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Dysfunction of the Mitochondrial Respiratory Chain in the Rostral Ventrolateral Medulla during Experimental Endotoxemia in the Rat

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Key Words

Mitochondrial respiratory chain · Rostral ventrolateral medulla · Experimental endotoxemia · Respiratory enzymes · Complex I · Complex IV

Abstract

We investigated the functional changes in the mitochondrial respiratory chain at the rostral ventrolateral medulla (RVLM), the medullary origin of sympathetic vasomotor tone, in an experimental model of endotoxemia that mimics systemic inflammatory response syndrome. In Sprague-Dawley rats maintained under propofol anesthesia, intravenous administration of *Escherichia coli* lipopolysaccharide (LPS; 30 mg/kg) induced a reduction (Phase I), followed by an augmentation (Phase II) and a secondary decrease (Phase III) in the power density of vasomotor components (0–0.8 Hz) in systemic arterial pressure signals. LPS also elicited progressive hypotension, and death ensued within 4 h. Enzyme assay revealed significant depression of the activity of nicotinamide adenine dinucleotide cytochrome *c* reductase (Complexes I + III) and cytochrome *c* oxidase (Complex IV) in the RVLM during all three phases of endotoxemia. On the other hand, the activity of succinate cytochrome *c* reductase (Complexes II + III) remained unaltered. We conclude that selective dysfunction of respiratory enzyme Complexes I and IV in the mitochondrial respiratory chain at the RVLM, whose neuronal activity is intimately related to the death process, is closely associated with fatal endotoxemia in the rat.

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Introduction

Sepsis is a complex pathophysiologic state that results from an exaggerated whole-body inflammatory response to infection. It is characterized by circulatory insufficiency, altered substrate metabolism and a narrowed arterial-venous oxygen difference that may lead to multiple organ failure and death [2]. Despite advances in new-generation antibiotics, there is still substantially high mortality from sepsis and septic shock. Metabolic disturbances, abnormal regulation of blood flow and diminished utilization of oxygen at the cellular level may account for tissue damage and organ dysfunction during sepsis and septic shock [2, 19].

The primary site of cellular energy generation and oxygen consumption is the mitochondrion [16]. In the process of oxidative phosphorylation, electrons are passed along a transport chain that consists of four respiratory

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enzyme complexes arranged in a specific orientation in the mitochondrial inner membrane. These electrons are generated from the oxidation of fuel molecules by oxygen, and their transport through the respiratory chain leads to the generation of adenosine triphosphate (ATP) [16]. As the major machinery for cellular ATP production, the mitochondrion therefore presents itself as an important target for the septic cascade. Indeed, the notion of bioenergetic failure and contribution of organ failure due to mitochondrial dysfunction in sepsis has been put forth [13, 26–29, 32]. A pathogenic mechanism proposed recently [10] hypothesizes that sepsis entails cytopathic hypoxia, which involves an intrinsic derangement in cellular energy metabolism and impaired ATP biosynthesis despite sufficient oxygen supply in the vicinity of the mitochondria.

Numerous studies [9, 12, 13, 18, 23, 26, 28, 32] have examined the activity or concentration of mitochondrial respiratory enzymes during sepsis. These studies were usually based on animal models or isolated organs that have received insults of variable severity and for different durations. Understandably, conflicting results, including increased [9, 23], decreased [13, 26, 28, 32], unaltered [12] or even mixed changes [18] in mitochondrial function in sepsis, have been reported. In addition, the majority of studies on mitochondrial functions during sepsis have been carried out in liver, heart, skeletal muscle or other tissues [13, 18, 23, 26, 32], while very few reports have addressed the role of mitochondria in the central nervous system during sepsis.

In patients who succumbed to either systemic inflammatory response syndrome [38], severe brain injury [21] or organophosphate poisoning [36], we found that death is invariably preceded by a dramatic reduction or loss of the low frequency (0.004 to 0.15 Hz) components in the power spectrum of their systemic arterial pressure (SAP) signals. We further established that these low-frequency SAP signals are related to the sympathetic neurogenic vasomotor tone [35] and reflect the functional integrity of the brain stem [21]. Their origin in the brain stem was subsequently traced in animal studies [20] to the premotor sympathetic neurons in the rostral ventrolateral medulla (RVLM). As the legal definition of death in Taiwan and many developed countries is brain stem death [17], we thus have in our hands a neural substrate that is suitable for mechanistic evaluations of the death process during sepsis.

The present study was undertaken to evaluate whether changes in mitochondrial respiratory functions are associated with death due to sepsis. Based on a rat model that provides continuous information on changes in neuronal activity in the RVLM during the entire course of experimental endotoxemia [5], we found that respiratory enzyme Complexes I and IV in the mitochondrial respiratory chain of the RVLM exhibited dysfunction during the progression towards death in sepsis.

Materials and Methods

All experimental procedures were carried out in compliance with the guidelines for the care and use of experimental animals endorsed by our institutional animal care committee. All efforts were made to reduce the number of animals used and to minimize animal suffering during the experiments.

General Preparation of Animals

Experiments were carried out in 42 specific pathogen-free adult male Sprague-Dawley rats (280–350 g) that were obtained from the Experimental Animal Center of the National Science Council, Taiwan, ROC. They were housed in an animal room under conditions of controlled temperature (24–25 °C) and a 12-hour light/dark cycle. Standard laboratory rat chow and tap water were available ad libitum.

Animals were initially anesthetized with pentobarbital sodium (50 mg/kg, i.p.) for routine preparatory surgery. The trachea was intubated to facilitate ventilation, and the femoral artery was cannulated to measure SAP. Both femoral veins were also cannulated for the administration of test chemicals or anesthetic agent. After the completion of preparatory surgery, the animal was placed on a heating pad to maintain body temperature at 37 °C throughout the experiment. Thereafter, rats received an intravenous infusion of propofol (Zeneca, Macclesfield, UK) at 28 mg/kg/h. This management scheme provided satisfactory anesthetic maintenance while preserving the capacity of central cardiovascular regulation [34]. Animals were also paralyzed with intravenous infusion of pancuronium bromide (2 mg/kg/h) and were mechanically ventilated (90–100 stroke/min, 3–4 ml/stroke) by an animal ventilator (Harvard 683, South Natick, Mass., USA) to avoid hypoxia [20].

Recording and Power Spectral Analysis of SAP Signals

The arterial catheter was connected to a pressure transducer (Gould P23ID, Valley View, Ohio, USA; frequency range: DC to 200 Hz), and in turn to a universal amplifier (Gould 20-4615-58), via which SAP signals were amplified and filtered (frequency range: DC to 100 Hz). Heart rate (HR) was derived instantaneously from the SAP signals. On-line and real-time power spectral analysis of the SAP signals was carried out simultaneously according to procedures detailed previously [5, 6, 20, 34]. We quantified the spectral components of SAP signals by computing the power density of the low-frequency (BLF; 0.25–0.8 Hz) and very low-frequency (BVLF; 0–0.25 Hz) components. Our laboratory has demonstrated that these spectral components of SAP signals take origin in the RVLM [20] and reflect the prevalence of sympathetic neurogenic vasomotor tone [5, 6, 20, 34]. The power density of these vasomotor components was displayed on-line during the experiment, alongside pulsatile SAP, mean SAP and HR, in a real-time manner.

Experimental Endotoxemia

Escherichia coli lipopolysaccharide (LPS; serotype 0111:B4, Sigma, St. Louis, Mo., USA) was slowly administrated intravenously over 1–2 min at 30 mg/kg [5]. Injection of the same amount of 0.9% saline served as vehicle and volume control. Temporal changes in mean SAP, HR or power density of the vasomotor components of the SAP signals, and survival time were routinely followed for 6 h or until the animal succumbed to endotoxemia.

Isolation of Mitochondria from the RVLM

We previously reported [5] that, based on changes in the power density of vasomotor components in the SAP spectrum, rats undergo three distinct phases over the course of experimental endotoxemia. At the peak of each of these three phases (LPS group) or 15 min, 1.5 or 3 h after intravenous injection of saline (vehicle group), rats were perfused intracardially with 100 ml of warm (37 °C) saline containing heparin (100 U/ml). The brain was rapidly removed and placed on dry ice. Tissues were collected on both sides of the ventrolateral part of the medulla oblongata, at the level of the RVLM (0.5–2.5 mm rostral to the obex) [5, 6]. Samples were immediately placed in ice-cold buffer containing 0.25 *M* sucrose, 0.5 m*M* EGTA and 3 m*M* Hepes, pH 7.2 (SEH buffer). Control samples of RVLM were collected from rats before they received LPS or saline.

Isolation of rat mitochondria from the RVLM was carried out according to the procedures of Yen et al. [37]. The entire procedure was carried out at 4 °C and completed within 2 h. Tissue samples were gently homogenized in SEH buffer using a loose-fit 20-ml glass-Teflon homogenizer (Iwaki Glass, Tokyo, Japan). The homogenate was centrifuged at 800 *g* for 10 min at 4 °C, and the supernatant thus obtained was further centrifuged at 8,000 *g* for 10 min. The precipitate was collected and the above centrifugation was repeated. The final mitochondrial pellet was suspended in a minimal amount of SEH buffer and stored at −80 °C until measurement of mitochondrial respiratory enzyme activity.

Assays for Activity of Mitochondrial Respiratory Enzymes

All enzyme assays were performed using a thermostatically regulated UV/visible spectrophotometer (Utrospec 400, Amersham Pharmacia Biotech, Uppsala, Sweden). At least duplicate determination was carried out for each tissue sample in all enzyme assays. All reagents used in enzyme assays were purchased from Sigma.

Nicotinamide adenine dinucleotide (NADH) cytochrome *c* reductase (NCCR; Complexes I + III) activity was determined by the reduction of oxidized cytochrome *c* measured at 550 nm, and was calculated as the difference in the presence or absence of rotenone [8, 39]. The activity was assayed in 50 m*M* K_2HPO_4 buffer (pH 7.4) containing 1.5 m*M* KCN, 1.0 m*M* β-NADH and 20 µl (75–150 µg of protein) of mitochondrial suspension in the presence or absence of rotenone (20 µ*M*). The reaction was initiated after 2 min of stabilization by adding 0.1 m*M* cytochrome *c*, and absorbance at 550 nm was measured at 5-second intervals over the first 3 min at 37 °C. The molar extinction coefficient of cytochrome *c* at 550 nm is 18,500 M^{-1} cm^{-1}.

Determination of succinate cytochrome *c* reductase (SCCR; Complexes II + III) activity was performed in 40 m*M* K_2HPO_4 buffer (pH 7.4) containing 20 m*M* succinate, 1.5 m*M* KCN and 30 µl (120–250 µg of protein) of mitochondrial suspension [30, 39]. After 5 min of incubation at 37 °C, the reaction was initiated by adding 50 µ*M* cytochrome *c*, and absorbance at 550 nm was measured at 5-second intervals over the first 2 min at 37 °C.

Cytochrome *c* oxidase (CCO; Complex IV) activity was estimated by recording the oxidation of reduced cytochrome *c* at 550 nm [33, 39]. The activity of CCO is defined as the first-order rate constant and is calculated from the known concentration of ferrocytochrome *c* and the enzyme amount in the assay mixture [33]. The activity was assayed in 10 m*M* K_2HPO_4 buffer (pH 7.4) containing 30 µl (120–250 µg of protein) of mitochondrial suspension. After 5 min of incubation at 30 °C, 45 µ*M* ferrocytochrome *c* was added to start the reaction. The background rate was measured after the addition of $K_3Fe(CN)_6$ (1.0 m*M*). Ferrocytochrome *c* was prepared from 1 m*M* oxidized cytochrome *c* by reduction with an excess amount of $Na_2S_2O_4$ at 25 °C for 5 min. The mixture was applied to a Sephadex G-25 column to separate ferrocytochrome *c* from $Na_2S_2O_4$.

Determination of Protein Concentration

Total protein in the mitochondrial suspension was estimated by the method of Bradford [3] with a protein assay kit (Bio-Rad, Hercules, Calif., USA).

Statistical Analysis

All values are expressed as mean ± SE. One-way analysis of variance was used to assess group means, followed by the Dunnett or Scheffé multiple-range test for post hoc assessment of individual means. $p < 0.05$ was considered statistically significant.

Results

Temporal Changes in SAP, HR and Power Density of Vasomotor Components of SAP Signals during Experimental Endotoxemia

Intravenous injection of LPS (30 mg/kg) resulted in distinct temporal changes in SAP, HR and the power density of the BLF or BVLF component of SAP signals (fig. 1). As we reported previously [5], the sequence of cardiovascular events during this LPS-induced experimental endotoxemia can be divided into three phases. Phase I endotoxemia showed a reduction in the power density of vasomotor components in the SAP spectrum, which occurred immediately after injection of LPS. Phase II exhibited a reversal of these spectral signals, resulting in an augmentation of BLF and BVLF power. This was followed by Phase III, which was characterized by a secondary decrease in the power density of the vasomotor components of SAP signals.

In the present study, treatment with LPS induced an immediate hypotension, followed by a rebound increase and a secondary decrease in SAP. HR underwent an initial decrease, followed by a slight increase until shortly before the animal succumbed (fig. 1). The survival time after treatment with LPS at 30 mg/kg was 232.9 ± 12.5 min, and the durations of Phases I, II and III of endotoxemia were 27.2 ± 1.9, 117.9 ± 8.1 and 87.8 ± 8.7 min, respectively. Consistent with our observations in the

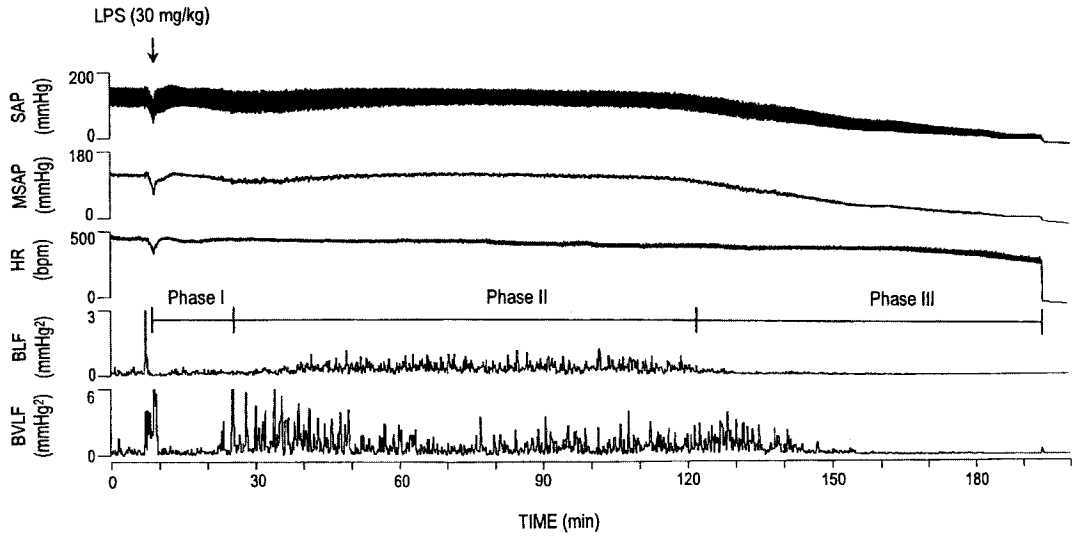


Fig. 1. Representative continuous tracings showing temporal changes in SAP, mean SAP and HR in an animal that received an intravenous injection of LPS (30 mg/kg; at arrow). Also included are displays of integrated values for the power density of BLF and BVLF components of SAP signals. Phases I, II and III denote the stages of the hemodynamic response based on changes in the power density of the BLF and BVLF components.

intensive care unit [36, 38], the commencement of Phase III endotoxemia preceded a discernible reduction in SAP.

Assessment of Mitochondrial Functions in the RVLM during Experimental Endotoxemia

We evaluated changes in mitochondrial functions in the RVLM during the progression towards death by examining the activity of key enzymes in respiratory Complexes I + III (NCCR), II + III (SCCR) and IV (CCO) during the three phases of experimental endotoxemia. The specific enzyme activity of NCCR or SCCR was expressed as nmol of reduced cytochrome *c*/min/mg of protein, and for CCO, as nmol of oxidized cytochrome *c*/min/mg of protein. It should be mentioned that the activity of all enzymes measured in control animals or during time intervals comparable to the three phases of endotoxemia in saline-treated groups displayed no significant alterations.

The activity of NCCR in the RVLM underwent a significant decrease during all three phases of experimental endotoxemia (fig. 2). Compared to the control group and saline-treated rats, the activity of NCCR in the LPS-treated rats showed an approximately 65% reduction during Phase I endotoxemia. There was a trend of reversal,

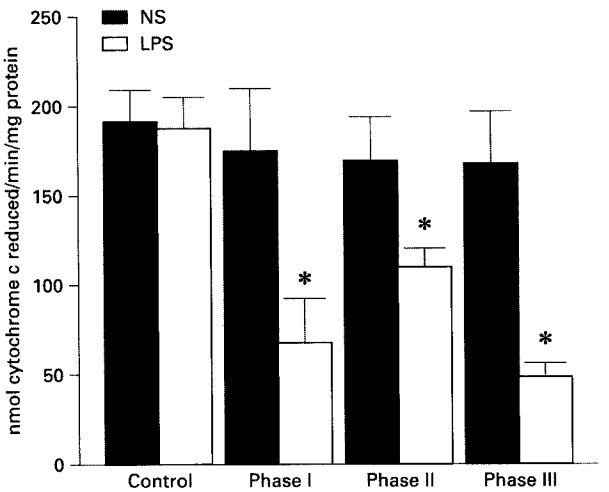


Fig. 2. Enzyme assay for activity of NCCR (Complexes I + III) in mitochondria isolated from the RVLM before and during each phase of experimental endotoxemia in LPS-treated rats or their saline controls (NS). Values are presented as mean ± SE from 3–6 samples. *$p < 0.05$ versus baseline control in the Dunnett analysis or saline-treated controls in the Scheffé analysis.

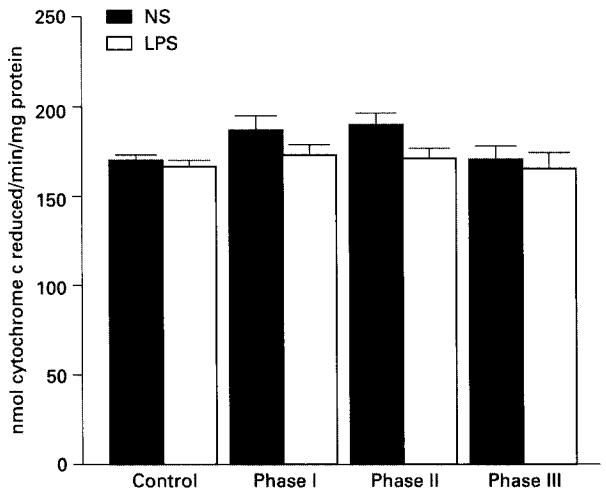


Fig. 3. Enzyme assay for activity of SCCR (Complexes II + III) in mitochondria isolated from the RVLM before and during each phase of experimental endotoxemia in LPS-treated rats or their saline controls (NS). Values are presented as mean ± SE from 3–6 samples. No significant difference was detected ($p > 0.05$) between the treatment groups.

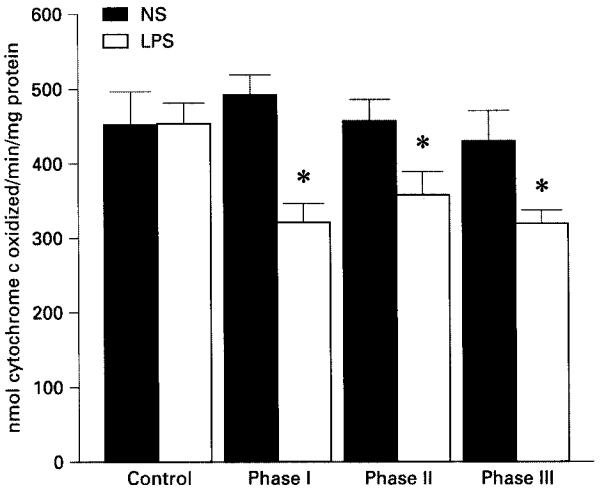


Fig. 4. Enzyme assay for activity of CCO (Complex IV) in mitochondria isolated from the RVLM before and during each phase of experimental endotoxemia in LPS-treated rats or their saline controls (NS). Values are presented as mean ± SE from 3–6 samples. *$p < 0.05$ versus baseline control in the Dunnett analysis or saline-treated controls in the Scheffé analysis.

albeit insignificant, of this reduction in Phase II, when the decline in NCCR activity amounted to 42%. This was, however, followed by a dramatic reduction in the activity of NCCR by 74% during Phase III endotoxemia.

Measurement of SCCR activity in the RVLM revealed an entirely different picture. There was no significant alteration during any of the three phases of experimental endotoxemia (fig. 3). On the other hand, the activity of CCO in the RVLM of LPS-treated rats was significantly decreased during Phases I, II and III of endotoxemia (fig. 4). The degree of reduction during each of these three phases was comparable (Phase I, 29%; Phase II, 21%; Phase III, 29%).

Discussion

The present study took advantage of an animal model of experimental endotoxemia [5] that closely resembles the clinical condition of sepsis. Our on-line monitoring of changes in the power density of vasomotor components of SAP signals allowed us to follow the initial reduction (Phase I), subsequent augmentation (Phase II) and secondary decrease (Phase III) in neuronal activity at the RVLM during the progression towards death. By determining the functions of the mitochondrial respiratory chain in the RVLM during these three phases, we provided a novel demonstration of a significant reduction in NCCR and CCO activity during fatal endotoxemia in a neural substrate that is intimately related to the death process.

A potential mechanism leading to cytopathic hypoxia in sepsis is mitochondrial uncoupling [10]. Under this condition, utilization of oxygen is not tightly linked to the phosphorylation of ADP to form ATP, leading to an intrinsic derangement in cellular energy metabolism. During oxidative phosphorylation [16], electrons from the reducing agent, NADH, move from Complex I to Complex III through coenzyme Q_{10}, and then to Complex IV through cytochrome *c*. Electrons from succinate enter the respiratory chain through flavin adenine dinucleotide, which is covalently linked to Complex II, and move to Complexes III and IV by way of coenzyme Q_{10}. The passage of electrons between these complexes releases energy that is stored in the form of a proton gradient across the membrane by Complexes I, III and IV and is used by ATPase to produce ATP from ADP. In the present study, we observed that while the activity of NCCR (Complexes I + III) and CCO (Complex IV) in the RVLM underwent a decrease during the course of endotoxemia, SCCR (Complexes II + III) remained essentially unchanged. It follows

that the fatal effects of LPS-induced endotoxemia probably arise from a dysfunction of Complexes I and IV in the mitochondrial electron transport chain at the RVLM. Furthermore, it is likely that pathways linked to NADH, but not flavin adenine dinucleotide, are primarily engaged in the speculated defects of mitochondrial respiratory function [13, 23].

Our results indicate a rapid onset of mitochondrial dysfunction after administration of LPS. The most likely candidate that fulfills this necessity of a short time frame is nitric oxide (NO) [31]. A number of studies [14, 18, 31] showed that endotoxin and various proinflammatory cytokines are capable of causing mitochondrial dysfunction via NO-dependent pathways. NO competes with oxygen for the same site in CCO and is a rapid, potent and completely reversible inhibitor of this mitochondrial enzyme at nanomolar concentrations [4]. NO additionally interacts with superoxide ion to form its congeners, peroxynitrite and peroxynitrous acid, which are potent oxidants with cytotoxic effects [24, 31]. Exogenously generated peroxynitrite [13, 24, 27, 31] may cause irreversible inhibition of ATPase, inhibition of Complexes I–IV in the mitochondrial respiratory chain or inhibition of aconitase in the citric acid cycle. Endogenous peroxynitrite also mediates mitochondrial dysfunction in the diaphragm during endotoxemia [1].

Recent work from our laboratory lent direct credence to the notion that overproduction of NO at the RVLM is directly related to the death process. In the same rat model of experimental endotoxemia used in the present study [5], which mimics systemic inflammatory response syndrome, we demonstrated that the reduction in the power density of vasomotor components in SAP signals during the progression towards death is associated with the progressive augmentation in both molecular synthesis and functional expression of inducible NO synthase (iNOS) in the RVLM. We also demonstrated that in a rat model of mevinphos intoxication [7], which mimics organophosphate poisoning, the cholinesterase inhibitor induces toxicity via NO produced by iNOS upon activation of the M_2 subtype of muscarinic receptors by the accumulated acetylcholine in the RVLM. Activated iNOS produces long-lasting generation of NO [15, 22] that is responsible for the sympathoinhibitory action of NO at the RVLM [6]. Results from a parallel study [Chan et al., unpubl. data] further identified that a crucial link between the overproduction of NO by iNOS in the RVLM and death is the formation of peroxynitrite via a reaction between NO and superoxide anion. It follows that a likely consequence of the cytotoxic effects of peroxynitrite is our observed dysfunction in enzymes associated with Complexes I and IV in the mitochondrial respiratory chain at the RVLM during endotoxemia. While this speculation must await confirmation, it should be noted that in a previous study, depression of mitochondrial enzyme activity, predominantly in Complexes I and IV, was detected in muscle biopsies and blood samples of patients in septic shock [27].

The mitochondrial dysfunction observed in the present study may also be achieved indirectly by LPS-induced release of inflammatory cytokines. Cytokine mediators such as tumor necrosis factor and interleukins are detected in the blood several minutes after delivery of endotoxin or an infectious challenge [11]. It is therefore possible that generation of mitochondrial reactive oxygen species after exposure to tumor necrosis factor or other cytokines is related to the dysfunction of the mitochondrial respiratory chain seen during sepsis [13, 27, 29]. Cytokines that influence metabolic pathways may also alter oxygen consumption and cause defective mitochondrial functions [10, 25–27]. Body oxygen consumption and metabolic rate fall progressively with increased severity of disease in septic patients [19].

Conflicting opinion exists regarding the role of mitochondrial dysfunction in sepsis [9, 12, 13, 18, 23, 26, 27, 32]. This conflict may arise from differences in experimental septic models, doses of endotoxin, time scales of observation and tissues or organs used [32]. Mitochondrial functions are generally depressed in models with longer duration and greater severity of sepsis, but are usually unchanged in short-term studies [27]. The present study evaluated changes in mitochondrial enzymes in a brain area that is intimately related to 'life and death' over the entire course towards death during endotoxemia. Based on this model, we were able to demonstrate that whereas the activity of respiratory enzyme Complexes I and IV was inhibited, that of Complexes II and III remained unaltered. We are aware that our experiments were carried out in animals that were maintained under anesthesia (propofol), a muscle relaxant (pancuronium) and mechanical ventilation. That these may not be confounding factors was indicated by the fact that the activity of all enzymes measured in saline-treated rats, which received the same preparatory and experimental procedures as our LPS-treated rats, was found to be comparable to that in the control animals.

In conclusion, the present study demonstrated that dysfunction of the mitochondrial respiratory chain, particularly in respiratory enzyme Complexes I and IV, occurred during LPS-induced endotoxemia in the rat. It is

significant that this mitochondrial dysfunction took place in the RVLM, whose neuronal activity is intimately related to the 'life and death' process. These findings therefore open up a new direction for future development of therapeutic strategies in the critical, complicated and highly fatal condition known as sepsis.

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