Melatonin protects against LPS-induced inflammation and oxidative stress in hepatocytes by enhancing mitophagy and mitochondrial biogenesis

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Research Article

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Abstract

Background: Melatonin have a protective effect in the liver during sepsis by counteracting oxidative stress and reducing inflammatory responses. Melatonin also regulates mitochondrial biogenesis. This study explored the mechanisms by which melatonin protects against liver injury in experimental sepsis with a focus on mitophagy and mitochondrial biogenesis.

Methods and Results: An *in vitro* model of sepsis-induced hepatocyte injury was established using AML12 cells. Indicators of oxidative stress, inflammation, mitophagy and mitochondrial biogenesis were assessed. Tumor necrosis factor (TNF)-α and interleukin (IL)-6 protein levels, intracellular reactive oxygen species (ROS) levels, lipid peroxidation (malondialdehyde [MDA] levels), and markers of mitophagy (PTEN-induced putative kinase 1 [PINK1] and Parkin) and mitochondrial biogenesis (peroxisome proliferator-activated receptor-gamma coactivator 1a [PGC-1α], nuclear respiratory factor 1 [NRF-1], mitochondrial transcription factor A [TFAM]) were significantly increased, while superoxide dismutase (SOD) activity and intracellular adenosine triphosphate (ATP) levels were significantly decreased in LPS-treated AML12 cells compared to controls. TNF-α and IL-6 protein levels and intracellular ROS and MDA levels were significantly decreased, while SOD activity, intracellular ATP levels, and markers of mitophagy and mitochondrial biogenesis were significantly increased by melatonin pre-treatment.

Conclusion: This study demonstrated that melatonin was involved in the maintenance of mitochondrial homeostasis in hepatocytes during sepsis. Mechanisms involved selective elimination of dysfunctional mitochondria through mitophagy and induction of mitochondrial biogenesis.

Introduction

Sepsis is a global public health concern that is responsible for high mortality rates in intensive care units [1]. The Sepsis-3 task force defines sepsis as ‘a life-threatening organ dysfunction caused by a dysregulated host response to infection’ [2]. The liver has a crucial role in the defense against sepsis as it clears bacteria and mediates inflammatory responses. In critically ill patients, early hepatic dysfunction is a risk factor for poor prognosis and mortality [3], but the morbidity and mortality associated with sepsis can be decreased by reducing liver injury and restoring liver function [4]. Currently, there remains an unmet clinical need to fully elucidate the molecular mechanisms underlying liver dysfunction during sepsis.

Mitochondria are life-sustaining organelles that have a critical role in stress responses [5]. Ultrastructural damage and functional impairment are observed in mitochondria during sepsis, resulting in the depletion of adenosine triphosphate (ATP), the production of reactive oxygen species (ROS), cell damage induced by oxidative stress, and organ failure [6].

In mammals, mitochondrial quality control is regulated by various mechanisms that result in mitochondrial turnover [7]. During sepsis, defective mitochondrial quality control mechanisms amplify

organ failure, while restoration of mitochondrial quality control mechanisms ameliorate organ failure [7]. In several *in vivo* and *in vitro* models of sepsis, augmented mitophagy and mitochondrial biogenesis increased the number of functional mitochondria and decreased mitochondrial ROS production, inflammation, and injury [8][9]. As mitochondrial damage and depletion are early manifestations of liver dysfunction during sepsis [10], mitophagy and mitochondrial biogenesis in hepatocytes represent potential therapeutic targets in sepsis.

Melatonin (N-acetyl-5-methoxytryptamine), a hormone produced by the pineal gland, contributes to numerous physiological functions, including stress, sleep-wake rhythm, body temperature cycles, and circadian rhythms [11]. Melatonin may have a protective effect in the liver during sepsis by directly scavenging ROS or upregulating antioxidant enzymes to counteract oxidative stress, reduce inflammatory responses, and increase ATP levels [12][13]. Melatonin may regulate mitophagy and mitochondrial biogenesis[14]. In a mouse model, melatonin induced mitophagy and reduced oxidative stress and inflammation in atherosclerotic lesions, and prevented atherosclerotic progression [15]. In HepG2 cells *in vitro*, melatonin enhanced mitochondrial biogenesis and had protective effects in cadmium-induced hepatotoxicity [16]. The protective effects of melatonin against sepsis-induced hepatocyte injury remain to be elucidated.

This study investigated the protective mechanisms of melatonin in an *in vitro* model of sepsis-induced hepatocyte injury, with a focus on mitochondrial quality control.

Methods

Cell culture

Mouse hepatocyte AML12 cells were cultured in DMEM/F12 medium (11330032, Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS; 10270106, Gibco), 100 U/mL penicillin, 100μg/mL streptomycin (15140122, Sigma-Aldrich; St. Louis, Mo, USA), insulin, transferrin, selenium (ITS) liquid media supplement (I3146, Sigma-Aldrich), and 40 ng/ml dexamethasone (D4902, Sigma-Aldrich) at 37°C and 5% CO2 in a humidified incubator.

Experimental Procedure

Melatonin (MEL) (M5250, Sigma-Aldrich) was dissolved in ethanol to a final concentration of 0.01%. When 80% confluence was reached, AML12 cells were divided into four groups: MEL, AML12 cells were treated with 1μM melatonin; LPS, AML12 cells were treated with 25μg/mL lipopolysaccharide (LPS from Escherichia coli O55:B5; L-2880, Sigma-Aldrich) for 24h; MEL+LPS, AML12 cells were pretreated with 1μM melatonin for 1h followed by 25μg/mL LPS for 24 h; and control, AML12 cells were treated with an equivalent volume of vehicle.

Measurement of intracellular ROS levels

Intracellular ROS levels were evaluated using a commercially available kit (S0033S, Beyotime Biotechnology, China), according to the manufacturer's recommendations. Briefly, AML12 cells were trypsinized, centrifuged, and incubated with 2',7'-dichlorofluorescein-diacetate (DCFH-DA) (10 μM in DMEM/F12). ROS was quantified as mean fluorescence intensity using a flow cytometer (BD Biosciences, San Jose, CA, USA) with 488 nm excitation and 525 nm emission.

Enzyme-linked immunosorbent assay (ELISA)

TNF-α and IL-6 protein levels were assessed in AML12 cell-free culture supernatants using a commercial ELISA kit (EMC102A, EMC004, Neobioscience Technology Company, China), according to the manufacturer's instructions.

Biochemical analyses

Malondialdehyde (MDA) levels, superoxide dismutase (SOD) activity, and intracellular ATP levels were assessed in AML12 cell cultures using appropriate kits (S0131S, S0101S, S0027, lipid peroxidation MDA assay kit, total SOD assay kit with WST-8, enhanced ATP assay kit [Beyotime Biotechnology, China]) according to the manufacturer's instructions.

Western blot analysis

AML12 cells were homogenized in radioimmunoprecipitation assay (RIPA) buffer (P0013B, Beyotime Biotechnology, China) supplemented with a protease and phosphatase inhibitor cocktail (P1045, Beyotime Biotechnology, China). Total protein was extracted and analyzed using a BCA protein assay kit (P0012S, Beyotime Biotechnology). After separation on 10% SDS-PAGE, proteins were transferred onto polyvinylidene difluoride membranes (Millipore), which were then blocked for 1 h at room temperature (5% BSA) and incubated with the following primary antibodies: PINK1 (1:1,000, 23274-1-AP, Proteintech), Parkin (1:1,000, 14060-1-AP, Proteintech), PGC-1α (1:1,000, 66369-1-Ig, Proteintech), NRF-1 (1:1,000, 12482-1-AP, Proteintech), TFAM (1:1,000, 19998-1-AP, Proteintech), and GAPDH (1:5,000, 60004-1-Ig, Proteintech) overnight at 4°C. Subsequently, membranes were incubated with horse radish peroxidase (HRP)-conjugated secondary antibodies. Protein levels were quantified using densitometry and Image J software.

Statistical analysis

Statistical analysis was performed with SPSS 20.0. Data are presented as the mean ± SD of three individual experiments using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). Comparisons were performed using one-way analysis of variance (ANOVA) followed by a post hoc pairwise comparison (least significant difference [LSD]). $P<0.05$ was considered statistically significant.

Results

Melatonin attenuated LPS-induced inflammatory response in hepatocytes

As proinflammatory cytokine levels are increased in sepsis, which may damage the liver, TNF-α and IL-6 protein levels were assessed in cell-free culture supernatants to investigate the effects of melatonin on the LPS-induced inflammatory response in hepatocytes. TNF-α and IL-6 protein levels were significantly increased in cell-free culture supernatants from LPS-treated AML12 cells compared to controls, and significantly decreased in cell-free culture supernatants from LPS-treated AML12 cells by melatonin pre-treatment (**Fig.1**).

Melatonin attenuated LPS-induced oxidative stress in hepatocytes

To investigate the redox status of hepatocytes during sepsis, intracellular ROS levels, lipid peroxidation (MDA level), and SOD activity were measured. Intracellular ROS and MDA levels were significantly increased in LPS-treated AML12 cells compared to controls, and significantly decreased in LPS-treated AML12 cells by melatonin pre-treatment. Conversely, SOD activity was significantly decreased in LPS-treated AML12 cells compared to controls, and significantly increased in LPS-treated AML12 cells by melatonin pre-treatment (**Fig.2**).

Melatonin increased intracellular ATP levels under septic conditions

To investigate mitochondrial function in hepatocytes during sepsis, intracellular ATP levels were assessed. Intracellular ATP levels were significantly decreased in LPS-treated AML12 cells compared to controls, and significantly increased in LPS-treated AML12 cells by melatonin pre-treatment (**Fig.3**).

Melatonin upregulated mitophagy-related protein levels

The PINK1-Parkin pathway is a main regulator of mitophagy. Western blot analysis showed that PINK1 and Parkin protein levels were significantly increased in LPS-treated AML12 cells compared to controls, and significantly increased in LPS-treated AML12 cells by melatonin pre-treatment (**Fig.4**).

Melatonin upregulated mitochondrial biogenesis-related protein levels

PGC-1α, NRF-1 and TFAM are the most relevant proteins in mitochondrial biogenesis. Western blot analysis showed that PGC-1α, NRF-1 and TFAM protein levels were significantly increased in LPS-treated AML12 cells compared to controls, and significantly increased in LPS-treated AML12 cells by melatonin pre-treatment (**Fig.5**).

Discussion

This study explored the protective mechanisms of melatonin in an *in vitro* model of sepsis-induced hepatocyte injury with a focus on mechanisms involved in mitochondria quality control. Findings showed that melatonin reduced LPS-induced inflammation and oxidative stress and enhanced mitophagy and mitochondrial biogenesis in hepatocytes.

Patients with liver dysfunction often experience sepsis, with symptoms occurring in the early stages [4]. Melatonin has anti-inflammatory, anti-oxidative stress and anti-tumor effects, and may have a protective role in the pathogenesis of sepsis. A prior report showed that melatonin reduced sepsis-induced liver inflammation and oxidative stress, thereby alleviating liver damage and reducing liver dysfunction [17]. In the present study, LPS was used to establish an *in vitro* model of hepatocyte injury induced by sepsis. Consistent with previous results, melatonin pretreatment decreased the LPS-induced release of inflammatory cytokines from hepatocytes, implying that melatonin may reduce hepatocellular inflammation during sepsis.

Oxidative stress, defined as an imbalance between the production of ROS and their elimination by protective mechanisms, contributes to the pathogenesis of sepsis. Sepsis can cause metabolic imbalance, mitochondrial dysfunction, and the production of large quantities of ROS, which accumulate and damage hepatocytes [18]. Mitochondria produce and are damaged by ROS within cells. Mitochondrial ROS production mainly takes place in the electron transport chain during oxidative phosphorylation. Altered mitochondrial function can lead to ROS overproduction, which damages mitochondrial proteins and DNA, and produces a continuous cycle of ROS generation [19]. In the present study, intracellular ROS level was elevated in LPS-treated hepatocytes vs. controls, and this effect was reversed by pre-treatment with melatonin. MDA is formed during lipid peroxidation and is considered a marker of oxidative stress. SOD is a cellular antioxidant and responsible for the elimination of ROS [20]. In the present study, MDA levels were elevated and SOD activity was reduced in LPS-treated hepatocytes vs. controls, and these effects were reversed by pre-treatment with melatonin. These data suggest that melatonin may alleviate LPS-induced oxidative stress in hepatocytes.

Hepatocytes have developed a variety of mechanisms to maintain mitochondrial homeostasis, including mitophagy and mitochondrial biogenesis [7]. Mitophagy, or selective autophagy of mitochondria, can remove damaged mitochondria and prevent excessive production of ROS [6]. The PINK1-Parkin pathway is a main regulator of mitophagy. In sepsis, PINK1 accrues on the outer membrane of dysfunctional mitochondria, which induces the translocation of Parkin from the cytoplasm to the outer mitochondrial membrane and triggers mitophagy [7]. Accumulating evidence implies that mitophagy may have a protective effect on liver function during sepsis. Enhancing mitophagy reduced LPS-induced cardiomyocyte injury, oxidative stress, and inflammation [21], while Parkin-knockout mice exhibited worse cardiac function and constant degradation of mitochondrial metabolic functions compared to wild-type mice during sepsis [22]. In the present study, LPS induced mitophagy in hepatocytes vs. controls, and these effects were enhanced by melatonin pre-treatment, suggesting that melatonin may augment mitophagy in hepatocytes during sepsis.

Mitochondrial biogenesis, or the formation of new mitochondria, is mainly regulated by the PGC-1a-NRF1-TFAM pathway. PGC-1a is a transcriptional co-activator that promotes mitochondrial DNA replication and transcription by activating the transcription factor NRF1 and subsequently upregulating the expression of TFAM [7]. Mitochondrial biogenesis has a role in sepsis. In astrocytes, sepsis can stimulate mitochondrial biogenesis, increase the number of mitochondria in cells, and recover mitochondrial ultrastructure to meet

the increased energy demands of the astrocytes under septic conditions [23][24]. One recent report showed that melatonin can enhance mitochondrial biogenesis in liver fibrosis and sustain mitochondria [14]. In the present study, PGC-1a, NRF1 and TFAM protein levels were elevated in LPS-treated hepatocytes vs. controls, and these effects were enhanced by melatonin pre-treatment. These results suggest that melatonin can promote mitochondrial biogenesis during sepsis.

Cellular ATP levels are a marker of mitochondrial function. In the present study, intracellular ATP levels were reduced in LPS-treated hepatocytes vs. controls, and this effect was reversed by melatonin pre-treatment. These results suggest that melatonin can improve mitochondrial function in hepatocytes during sepsis.

In conclusion, our study elucidated the role of melatonin in preserving mitochondrial homeostasis in hepatocytes during sepsis. Mechanisms involved selective elimination of dysfunctional mitochondria through mitophagy and induction of mitochondrial biogenesis. These data indicate melatonin has potential as a therapy for sepsis-induced liver injury.

Declarations

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Availability of data and material

The datasets used during the present study are available from the corresponding author upon reasonable request.

Code availability

Not applicable.

Author contributions

Bin Hu, Zhijiang Chen, Lili Liang, Meiyu Zheng, Xinxin Chen and Yaxin Wang performed the experiments; Bin Hu and Zhijiang Chen analyzed the data; Bin Hu drafted the manuscript; Qiyi Zeng contributed to the interpretation of the results and critical revision of the manuscript. All authors had final approval of the submitted versions.

Competing interests

The authors declare that there have no competing interests.

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Figures

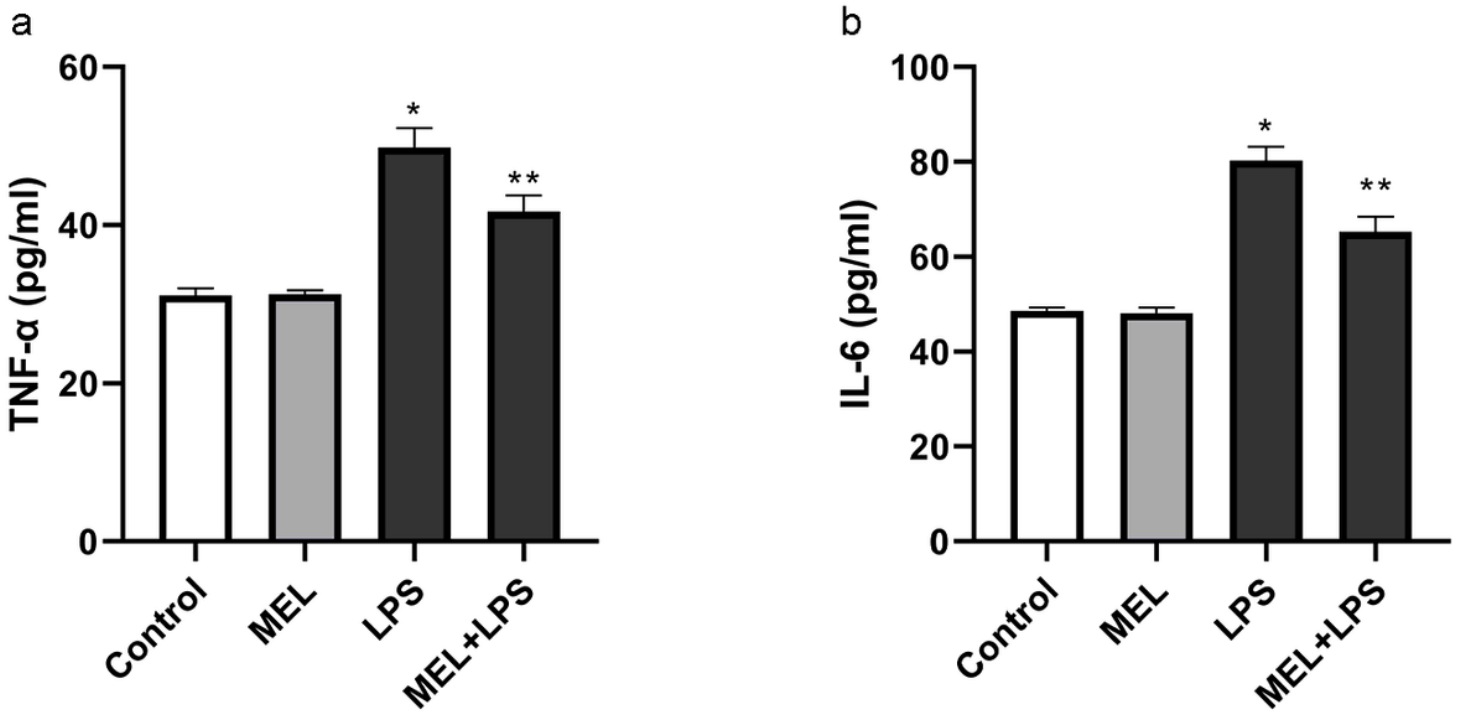

Figure 1

TNF-α (a) and IL-6 (b) protein levels in cell-free culture supernatants from LPS-treated AML12 cells were significantly decreased by melatonin pre-treatment. TNF-α and IL-6 levels were determined on ELISA. Data are the mean ± SD of three independent experiments. *P<0.05 versus Control; **P<0.05 versus LPS.

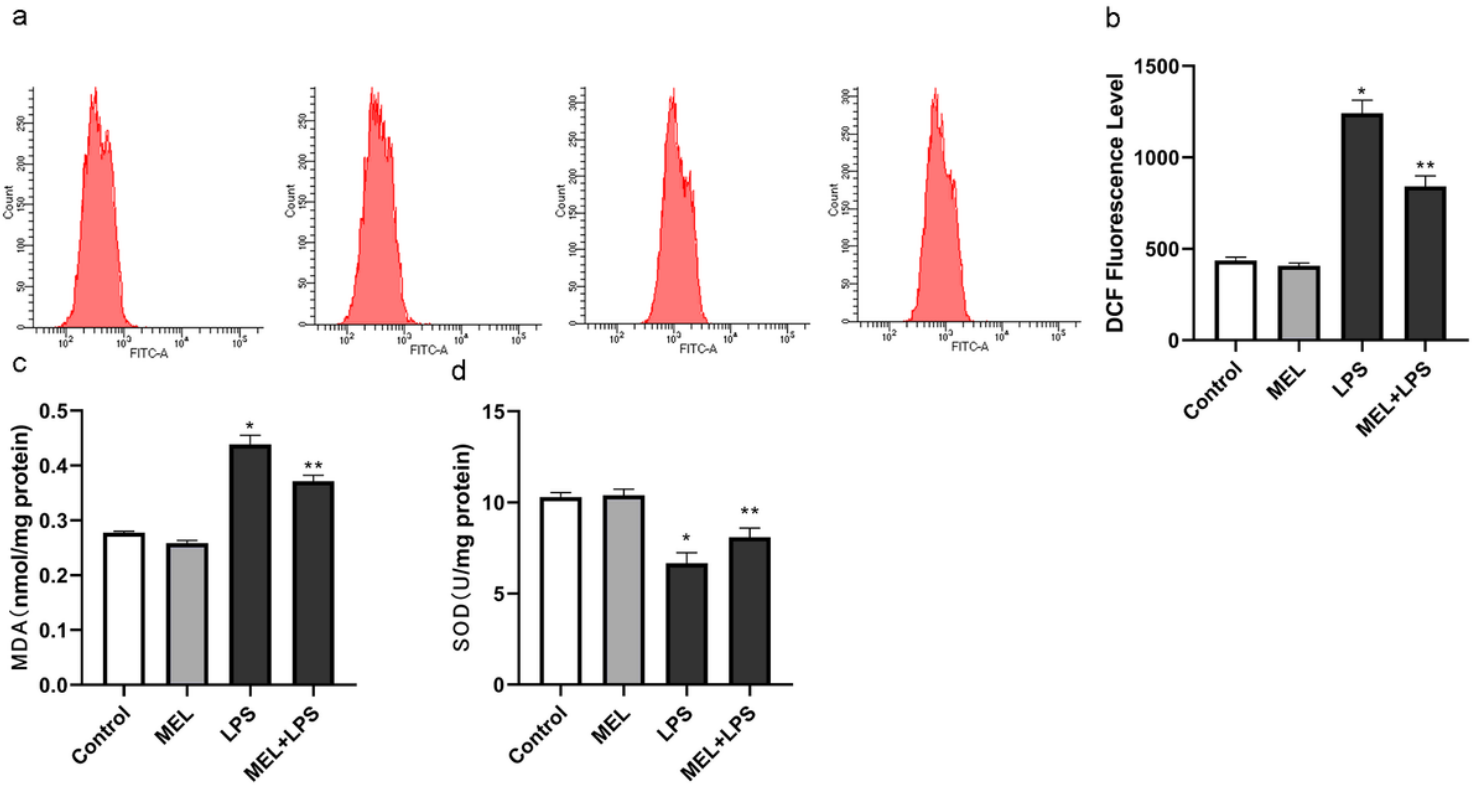

Figure 2

Intracellular ROS (a, b) and MDA (c) levels in LPS-treated AML12 cells were significantly decreased by melatonin pre-treatment Conversely, SOD activity (d) in LPS-treated AML12 cells was significantly increased by melatonin pre-treatment. Intracellular ROS levels in DCFH-DA stained AML12 cells were determined by flow cytometry. MDA levels and SOD activity were measured using appropriate kits. Data are the mean ± SD of three independent experiments. *P<0.05 versus Control; **P<0.05 versus LPS.

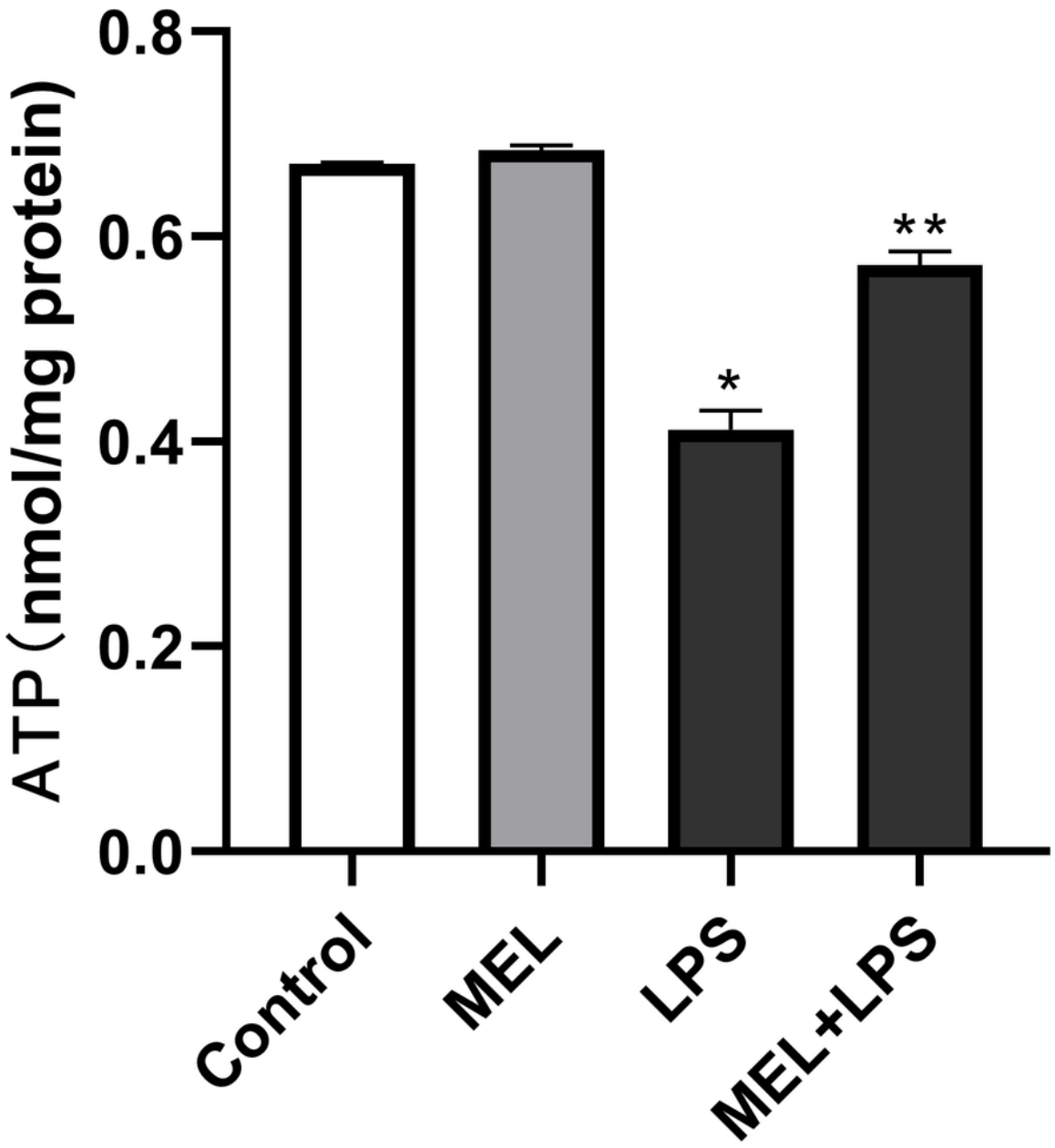

Figure 3

Intracellular ATP levels in LPS-treated AML12 cells were significantly increased by melatonin pre-treatment. Intracellular ATP levels were measured using an appropriate kit. Data are the mean ± SD of three independent experiments. *P<0.05 versus Control; **P<0.05 versus LPS.

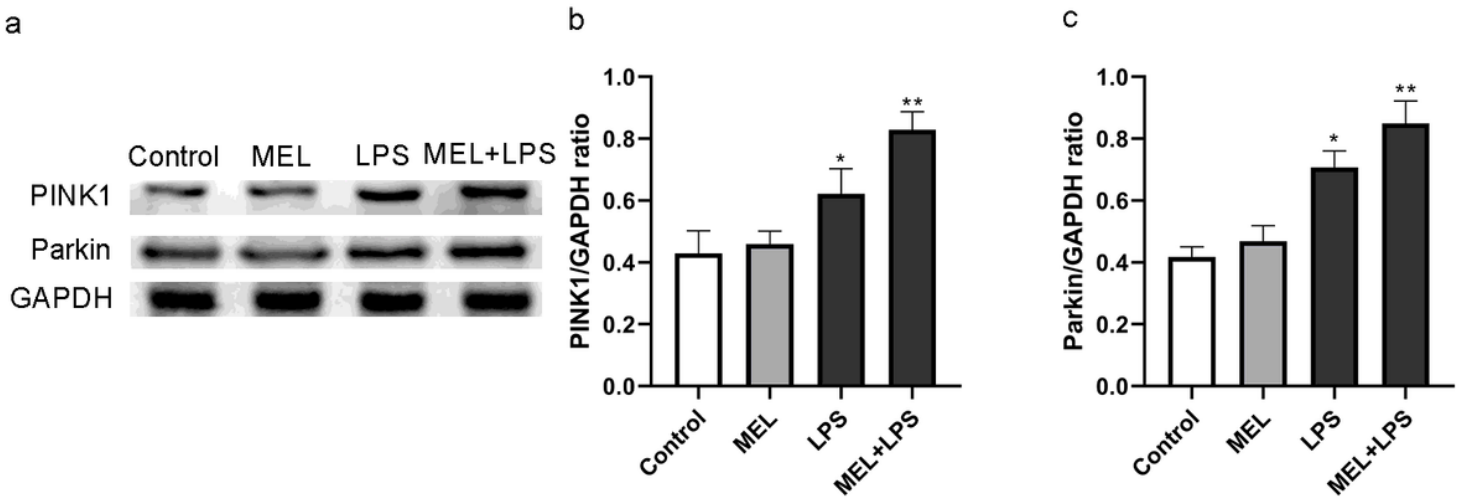

Figure 4

PINK1 (a, b) and Parkin (a, c) protein levels in LPS-treated AML12 cells were significantly increased by melatonin pre-treatment. PINK1, Parkin, and GAPDH protein levels were assessed using Western blotting and were quantified by densitometry. Data are the mean ± SD (n=3 in each group). *P<0.05 versus Control group; **P<0.05 versus LPS group.

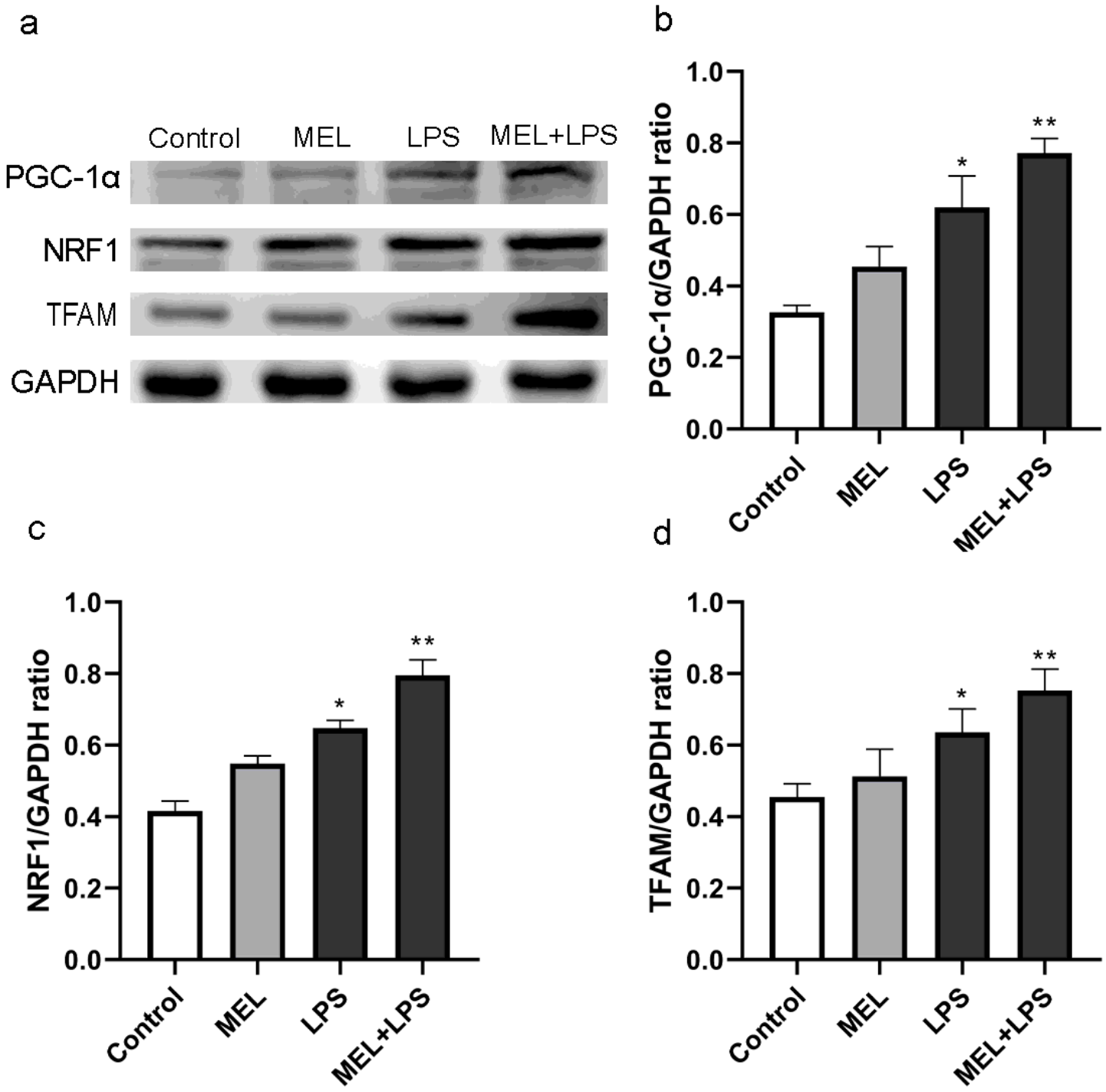

Figure 5

PGC-1α (a, b), NRF-1 (a, c), and TFAM (a, d) protein levels in LPS-treated AML12 cells were significantly increased by melatonin pre-treatment. PGC-1α, NRF1, and TFAM protein levels were assessed using Western blot. Data are the mean ± SD (n=3 in each group). *P<0.05 versus Control group; **P<0.05 versus LPS group.