



## Indirect evidence for stimulation of nitric oxide release by tumour necrosis factor- $\alpha$ in human veins in vivo

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### Abstract

**Objectives:** The detrimental haemodynamic changes observed in septicemia are generalised vasodilation, arterial hypotension, and hyporesponsiveness to vasopressor compounds, all of which could be explained by the release of an endogenous vasodilator. Experimental and clinical evidence suggests that tumour necrosis factor- $\alpha$  (TNF) induces the expression of vascular nitric oxide (NO) synthase within hours and that NO released from smooth muscle cells could be involved in the pathogenesis of septic shock. The aim of this study was to investigate the role of NO in the vascular effects of TNF. **Methods:** Using the dorsal hand vein compliance technique, the effect of the NO synthase inhibitor L-N<sup>G</sup>-monomethyl-arginine (L-NMMA) on  $\alpha_1$ -adrenergic responsiveness (phenylephrine 1.25–8000 ng/min) was studied after prolonged local venous infusion of TNF (8.7  $\mu$ g in 5 h) in 9 volunteers and in 6 volunteers without previous cytokine exposure. **Results:** Mean ( $\pm$  s.e.) maximum phenylephrine constriction ( $E_{\max}$ ) was  $73 \pm 6\%$  and log dose-rates exerting 50% of  $E_{\max}$  (log ED<sub>50</sub>) were  $3.2 \pm 0.09$  (geometric mean: 1535 ng/min). Local co-administration of L-NMMA at a dose sufficiently high to block NO formation (3.4  $\mu$ mol/min) increased venous sensitivity to phenylephrine threefold (log ED<sub>50</sub>  $2.8 \pm 0.1$ ,  $P < 0.015$ ; geometric mean: 574 ng/min) whereas  $E_{\max}$  was similar ( $73 \pm 5\%$ ). In the controls the phenylephrine dose–response relationship remained unaffected by simultaneous administration of L-NMMA. **Conclusions:** As no basal release of NO occurs in hand veins without previous exposure to TNF these results provide direct evidence for induction of NO formation in the human vasculature and consecutive resistance to  $\alpha$ -adrenergic venoconstriction. NO might, therefore, be a key mediator of haemodynamic impairment in humans under conditions with known elevations of circulating TNF, such as a septic shock.

**Keywords:** Nitric oxide; Tumor necrosis factor; Endotoxins; L-NMMA; Human, veins

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### 1. Introduction

The haemodynamic characteristics of patients with septic shock are consistent with the release of large amounts of vasodilator compounds resulting in a reduction of peripheral vascular resistance and refractoriness to vasoconstrictors [1]. In vitro data and animal experiments suggest that endotoxin and endotoxin-induced release of interleukin-1 $\beta$  and tumour necrosis factor- $\alpha$  (TNF) are involved in the pathogenesis of generalised vasodilation [2–6]. A causal relationship is further supported by the similarity of haemodynamic changes observed after systemic infusion of TNF in humans [7,8]. There is good evidence that vascular smooth muscle cells release a vasodilator after exposure to TNF [9] and that this vasodilator is most probably nitric oxide (NO) enzymatically formed from L-arginine by inducible NO synthase [10].

NO acts through the stimulation of soluble guanylate cyclase and subsequent formation of cyclic GMP which ultimately leads to relaxation of smooth muscle cells [11]. In vivo administration of specific inhibitors of NO synthase [12–14] or methylene blue, an inhibitor of NO-induced activation of guanylate cyclase [15], to patients with refractory septic shock has consistently increased systemic vascular resistance and blood pressure, suggesting that NO might be the cause. However, as NO is also released from arteries under basal conditions, L-N<sup>G</sup>-monomethyl-arginine (L-NMMA), a competitive, reversible inhibitor of NO synthesis [16–18], also exerts pressor responses in healthy subjects [19]. To date it has not been confirmed that NO is released locally from the human vasculature after cytokine exposure in vivo. The results of these studies in superficial hand veins without basal NO release [20] confirm the vascular release of a vasodilator, which is most likely NO, in response to local TNF administration.

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## 2. Material and methods

### 2.1. Subjects and protocol

Fifteen healthy male non-smokers with a mean age of 25 years (range 20–34 years) and a mean weight of 73 kg (63 to 84 kg) entered the study after giving written informed consent. No subject had a previous history of severe medical illness or used any chronic medication. Volunteers had not taken any medication within one week prior to the study. The current status of health was verified by a complete physical examination and routine laboratory tests. The volunteers were allowed to eat a light breakfast but were asked to refrain from methylxanthine-containing beverages and alcohol for at least 12 h before the study. The protocol was reviewed and approved by the local Ethical Committee; the investigation conforms with the principles outlined in the Declaration of Helsinki.

### 2.2. Hand vein compliance technique

The dorsal hand vein compliance technique [21] was used with modifications as previously described [22]. Briefly, a continuous infusion (0.25 ml/min) of normal saline was delivered through a 23 G needle into a suitable dorsal hand vein; a high precision infusion pump (Harvard Apparatus Inc., South Natick, MA, USA) was used at a constant delivery rate (0.25 ml/min). The arm was placed on a vacuum pillow above heart level sloping upwards at an angle of 30 degrees from the horizontal to allow for complete emptying of the vein. A tripod holding a linear variable differential transformer (Schaevitz Engineering, Pennsauken, NJ, USA) was mounted on the dorsum of the hand approximately 10 mm down-stream from the tip of the needle. The freely-movable core of the transformer was placed over the centre of the vein under investigation. The vertical movement of the core is directly proportional to the signal output of the transformer, which was recorded on a strip-chart recorder. The difference between the positions of the core before and during inflation of a sphygmomanometer cuff on the same arm to 40 mmHg gives a measure of the diameter changes under a given congestion pressure. Each phenylephrine dose was administered for at least 6 min to allow sufficient time for equilibration. The maximum constriction by phenylephrine was expressed as percentage change from baseline compliance which was set to 100%. Blood pressure, heart rate and axillary temperature were monitored on the opposite arm. Throughout the study the volunteers remained semirecumbent in a quiet room with a constant temperature of  $23 \pm 1^\circ\text{C}$ .

### 2.3. Study design

To study the potential release of vasodilator compounds induced by TNF, two dose–response curves to the  $\alpha_1$ -adrenergic vasoconstrictor phenylephrine were constructed in 9 volunteers in the absence and presence of L-NMMA, a competitive inhibitor of NO formation by NO synthase [23,24]. TNF (28.9 ng/min) in 0.5% human serum albumin in normal saline was administered locally over 5 h into the vein under investigation (total dose: 8.7  $\mu\text{g}/$

experiment). The duration of TNF infusion was chosen according to in vitro studies in which it was shown that induction of NO synthase by TNF is dose-dependent and usually does not occur until several hours after exposure [25,26]. In two subjects, the infusion rate was higher (250 ng/min) and the duration of TNF administration shorter (34 or 22 min) resulting in a total dose of 8.5 or 5.5  $\mu\text{g}$ , respectively. After completion of the TNF infusion, the vein was kept patent by a continuous infusion of normal saline. Approximately 6 h after the beginning of TNF administration and after establishing a stable baseline, cumulative dose-rates of the full  $\alpha_1$ -selective agonist phenylephrine (1.25–8000 ng/min) were administered locally into the vein under investigation to construct a first dose–response curve. After a sufficiently long washout period (120 min) to negate the effect of phenylephrine, L-NMMA (3.4  $\mu\text{mol}/\text{min}$ ) was infused for 20 min to allow the inhibitor to act. Then, while continuing administration of L-NMMA, a second dose–response curve to phenylephrine was constructed. The dose of L-NMMA in these experiments is in a range of its maximum inhibitory effect as established recently in hand vein experiments using bradykinin as a NO donor [27].

In a separate control group of 6 volunteers, the effect of the same L-NMMA dose on the phenylephrine dose–response curve was assessed without previous administration of TNF.

### 2.4. Drugs

L-NMMA was purchased from Clinalfa AG (Läufelfingen, Switzerland) and phenylephrine (Neo-Synephrine®) from Winthrop (Brussels, Belgium). TNF was a generous gift from Knoll AG (Ludwigshafen, Germany). To prevent TNF from sticking to tubing, 0.5% human serum albumin in normal saline was used as solvent.

### 2.5. Data analysis

Data are expressed as means  $\pm$  s.e. Individual dose–response curves were fitted to a four-parameter logistic equation [28] by means of a computerised non-linear least-squares regression program (Allfit®, version 2.7), which provides estimates for the maximum effect ( $E_{\text{max}}$ ) and the dose-rate producing a half-maximal response ( $ED_{50}$ ). Paired Student's *t*-test was used to evaluate the difference between pairs of individual  $E_{\text{max}}$  and  $\log ED_{50}$  values. The significance of the effect of L-NMMA on  $ED_{50}$  values of mean phenylephrine dose–response curves was evaluated by F-test as previously described [22]. A two-tailed *P* value of less than 0.05 was considered to indicate statistical significance.

## 3. Results

In all 9 volunteers TNF was well tolerated. During the last hour of TNF administration a mild increase of temperature and heart rate, without effect on blood pressure values, was observed (Table 1). Occasionally, this was preceded by subjective symptoms of shivering and

Table 1  
Effects of TNF on body temperature and haemodynamics

	Before TNF	During TNF	Before phenylephrine
Temperature (°C)	36.4 (0.2)	37.7 (0.1) **	37.2 (0.2)
Systolic BP (mmHg)	112 (3)	112 (4)	114 (4)
Diastolic BP (mmHg)	69 (2)	70 (2)	69 (1)
Heart rate (beats/min)	62 (2)	74 (3) *	67 (3)

\*  $P < 0.05$  vs baseline values before TNF.

\*\*  $P < 0.01$  vs baseline values before TNF.

BP, blood pressure; all values are means ( $\pm$ s.e.).

headache. All symptoms were transient and resolved within one hour after the end of infusion, i.e. before the effects of phenylephrine were assessed.

Pilot studies by our group in hand veins precontracted with phenylephrine have shown that TNF, administered at dose rates up to 10 ng/min over 6 min, did not exert any immediate direct venodilator effects (data not shown). In 8 of 9 volunteers phenylephrine constricted hand veins after pretreatment with TNF. Mean  $E_{max}$  in these 8 volunteers was  $73 \pm 6\%$  and mean  $\log ED_{50}$  was  $3.2 \pm 0.09$  (geometric mean: 1535 ng/min). In one volunteer, doses of phenylephrine as high as 8000 ng/min did not constrict the hand vein at all. Blood pressure, heart rate and baseline venous compliance were unaffected by L-NMMA administration after 20 min ( $P > 0.2$ ). Co-administration of phenylephrine with L-NMMA resulted in potent venoconstriction in all 9 volunteers including the subject who did not have a constrictor response previously. During L-NMMA,  $E_{max}$  values of these second phenylephrine dose-response curves were similar ( $73 \pm 5\%$ ; n.s.) but mean  $\log ED_{50}$  values were significantly lower,  $2.8 \pm 0.1$  (geometric mean: 574 ng/min;  $P = 0.015$ ), suggesting that NO had shifted the dose-response curve towards higher dose-rates (Fig. 1). When only dose-response curves of the 6 volunteers were compared who had received TNF over 5 h, mean  $E_{max}$  and  $\log ED_{50}$  values were similar and the shift of the phenylephrine dose-response relationship was almost identical (geometric mean before and during L-NMMA: 1832 and 697 ng/min, respectively,  $P = 0.033$ ).

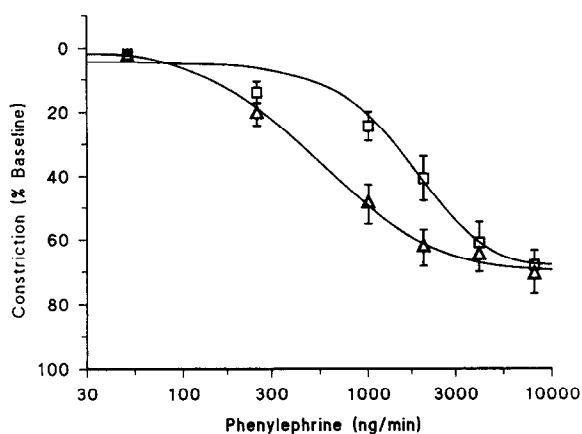


Fig. 1. Semilogarithmic plot of phenylephrine dose-response curves constructed 6–8 h after local exposure to TNF (8.7  $\mu$ g) before ( $\square$ ) and during ( $\Delta$ ) administration of L-NMMA in 8 healthy volunteers.

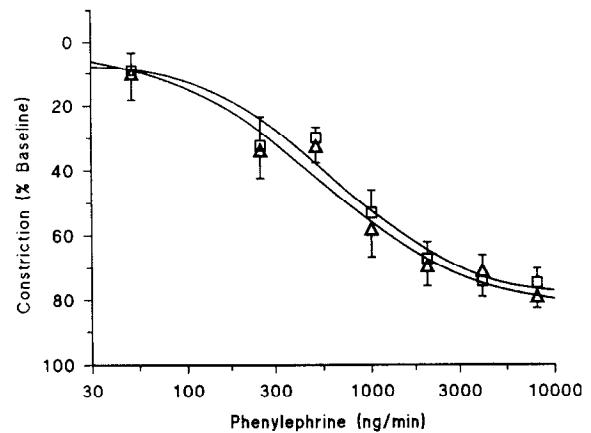


Fig. 2. Semilogarithmic plot of phenylephrine dose-response curves before ( $\square$ ) and during ( $\Delta$ ) administration of L-NMMA in 6 healthy volunteers constructed according to the same protocol as in Fig. 1 but without previous administration of TNF.

The 6 volunteers in the L-NMMA control group did not differ with respect to age and weight from the group in which TNF was administered. The administration of L-NMMA before the second phenylephrine dose-response curve was again without any effect on baseline venous diameter ( $P = 0.19$ ) and the second dose-response curve was not different from the curve constructed immediately before L-NMMA administration (Fig. 2). Mean  $E_{max}$  values before and during L-NMMA were  $76 \pm 4\%$  vs.  $77 \pm 5\%$  (n.s.), respectively, and mean  $\log ED_{50}$  values were  $2.8 \pm 0.1$  (geometric mean: 638 ng/min) vs.  $2.7 \pm 0.2$  (geometric mean: 533 ng/min), ( $P = 0.72$ ), respectively.

#### 4. Discussion

The cytokine TNF is an endogenous polypeptide produced by macrophages and monocytes in response to exposure to endotoxin [1]. Administered systemically, TNF induces many of the classic cardiovascular changes observed in septic shock [7] and there is good experimental evidence for the critical involvement of the L-arginine-NO pathway in hypotension and vascular hyporeactivity under these conditions [2,10,29]. TNF and other cytokines have been shown to promote the expression of the inducible isoform of NO synthase in the vascular wall and to stimulate synthesis and release of large amounts of NO [11]. In line with the theory of causal participation of NO in septic shock, systemic administration of inhibitors of NO formation (e.g. L-NMMA) has consistently increased blood pressure and peripheral vascular resistance in these patients [12–14]. However, such findings only suggest but do not prove, the induction of NO synthase in humans, because the administration of L-NMMA or its analogues can also increase vascular tone and blood pressure in the absence of septicemia [19,23].

For two reasons, therefore, the dorsal hand vein compliance technique was deemed particularly useful to indirectly test the hypothesis that local administration of small amounts of TNF might result in the expression of the inducible isoform of NO synthase, and consecutively lead to the release of NO. First, this method allows the con-

struction of full dose–response curves *in vivo* without activation of cardiovascular reflexes. Second, the peripheral veins are devoid of basal NO release [20]. Hence, the administration of inhibitors of NO synthase, or of formation of its second messenger cGMP, does not modify basal venous compliance or affect the dose–response curve of  $\alpha$ -adrenoceptor agonists in native hand veins [20,22]. These previous findings are confirmed in this study by the lack of interaction of L-NMMA with the phenylephrine dose–response relationship under control conditions. As under optimal experimental conditions hand veins are devoid of significant tone, the local release of a venodilator will result in only minor changes of its congested diameter unless the vein is precontracted with phenylephrine. This may explain why L-NMMA did not change baseline venous diameter whereas it substantially shifted the dose–response relationship of phenylephrine.

The results of the present study suggest that exposure to locally elevated TNF levels may induce vascular hyposensitivity to  $\alpha$ -adrenergic constrictors while TNF was devoid of an acute vasodilator effect. Assuming local flow rates of 2.5 ml/min [30], local TNF concentrations were in a range similar to values reported in serum of patients with severe meningococcal septic shock [31]. In the present study, vascular responsiveness was markedly increased by co-administration of L-NMMA and, in accordance with previous studies in animals and humans, L-NMMA restored vascular responsiveness to adrenergic vasoconstrictors [6,12–14,32]. Unlike other studies, however, these *in vivo* experiments assessed the effect of L-NMMA in a setting in which the inhibitor did not exert constrictor effects under basal conditions. Hence, it can be concluded that the potentiation of  $\alpha$ -adrenergic venoconstriction by L-NMMA reflects TNF-induced release of a local vasodilator which is most likely NO formed by the cytokine-inducible isoform of NO synthase in smooth muscle cells. In addition to induction of NO release in vascular smooth muscle cells, TNF has also been reported to modulate NO release from the endothelium [33] and leukocytes [34], and to promote leukocyte adhesion to endothelial cells [35]. In addition to the well documented and quantitatively relevant NO release from smooth muscle cells, it appears therefore possible that also other cells within or adhering to the venous wall may have contributed to vasodilation induced by TNF in these experiments.

In addition to its stimulatory effect on NO production, TNF was also shown to induce prostacyclin synthesis in cultured vascular endothelial cells [36] and to increase plasma 6-keto-PGF<sub>1 $\alpha$</sub>  in dogs [5]. As it was impossible to construct a third dose–response curve to phenylephrine while additionally blocking prostanoid effects in these long-lasting experiments, the eventual role of prostanoids remains open. However, the phenylephrine ED<sub>50</sub> value obtained during L-NMMA administration after TNF infusion was quite similar to values previously reported in native hand veins [22] and to those measured in the absence of TNF in the control L-NMMA group. This suggests that a second vasodilator — if present at all — did not substantially modulate vascular sensitivity during the evaluated time period under these experimental conditions.

Temporary changes in haemodynamic parameters and in axillary temperature have been observed during administration of TNF in these studies. Mean heart rate and body temperature increased by 12 beats/min and 1.3°C, respectively, until discontinuation of TNF. These changes might reflect activation of the sympathetic nervous system as has been reported for other cytokines such as interleukin-1 $\beta$  [37]. It is, however, unlikely that these changes have modified the response to phenylephrine and/or L-NMMA as both haemodynamic parameters and body temperature had returned to baseline values when dose–response curves to phenylephrine were constructed.

The present study indicates that, within hours after TNF exposure, basal NO release also occurs in human capacitance vessels. Venous responsiveness was substantially impaired and it appears probable, therefore, that part of the haemodynamic changes in septic shock occur at the venous side of the circulation. As the subcutaneous venous vasculature contains a large fraction of the total blood volume, vasodilation in these vessels may further promote haemodynamic shock by venous pooling. Although this study provides support for the notion that NO is a key element in the pathogenesis of cytokine-associated vasodilation in humans, blockade of NO synthesis with L-NMMA may not be the optimal therapy. It has been shown previously that complete reversal of NO effects by high systemic doses of L-NMMA might be detrimental for recovery from a shock-like state as both constitutive and inducible isoforms of NO synthases are inhibited [29]. The development of NO synthase inhibitors [38] selective only for the inducible isozyme in the vascular wall would enable the treatment of septic shock states thereby eliminating the hyposensitivity to vasoconstrictors while preserving endothelial function.

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