In Vivo Administration of Endotoxin and Tumor Necrosis Factor-α Produce Different Effects on Constitutive and Inducible Nitric Oxide Synthase Activity in Rat Neutrophils and Aorta *Ex Vivo* (43852)

STAN S. GREENBERG,*,†,§,[1] JIANMING XIE,*,§ KEVIN O. JOSEPH,* JAY KOLLS,*,‡,§ AND WARREN SUMMER*,†,§

Section of Pulmonary and Critical Care Medicine Departments of Medicine, Physiology,† and Pediatrics,‡ and NIAAA Center on Alcohol, Infection and Host Defense,§ Louisiana State University Medical Center, New Orleans, Louisianna 70112*

Abstract. **Tumor necrosis factor-α (TNF-α) inhibits release of nitric oxide (NO) *in vitro* by stimulating the degradation of constitutive NO synthase (cNOS III) mRNA. However, TNF-α is believed to be the cytokine mediator of the hypotension and upregulation of inducible NO synthase (iNOS II) produced by gram-negative bacterial endotoxin (LPS). Some *in vivo* effects of TNF-α are opposite to those which occur *in vitro*. This study tested the hypothesis that *in vivo* administration of exogenous TNF-α and endogenously released TNF-α induce iNOS II activity and inhibit cNOS III activity, and thereby mediate the acute phase effects of LPS on blood pressure and the NO system in the rat. We show that LPS produces acute phase hypotension in ketamine anesthetized rats. The hypotension was associated with elevation of biologically active TNF-α in plasma, increased production of RNI (NO_2^- and NO_3^- anion) in rat neutrophils (PMN) and suppression of RNI production by A23187 (1 μM) stimulated thoracic aorta (RTA) ex *vivo*. TNA-α (10^6 U/ml, iv) did not produce acute phase hypotension but initially raised arterial blood pressure and heart rate (HR), did not increase RNI production by PMN, and inhibited RNI production by A23187 stimulated RTA ex *vivo*. Pretreatment of rats with the Immunex monomeric soluble P75 receptor binding protein for TNF-α (TNFsr, 0.5 mg/kg, iv) 15 min prior to LPS administration decreased circulating TNF-α from 92,137 ± 12,456 U/ml to undetectable levels as determined by the L929 bioassay. However, LPS-induced increases in RNI in PMN was enhanced and LPS-induced decreases in RNI production by RTA was inhibited by TNFsr. Thus, *in vivo* administration of TNF-α does not mimic the hemodynamic and NO-inducing effects of LPS. However, TNF-α mediates in part LPS-induced inhibition of RNI production by RTA. Thus, endogenous TNF-α is not required for LPS-induced acute phase hypotension or iNOS II activity. The importance of TNF-α in sepsis resides in systems other than iNOSII and blood pressure.** [P.S.E.B.M. 1995, Vol 208]

[1] To whom requests for reprints should be addressed at Department of Medicine, Room 331A Medical School Building, Louisiana State University Medical Center at New Orleans, 1542 Tulane Avenue, New Orleans, LA 70112.

Received May 19, 1994. [P.S.E.B.M. 1995, Vol 208]
Accepted September 2, 1994.

0037-9727/95/2082-0199$10.50/0

Tumor necrosis factor-α (TNF-α) is believed to play a pivotal role in mobilizing the host defense response to infection and sepsis. TNF-α is believed to be a mediator of the tissue injury, cachexia, fever, and metabolic dysfunction associated with gram-negative bacterial endotoxin (LPS)-mediated sepsis and shock (1–3). Moreover, LPS induces the release of TNF-α from macrophages, hepatic Kupffer and endothelial cells, and vascular en-

dothelium, which in turn is believed to act in an autocrine and paracrine manner to stimulate the production of other cytokines and the inducible form of nitric oxide synthase (iNOS II) in phagocytes, smooth muscle, endothelium, and epithelium (4–6). *In vitro* studies using isolated vascular endothelial cells in culture demonstrate that TNF-α increases production of nitric oxide (NO) by inducing the enzyme iNOS II (6–9). This enzyme differs from the constitutive form of the enzyme found in vascular endothelium (cNOS III) in that iNOS II is Ca^{2+} independent, inducible rather constitutive, requires new protein synthesis, is inhibited by dexamethasone and other corticosteroids, and is associated with the production of nanograms, rather than picograms, of NO/min (10–14). As such, TNF-α-induced increases in NO occur with a lag time of 4–6 hr after incubation of endothelial cells or blood vessels with TNF-α (4–9, 12–14). Despite the *in vitro* ability of TNF-α to induce NO production in endothelial and smooth muscle cells in culture, *in vitro* studies demonstrated that TNF-α inhibited endothelium-dependent relaxation to ACh in isolated cat carotid artery (15) and rat (16), sheep (17), bovine (18, 19), and porcine (20) pulmonary artery, and release of NO from the vascular endothelium (19, 20), in part by stimulating the degradation of mRNA for cNOS III (21).

The acute phase hypotension response to LPS is believed to be mediated by NO (6, 22, 23). TNF-α is believed to be the primary cytokine which induces release of NO following administration of LPS to animals (24, 25) and administration of TNF-α to rats and rabbits produced hypotension and shock which were reversible by l-arginine-derived inhibitors of NO synthase (6, 22–26). However, if TNF-α was the mediator of LPS-induced acute phase hypotension and NO production, then it should mimic the actions of LPS. Nielsson *et al.* (27) demonstrated that exogenously administered TNF-α did not produce hypotension in the absence of concomitant exposure to LPS. Moreover, *in vitro* studies suggest that LPS-induced stimulation of TNF-α and NO are temporally related but may occur by independent and unrelated pathways (28, 29). Finally, LPS-induced gene expression for iNOS II and its translation into iNOS II activity has recently been shown to occur rapidly and in a time frame to account for LPS-induced acute phase hypotension (30). However, *in vitro* studies suggest that the inhibitory effect of TNF-α on endothelium-dependent relaxation and production of NO predominates at a time when no detectable iNOS II activity is evident (15–20), and *in vivo* studies indicate that monoclonal (6, 31) and polyclonal (32) neutralizing antibodies against TNF-α have little to no inhibitory effect on LPS-induced hypotension and lethality. Thus, despite the documented role of TNF-α as a mediator of the late phase shock and tissue injury of experimental and human sepsis (1–5, 33–36), it is difficult to understand how TNF-α may upregulate the iNOS II system and produce the acute phase hypotension response of LPS. However, recent studies have demonstrated that TNF-α produces effects *in vivo* opposite to those produced *in vitro* (37). This study tested the hypothesis that *in vivo* administration of exogenously administered TNF-α and endogenously released TNF-α mediated the acute phase effects of LPS on the NO system and blood pressure and heart rate (HR) in the rat.

Methods and Materials

All experiments were performed with an approved (LSU) Institutional Animal Care and Use Committee Protocol (LSU-NO-859). Male, Sprague-Dawley rats (325–350 g) were anesthetized with ketamine-xylazine (25 mg/kg, ip) and catheters inserted into the carotid artery and jugular vein for measurement of blood pressure and drug administration, respectively, as described previously in detail (30, 38, 39). After a 30-min equilibration period they were given either PBS (0.1 ml/kg, iv) or the Immunex, Inc., monomeric soluble 975 TNF-α receptor (TNFsr, 0.5 mg/kg, iv; Immunex, Inc., Seattle, WA) followed 15 min later by either LPS (0.5 mg/kg, iv, GIBCO, Inc., Long Island, NY), PBS (0.1 ml/kg, iv) or murine recombinant TNF-α (1,000,000 U/kg, iv; Specific activity of 1.2×10^8 U/mg [8.3 μg/kg], Genentech Inc., South San Francisco, CA). In each of the rats, a 0.1-ml samples of venous blood was obtained 90 min after administration of PBS, LPS, or TNF-α. This represents the time at which plasma levels of TNF-α reach their peak levels (30, 38, 39). Plasma levels of bioactive TNF-α were measured by the L929 bioassay (30, 31, 38). In some experiments blood pressure, heart rate (HR) obtained from the arterial pressure pulse, and rate-pressure product (RPP, obtained by multiplying heart rate by mean systolic arterial pressure) were monitored for 2 hr after PBS, LPS, or TNF-α administration, after which a thoracotomy was performed. In the remaining experiments, a thoracotomy was performed 2 hr after administration of LPS or PBS to the TNFsr-treated rats. At the end of the 2-hr period, blood (8–10 ml) was obtained from each of the rats by cardiac puncture into heparin-treated tubes (1000 U/ml) for measurement of plasma reactive nitrogen intermediates (nitrate and nitrite anion; RNI) by ozone chemiluminescence. The neutrophils (PMN) were isolated and a purified fraction obtained by density gradient centrifugation and assayed for spontaneous RNI production *ex vivo* (30, 38, 39). The thoracic aorta was also isolated, removed and prepared for incubation and measurement of RNI as described previously in detail (30, 38, 39). The TNF-α contained 1.59×10^8 U/mg protein and 5.68 ng of endotoxin/mg of protein as determined by the Lim-

ulus assay. TNFsr (0.2 mg/kg, iv) was capable of completely neutralizing the TNF-α produced by 2 mg/kg of LPS (Immunex, Inc., personal communication).

RNI Production by Rat Neutrophils. Rat circulating neutrophils (PMN) were isolated from heparin (1000 U/ml)-treated blood obtained by cardiac puncture and prepared to a purity of greater than 90%–95% with PolymorphPrep (Nycomed, Gibco/BRL, Gaithersburg, MD) following the manufacturer's protocol (30, 38). The percentage of viable cells determined by trypan blue exclusion was greater than 98%. PMN (10^6/0.5 ml) obtained from PBS- and LPS-treated rats were incubated in HEPES buffered salt solution containing (m*M*): NaCl (128), KCl (4.9), $MgCl_2$ (1.2), $CaCl_2$ (1.6), dextrose (10), NaHEPES/HEPES buffer (18.7), NaH_2PO_4 (1.18), and hydrocortisone hemisuccinate (10 μ*M*) to prevent artifactual *ex vivo* induction of iNOS II. Following a 30-min incubation at 37°C, the cellular response was stopped by placing the reaction tubes in ice water. The cells were removed by centrifugation and the incubate assayed for RNI (18, 20, 30, 38, 39).

Measurement of Aorta RNI Production. In paired rings of rat thoracic aorta (RTA, n = 3–5 rings/treatment group) we evaluated the effects in *in vivo* administration of PBS, LPS, and TNF-α on basal and A23187 (1 μ*M*)-induced production of the reactive nitrogen intermediates nitrate and nitrite anions, with ozone chemiluminescence, as described previously in detail (18, 20, 30, 38, 39). Briefly, the rings of aorta (4 mm long) were rinsed in HEPES buffered salt solution and either subjected to endothelium rubbing or sham rubbing. After a 15-min equilibration period, adjacent rings of intact and endothelium-rubbed aorta (22.4 ± 1.8 mg) were placed into sealed vials with 3 ml of Krebs solution modified so that the phosphate and bicarbonate were replaced with HEPES buffer (22 mM; pH 7.4) and hydrocortisone 10 μM was present to prevent induction of iNOS II activity *in vitro* during the incubation period. The HEPES buffered solution (PSS) contained either the mixed cNOS III/iNOS II inhibitor 1-N^G-monomethylarginine (LNMMA, 0.3 m*M*), the iNOS II inhibitor 3-amino, 1,2,4-triazine (3-ATINE, 5 m*M*) or A23187 (1 μ*M*) (30, 38, 39). The vials were incubated for 60 min at 37°C. After the incubation the blood vessels were removed, blotted dry, and weighed. The solutions were centrifuged and assayed for RNI-derived NO by ozone chemiluminescence (18, 20, 30, 38, 39).

Measurement of RNI. Briefly, 10–50 μl aliquots of plasma, PMN incubate, or aorta incubates were placed into a purge chamber containing 100 ml of 2.8% vanadium chloride in 2 *N* HCl at 100°C under a constant stream of nitrogen gas with constant stirring. The total volume of gas in the purge chamber which contained the RNI-derived NO in nitrogen was taken up by vacuum into a Dasibi Model 803 NO/NO_X analyzer (Dasibi, Inc., Glendale, CA). This analyzer measures dissolved NO and NO derived from NO_2^- and NO_3^- in the incubate which has been generated by the reducing solution and stripped from the solution using the inert carrier gas (nitrogen). The NO reacts with machine generated ozone to form excited NO which releases light at 6500–8000 A°, in the red region. The amount of light generated is concentration dependent and was measured with a photomultiplier tube. The lower reproducible limit of detection of NO over background was 17 pg using 10 μl injectate and standard solutions of NO_2^- and NO_3^- prepared fresh daily and treated in a manner identical to the samples. The extent of conversion of NO_2^- and NO_3^- to NO was 94.8% ± 0.3% (n = 69) when compared with standard solutions of NO and NO calibration gases.

Data Analyses. Data were analyzed with ANOVA using Bonferroni's correction for time series measurements (4). A P value of 0.05 or less was accepted for statistical significance of differences between and among means.

Results

Hemodynamics. Mean arterial blood pressure, heart rate, and the rate-pressure product were stable throughout the 2-hr observation period in rats treated only with PBS as described previously (30, 38, 39) (data not shown). Administration of LPS to rats pretreated with PBS produced an immediate increase in blood pressure which then rapidly decreased to reach its nadir within 6–10 min (Fig. 1, Top) and which slowly returned towards pre-LPS values over the 2-hr period of evaluation (Fig. 1, Top). However, mean arterial pressure remained 32% ± 4% below control values ($P < 0.05$) 2 hr after administration of LPS (Fig. 1, Top). HR decreased slightly immediately after LPS, whereas RPP increased. RPP subsequently declined after administration of LPS and then paralleled the changes in arterial pressure. HR subsequently returned to and then above pre-LPS values, whereas RPP remained below the initial level of RPP (Fig. 1, Bottom). In contrast to these findings, bolus administration of TNF-α (10^6 U/kg, iv) initially did not affect systemic pressure. However, 15 min after the bolus injection of TNF-α systolic, diastolic and mean arterial pressures increased and remained elevated for the next 30 min, after which these pressures decreased by 10–14 mm Hg (Fig. 2, Top). Heart rate slowly increased during the 2-hr period following the administration of TNF-α whereas RPP transiently increased and then returned to control values within 1 hr after the bolus injection of TNF-α and then decreased by 4%–11% from pre-TNF-α values over the remaining 60 min of the experiment (Fig. 2, Bottom). Thus, TNF-α did not mimic the hemodynamic responses of LPS.

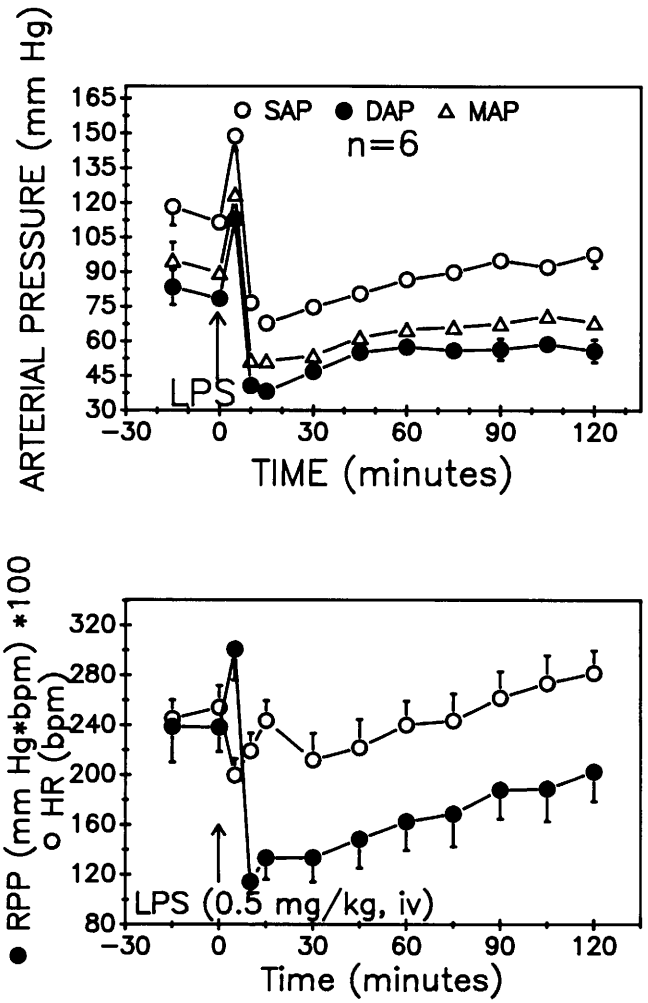

Figure 1. Time course of the hemodynamic changes in rats following administration of LPS (0.5 mg/kg, iv). (Top Panel) The ordinate is the systolic (SAP), diastolic (DAP), or mean (MAP) arterial pressure in mm Hg (±SEM). (Bottom Panel) Heart Rate and rate-pressure product. The abscissa is the time of the experiment. Rats were given LPS at the arrow. Vertical lines are the SEM. Each data point is the mean of six rats. Each mean for arterial pressure and rate-pressure product from 5 to 120 min differs from Time 0 ($P < 0.05$). The heart rate responses at 5, 10, 30, and 45 min and at 90, 105, and 120 min differ ($P < 0.5$) from Time 0.

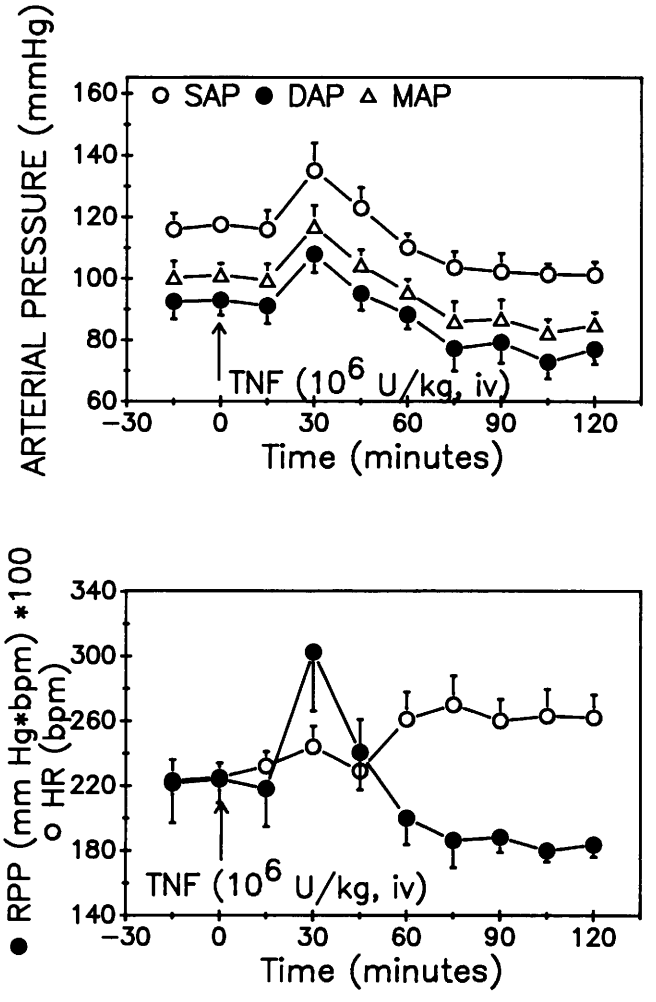

Figure 2. Time course of the hemodynamic changes in rats following administration of TNF-α (1,000,000 u/kg, iv). (Top Panel) The ordinate is the systolic (SAP), diastolic (DAP), or mean (MAP) arterial pressure in mm Hg (±SEM). (Bottom Panel) Heart rate and rate pressure product. Rats were given TNF-α at the arrow. Each data point is the mean of five rats. Each mean for arterial pressure and rate-pressure product at 30 min and 90, 105, and 120 min differs from Time 0 ($P < 0.05$). The heart rate responses at 60, 75, 90, 105, and 120 min differ ($P < 0.5$) from Time 0.

Effects of TNFsr. TNFsr (0.5 mg/kg) did not affect resting mean arterial pressure (Fig. 3, Bottom). However, pretreatment of rats with TNFsr abolished the hemodynamic effects of TNF-α while only partially attenuating the transient pressor response to LPS (Fig. 3, Bottom).

Plasma Levels of TNF-α. Plasma levels of bioactive TNF-α were undetectable 90 min after administration of PBS to rats. In contrast, LPS increased bioactive plasma levels of TNF-α to over 90,000 U/ml. Similarly, within 90 min after the bolus injections of exogenous TNF-α (10^6 U/ml) the mean plasma level of bioactive TNF-α-induced release of TNF-α (4, 5). Bioactive levels of TNF-α were undetectable in rats given TNFsr (0.5 mg/kg, iv). However, in rats pretreated with TNFsr and subsequently administered TNF-α, the level of plasma TNF-α was reduced to 2384 U/ml. (Fig. 4). This level of TNF-α was only measured when the plasma samples from the TNFsr + TNF-α treated rats were subjected to the same dilution factor as the plasma samples obtained from the LPS or TNF-α treated animals. When lesser dilutions of plasma were utilized measureable bioactive TNF-α was undetectable. The small amount of TNF-α measured in the plasma of TNFsr + TNF-α-treated rats represents an artifact of dilution. Upon high dilution, the TNFsr releases bound inactive TNF-α into the medium, at which point it becomes active TNF-α (4, 5). Thus, the bolus injection of exogenous TNF-α which failed to mimic the hemodynamic effects of LPS produced plasma levels of bioactive TNF-α equal to or greater

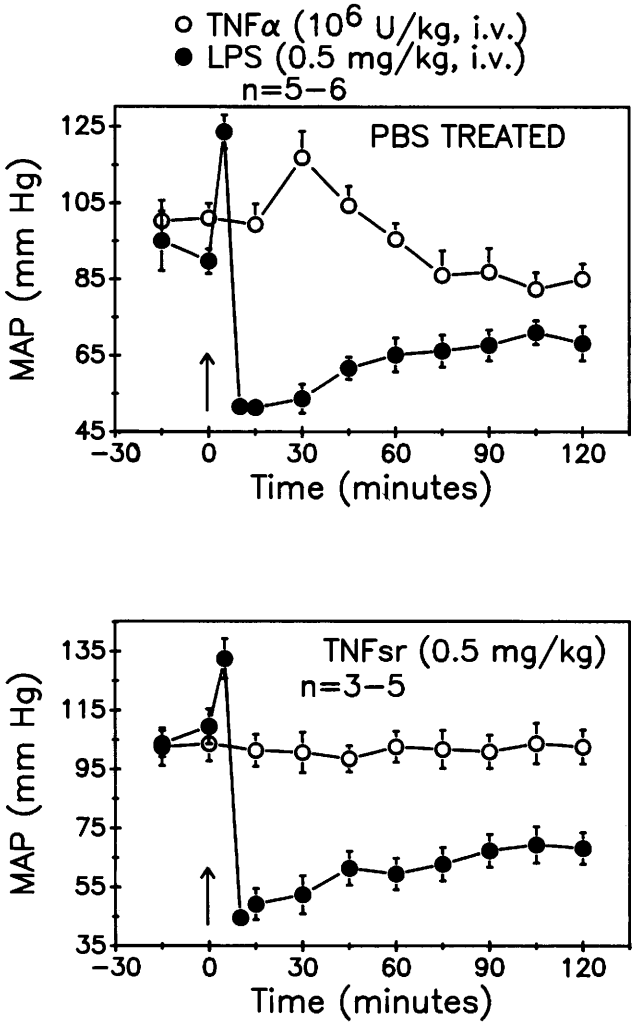

Figure 3. Time course of the hemodynamic changes in rat mean arterial pressure following administration of TNF-α (10^6 U/kg, iv) or LPS (0.5 mg/kg, iv) in phosphate buffered saline (PBS) (Top Panel) or the Immunex monomeric soluble TNF-α P75 receptor (TNFsr) (Bottom Panel). The ordinate is the mean arterial pressure (MAP) in mm Hg (±SEM). Rats were given TNFsr 30 min before TNF-α or LPS which were given at the arrow. Each data point is the mean of three to six rats.

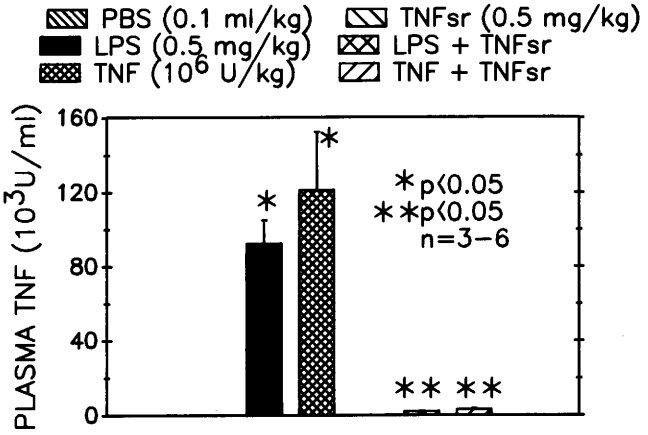

Figure 4. Plasma TNF-α levels determined 90 min after iv administration of PBS (0.1 ml/kg, iv), LPS (0.5 mg/kg, iv), TNF-α (1,000,000 U/kg, iv), TNFsr (0.5 mg/kg, iv), and LPS (0.5 mg/kg, iv) in rats pretreated with TNFsr. Bioactive TNF-α was assayed with the L929 bioassay. The ordinate represents the plasma TNF-α in Units/ml · 1000. Vertical lines are the SEM. n = 3–6.

than that produced by LPS. Moreover, TNFsr decreased LPS-induced circulating TNF-α levels by 97.5% (Fig. 4) ($P < 0.05$).

RNI in Plasma and Rat Neutrophils. Plasma obtained from rats treated with PBS contained low concentrations of RNI (Fig. 5, Top). LPS increased plasma levels of RNI, whereas plasma RNI levels did not increase with TNF-α or with TNFsr. Moreover, TNFsr pretreatment did not prevent LPS-induced increases in plasma RNI (Fig. 5, Top). Similarly, RNI was not detectable in each of the incubates of PMN obtained from rats treated with PBS or TNFsr and in four of the five incubates obtained from rats treated with TNF-α (Fig. 5, Bottom). However, LPS administration *in vivo* induced spontaneous RNI production by PMN incubated *ex vivo* and the production of RNI was not suppressed when PMN were obtained from rats pretreated with TNFsr prior to administration of LPS (Fig. 4, Bottom). Previous studies demonstrated that basal plasma levels of RNI were unaffected by inhibitors of iNOS II whereas pretreatment of animals with prednisolone or 3-amino, 1,2,4-triazine, inhibitors of iNOS II prevented LPS-induced increases in plasma and PMN RNI (30, 38, 39). Since the HEPES buffer in which the PMN were incubated contained 10 μM of hydrocortisone and RNI production was not present in PMN from PBS-, TNFsr-, or TNFα-treated rats, it is unlikely that the spontaneous production of RNI resulted from endotoxin-mediated induction of iNOS in PMN *ex vivo*. Thus, LPS, but not TNF-α, rapidly induces RNI production in PMN and increases plasma levels of RNI (Fig. 5).

Changes in Aorta RNI. Rings of aorta obtained from rats treated with PBS *in vivo* exhibited basal and A23187-stimulated release of RNI *ex vivo* which was abolished by endothelium rubbing and by L-N^G-monomethylarginine (Fig. 6). In contrast, basal, A23187- , and bradykinin-stimulated release of RNI was diminished or abolished in rat aorta obtained 2 hr after *in vivo* administration of LPS or TNF-α (Fig. 7 and 8). TNFsr by itself did not affect RNI production by the aorta but inhibited LPS-induced inhibition of aortic RNI production (Fig. 7 and 8). Thus, the data suggest that LPS and TNF-α both inhibit cNOS III activity in aorta and that the inhibitory effect of LPS on aortic RNI production is prevented by TNFsr (Fig. 7 and 8).

Discussion

This study demonstrates that exogenous administration of a dose of TNF-α devoid of endotoxin fails to mimic the hemodynamic effects of exogenously administered endotoxin. Moreover, exogenously admin-

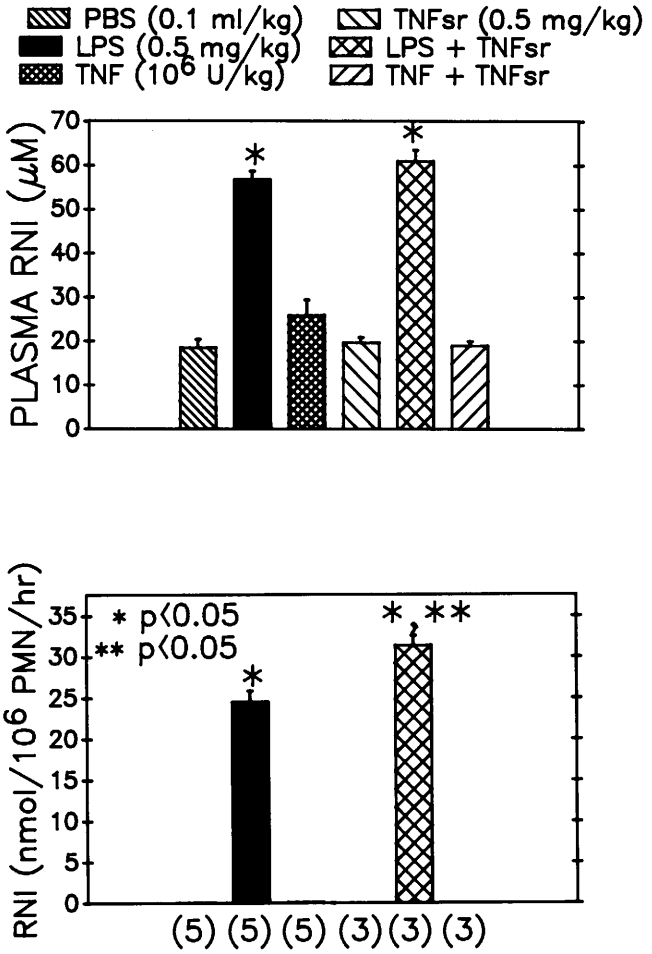

Figure 5. (Top Panel) Peak plasma RNI (μM) determined 2 hr after iv administration of PBS, LPS, TNF-α, TNFsr, or LPS to rats pretreated with TNFsr. (Bottom Panel) Peak changes in circulating PMN RNI (nmoles/10^6cells/hr) incubated *ex vivo* in HEPES buffered salt solution when obtained 2 hr after iv administration of PBS, LPS, TNF-α, TNFsr, or LPS + TNFsr. Vertical lines are the SEM. *Means differ from that obtained in animals given PBS ($P < .05$). **Measurement in TNFsr-pretreated rats differs from that in LPS-treated rats by ANOVA ($P < 0.05$). The number of animals tested are shown in the brackets.

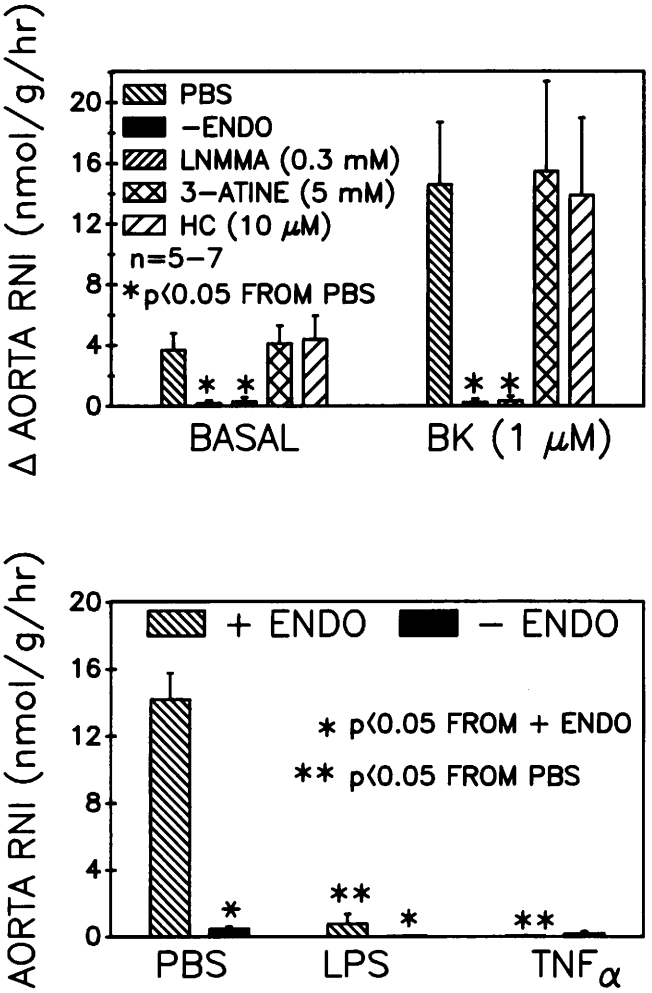

Figure 6. Effect of pretreatment with PBS (0.1 ml/kg, iv) on aorta production of RNI *ex vivo*. The ordinate is the spontaneous (BASAL), A23187- (Top Panel), or bradykinin-stimulated (Bottom Panel) increase in RNI in normal (PBS) and endothelium rubbed aorta (-ENDO). The drugs indicated were added *in vitro* to the incubate. HC is hydrocortisone (10 μM), 3-ATINE is 3-amino, 1,2,4-triazine, an inhibitor of iNOS II. LNMMA is 1-N^G-monomethylarginine, an inhibitor of iNOS II and cNOS III.

istered TNF-α does not mimic the acute induction of iNOS II activity (30, 38) produced in PMN and lung and liver (39) by LPS. However, both exogenously administered endotoxin and TNF-α produced equivalent plasma levels of biologically active TNF-α. Moreover, we also show for the first time that *in vivo* administration of exogenous TNF-α and endogenously produced TNF-α suppress *ex vivo* basal and A23187-induced RNI production by the aortic endothelium. This must represent cNOS III activity, since the activity was stimulated by the calcium ionophore A23187 and abolished by endothelium rubbing. Moreover, LNMMA inhibited these effects, whereas the basal and stimulated activity was unaffected by hydrocortisone which inhibits gene expression for iNOS II or by 3-ATINE which inhibits iNOS II activity (30, 41). Thus, we conclude that although the plasma levels of TNF-α are important contributors to tissue damage and long-term morbidity and mortality in gram-negative sepsis (1–5, 33–36), TNF-α does not mediate or is not required for the acute phase hypotension and induction of iNOS II activity associated with endotoxin administration. The data also support the conclusions that exogenously administered LPS and TNF-α and endogenously produced TNF-α *in vivo* rapidly (within 2 hr) suppress aortic endothelium production RNI and that TNF-α appears to mediate LPS-induced inhibition of endothelium production of RNI. Speculatively, this may be important for the rapid upregulation of phagocyte adhesion to the vascular endothelium observed following administration of both TNF-α and LPS (1–5, 33–36).

Current evidence suggests that LPS induces TNF-α production which in turn acts in an autocrine

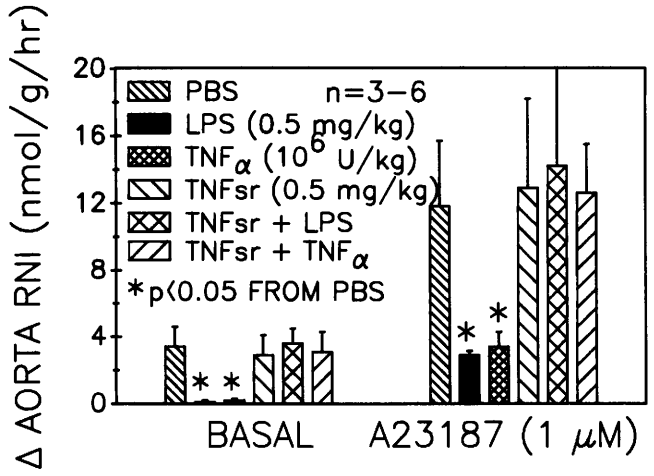

Figure 7. Effect of *in vivo* treatment of rats with PBS (0.1 ml/kg, iv), LPS (0.5 mg/kg, iv). TNF-α (1,000,000 U/kg, iv), TNFsr (0.5 mg/kg, iv), and LPS in rats pretreated with TNFsr on aorta production of RNI *ex vivo*. The ordinate is the spontaneous (BASAL) or A23187-stimulated increase in RNI in normal (PBS) aorta. Vertical lines are the SEM from three to six experiments. *Differs from PBS; **differs from LPS.

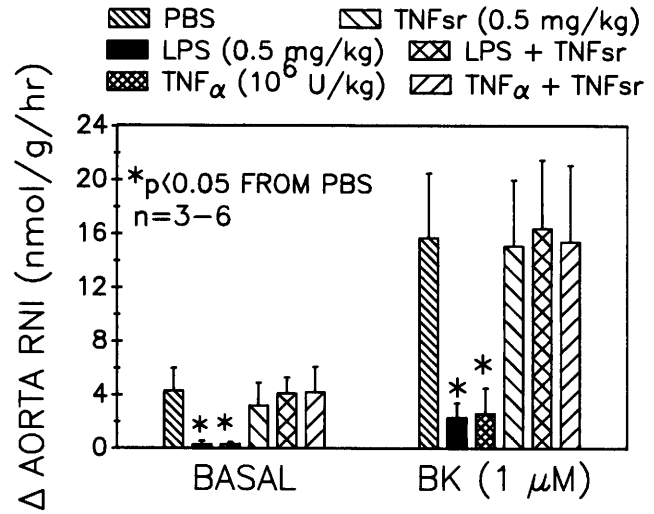

Figure 8. Effect of *in vivo* treatment of rats with PBS (0.1 ml/kg, iv), LPS (0.5 mg/kg, iv). TNF-α (1,000,000 U/kg, iv), TNFsr (0.5 mg/kg, iv), and LPS in rats pretreated with TNFsr on aorta production of RNI *ex vivo*. The ordinate is the spontaneous (BASAL) or bradykinin-stimulated increase in RNI in normal (PBS) aorta. Vertical lines are the SEM from three to six experiments. *Differs from PBS; **differs from LPS.

and paracrine fashion to induce iNOS activity and RNI production (4–9, 22–24). TNF-α and NO are believed to mediate the hemodynamic sequellae associated with endotoxemia and sepsis in animals and humans (4–6, 22–24, 33). Early studies demonstrated that (i) TNF-α produced hypotension associated with an increase in plasma RNI levels similar to LPS, (ii) LNMMA reversed these effects of TNF-α, and (iii) monoclonal antibodies to TNF-α partially attenuated but did not abolish LPS-induced hypotension and production of RNI (6, 22–24). Thus, the results reported herein initially appear to be in conflict with existing data. However, Neilson *et al.* (27) demonstrated the inability of exogenously administered TNF-α to produce hypotensive shock in the absence of contaminating endotoxin. Thus, our data are in agreement with that of Nielsson *et al.* (27). Our data also show that both LPS and exogenous TNF-α produce a transient hypertension. However, the time course and duration of the hypertension also differ. This study was not designed to evaluate the mechanism of the hypertension produced by LPS or TNF-α. Nevertheless, both LPS and TNF-α inhibited endothelium production of RNI. TNFsr inhibited LPS-mediated inhibition of RNI production by the endothelium. TNFsr completely prevents TNF-α-induced hypotension (Cindy Jacobs, personal communication). Speculatively, TNF-α-mediated hypertension may be mediated by inhibition of endothelium production of NO (Fig. 6). However, we previously reported that a polyclonal antibody against TNF-α only suppressed the pressor response to LPS by 26% + 6% (41). Therefore, inhibition of TNF-α only partially mediates LPS-induced hypertension and factors other than TNF-α must be involved in the acute pressor response to LPS. In agreement with this conclusion is the study in which LPS directly inhibited RNI production by isolated endothelial cells in culture (42).

Thiemermann *et al.* (6) using a monoclonal antibody to TNF-α in rats demonstrated only partial (25%) inhibition of LPS-induced hypotension and approximately the same inhibition of ^{14}C-citrulline production by lung homogenates. TNF-α is a trimeric protein which contains two binding sites which interact with either a 55-kDa and 70-kDa receptor on their effector tissues (4, 5). Monoclonal antibodies to TNF-α only bind to one of these sites. In contrast, the polyclonal antibody to TNF-α binds to both sites on the TNF-α moiety, thereby preventing its biologic activity by abrogating its ability to interact with either the 55-kDa or 75-kDa receptor (4, 5). It is presently uncertain if the 55-kDa and 75-kDa receptors are coupled to the same, different, or reciprocal functions on their effector tissue (4, 5). Bagby *et al.* (43) presented evidence which initially suggested that TNF-α mediated the change in glucose kinetics in an intravascular model of endotoxemia. Subsequent studies using a polyclonal antibody to TNF-α demonstrated complete neutralization of bioactive circulating TNF-α without any major effect on glucose metabolism and concluded that regulation of glucose metabolism after endotoxin administration was independent of TNF-α (44). In addition, several studies have shown that both monoclonal and polyclonal antibodies against TNF-α fail to modify the hypotension or lethality of cecal ligation, peritonitis, and intravascular models of endotoxemia and sepsis (31, 32, 44). Thus, the differences between the data obtained here and elsewhere (6, 22–24) may merely re-

flect quantitative rather than qualitative differences, and this may relate to the different strains of rats used.

The data reported herein that *in vivo* administration of TNF-α and LPS exhibit different effects on blood pressure and production of RNI confirm and extend previously published findings which demonstrated that *in vivo* administration of a monoclonal antibody against TNF-α only partially suppressed LPS-induced hepatic iNOS II activity (29) and that LPS and γ-interferon stimulated iNOS II activity and TNF-α by independent and distinct mechanisms or pathways (28, 29). LPS-induced increases in plasma TNF-α and RNI were shown to be temporally related (38, 41). Nevertheless, TNF-α did not increase PMN RNI production and TNFsr did not inhibit LPS-mediated increases in PMN RNI production. A previous study demonstrated that the rapid induction of gene expression and RNI production in rat PMN was inhibited by prednisolone and 3-ATINE (30), and accounted for the acute phase hypotension to LPS (38). In this study, LPS also increased RNI production by PMN and produced acute phase hypotension, whereas TNF-α was devoid of these effects and TNFsr failed to suppress LPS-induced increases in RNI production by PMN. Thus, we conclude that LPS-mediated increases in iNOS II in PMN and acute phase hypotension are not mediated by TNF-α. Alternatively, TNF-α may contribute to the response, but sufficient redundancy exists in the pathway so that in the absence of TNF-α, other cytokines can effectively accomplish the functions of TNF-α. This could explain, in part, the lack of clinical success of antibodies to TNF-α and TNFsr in patients with sepsis (Jeff Norton, personal communication).

It could be argued that the inability of TNF-α to mimic the effect of LPS on blood pressure and RNI production resulted from administration of an insufficient dose of TNF-α to the rats. Tracey *et al.* (1) and others (for references see 2–4, 16, 17, 24) observed hypotension with doses of TNF-α significantly higher than the 8.8 μg/kg used herein. It is currently unclear what dose of TNF-α is required to mimic the effects of LPS, since some TNF-α production may occur at extravascular sites. Therefore, simple reproduction of the concentration of TNF within the plasma compartment may not be sufficient to mimic LPS-like effects. However, it is unlikely that the rapid effects of TNF-α on blood pressure and heart rate can be mediated by extravascular TNF-α. In a fecal implant model of endotoxemia in the rat, TNF-α levels are significantly elevated in peritoneal fluid at a time when plasma TNF-α is elevated and the blood pressure is essentially normal (32). Thus, plasma TNF-α appears to reflect the systemic compartment of TNF-α. Peak increases in plasma TNF-α occur 90 min after LPS administration, almost 1 hr after blood pressure has reached its nadir and is slowly returning towards normal, despite the capacity of the systemic blood pressure to undergo further decline. Finally, this study clearly shows that iv administration of TNF-α producing bioactive levels of plasma TNF-α equal to or greater than that produced by iv administration of LPS cannot produce equivalent hypotension and TNFsr cannot attenuate LPS-induced hypotension. Thus, we are forced to conclude that despite the importance of TNF-α as an important biologic mediator of endotoxemia and the systemic inflammatory response syndrome, it does not appear to play a significant role as the mediator of acute phase hypotension and RNI production in response to LPS.

The present data do not allow us to elucidate the pathway by which LPS induces iNOS II activity in PMN. The experiments were designed to test the concept that exogenous TNF-α inhibited endothelium production of RNI as it did *in vitro* (15, 21). Moreover, the study was designed to determine if TNF-α mimics, and thereby could mediate, the acute phase hemodynamic profile produced by LPS and the effects of LPS on the NO system. The data clearly show that exogenously administered TNF-α and endogenously produced TNF-α cannot account for the acute phase hemodynamic effects of LPS or the ability of LPS to rapidly induce iNOS II activity to PMN. Nevertheless, *in vivo* administration of both LPS and TNF-α both suppress the *ex vivo* production of RNI by the aortic endothelium. This differs from the inability of TNF-α when administered in muscle chambers to inhibit basal production of RNI and A23187-induced RNI production by bovine pulmonary artery (18). However, in this study, TNF-α was given *in vivo*. It has been established that some of the effects of TNF-α on the vascular endothelium when given *in vitro* and *in vivo* differ (37). Thus, the inhibitory effect of TNF-α on endothelium production of RNI exists when TNF-α is given *in vivo*. However, TNF-α is less selective as an inhibitor of cNOS III activity when given *in vivo* than *in vitro* (18). Since LPS and TNF-α rapidly inhibit vascular endothelial cell production of RNI, we speculate that this effect may contribute in part to their ability to increase phagocyte adhesion to the endothelium, the first step in phagocyte migration into sites of infection and inflammation. Further studies are in progress to test this postulate.

This work was supported by research funds from the Department of Medicine, LSU, and National Institutes of Health Grant NIAAA 09816-01 and NIAAA IP50AA09803-01.

1. Tracey KJ, Stephen FL, Cerami A. Catechin/TNFα in septic shock and septic adult respiratory distress syndrome. Am Rev Respir Dis **138**:1377–1379, 1988.
2. Cerami A, Beutler B. The role of cachectin/TNF in endotoxic shock and cachexia. Immunol Today **9**:28–31, 1988.

3. Beutler B, Cerami A. The biology of cachectin/TNF—A primary mediator of the host response. Annu Rev Immunol **7**:625–655, 1989.
4. Tracey KJ, Cerami A. Tumor necrosis factor: An updated review of its biology. Crit Care Med **21**:S415–S422, 1993.
5. Beutler B, Grau GE. Tumor necrosis factor in the pathogenesis of infectious disease. Crit Care Med **21**:S423–S434, 1993.
6. Thiemermann C, Chin-Chen W, Szabo C, Perretti M, Vane JR. Role of tumor necrosis factor in the induction of nitric oxide synthase in a rat model of endotoxin shock. Br J Pharmacol **110**:177–181, 1993.
7. Busse R, Mulsch A. Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. FEBS Lett **275**:87–90, 1990.
8. Kilbourn RG, Belloni P. Endothelial cell production of nitrogen oxides in response to interferon gamma in combination with tumor necrosis factor, interleukin-1, or endotoxin. J Natl Cancer Inst **82**:772–776, 1990.
9. Marsden PA, Ballermann BJ. Tumor necrosis factor alpha activates soluble guanylate cyclase in bovine glomerular mesangial cells via an L-arginine-dependent mechanism. J Exp Med **172**:1843–1852, 1991.
10. Gross SS, Jaffe EA, Levi R, Kilbourn RG. Cytokine-activated endothelial cells express an isotype of nitric oxide synthase which is tetrahydrobiopterin-dependent, calmodulin-independent and inhibited by arginine analogs with a rank-order of potency characteristic of activated macrophages. Biiochem Biophys Res Commun **178**:823–829, 1991.
11. Bredt DS, Snyder SH, Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proc Natl Acad Sci USA **87**:682–689, 1990.
12. Forstermann U, Schmidt HH, Pollack JS, Sheng H, Mitchell JA, Warner TD, Nakane M, Murad F. Isoforms of nitric oxide synthase. Characterization and purification from different cell types. Biochem Pharmacol **42**:1849–1857, 1991.
13. Radomski MW, Palmer RM, Moncada S. Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. Proc Natl Acad Sci USA **87**:10043–10047, 1990.
14. Pohlman T, Harlan JM. Human endothelial cell response to lipopolysaccharide, interleukin-1, and tumor necrosis factor is regulated by protein synthesis. Cell Immunol **119**:41–52, 1989.
15. Aoki N, Siegfried M, Lefer AM. Anti-EDRF effect of tumor necrosis factor in isolated, perfused cat carotid arteries. Am J Physiol **256**:H1509–H1512, 1989.
16. Liu S, Dewar A, Crawley DE, Barnes PJ. Effect of tumor necrosis factor on hypoxic pulmonary vasoconstriction. J Appl Physiol **72**:1044–1049, 1992.
17. Wheeler AP, Jesmok G, Brigham KL. Tumor necrosis factor's effects on lung mechanics, gas exchange, and airway reactivity in sheep. J Appl Physiol **68**:2542–2549, 1990.
18. Xie J, Wang Y, Kolls J, Nelson S, Summer WR, Greenberg S. Tumor necrosis factor alpha selectively suppresses acetylcholine and bradykinin-induced nitric oxide release from pulmonary vascular endothelium. Am Rev Respir Dis **147**:1072–1083, 1993.
19. Greenberg S, Xie J, Wang Y, Nelson S, Cai B, Hyman A, Lippton HE, Summer WR. Tumor necrosis factor$_\alpha$ inhibits endothelium dependent relaxation. J Appl Physiol **74**:2394–2403, 1993.
20. Xie J, Rust B, DeBoissblanc B, Summer WR, Greenberg S. Endotoxin-mediated increases in pulmonary artery pressure are associated with suppression of agonist-mediated release of nitric oxide from porcine pulmonary arteries. J Appl Physiol (in press).
21. Yoshizumi M, Perrella MA, Burnett JC, Lee ME. Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life. Circ Res **73**:205–209, 1993.
22. Lorente JA, Landin L, Renes E, De Pablo R, Jorge P, Rodena E, Liste D. Role of nitric oxide in the hemodynamic changes of sepsis. Crit Care Med **21**:759–768, 1993
23. Wright CE, Rees DD, Moncada S. Protective and pathological roles of nitric oxide in endotoxin shock. Cardiovasc Res **26**:48–57, 1992.
24. Kilbourn RG, Gross SS, Jubran A, Adams J, Griffith OW, Levi R, Lodato RF. NG-methyl-L-arginine inhibits tumor necrosis factor-induced hypotension: Implications for the involvement of nitric oxide. Proc Natl Acad Sci USA **87**:3629–3632, 1990.
25. Ignarro LJ. Biosynthesis and metabolism of endothelium-derived nitric oxide. Annu Rev Pharmacol Toxicol **30**:535–560, 1990.
26. Moncada S, Palmer RM, Higgs EA. Nitric oxide: Physiology, pathophysiology, and pharmacology. Pharmacol Rev **43**:109–142, 1991.
27. Neilson IR, Neilson KA, Yunis EJ, Rowe MI. Failure of tumor necrosis factor to produce hypotensive shock in the absence of endotoxin. Surgery **106**:439–443, 1989.
28. Leu RW, Leu NR, Shannon BJ, Fast DJ. IFNgamma differentially modulates the susceptibility of L1210 and P815 tumor targets for macrophage-mediated cytotoxicity. Role of macrophage-target interaction coupled to nitric oxide generation, but independent of tumor necrosis factor production. J Immunol **147**:1816–1823, 1991.
29. Evans T, Carpenter A, Silva A, Cohen J. Differential effects of monoclonal antibodies to tumor necrosis factor alpha and gamma interferon on induction of hepatic nitric oxide synthase in experimental gram-negative septis. Infect Immun **60**:4133–4139, 1992.
30. Kolls J, Xie J, Warner A, Leblanc R, Malinski T, Nelson S, Summer WR, Greenberg, SS. Rapid induction of mRNA for nitric oxide synthase II by endotoxin and inhibition of its transcription by prednisolone. Proc Soc Exp Biol Med **205**:220–229, 1994.
31. Eskandari MK, Bolgos G, Miller C, Nguyen CT, DeForge LE, Remick DG. Anti-tumor necrosis factor antibody therapy fails to prevent lethality after cecal ligation and puncture or endotoxemia. J Immunol **148**:2724–2728, 1992.
32. Bagby GJ, Plessala KJ, Wilson LA, Thompson JJ, Nelson S. Divergent efficacy of antibody to tumor necrosis factor-alpha in intravascular and peritonitis models of sepsis. J Infect Dis **163**:83–92, 1991.
33. Mathison JC, Wolfson E, Ulevitch RJ. Participation of tumor necrosis factor in the mediation of gram negative bacterial lipolysaccharide-induced injury in rabbits. J Clin Invest **81**:1925–1931, 1988.
34. Beutler B, Milsark IW, Cerami AC. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science **229**:869–873, 1985.
35. Beutler B, Cerami A. Cachectin/tumor necrosis factor: An endogenous mediator of shock and inflammation. Immunol Res **5**:281–287, 1986.
36. Tracey KJ, Beutler B, Lowry SF, Merryweather J, Wolpe S, Milsark IW, Hariri RJ, Fahey TJ III, Zentella A, Albert JD, et al. Shock and tissue injury induced by recombinant human cachectin. Science **234**:470–475, 1986.
37. Frater-Schroder M, Risau W, Hallmann R, Gautschi P, Bohlen P. Tumor necrosis factor type alpha, a potent inhibitor of endothelial cell growth *in vitro*, is angiogenic *in vivo*. Proc Natl Acad Sci USA **84**:5277–5281, 1987.
38. Xie J, Wang Y, Leblanc R, Kolls J, Summer WR, Greenberg S. Rapid induction of mRNA for nitric oxide synthase II by *Escherichia coli* endotoxin and its relationship to acute phase hypotension. J Appl Physiol (in press).
39. Greenberg SS, Xie J, Wang Y, Malinski T, Summer WR, McDonough K. Ethanol opposes the action of *Escherichia coli* on

endothelium-dependent relaxation, nitric oxide and gene expression for NOS I in rat aorta. Alcohol (in press).
40. Sig-Stat V 2.1. A BMDP statistical package for the IBM personal computer. Provo, UT: Significant Statistics, 1992.
41. Xie J, Kolls J, Joseph KO, Bagby GJ, Summer WR, Nelson S, Greenberg S. A polyclonal antibody to tumor necrosis factor alpha fails to suppress endotoxin-induced hypotension. J Exp Med (in press).
42. Myers PR, Wright TF, Tanner MA, Adams HR. EDRF and nitric oxide production in cultured endothelial cells: Direct inhibition by *E. coli* endotoxin. Am J Physiol **262**:H710–H718, 1992.
43. Bagby GJ, Lang CH, Hargrove DM, Thompson JJ, Thompson JJ, Wilson LA, Spitzer JJ. Glucose kinetics in rats infused with endotoxin-induced monokines or tumor necrosis factor. Circ Shock **24**:111–118, 1988.
44. Bagby GJ, Lang CH, Skrepnik N, Golightly G, Spitzer JJ. Regulation of glucose metabolism after endotoxin and during infection is largely independent of endogenous tumor necrosis factor. Circ Shock **39**:211–221, 1993.