Journal of Surgical Research **171,** 108–113 (2011)
doi:10.1016/j.jss.2010.01.019

Melatonin Attenuates I/R-Induced Mitochondrial Dysfunction in Skeletal Muscle

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Submitted for publication October 13, 2009

Background. **Our recent studies have shown that ischemia/reperfusion (I/R) produces significant necrosis and apoptosis in the cells of skeletal muscle. Our previous studies also demonstrated that melatonin provides significant protection against superoxide generation, endothelial dysfunction, and cell death in the skeletal muscle after I/R. Mitochondria are essential for cell survival, because of their roles as ATP producers as well as regulators of cell death. However, the efficacy of melatonin on I/R-induced mitochondrial dysfunction in the skeletal muscle *in vivo* has not been demonstrated in the literature.**

Materials and Methods. **Vascular pedicle isolated rat gracilis muscle model was used. After 4 h of ischemia followed by 24 h of reperfusion, gracilis muscle was harvested, and mitochondrial as well as cytosolic fractions were isolated. Mitochondrial dysfunction was determined by the alteration of mitochondrial membrane potential and the release of the proapoptotic protein, cytochrome *c*. Three groups were designed; sham I/R, I/R-V (I/R with vehicle), and I/R-Mel (I/R with melatonin). Melatonin or vehicle was given intravenously 10 min prior to reperfusion and 10 min after reperfusion.**

Results. **We found that the capability of uptake of fluorescent JC-1 dye in skeletal muscle cells was substantially improved in I/R-Mel group compared with I/R-V group. Melatonin significantly inhibited the outflow of cytochrome *c* from mitochondria to cytoplasm, which was demonstrated in the I/R-V group.**

Conclusions. **Melatonin significantly attenuates I/R-induced mitochondrial dysfunction, such as the depolarization of mitochondrial membrane potential and the release of the proapoptotic protein, cytochrome *c*, from the mitochondria.**

Key Words: **melatonin; mitochondrial membrane potential; cytochrome *c*; ischemia/reperfusion; skeletal muscle.**

INTRODUCTION

Our previous studies [1, 2] have shown that prolonged ischemia followed by reperfusion not only induces necrosis but also accelerates apoptosis in the cells of skeletal muscle. Mitochondria are essential for cell survival, because of their roles as generators of energy as well as regulators of cell death [3]. Mitochondria are a major source of reactive oxygen species (ROS) that could be the toxic stimuli produced during reperfusion and the key components of I/R injury [4, 5]. Our previous study [6] found that ischemia/reperfusion (I/R) causes a significant mitochondrial dysfunction, which could be an underlying mechanism of I/R-induced cell death. The present study is the continuation of our long-term project focused on I/R injury in skeletal muscle.

Melatonin (N-acetyl-5-methoxytryptamine), released from the pineal gland, is a highly efficient scavenger of ROS [7]. The highest melatonin concentrations are found in the mitochondria [8–10]. Mitochondria have been identified as a target for melatonin actions [11, 12]. In the present study, we hypothesized that melatonin might be able to attenuate I/R-induced mitochondrial dysfunction in skeletal muscle. Mitochondria dysfunction was determined by the depolarization of mitochondrial membrane potential and the release

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0022-4804/$36.00

of the proapoptotic protein, cytochrome *c*, from the mitochondria. The mitochondrial membrane potential was measured by JC-1 assay in the mitochondrial fractions. The concentrations of cytochrome *c* were determined by cytochrome *c* immunoassay in both mitochondrial and cytosolic fractions.

METHODS

All experimental procedures and care of the animals were approved by the Institutional Animal Care and Use committee. Male Sprague-Dawley rats weighing 400 to 450 g were used. Anesthesia was accomplished using intraperitoneal sodium pentobarbital (50 mg/kg).

Experimental Protocol

The vascular pedicle isolated gracilis model was used [1, 2, 6]. The right thigh musculature and femoral vasculature were exposed and the gracilis muscle was dissected free upon its vascular pedicle using standard microsurgical techniques. Surgical dissection resulted in the gracilis muscle having no attachment to the body except through the femoral vasculature. After surgical preparation, two microvascular clamps were placed across the iliac and deep femoral vessels respectively to create global ischemia in the skeletal muscle and then released to allow for reperfusion. After 4h of ischemia, Melatonin 10 mg/kg (I/R-Mel) or vehicle saline (I/R-V) was given by intravenous injection at 10 min prior to reperfusion and a second dose at 10 min after reperfusion. The vascular clamping was omitted and served as a control (sham I/R). Muscle samples were harvested after 24 h of reperfusion. The central portion of the muscle flap (500 mg) was taken for sampling. The edges of muscle flap were not included in order to avoid the complication of surgical trauma.

Isolation of Mitochondrial and Cytosolic Fractions

The isolation of mitochondrial and cytosolic fractions was performed by using a mitochondria isolation kit provided by Sigma (St. Louis, MO) according to the manufacturer's instruction. Briefly, the muscle sample was washed and suspended with extraction buffer containing 0.25 mg/mL trypsin. After 3 min of incubation on ice, the supernatant was removed. Muscle was then resuspended with buffer containing trypsin for 20 min. Albumin solution 10 mg/mL was added to quench the photolytic reaction. The supernatant was removed. the pellet was washed again with extraction buffer and then homogenized with 25 strokes. Sample was centrifuged at 600× *g* for 5 min. The supernatant (containing both cytosolic and mitochondrial fractions) was transferred to a new tube and centrifuged at 11,000× *g* for 10 min. The supernatant (containing cytosolic fraction) was transferred to a new tube and spun at 16,000× *g* for 20 min at 4 °C to remove any residual mitochondria. The supernatant was then transferred to a fresh tube and served as the cytosolic fraction. The pellet (containing mitochondrial fraction) was suspended with storage buffer (40 μL per 100 mg tissue). The total protein concentration of each fraction was then measured by Bio-Rad Protein Assay Kit II and determined by SmartSpec 3000 Spectrophotometer (Bio-Red Laboratories, Hercules, CA) with the wavelength set at 595 nm with a BSA standard curve. The extract was then stored at –80 °C.

Mitochondrial Membrane Potential

Mitochondrial membrane potential was determined by JC-1 assay provided by Sigma (St. Louis, MO). After the isolation of mitochondrial and cytosolic fractions was complete, the pellet (containing mitochondrial fraction) was diluted with storage buffer to 1 mg of protein per mL. We used 3 μg protein from each sample (based on a dose-response test) for the JC-1 assay. JC-1 assay buffer 1.9 ml and an appropriate volume of sample were added to a tube and brought the volume to 2 mL with storage buffer. JC-1 stain 2 μL was added to start the reaction. The tubes were incubated at room temperature in the dark for 7 min. The samples were read in a spectrofluorometer with emission setting at 590 nm and excitation at 490 nm. All of the standards, controls, and samples were read in duplicate.

Concentration of Cytochrome *c*

The concentration of cytochrome *c* in both mitochondrial and cytosolic fractions was determined by the Quantikine Rat/Mouse Cytochrome *c* Immunoassay provided by R and D Systems, Inc.. (Minneapolis, MN). This assay employs the quantitative sandwich enzyme immunoassay technique and was designed to replace the Western blot. A monoclonal antibody specific for rat/mouse cytochrome *c* was precoated onto a microplate. The conjugate (75 μL containing monoclonal antibody specific for rat/mouse cytochrome *c* conjugated to horseradish peroxidase) was added to each well of microplate. This was followed by adding 50 μL of standards and positive control (containing natural cytochrome *c*) into each well. Three hundred nanograms (300 ng) of protein from each mitochondrial fraction or one microgram (1 ug) of protein from each cytosolic fraction were added to the sample wells. All of the standards, controls, and samples added to the microplate were in duplicate. The determination of using these amounts of protein was based on several pilot tests. After 2 h of incubation at room temperature, each well was washed five times with 400 uL of wash buffer. The substrate solution (100 μL) was added to each well and incubated for 30 min at room temperature. The optical density of each well was determined by a microplate spectrophotometer (Spectra Max Plus 384; Molecular Devices Corp., Sunnyvale, CA) set to 450 nm after 100 μL of the stop solution was added to each well.

Statistics

All measurements were compared by using ANOVA followed by *t*-test. $P \leq 0.05$ was considered significant.

RESULTS

The result of mitochondrial membrane potential (Fig. 1): The average uptake of the fluorescent JC-1 dye (FLU ± SEM/mg protein) in the mitochondrial fraction of muscle cells was significantly decreased in I/R-V group (80697 ± 5355/mg protein) compared with sham I/R group (141303 ± 10986/mg protein) with $P < 0.001$. This 43% reduction of uptake of JC-1 dye in I/R-V group is an indicator of the depolarization of mitochondrial membrane potential. However, the treatment of melatonin (I/R-Mel) significantly increased the uptake of JC-1 dye (122766 ± 8125/mg protein) in the mitochondrial fraction compared with I/R-V group (80697 ± 5355/mg protein) with $P < 0.001$.

The result of cytochrome *c* immunoassay (Fig. 2): in the mitochondrial fraction, the average concentration of cytochrome *c* (ng ± SEM) was significantly decreased in I/R-V group (8.0 ng ± 0.78) compared with sham I/R group (25.8 ng ± 01.3) with $P < 0.001$. This substantial reduction of cytochrome *c* in the mitochondrial fraction was associated with a significant increase in the concentration of cytochrome *c* in the cytosolic fraction in