Nitric oxide synthase is downregulated, while haem oxygenase is increased, in patients with septic shock

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Background. The vasodilatation characteristic of human septic shock is conventionally attributed to increased nitric oxide production, primarily by extrapolation of animal and human in vitro studies. There are no conclusive studies of human disease, and the cellular source of nitric oxide in human sepsis is not known. Haem oxygenase is upregulated by oxidative stress, but little is known about haem oxygenase expression in human sepsis. Haem oxygenase may modulate nitric oxide production, and may also have a direct effect on vascular tone.

Methods. Mesenteric arterial smooth muscle (ASM) (obtained during laparotomy) and peripheral blood mononuclear cells (PBMCs) were obtained from patients with early septic shock and from control patients. mRNA levels were determined by real-time RT-PCR.

Results. mRNA for inducible and endothelial nitric oxide synthase was reduced in both ASM and PBMCs from septic patients. In contrast, inducible haem oxygenase mRNA was increased in sepsis in both cell types.

Conclusions. These results suggest that, rather than being induced, the enzymes which produce nitric oxide are reduced at this time point in human septic shock. Thus many of the in vitro models of sepsis studied to date may not fully replicate human disease. The increase in haem oxygenase expression confirms that these cells have been subjected to oxidative stress in sepsis. The activity of induced haem oxygenase may limit nitric oxide production, while possibly causing vasodilation through production of carbon monoxide.

Keywords: blood, peripheral blood mononuclear cells; enzymes, haem oxygenase; measurement techniques, PCR; muscle, vascular, arterial; pharmacology, nitric oxide

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Vasodilatory shock due to overwhelming infection is the most common cause of death in adult intensive care units. Nitric oxide is an inflammatory mediator often implicated in septic shock. It can interfere with cellular oxygen utilization, form cytotoxic free radicals, act as a negative inotrope and further stimulate inflammation. It is also a potent vasodilator.1

It is now clear that whole-body nitric oxide production is increased in human sepsis,2 though perhaps more modestly than animal models suggest. This additional nitric oxide may originate in the cells of the immune system, which synthesize nitric oxide as a means of killing microorganisms. We recently reported that peripheral blood mononuclear cells (PBMCs) from patients with septic shock produce more nitric oxide than those from healthy controls, and that the membrane transport mechanism which supplies arginine, the substrate for nitric oxide synthase (NOS), is both more active and more highly expressed in these cells in sepsis.3 It is assumed, by extrapolation from animal4 and human in vitro5 data, that PBMCs from patients with clinical sepsis producing increased nitric oxide will have increased expression of the NOS enzymes (the inducible form NOS2, and perhaps the constitutive endothelial form NOS3). However, to date this has not been demonstrated.

Whether nitric oxide from circulating inflammatory cells could cause significant hypotension is unclear. Nitric oxide overproduced in the vessel wall would be likely to be more significant physiologically. In health, nitric oxide is produced mainly by endothelial cells, but endothelial nitric oxide production appears to be decreased in sepsis.6
Until recently, vascular smooth muscle was considered merely the target of nitric oxide, but there is growing evidence from animal studies, as well as from in vitro experiments on human tissue, that vascular smooth muscle is itself a source of nitric oxide. Whether this is true in clinical human sepsis is not known.

Another possible mediator linking sepsis to shock is carbon monoxide. Carbon monoxide activates guanylyl cyclase in a manner similar to nitric oxide, and also opens calcium-sensitive potassium channels. Constitutive production of carbon monoxide has a role in the physiological control of vascular tone in rat small arteries and arterioles. Carbon monoxide is formed by haem oxygenase as part of the catabolic pathway for haem, which also produces the anti-oxidant bilirubin. For this reason, haem oxygenase activity is generally considered protective in sepsis. The amount and activity of the inducible form of haem oxygenase (HO-1) is increased by oxidative stress in many cell types. The exhaled breath of critically ill patients contains increased concentrations of carbon monoxide, and their plasma carboxyhaemoglobin and carbon monoxide concentrations are also increased.

Despite the strong likelihood that the haem oxygenase–carbon monoxide system is important in human septic shock, essentially nothing is currently known about HO-1 expression in clinical sepsis.

This study examines the levels of mRNA for NOS enzymes (NOS2 and NOS3) in peripheral blood mononuclear cells (PBMCs) and mesenteric arterial smooth muscle (ASM) from patients with septic shock and from non-septic controls. The expression of HO-1 mRNA in these cells and tissues is also quantified.

Methods

Patients

We took 20 ml of blood from the arterial cannulae of patients between 12 and 72 h after they initially satisfied the American College of Chest Physicians–Society of Critical Care Medicine definition of septic shock. Control blood was obtained by venepuncture of healthy volunteers. Blood was taken between July 2000 and July 2001, with control and patient sampling spread evenly over this time.

ASM was dissected from the mesentery of bowel sections taken from two patient groups: patients undergoing laparotomy for peritonitis secondary to bowel perforation who also met the definition of severe sepsis or septic shock at the time of surgery or whom it was suspected would meet these criteria in the first 24 h post-surgery (only those patients who subsequently met these criteria were included in the study); and patients who were undergoing large bowel resection for carcinoma but who were otherwise well.

ASM from both septic and control patients was collected concurrently from May 2000 to December 2001. Mesenteric artery was collected as a single piece from each patient, and the ASM was isolated by scraping the endothelial and adventitial surfaces with a blade. Histological examination of a number of trial specimens confirmed the removal of virtually all endothelium and adventitia.

Exclusion criteria for both studies included a premorbid history of a systemic inflammatory condition or treatment with systemic corticosteroids at any time within the preceding 6 months. There were no significant differences in the ages of the control and septic groups in any of the studies (Table 1).

The Central Oxfordshire Research Ethics Committee advised that informed consent was not required for the studies of ASM as the tissue was taken from samples which had been removed for clinical pathological analysis. In the studies of PBMCs, informed consent was obtained from patients who were competent to give it, and whenever possible assent was asked of close relatives of patients unable to give consent. Nonetheless, as the amount of blood taken was substantially less than 10% of that drawn for clinical purposes, we were similarly advised that informed consent was not required. No tissues or cells were retained, and no identifying clinical data (other than confirmation that the patient met the study inclusion and exclusion criteria) were kept.

Quantitation of mRNA levels: real-time RT-PCR

Blood was diluted with aerated Ringer’s physiological salt solution, layered over Ficoll–sodium diatrizoate (LSM, Cappel) and centrifuged; the PBMC layer was aspirated and then washed twice more with Ringer’s solution. The PBMCs were again concentrated by centrifugation, suspended in proprietary cell lysis solution containing citric acid, EDTA and sodium dodecyl sulphate (Gentra, Minneapolis, USA), and immediately frozen at −80°C. Samples were stored until a sufficient number had been accumulated; they were then thawed and the remainder of the RNA extraction procedure (involving protein precipitation with citric acid and NaCl followed by isopropanol precipitation of RNA) was completed.

Mesenteric ASM was collected as described above and stored at −80°C in ‘RNA Later’ (Ambion Ltd, Huntingdon, UK) until sufficient samples had been accumulated. When thawed, the same proprietary cell lysis solution (Gentra) was

| Table 1 | Ages of patients in the various studies. Data are expressed as mean (range) |
|---------|-------------------|-------------------|------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Experimental series | Age (yr) | Controls | Septic patients |
|----------------------|----------|---------|----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| PBMC                 | 48 (30–58) | 64 (43–76) |
| ASM (all except HO-1) | 64 (36–88) | 69 (54–89) |
| ASM (HO-1)            | 65 (36–88) | 70 (54–89) |
added and the remainder of the RNA extraction protocol completed.

RNA samples from both ASM and PBMCs were DNase treated (‘DNA-free’, Ambion) and, after dilution to 25 mg ml\(^{-1}\), the RNA was reverse transcribed using random hexamer primers (TaqMan Gold RT kit, Applied Biosystems, Warrington, UK).

Segments of the cDNA produced were amplified using cDNA specific primers for HO-1, NOS2 and NOS3. The sequences of the primers and probes used are shown in Table 2.

The quantity of PCR product was detected with an Applied Biosystems 7700 Sequence Detector. Sample quantities were standardized by reference to amplification of the 18S component of ribosomal RNA. In a preliminary experiment, the expression of 10 commonly used reference ‘housekeeper’ genes and 18S was measured in PBMCs from one septic patient and one control. 18S showed the least change in sepsis. PCR reactions identical to those described above were run for aliquots of each sample which had not been reverse transcribed. The identity of the products of the reactions was confirmed by visualization of a single amplicon of the expected size when the PCR product was subjected to gel electrophoresis.

The amount of specific cDNA in each sample was expressed as a proportion of the expression of that mRNA in placenta, a tissue known to express all these mRNA species at high levels.

**Statistical analysis**

Data are presented as mean (sd). Unpaired Student’s \(t\)-tests were used to determine the significance of differences between two groups, and the exact \(P\)-value is reported where it is \(\leq 0.05\).

**Results**

Each primer–probe combination produced a detectable signal when the placental cDNA standard was diluted up to 1:100 or 1:1000. There was a linear relationship between dilution factor and number of PCR cycles to threshold \((r > 0.94 \text{ in each case})\), confirming the validity of the assays (data not shown). There was insignificant or undetectable amplification in the 18S no-RT control for each sample, demonstrating minimal genomic DNA contamination.

**Peripheral blood mononuclear cells**

PBMCs were collected from eight septic patients and eight controls. RNA of insufficient quality was extracted from one of the septic samples (most likely because of RNA degradation before the isolation of the PBMCs and freezing), and so results from this sample were discarded.

The mRNA for both NOS2 and NOS3 was significantly reduced in PBMCs from the seven septic patients studied compared with those from the eight healthy controls. In contrast, the mRNA for HO-1 in the same cells was significantly increased (Table 3).

**Arterial smooth muscle**

Initially, RNA from eight septic patients and eight controls was studied. The mRNA for both NOS2 and NOS3 was significantly reduced in sepsis (Table 4).

There was a trend towards increased HO-1 expression in ASM from septic patients; however, the difference was not significant.
statistically significant. To clarify this result, a further septic sample and three control samples were collected. More patients underwent elective laparotomy for cancer than had a perforated bowel during this second collection period, which explains the unequal numbers of samples in each group. These extra samples were only analysed for HO-1 expression. When these extra data were taken into account, mRNA for HO-1 in ASM was significantly increased in sepsis (Table 4).

Discussion
Expression of mRNA for NOS2 and NOS3 is decreased in both ASM and PBMCs from patients in the early stages of septic shock. In contrast, the expected increase in HO-1 expression was observed in both cell types, suggesting these were indeed cells which had been subjected to the oxidative stress of sepsis.

Our results in PBMCs contrast with those of most animal and human in vitro studies. Rat macrophages cultured with lipopolysaccharide (LPS) showed increased expression of NOS2 mRNA, as did mouse macrophages and human monocytes. Rats given i.v. LPS rapidly increased their neutrophil NOS2 mRNA expression. In contrast, our finding of increased HO-1 expression in sepsis is consistent with previous work. LPS increases HO-1 mRNA expression in cultured rat macrophages. Monocytes from children with acute inflammatory illnesses also have raised HO-1 mRNA.

The reduction in NOS mRNA expression in septic ASM is equally surprising in the light of previous studies. The aorta of rats given endotoxin in vivo showed increased NOS2 mRNA expression. Human smooth muscle cells activated in vitro also showed increased NOS2 mRNA and protein.

All the studies cited were performed in vitro or in very short term in vivo models. The activating stimuli were artificially high levels of LPS and pro-inflammatory cytokines, and the effect of anti-inflammatory cytokines produced by other tissues may not have been present or have had time to develop. Studies of animals exposed to LPS at lower levels for more prolonged periods in vivo may be more pathophysiologically relevant. The haemodynamics of rats given a constant infusion of LPS changed markedly over the first 24 h. After 2 h of LPS exposure there was modest hypotension and vasodilatation; the blood pressure returned to baseline after 6 h, and only after 24 h was there the expected hypotension, tachycardia and generalized vasodilatation, which resembles clinical human sepsis. This same experimental model was used to assess NOS2 and HO-1 protein expression. At 6 h there was an increase of up to 6-fold in NOS2, but at 24 h this had returned to control values in all tissues. In contrast, HO-1 expression remained increased at 24 h. Rats given i.p. LPS initially had increased NOS2 mRNA in their myocardial arterioles, but this had reached a plateau by 8 h and had substantially decreased by 24 h. IP LPS also stimulated NOS2 protein expression in liver, which peaked at 6 h but then decreased by 24 h. Thus it appears that in animal models which are pathophysiologically more similar to human septic shock than the in vitro models described, NOS2 decreases after an initial increase, which agrees entirely with the results of our study.

Very few data are available from cells (either ASM or PBMCs) from patients with clinical septic shock. There are only two published studies of mesenteric arterial tissue from patients with sepsis. These suggested, but did not statistically confirm, that mesenteric arteries might produce more nitric oxide in sepsis. Our recent report of increased nitric oxide production by septic PBMCs would also at first seem inconsistent with the results presented in the current study. However, the rate of nitric oxide production is determined by the availability of cofactors and the supply of substrate, as well as by the quantity of NOS present. It is possible that the elevated nitric oxide production by septic PBMCs in our previous study was due to increased arginine transporter function despite reduced enzyme levels.

A number of possible mechanisms may reduce NOS mRNA expression at this time point in sepsis. Nitric oxide is known to negatively feed back on its own production. Alternatively, the net effect of the immunoregulatory cytokines may shift from pro- to anti-inflammatory, a suggestion which has recently been extensively reviewed. Another possible anti-inflammatory mechanism involves haem oxygenase, which we observed to be increased in our septic patients. There are several means whereby haem oxygenase can reduce nitric oxide production either directly or via carbon monoxide. Haem oxygenase degrades haem, an essential component of the NOS enzyme structure. Haem oxygenase and NOS compete for NADPH. Compounds which induce HO-1 prevented the induction of NOS2 mRNA by cytokines. Conversely, inhibition of haem oxygenase increased nitric oxide production by endotoxin-stimulated mouse macrophages. Of additional significance, carbon monoxide is a recognized vasodilator, and if it is indeed being produced in sufficient quantities to decrease nitric oxide production by ASM, it may also exert an effect on vascular tone.

The controls in our study of ASM were patients with large bowel carcinoma, and hence were 'non-septic' rather than strictly 'healthy'. Unfortunately, it is not possible to obtain mesenteric artery from healthy subjects. If cancer causes an activation of the nitric oxide pathway, it is possible that what we have interpreted as 'decreased' activation in sepsis might in reality be activation, but to a lesser degree than with cancer. There is reason to believe that this is not the case; the ASM from septic patients expressed HO-1 mRNA at a higher level than that from controls, and HO-1 expression is known to be very sensitive to oxidative stress. The control vessels were sampled as far away as possible from the tumour, and there is no evidence that the systemic haemodynamics of cancer patients is abnormal.

We sampled PBMCs at a single point at any time within a relatively long time period (12–72 h from the onset of 'septic...
shock’). The pathological processes leading to septic shock are highly variable; some patients become ill rapidly, while others display signs of infection for a longer period before meeting the required criteria. Therefore we felt that the advantage of comparing samples taken within a more restricted timeframe in the development of the disease was outweighed by the practical difficulties that this would entail.

In summary, mesenteric ASM from septic patients and PBMCs show decreased expression of mRNA encoding the enzymes producing nitric oxide in early human septic shock. These results are at variance with most in vitro cellular studies, but are reinforced by the expected finding of increased HO-1 mRNA in the same tissues. Moreover, they agree entirely with the results of animal studies involving chronic in vivo LPS exposure. Our results might be due to specific characteristics of PBMCs and the mesenteric vascular bed, but more probably indicate that the role of nitric oxide as a vasodilator at this and later time points in human sepsis has been overstated.

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