Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 3629–3632, May 1990
Medical Sciences

N^G-Methyl-L-arginine inhibits tumor necrosis factor-induced hypotension: Implications for the involvement of nitric oxide

(endothelium-derived relaxing factor/septic shock/arginine)

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Communicated by Alton Meister, February 22, 1990 (received for review December 30, 1989)

ABSTRACT **Clinical assessment of the activity of tumor necrosis factor (TNF) against human cancer has been limited by a dose-dependent cardiovascular toxicity, most frequently hypotension. TNF is also thought to mediate the vascular collapse resulting from bacterial endotoxin. The present studies address the mechanism by which TNF causes hypotension and provide evidence for elevated production of nitric oxide, a potent vasodilator initially characterized as endothelium-derived relaxing factor. Nitric oxide is synthesized by several cell types, including endothelial cells and macrophages, from the guanidino nitrogen of L-arginine; the enzymatic pathway is competitively inhibited by N^G-methyl-L-arginine. We found that hypotension induced in pentobarbital-anesthetized dogs by TNF (10 μg/kg, i.v., resulting in a fall in mean systemic arterial pressure from 124.7 ± 7 to 62.0 ± 22.9 mmHg; 1 mmHg = 133 Pa) was completely reversed within 2 min following administration of N^G-methyl-L-arginine (4.4 mg/kg, i.v.). In contrast, N^G-methyl-L-arginine failed to reverse the hypotensive response to an equivalent depressor dose of nitroglycerin, a compound that acts by forming nitric oxide by a nonenzymatic, arginine-independent mechanism. The effect of N^G-methyl-L-arginine on TNF-induced hypotension was antagonized, and the hypotension restored, by administration of excess L-arginine (100 mg/kg, i.v.). Our findings suggest that excessive nitric oxide production mediates the hypotensive effect of TNF.**

Tumor necrosis factor (TNF) is a cytotoxic protein produced by macrophages upon activation by bacterial endotoxin (1, 2). In addition to a spectrum of cytotoxic and immunologic actions, TNF causes marked hypotension in mammals (1, 3). The observations that bacterial endotoxin elicits TNF production (4, 5) and that pretreatment of animals with anti-TNF antibodies abolishes the hypotensive action of endotoxin (6) suggest that TNF is the key mediator of endotoxic shock *in vivo*. Although TNF is known to promote hemorrhagic necrosis of some animal tumors (7), its clinical promise as an antineoplastic agent is limited by severe dose-dependent side effects, predominantly hypotension (8, 9). Despite the clinical importance of TNF-induced hypotension, its mechanism is unknown.

The present study addresses the possibility that increased nitric oxide production accounts for TNF-induced hypotension. Earlier studies established that endothelium-derived nitric oxide is a labile modulator of vascular tone (10, 11). Originally termed endothelium-derived relaxing factor (EDRF, ref. 12), nitric oxide is responsible for the vascular smooth muscle relaxation elicited by acetylcholine, bradykinin, and many other endogenous vasorelaxants. L-Arginine is the biosynthetic precursor of endothelium-derived nitric oxide (13–16), and N^G-methyl-L-arginine (L-MeArg) is a competitive inhibitor of this pathway (14, 15). The finding that administration of L-MeArg causes a moderate increase in blood pressure by an arginine-reversible mechanism in the anesthetized guinea pig (17), rabbit (18), and rat (R.L. and S.S.G., unpublished data) indicates that nitric oxide is normally produced at a significant basal rate and plays a role in blood pressure homeostasis.

Once formed, nitric oxide is a short-lived species that rapidly oxidizes to nitrite and nitrate. Although the implications were not understood at the time, Wagner *et al.* (19) reported a 9-fold increase in urinary nitrate excretion following endotoxin treatment in the rat. Additionally, cultured macrophages (20, 21) and endothelial cells (22) in the presence of γ-interferon secrete large quantities of arginine-derived nitrogen oxides after activation by TNF or endotoxin. These findings raised the possibility that increased synthesis of the potent vasodilator nitric oxide may underlie endotoxin- and TNF-induced hypotension.

We report here that the hypotension elicited by TNF administration in the dog is reversed, and blood pressure returns to baseline, after selective inhibition of nitric oxide synthesis with L-MeArg. Once reversed, the hypotension is restored by L-arginine administration. In contrast, L-MeArg has only a modest effect and L-arginine is without effect on blood pressure in dogs not given TNF. These results imply that L-arginine-derived nitric oxide is a principal mediator of TNF-induced shock in the dog.

MATERIALS AND METHODS

Reagents. Recombinant human TNF (specific activity 10^7 units/mg) was a gift from Nippon Chemical (Tokyo). Nitroglycerin was purchased from DuPont. L-MeArg was synthesized according to the method of Corbin and Reporter (23). The flavianate salt of L-MeArg was converted to the hydrochloride salt by stirring with Dowex 1 (OH^-) and titrating the resulting free base to pH 7.2 with HCl. The concentration of L-MeArg was determined by amino acid analysis using a standard solution prepared from the crystalline flavianate salt. L-Arginine was obtained from Sigma.

Animals. Experiments were carried out on conditioned mongrel dogs weighing 28–30 kg. Care of the animals was in accordance with the recommendations of the American Association for Accreditation of Laboratory Animal Care and met all standards prescribed by the *Guide for the Care and Use of Laboratory Animals* (24). All protocols were approved by the University of Texas Animal Welfare Committee. The

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Abbreviations: TNF, tumor necrosis factor; L-MeArg, N^G-methyl-L-arginine.

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dogs were fasted overnight before the day of the experiment. They were anesthetized with pentobarbital (25 mg/kg, i.v.), orotracheally intubated, and ventilated with a Harvard pump at a nominal rate of 12 breaths per minute and a tidal volume of 15 ml/kg. An arterial line was percutaneously placed in the femoral artery, and before data collection was initiated, the ventilator rate and tidal volume were adjusted to obtain a normal arterial pH and Pco_2 by using an IL1302 pH/blood gas analyzer (Instrumentation Laboratory, Lexington, MA).

Physiological Measurements. Mean systemic arterial pressure was continuously recorded on a Hewlett–Packard recording system (model 7758B), using a strain-gauge manometer (Hewlett–Packard model 1290A) connected to an arterial line, as described by Lodato (25). Heart rate was determined by electrocardiography and a calibrated cardiotachometer. Oxyhemoglobin saturation was continuously monitored with a pulse oxymeter (BIOX 111, Boulder, CO). Continuous time-series records of systemic arterial pressure, heart rate, and oxyhemoglobin saturation were obtained using a Lab Master analog-to-digital converter (16-channel, 12-bit, 30-kHz; Scientific Solutions, Salon, OH), sampling at 55 Hz and storing 6-sec averages on a magnetic disk with an AST Premium 286 personal computer.

Protocol. After the blood pressure and heart rate were stabilized, TNF was administered at a dose of 10 μg/kg, i.v., in 10 ml of phosphate-buffered saline (PBS, pH 7.4), containing dog albumin at 2 mg/ml. Blood pressure was monitored until a nadir was reached; after an additional 10 min, L-MeArg was administered (4.4 mg/kg, i.v., in 10 ml of PBS). Ten minutes later L-arginine was administered as a bolus injection (100 mg/kg, i.v., in 20 ml of PBS). A total of four experiments were carried out on three dogs (one male and two females).

Statistics. Statistical analysis of the data was by Student's *t* test using a one-tailed, paired analysis.

RESULTS

A representative tracing depicting TNF-induced hypotension, its inhibition by L-MeArg, and its restoration by L-arginine is shown in Fig. 1. Following TNF administration, mean systemic arterial blood pressure transiently increased (42 mmHg; 1 mmHg = 133 Pa) concomitant with an increase in heart rate. However, after 30 min, blood pressure declined precipitously before stabilizing at a level 41 mmHg lower than the pre-TNF baseline value of 121 mmHg. Following 10 min of stable hypotension, L-MeArg was administered; blood pressure rose immediately and attained the pre-TNF level within 2 min. Subsequent administration of L-arginine fully restored TNF-induced hypotension to the level prior to L-MeArg administration.

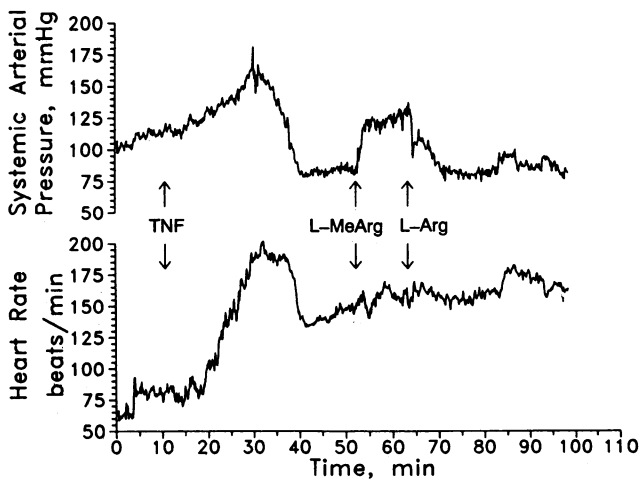

FIG. 1. Time course of changes in mean systemic arterial pressure and heart rate in a pentobarbital-anesthetized dog following i.v. administration (arrows) of TNF, (10 μg/kg), L-MeArg (4.4 mg/kg), and L-arginine (100 mg/kg). Data from this and additional experiments are summarized in Table 1.

Table 1. Hemodynamic changes after sequential administration of TNF, L-MeArg, and L-arginine

Period	Systemic arterial blood pressure, mmHg	Change from previous period, mmHg	*P* value*
Before TNF	126.8 ± 13.4	—	—
After TNF	66.3 ± 20.5	−60.5	0.012
After L-MeArg	120.5 ± 12.8	+54.2	0.010
After L-Arg	70.8 ± 25.3	−49.7	0.019

Values represent means ± SD, n = 4. Drugs were administered sequentially by i.v. bolus as described in *Materials and Methods*.
*Comparison with previous period.

Mean hemodynamic changes elicited by administration of TNF are shown in Table 1. TNF caused a decrease in blood pressure in all animals; the magnitude of the decrease in blood pressure varied considerably (mean, 60.7 mmHg; range, 45–89 mmHg), as did the time at which the nadir of the hypotension occurred (mean, 74 min; range, 30–136 min).

In one experiment, the hypotensive response to TNF was especially severe. At 36 min after TNF administration, blood pressure fell from a baseline of 125 mmHg to 36 mmHg. Administration of L-MeArg resulted in the return of blood pressure to 115 mmHg. This amounted to a remarkable 79-mmHg increase in blood pressure following L-MeArg administration. This increase in blood pressure was completely reversed by administering L-arginine, which caused the blood pressure to fall again to 37 mmHg.

Results of administering L-MeArg alone to an untreated dog are shown in Fig. 2. Within 2 min after L-MeArg administration, the blood pressure increased by only 12 mmHg; this was associated with a decrease in the heart rate from 101 to 92 beats per min. Mean values for the L-MeArg-induced increase in mean systemic arterial pressure and decrease in heart rate were 15.3 ± 2.6 mmHg and 21.6 ± 9.6 beats per min, respectively (n = 3). Subsequent administration of L-arginine reversed these changes. Administration of L-arginine alone had no effect on blood pressure (Fig. 3) or heart rate (data not shown).

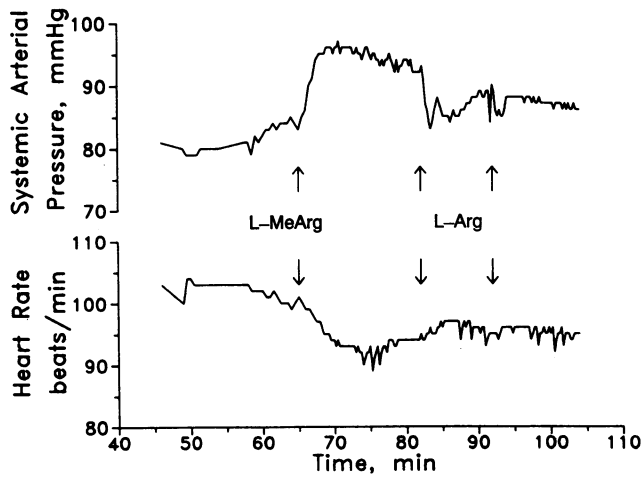

FIG. 2. Time course of changes in mean systemic arterial pressure and heart rate in a pentobarbital-anesthetized dog following i.v. administration (arrows) of L-MeArg (4.4 mg/kg) and L-arginine (100 mg/kg).

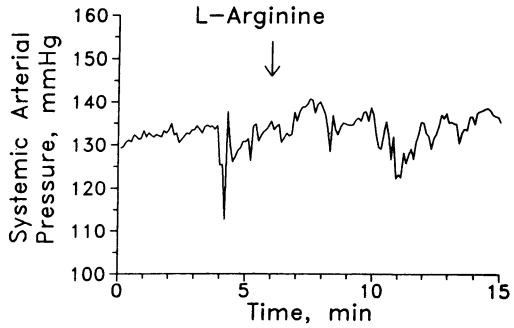

FIG. 3. Lack of effect of L-arginine (100 mg/kg, i.v.) on mean systemic arterial pressure in a pentobarbital-anesthetized dog.

In a control study nitroglycerin was infused at a rate (28 μg/kg per min, i.v.) selected to lower the blood pressure to the same level as with TNF (Fig. 4). After 10 min of nitroglycerin-induced hypotension, L-MeArg was administered and resulted in only a 14-mmHg rise in blood pressure. Subsequent administration of L-arginine reversed this modest effect of L-MeArg.

DISCUSSION

Injection of L-MeArg into anesthetized dogs (i.e., in the absence of TNF) produced an increase in mean arterial blood pressure, as previously observed in guinea pigs (17) and rabbits (18). Since L-MeArg specifically blocks nitric oxide synthesis (14, 15), these observations suggest a physiological role for nitric oxide in normal blood pressure homeostasis. Whereas the increase in blood pressure following L-MeArg administration was relatively small in untreated dogs (10–20 mmHg), it was greatly potentiated in animals made hypotensive with TNF (40–80 mmHg). That L-MeArg completely reversed the marked hypotension induced by TNF implicates an overproduction of nitric oxide in this form of shock. Administration of L-arginine overcomes the competitive inhibition effected by L-MeArg and provides an excess of required precursor for additional nitric oxide synthesis. Thus, the complete restoration of TNF-induced hypotension by L-arginine supports the view that the TNF-induced hypotension is mediated by excessive nitric oxide synthesis. The reversal of hypotension by L-MeArg appears to be selective for TNF-induced hypotension. Indeed, reduction in blood pressure by nitroglycerin, an agent that acts by releasing nitric oxide via an arginine-independent pathway, was not antagonized by L-MeArg (see Fig. 4). This further supports a specific role for arginine in the production of TNF-induced hypotension.

The response of the dog to TNF is similar to that observed during clinical trials in which TNF was administered to cancer patients for evaluation of its antitumor activity; an early hypertensive phase associated with tachycardia is followed by hypotension (8). As observed in the dog, the time of onset and the degree of subsequent hypotension that develops in patients is quite variable. Indeed, the cardiovascular responses to repeated TNF administrations may differ substantially in an individual patient.

The hypotension that results from TNF administration restricts the dose of TNF that can be administered to humans and may account for the limited antitumor activity observed in clinical trials (8). Our findings suggest that cotreatment of patients with an inhibitor of nitric oxide synthesis may allow the administration of higher, potentially therapeutic doses of TNF, thereby increasing TNF's therapeutic index.

Most forms of septic shock are believed to be caused by exposure to endotoxin, a component of the cell wall of Gram-negative organisms (26). Many of the effects of endotoxin are mimicked by TNF (27). Nathanson *et al.* (28) have demonstrated that administration of TNF to dogs results in hemodynamic and metabolic derangements similar to those observed with endotoxin-induced shock. Their observations are consistent with previous work showing that the administration of endotoxin to animals and humans elicits an increase in serum TNF (4, 5). Furthermore, pretreatment with anti-TNF antibodies has been shown to protect against the deleterious effects of endotoxin (6). Collectively, these findings implicate TNF as a necessary factor in the development of endotoxic shock. It is interesting that administration of anti-TNF antibodies after endotoxin exposure does not protect against hypotension, implying that TNF activates the synthesis of a secondary product that mediates the hypotensive effect. The results presented here suggest that nitric oxide is the hypotensive mediator elicited by TNF. Accordingly, pharmacological interventions that inhibit nitric oxide synthesis may be of therapeutic utility in the treatment of septic shock.

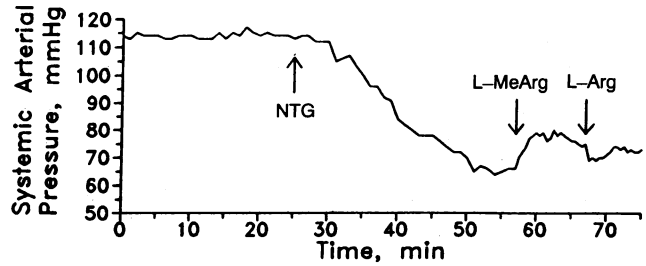

FIG. 4. Time course of changes in systemic arterial pressure in a pentobarbital-anesthetized dog after continuous i.v. infusion of nitroglycerin (NTG, 28 μg/kg per min) and i.v. bolus administration of L-MeArg (4.4 mg/kg) and L-arginine (100 mg/kg).

While our data indicate that nitric oxide production increases in response to TNF, the cell type responsible remains to be determined. The induction of nitrogen oxide synthesis by cytokines, including TNF, has been documented in cultured macrophages (20, 21, 29). Kilbourn and Belloni (22) have recently shown that the nitric oxide biosynthetic pathway is induced in mouse brain microvascular endothelial cells cultured in the presence of both TNF and γ-interferon; neither agent has a significant action alone. In view of the short half-life of nitric oxide *in vivo*, an endothelial cell origin of nitric oxide acting on vascular smooth muscle is an attractive possibility. It is curious that TNF induction of nitric oxide biosynthesis exhibits a 6- to 8-hr lag in cultured macrophages and endothelial cells, whereas the hypotensive effect of TNF in the dog occurs within as little as 30 min. It is plausible that in the intact animal TNF and endogenous cytokines act synergistically, resulting in an accelerated increase in nitric oxide production. Indeed, Rothstein and Schreiber (30) have reported that TNF and bacterial endotoxin, an inducer of cytokine production, are synergistic in their ability to cause hemorrhagic necrosis and lethal shock in mice.

In addition to elucidating the role of nitric oxide in the control of vascular tone and blood pressure under normal and pathological conditions, the present studies strongly suggest that inhibitors of nitric oxide synthesis may constitute an important class of pharmacologic agents for the treatment of cytokine-mediated shock.

Note Added in Proof. Tumor necrosis factor is believed to be a major mediator of endotoxic shock. In recent preliminary studies we have shown that L-MeArg administration reverses the severe hypotension seen in dogs given bacterial endotoxin. This result is consistent with *in vitro* studies reported by Kilbourn and Belloni (Conference on Nitric Oxide from L-Arginine, Royal Society, London, September 1989; ref. 31) showing that endothelial cells produce nitrogen oxides

in response to endotoxin and γ-interferon. Salvemini *et al.* (32) subsequently reported *in vitro* studies leading to a similar conclusion.

We thank Ernest B. Campbell for expert technical assistance. This work was supported in part by Grants HL34215 (S.S.G. and R.L.) and DK37116 (O.W.G.) from the National Institutes of Health and grants from the American Lung Association (R.F.L.), American Heart Association–Texas Affiliate (R.F.L.).

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