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In vivo measurement of nitric oxide production in porcine gut, liver and muscle during hyperdynamic endotoxaemia

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1 During prolonged endotoxaemia, an increase in arginine catabolism may result in limiting substrate availability for nitric oxide (NO) production. These effects were quantitated in a chronically instrumented porcine endotoxaemia model.

2 Ten days prior to the beginning of the experiments, pigs were catheterized. On day 0, pigs received a continuous infusion of endotoxin (3 μg kg^{-1} h^{-1}) over 24 h and were saline resuscitated. Blood was drawn from the catheters at 0 and 24 h during primed-infusion of $^{15}N_2$-arginine and P-aminohippurate to assess $^{15}N_2$-arginine to ^{15}N-citrulline conversion and plasma flow rates, respectively, across the portal-drained viscera, liver and hindquarter.

3 During endotoxin infusion a hyperdynamic circulation with elevated heart rate, cardiac index and decreased mean arterial pressure was achieved, characteristic of the human septic condition.

4 Endotoxin induced NO production by the portal-drained viscera and the liver. The increased NO production was quantitatively matched by an increase in arginine disposal. Nitrite/nitrate levels remained unchanged during endotoxaemia.

5 Despite an increased arginine production from the hindquarter and an increased whole-body arginine appearance rate during endotoxin infusion, the plasma arginine concentration was lower in endotoxin-treated animals than in controls.

6 On a whole-body level, the muscle was found to serve as a major arginine supplier and, considering the lowered arginine plasma levels, seems critical in providing arginine as precursor for NO synthesis in the splanchnic region.

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Abbreviations: AA_A, arterial concentration; AA_V, venous concentration; A1, abdominal aorta catheter proximal to the bifurcation; A2, abdominal aorta catheter proximal to the right renal vein; HCO_3^-, bicarbonate concentration; I, tracer infusion rate; LC-MS, liquid chromatography-mass spectrometry; MAP, mean arterial pressure; NB, net balance; NO, nitric oxide; NOS I, neuronal nitric oxide synthase; NOS II, inducible nitric oxide synthase; NOS III, endothelial nitric oxide synthase; NO_X, sum of NO_2^- and NO_3^- concentrations; $PaCO_2$, arterial carbon dioxide pressure; PAH, P-aminohippurate; PaO_2, arterial oxygen dioxide pressure; Q_{ARG}, whole-body turnover rate of arginine; SaO_2, arterial oxygen saturation; TCA, trichloroacetic acid; TTR, tracer-to-tracee ratio; V1, caval vein catheter proximal to the bifurcation; V2, caval vein catheter proximal to the right renal vein; LPS, lipopolysaccharide

Introduction

Septic shock is an important cause of death in the intensive care unit. The main features of septic shock are hypotension and vascular hyporeactivity to traditional vasopressors (Bone *et al*., 1992). When blood pressure is not maintained in septic shock, this results in compromised cardiac output and organ perfusion which can lead to hypovolaemia and irreversible septic shock. Persistent arterial hypotension is a major risk factor for mortality in patients with septic shock (Groeneveld *et al*., 1986; Bernardin *et al*., 1996). However, in patients with compensated sepsis, cardiac output is typically elevated and systemic vascular resistance is usually abnormally low. Because bacterial endotoxin is able to provoke a generalized proinflammatory response in the infected host (Qureshi *et al*., 1999), it is often used to produce a sepsis-like condition in experimental models.

The endotoxins and cytokines that are abundantly produced during septicaemia induce the inducible nitric oxide (NO) synthase isoform (NOS-II) in rats (Salter *et al*., 1991) and mice (Ter Steege *et al*., 2000). The NOS-II isoform can be expressed in virtually all tissues and is capable of producing large amounts of NO. High local concentrations of NO may contribute to the haemodynamic effects that accompany sepsis by inducing vascular leak and subsequent hypotension (Kirkeboen & Strand, 1999). However, most evidence of increased NO production during sepsis derives from rodent

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models, whereas only few studies in humans (Pastor & Suter, 1998) and larger experimental animal models (Pastor *et al*., 2000) show direct evidence for increased NO production during sepsis. The haemodynamic features of rodent models of sepsis differ substantially from those in large animals. In rodents, endotoxin is often administered intraperitoneally in large doses whereas in most porcine models of sepsis, endotoxin is continuously infused in moderate amounts. In addition to differences that are intrinsic to both species, these haemodynamic aspects may confound the evidence for increased NO during experimental sepsis.

To assess the involvement of NO in hyperdynamic sepsis, we measured NO synthesis in three main organs in a clinically relevant porcine sepsis model with haemodynamic features characteristic of compensated human sepsis. The pig was chosen as a chronically instrumented large animal model, because organ physiology and metabolic changes in this animal are in many respects comparable with those observed in humans (Dodds, 1982; Miller & Ullrey, 1987). Arginine and NO metabolism were measured in the portal-drained viscera, liver and hindquarter of endotoxaemic pigs using the stable isotope L-[*guanidino*-$^{15}N_2$]-arginine and its conversion to L-[*ureido*-^{15}N]-citrulline.

Methods

All procedures were performed according to the guidelines of the Animal Care Committee of the Maastricht University, and the study was approved by the Local Animal Ethics Committee. Female pigs were the offspring of Yorkshire and Dutch Landrace species and weighed 20–22 kg. Pigs were individually housed, had free access to water and were fed 1 kg of regular pig feed (149 g crude protein kg^{-1} body weight; Landbouwbelang, Roermond, The Netherlands) daily, which supported a growth rate of $\approx$300 g d^{-1}.

Surgical procedure

Animals were overnight fasted with free access to water. After the pigs were premedicated with 10 mg kg^{-1} azoperone (Stresnil, Janssen Pharmaceutica, Besse, Belgium) intramuscularly, anaesthesia was induced with a mixture of nitrous oxide/oxygen (1 : 2, by v v^{-1}) and halothane (0.8%). After intubation, the pigs were intravenously administered 6.25 mg kg^{-1} Lincomycin.2HCl (A.U.V., Cuyk, The Netherlands) as bactericidal prophylaxis and 12.5 mg kg^{-1} Spectinomycin.HCl (A.U.V.) as bacteriostatic prophylaxis. Flunixine (50 mg kg^{-1}, Finadyne, Schering-Ploegh, Brussels, Belgium) was given as postoperative analgesic. During surgery, anaesthesia was maintained with a mixture of nitrous oxide/oxygen and halothane and with intravenous Lactetrol (Janssen Pharmaceutica). The surgical procedure has previously been described in detail (Deutz *et al*., 1992). In brief, seven catheters were cannulated after a midline incision was performed as depicted in Figure 1. Two catheters were inserted in the abdominal aorta; one proximal to the bifurcation (A1) and one proximal to the right renal vein (A2). Two catheters were inserted in the inferior caval vein at the corresponding positions (V1 and V2, respectively).

Furthermore, catheters were inserted in the portal vein (*via* the v. lienalis), the hepatic vein and a splenic vein. The abdominal aorta (A1) and the splenic vein catheters were used for the infusion of P-aminohippurate (PAH) to measure plasma flow, and the caval vein (V2) catheter was used for isotope and endotoxin infusion. Blood collected from the abdominal aorta (A2) in combination with the portal, hepatic and caval vein (V1) catheters, was used to measure across the portal-drained viscera, splanchnic area and the hindquarter, respectively. Also, a gastrostomy catheter was inserted. All catheters were tunnelled through the abdominal wall and skin.

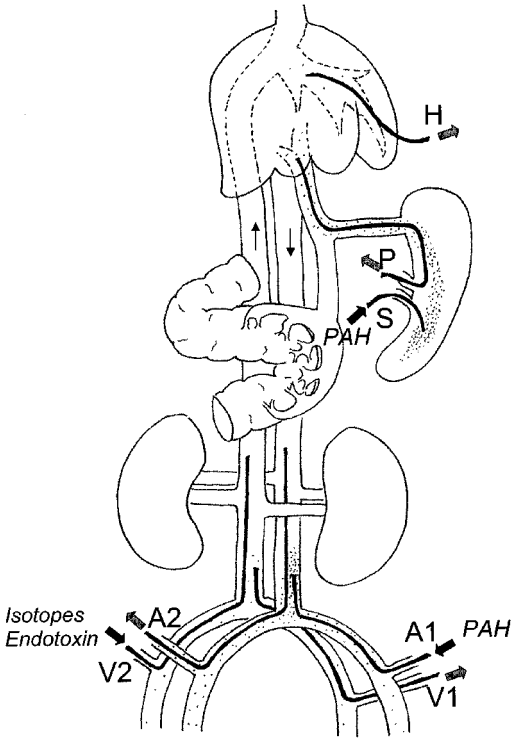

Figure 1 Schematic illustration of the catheter implantation. Catheters were placed in the abdominal aorta just above the bifurcation (A1) and just above the right renal vein (A2) and in the inferior caval vein at the corresponding positions (V1 and V2, respectively). Catheters were also placed in the portal (P) vein (*via* the *vena lienalis*), the hepatic (H) vein and a splenic (S) vein. The P-Aminohippurate (PAH) dye solution was infused through the A1 and S catheters while isotopes and endotoxin were infused through the V2 catheter. Blood was sampled from the A2, P, H and V1 catheters.

Postoperative care

Every 2 days, catheters were flushed with heparinized (50 U ml^{-1}) 0.9% saline (saline) to maintain patency (Palm *et al*., 1991). Postoperative care was standardized and as previously described (Ten Have *et al*., 1996). Ten days after surgery, when pigs were fully recovered, liquid enteral nutrition (Nutrison Steriflo High-Protein, Nutricia, Zoetermeer, The Netherlands) was infused into the gastrostomy catheter *via* a swivel system connected to a pump for a period of 4 days to standardize daily food intake. The liquid nutrition contained no nitrite (NO_2^-) or nitrate (NO_3^-). During postoperative handling, the pigs were placed in a movable cage to get accustomed to their experimental condition.

Experimental protocol

The night prior to the endotoxin infusion, the enteral nutrition was stopped. The next morning, the pigs were placed in a movable cage and experiments were conducted. Seven pigs received 3 μg kg^{-1} h^{-1} lipopolysaccharide endotoxin from *Escherichia coli* (055:B5, Sigma Chemicals Co., St. Louis, MO, U.S.A.) dissolved in saline over 24 h *via* the caval vein (V2) catheter while seven control animals received saline at an equivalent infusion rate. During endotoxin infusion, all pigs were infused with saline to secure replenishment of intravascular volume losses: 30 ml·kg bw^{-1}·h^{-1} during the first 8 h and 20 ml·kg bw^{-1}·h^{-1} during the following 16 h. Samples were taken before and 24 h after the start of endotoxin or saline infusion. Pigs were fasted during the whole experimental period. Rectal temperature, weight, urinary output, mean arterial pressure (MAP) and heart rate were regularly monitored in both groups. Changes in cardiac output in this protocol were assessed using the bolus thermodilution method in an independent group of six animals, which were equipped with a balloon-tipped thermodilution catheter (9520A, 7F, Baxter Healthcare, Irvine, CA, U.S.A.) in the pulmonary artery. Triplicate bolus of 5 ml ice-cold saline were used to estimate the cardiac output and index.

Infusion protocol

Before the start of the infusions, background blood samples were collected. On the morning of the trial, 1 h before blood sampling began, a primed infusion of 25 mM PAH (A 1422; Sigma Chemicals Co.) was administered at a rate of 40 ml h^{-1} per catheter through the splenic vein and the abdominal aorta (A1) catheters after an initial prime of 5 ml. Directly after the primed, constant infusion of PAH, a priming dose (0.1 μmol kg^{-1}) followed by a constant infusion (0.1 μmol kg^{-1} h^{-1}) of L-[*guanidino*-$^{15}N_2$]-arginine ($^{15}N_2$-arginine, Mass Trace, Woburn, MA, U.S.A.) was started *via* the caval vein (V2) catheter. One hour after the start of the infusions, steady state conditions for PAH (Ten Have *et al*., 1996) and $^{15}N_2$-arginine (Van Eijk *et al*., 1999) were obtained and blood was sampled.

Sample processing

Immediately after the blood was sampled, blood was transferred into heparin-containing tubes (Sarstedt, Nümbrecht, Germany) on ice. Haematocrit was obtained with a microfuge. For arterial blood gas analysis (pH, bicarbonate, PaO_2 and $PaCO_2$), 200 μl blood was sealed airtight in heparinized 1 ml syringes and immediately analysed on an automatic blood gas system (Acid Base Laboratory, Radiometer, Copenhagen, Denmark). For the measurement of PAH concentrations, 300 μl whole blood was added to 600 μl of 120 g l^{-1} trichloroacetic acid (TCA; Merck, Darmstadt, Germany) solution. The remaining blood was centrifuged at 4°C for 5 min at 8500 × *g*, plasma collected and kept on ice. For the amino acid analysis, 500 μl plasma was deproteinized by mixing it with 20 mg dry sulfosalicylic acid. To measure ammonia and urea concentrations, 400 μl plasma was mixed with 40 μl of 3.5 mol l^{-1} TCA solution. The solutions were thoroughly mixed and centrifuged (4°C, 5 min at 8500 × *g*) followed by the collection of supernatants. All samples were frozen in liquid nitrogen and stored at −80°C until use.

Biochemical analysis

To determine PAH concentrations, the supernatant fluid of the deproteinized whole blood was deacetylated at 100°C for 45 min. The PAH concentration was detected by standard enzymatic methods and spectrophotometrical analysis with an automated Cobas Mira-S system (Hoffmann-La Roche, Basel, Switzerland). To calculate the plasma PAH concentrations, the whole blood PAH concentration was corrected for haematocrit values. Before urea measurements, the plasma ammonia was removed by conversion into glutamate. The urea was enzymatically converted into ammonia by the addition of urease. The ammonia formed in this reaction was quantified by measuring the extinction of NADPH utilized in the ammonia conversion into glutamate. Plasma NO_2^- and NO_3^- concentrations were determined using a fully automated HPLC method. Hereto, 100 μl acetonitrile was added to 50 μl plasma, mixed and centrifuged. From the clear supernatant, 60 μl was mixed with 140 μl Super-Q water and, subsequently, 100 μl of the solution was injected using a WISP Model 717 autosampler with chilled (10°C) sample compartment (Waters, Etten-Leur, Netherlands) into a low pressure gradient HPLC system consisting of a Model PU980 pump and a Model LG980 gradient former Jasco Benelux, Maarssen, Netherlands). Plasma anions were separated isocratically within 18 min on an IC-Pack HR column (75 × 4.6 mm, I.D., Waters) equipped with a 10 × 4.6 mm I.D. Allsphere ODS II 3-μm precolumn (Alltech, Breda, Netherlands) using solvent A containing 10 mM sodium chloride and 1 mM potassium dihydrogenphosphate (pH 6.0) pumped at 1 ml min^{-1}. Next, the column was regenerated by pumping solvent B, consisting of 100 mM sodium chloride in water, 12% acetonitril and 2% iso-propanol (v v v^{-1}) for 4 min, followed by a re-equilibration of 13 min using solvent A. The column effluent was monitored using a Model UV-975 intelligent UV-VIS detector, set at 205 nm (Jasco, B&L-systems, Wassenaar, The Netherlands). Data were collected and integrated using Turbochrom version 3.2 software (Perkin-Elmer, Gouda, The Netherlands). Data are expressed as NO_X, which was calculated as the sum of NO_2^- and NO_3^- concentrations. The plasma amino acid concentrations were determined by HPLC after pre-column derivatization with *o*-phthaldialdehyde (Van Eijk *et al*., 1993). Enrichments of amino acids were determined by a fully automated liquid chromatography-mass spectrometry (LC-MS, Thermoquest LCQ, Veenendaal, The Netherlands) system and calculated as tracer-to-tracee ratios (TTR) as has previously been described (Van Eijk *et al*., 1999).

Calculations

The portal-drained viscera represent all portal-drained organs, which, apart from the spleen, stomach and pancreas, largely represent the intestines. The splanchnic area is the sum of the portal-drained viscera and liver, therefore, calculations on liver are made by subtracting portal-drained viscera from splanchnic values. Calculations on muscle kinetics were made by assuming that the hindquarter represents 50% of whole-body muscle (Deutz *et al*., 1999).

Substrate metabolism across the organs was calculated in a two-compartment model as was described previously (Wolfe, 1992). The plasma TTR values were corrected for background TTR values.

The $^{15}N_2$-arginine isotope is used to calculate the whole-body turnover rate of arginine (Q_{ARG}, μmol kg^{-1} min^{-1}). Under steady state conditions, the Q_{ARG} represents the arginine appearance and also disappearance rate in the circulation. Q_{ARG} is calculated using the tracer infused (I, μmol kg^{-1} min^{-1}) and the tracer to tracee ratio of $^{15}N_2$-arginine in the arterial plasma (TTR_A):

$$Q_{ARG} = I/TTR_A \quad (1)$$

The plasma flow rate (ml kg^{-1} min^{-1}) was calculated using the PAH indicator-dilution technique (Ten Have *et al*., 1996). Substrate fluxes or net balance (NB; nmol kg^{-1} min^{-1}) was calculated by multiplying the mean plasma flow with the difference between the venous and arterial plasma concentration of the amino acid, AA_V and AA_A respectively:

$$NB = flow_{plasma} \times (AA_V - AA_A). \quad (2)$$

A positive NB represents net efflux from the organ and a negative NB net influx into the organ. The disposal rate (nmol kg^{-1} min^{-1}) of an amino acid represents its total rate of disappearance by conversion into other compounds or incorporation into protein. Disposal can be calculated by using the tracer disappearance across that organ. For that purpose, the NB of the tracer (tracer NB) across an organ is calculated from arterial-venous tracer concentration differences in the plasma. Tracer concentrations are the product of tracer to tracee ratio (TTR) and tracee (or amino acid) concentration in the plasma, respectively:

$$\text{Tracer NB} = flow_{plasma} \times (AA_A \times TTR_A - AA_V \times TTR_V) \quad (3)$$

Amino acid disposal is subsequently calculated by dividing the tracer NB by the venous TTR:

$$\text{Disposal} = \text{tracer NB}/TTR_V \quad (4)$$

The venous TTR is used for the calculation of the disposal rate because it best approaches the intracellular enrichment (precursor pool) of the organ (Biolo *et al*., 1995). Since the NB of a substrate across an organ is the net difference between production and disposal by that organ, the production is presented as.

$$\text{Production} = \text{NB} + \text{disposal} \quad (5)$$

The rate of organ NO synthesis can be calculated from conversion of $^{15}N_2$-arginine to ^{15}N-citrulline (Castillo *et al*., 1995) across that organ, therefore, is calculated by using the venous-arterial organ tracer NB of ^{15}N-citrulline divided by the arterial TTR of $^{15}N_2$-arginine. The organ ^{15}N-citrulline net balance is corrected for the ^{15}N-citrulline extracted by that organ, which is estimated from L-[*ureido*-^{13}C;5,5-$^{2}H_2$]-citrulline isotope primed-infusion values obtained from a different study (Bruins *et al*., 2002).

Statistics

Results are presented as means ± s.e.mean. SPSS v 7.5 software was used to perform statistics. When the normality or equal variance test failed, data were transformed or log-transformed where appropriate. The data were subjected to an analysis of variance (general factorial ANOVA). Haemodynamics, were analysed using a two-factor (temperature, heart rate, MAP) or one-factor (cardiac output) repeated measures ANOVA. When an overall significance for time was observed, the univariate *F*-test was used to evaluate contrasts with baseline values. When interaction of time and treatment group (time × group) was observed, a nonpaired Student's *t*-test was used to compare individual groups. A paired Student's *t*-test was used to assess difference from zero. Levels of significance were set at $P<0.05$.

Results

Haemodynamics (Figure 2)

No pigs died in this endotoxaemia model. The body temperature (Figure 2A) and heart rate (Figure 2B) of the endotoxin-treated pigs rose to a maximum at 4–6 h after initiation of endotoxin ($P_{GXT}=0.00$) and was still elevated above control values at 24 h endotoxin infusion. The MAP transiently decreased ($P_{GXT}=0.00$) during the first 4 h of endotoxin infusion, reaching a minimum at 1.5 h (Figure 3C). At 5 h, MAP had returned to control values. Thereafter, MAP again fell below baseline values to a plateau at 8 h. In a separate group of animals ($n=6$ pigs), the effect of endotoxin and fluid resuscitation on cardiac output was monitored during 24 h. The cardiac output of these pigs revealed reciprocal changes compared to the changes in temperature and MAP. The cardiac output transiently increased ($P_T=0.00$) during the first 4 h with a peak at 1 h (Figure 2D). Thereafter, output index increased to clearly elevated values at 24 h. These findings indicate that the experimental sepsis protocol induced the characteristics of hyperdynamic endotoxaemia.

Body weight, urinary output, and organ plasma flow (Table 1)

Table 1 shows that the body weight of the pigs did not change in time and did not differ between the endotoxin and control groups. The urinary output (Table 1) of the endotoxin and control groups increased ($P_T=0.05$) with time (as a result of the fluid infusions), but these changes were similar in both groups. The plasma flow across the hindquarter, the portal-drained viscera and liver was not significantly altered by endotoxin treatment ($P_{GXT}>0.05$). In both groups, the liver flow tended to increase in time ($P_T=0.06$).

Arterial blood gas values (Table 2)

No time-dependent decreases in arterial blood gas values were observed. The haematocrit was not significantly altered ($P_{GXT}=0.08$) in the endotoxin-treated animals. The carbon dioxide pressure ($Paco_2$) was lower ($P_{GXT}=0.058$) and the pH significantly higher ($P_{GXT}=0.03$) in the endotoxin-infused animals, indicating that pigs developed respiratory alkalosis.

Arterial plasma concentrations and whole-body arginine turnover rate (Table 3)

Arterial plasma concentration of ornithine time-dependently decreased but not when correcting for possible dilutional

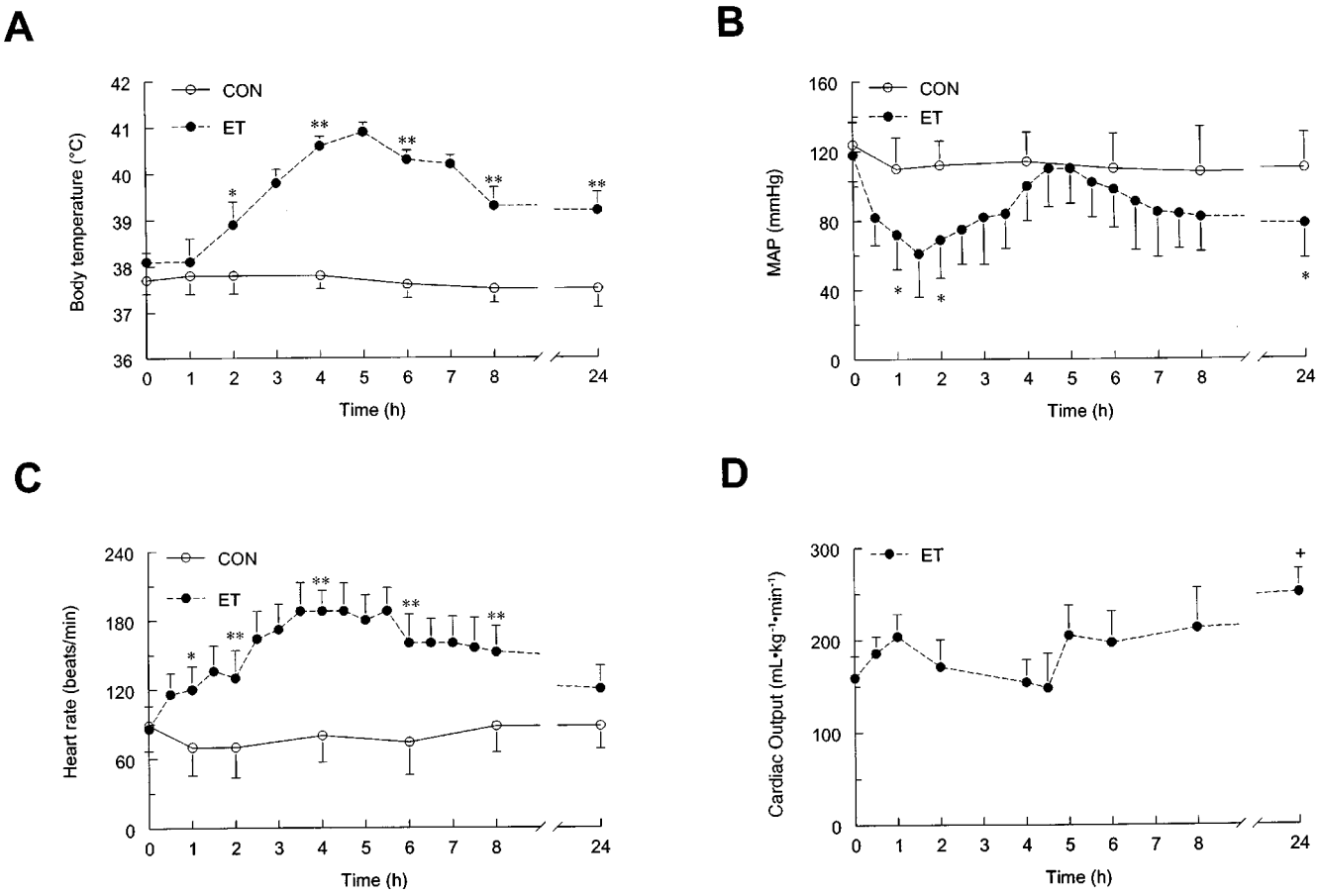

Figure 2 Haemodynamics in endotoxin-treated pigs. Data are mean ± s.e.mean. (A) Body temperature (control: $n=7$; endotoxin: $n=7$), (B) heart rate (control: $n=4$, endotoxin, $n=5$) and (C) mean arterial blood pressure (MAP; control: $n=3$, endotoxin: $n=3$) were followed during 24-h saline or endotoxin infusion. (D) Cardiac output was measured in a separate endotoxin-treated pig group ($n=6$). Statistics by repeated measures ANOVA: (A, B, C) $^{**}P<0.01$, $^{*}P<0.05$; significantly different from control group and (D) $^{+}P<0.05$; significantly different from baseline values (0 h).

effects from infusions by correcting for plasma levels of total protein. There was no effect of endotoxin infusion on plasma concentrations of NO_X, ornithine and citrulline. The plasma concentration of urea increased ($P_{GXT}=0.01$) whereas the plasma concentration of arginine decreased ($P_{GXT}=0.01$) in the endotoxin-treated pigs compared to controls, also when taking dilutional effects into account ($P_{GXT}=0.01$ and $P_{GXT}=0.04$, respectively). Infusion with endotoxin resulted in a significantly higher ($P_{GXT}=0.05$) whole-body arginine turnover (appearance and disappearance) rate than in controls.

Portal-drained viscera (Figure 3)

Net fluxes of urea (not shown) and ornithine across the portal-drained viscera were not significantly different from zero ($P>0.05$). Intervention with endotoxin had no effect on either urea, citrulline or ornithine net fluxes whereas it reduced ($P_{GXT}=0.04$) the visceral arginine efflux 6 fold (Figure 3A). Figure 3B shows this reduction in arginine efflux to be due to higher ($P_{GXT}=0.04$) arginine disposal (615 ± 49) in the endotoxin animals compared to controls (366 ± 90 nmol kg^{-1} min^{-1}), and unchanged arginine production in the endotoxin-treated compared to control animals. Moreover, the visceral arginine production decreased over time ($P_T=0.05$) in both treatment groups. The production of NO by the portal-drained viscera (Figure 3C), measured from the conversion of $^{15}N_2$-arginine to ^{15}N-citrulline, was not significantly different from zero ($P>0.05$) in the control group and at 0 h. The visceral NO production in the endotoxin-treated pigs (235 ± 43) was significantly higher ($P_{GXT}=0.05$) than in the control pigs (63 ± 55 nmol kg^{-1} min^{-1}). The endotoxaemia-induced increase in arginine disposal matched the increase in NO production ($\sim$170 nmol kg^{-1} min^{-1}).

Liver (Figure 4)

The endotoxin infusion doubled the hepatic efflux of urea, but this difference did not reach significance ($P_{GXT}=0.08$). The ornithine efflux was significantly increased ($P_{GXT}=0.03$) by endotoxin infusion (Figure 4A) while no significant citrulline net flux across the liver was detectable ($P>0.05$) in any of the groups. The arginine influx into the liver (Figure 4A) was higher ($P_{GXT}=0.02$) in the endotoxin than in the control group. Figure 4B shows that these effects could be ascribed to increased ($P_{GXT}=0.00$) arginine disposal (from 913 ± 23 (controls) to 1150 ± 71 nmol kg^{-1} min^{-1}) and unchanged arginine production. The 24-h endotoxin infusion induced a significant increase ($P_{GXT}=0.03$) in NO synthesis

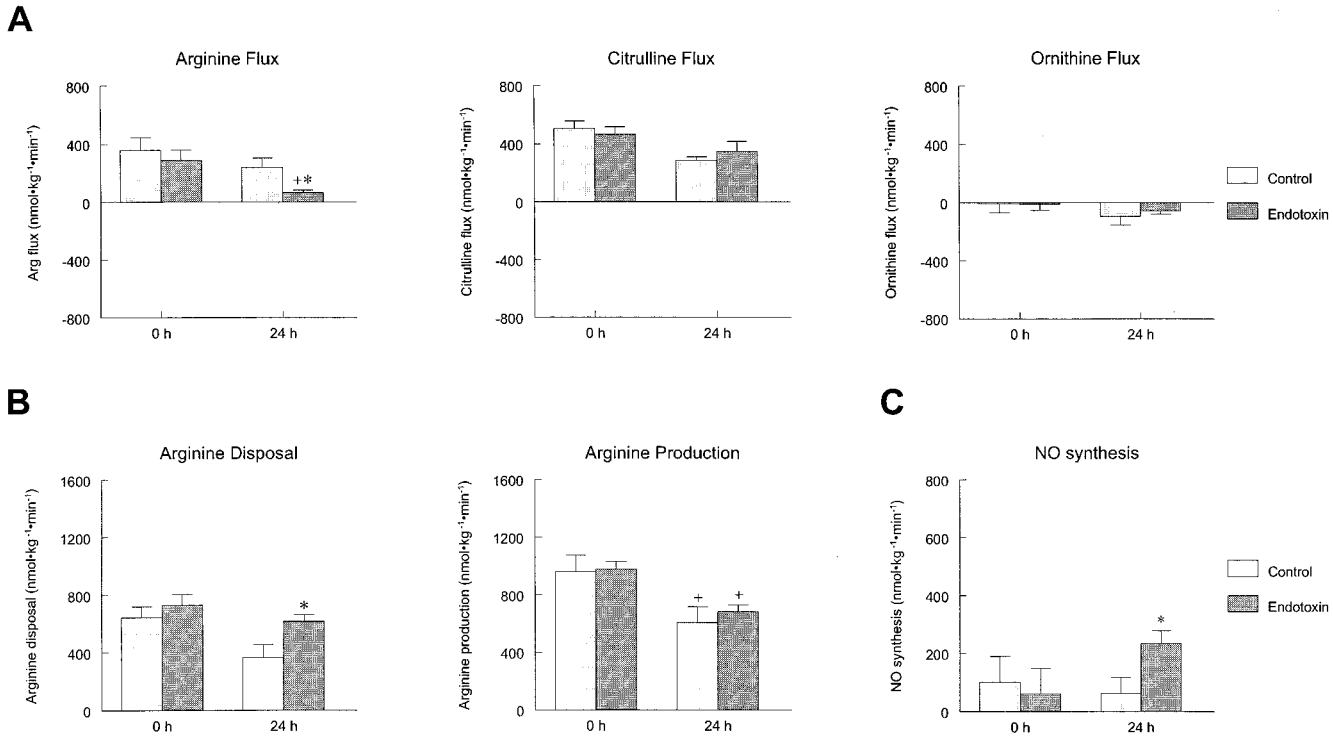

Figure 3 Portal-drained viscera (A) net fluxes (+ = efflux, − = influx) of arginine, citrulline and ornithine, (B) arginine disposal and production rates, and (C) nitric oxide synthesis rate in the postabsorptive state before (0 h) and 24 h after starvation plus infusion of saline (control) or endotoxin. Statistics by ANOVA: $*P<0.05$; significantly different from control group and $^{+}P<0.05$; significantly different from 0 h.

Table 1 Body weight, urinary output, and organ plasma flow in fasting pigs, before and 24 h after saline (control) or endotoxin infusion

	0 h		*24 h*		*ANOVA*		
	Control	*Endotoxin*	*Control*	*Endotoxin*	P_G	P_T	$P_{T\times G}$
Body weight (kg)	22.5±0.7	22.5±0.5	23.6±1.9	23.9±1.6			
Urinary output (L day^{-1})	1.2±0.2	1.0±0.1	2.3±0.1^{+}	2.0±0.3^{+}		0.05	
Flow (mL·kg^{-1}·min^{-1})							
PDV	28±4	36±5	26±4	34±1	0.02		
Liver	39±6	46±3	48±4	56±4	0.06	0.06	
Muscle	25±3	30±6	32±7	43±4			

Data are mean±s.e.mean. ANOVA: significant group (P_G), time (P_T) or group × time ($P_{G\times T}$) effect. Student's *t*-test: $^{+}P<0.05$; significantly different from 0 h within group.

Table 2 Arterial blood values and plasma flow in fasting pigs, before and 24 h after saline (control) or endotoxin infusion

	0 h		*24 h*		*ANOVA*		
	Control	*Endotoxin*	*Control*	*Endotoxin*	P_G	P_T	$P_{T\times G}$
Haematocrit (%)	29.1±1	28.7±0.8	27.9±1.3	24.5±0.5			
pH	7.44±0.02	7.44±0.01	7.40±0.01	7.46±0.01			0.03
$PaCO_2$ (kPa)	5.64±0.46	5.58±0.12	5.36±0.29	4.69±0.22			0.06
PaO_2	12.0±0.8	12.7±0.6	11.9±0.8	13.0±0.5			
HCO_3^- (mM)	27.3±1.8	27.7±1.4	21.8±3.2	24.1±1.1			
SaO_2 (%)	97.0±0.6	97.3±0.4	96.7±0.9	97.2±0.4			

$PaCO_2$ and PaO_2, carbon dioxide and oxygen pressure; HCO_3^-: bicarbonate concentration, SaO_2: oxygen saturation. Data are mean±s.e.mean. ANOVA: group (P_G), time (P_T) or group × time ($P_{G\times T}$) effect.

by the liver (from 104±65 (controls) to 392±54; Figure 4C). Increases in arginine disposal and NO synthesis (both ~250 nmol kg^{-1} min^{-1}) nicely balanced each other (Figure 4B,C).

Hindquarter (Figure 5)

The net flux of urea across the hindquarter was not significantly different from zero ($P>0.05$) in control and

Table 3 Arterial plasma concentrations, concentrations taking dilutional effects into account and whole-body arginine turnover rate in fasting pigs, before and 24 h after saline (control) or endotoxin infusion

	0 h		24 h		ANOVA		
	Control	*Endotoxin*	*Control*	*Endotoxin*	P_G	P_T	$P_{T\times G}$
			μM				
NO_X^-	42±10	39±7	42±15	33±6			
Urea	3205±478	3282±678	1368±457	2729±227			0.01
Ornithine	118±15	98±10	79±10$^+$	58±4^{++}	0.01	0.01	
Citrulline	79±7	79±6	58±3	42±4			
Arginine	90±8	94±5	106±4	70±3			0.00
			μmol^{-1} g^{-1} protein				
NO_X^-	1.4±0.4	1.5±0.3	1.4±0.4	1.8±0.3			
Urea	90±13	92.2±19	47±17	122±17			0.01
Ornithine	3.2±0.5	3.6±1.7	2.6±0.9	2.7±0.8			
Citrulline	2.3±0.3	2.9±1	1.9±0.6	2.1±0.9			
Arginine	2.6±0.3	3.3±0.7	3.4±0.6	2.7±0.7			0.04
			μmol kg^{-1} min^{-1}				
Q_{ARG}	2.3±2.6	2.6±0.4	2.0±0.3	2.8±0.1			0.05

NO_X^-: sum of nitrite (NO_2^-) and nitrate (NO_3^-), Q_{ARG}: whole-body turnover rate of arginine. Data are mean±s.e.mean. ANOVA: group (P_G), time (P_T) or group×time ($P_{G\times T}$) effect. Student's *t*-test: $^+P<0.05$, $^{++}P<0.01$; significantly different from 0 h within group.

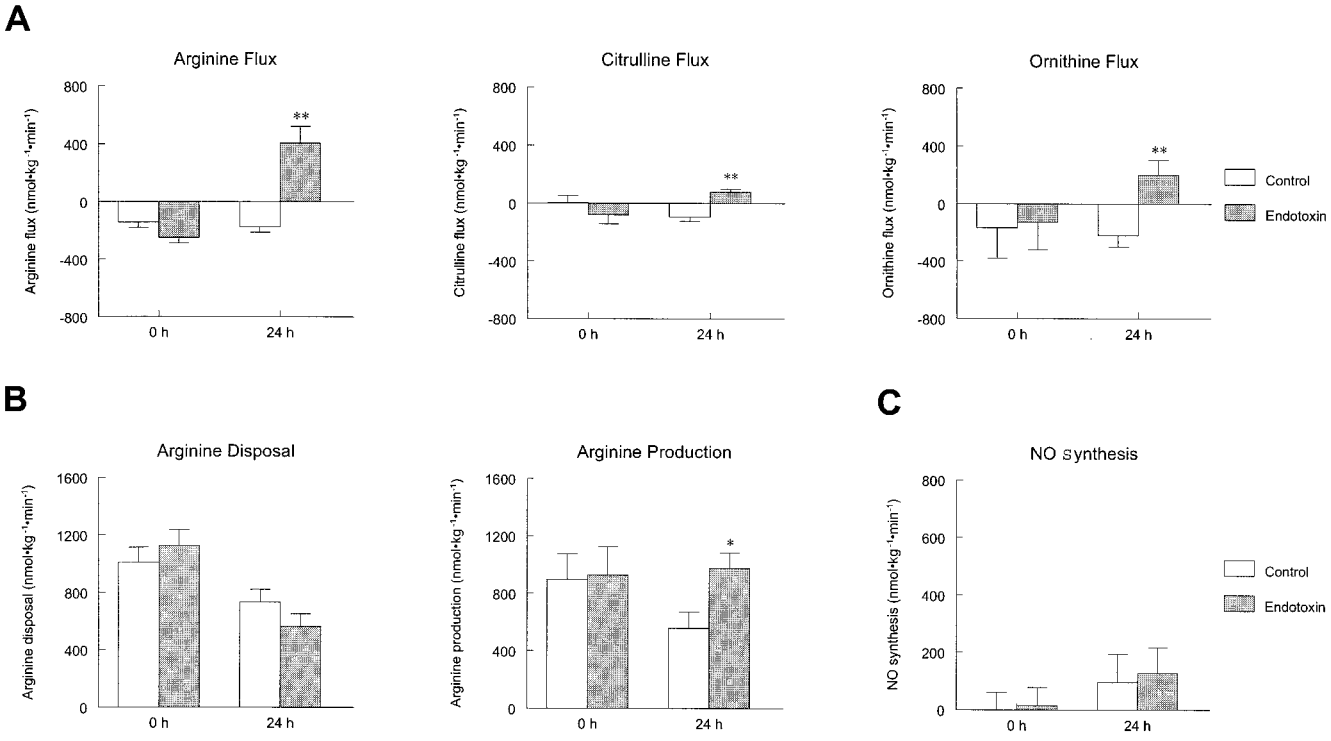

Figure 4 Liver (A) net fluxes (+ = efflux, − = influx) of arginine, citrulline and ornithine, (B) arginine disposal and production rates, and (C) nitric oxide synthesis rate in the postabsorptive state before (0 h) and 24 h after starvation plus infusion of saline (control) or endotoxin. Statistics by ANOVA: $^{**}P<0.01$, $^*P<0.05$; significantly different from control group.

endotoxin groups (not shown). When pigs were treated with endotoxin, the hindquarter influx of ornithine, citrulline, and arginine significantly ($P_{GXT}<0.01$) turned into an efflux (Figure 5A). The change from arginine influx to efflux by endotoxin intervention was due to a significant (almost 2 fold) higher ($P_{GXT}=0.04$) arginine production compared to controls (Figure 5B). Assuming that the hindquarter represents 50% of the whole-body muscle, the hindquarter arginine production in both the basal and endotoxaemic state (~900 nmol kg^{-1} min^{-1}) accounted for approximately 70–80% of the systemic whole-body arginine turnover (Table 3). The net release of arginine from the hindquarter muscle (~400 nmol kg^{-1} min^{-1}) covered the entire net uptake of arginine by the splanchnic region. The NO synthesis measured by the hindquarter was not significantly different from zero ($P>0.05$) in any of the groups and was not affected by endotoxin infusion (Figure 5C).

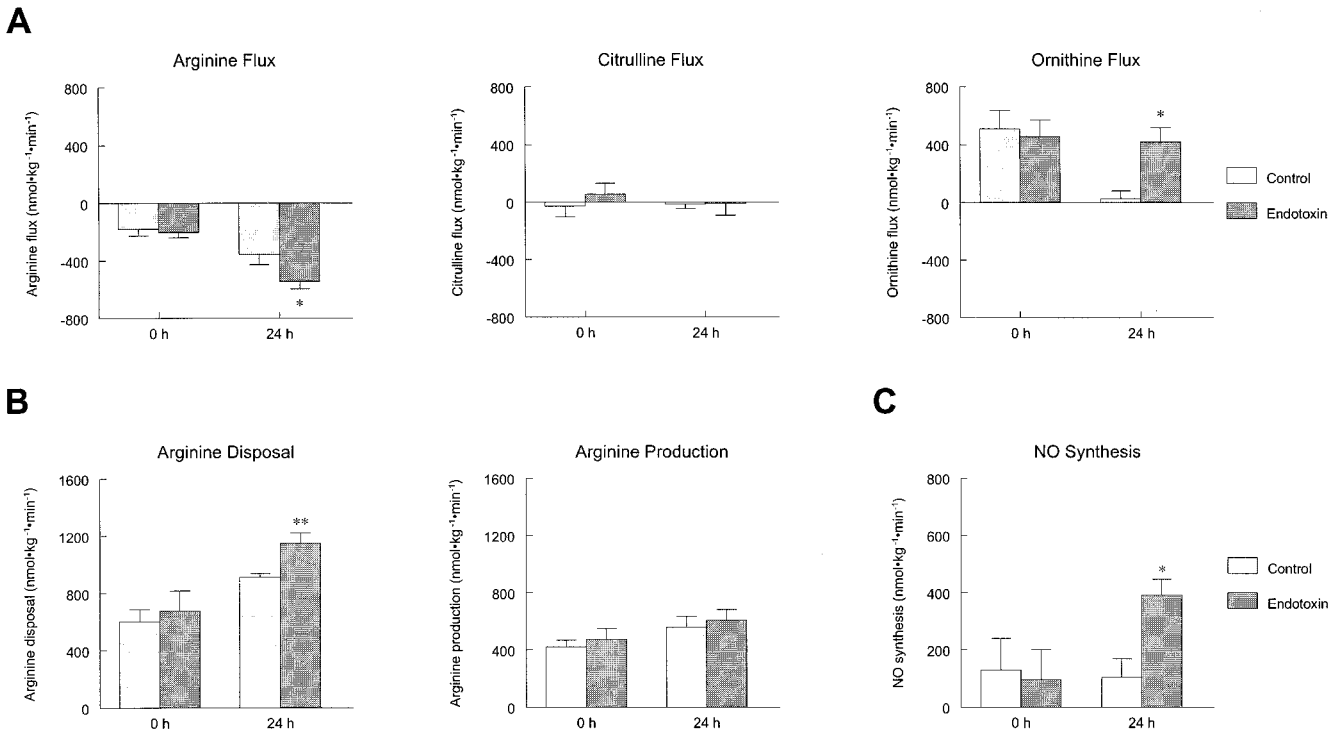

Figure 5 Hindquarter (A) net fluxes (+ = efflux, − = influx) of arginine, citrulline and ornithine, (B) arginine disposal and production rates, and (C) nitric oxide synthesis rate in the postabsorptive state before (0 h) and 24 h after starvation plus infusion of saline (control) or endotoxin. Statistics by ANOVA: $^{**}P<0.01$, $^{*}P<0.05$; significantly different from control group.

Discussion

In order to understand organ NO production and requirements for arginine as substrate for NO during sepsis, we performed a study in which stable isotope techniques were used to quantify arginine and NO metabolism in the portal-drained viscera, liver and hindquarter in pigs. Our observation that endotoxin challenge increases the $^{15}N_2$-arginine to ^{15}N-citrulline conversion in the portal-drained viscera and liver indicates that the NO production in these organs is increased during endotoxaemia.

Haemodynamics

In severely ill patients splanchnic hypoperfusion causes slow endotoxin release from the gut (Hynninen *et al*., 2000; Withlin *et al*., 1997). This slow leakage of small amounts of endotoxin into the circulation elicits a hyperdynamic sepsis response. Therefore, very small doses of endotoxin are more representative for the hyperdynamic circulation seen in human sepsis receiving modern intensive care, whereas the hypodynamic circulation (i.e. hypotension) that is elicited by large doses of endotoxin used in experimental rodent models of sepsis is more representative for septic shock (Fink & Heard, 1990). In this porcine model of endotoxaemia, a relatively small dose of endotoxin was used (Schrauwen *et al*., 1988) in combination with generous fluid administration. The endotoxaemia model is associated with haemodynamic features that are characteristic of the hyperdynamic state of compensated human sepsis, including increased temperature, reduced MAP, elevated heart rate and cardiac output and slightly increased blood flow to the splanchnic organs. Increased lactate levels in the endotoxaemic pigs indicate lactate acidosis. The slight increase in pH in the endotoxin-infused animals indicates that respiratory alkalosis developed as a compensatory mechanism to lactic acidosis (MacKenzie, 2001). The modest decrease in arterial $PaCO_2$ during endotoxaemia may be attributed to hyperventilation resulting from increased body temperature (Frankel *et al*., 1980).

Species differences in NO production

In the rat, NOS-II expression has been described in several tissues including the liver, spleen and intestine (Cook *et al*., 1994). Whereas NOS-II mRNA expression in the intestine (Morin *et al*., 1998; Chen *et al*., 1996) and liver (Tabuchi *et al*., 2000) of rats normally peaks 2 to 6 h after intraperitoneal injection of LPS, the NOS-II protein may still be present up to 24 h after stimulation (Tabuchi *et al*., 2000). In porcine endotoxaemia, evidence for NOS-II expression is relatively scarce. Saetre *et al*. (2000) did not detect any NOS-II activity in liver, gut, and lung after an infusion with live bacteria for 5 h despite the development of haemodynamic alterations that are typical of sepsis. Thus far, only minimal increases in NOS-II were detected in the liver, lung, inflammatory cells, and small vessels of endotoxin-stimulated pigs (Javeshghani & Magder, 2001a). Pastor *et al*. (2000) reported direct evidence for induction of NOS-II expression in the liver and in the wall of the portal and inferior caval vein after intravenous endotoxin infusion. Moreover, administration of a selective NOS-II inhibitor in pigs blunted the increase in expired NO after 24-h intravenous endotoxin infusion (Ploner *et al*., 2001), indicating that NOS-II activity is present at 24 h. The presence of elevated NOS levels at 24-h of endotoxin infusion

agrees with our observation in hyperdynamic porcine endotoxaemia in which we were able to demonstrate elevated NO synthesis by the visceral organs and the liver but not by the hindquarter at 24-h endotoxin infusion. Moreover, arginine supplementation at that time point was capable of increasing the NO production by the portal-drained viscera, the liver and the kidney (Bruins *et al*., 2002). These findings imply a minor role for inducible NO production in the muscle during sepsis. Similarly, no expression of NOS-II was observed in the skeletal muscle of rats (El-Dwairi *et al*., 1998), although NOS-I and III were increased up to 24 h after endotoxin injection. A study from Javeshghani & Magder (2001b) in pigs showed that it was not the NOS-II isoform, but the constitutive NOS isoforms (NOS-I and III) that were increased in the caval vein in response to endotoxin challenge. NOS-I and III could, therefore, contribute to the increase in NO synthesis in sepsis and play a significant role in the haemodynamic alterations resulting from endotoxin challenge. On the other hand, we did observe a reduction in endotoxin-induced whole-body NO synthesis in this porcine model of endotoxemia by administering the selective NOS-II inhibitor aminoethyl-isothiourea (article in preparation). Assuming that the inhibitor has a similar specificity towards porcine as towards rat NOS isoforms, this finding still argues for a role of NOS-II in the endotoxemia-induced upregulation of NO synthesis.

Comparison with other methods to assess NO production rates in the pig

Using the $^{15}N_2$-arginine to ^{15}N-citrulline conversion method we measured a basal NO synthesis rate of $\sim$190 nmol kg^{-1} min^{-1} by the sum of muscle (estimated from 2 $\times$ hindquarter) and splanchnic organs. Although not reflecting the whole-body synthesis rate, there was a good agreement between this NO synthesis rate by the sum of organs and the basal whole-body NO synthesis rate measured in pigs (Bruins *et al*., 2002) and mice (Hallemeesch *et al*., 2002) that were both $\sim$300 nmol kg^{-1} min^{-1} using the same methodological approach. An approximately 18 fold lower basal whole-body NO synthesis rate was reported for healthy fasted human volunteers measured by the same isotope conversion method (Castillo *et al*., 1996). Higher whole-body metabolic turnover rates, and thus, NO synthesis rates, in smaller species relative to humans may only partly explain the dissimilarity in findings. A 3 fold higher basal NO synthesis rate by the sum of measured organs in our pigs compared to whole-body NO synthesis rate in pigs measured by $Na^{15}NO_3$ infusion (Santak *et al*., 1997), is probably due to formation of NO metabolites other than nitrite. These values for basal rate of NO synthesis as measured by isotope techniques are still quantitatively higher than values determined by chemiluminescence in expired air. In pigs, the latter method found NO synthesis rates of 7 (Ploner *et al*., 2001) to 70 pmol kg^{-1} min^{-1} (Mehta *et al*., 1999). In all likelihood, a large fraction of this difference can be accounted for by NO-derived products which are not expired.

Plasma NO_x measurements

In contrast to the increase in visceral and liver NO production measured by isotope conversion, we did not measure any change in plasma levels of NO_X during endotoxin treatment. Although plasma levels of NO_X were postulated to reflect NO production in sepsis (Evans *et al*., 1993), these conclusions were largely based on rodent sepsis models. In porcine sepsis models, no changes in NO metabolites were found despite direct evidence for NOS activation, such as expired NO production (Ploner *et al*., 2001), and NOS-II mRNA induction in the liver (Pastor *et al*., 2000; Matejovic *et al*., 2001) and indirect evidence such as increased labelling of proteins with nitrotyrosine (Javeshghani & Magder, 2001b), haemodynamic changes (Cohen *et al*., 2000; Saetre *et al*., 2000), and increased ^{15}NO-labelled nitrate (Santak *et al*., 1997). Many of the rodent models of endotoxaemia to study NO production use large single doses of endotoxin (in the micro-to-milligram range) reproducing haemodynamic alterations that resemble hypodynamic circulatory shock rather than hyperdynamic sepsis. In the current hyperdynamic endotoxaemia model, pigs were challenged with a much lower dose of endotoxin that is comparable with endotoxin doses (in the nano-to-microgram range) used in other porcine models of endotoxaemia. Another complicating factor is the fluid resuscitation often applied in porcine but not in rodent sepsis models. Fluid resuscitation will reduce the residence time of NO_X in the plasma by enhancing the renal plasma flow and, with that, the clearance rate of NO_X. Accordingly, a rise in plasma NO_X concentration was observed 5 h after endotoxin stimulation in non-resuscitated mice (Hallemeesch *et al*., 2002) but not in rats that were given generous fluid resuscitation (Hallemeesch *et al*., 2000). Finally, differences in pH were reported to affect NO production although the relationship between NO production and pH remains controversial (Matejovic *et al*., 2001). In pigs, alkalosis was associated with higher nitrate levels (Ploner *et al*., 2001) whereas moderate acidosis in rats also increased exhaled NO and plasma NO_X concentrations (Pedoto *et al*., 1999). Because the alkalosis in our endotoxaemic pigs was relatively mild, we do not expect any influence on organ NO synthesis or plasma NO_X levels.

Whole-body turnover rate of arginine

In the pig, the increase in arginine turnover at whole-body level is predominantly determined by the increase in arginine production by the hindquarter. The latter is expectedly the result of increased protein catabolism in the skeletal muscle, which is a prominent feature of the metabolic response to inflammatory stimuli (Bruins *et al*., 2000; Gore *et al*., 1995). The arginine released from the hindquarter largely accounts ($\sim$70–80%) for the arginine appearing at whole-body level. The arginine net released from the hindquarter matched the arginine net taken up by the splanchnic region ($\sim$400 nmol kg^{-1} min^{-1}) implying that the muscle is an important supplier of arginine for splanchnic arginine consumption. Although arginine derived from accelerated muscle protein breakdown is a large source of increased whole-body arginine turnover, increased *de novo* synthesis from citrulline in the kidney may also provide extra circulating arginine as was demonstrated in endotoxaemic mice (Hallemeesch *et al*., 2002). It may be speculated that the severity of an insult plays an important role in the quantitative alterations in arginine fluxes. The increase in whole-body arginine turnover from 2.0 to 2.8 μmol kg^{-1} min^{-1} that

was observed in our porcine model of endotoxaemia is similar to the 0.8 μmol kg^{-1} min^{-1} increase in arginine flux reported in fasted severely burned patients (Yu *et al*., 2001). This may be due to the fact that trauma and sepsis share many potential mediators (increases in stress hormones and cytokine secretions). Moreover, it is of interest that these patients with burns showed a higher whole-body arginine to ornithine conversion (Yu *et al*., 2001). Besides for the accelerated arginine disposal through the NOS pathway, the arginase pathway may therefore, under stress conditions, importantly contribute to an increased arginine turnover rate.

Arginine availability and source of NO

The increased flux through the arginase and, to a lesser degree, NOS pathways by inflammatory stimuli such as endotoxin and cytokines may, in the long run, contribute to the frequently observed arginine depletion of plasma arginine levels in endotoxin-treated rats (Desmukh *et al*., 1997; Roland *et al*., 1999; Lortie *et al*., 2000) and septic humans (Freund *et al*., 1979). We showed a significant decline (from 120 to 70 μM) in plasma concentration of arginine under endotoxaemic conditions, even though whole-body arginine production was increased. Formation of NO by NOS is linearly dependent on the extracellular arginine substrate concentration (Granger *et al*., 1990; Wu *et al*., 1999), probably reflecting the kinetics of arginine transport across the cell membrane (Traber, 2002). Extracellular arginine availability is therefore a critical step in NO formation. Under conditions of low circulating arginine levels, the availability of arginine from blood may become rate-limiting for cellular NO synthesis (Schott *et al*., 1993; Durante *et al*., 1996; Nicholson *et al*., 1998; Lorente *et al*., 1999). Although arginine levels were near the half-saturating concentration of 60–70 μM for NOS-dependent NO synthesis (Granger *et al*., 1990), visceral and hepatic NO synthesis were clearly increased after endotoxin infusion. Many cell types, including endothelial cells (Hecker *et al*., 1990), smooth muscle cells (Hattori *et al*., 1994) and macrophages (Nawabi *et al*., 1990) have the capacity to regenerate arginine from citrulline. This capacity, to some extent, predicts intracellular arginine for cellular NO production. Considering the low circulating arginine levels, increased arginine *de novo* synthesis from citrulline may be a tool for NOS-containing cells to meet the increased demand for arginine. The increase in arginine disposal by the portal-drained viscera and liver during endotoxaemia matched the increase in NO synthesis by these organs. NO synthesis made up $\sim$35% of total arginine metabolism in both the portal-drained viscera and the liver. Because endotoxin or endotoxin-associated increases in cytokines can induce expression of arginase-I in rat liver (Tabuchi *et al*., 2000), mouse macrophages (Granger *et al*., 1990; Wang *et al*., 1995), spleen and kidney (Ochoa *et al*., 2000), and of arginase-II in mouse peritoneal macrophages (Salimuddin *et al*., 1999), the increase in arginine consumption by the portal-drained viscera and liver may not only represent increased arginine conversion by NOS, but also by arginase.

The current findings show that during hyperdynamic endotoxemia in the pig, NO synthesis by the splanchnic organs is increased and associated with enhanced arginine disposal, while the hindquarter functions as a major supplier of extracellular arginine. In contrast to NO_X measurements in plasma, the $^{15}N_2$-arginine to ^{15}N-citrulline conversion method appears to be a reliable quantitative measure of NO synthesis rates.

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