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NITRIC OXIDE PRODUCTION AND HEPATIC DYSFUNCTION IN PATIENTS WITH POSTOPERATIVE SEPSIS

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SUMMARY

1. Although hepatic function is well known to deteriorate following bacterial infection, the underlying mechanisms remain poorly understood. We have previously reported that nitric oxide (NO) radical leads to a decrease in the ketone body ratio (KBR) and in ATP content due to the inhibition of mitochondrial electron transport in primary cultured rat hepatocytes.

2. To evaluate the effects of NO radical on the liver in patients with postoperative sepsis, we analysed both the stable end-product of nitric oxide radical (NOx) as well as the arterial KBR (AKBR), which reflects liver tissue NAD^+/NADH.

3. Twenty patients who had undergone general abdominal surgery and who developed postoperative sepsis were divided into two groups: (i) surviving; and (ii) non-surviving. Blood samples were collected before the development of postoperative sepsis and every 3 days until the patient either died or was discharged from hospital.

4. Plasma NOx levels in seven patients who subsequently died became progressively higher than those in the 13 surviving patients over the clinical course of postoperative sepsis.

5. In the non-surviving group, the AKBR was significantly lower than in surviving patients, indicating impaired hepatic function. In contrast, plasma NOx levels in non-surviving patients were significantly higher than in surviving patients.

6. Decreases in AKBR to levels below 0.7 in non-surviving patients followed high NOx levels. Moreover, plasma NOx levels were closely correlated with the AKBR, indicating that NO radical is associated with mitochondrial dysfunction in the liver.

7. It is likely that the overproduction of NO radical plays an important role in causing fatal metabolic disorders in patients with postoperative sepsis.

Key words: cytokine, end-product of nitric oxide radical, hepatic failure, ketone body ratio, metabolic disorder, mitochondrial dysfunction, NAD^+/NADH ratio, systemic inflammatory response syndrome.

INTRODUCTION

The liver is involved in many processes and its failure has severe consequences for metabolism, immune responses, detoxification and antimicrobial defence. In addition to cytokines and eicosanoids, nitric oxide (•NO) may be involved in the modification of liver function during sepsis.[1–5] Nitric oxide is a highly reactive, diffusible gas that is produced in a number of tissues and exerts a wide range of physiological and pathophysiological effects. Inducible nitric oxide synthase (iNOS) is produced in the liver and •NO may protect the liver under some circumstances,[4] but also has the potential to promote injury.[5]

The septic response in humans appears to be a disease state in which an infectious agent induces a state of disordered metabolic control in the host. The abnormal regulation of metabolic pathways causes a diversion of substrate use towards gluconeogenesis, ketone body formation and a reduction in oxidative energy producing metabolism. Clinical studies have shown that the arterial ketone body ratio (AKBR; the ratio of acetoacetate to β-hydroxybutyrate) is closely correlated with the NAD^+/NADH ratio of liver tissue[6] and that changes in the AKBR are well correlated with the metabolic capacity of the diseased liver. This ratio has been used as a clinical tool that predicts the outcome of patients with shock[7] and postoperative organ failure[8] and the postoperative outcome of patients who have undergone major hepatic[9] or cardiac surgery.[10] In particular, Ozawa *et al*.[8] have suggested that patients with an AKBR below 0.7 or 0.4 show a high incidence of the development of postoperative organ failure and a high mortality rate.

Previous studies in other laboratories have demonstrated that •NO affects mitochondrial energy metabolism.[11–14] We have reported that •NO formation is stimulated by interleukin (IL)-1β in cultured hepatocytes and by lipopolysaccharide in Kupffer cells[15] and that •NO released by IL-1β inhibited ATP synthesis by causing mitochondrial dysfunction.[16] Thus, our *in vitro* study demonstrated a decrease in the ketone body ratio (KBR) and an inhibition of ATP synthesis mediated by •NO via the following pathway: IL-1β → increase in iNOS mRNA → •NO formation → mitochondrial dysfunction → decrease in KBR and inhibition of ATP synthesis.[15,16] Therefore, it is likely that augmented •NO formation induced by the initial inflammatory mediator in the pathway causes the decrease in the KBR and the inhibition of ATP synthesis through mitochondrial dysfunction. Recently, we have found that the greater the surgical stress, the longer the duration of decreased plasma levels of the stable end-product of nitric oxide radical (NOx) in patients without infectious complications, thus indicating that surgical stress does not lead to increased plasma NOx levels.[17]

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Although a number of studies have reported that •NO plays a major role in circulatory shock, there is limited knowledge regarding the effects of •NO on metabolic alterations, particularly in the clinical setting. Thus, we hypothesized that •NO may cause hepatic mitochondrial dysfunction in patients with postoperative sepsis. To evaluate the effects of •NO on the liver, the present study was designed to measure both the stable end-product of •NO, plasma NOx anions, as well as the AKBR, which is an index of the hepatic mitochondrial NAD^+/NADH ratio, in patients with postoperative sepsis.

METHODS

Patients

This study was performed according to the principles of the Declaration of Helsinki. Informed consent was obtained in all cases. From June 1996 to August 1997, 179 laparotomies were performed in the surgical unit of the Kansai Medical University. We had routinely measured the AKBR and NOx in critically ill patients, including those with sepsis, haemorrhagic shock and hepatic failure, as well as in patients subjected to hepato–pancreato–biliary or gastrointestinal surgery. Of these patients, 20 had postoperative sepsis. These 20 patients, who met the criteria of a systemic inflammatory response syndrome (SIRS) and had a documented bacterial infection, were examined in the present study (mean (±SEM) patient age 65.0±2.4 years; male : female ratio 15 : 5).

These 20 patients were divided into two groups based on their clinical courses (Table 1). The surviving group consisted of 13 patients, 12 with postoperative intra-abdominal abscesses and one with pneumonia. The non-surviving group consisted of seven patients, six with intra-abdominal abscesses and one with pneumonia.

Systemic inflammatory response syndrome and sepsis were defined according to the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference.[18] Systemic inflammatory response syndrome was defined as two or more of the following: a temperature > 38 or < 36°C; heart rate > 90 b.p.m.; respiratory rate > 20 /min or P_{aCO_2} < 32 mmHg; white blood cell count > 12.0×10^9/L, < 4.0×10^9/L or the presence of > 0.10 immature forms. Sepsis included the criteria for SIRS plus a clinically identified site of infection. The site of infection was recorded from the patient's medical records, including bacteriological and radiological studies. Treatments for the infections included fluid resuscitation and empirical antibiotic therapy. Operative management or percutaneous drainage included the elimination of infectious foci by closing, excluding or resecting the primary source and by evacuating any contaminated fluids.

Table 1 Demographic characteristics, operative procedures, APACHE scores and biological data on sepsis day 0

	Surviving group	Non-surviving group	*P*
No. patients	13	7	
Age (years)	62.5±12	69.7±5	NS
Gender (male : female)	9 : 4	6 : 1	
Operative procedure			
Hepatectomy	6	2	
Pancreatectomy	3	1	
PD	2	1	
Extended cholecystectomy	0	2	
Gastrointestinal surgery	2	1	
APACHE score	9.9±1.5	12.7±2.1	NS
Biological data			
WBC (/μL)	13 700±2800	10 900±1800	NS
PLT ($\times 10^4$/μL)	28.3±5.0	20.2±5.0	NS
BUN (mg/dL)	24.5±6.0	34.6±4.0	< 0.05
Creatinine (mg/dL)	1.0±0.2	1.5±0.2	< 0.05
Total protein (g/dL)	6.7±0.2	6.4±0.5	NS
Total bilirubin (mg/dL)	1.6±0.4	3.5±1.2	NS
CRP (mg/dL)	8.5±2.4	7.8±1.1	NS

Data are the mean±SEM.

PD, pancreatoduodenectomy; APACHE, acute physiology and chronic health evaluation; WBC, white blood cell count; PLT, platelet count; BUN, blood urea nitrogen; CRP, C-reactive protein.

Samples

All blood samples were obtained under overnight fasting or continuous fasting and were drawn on the pre-operative day and continued to be collected until either the day of patient's discharge or death. Because we have reported that there were no significant differences in plasma NOx levels between the peripheral vein and artery, blood samples were obtained from the peripheral vein or artery.[17] Blood samples were drawn into a heparinized syringe. We designated the day when sepsis was initially diagnosed as 'sepsis day 0' and blood samples were collected from then once every 3 days until the patient's discharge or death. Plasma NOx levels, as the end-product of •NO, and the AKBR, which is an index of the hepatic mitochondrial NAD^+/NADH ratio, were measured, together with the usual biological data, and evaluated between surviving and non-surviving groups. The time course in the present study was from the pre-sepsis day to sepsis day 9, because one case in the surviving group was discharged on sepsis day 11. Reference values for the plasma NOx levels in human blood were determined by measuring eight samples from healthy volunteers.

Measurements of plasma NOx

Plasma samples obtained using heparin as an anticoagulant were used for NOx determination. Plasma was obtained by centrifugation for 10 min at 1000 *g* and samples were then stored at –70°C until use. All samples were measured within 2 weeks of sampling. We measured nitrite plus nitrate anions using the chemiluminescence method, as reported previously.[15–17] With respect to sample preservation, we have confirmed that plasma NOx levels are stable at –70°C for at least 6 months (S Satoi *et al*., unpubl. obs. 1998).

Measurements of the AKBR

The arterial ketone bodies (acetoacetate and β-hydroxybutyrate) were measured enzymatically using a commercial kit (Sanwa Chemical, Nagoya, Japan) and an automatic analyser (KETO-340; Ihara Electric Co., Kasugai, Japan).[19] Hypoxic subjects (arterial oxygen tension < 70 mmHg) and subjects with hypotension (systolic pressure < 80 mmHg) were excluded from the study because hypoxia and hypotension lead to decreases in AKBR. The AKBR was calculated only when plasma glucose levels were sufficient (> 120 mg/dL) in order to avoid fasting-induced increases in ketone bodies.

Biological data

On sepsis day 0, biological data were compared between the surviving and non-surviving groups with respect to red and white blood cell (RBC and WBC, respectively) and platelet counts and levels of serum urea nitrogen (BUN), creatinine, transaminase, total protein, bilirubin, glutamate pyruvate transaminase (GPT), lactate dehydrogenase (LDH), C-reactive protein (CRP), prothrombin time and plasma sugar. In addition, the Acute Physiology and Chronic Health Evaluation (APACHE) score[20] was investigated in both groups. Biological data were evaluated once every 3 days after sepsis day 0.

Criteria of organ failure

Organ systems considered were the lungs, liver, kidneys, heart and gastrointestinal tract and the blood clotting mechanisms. Bleeding stress ulcers and failure of the ventilatory system, requiring mechanical respiratory support, provided objective evidence of organ failure. Hepatic failure was defined as serum total bilirubin levels > 3 mg/dL, glutamic oxaloacetic transaminase > 100 IU/L and glutamic pyruvic transaminase > 100 IU/L; renal failure was defined as a BUN level > 50 mg/dL and a serum creatinine level > 3 mg/dL;

coagulopathy was defined as a prothrombin time < 70% and a fibrinogen level < 100 mg/dL; cerebral failure was determined by investigating responses to painful stimuli; heart failure was defined as no response to inotropic agents.[8] A number of failing organs were observed in both of the groups.

Statistical analysis

Data are expressed as the mean ± SEM. For the analysis of time-dependent variations in parameters within the same group, a one-way analysis of variance (ANOVA) for multiple comparisons with the Scheffé's correction was used. Similarly, for the comparisons of data between two groups, a two-way repeated-measures ANOVA was performed using the Mann–Whitney *U*-test. Correlations were analysed by Pearson's test and line, cubic or multiple regression analyses were performed. $P < 0.05$ was considered statistically significant. The StatView 4.51 program (Abacus Concepts Inc., CA, USA) was used for all statistical analyses.

RESULTS

Plasma NOx levels in healthy volunteers averaged 30.3 ± 3.6 μmol/L, which was comparable to levels reported previously.[21] Plasma NOx levels were significantly higher in patients with postoperative sepsis on sepsis day 0 compared with healthy volunteers (83.1 ± 13.8 *vs* 30.3 ± 3.6 μmol/L, respectively; $P < 0.005$). During the period of observation, plasma NOx levels in the non-surviving group were significantly higher than in the surviving group (102.2 ± 10.2 *vs* 44.0 ± 5.0 μmol/L (range 15.3–251.8 and 9.2–116.9), respectively; $P < 0.0001$). The time course of changes in plasma NOx levels between the non-surviving and surviving groups was significantly different over sepsis days 0–9 ($P = 0.0003$; Fig. 1). On sepsis days 0 and 3, 6 and 9, plasma NOx levels in the non-surviving group were higher than in the surviving group ($P < 0.05$ and 0.005, respectively). After sepsis day 9, plasma NOx levels did not increase in the surviving group, because treatments for infection were successful. In contrast, these levels changed together with clinical events in the non-surviving patient group.

During the period of observation, the AKBR in the non-surviving group was significantly lower than in the surviving group (1.06 ± 0.10 *vs* 1.43 ± 0.10 (range 0.29–2.65 and 0.51–2.26), respectivelt; $P < 0.01$). The lowest AKBR values in the non-surviving and surviving groups were 0.48 ± 0.09 and 1.61 ± 0.20, respectively and the lowest AKBR in the non-surviving group was significantly lower than that in the surviving group ($P < 0.0005$). Levels of the AKBR in the non-surviving group decreased below 0.7; patients in the surviving group had transient decreases in the AKBR below 0.7. Thus, increased NOx levels and decreased AKBR were observed in patients in the non-surviving group.

With respect to the relationship between plasma NOx levels and the AKBR, when the AKBR was below 1.0, as was normally observed in healthy volunteers,[19] plasma NOx levels were inversely correlated with the AKBR ($r = -0.481$; $P = 0.008$ linear regression analysis; Fig. 2). In the non-surviving group, AKBR on sepsis day 9 were lower than those on the presepsis day (0.50 ± 0.13 *vs* 1.15 ± 0.06, respectively; $P < 0.05$). Moreover, in the non-surviving group, increases in NOx levels occurred before decreases of the AKBR below 0.7, which is thought to be the critical point for survival.[8]

However, renal function had to be taken into account for the evaluation of NOx, because the end-products of ·NO metabolism are excreted into the urine. With respect to NOx and various independent variables (WBC count, platelet count, blood creatinine, blood total bilirubin, blood GPT, blood LDH, CRP levels and the AKBR), multiple regression analyses revealed a very close correlation ($r = 0.661$; $P < 0.05$) when the AKBR was below 1.0. Moreover, the partial *F* ratio exceeded the significant level only for the AKBR ($P < 0.05$), but was not significant for serum creatinine (Table 2). This indicates that NOx is independently correlated with only the AKBR of variables analysed. Multiple regression analysis, including liver

Table 2 Multiple regression analysis between nitrate and nitrite levels and independent variables

Independent variables	*n*	*r*	*P*
AKBR	29	−0.511	0.012
WBC	29	−0.175	NS
PLT	29	0.142	NS
Creatinine	29	0.162	NS
Total protein	29	0.062	NS
Total bilirubin	29	−0.100	NS
CRP	29	0.337	NS

Data show the results of multiple regression analysis between nitrate and nitrite levels (NOx) and independent variables (arterial ketone body ratio (AKBR), white blood cell count (WBC), platelet count (PLT), creatinine, total protein, total bilirubin and C-reactive protein (CRP)) when the AKBR was below 1.0.

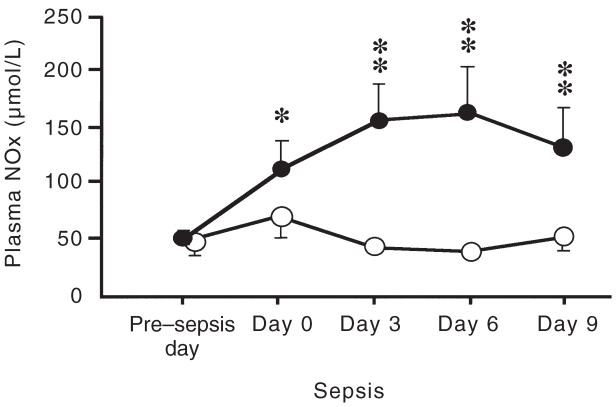

Fig. 1 Time course of changes in plasma nitrate and nitrite (NOx) levels in non-surviving (●) and surviving (○) groups. The time course was significantly different over sepsis days 0–9 ($P = 0.0003$). On sepsis days 0 and 3, 6 and 9, plasma NOx levels in the non-surviving group were significantly higher than those in the surviving group (*$P < 0.05$, **$P < 0.005$).

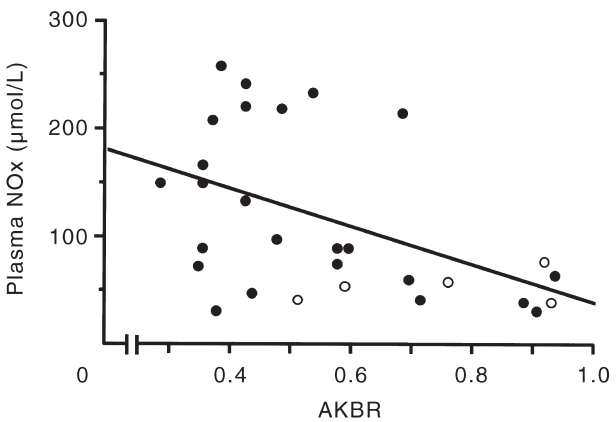

Fig. 2 When the arterial ketone body ratio (AKBR) was below 1.0, plasma NOx levels correlated highly with the AKBR ($r = -0.481$; $P = 0.008$ linear regression analysis). (●), non-surviving patients; (○), surviving patients.

function tests, such as blood GPT and blood LDH, also showed the same results.

The period of observation of the NOx and AKBR in surviving and non-surviving groups was 26±5.5 and 33±7.4 days (range 11–69 and 15–65), respectively, and the difference was not significant. On sepsis day 0, serum creatinine levels in the non-surviving group were significantly higher than in the surviving group (1.5±0.3 *vs* 1.0±0.2 mg/dL, respectively; $P < 0.05$). There were no significant differences in the APACHE score between the surviving and non-surviving groups (9.9±1.5 *vs* 12.7±2.1, respectively). The maximum number of failing organs in the surviving and non-surviving groups during the entire period was > 3 (including hepatic failure) and < 3 organs, respectively.

DISCUSSION

On the day when sepsis was initially diagnosed, plasma NOx levels were higher in septic patients than in healthy volunteers. Within septic patients, plasma NOx levels in non-surviving patients became progressively higher than in the surviving patients. These results show that continuously high plasma NOx levels may be associated with the clinical course of patients with postoperative sepsis.

Bacterial infections are often complicated by hepatic dysfunction in humans. Despite extensive efforts, the mechanisms responsible for this complication remain poorly understood. A number of *in vitro* studies, which have been recently reviewed,[22] have suggested •NO as one of the possible mediators for this hepatic dysfunction. The liver is thought to be one of the main sources of •NO and cytokine production, because a large portion of the reticuloendothelial system is present in the liver. During acute inflammation, the expression of iNOS leads to increased production of •NO in the whole body. We have previously reported that •NO can be formed in the liver, based on observations using cultured hepatocytes and Kupffer cells,[15] which is in agreement with other reports.[23,24] Moreover, •NO release stimulated by IL-1β has been shown to lead to a decrease in the KBR, which was probably caused by an inhibition of electron transport in the mitochondria, thus resulting in a decreased ATP content in hepatocytes.[16] Kurose *et al.*[12] have also reported that •NO directly inhibits hepatocyte mitochondrial function and causes lethal hepatocyte injury. Moreover, Stadler *et al.*[14] have suggested that tumour necrosis factor-α and NO inhibit mitochondrial state 3 respiration.

The end-products of •NO metabolism have been shown to be increased in patients with sepsis,[21,25] cancer[26] and liver transplantation.[27] Furthermore, Doughty *et al.*[28] have reported that increased plasma nitrite and nitrate concentrations are associated with the development of multiple organ failure in paediatric sepsis. However, there have been no reports on the relationship between •NO and hepatic mitochondrial function and, therefore, we examined the relationship between plasma NOx levels and metabolic derangement in the liver clinically.

In order to assess hepatic mitochondrial function clinically, Ozawa *et al.* proposed the redox theory, in which the AKBR reflects the hepatic mitochondrial redox potential (NAD^+/NADH).[6–10,19,29–31] Because the enzyme that catalyses the conversion of these ketone bodies is localized exclusively in hepatic mitochondria, a decrease in the acetoacetate/β-hydroxybutyrate ratio in the hepatic mitochondria is rapidly reflected by the AKBR.[9] Numerous clinical studies have confirmed the usefulness of AKBR measurements in patients with haemorrhagic shock,[7] acute heart failure,[10] hepatic failure[30] and multiple organ failure[8] and those undergoing hepatectomy[9,31] and liver transplantation.[32,33] Although Matsushita *et al.*[34] suggested that the AKBR can be affected by many factors, Ozawa *et al.*[6] have reported that the NAD^+/NADH ratio in the liver tissues of surgical patients was reflected by the AKBR. Moreover, Schlichtig *et al.*[35] have reported that the hepatic venous KBR was independent of changes in the absolute ketone body levels associated with various degrees of lipolysis and that a close correlation exists between hepatic parenchymal KBR, hepatic venous KBR and the AKBR. Thus, it is believed that the AKBR accurately reflects the hepatic KBR, indicating the hepatic mitochondrial redox potential (NAD^+/NADH).

In the present study, the cause of death in non-surviving group was multiple organ failure, including hepatic failure. In the non-surviving group, the AKBR were markedly decreased, which indicated impaired hepatic function, whereas plasma NOx levels were markedly increased. Moreover, increased NOx levels occurred before decreases of AKBR below 0.7. With respect to the relationship between plasma NOx levels and AKBR, plasma NOx levels were inversely correlated with the AKBR in all septic patients. Thus, in patients with high NOx levels throughout the whole body, it is likely that •NO is one of the inhibitory factors of electron transport in the liver mitochondria in patients with liver failure following postoperative sepsis.

Renal dysfunction, which leads to the retention of end-products of •NO metabolism, must be taken into account in order to interpret the results from the present study. However, our results showed a close correlation only between AKBR and plasma NOx levels. Thus, renal function did not appear to influence the relationship between the AKBR and plasma NOx levels in the present study.

In conclusion, we believe that •NO plays an important role in causing fatal metabolic derangements in patients with postoperative sepsis clinically. However, we could not show a direct cause and effect relationship between plasma NOx levels and the AKBR in the present study. Further studies are necessary to determine the regulatory role of •NO in inducing metabolic alterations in patients with postoperative sepsis.

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