hybridized with the sense iNOS probe (Fig. 5 *C* and *D*, respectively).

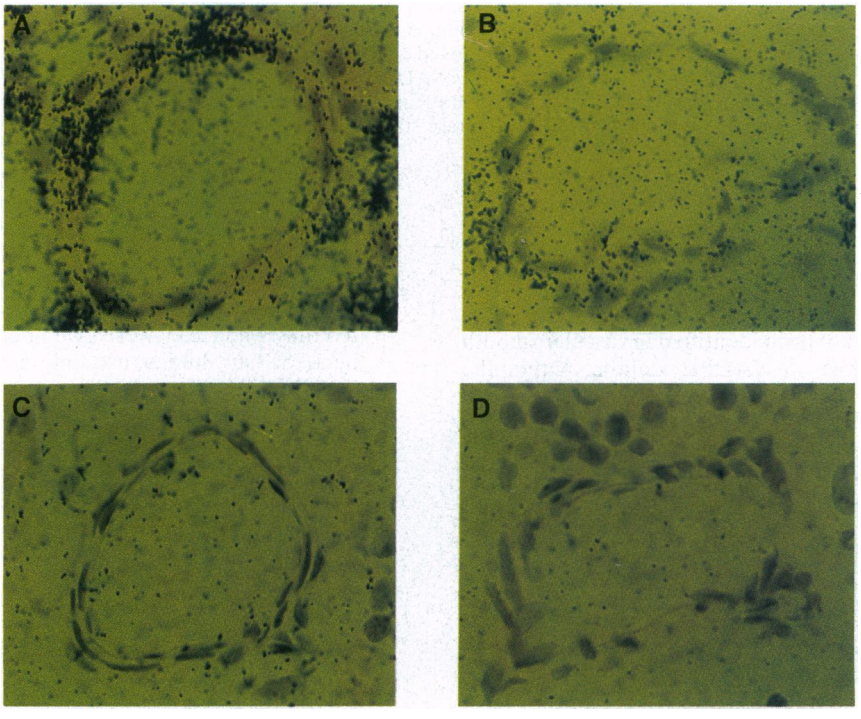

FIG. 5. Effect of TGF-β1 on LPS-induced smooth muscle cell iNOS mRNA. At the completion of the experiments described in Fig. 4, a subset of rats was perfused with 4% paraformaldehyde, and the kidneys were removed and sectioned as described (27). Sections from the kidneys of rats receiving LPS (*A*) and LPS/TGF-β1 (*B*) were hybridized with a ^{35}S-labeled UTP-labeled antisense RNA probe (28) for iNOS. Renal tissue sections of rats receiving LPS (*C*) and LPS/TGF-β1 (*D*) were also hybridized with a sense RNA probe for iNOS. Vessels with a diameter of <300 μm were examined for smooth muscle cell iNOS message. (×325.)

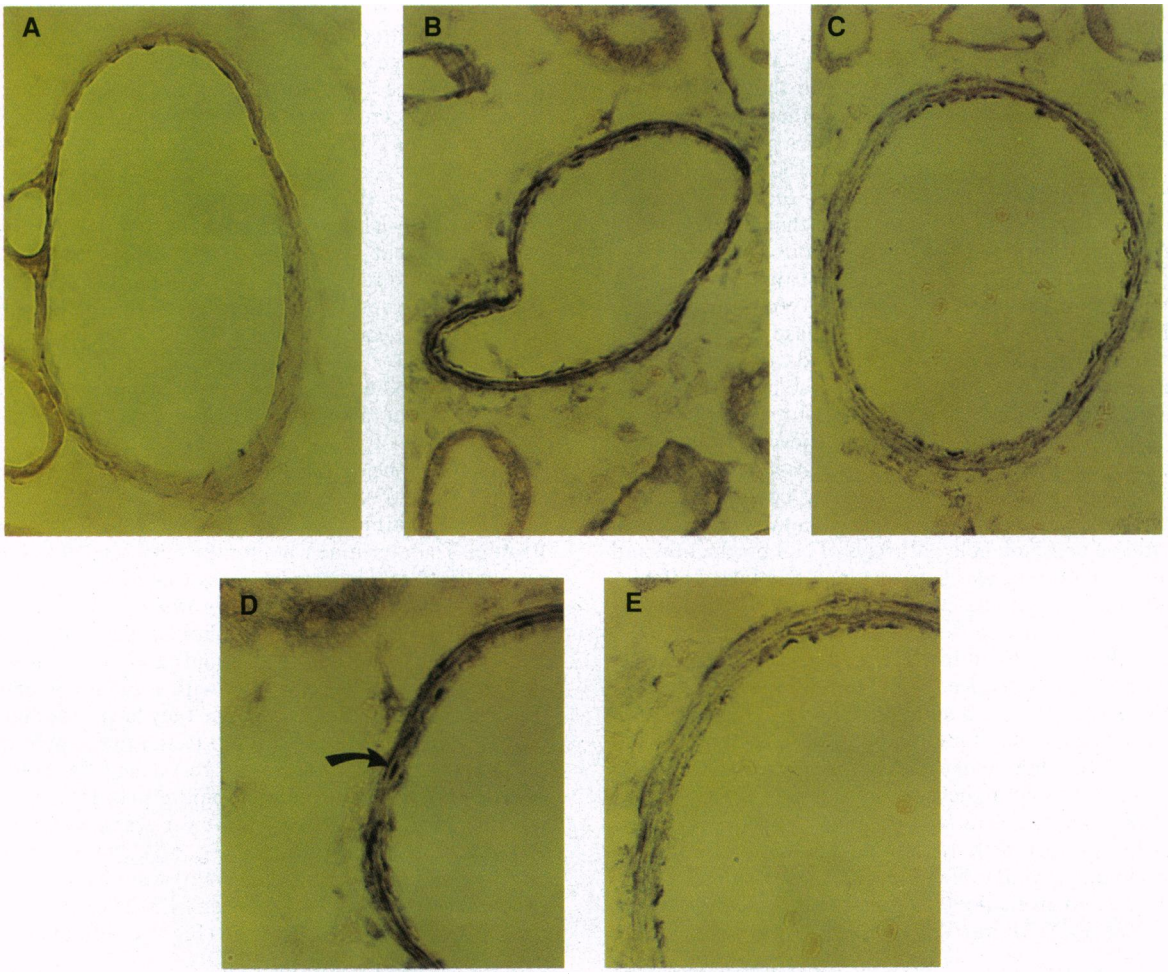

FIG. 6. Effect of TGF-β1 on LPS-induced vascular NOS. At the completion of the experiments described in Fig. 4, a subset of rats was perfused with 4% paraformaldehyde and the kidneys were removed and sectioned as described (27). Sections from the kidneys of rats receiving vehicles (control group) (*A*), LPS (*B*), and LPS/TGF-β1 (*C*) were stained for NADPH diaphorase activity as described (23). Vessels with a diameter of <300 μm were examined for smooth muscle cell NOS activity. (×500.) Higher magnification views (×750) (*D* and *E*) of the arterioles shown in *B* and *C*, respectively. Arrow (*D*) points to positive NADPH diaphorase staining in a vascular smooth muscle cell.

NADPH diaphorase staining was not evident in the smooth muscle cells of arterioles from control rats (Fig. 6*A*); however, in rats receiving LPS alone intense staining for NADPH diaphorase activity was visible (Fig. 6 *B* and *D*). Staining within smooth muscle cells was less intense in the arterioles of rats receiving LPS/TGF-β1 (Fig. 6 *C* and *E*). Because a constitutive isoform of NOS has not been identified in vascular smooth muscle cells, this decrease in NADPH staining within the smooth muscle cell layer appears to represent a decrease in the inducible isoform of NOS.

It has been suggested that NO may have both beneficial and detrimental effects during septic shock (37). NO produced through the inducible NO pathway contributes to the hypotension of septic shock; however, constitutively produced NO may help to maintain visceral and microvascular blood flow by counteracting the effects of vasoconstrictors released during septic shock (37). Moreover, previous animal studies using inhibitors that completely block the NO pathway have demonstrated detrimental effects including myocardial ischemia (13), cardiovascular collapse, and death (29). Thus, an ideal agent for use in the treatment of septic shock would inhibit iNOS but not ecNOS. In these studies, we demonstrate that the beneficial effects of TGF-β1 were associated with a down-regulation of iNOS, but not ecNOS, in organs critical to the pathogenesis of septic shock.

Because the hypotension of septic shock is present at the time of diagnosis, a therapeutic agent must also be effective after initiation of the sepsis cascade. Inhibitors of tyrosine kinase (38) and tumor necrosis factor processing (39) have been used to treat endotoxin-induced septic shock in mice. However, the beneficial effects of these agents were demonstrated only when they were given before or at the same time as the LPS. Our studies not only demonstrate that TGF-β1 selectively inhibits the inducible pathway of NOS (even though NO levels were not directly assessed) but also show that TGF-β1 arrests hypotension after the initiation of endotoxic shock. These properties of TGF-β1 suggest that it may be of therapeutic benefit by stopping hypotension and preserving perfusion of vital organs.

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