# Tumor Necrosis Factor$_\alpha$ Inhibits Contractions to Sympathetic Nerve Stimulation by a Nitric Oxide-Dependent Mechanism (43621)

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*Abstract.* **Gram-negative sepsis and administration of tumor necrosis factor$_\alpha$ (TNF$_\alpha$) are associated with hypotension and peripheral neuropathies suggestive of impaired sympathetic neurotransmission. We examined the effect of TNF$_\alpha$ on the responses of the bovine pulmonary artery (BPA) to transmural sympathetic nerve stimulation (SNS). BPA contracted to SNS (0.5–32 Hz, 5–10 V, 2-msec duration, 2-msec delay) in a frequency-dependent manner. The contractions of the BPA to SNS were mediated by norepinephrine and activation of postsynaptic $\alpha_1$-adrenoceptors, since they were attenuated by prazosin. Maximum contraction of the BPA to SNS was significantly enhanced (148 ± 37% increase, $n$ = 6) after inhibition of nitric oxide synthase with L-N$^G$-monomethylarginine (LNMMA, 500 $\mu M$), an effect abrogated by L-arginine (1 m$M$). TNF$_\alpha$ (0.0042, 0.042, and 0.42 $\mu$g/ml) selectively inhibited contractions of the BPA to SNS without affecting the contraction of the BPA to exogenous norepinephrine. In BPA incubated with LNMMA (5–500 $\mu M$), TNF$_\alpha$ facilitated rather than inhibited SNS. TNF$_\alpha$ increased the formation of amperiometrically measured free nitric oxide in bovine adrenal chromaffin cells in primary culture. The data show that in the absence of LNMMA, TNF$_\alpha$ releases free nitric oxide from a sympathetic neuron and selectively inhibits the contractions of the BPA to SNS. In BPA in which nitric oxide synthase I is inhibited by LNMMA, TNF$_\alpha$ amplifies the contractions to SNS, even in the absence of endothelium. Thus, TNF$_\alpha$ can modify vascular smooth muscle tone by affecting SNS. TNF$_\alpha$ inhibits SNS at the level of the neuron by a mechanism involving the L-arginine-nitric oxide pathway. TNF$_\alpha$-induced suppression of SNS and neurotransmission may contribute to the hypotension and peripheral neuropathy of sepsis.**

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Bacteremia and sepsis elevate animal and human plasma concentrations of tumor necrosis factor$_\alpha$ (TNF$_\alpha$) (1, 2). Administration of antibodies to TNF$_\alpha$ to rats attenuates the hypotension and prevents the cardiovascular collapse associated with sepsis of bacterial origin (3). Thus, TNF$_\alpha$ has been implicated as a mediator of the hypotension and diminished responsiveness of vascular smooth muscle to vasoconstrictor stimuli (4–6) of gram-negative sepsis and endotoxic shock. It has been suggested that the vascular depressant effects of TNF$_\alpha$ are mediated by TNF$_\alpha$-induced gene expression for the inducible form of nitric oxide synthase (NOS II) within the smooth muscle and vascular endothelium (7). Nitric oxide normally produced within the endothelium by the constitutive enzyme NOS III as well as from the inducible NOS II in endothelium and smooth muscle can stimulate guanylate cyclase and elevate the cyclic GMP content of the smooth muscle (8). The increased cyclic GMP has been suggested to mediate the depressed vascular function associated with sepsis and administration of endotoxin

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and $TNF_\alpha$ (9). However, in the early phase of sepsis and within 30 min after administration of endotoxin or $TNF_\alpha$ to rats, isolated endothelial cells in culture, or intact blood vessels, endothelium-derived nitric oxide synthesis is suppressed (10–14). Since several hours are required for $TNF_\alpha$ to stimulate gene expression for NOS II in endothelium and smooth muscle, it is unlikely that induction of NOS II and $TNF_\alpha$-mediated suppression of nitric oxide synthesis from constitutive NOS III within the endothelium could mediate the acute hypotension observed after administration of endotoxin or $TNF_\alpha$.

Neurogenic tone is an important component of vascular resistance under resting conditions, *in vivo*. Moreover, it plays a key role in blood pressure maintenance in bacteremic conditions and sepsis (2). Recent studies (15–19) demonstrated that nitric oxide (NO) and substances that elevate cGMP inhibit norepinephrine (NE) release from peripheral postganglionic sympathetic nerve endings innervating the myocardium and vascular smooth muscle. The sympathetic neurons contain a distinct isoform of constitutive nitric oxide synthase (NOS I). Stimulation of NO within the neuron could result in suppression of sympathetic neurotransmission and hypotension. However, to our knowledge, the effects of $TNF_\alpha$ on sympathetic neurotransmission to blood vessels and free NO derived from constitutive NOS I within neurons have not been examined. Thus, we examined the effect of $TNF_\alpha$ on the contractions of the bovine pulmonary artery to transmural sympathetic nerve stimulation (SNS) and exogenously administered norepinephrine, and on the production of free nitric oxide from the constitutive NOS I in bovine adrenal chromaffin cells, a model for the sympathetic neuron (20).

## Methods and Materials

Animals were treated according to the *Guiding Principles for Laboratory Animal Care* of the American Physiological Society. All experiments were conducted after an approved protocol from the Animal Institutional Care and Use Committee of the Louisiana State University Medical Center. Bovine lungs and adrenal glands were obtained from an abattoir at the time of animal slaughter and transported to the laboratory in pouches of aerated (95% $O_2$ and 5% $CO_2$) Krebs-Henseleit solution packed in ice. Transit time to the laboratory did not exceed 1 hr. Fourth-order branches of bovine pulmonary artery (BPA) were carefully cleaned, cut into four paired rings (2–3 mm wide), and prepared for transmural sympathetic nerve stimulation with established techniques (18, 19).

**Studies in Pulmonary Arteries.** Briefly, paired rings (four per blood vessel) of normal and endothelium-rubbed BPA were mounted on stainless steel wires between two platinum plate electrodes (1 mm × 3 mm) in 20-ml capacity muscle chambers and connected to Grass FT.03C force transducers. The Krebs-Henseleit solution (PSS) in the muscle chamber was maintained at 37°C and aerated with 95% and $O_2$ and 5% $CO_2$ (pH 7.37–7.42), and contained (m*M*): NaCl (118), $CaCl_2$ (4.9); KCl (4.8); $MgCl_2$ (1.2); $NaHCO_3$ (24.7); $NaH_2PO_4$ (1.18); dextrose (10); sucrose (50); and $CaNa_2EDTA$ (0.026), cocaine (2 $\mu M$) and ibuprofen (5 $\mu M$) to inhibit prostaglandin biosynthesis (18, 19). Experiments conducted in our laboratories have established that these concentrations of inhibitor maximally potentiate the responses of the BPA to SNS and exogenous NE and abolish all contractile and relaxant responses to arachidonic acid. Each blood vessel was stretched to the optimal portion of its length-tension curve by the incremental increase in applied preload and evaluation of the contraction to 60 m*M* KCl until two successive incremental increases in applied preload resulted in contractions to KCl that did not differ by more than 5% (11, 13, 14). The mean applied preload for maximal force output was 5.5 ± 0.3 g for the BPA ($n = 3$). The rings of blood vessel were equilibrated at 37°C for 3 hr before further experimentation. The endothelium was damaged by rubbing the intimal surface of the blood vessels with a small wooden applicator stick. If bradykinin (1 $\mu M$) or acetylcholine (ACh 1 $\mu M$) produced greater than 5% relaxation of the rubbed BPA, the rubbing procedure was considered ineffective and the tissues were eliminated from consideration (11–13). At the end of each experiment, the blood vessels were removed from the muscle chambers, measured to the nearest millimeter, and weighed to the nearest milligram.

A contractile response to 40 m*M* KCl was obtained in each blood vessel. The KCl was washed from the muscle chamber, the BPA was allowed to re-equilibrate for 15 min, and then cumulative, equilibrium responses to SNS (0.5, 1, 2, 4, 8, 16, and 32 Hz; square wave pulses, 2-msec duration, 2-msec delay, 9–10 V) or to exogenous NE (0.1 n*M* to 100 $\mu M$) were obtained on paired rings of BPA. Stimulation was then terminated and contractile force allowed to return to prestimulation values. To determine whether the contractile responses to SNS and exogenous NE were mediated by an interaction with $\alpha_1$-adrenoceptors on the smooth muscle, the contractions to SNS or exogenous NE were obtained before and 30 min after incubation of BPA with cumulative, increasing concentrations of either vehicle or prazosin (1, 10, and 100 n*M*). The response to KCl (40 m*M*) was repeated at the end of each experiment to test for nonspecific changes in the contractility of the smooth muscle attributable to time or test compound. The contractions to KCl were within 5% of each other at the beginning and end of the experiment.

The effects of $TNF_\alpha$ (0.0042, 0.042, and 0.42 $\mu$g/

ml) and L-$N^G$-monomethylarginine (LNMMA, 5, 50, and 500 $\mu M$) on the contractions of the normal and rubbed BPA to SNS and NE were evaluated by repeating the above paradigm on separate groups of BPA with one log molar increments of test compound or an equivalent volume of vehicle (PSS, 10 $\mu$l). Each frequency or concentration response curve was repeated after a 30-min rest period between test cycles and a 60-min equilibration period with each concentration of vehicle or test compound. Each blood vessel was exposed to either SNS or NE and to one test compound, a combination of test compounds, or vehicle.

**Measurement of Reactive Nitrogen Intermediates in BPA.** In another group of pulmonary arteries ($n$ = 5/treatment group), we evaluated the effects of $TNF_\alpha$, LNMMA, or vehicle on basal and ACh (100 n$M$ and 10 $\mu M$)-stimulated release of the total reactive nitrogen intermediates (RNI) nitrite ($NO_2^-$) and nitrate ($NO_3^-$) in normal and endothelium-rubbed BPA. After a 2-hr equilibration period, adjacent rings of blood vessel (23 ± 2 mg) were placed into sealed vials with 2 ml of physiologic salt solution buffered with HEPES (18 m$M$; pH 7.4) and containing 0.42 $\mu$g/ml of $TNF_\alpha$, 100 $\mu M$ of LNMMA, or PSS alone or in combination with PSS or ACh. 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 (11, 13, 14). Briefly, 100-$\mu$l aliquots 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 that contained the RNI-derived NO in nitrogen was taken up by vacuum into a Dasibi model 821 NO analyzer (Dasibi, Inc., Glendale CA). This analyzer measures dissolved NO and NO derived from $NO_2^-$ and $NO_3^-$ in the incubate that 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 Å 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 3 pg using 100 $\mu$l of 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 95 ± 1% ($n$ = 40) when compared with standard solutions of NO and NO calibration gases.

**Isolation of Bovine Adrenal Medullary Chromaffin Cells.** To evaluate the possibility that $TNF_\alpha$ may modulate SNS by affecting nitric oxide synthesis within the neuron, we measured free nitric oxide production in bovine adrenal chromaffin cells (BACC), a model of a sympathetic neuron (20). The adrenal medullary cells were obtained by collagenase digestion of adrenal medullary tissue from five cows/experiment. BACC were isolated on Renografin gradients (20, 21). The gradients were centrifuged at 15°C for 20 min at 7700$g$ in a Sorvall SS-34 rotor. The cells present in a diffuse band at the interface of the two Renografin solutions were washed twice with $Ca^{2+}$- and $Mg^{2+}$-free HEPES buffered solution, placed into a 1:1 mixture of Dulbecco's modified Eagle's medium with Ham's F12 medium, placed on ice, and shipped overnight to Oakland University (Rochester, MI). This layer had a phenethanolamine-$N$-methyl transferase activity of 32 ± 5 units/mg protein/min, whereas dopamine $\beta$-hydroxylase activity was 86 ± 6 units/mg protein/min and contained 83 ± 4 × $10^6$ cells/tube. This indicated that the BACC were viable and, consistent with the microscopic evaluation, reflected a preparation of BACC with greater than 98% purity. The BACC were placed in T-75 flasks in Dubelco's modified Eagle's medium-Ham's F12 with 1% fetal calf serum containing 1 m$M$ L-arginine, 50 $\mu$g of streptomycin, and 50 units/ml of penicillin G (95% air and 5% $CO_2$) at a density of 2 × $10^5$ cells/ml and incubated at 37°C (95% air and 5% $CO_2$) for 24 hr, at which time the antibiotics and fetal calf serum were removed and the 3-day-old BACC isolates used 24 hr later for the differential pulsed voltametric measurement of free NO at the surface of single BACC within different clusters of BACC. The percent viability of cells was determined by exclusion of trypan blue and averaged 95–97%.

**Measurement of Free Nitric Oxide in Single BACC.** Malinski and Taha (22) developed a porphyrinic microsensor for rapid detection of NO released by single cells in the presence of oxygen and/or nitrite ($NO_2^-$) and nitrate ($NO_3^-$) anions. The release, distribution, and reactivity of endogenous free NO in biologic systems can now be analyzed. We measured NO release from single cells within clusters of BACC with microelectrodes with a response time of less than 10 msec. The microsensor consisted of p-type semiconducting polymeric porphyrin and a cationic exchanger (Nafion) deposited on a thermally sharpened carbon fiber with a tip diameter of approximately 0.5 microns. The microsensor, operated in either the amperiometric or voltametric mode, is characterized by a linear response to NO from 10 n$M$ to 300 $\mu M$ (22). Since the sensor measures NO in 0.1 nl, it can theoretically sense NO at $10^{-20}$ moles in a single cell (22). The electrode was calibrated by addition of standard concentrations of NO to a solution of culture medium. The electrode was then placed under microscopic guidance on the surface of a single BACC within a cluster of BACC. The change in current at 0.68 V of holding potential was determined over the next 100 min. The experiment was repeated on BACC incubated with bradykinin (BK; 1 $\mu M$),

A23187 (1 $\mu M$), and $TNF_\alpha$ (0.42 $\mu$g/ml). Each experiment was repeated on BACC from three different cows. At the end of each experiment, the calibration of the electrode was retested with the addition of standard concentration of NO to the culture medium. The data are expressed as nanomolar concentrations of NO.

**Drugs Used.** Bradykinin triacetate, A23187, ATP, LNMMA, norepinephrine, and KCl were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant human $TNF_\alpha$ was a gift from Genentech, Inc. (South San Francisco, CA). All drugs were made up fresh daily.

**Data Analyses.** Data were analyzed with analysis of variance using Bonferroni's correction for time series analysis and log-linear regression. Means were compared between and among groups with Duncan's new multiple range test and Tukey's procedure. A $P$-value of 0.05 or less was chosen for significance of differences between and among means.

## Results

**Effect of Prazosin.** The contractions of the BPA to SNS were frequency dependent. Maximum contraction occurred at 16 Hz or 32 Hz SNS. Prazosin produced concentration-related inhibition of the contractions of the BPA to SNS (Fig. 1) and NE (data not shown). The inhibition was mediated by $\alpha_1$-adrenoceptor blockade and not smooth muscle depression, since the contractions to KCl (40 m$M$) did not differ before (8.6 ± 1.2 g) or after (8.4 ± 1.3 g) incubation with prazosin (100 n$M$).

**LNMMA and $TNF_\alpha$ on NE.** The contractions of the BPA with an intact endothelium (Fig. 2A) and with a rubbed endothelium (data not shown; 11, 14) to NE were reproducible over the time course of the experiment (Fig. 2A). Resting tension of the BPA with an intact endothelium was increased in a concentration-related manner during incubation with LNMMA. Basal tension increased from 3.2 ± 0.3 to 5.4 ± 0.6 ($n$ = 6) after incubation with 500 $\mu M$ LNMMA. LNMMA enhanced the contractions of the BPA to NE (Fig. 2B).

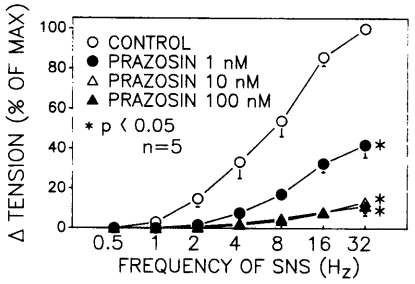


**Figure 1.** Effect of prazosin on the contractions of the BPA to SNS. The ordinate is the change in tension produced by SNS, expressed as a percentage of the control contraction at 32 Hz. The abscissa is the frequency of SNS (2-msec duration and delay, 9–10 V). Vertical lines are the SE from five blood vessels, each obtained from an individual cow.

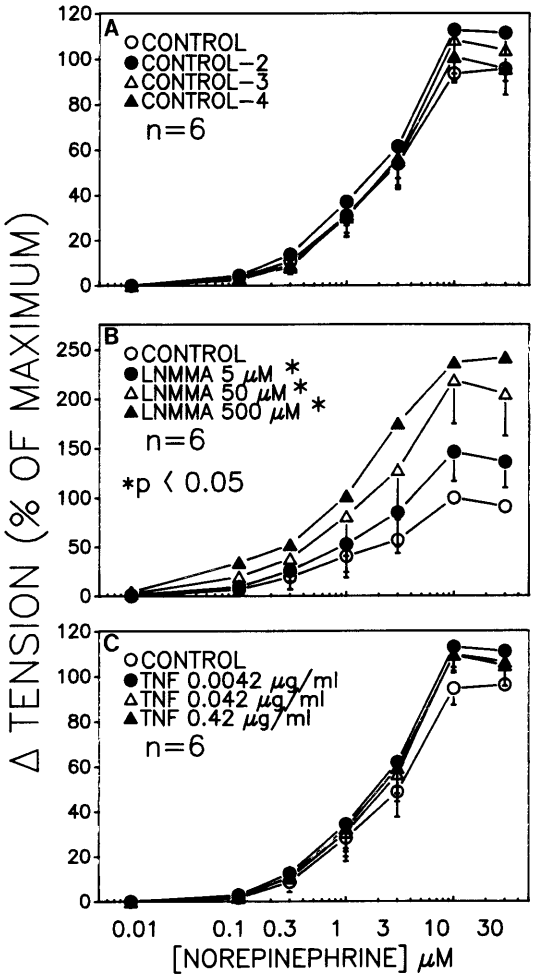


**Figure 2.** (A) Effect of vehicle, (B) LNMMA, and (C) $TNF_\alpha$ on the cumulative contractions of the normal BPA to NE. The ordinate is the change in tension produced by NE, expressed as a percentage of the maximum control contraction to NE (10 or 30 $\mu M$). The abscissa is the concentration of NE. Vertical lines are the SE from six blood vessels, each obtained from an individual cow. Asterisk indicates that the responses to NE differed from control ($P < 0.05$) at 1 $\mu M$ NE or greater (LNMMA 5 and 50 $\mu M$) or at all concentrations of NE (LNMMA 500 $\mu M$). In (A), Controls 2, 3, and 4 are the second, third, and fourth series of concentration effect curves for NE in the presence of PSS (vehicle).

In th dothelium-rubbed BPA, basal force was 3.4 ± 0.5 g before incubation with LNMMA and 3.6 ± 0.5 g during incubation with LNMMA ($P > 0.05$). In the endothelium-rubbed BPA, LNMMA did not affect the contraction to NE (Fig. 3). $TNF_\alpha$ (0.0042–0.42 $\mu M$) did not affect resting tension in either the normal or endothelium-rubbed BPA (data not shown). This finding was in agreement with previously published data (11, 14). Moreover, $TNF_\alpha$ did not affect the contractions of the normal or endothelium-rubbed BPA to exogenous NE (Fig. 2C; Fig. 3).

**LNMMA and $TNF_\alpha$ on SNS.** The contractions of the BPA to SNS were also reproducible over the time course of the experiment (Fig. 4A). LNMMA enhanced the contractions of the endothelium-rubbed (Fig. 3) and

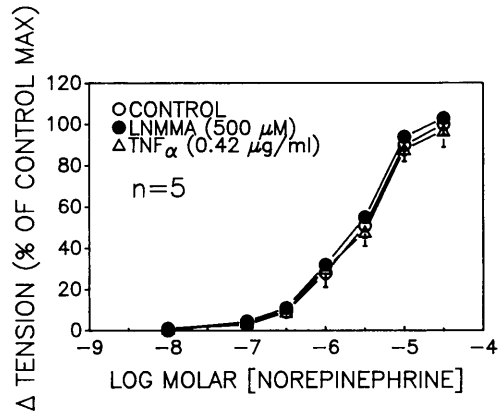


**Figure 3.** Effect of vehicle, LNMMA (500 $\mu M$), and $TNF_\alpha$ (0.42 $\mu M$) on the contraction of the endothelium-rubbed BPA to NE. The ordinate is the change in tension produced by NE, expressed as a percentage of the maximum control contraction to NE (10 or 30 $\mu M$). The abscissa is the concentration of NE. Vertical lines are the SE from five blood vessels, each obtained from an individual cow.

normal (Fig. 4B) BPA to SNS in a concentration-dependent manner. The effect of LNMMA on the contractions to SNS were attenuated in the endothelium-rubbed BPA when compared with the normal BPA. In the normal BPA, LNMMA increased the absolute magnitude of tension development at the higher frequencies of SNS more than at the lower frequencies of SNS. However, since the initial contractions to SNS were smaller at the lower frequencies, the percentage of enhancement of SNS was greater at the lower frequencies of SNS than at the higher frequencies. Maximal enhancement of SNS was 138 ± 19% of the control response at 16 Hz SNS and 627 ± 34% at 2 Hz SNS ($n = 6$, $P < 0.05$). In the endothelium-rubbed BPA, the percentage of enhancement at 2, 8, and 16 Hz was ($n = 5$) 123 ± 26% ($P < 0.05$), 43 ± 8% ($P < 0.05$), and 12 ± 13% ($P > 0.2$), respectively.

$TNF_\alpha$ (0.042 and 0.42 μg/ml) produced small, but significant ($P < 0.05$), inhibition of the contractions of the BPA to SNS (Fig. 4C). The response to SNS was 64 ± 6% of the control response at 32 Hz SNS, 48 ± 11% at 8 Hz SNS, and 0 ± 0% at 1 Hz ($n = 6$, $P < 0.05$). In BPA pretreated with LNMMA (500 $\mu M$), $TNF_\alpha$ did not inhibit SNS, but significantly enhanced the contractions of the BPA to SNS (Fig. 5). Similarly, in endothelium-rubbed BPA, $TNF_\alpha$ inhibited the contraction to 8 Hz SNS by 45 ± 7% (Fig. 6). In the endothelium-rubbed BPA treated with LNMMA, $TNF_\alpha$ (0.42 μg/ml) enhanced the contractions to SNS (Fig. 6).

**RNI Production by BPA.** The basal output of RNI from normal BPA was 3.2 ± 0.8 pg/mg/min and undetectable in endothelium-rubbed BPA. Acetylcholine increased the output of RNI from the endothelium of normal BPA but not from endothelium-rubbed BPA (Fig. 7). We demonstrated previously that both basal and agonist-stimulated RNI was inhibitable by L-$N^G$-

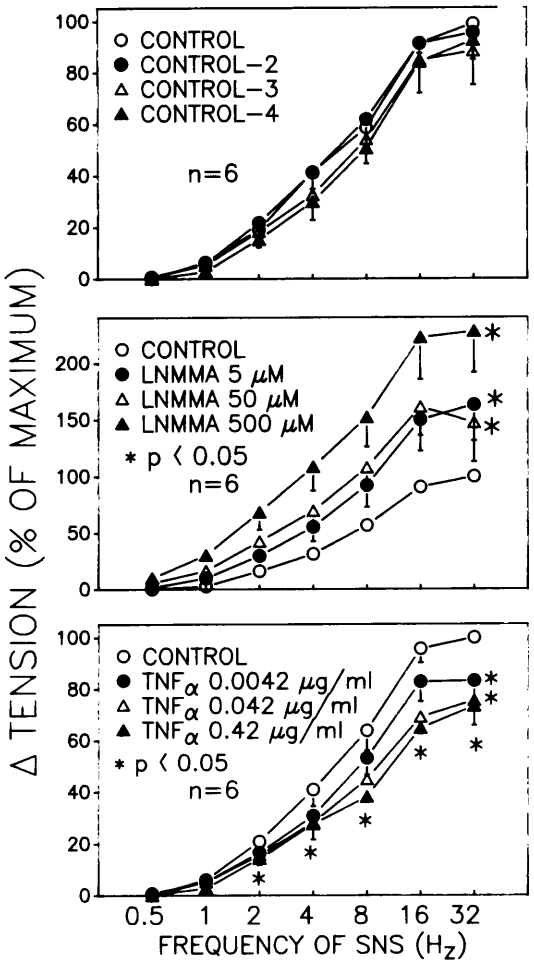


**Figure 4.** (A) Effect of vehicle, (B) LNMMA, and (C) $TNF_\alpha$ on the cumulative contractions of the BPA to SNS. The ordinate is the change in tension produced by SNS, expressed as a percentage of the maximum control contraction at 32 Hz. The abscissa is the frequency of SNS. Vertical lines are the SE from six blood vessels, each obtained from an individual cow. The asterisk at the end of the frequency response curve indicates that the responses to SNS differed from control ($P < 0.05$) at all Hz of SNS. In (A), Controls 2, 3, and 4 are the second, third, and fourth series of frequency response curves for SNS in the presence of PSS (vehicle).

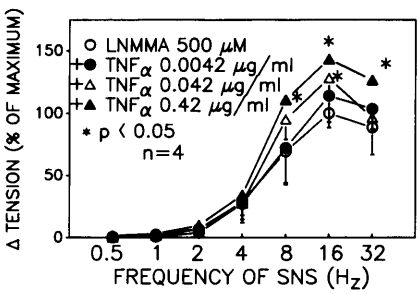


**Figure 5.** Effect of $TNF_\alpha$ on the contractions of the BPA pretreated with LNMMA (500 $\mu M$) to SNS. The ordinate is the change in tension produced by SNS, expressed as a percentage of the control contraction at 32 Hz. The abscissa is the frequency of SNS (2-msec duration and delay, 9–10 V). Vertical lines are the SE from four blood vessels, each obtained from an individual cow. *Differs from control ($P < 0.05$).

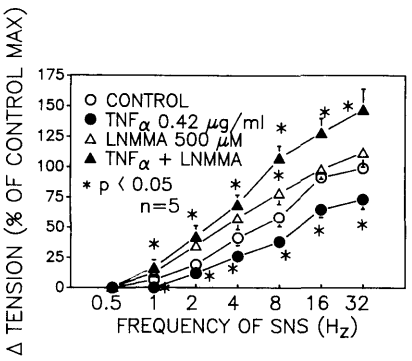


**Figure 6.** Effect of vehicle, LNMMA (500 $\mu M$), $TNF_\alpha$ (0.42 $\mu M$), and a combination of LNMMA together with $TNF_\alpha$ on the contraction of the endothelium-rubbed BPA to SNS. The ordinate is the change in tension produced by SNS expressed as a percentage of the maximum control contraction to 32 Hz SNS. The abscissa is the frequency of SNS. Vertical lines are the SE from five blood vessels, each obtained from an individual cow. *Differs from control ($P < 0.05$).

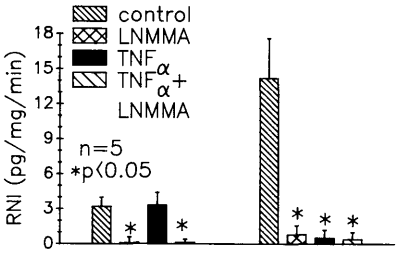


**Figure 7.** Effect of vehicle (PSS; control), $TNF_\alpha$ (0.42 $\mu$g/ml), and LNMMA (100 $\mu M$) on basal and ACh-induced stimulation of RNI-derived NO by normal BPA. The ordinate is the amount of RNI-derived NO (pg/mg blood vessel/min). Vertical lines are the SE from five experiments. An asterisk indicates that the responses in the presence of test compound differ from vehicle ($P < 0.05$). In endothelium-rubbed BPA, we could not detect any basal RNI-derived NO. The ACh-stimulated values in control, LNMMA-, TNF-, and TNF + LNMMA-treated BPA were 0.1 ± 0.5, −0.6 ± 0.8, −0.3 ± 0.6, and 0.5 ± 0.8 pg/mg/min, respectively ($P > 0.3$) ($n = 4$).

monomethylarginine (50 $\mu M$) and L-$N^G$-nitroarginine (10 $\mu M$) (23). $TNF_\alpha$ did not affect basal release of RNI but inhibited ACh-induced release of RNI from BPA (Fig. 7). In the endothelium-rubbed BPA, ACh and $TNF_\alpha$ did not stimulate production of RNI (Fig. 7). Thus, it is unlikely that any significant muscle-derived NO was released by $TNF_\alpha$.

**Nitric Oxide in Single BACC.** Figure 8 is a differential pulse voltamogram of BACC incubated in the absence and presence of increasing concentrations of bradykinin (1 $\mu M$). A downward displacement of the curve indicates an increase of the current at the holding voltage and therefore a higher concentration of NO. Within 30 sec after addition of BK, NO increased from undetectable limits to 45 n$M$/sec/2 × $10^5$ cells and stabilized at 75 n$M$/sec at 25 min after addition of BK. Similar results were found with $TNF_\alpha$ (Fig. 8) and the calcium ionophore A23187, which increased NO production significantly more than BK (data not shown). Figure 9 summarizes the effect of bradykinin, A23187,

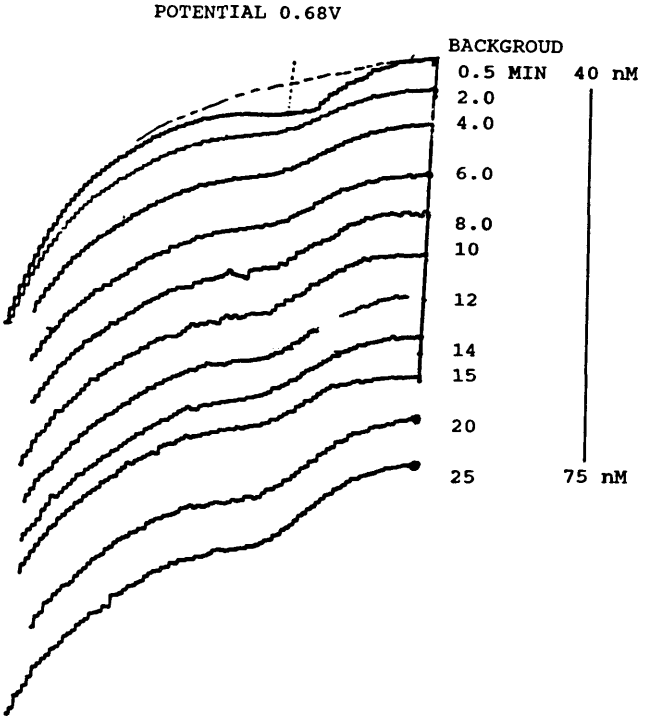


**Figure 8.** A differential pulsed voltamogram of free nitric oxide production by bradykinin (1.0 $\mu M$) in isolated single bovine adrenal chromaffin cells in primary culture containing 1000 cells. The peak current at 0.68 V holding potential represents the concentration of free NO shown on the right side. A downward shift indicates an increase in free NO. Each line represents a scan at different times after addition of bradykinin to the culture medium.

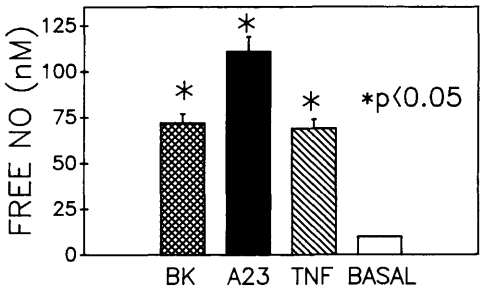


**Figure 9.** Effect of bradykinin (1 $\mu M$), A23187 (1 $\mu M$), and $TNF_\alpha$ (0.42 $\mu$g/ml) on the maximal production of free NO by 3-day-old bovine adrenal chromaffin cells in primary culture in Dulbecco's modified Eagle's medium-Ham's F12 medium. The ordinate is the concentration of free NO (n$M$). Vertical lines are the mean and SE from three replicates. The time required for maximal output of NO was 55 min for bradykinin and A23187 and 100 min for $TNF_\alpha$. *Differs from control ($P < 0.05$).

and $TNF_\alpha$ on release of free NO from BACC. BK, A23187, and $TNF_\alpha$ increased the release of free NO, with A23187 producing greater concentrations of free NO than either $TNF_\alpha$ or BK, which did not differ.

## Discussion

The effect of $TNF_\alpha$ on neurogenically mediated contraction of pulmonary vascular smooth muscle has not been examined previously. This study demon-

strated that the contractions of the BPA to SNS and exogenous NE are mediated by stimulation of prazosin-sensitive $\alpha_1$-adrenoceptors. Moreover, concentrations of $TNF_\alpha$ associated with bacterial sepsis and septic shock (1–3) selectively inhibit the contractions of the BPA to SNS in the absence of LNMMA and enhance the neurogenic contractions of the BPA in the presence of LNMMA. These effects of $TNF_\alpha$ on neurogenically mediated contraction of the BPA are independent of the presence of a functional endothelium. Moreover, $TNF_\alpha$ in isolated bovine adrenal chromaffin cells increases the release of free NO from isolated BACC, a model of a sympathetic neuron, with a time course similar to that required for inhibition of the contractions to SNS. Moreover, when the magnitude of inhibition of SNS produced by $TNF_\alpha$ (Figs. 3 and 6), bradykinin (19, 24), and A23187 (24) is compared with the magnitude of release of NO from the BACC, it is clear that a correlation exists between the magnitude of stimulation of NO from BACC and the inhibition of SNS within pulmonary vascular smooth muscle. Correlation does not prove causality. However, after inhibition of NO synthase with LNMMA, $TNF_\alpha$ selectively enhanced the contractions of the BPA to SNS; this results is similar to that observed in canine pulmonary arteries with BK (19). Thus, the data suggest that $TNF_\alpha$ inhibits neurogenically mediated contraction by an indirect effect involving neural NO and enhances SNS by an effect independent of NO.

The mechanism by which $TNF_\alpha$ affects SNS and NO is beyond the scope of this investigation. However, several possibilities can be ruled out from our studies. It is unlikely that the effects of $TNF_\alpha$ resulted from prostaglandins acting on the sympathetic nerve terminals or junctional $\alpha_1$-adrenoceptors of the BPA, since the prostanoid system was inhibited by concentrations of ibuprofen that inhibit the prostanoid system (19, 24). Since cocaine was present in the PSS and the contractions of the BPA to exogenous NE were unaffected by $TNF_\alpha$, it is also unlikely that $TNF_\alpha$ stimulated neuronal reuptake of NE or inhibited the junctional $\alpha_1$-adrenoceptor, similar to its documented ability to downregulate myocardial $\beta_1$-adrenoceptors in rabbits (25). Downregulation of $\alpha_1$-adrenoceptors would be associated with inhibition of contraction of the BPA. However, inhibition of NO production by LNMMA unmasked $TNF_\alpha$-mediated enhancement of the contraction to SNS. It is also unlikely that $TNF_\alpha$ induces NOS II in the sympathetic neuron, endothelium, or smooth muscle of the BPA or stimulates constitutive NOS III in the vascular endothelium. Induction of the macrophage-isozyme NOS II requires 6–8 hr to occur (26), rather than the 15–100 min observed herein. Also, $TNF_\alpha$ inhibits, rather than activates, NOS III in the vascular endothelium. This inhibition is associated with decreased production of NO by the vascular endothelium (11, 14). Thus, the data obtained support the conclusion that $TNF_\alpha$-mediated selective inhibition of the contractions of the bovine pulmonary artery to SNS is probably mediated at the level of the sympathetic nerve terminal innervating the smooth muscle.

$TNF_\alpha$-mediated inhibition of contraction to SNS is small relative to that produced by prazosin. However, it is selective for neurally induced vasoconstriction and is not observed with NE-induced contractions (18, 19, 27). Nitric oxide appears to be the mediator of $TNF_\alpha$-induced inhibition of SNS, since LNMMA inhibits nitric oxide synthesis, prevents $TNF_\alpha$-induced inhibition of the contractions of the BPA to SNS, and unmasks $TNF_\alpha$-induced facilitation of the contractions of the BPA to SNS. Moreover, similar effects are found in endothelium-rubbed PA. Finally, $TNF_\alpha$ increases NO release from BACC. Endothelium-derived and neural NO inhibits the efflux of NE from sympathetic nerve endings (15–19). Thus, the selective inhibitory effect of $TNF_\alpha$ on the contractions to SNS may reflect a selective stimulation of the neural constitutive enzyme, NOS I, and increased neural production of NO either in nitroxidergic nerves or within the sympathetic nerve terminal itself. Nitric oxide stimulates the production of cGMP, which delays neuronal recovery from inactivation (28). Soliven and Albert (28) concluded that $TNF_\alpha$ inhibited NE secretion from rat superior cervical ganglia by causing a prolonged neuronal recovery from inactivation. Further studies are now required to define the mechanism by which $TNF_\alpha$ modulates neurogenically mediated contraction of vascular smooth muscle.

LNMMA, an L-arginine derived inhibitor of NOS I, II, and III, reverses the hypotension of animals given $TNF_\alpha$ and endotoxin. Nevertheless, the animals exhibit more severe organ damage and increased mortality (2). It is possible that inhibition of NOS in sepsis may unmask $TNF_\alpha$-mediated enhancement of neurogenically mediated contraction. This could result in augmented small vessel vasoconstriction superimposed on the reduction in blood flow produced by the abolition of the vasodilator component of NO, further compromising organ blood flow and amplifying the ischemic insult to vital organs. Further studies are required to test this postulate.

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