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The Effect of Melatonin on Mitochondrial Function in Endotoxemia Induced by Lipopolysaccharide

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ABSTRACT : This study examined the metabolism of free radicals in hepatic mitochondria of goats induced by lipopolysaccharide (LPS), and investigated the effects of melatonin (MT). Forty-eight healthy goats (10±1.2 kg) were randomly selected and divided into four groups: saline control, LPS, MT+LPS and MT. The goats within each group were3 sacrificed either 3 or 6 h after treatment and the livers removed to isolate mitochondria. The respiration control ratio (RCR), the ADP:O ratio, the oxidative phosphorylation ratio (OPR), the concentration of H_2O_2 and the activities of Complex I-IV were determined. The mitochondrial membrane potential ($\Delta\psi_m$) was analyzed by flow cytometry. The results showed that RCR, O/P and OPR of the LPS group decreased ($p<0.05$), as well as activities of respiratory complexes, whereas the generation of H_2O_2 in Complex III increased ($p<0.05$) after 3 h, while Complex II and III increased after 6 h. Also, it was found that the mitochondrial membrane potential of the LPS group declined ($p<0.05$). However, pre-treatment with MT attenuated the injury induced by LPS, which not only presented higher ($p<0.05$) RCR, O/P, OPR, and respiratory complex activities, but also maintained the $\Delta\psi_m$. Interestingly, it is revealed that, in the MT+LPS group, the generation of H_2O_2 increased firstly in 3 h, and then significantly ($p<0.05$).decreased after 6 h. In the MT group, the function of mitochondria, the transmenbrane potential and the generation of H_2O_2 were obviously improved compared to the control group. Conclusion: melatonin prevents damage caused by LPS on hepatic mitochondria of goats. (**Key Words :** Goat, Endotoxemia, Mitochondrial Function, Free Radicals, Melatonin)

INTRODUCTION

Recently, data indicating that mitochondrial deficiency was associated with many diseases and induced multiple organ failure has been published (Hartmut et al., 2002; Michael, 2004; Jane et al., 2005; Hiroyuki, 2006). Mitochondria not only generate cellular energy but are also the source of reactive oxygen species (ROS). About 2% of electrons from their substrates leak from the respiratory chain and bind to other species to form ROS, such as superoxide anion ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2) or reactive nitric species (RNS, e.g., $ONOO^-$, NOO^-) (Andreyev et al., 2005). ROS attack the mitochondrial membrane and either induce lipid oxidation followed by Ca^{2+} influx (Michael, 2000) and mitochondrial transmembrane potential ($\Delta\psi_m$) depletion (Tatyana et al., 2001), or induce $_{mt}$DNA fragmentation (Haider et al., 2008). Tutsi et al. (2006) pointed out that ROS induce myocyte hypertrophy, apoptosis, and interstitial fibrosis by activation of maladaptive cardiac remodeling and failure. Hartmut et al. (2002) believed that the increasing peroxynitrite in drug-induced hepatotoxicity affected mitochondrial function and led to microvesicular steatosis, nonalcoholic steatohepatitis (NASH), and cytolytic hepatitis.

Although mitochondrial dysfunction has been studied in many diseases, such as Barth Syndrome (Matthew, 2006), heart failure (Hiroyuki, 2006), diabetes (Michael, 2004) and alcoholic hepatitis (Hartmut, 2002), the mechanism of endotoxic shock remains unclear. Lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria, plays a key role in sepsis by increasing the diameter of the intravascular space through nitric oxide (NO) (Rongen et al., 1994), recruiting leukocytes to the site of inflammation (Kubes, 2002), and reducing expression of anticoagulatory molecules thereby promoting disseminated intravascular coagulation (Wu and Thiagarajan, 1996). Recently, studies have shown that LPS activates mitochondria to increase the generation of ROS, which induce lipid peroxidation and decrease the ratio of ADP/O, as well as the respiration control ratio (RCR) (Bi et al., 2004; Wang et al., 2006). It has been reported that LPS

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induces rat liver mitochondria to generate excessive ROS and increases the activity of ATP synthesis at a low phosphate-to-oxygen ratio (P/O) (Andrey et al., 2006); however, the ATPase activity was lower than in control samples, and the level of ROS and the P/O value were not significantly different from the controls. Furthermore, another study has suggested that LPS induces apoptosis through signaling pathways associated with LPS receptors (Shauna and Aly, 2006).

Melatonin (MT), a hormone secreted by the pineal gland in the brain, has been shown to function as a direct free radical scavenger (Tan et al., 1993) and an indirect antioxidant via its stimulatory actions on antioxidative enzymes (Reiter et al., 2000; Rodriquez, 2004). It has been used in both experimental and clinical studies for its antioxidative effects. MT possesses an electron-rich aromatic indole ring and functions as an electron donor, thereby reducing and repairing electrophilic radicals (Martinez et al., 2005). Since detoxification of the highly toxic hydroxyl radical (·OH) was demonstrated (Tan et al., 1993), it has been shown that MT scavenges many ROS including hydrogen peroxide (H_2O_2) (Tan et al., 2000a), hypochlorous acid (HClO) (Zavodik et al., 2004), the superoxide anion radical ($O_2^{-}\cdot$), nitric oxide (NO^{-}) (Ximenes et al., 2005; Aydogan et al., 2006), and the peroxynitrite anion ($ONOO^{-}$) (Reiter et al., 2001a). It has been reported that MT is a highly effective antioxidant, which a potency 4-fold higher than glutathione, 14-fold higher than mannitol and 2-fold higher than vitamin E (Tan et al., 1993).

In present study, we pre-treated MT to the goats with endotoxemia, and revealed that MT protect the hepatic mitochondria from damage by enhanced the function of respiration complexes, increased the RCR and $\Delta\psi_m$, while reduced the generation of H_2O_2 from the respiration chain.

MATERIAL AND METHODS

Animals and treatments

Twenty-four male and 24 female goats aged from 65-75 days old were randomly divided into 4 groups: control group (NS JV injection), LPS group (LPS 1 mg/kg weight JV injection), MT pre-treated group (MT 1 mg/kg wt JV injection before LPS treatment) and MT group (MT 1 mg/kg wt JV).

Isolation of liver mitochondria

Liver mitochondria were isolated by the modified method of Tang et al. (2002) at 3 and 6 h after the injection of LPS. Approximately 15 g of liver was excised rapidly and finely minced in an initial liver isolation medium (220 mM D-Mannitol, 70 mM Sucrose, 2 mM HEPES, 1 mM EGTA, pH 7.4) containing 0.5 mg/ml BSA. The minced tissue was homogenized using a homogenizer (Glas-Col, USA). The resulting homogenate was then centrifuged at 1,000×g for 10 min. The pellet was discarded and the supernatant centrifuged at 10,000×g for 14 min to obtain the mitochondrial pellet. After washing in 10 ml of initial liver isolation medium, the pellet was centrifuged at 10,000×g in a second liver isolation medium (220 mM D-Mannitol, 70 mM Sucrose, 2 mM HEPES, pH 7.0) containing 0.5 BSA mg/ml. The protein content of the final mitochondrial pellet was determined by Coomassie brilliant blue G520 (Marion, 1976) and then resuspended in the third liver isolation medium (220 mM D-Mannitol, 70 mM Sucrose, 2 mM HEPES, pH 7.0).

Mitochondrial function

Mitochondrial function was assessed by monitoring oxygen consumption with a Clark-type electrode with a thermostatically controlled respiration chamber set at 35°C as described by Estabrook (1967). The oxygen consumption measurements were carried out in duplicate and all measurements were completed within 3 h of mitochondrial isolation. Mitochondria (~10 mg mitochondrial protein) were added to the chamber containing an RCR reaction buffer composed of 74.6 mM KCl, 209.3 mM MOPS, 136.1 mM KH_2PO_4, 380.4 mM EGTA and 1 g/L BSA, with 10 mM succinate and 5 μM rotenone (to block Complex I when succinate was used as substate). State 3 was induced by adding 155 μM ADP (final concentration) and the RCR (an index of electron transport chain coupling) calculated by dividing State 3 by State 4 respiration. The efficiency of ATP synthesis coupled to cell respiration, the ADP:O ratio, was determined by dividing the quantity of ADP added by the amount of oxygen consumed during State 3 respiration. The oxidative phosphorylation ratio (OPR) was calculated by multiplying State 3 by the ADP:O ratio in control and other groups mitochondria as described previously (Cawthon et al., 1999; Iqbal et al., 2001a).

Determination of mitochondrial H_2O_2 production

Mitochondrial H_2O_2 generation was determined using 2', 7'-dichlorofluorescin diacetate (DCFH-DA, Molecular Probes Inc., Eugene, OR 97402) according to Bass et al. (1983) with modifications (Iqbal et al., 2001b). Briefly, H_2O_2 was measured in 96-well microplates with a photofluorometric detector (Elx 808 Ultra Microplate Reader, BIO-TEK INSTRUMENTS, USA) set at a sensitivity of 3 and excitation/emission wavelength at 480/530 nm. To each well, 0.1 mg mitochondrial protein was added to 124 μl RCR buffer (145 mM KCl, 30 mM Hepes, 15 mM KH_2PO_4, 3 mM $MgCl_2$ and 0.1 mM EGTA, pH 7.2) containing 51 μM DCFH-DA. The RCR buffer also contained succinate (12 mM) for liver mitochondria. Ten units of superoxide dismutase were added to convert all $O_2^{-}\cdot$

to H_2O_2. Fluorescence that remained following catalase treatment (225 Sigma units per well) was subtracted to correct fluorescence not attributed to H_2O_2 (Iqbal et al., 2001b). The microplate was incubated at room temperature and read sequentially at 0, 10 and 30 min using a Cytoflour photofluorimeter. Final values of H_2O_2 were calculated from a standard curve with known amounts of H_2O_2 and expressed as nmol H_2O_2/min per mg of mitochondrial protein.

Substrate-inhibitor studies

Generation of H_2O_2 in liver mitochondria was monitored with and without several electron transport chain inhibitors that block electron transfer at specific sites in the respiratory chain as follows: rotenone (Complex I); 4,4,4-Trifluoro-1-[2-thienyl-1,3-butanedione (TTFA) and malonate (Complex II); myxothiazol (Complex III, from ubiquinol to the iron-sulfur center (FeS) of Complex III), and antimycin A (inhibits electron transport within Complex III at cytochrome b_{560}). Final concentrations used were: rotenone, 10 μM; antimycin A, myxothiazol, and TTFA, 13 μM; and malonate, 7 μM, under the reaction conditions mentioned above. To avoid any variability between treatments with regard to background fluorescence, appropriate controls were used for all wells of the microplate, e.g., blanks for mitochondria, all inhibitors, and catalase with both substrates. The final values were corrected with these blanks.

Respiratory chain complex activities

Activities of respiratory chain complexes were determined by spectrophotometry (Glamour 8000, US) according to previously described methods (Galante and Hatefi, 1978; Hatefi and Stiggall, 1978; Ojano-Dirain et al., 2005). Briefly, the activity of Complex I (NADH ubiquinone: oxidoreductase) was measured by following oxidation of NADH. The activity of Complex II (succinate: ubiquinone oxidoreductase) was measured by following the reduction of dihydroubiquinone-2 (Coenzyme Q_2) by DCIP. The activity of Complex III (ubiquinone: cytochrome (cyt) c oxidoreductase) was measured by monitoring cyt c reduction by ubiquinol-2, and the activity of Complex IV (cyt c oxidase) was measured by the oxidation of reduced cyt c. All assays were performed in duplicate, and values were expressed in units of activity/min/mg mitochondrial protein.

Flow cytometry for mitochondrial transmembrane potential

Mitochondrial transmembrane potential was assessed by 5,5',6,6'- tetrachloro -1,1', 3, 3'- tetraethylbenzimidazoly lcarbocyanine iodide (JC-1). JC-1 was diluted by DMSO into 1 g/L and stored at -20°C. Before analysis, 900 μl JC-1 was added to the sample which contained 50 μg mitochondria, and incubated for 10 min at room temperature. Samples were immediately analyzed by flow cytometry (FACS Calibur, Immunochemistry Systems; San Jose, CA, USA) and data from 10,000 mitochondria were recorded. JC-1 green fluorescence associated with JC-1 monomers was collected in the FL-channel (530 nm). JC-1 red fluorescence associated with the formation of J-aggregates was collected in the FL-2 channel (585 nm). Spectral overlap was avoided by an adjusting compensation network.

Statistics

Data were analyzed by multiple-test and one-way ANOVA using SPSS and are presented as the mean±SEM. A probability of <0.05 was considered statistically significant.

RESULTS

Mitochondrial function

Mitochondrial respiratory function was measured and the results are shown as FADH-linked substrate (Table 1). Respiration ratios decreased dramatically in the LPS group compared to the control ($p<0.05$). In the MT+LPS group, RCR, P/O and OPR remarkably ($p<0.05$) increased in 3 h, but there was no significant difference ($p>0.05$) between the LPS group and the control group in 6 h. Although RCR, P/O and OPR increased in the MT group, there was no significance ($p>0.05$) when it compared to the control group.

Table 1. Oxygen consumption (State 3 and State 4) and function of liver mitochondria provided FADH-linked substrate succinate isolated from the 4 groups of goats

	RCR (n = 6)		P/O (n = 6) nmol ADP/nmol O		OPR (n = 6) nmol/min mg prot.	
	3 h	6 h	3 h	6 h	3 h	6 h
Control	2.80±0.28^{b}	3.17±0.36^{b}	2.65±0.19^{b}	2.74±0.57^{b}	120.31±8.42^{b}	126.90±7.29^{b}
LPS	2.01±0.12^{a}	2.10±0.13^{a}	1.73±0.56^{a}	1.80±0.26^{a}	79.45±12.72^{a}	72.78±10.13^{a}
MT+LPS	2.82±0.38^{b}	2.78±0.66^{ab}	2.69±0.68^{b}	2.21±0.45^{ab}	113.16±11.62^{b}	101.84±12.47^{ab}
MT	3.58±0.28^{c}	3.38±0.73^{b}	2.81±0.29^{b}	2.60±0.51^{b}	126.69±16.75^{b}	114.31±15.76^{b}

Superscript letters indicate values significantly different from each other ($p<0.05$) in each column, the same as followed.

Table 2. Hydrogen peroxide (H_2O_2) generation in liver mitochondria provided FADH-linked substrate succinate as substrate isolated from the different treatment groups after 3 h

		Noinhibitor	Complex I	Complex II		Complex III		Complex IV	Complex V
			Rot	TTFA	Mal	Myxo	AA	KCN	Olig
3 h	Control	3.13±0.28^{b}	3.88±0.27^{b}*	3.24±0.78^{b}	3.00±0.14^{b}	2.67±0.18^{b}	4.21±0.17^{b}*	3.12±0.22^{bc}	4.77±0.56^{b}*
	LPS	3.52±0.46^{b}	4.13±0.38^{b}*	1.99±0.53^{a}	3.77±0.74^{b}	3.06±0.59^{bc}	5.64±0.58^{c}*	2.62±0.41^{b}	2.53±0.50^{a}
	MT+LPS	3.49±0.51^{b}	7.06±1.39^{c}*	3.22±0.62^{b}	5.22±0.20^{c}	6.14±0.58^{c}*	6.01±0.75^{c}*	3.59±0.35^{c}	5.48±0.36^{b}
	MT	1.08±0.34^{a}	2.01±0.38^{a}*	1.32±0.23^{a}	0.90±0.30^{a}	1.90±0.75^{a}*	1.58±0.38^{a}	0.90±0.25^{a}	1.83±0.45^{a}*

Each value represents the mean±SEM (n = 6), considered as nmol per minute per microgram of protein. * Inhibitor values within columns are significantly different from the no inhibitor values ($p<0.05$), the same as followed.

Table 3. Hydrogen peroxide (H_2O_2) generation in liver mitochondria provided FADH-linked substrate succinate as substrate isolated from the different treatment groups after 6 h

		Noinhibitor	Complex I	Complex II		Complex III		Complex IV	Complex V
			Rot	TTFA	Mal	Myxo	AA	KCN	Olig
6 h	Control	3.21±0.10^{b}	4.01±0.35^{b}*	3.12±0.18^{b}	3.12±0.28^{b}	2.56±0.32^{a}	4.55±0.07^{b}*	3.15±0.28^{bc}	4.87±0.45^{b}*
	LPS	4.12±0.33^{c}	4.87±0.51^{b}*	4.42±0.58^{c}	3.89±0.46^{c}	5.68±0.46^{c}*	4.02±0.32^{bc}	3.99±0.14^{c}	5.16±0.31^{b}*
	MT+LPS	1.68±0.42^{a}	4.06±0.47^{b}*	2.32±0.47^{a}	2.22±0.86^{ab}	4.02±0.15^{b}*	2.84±0.85^{a}	1.88±0.58^{a}	4.11±0.47^{b}*
	MT	1.83±0.50^{a}	1.82±0.44^{a}	2.57±0.30^{ab}	1.80±0.34^{a}	2.71±0.32^{a}*	3.06±0.73^{ab}*	2.81±0.59^{b}	2.87±0.45^{a}*

Specific site of H_2O_2 generation

The generation of H_2O_2 in mitochondrial specify sites by different inhibitors were shown in Table 2. In 3 h, basal H_2O_2 production (no inhibitor) was about 3 fold lower in the MT group than the control group, while there was no significant difference ($p>0.05$) among the other groups. Incubated with Rot, Mal, Myxo, AA and Olig, the generation of H_2O_2 was remarkably ($p<0.05$) increased in the MT+LPS group, and there was no difference incubated with TTFA and KCN. In the LPS group, the generation of H_2O_2 significantly ($p<0.05$) increased incubated with AA while decreased ($p<0.05$) with TTFA and Olig, but there was no difference ($p>0.05$) with other inhibitors. In the MT group, the generation of H_2O_2 remarkably ($p<0.05$) decreased with all inhibitors when it compared to the control group.

It can be seen from the Table 3 that, in 6 h group, the generation of H_2O_2 significantly ($p<0.05$) increase incubated with TTFA, Mal and Myxo while there was no difference ($p<0.05$) with Rot, AA and KCN. In the MT+ LPS group, the generation of H_2O_2 significantly ($p<0.05$) decreased with TTFA, Mal, AA and KCN, while increased ($p<0.05$) in Myxo, and there was no difference with Rot and Olig. Similar result to the 3 h, the MT group in 6 h produced much lower ($p<0.05$) H_2O_2 than the control group.

Activities of mitochondrial respiration complexes

Activities of mitochondrial respiration chain in liver were shown in Figure 1 and 2. The activities of Complex I, Complex II and Complex IV dramatically ($p<0.05$) decreased in LPS group both in 3 h and 6 h. After pre-injected MT, the activities of mitochondrial respiratory complexes increased by presented different degrees in both 3 h and 6 h. Remarkably, the activities of Complex III increased ($p<0.05$) in the MT group, and remained no significant difference ($p>0.05$) in other complexes when it compared to the control group in 3 h and 6 h.

Mitochondrial transmembrane potential analysis

The evaluation of mitochondrial membrane potential by flow cytometry was shown in Figure 3. The population of liver mitochondria staining bright red in the control group (R2 = 2.96%, R3 = 90.44%) was significantly higher ($p<0.05$) than the LPS group (R2 = 4.38%, R3 = 17.09%), which represented dramatically higher in green (R4 = 78.43%). By pre-injected MT, the population of liver mitochondria staining green was decreased while the red one was increased (R2 = 46.57, R3 = 21.17%, R4 = 32.86%). In the MT group, there was no difference ($p>0.05$) compared to the control group.

DISCUSSION

It is well known that impaired mitochondrial function contributes to altered tissue oxygen metabolism during endotoxemia (Crouser et al., 1999; Crouser et al., 2002; Lobo et al., 2003). Brealey et al. (2002) recently showed that impaired mitochondrial function and attendant depletion of ATP in skeletal muscle is associated with increased mortality during sepsis. Because the liver is the significant organ of detoxication, the hepatocytes are likely

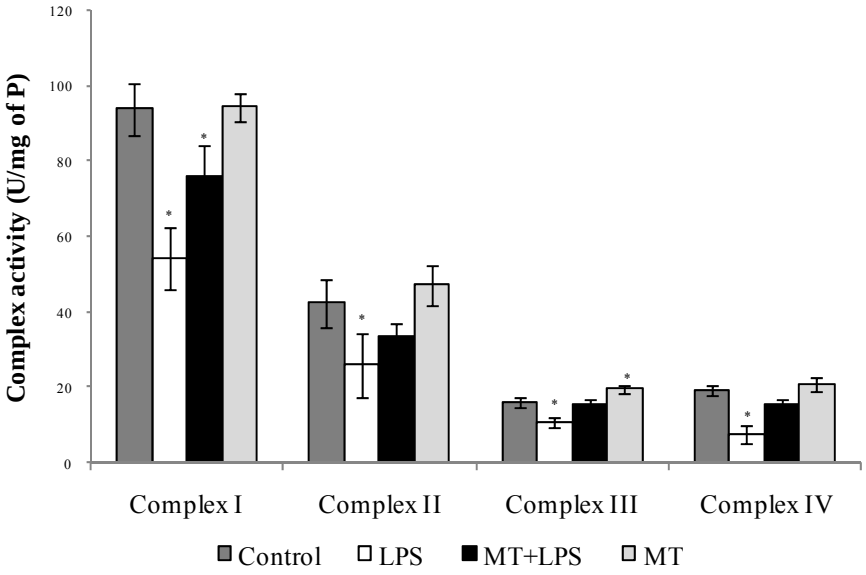

Figure 1. Activities (units/mg mitochondrial protein) of respiratory chain complexes I, II, III and IV in liver mitochondria isolated from goats with injected LPS for 3 h. Each bar represents the mean±SEM (n = 6), * $p<0.05$ compared to the control group, the same as followed.

to be the main target in endotoxemia. Based upon these observations, it is postulated that sepsis-induced reductions in mitochondrial bioenergetic capacity contribute significantly to acute incapacitation of the cell and attendant organ dysfunction (Fink, 2001; Crouser, 2004).

In this study, it is shown that the RCR of liver mitochondria significantly decreased in the LPS group compared to the controls ($p<0.05$). Together with the evidence that the O/P was also lower during endotoxemia, the results demonstrated that LPS reduced oxidative phosphorylation in liver mitochondria. Recently, Andrey and his colleagues (2006) have shown that rat liver mitochondria were impaired during endotoxic shock because of the increase in ROS and the decrease in P/O. However, there was no effect on heart mitochondria except for reduced ATPase activity. Furthermore, endotoxin induced a decrease in succinate-dependent State 3 respiration, while the State 4 respiration did not change (Francesca et al., 2006). The respiratory efficiency (RCR) consequently decreased, together with both a reduced ADP:O ratio and maximal ATP production, which was lower in the endotoxin-infused group than controls.

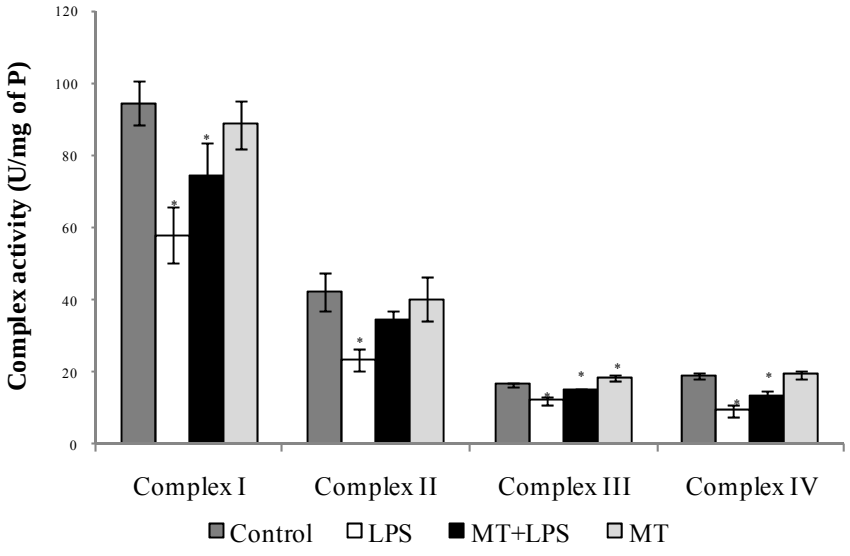

Figure 2. Activities (units/mg mitochondrial protein) of respiratory chain complexes I, II, III and IV in liver mitochondria isolated from goats with injected LPS for 6 h.

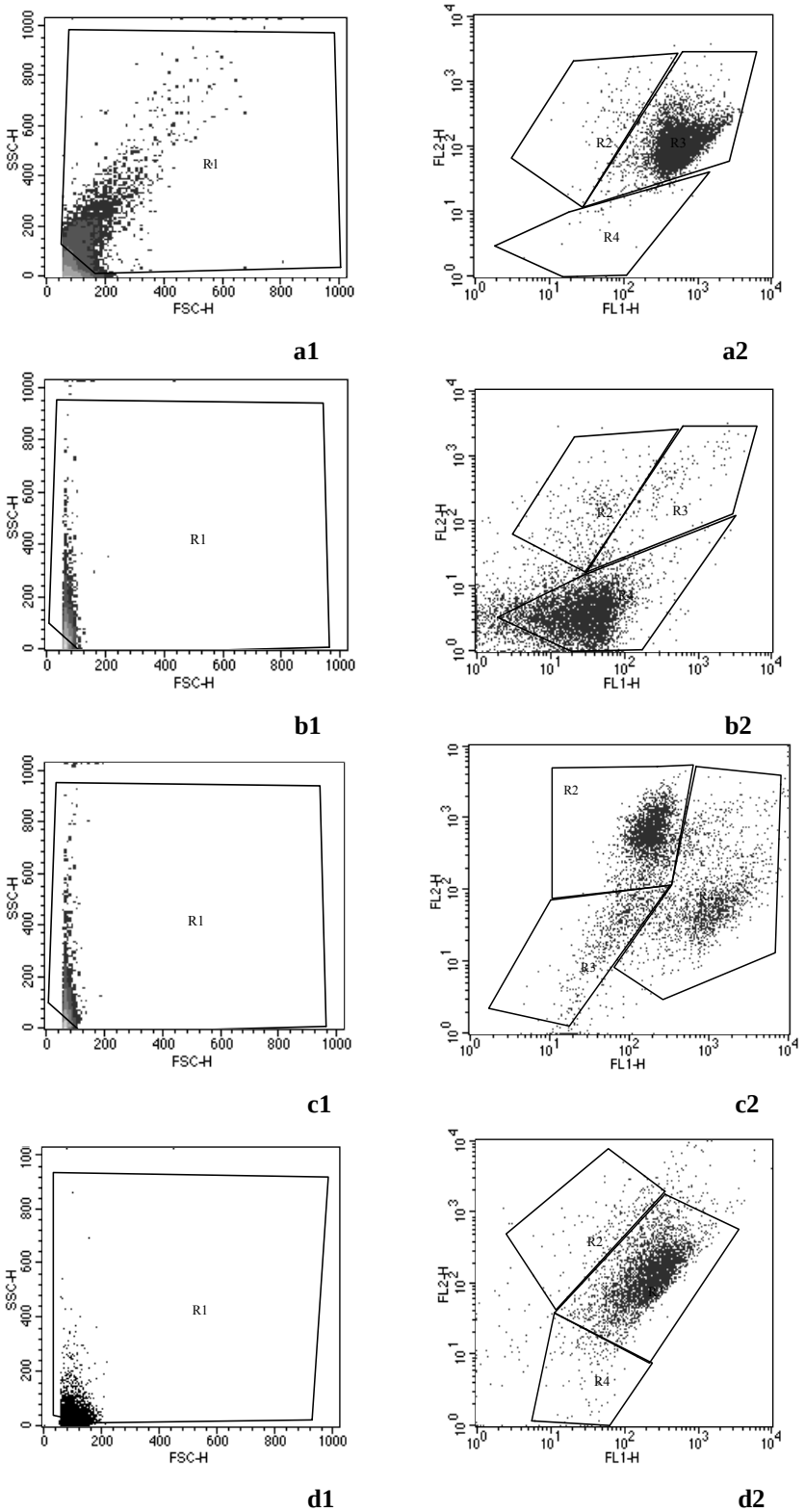

Figure 3. Representative flow cytometry results of liver mitochondrial membrane potential change (6 h). a. control group, b. LPS group, c. MT+LPS group, d. MT group. a1, b1, c1, d1: R1 = 10,000; a2: R2 = 2.96%, R3 = 90.44%, R4 = 6.60%; b2: R2 = 4.38%, R3 = 17.09%, R4 = 78.43%; c2: R2 = 46.57%, R3 = 21.17%, R4 = 32.86%; d2: R2 = 3.69%, R3 = 90.16%, R4 = 6.1.

Moreover, a higher level of electron leakage was demonstrated in liver mitochondria that was due to site-specific defects at Complex I and III (antimycin A sensitive site) in the 3 h group and at Complex I, III (myxothiazol sensitive site) and V in the 6 h group. This contrasts with the recent reports that Complex I and III were the major site-specific electron leakage in mitochondria (Barja, 1999; Qun et al., 2003; Lothar and Judy, 2006). Also, contrary to previous studies which concluded that the activities of Complex I, III and IV were sensitive to oxidative stress (Elizabeth and Karam, 2004; Raam et al., 2008), we found that the activities of Complex II decreased by nearly 60% except for Complex I (58%) and Complex IV (38%). The possible explanation for this may be reverse electron transfer (RET) (Andreyev et al., 2005; Vera and Christos, 2006), which is a set of reactions in the respiratory chain that allow electrons to be transferred from $FADH_2$-linked substrate (e.g. succinate in our study) to Complex I, which induced the large amount of ROS generated on Complex I and attacked the other complexes, such as Complex II. Also, Complex III generates superoxide on the matrix side of the mitochondrial inner membrane space (Turrens, 1997). Qun et al. (2003) suggested that Complex III was the principal site for ROS generation during the oxidation of Complex I substrates, and rotenone protects by limiting electron flow into Complex III. However, in our study, we found that the generation of H_2O_2 increased in Complex III while activity of the complex presented no difference with the control group. Generally, it is believed that the increasing ROS of respiratory chain caused the decreasing activities of respiratory complexes, while the higher respiratory complex activities were thought related with the lower generation of ROS (Marieke, 1996; Guiseppe et al., 2000). It might be another mechanism to exist and explain such phenomenon. Recent data suggested that the typical model of mitochondrial membrane has been challenged by the existence of supramolecular associations of the respiratory complexes (Giorgio et al., 2010). Therefore, advanced technology must be introduced to investigate the supercomplexes in further studies, such as Native Blue Gel Electrophoresis. Complex V, ATP synthase (ATPase), is rarely mentioned its defeats of electron leakage in mitochondrial electron transport chain. However, there were still some researches indicated that state 3 respiration and ATP synthesis decreased after intravenous injection/infusion of endotoxin (Kantrow et al., 1997; Crouser et al., 2002; Lu et al., 2003; Crouser et al., 2004). The other important observations pointed out that mitochondrial respiration increased in the early phase of acute critical illness, including septic/endotoxic shock, but consistently falls during the late phase of illness (Singer and Brealey, 1999; Lu et al., 2003; Singer M et al., 2004). That is why electron leakage decreased in 3 h group and then increased in 6 h group. We supposed that liver mitochondria enhanced its respiration in the early phase of endotoxemia to defend the invasion of LPS.

Generally, mitochondrial membrane potential is associated with the generation of ROS (Tatyana and Ian, 2001; Anatoly and Gary, 2003; Ly et al., 2003; Octavio et al., 2005). Anatoly and his colleagues (2003) indicated that there is a relationship between $\Delta\psi_m$, Complex I and mitochondrial ROS production, when the $\Delta\psi_m$ of brain mitochondria reduced, the production of H_2O_2 also decreased. In this study, analyzed by the flow cytometry, the $\Delta\psi_m$ presented dramatically reduced in the LPS group ($p<0.05$), while the production of H_2O_2 increased in 6 h. This is consistent with the result in previous studies (Tatyana and Ian, 2001; Octavio et al., 2005).

Melatonin (MT) is metabolized primarily in the liver, and secondarily in the kidney. It is believed that MT functions as a free radical scavenger directly and indirectly. On one hand, MT reacts with ·OH, singlet oxygen ($^1O_2$), H_2O_2, hypochlorous acid (HClO), NO·, $ONOO^-$ and oxoferryl-derived species (Tan et al., 1993a, b; Reiter et al., 2002; Allegra et al., 2003). On the other hand, data *in vitro* supported that MT inhibits nNOS activity in cerebellum (Pozo et al., 1994), hypothalamus (Bettahi et al., 1998) and striatum (Leon et al., 1998) due its binding to the calcium-calmodulin complex (Leon et al., 2000). Also, *in vivo* studies demonstrated that MT inhibits iNOS and mtNOS expression and activity in an experimental model of sepsis in rats (Crespo et al., 1999; Escames et al., 2003). Because of its highly lipophilic molecular nature, MT crosses cell membranes to reach mitochondria (Menendez-Pelaez and Reiter, 1993), and interacts with lipid bilayers (Costa et al., 1997) and stabilizes mitochondrial inner membranes (Garcia et al., 1999), reduces electron leakage from the mitochondrial electron transport chain (Leon et al., 2005), as an effect that may improve ETC activity (Acuna-Castroviejo et al., 2001). Moreover, MT also influences oxidative phosphorylation by increasing electron transport and ATP production in normal cells. These actions of MT may not relate to its antioxidant activities since the indoleamine specifically interacts with complex I and IV of the mitochondrial respiration chain to achieve these changes (Acuna-Castroviejo et al., 2003). In this study, MT obviously reduced the electron leakage from the liver mitochondrial respiration chain and increased the synthesis of ATP through raising RCR, O/P and OPR. During endoxemia, pre-injected with MT resulted in the decreasing electron leakage of Complex II, III and IV in 6 h groups, but not in 3 h groups, which presented higher generation of H_2O_2 than the LPS group. We assumed that MT enhanced mitochondrial function to attenuate the injury of LPS

through increasing the electron transmission and the activities of mitochondrial respiration complexes, as well $\Delta\psi_m$.

IMPLECATIONS

During endotoxemia in goats, the function of liver mitochondria was impaired due to the increasing production of free radical and the decreasing activities of mitochondrial respiration complexes, which induced the mitochondrial respiration dysfunction and the collapse of membrane potential. Further, melatonin was showed efficient protection effects on the injury induced by lipopolysaccharide and repaired the damage of liver mitochondria. To explain the specific site of free radical production in mitochondria, advanced technology should be introduced into further study.

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