Systemic side effects of isolated limb perfusion with tumor necrosis factor alpha
Zwaveling, Jan Harm

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1997

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Role of nitric oxide in recombinant tumor necrosis factor alpha-induced circulatory shock: A study in patients treated for cancer with isolated limb perfusion

In press as:
Role of nitric oxide in recombinant tumor necrosis factor-α-induced circulatory shock: A study in patients treated for cancer with isolated limb perfusion
Crit Care Med
Jan H. Zwaveling MD†, Jan K. Maring MD†, Han Moshage PhD§, Robert J. van Ginkel MD‡, Harald J. Hoekstra MD‡, Heimen Schraffordt Koops MD‡, Irene F. Donse MD†, Armand R. J. Girbes MD†.

†Division of Intensive Care, Department of Surgery, University Hospital Groningen
‡Division of Surgical Oncology, Department of Surgery, University Hospital Groningen
§Department of Internal Medicine, University Hospital Groningen
Summary

This chapter aims to analyze the mechanism of vasodilation and circulatory shock in patients who were treated with isolated limb perfusion with melphalan and recombinant tumor necrosis factor α (r-TNF-α) for locally advanced malignant tumors. The role of nitric oxide, if any, was determined by measuring plasma nitrite and nitrate levels.

Eight consecutive patients developed sepsis syndrome due to leakage of r-TNF-α from the perfusion circuit to the systemic circulation. Despite the presence of very high systemic TNF-α levels during and immediately after perfusion and definite signs of hyperdynamic circulatory shock (increased heart rate, increased cardiac index, decreased systemic vascular resistance) nitrite and nitrate levels, measured in plasma at several time points, were not elevated.

The hypothesis that, in humans, TNF-α induces vasodilation and shock through activation of inducible nitric-oxide synthase and subsequent formation of excessive quantities of nitric oxide is not substantiated by our results. Normal nitric oxide metabolite levels were found in the presence of high TNF-α levels and shock. Other mechanisms that do not involve the nitric oxide pathway are likely to play a role in the generation of hypotension and septic shock in this setting.
Nitric oxide in isolated limb perfusion with TNF-α

Introduction

Isolated limb perfusion with melphalan and r-TNF-α, used in the treatment of locally advanced soft tissue sarcomas or melanomas of a limb, has been shown to result in a severe, but short-lived sepsis syndrome, characterized by fever, low blood pressure and the need for fluid resuscitation and inotropic support [1,2]. The occurrence of this syndrome is explained by leakage of r-TNF-α from the perfusion circuit into the systemic circulation; high levels of TNF-α are found in arterial blood during and directly after the procedure. Since TNF-α can induce nitric oxide synthase leading to a sustained release of nitric oxide and catecholamine-refractory vasodilation in animal models [3], we speculated nitric oxide to be an important mediator of the circulatory shock observed in this setting. To test this hypothesis we have measured metabolites of nitric oxide in eight patients during and after perfusion.

Subjects and Methods

Subjects

Eight patients received hyperthermic isolated limb perfusion at the division of surgical oncology of the Groningen University Hospital after approval of the medical ethical committee and informed consent had been obtained. Tumor histology is summarized in the table.

Normal values for nitrate and nitrite were obtained from studies in 26 healthy volunteers.

Anesthesia and intensive care

Anesthesia was induced with thiopental, after which the patients were paralyzed with vecuronium and the trachea intubated. Anesthesia was maintained with midazolam, sufentanyl, nitrous oxide and isoflurane. After induction a radial artery catheter and a pulmonary artery catheter were inserted. Blood pressure, ECG, urine output, venous and pulmonary pressures, as well as pulmonary artery wedge pressures and arterial blood gas values were checked at standard intervals. All patients were admitted to the intensive care unit following surgery. Patients received mechanical ventilation until hemodynamically stable. Fluid resuscitation with crystalloid and colloid solutions was given to maintain pulmonary artery wedge pressures above 12 mm Hg, and a dopamine infusion was added if systolic arterial blood pressure fell to 90 mm Hg or decreased by > 30 mm Hg from preoperative values despite adequate fluid replacement.

Isolated Limb Perfusion

The perfusion technique employed at the Groningen University Hospital is based on the technique developed by Creech and Krementz [4]. Briefly, after ligation of all collateral vessels and heparinization of the patient with 3.3 mg heparin/kg bodyweight (Thromboliquine, Organon BV, Oss, the Netherlands) the axillary, iliac, femoral or popliteal vessels were dissected, cannulated and connected to the extracorporeal circuit. The perfused limb was wrapped in a thermal blanket to reduce heat loss and four thermistor probes were inserted subcutaneously and intramuscularly for continuous monitoring of the temperature during perfusion. A tourniquet was applied to the proximal limb in an attempt to minimize leakage of the perfusate into the systemic circulation through skin collaterals. Perfusion was performed during 90 minutes under mild hyperthermic conditions (39-40°C). The perfusate consisted of 350 ml 5% dextran 40 in glucose 5% (Isodex, Pharmacia AB, Uppsala, Sweden), 500 ml blood (250 ml red blood cells, 250 ml plasma), 30 ml 8.4% NaHCO₃ and 0.5 ml
5000 IU/ml heparin (Thromboliquine). The perfusate was oxygenated with a bubble oxygenator and driven by a roller pump. At the start of perfusion r-TNF-α (Boehringer, Ingelheim, Germany, 4 mg for leg perfusions and 3 mg for arm perfusions) was injected as a bolus into the arterial line of the perfusion circuit. Melphalan (Burroughs Wellcome, London, England, 10 mg/L volume of an affected leg and 13 mg/L volume of an affected arm) was administered 30 minutes later. During perfusion potential leakage to the systemic circulation was monitored with I\textsuperscript{131} labeled albumin [5]. After 90 minutes of perfusion, the limb was flushed with 2 L dextran 40 in glucose 5% (Isodex) and 500 ml blood (250 ml red blood cells, 250 ml plasma), catheters were removed, the circulation restored and the heparin antagonized with protamine chloride. A lateral fasciotomy of the anterior compartment of the lower leg was performed in leg perfusions or a fasciotomy of the forearm in arm perfusions to prevent a compartment syndrome.

**Hemodynamic measurements**

Hemodynamic variables were measured during perfusion and after the patient had arrived in the intensive care unit. Measured variables included heart rate and mean arterial pressure. Cardiac output, cardiac index and systemic vascular resistance were determined at two hourly intervals. Pressure transducers were set to zero at the level of the midaxillary line. Cardiac output was measured in triplicate by the thermodilution method, with the use of a cardiac output computer and cold saline.

**Assay for TNF-α**

TNF-α levels were determined by specific immunoradiometric assay (Medgenix Diagnostics, Soesterberg, the Netherlands). Samples were processed according to the guidelines of the manufacturer. Arterial blood samples (3 ml) were collected in EDTA Vacutainer tubes, and kept on melting ice during transport to a centrifuge. Samples were centrifuged for 10 min at 3000 rpm at 0°C and the separated plasma kept at -20°C until analysis. A baseline sample was taken for TNF-α assay after the insertion of the arterial line, then at 5, 30, 60, and 89 minutes after the start of the perfusion. After restoration of the circulation to the perfused limb, systemic samples were taken at 1, 5, 10, 30 and 60 minutes after removal of arterial clamps, hourly thereafter for at least eight hours and finally the next morning.

**Assay for nitric oxide metabolites** [6]

Nitrite was measured using the Griess reaction [7]. Briefly, plasma samples were diluted fourfold with distilled water and deproteinized by adding zinc sulfate (300 g/L) to give a final concentration of 15 g/L. After centrifugation at 10.000 g for 5 min at room temperature (or 1000 g for 15 min), 100 ml of supernate was applied to a microtiter plate well, followed by 100 ml of Griess reagent (1 g/L sulfanilamide, 25 g/L phosphoric acid, and 0.1 g/L N-1-naphthylethylenediamine). After 10 min of color development at room temperature, the absorbance was measured on a microplate reader (Titertek Multiskan MCC/340; Flow Lab, McLean, VA) at wavelength of 540 nm. Each sample was assayed in duplicate wells.

Nitrate was measured as nitrite after enzymatic conversion by nitrate reductase. Briefly, 100 ml of plasma was diluted fourfold with distilled water, nicotine adenine dinucleotide phosphate (reduced), FAD, and nitrate reductase from *Aspergillus* spp. (Boehringer Mannheim, Mannheim, Germany) were added to yield final concentrations of 50 mmol/L, 5 mmol/L, and 200 U/L, respectively. Samples were subsequently incubated for 20 min at 37°C, and then mixed with lactate dehydrogenase from rabbit muscle (Boehringer Mannheim) at a final concentration of 10 mg/L and sodium pyruvate at a final
Nitric oxide in isolated limb perfusion with TNF-α

centration of 10 mmol/L. Samples were further incubated for 5 min at 37°C to oxidize nicotine adenine dinucleotide phosphate (reduced), deproteinized, and assayed with Griess reagent as described above. Values obtained by this procedure represent the sum of nitrite and nitrate. Nitrate concentrations were obtained by subtracting nitrite concentration from the total nitrate + nitrite concentrations.

Plasma samples for nitrate and nitrite measurements were taken at the following time points: 1 hour before perfusion, during perfusion at 5, 30, 60 and 89 minutes and after perfusion at 5, 30, 60 minutes and after 2, 3, 4, 5, 6, 7, 8, 12, and 24 hours.

Samples were assessed for the presence of nitric oxide metabolites in two independent laboratories with similar results.

Statistical analysis

Data were analyzed using SPSS for MS WINDOWS (release 5.0). To analyze differences in heart rate, mean arterial pressure, cardiac output, systemic vascular resistance and TNF-α-levels before and after perfusion the Wilcoxon Matched-Pairs Signed-Ranks Test was used. A p-value < 0.05 was considered significant. To analyze the effect of perfusion on the sum of nitrate and nitrite an Analysis of Variance was carried out, comparing patients and controls.

Results

Clinical details, TNF-α levels and nitric oxide metabolites of the patients are summarized in the table, where the highest values of plasma TNF-α and the sum of nitrite and nitrate are shown.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Heart Rate</th>
<th>Cardiac Index</th>
<th>Systemic Vascular Resistance</th>
<th>TNF-α</th>
<th>NOx</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>synovia</td>
<td>120 (76)</td>
<td>5.1 (2.9)</td>
<td>585 (1072)</td>
<td>1393(43)</td>
<td>39.9 (22.2)</td>
</tr>
<tr>
<td>2</td>
<td>melanoma</td>
<td>100 (64)</td>
<td>5.5 (2.8)</td>
<td>334 (856)</td>
<td>38500(66)</td>
<td>35.4 (40.0)</td>
</tr>
<tr>
<td>3</td>
<td>synovia</td>
<td>150 (75)</td>
<td>8.0 (3.8)</td>
<td>429 (890)</td>
<td>21065(12)</td>
<td>22.2 (22.2)</td>
</tr>
<tr>
<td>4</td>
<td>sarcoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>liposarcoma</td>
<td>145 (75)</td>
<td>9.4 (3.9)</td>
<td>296 (749)</td>
<td>134000(1)</td>
<td>28.8 (33.2)</td>
</tr>
<tr>
<td>6</td>
<td>schwannoma</td>
<td>140 (80)</td>
<td>5.8 (3.6)</td>
<td>572 (1119)</td>
<td>17109(6)</td>
<td>35.0 (42.0)</td>
</tr>
<tr>
<td>7</td>
<td>histiocytoma</td>
<td>150 (110)</td>
<td>5.8 (3.6)</td>
<td>650 (908)</td>
<td>51000(10)</td>
<td>19.0 (18.6)</td>
</tr>
<tr>
<td>8</td>
<td>melanoma</td>
<td>155 (88)</td>
<td>6.2 (4.0)</td>
<td>501 (1000)</td>
<td>8200 (1)</td>
<td>32.2 (23.6)</td>
</tr>
</tbody>
</table>

Table 1: Clinical details, peak systemic TNF-α levels and nitric oxide metabolites. Tumor necrosis factor α is abbreviated as TNF-α, the sum of plasma nitrite and nitrate as Nox. Values are presented as highest value after perfusion (heart rate, cardiac index, TNF-α, NOx), or lowest value after perfusion (systemic vascular resistance), while the value 1 hour before perfusion is given between brackets.
Chapter VI

**Fig. 1** The black rectangles (dotted line) show the change in mean systemic vascular resistance (SVR) with time. The open rectangles (interrupted line) represent the mean cardiac output (CO). The open circles (solid line) show the mean sum of nitrite and nitrate (NO$_x$). Hyperthermic isolated limb perfusion was started at time point 0 and stopped after 1.5 hrs. as indicated by the large closed triangle. Error bars represent standard deviation.

Figure 1 shows the course of mean systemic vascular resistance, cardiac output and mean nitrite + nitrate levels over time.

**Fig. 2** The black rectangles (dotted line) show the change in mean arterial blood pressure (MAP) with time. The open rectangles (interrupted line) represent the mean dose of dopamine administered. The open circles (solid line) show the mean heart rate. Hyperthermic isolated limb perfusion was started at time point 0 and stopped after 1.5 hrs. as indicated by the large closed triangle. Error bars represent standard deviation.

Figure 2 shows the trend of mean values for heart rate, mean arterial pressure and dopamine requirements.
All patients developed a clinical picture of septic shock with hypotension, a significant increase in heart rate and cardiac index and a drop in systemic vascular resistance. They all needed volume resuscitation and dopamine in a dose of up to 6 mg/kg/min to maintain adequate blood pressure. Signs of sepsis abated within 12 hours and all patients could be discharged from the intensive care unit within two days following perfusion. Systemic TNF-α levels were very high during and immediately after the perfusion; they returned to baseline levels within 8 hours.

The clinical picture of septic shock in these patients is attributed to leakage of r-TNF-α from the perfusion circuit to the circulation of the patient, mainly through collateral blood flow. Leakage was confirmed by adding I¹³¹-labeled albumin to the perfusate, up to 2% of which was subsequently localized in the central circulation of the patients. All patients showed a clinically and statistically significant rise in heart rate and cardiac index (p < 0.01) and a decrease in mean arterial pressure and systemic vascular resistance (p < 0.01). The rise in TNF-α levels was statistically significant in all patients.

None of the patients showed any increase in nitrite or nitrate level at any time point. The mean value of the sum of nitrite and nitrate following perfusion in the 8 patients was 24.4 mmol/L (SD 5.9 mmol/L, 95% CI 23.4 - 25.4 mmol/L). This did not differ significantly from the mean value in 26 healthy controls of 23.9 mmol/L (SD 9.7 mmol/L, 95% CI 20.0 - 27.8 mmol/L).

**Discussion**

Nitric oxide is synthesized from L-arginine by nitric oxide synthase in many different cell types including endothelial cells. Two forms of nitric oxide synthase have been distinguished. In its constituent, calcium-dependent form nitric oxide synthase enzymatically produces small quantities of nitric oxide to act as an important modulator of vascular tone, in normal animals as well as in humans. In some animal models excessive amounts of nitric oxide can be produced by induction of a second type of nitric oxide synthase, the so-called cytokine-inducible nitric oxide synthase. Overproduction of nitric oxide by stimulation of this alternative, calcium-independent pathway is considered to explain the inappropriate vasodilation which is the hallmark of septic shock [8]. Endotoxin, but also cytokines like TNF-α are believed to induce nitric oxide synthase, leading to a sustained release of nitric oxide and catecholamine-refractory vasodilation. A rapidly expanding literature has implicated a role for cytokine-inducible nitric oxide synthesis in the pathogenesis of septic shock in animal models. IL-1, TNF-α and endotoxin induce nitric oxide synthase activity in vascular smooth muscle cells from rat aorta in vitro [9]. Studies in mice have shown that the administration of anti-TNF-α antibodies markedly reduces endotoxin-induced shock and nitric oxide synthesis in vivo [10]. Kilbourn and coworkers have induced hypotension in dogs by administering recombinant human TNF-α. NG-monomethyl-L-arginine, a competitive inhibitor of nitric oxide formation from L-arginine, completely reversed this fall in blood pressure, which reappeared after the administration of excess L-arginine. Though levels of nitrite or nitrate were not measured directly, the authors conclude that excessive nitric oxide production mediates the hypotensive effect of TNF-α [3].

The role of a cytokine-inducible nitric oxide synthase in *human* septic shock is less clear. Cytokine-inducible nitric oxide synthase has been demonstrated in only a few human cell types in vitro, in contrast to the abundance of animal cell types shown to have this activity. TNF-α failed to induce nitric oxide in vascular smooth muscle cells from human saphenous vein [11]. On the other hand clinical studies in human bacterial sepsis have shown signs of increased nitric oxide production and a
correlation between the concentration of endotoxin in plasma, the plasma levels of nitrite and nitrate and the severity of circulatory shock [12, 13]. Furthermore L-arginine analogues that block nitric oxide production have been effective at increasing blood pressure in septic patients [14]. In patients treated with interleukin-2 plasma concentrations of nitric oxide metabolites have been shown to rise significantly [15]. On the other hand Ochoa and coworkers have described normal levels of nitrite and nitrate in trauma patients even when they developed signs of sepsis [16, 17].

In this study we have looked at the mechanism of vasodilation in cancer patients, who were treated with isolated limb perfusion with r-TNF-α and melphalan, by measuring plasma levels of TNF-α, nitrite and nitrate. Our study has several limitations. First, it may well be argued that plasma levels of nitrite and nitrate do not reliably reflect activation of the inducible form of nitric oxide synthase. Local levels of nitric oxide may have been sufficiently increased to generate profound vascular effects without producing a demonstrable rise of nitrite or nitrate in the plasma. Conclusive data would perhaps require a combination of plasma levels with other parameters such as excretion of nitrate in urine, nitric oxide levels in exhaled air, or accumulation of ^15N-nitrite and ^15N-nitrate in plasma and urine following administration of ^15N-labeled arginine. It should be noted however, that most of the scientific evidence for a pivotal role of nitric oxide in various types of vasodilation has been based on the demonstration of elevated levels of nitrite and nitrate in plasma or serum [12, 13, 15, 16, 18, 19]. Moreover, alternatives to measurement of plasma levels of nitrite and nitrate, like measurement of urinary excretion of nitrate, have not been shown to be better markers of the septic state. On the contrary, Jacob and coworkers have found that in trauma patients the mean plasma nitrate concentration on days with evidence of infection was significantly increased compared with days without active infection; mean urinary excretion of nitrate was not increased on infected days as compared with days without infection [17]. We feel that activation of the inducible nitric oxide synthase by r-TNF-α would at least be partially reflected in a rise of serum levels of nitrite and nitrate. Another limitation of our study is that we did not investigate the effect on hemodynamic parameters of nitric oxide inhibitors like N^G^-methyl-L-arginine. Although important scientifically, this was considered to be unsuitable since it would imply treating our patients with two different investigational drugs: r-TNF-α and a nitric oxide synthase blocker. Furthermore, hypotension and shock in these patients could well be managed with conventional therapy and did not warrant the use of investigational drugs with possible harmful side effects. Potential drawbacks of the use of non-selective nitric oxide synthase blockers have been pointed out by a number of authors [20, 21, 22, 23, 24].

Our findings in human subjects show that despite very high TNF-α levels and distinct signs of sepsis syndrome, increased nitric oxide-production could not be demonstrated in plasma. It is possible that, in human subjects, the presence of endotoxin is mandatory to trigger excessive nitric oxide production, despite high levels of TNF-α. The assumed absence of relevant amounts of endotoxin in our model may also explain the relative mildness and short duration of the clinical syndrome; animal experiments have shown that the toxicity of TNF-α is greatly enhanced in the presence of endotoxin. Endotoxin levels in blood were not measured in our study. Alternatively, there may be a nitric-oxide-independent pathway through which TNF-α induces vascular relaxation, possibly by direct activation of soluble guanylate cyclase. The existence of such a pathway was postulated earlier by Beasley and coworkers on the basis of their experiments in human vascular smooth muscle cells [11]. They could show that interleukin 1, TNF-α, interferon-γ and Escherichia coli lipopolysaccharide increased cyclic guanosine monophosphate in human saphenous vein vascular smooth muscle cells and that this effect was not reversed by adding L-arginine analogues. Moreover, analysis of nitric oxide synthase mRNA with the
Nitric oxide in isolated limb perfusion with TNF-α

Use of polymerase chain reaction indicated that levels of mRNA for inducible nitric oxide synthase were low.

Alternatively, hypotension in the setting of isolated limb perfusion with TNF-α could also be explained by the generation of cyclooxygenase products like prostacyclin. Bernard et al. could show high levels of both prostacyclin and thromboxane A₂-metabolites in a group of patients with sepsis syndrome [25]. Calcitonin gene-related peptide is yet another possible mediator of hypotension in sepsis. It is increased in patients with sepsis and has been shown to be the most potent vasodilator and hypotensive agent in humans to date [26]. Adenosine triphosphate-regulated K⁺ channels have also been shown to be important mediators of vascular smooth muscle tone [27]. These channels are activated by decreased intracellular adenosine triphosphate, by cytosolic acidosis and increased cytosolic lactate conditions, that may well have occurred in our patients. Activation of adenosine triphosphate-sensitive K⁺ channels hyperpolarizes vascular smooth muscle and reduces Ca²⁺ entry into the cell, thereby inducing relaxation and vasodilation. These Ca²⁺ channels can also be downregulated by oxygen radicals produced by an oxidative burst of endothelial cells in response to the presence of endotoxin [28].

In conclusion, the results presented in this study do not support an important role for nitric oxide in producing vasodilation and shock in cancer patients treated with isolated limb perfusion with r-TNF-α, in whom a considerable leak of r-TNF-α from the perfusion circuit leads to high systemic levels of this cytokine. Further studies will be needed to clarify the exact mechanism of septic vasodilation in these patients.

References


