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Systematic evaluation of nitric oxide, tetrahydrobiopterin, and anandamide levels in a porcine model of endotoxemia

Tetsuaki Hashiguchi[1], Yasuyuki Kakihana[2], Sumikazu Isowaki[1], Tamotsu Kuniyoshi[1], Tatsuya Kaminosono[1], Etsuro Nagata[1], Kazumi Tobo[1], Masamichi Tahara[1], Naoko Okayama[1], Yuki Arakawa[3], Yoshie Kakihara[3], Tsuyoshi Goromaru[3], Nobuo Nakanishi[4], Hiroe Nakazawa[5], and Yuichi Kanmura[1]

[1] Department of Anesthesiology and Critical Care Medicine, Kagoshima University Graduate School of Medical and Dental Science, Kagoshima, Japan
[2] Division of Intensive Care Medicine, Kagoshima University Hospital, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan
[3] Department of Radiopharmaceutics, Fukuyama University, Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama, Japan
[4] Department of Biochemistry, Meikai Dental University, Sakado, Japan
[5] Department of Physiology, Tokai University School of Medicine, Isehara, Japan

Abstract
Purpose. Using a lipopolysaccharide (LPS)-treated porcine model, we examined: (1) whether nitric oxide (NO), anandamide, and tetrahydrobiopterin (BH4) increased or not in early endotoxic shock; and (2) the location of the major site of production of these molecules, by comparing their concentrations in arteries and the portal and hepatic veins.
Methods. Ten pigs received an infusion of LPS at 1.7 $\mu g \cdot kg^{-1} \cdot h^{-1}$ via the portal vein for 240 min. Consecutive changes in systemic hemodynamics, hepatosplanchnic circulation, and oxygen delivery were measured. Furthermore, the variable changes in the concentrations of nitrite and nitrate (NOx), anandamide, and BH4 were measured. To access the effects of surgery, anesthesia, and fluid management on BH4, an experiment without LPS infusion was performed in two other animals.
Results. Mean arterial pressure and cardiac index started to decrease at 60 min after LPS infusion. However, systemic vascular resistance remained unchanged. Total hepatic blood flow and hepatic oxygen delivery also decreased significantly. NOx and anandamide did not change during LPS infusion. BH4 values did not change without LPS infusion. However, BH4 values increased significantly in the arterial, portal, and hepatic circulation during LPS infusion, especially in the hepatic vein (from 136.8 ± 27.5 to 281.3 ± 123.2 mol/ml; $P < 0.01$).
Conclusion. Our data suggest that the BH4 values were significantly increased in several organs, especially in the liver during endotoxic shock. Impaired cardiac output and decreased blood pressure appeared in the early phase of porcine endotoxemia. Longer-term observation of these parameters after LPS treatment should be performed as the next step in future studies.

Address correspondence to: Y. Kakihana
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Key words Septic shock · Nitric oxide · Tetrahydrobiopterin · Anandamide

Introduction

Septic shock is a frequently encountered serious clinical condition and still remains a major cause of multiple organ dysfunction/failure and death in intensive care units [1–3]. The potentially lethal inflammatory response is initiated by the release of a cell-wall component of Gram-negative bacteria—lipopolysaccharide (LPS) [4–6]. Marked vasodilation is one of the main features, and blood pressure progressively decreases thereafter despite adequate restoration of circulating volume and vasoconstrictor therapy. It has been shown that the enhanced formation of nitric oxide (NO), due to the induction of the inducible isoform of NO synthase (iNOS), plays an important role in the development of vascular hyporeactivity to vasopressor agents [7,8] and the hypotension associated with endotoxic shock [9,10]. In addition to this well-known effect of NO, anandamide (N-arachidonoylethanolamine), an endogenous cannabinoid agonist, has been proposed as being responsible for intractable hypotension, as it elicits vasodilation via the peripherally located cannabinoid receptor (CB1) [11,12]. The production of anandamide has been shown to increase in various pathophysiological conditions, including sepsis, and the induction of CB1 has also been demonstrated in septic shock [13–15].

The production of large amounts of NO from iNOS has been shown to be associated with a simultaneous increase in the de novo biosynthesis of tetrahydrobiopterin (BH4) with iNOS induction, because BH4 is an

essential cofactor of iNOS activity [16,17]. Upregulation of the expression of iNOS, concomitant with an increase in BH4 synthesis, was demonstrated in patients with sepsis [18] and BH4 was proposed as another endothelium-derived relaxing factor (EDRF) in humans [19]. The increase in BH4 was shown in septic models of rats and beagles [18,20]. Thus, BH4 was suggested to be an important target for pharmacological interventions aimed at controlling excessive vasodilatation by inhibiting its biosynthesis during septic shock [18].

However, systematic investigation of the production of NO, anandamide, and BH4 in sepsis has not been studied. The purposes of this study were: (1) to examine whether these molecules were produced in early sepsis, and (2) to determine the major site of production of these molecules by comparing their concentrations in the arteries and portal and hepatic veins.

Methods

Animal preparation

All procedures were performed in accordance with the guidelines for the care and use of laboratory animals at Kagoshima University research laboratories and were approved by the local ethics committee of Kagoshima University. Studies were performed in 12 male pigs (25–30 kg). The animals were fasted overnight but allowed free access to water. They were premedicated with an IM injection of 25 $mg \cdot kg^{-1}$ ketamine hydrochloride and 0.5 mg atropine sulfate. Anesthesia was induced by IV pentobarbital (20 $mg \cdot kg^{-1}$) and maintained with continuous inspiration of 1.0% isoflurane. After intubation, the animals were mechanically ventilated with a respirator at a tidal volume of 10 $ml \cdot kg^{-1}$ and a respiratory rate of 15 times per minute initially, but later the tidal volume was adjusted to keep the arterial carbon dioxide tension within the range of 35 to 40 mmHg. Inspired oxygen was maintained at 40% throughout the experiment. ECG leads were placed. Acetate Ringer solution was continuously infused intravenously at 20 $mg \cdot kg^{-1} \cdot h^{-1}$ through a catheter inserted from the right femoral vein. A Swan-Ganz thermodilution catheter was inserted from the right internal jugular vein into the pulmonary artery to measure cardiac output (CO). A catheter was inserted into the right femoral artery for blood pressure (BP) measurement. Through an abdominal midline incision, ultrasound transit-time flow probes (Transonic Systems, Ithaca, NY, USA) were placed around the hepatic artery and the portal vein for continuous measurement of hepatic arterial flow and portal venous flow. A double-lumen catheter (3-cm tip distance) was positioned within the portal vein via a branch of the superior mesenteric vein for the infusion of LPS (via the distal lumen) and for blood sampling (via the proximal lumen).

Experimental protocol

After a 60-min stabilization period, basal measurements of hemodynamics and blood samplings were performed in all 12 animals. Then, in 10 animals, LPS (lipopolysaccharide of *Escherichia coli* serotype 0111:B4; Sigma, St. Louis, MO, USA) was administered intravenously via the portal vein, at a dose of 1.7 $\mu g \cdot kg^{-1} \cdot h^{-1}$ continuously for 240 min. To access the effects of surgery, anesthesia, and fluid management on BH4, the same experiment, but without LPS infusion was performed in the other two animals. Blood pressure (BP), ECG, CO, pulmonary arterial pressure, central venous pressure, hepatic arterial flow (HAF), and portal venous flow (PVF) were continuously monitored. Three milliliters of blood was withdrawn from each catheter in the femoral artery, hepatic vein, and portal vein to measure concentrations of nitrate (NOx), anandamide, BH4, and lactate at the following times: basal, before LPS infusion; 150 min, and 240 min after LPS infusion. Blood gases were also measured. Systemic vascular resistance (SVR), systemic oxygen delivery (DO_2), and hepatic oxygen delivery (hDO_2) were calculated.

Quantification of NOx, anandamide, and BH4

We measured NOx with the Griess reagent after reducing nitrate to nitrite with an on-line cadmium column (JASCO, Tokyo, Japan) [18], as direct measurement of NO is difficult because NO rapidly metabolizes to nitrite and nitrate (NOx). Plasma anandamide was measured using liquid chromatographic-atmospheric pressure chemical ionization mass spectrometric (LC-MS) determination, as previously described [21]. The BH4 concentration was obtained by measuring total biopterin, using reverse-phase high performance liquid chromatography (HPLC) with fluorescence detection after acidic oxidation with iodine, as previously described [18,22].

Statistical analysis

Values for all results are expressed as means ± SD, with statistical evaluation done by repeated-measure one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) for multicomparisons, each value being compared with the basal value (0 min). The criterion of significance was $P < 0.05$.

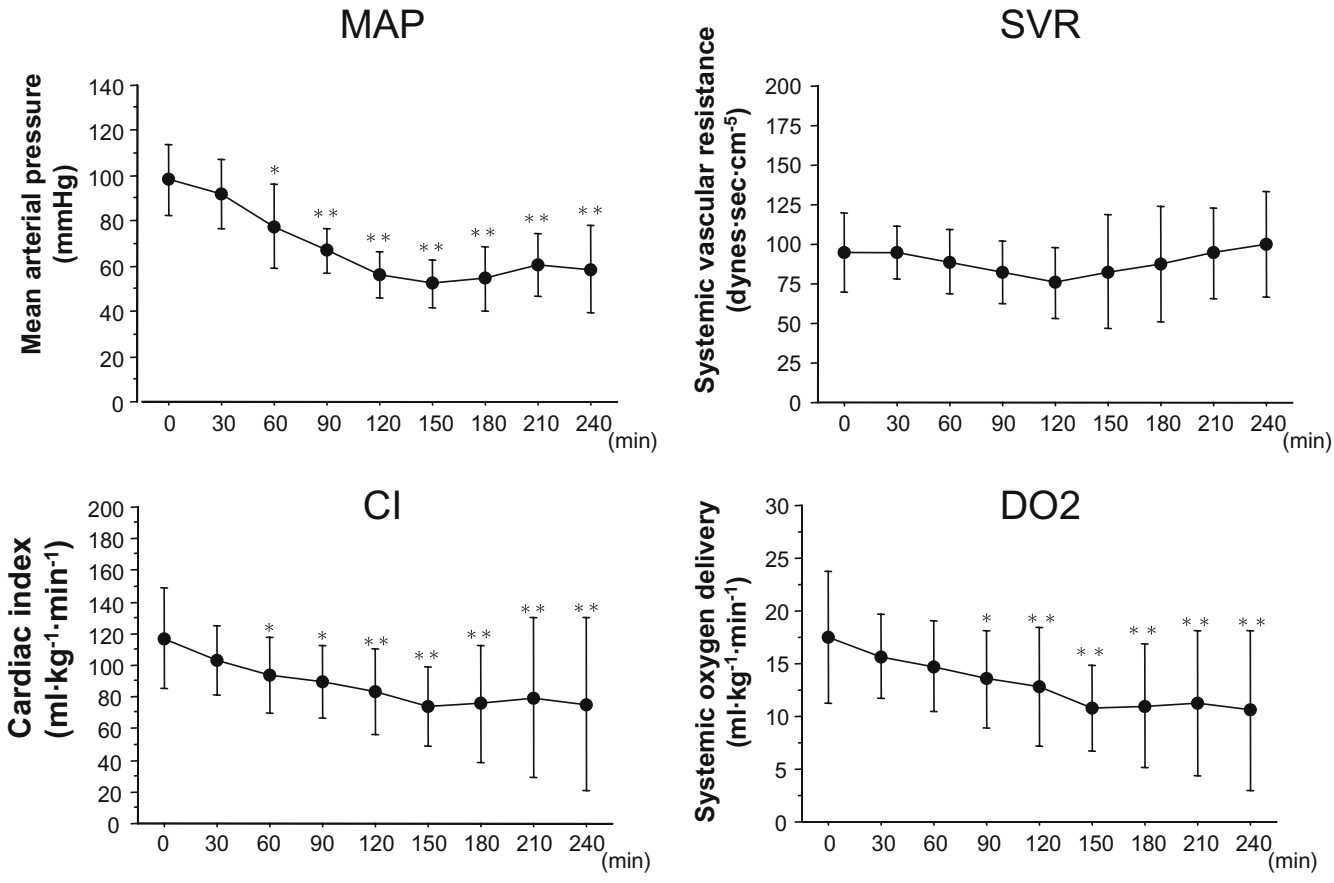

Fig. 1. Effects of lipopolysaccharide (LPS) on systemic hemodynamics and systemic oxygen delivery. LPS was infused via the portal vein at a rate of 1.7 $\mu g \cdot kg^{-1} \cdot h^{-1}$ for 240 min. *P < 0.05 vs basal values (0 min); **P < 0.01 vs basal values (0 min). Data values are expressed as means ± SD. *MAP*, Mean arterial pressure; *CI*, cardiac index; *SVR*, systemic vascular resistance; *DO2*, systemic oxygen delivery

Results

Hemodynamic changes

Continuous infusion of LPS (1.7 $\mu g \cdot kg^{-1} \cdot h^{-1}$, via the portal vein) for 240 min caused a significant decrease in mean arterial pressure (MAP; from 98.3 ± 15.7 to 58.6 ± 19.3 mmHg; $P < 0.01$) and cardiac index (CI; from 117 ± 32 to 75 ± 55 $ml \cdot kg^{-1} \cdot min^{-1}$; $P < 0.01$), while systemic vascular resistance (SVR) showed no significant changes during LPS infusion. Systemic oxygen delivery (DO_2) decreased significantly, from 17.5 ± 6.3 to 10.5 ± 7.5 $ml \cdot kg^{-1} \cdot min^{-1}$ ($P < 0.01$; Fig. 1). Arterial lactate concentration increased significantly during LPS infusion (from 1.4 ± 0.3 to 3.0 ± 1.1 $mmol \cdot l^{-1}$; $P < 0.01$; Fig. 2).

Splanchnic vascular bed (especially liver blood flow)

Portal venous flow (PVF) showed a progressive reduction, and hepatic arterial flow (HAF) showed a transient increase, from 60 to 90 min, followed by a maintained decrease during LPS infusion. PVF + HAF (total hepatic blood flow; THBF) showed a significant decrease, from 16.5 ± 4.0 to 11.7 ± 5.2 $ml \cdot kg^{-1} \cdot min^{-1}$ ($P < 0.01$). Furthermore, hepatic oxygen delivery (hDO_2) showed a significant decrease, from 1.99 ± 0.67 to 1.09 ± 0.63 $ml \cdot kg^{-1} \cdot min^{-1}$ ($P < 0.01$), and lactate concentration increased significantly in the portal vein (from 1.6 ± 0.3 to 3.2 ± 1.1 $mmol \cdot l^{-1}$; $P < 0.01$) and in the hepatic vein (from 0.8 ± 0.4 to 2.3 ± 1.2 $mmol \cdot l^{-1}$; $P < 0.01$) (Figs. 2, 3).

Changes in plasma BH4 levels

At the basal condition (0 min), there was a slight difference among the arterial, portal, and hepatic venous BH4 concentrations (0 min, 107.6 ± 29.6 $pmol \cdot ml^{-1}$, 120.8 ± 29.2 $pmol \cdot ml^{-1}$, and 136.8 ± 27.5 $pmol \cdot ml^{-1}$, respectively), and all these values increased significantly during LPS infusion (240 min, 221.5 ± 113.6 $pmol \cdot ml^{-1}$;

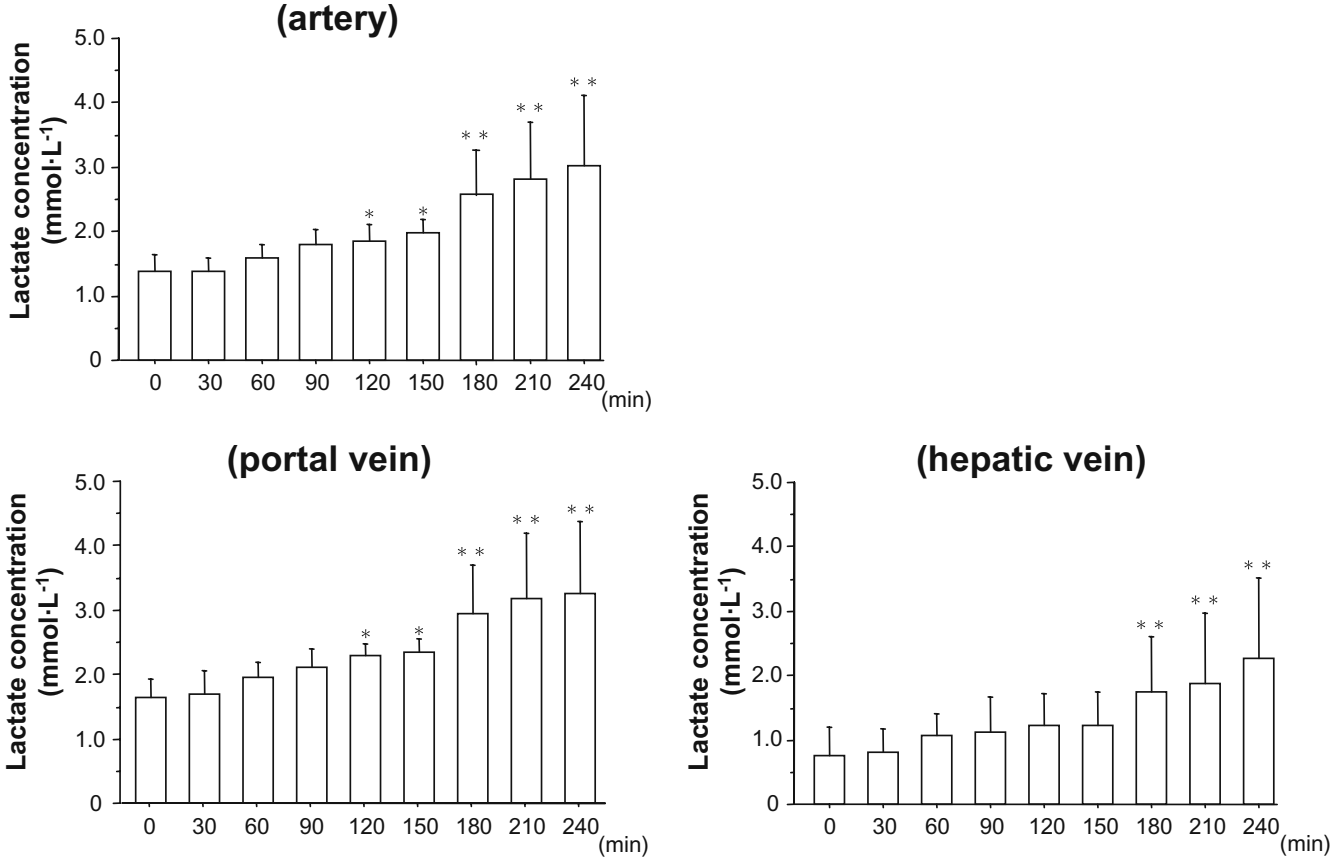

Fig. 2. Changes in lactate concentration in the arterial, portal, and hepatic venous circulatory systems during lipopolysaccharide (LPS) infusion. LPS was infused via the portal vein at a rate of 1.7 $\mu g \cdot kg^{-1} \cdot h^{-1}$ for 240 min. Data values are expressed as means ± SD. *$P < 0.05$ vs basal values (0 min); **$P < 0.01$ vs basal values (0 min)

$P < 0.01$, 241.4 ± 109.7 $pmol \cdot ml^{-1}$; $P < 0.01$, and 281.3 ± 123.2 $mol \cdot ml^{-1}$; $P < 0.01$, respectively; Fig. 4A). On the other hand, the mean values of BH4 in the two animals without LPS infusion scarcely changed during the experiment (Fig. 4B).

Changes in plasma anandamide levels

Anandamide concentrations showed no significant changes during LPS infusion in artery, portal vein, and hepatic vein (0 min, 1.54 ± 1.06 $pmol \cdot ml^{-1}$, 1.79 ± 1.00 $pmol \cdot ml^{-1}$, and 1.87 ± 1.29 $pmol \cdot ml^{-1}$; 240 min, 1.79 ± 0.79 $pmol \cdot ml^{-1}$, 2.01 ± 1.27 $pmol \cdot ml^{-1}$, and 2.08 ± 1.16 $pmol \cdot ml^{-1}$, respectively; Fig. 5).

Changes in plasma NOx levels

Arterial, portal, and hepatic venous plasma NOx levels did not show any significant changes during LPS infusion (0 min, 78.8 ± 68.0 μM, 78.2 ± 76.0 μM and 67.4 ± 71.3 μM; 240 min, 82.3 ± 70.0 μM, 84.4 ± 63.6 μM, and 73.8 ± 66.9 μM, respectively; Fig. 6).

Discussion

In porcine models of endotoxemia, it has been reported that the primary hemodynamic effect of LPS infusion is an increase in pulmonary artery pressure and an increase in systemic vascular resistance, followed by a reduction in cardiac output (CO) [23–26]. In porcine endotoxemia, an increase in pulmonary vascular resistance is also typical [27], which indicates that the porcine pulmonary circulation is very sensitive to vasoconstrictors, or that it produces considerable amounts of vasoconstrictors such as endothelin-1. This well-known porcine pulmonary hypertension, which is unusual in human sepsis, may be a limitation regarding the relevance of the pig as a model of human sepsis. Further, in resuscitated human sepsis, there is regularly a hyperdynamic circulation, in which systemic vascular resistance is typically low and CO is high [28]. In the present study, systemic vascular resistance showed no significant changes, and CO was steadily reduced during LPS infusion (hypodynamic circulation). Despite the differences between human and porcine hemodynamic response

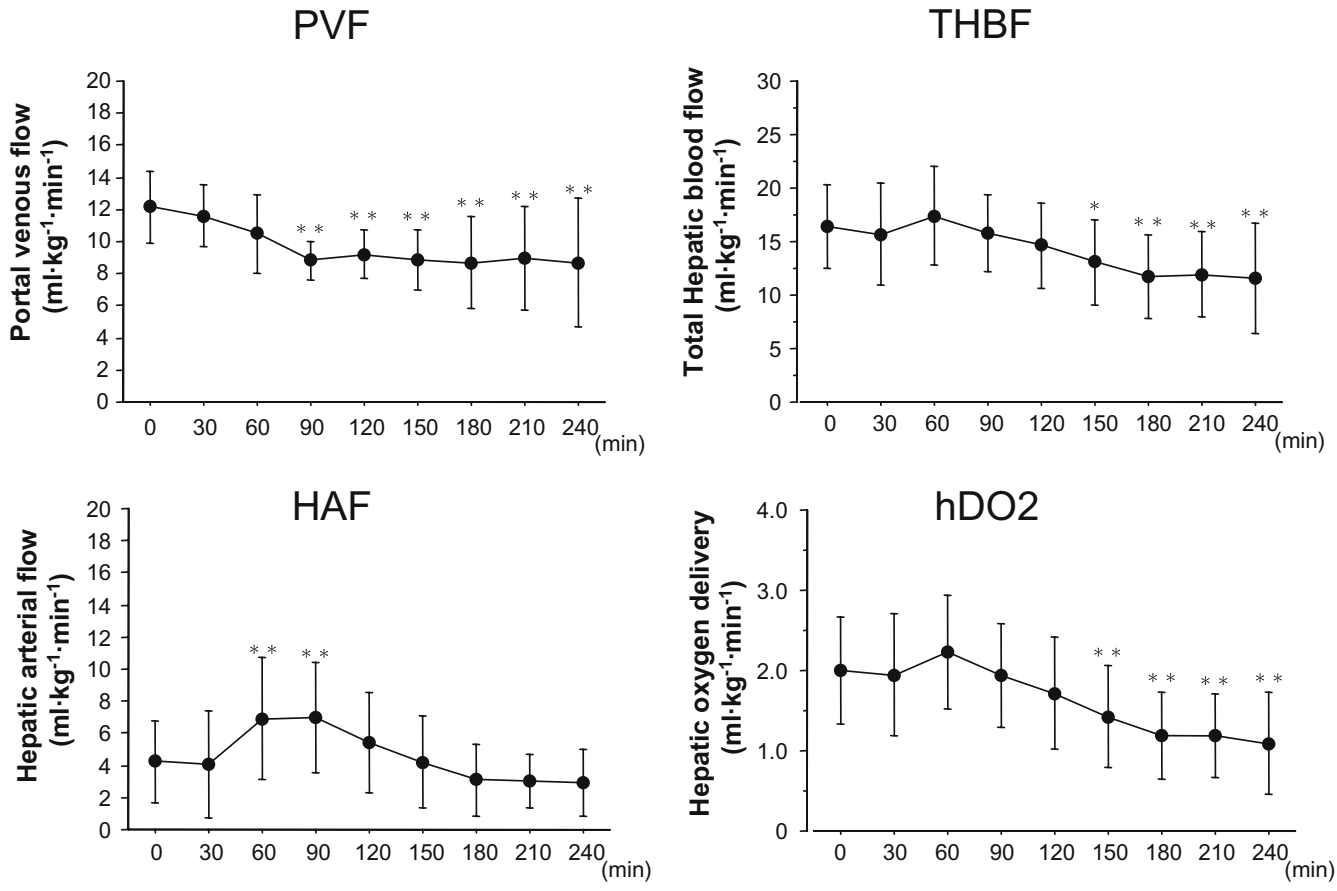

Fig. 3. Effects of lipopolysaccharide (LPS) on splanchnic hemodynamics and hepatic oxygen delivery. LPS was infused via the portal vein at a rate of 1.7 μg·kg^{-1}·h^{-1} for 240 min. *P < 0.05 vs basal values (0 min); **P < 0.01 vs basal values (0 min). Data values are expressed as means ± SD. *PVF*, Portal venous flow; *HAF*, hepatic arterial flow; *THBF*, total hepatic blood flow; hDO_2, hepatic oxygen delivery

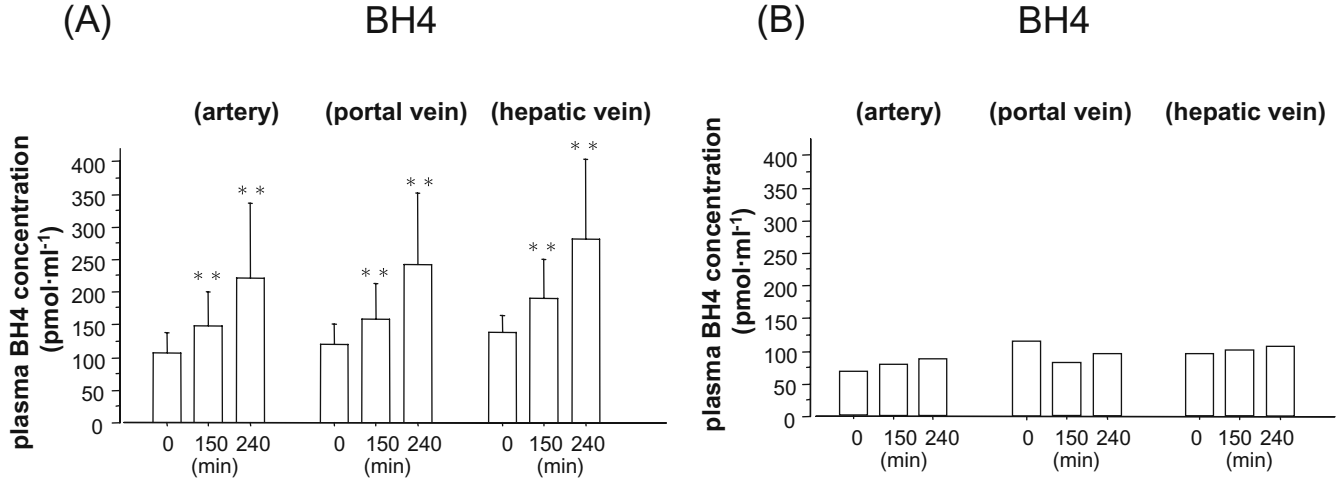

Fig. 4A,B. Changes in plasma tetrahydrobiopterin (*BH4*) levels in the porcine model with lipopolysaccharide (LPS) infusion (**A**) or without LPS infusion (**B**) in the artery and portal and hepatic veins. LPS infusion was done at a rate of 1.7 μg·kg^{-1}·h^{-1} for 240 min. Data values are expressed as means ± SD. **P < 0.01 vs basal values (0 min)

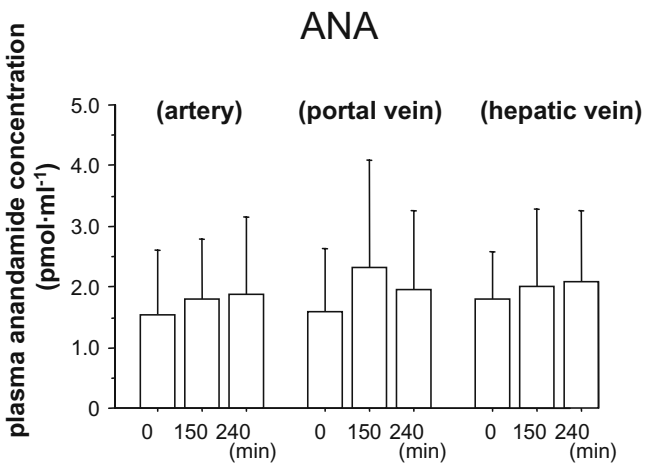

Fig. 5. Changes in plasma anandamide (*ANA*) levels in the arterial, portal, and hepatic venous systems during lipopolysaccharide (LPS) infusion. LPS was infused via the portal vein at a rate of 1.7 $\mu g \cdot kg^{-1} \cdot h^{-1}$ for 240 min. Data values are expressed as means ± SD

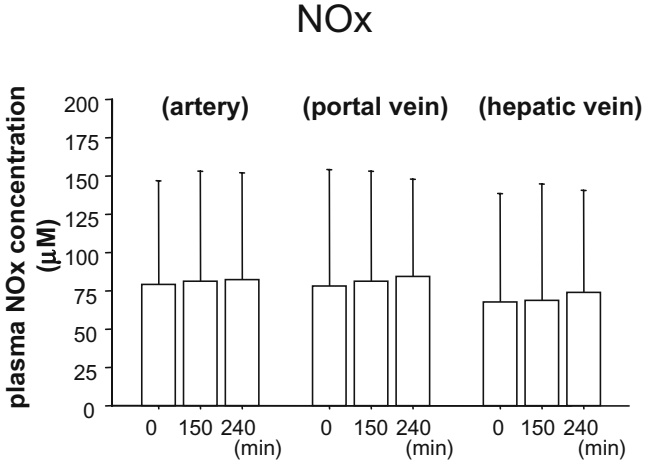

Fig. 6. Changes in plasma nitrate (*NOx*) levels in the arterial, portal, and hepatic venous systems during lipopolysaccharide (LPS) infusion. LPS was infused via the portal vein at a rate of 1.7 $\mu g \cdot kg^{-1} \cdot h^{-1}$ for 240 min. Data values are expressed as means ± SD

patterns, the porcine model of septic shock has been widely used in cardiovascular research and to examine the pathophysiology of multiple organ dysfunction/failure [29–31]. The use of large animals has several advantages compared with shock models in small animals, including mice, rats, and rabbits. The major advantage is the availability of information from local organs or circulation. Thus, we selected the porcine model, because measurements of the time course of NOx, anandamide, and BH4 concentrations in systemic and splanchnic blood could be performed during the continuous monitoring of the systemic and local hemodynamics. We used the portal vein as the site of LPS administration to simulate human sepsis, because inadequate removal of LPS in the liver plays a major role in sepsis [32,33]. Mean arterial pressure (MAP) started to decrease 60 min after LPS administration, concomitant with the decrease in CO, evaluated as the cardiac index (CI), as shown in Fig. 1. The vascular resistance remained unchanged and even increased during the later stage of monitoring. Lactate concentrations remained unchanged until 60 min after LPS administration, but doubled at 240 min. Total hepatic blood flow (hepatic arterial flow + portal venous flow) remained unchanged until 120 min after LPS administration and declined thereafter, and hepatic oxygen delivery (hDO_2) decreased significantly. The above findings indicate that this model is representative of typical septic shock with splanchnic vascular flow failure.

Regarding the three molecules—NOx, anandamide, and BH4—the former two showed no increase in blood taken from the artery or hepatic or portal veins. In contrast, concentrations of BH4 were significantly increased in blood taken from all three circulatory systems at the 240-min assessment point. Our results suggested that the significant increase in BH4 was caused by LPS infusion (but not by surgery, anesthesia, or fluid management; Fig. 4B). The concentration of BH4 tended to be highest in the hepatic vein. In a previous study, it was shown that both BH4 and NOx concentrations were significantly elevated in patients with septic shock compared with the concentrations in healthy volunteers [18]. The discrepancy regarding BH4 and NOx levels in this study may be explained by the following pieces of evidence: (1) the plasma NOx concentration may not reflect dynamic changes of NO released into the blood-stream, because NO reacts rapidly with hemoglobin; (2) it has been shown that NOx was not an appropriate parameter to study the course of septicemia in a rabbit model [34] and the induction of iNOS by LPS was not significant in swine sepsis, though it was marked in rats and mice [35]; and (3) the induction of BH4 production may precede iNOS induction and NOx production, which do not peak until 6 h after LPS treatment [36].

The de-novo synthesis of BH4 requires GTP cyclohydrolase 1, the rate-limiting enzyme of BH4, and BH4 increases in tissues and plasma in animal models of septic shock through the induction of GTP cyclohydrolase 1 [37,38]. However, the cascade of BH4 induction differs markedly among various mammalian species and tissues, and basal BH4 levels vary markedly in various organs. Increased BH4 production and the physiology of hypotensive shock may indicate a role not only of iNOS but also of the constitutive isoform of the enzyme in the vascular endothelium [39]. Endothelial NOS (eNOS) plays a fundamental role in organ blood flow

distribution and is involved in the regulation of microvascular permeability and platelet, leukocyte, and endothelial interaction, without significantly increasing plasma NOx levels. In fact, eNOS activation has been implicated as a cause of early septic shock. The liver showed the highest value of BH4 compared with the aorta, heart, kidney, and lungs [19,38–40], and both isoforms (iNOS and eNOS) are involved in the hepatic vasodilation that occurs after LPS infusion. Thus, in the present study, abundant basal BH4 in the liver may have been responsible for the increase of BH4 in the hepatic vein without elevation of NOx.

Anandamide is an endogenous agonist of the cannabinoid receptor (CB1) that exhibits cannabinoid-like activity, and vasodilation is one of its several physiological roles [11–15]. Recent studies using rodent models of LPS-induced shock have suggested that anandamide is synthesized by activated macrophages and platelets, and contributes to the hypotension in such shock [11]. In addition, the CB1 exposed to anandamide was shown to be coupled to NO release [41,42]. We expected to find an association between anandamide and NOx in the present study. However, there was no change in the levels of either molecule. Taken together, the results of the present study showed that the induction of iNOS and anandamide did not occur in the early stage of the LPS-induced hypotensive condition; the increase of BH4, with a higher tendency seen in the hepatic vein, suggested that the amount of BH4 present in the basal condition may have been responsible for this result. Considering that the systemic vascular resistance increased even though systemic BP decreased, the mechanism underlying the decrease in BP appears to be decreased CO, possibly due to impaired myocardial contractility induced by the direct effect of LPS [43].

Summary

In this porcine model of early septic shock we showed the following: (1) systemic pressure decreased, starting from 60 min after LPS administration, which coincided with a decrease in CO; (2) the total hepatic blood flow (hepatic arterial + portal flows) was significantly decreased from 150 min after LPS administration; (3) oxygen delivery to the liver remained until 120 min after LPS administration; (4) NOx and anandamide did not change; (5) BH4 gradually increased in blood taken from the arterial, portal, and hepatic venous circulation and tended to show the highest value in the hepatic circulation; and (6) impaired CO appeared to be responsible for the decreased BP in the early phase of LPS treatment in this porcine model. Longer-term observation of these parameters after LPS treatment should be performed as the next step in future studies.

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