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Metformin Alleviated the Neuronal Oxidative Stress in Hippocampus of Rats under Single Prolonged Stress

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Abstract In an animal model of post-traumatic stress disorder (PTSD), our previous studies showed mitochondrial stress-induced apoptosis in the hippocampus. Metformin, the most commonly prescribed anti-diabetic drug, exerts its effects through 5′-adenosine monophosphate-activated protein kinase (AMPK) activation. It was shown that a neuroprotective role was gradually established against stroke, spinal cord injury and Parkinson's disease. The aim of this study was to explore the role of the AMPK pathway in neuronal apoptosis in the hippocampus using a rat model of PTSD. The model PTSD rats received acute exposure to prolonged stress (single prolonged stress, SPS), followed by examination of the effects of genes and/or proteins related to the AMPK and oxidative stress pathways in the hippocampus with or without metformin preconditioning. The results indicated that the level of phosphorylated AMPK was markedly increased after SPS. Metformin protected the hippocampus as evidenced by abolishing down-regulation of the AMPK pathway and up-regulating expression of oxidative stress-related genes. These results indicated that metformin attenuated oxidative stress in the hippocampus in rats under SPS. AMPK pathway activation may be a novel therapeutic protocol for PTSD patients.

Keywords Post-traumatic stress disorder · Single prolonged stress · Hippocampus · Oxidative stress

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Introduction

Post-traumatic stress disorder (PTSD) is an extended and delayed psychiatric disorder. It often develops after experiencing or witnessing life-threatening events such as warfare, natural disasters, terrorist incidents, serious accidents, or violent assaults (Zhang and Ho 2011; Al-Hadethe et al. 2014).

The pathophysiology of PTSD has been intensively investigated (Kessler 2000). As shown in previous studies, the amygdaloid nucleus, hippocampus, and medial prefrontal cortex (mPFC) are closely related to the experience of PTSD (Shin et al. 2006). Shin and Liberzon have shown that single prolonged stress (SPS) induces inhibition of the hypothalamic–pituitary–adrenal (HPA) axis (Knox et al. 2012; Hughes and Shin 2011) . The HPA is a putative endocrinological marker of PTSD (Yehuda 2005). SPS models have therefore been extensively applied in the investigation of PTSD. In a previous study, we revealed that apoptotic cells were significantly increased in the hippocampus in rats after SPS, which was accompanied by release of cytochrome C from the mitochondria into the cytosol, indicating malfunction of mitochondria may be involved in the atrophy and cell death of the hippocampus during PTSD (Li et al. 2010).

Metformin, one of first-line drugs for type 2 diabetes and an agonist of AMPK pathway, which is known for autophagy promotion (Meijer and Codogno 2007), anti-inflammation (Isoda et al. 2006; Kim et al. 2014; Cameron et al. 2016) and anti-cancer effects (Jalving et al. 2010; Dowling et al. 2012), has extensive anti-apoptotic properties in the nervous system. Wang et al. have demonstrated that metformin pretreatment prevented spinal cord apoptosis through enhancement of autophagy and oppression of inflammation (Wang et al. 2016).

Ashabi et al. showed that pretreatment with metformin protects against global cerebral ischemia in male rats by intervening with the AMPK/PGC1α pathway (Ashabi et al. 2014). Deng et al. have revealed that pre-stroke metformin treatment is neuroprotective for ischemic brains (Deng et al. 2016).

AMPK pathway is involved in anti-obesity (Kim et al. 2004; Ahn et al. 2008; Chen et al. 2015) and has anti-cancer (Park et al. 2010; Shao et al. 2012; Chen et al. 2013) effects. AMPK, a serine-threonine kinase and an evolutionarily conserved energy sensor, is activated by reductions in the cellular ATP/AMP ratio. The ATP/AMP ratio turns off anabolic processes and activates catabolic pathways. AMPK activation can therefore restore an imbalance in cellular energy. AMPK senses energy deficiency and is triggered by metabolic insults, such as oxygen or glucose deficiency (Rutter et al. 2003; Ramamurthy and Ronnett 2012). Activated AMPK phosphorylates various substrates that generally result in reduced ATP consumption activities and increased ATP production. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1a) is a downstream regulator of phosphorylated AMPK and a transcriptional coactivator that represents a master regulator of mitochondrial biogenesis. It promotes the transcription of mitochondrial genes and plays a key role in coordinating the oxidation of intracellular fatty acids with mitochondrial remodeling. These coactivators target multiple transcription factors including NRF1, NRF2 and the orphan nuclear hormone receptor (Scarpulla 2011; Ventura-Clapier et al. 2008). Mitochondrial transcription factor A (TFAM) performs regulation of mitochondrial biogenesis by interactions between TFAM and mtDNA (Picca and Lezza 2015).

During oxidative stress, in DNA repair processes, the poly(ADP-ribose) polymerase family plays an important role in cell death mechanisms and the regulation of transcription factors. It has been reported that the lipopolysaccharide (LPS)-evoked systemic inflammatory response induces expression of PARP family genes, including PARP1, -3, -9, -12 and -14 gene expressions in the mouse hippocampus (Czapski et al. 2013). DNA damage-inducible protein 45 beta (GADD45B) (Liu et al. 2015) and thioredoxin reductase 1 (TXNRD1) (Miranda-Vizuete et al. 2000) are common oxidative stress markers, and they denote the levels of stresses.

Currently, detailed understanding of the AMPK pathway as possibly being involved in apoptosis of the hippocampus due to PTSD is lacking. Therefore, we assessed the role of the AMPK pathway in the hippocampus under SPS including neuronal changes in SPS rats with or without treatment with metformin to illuminate whether metformin attenuates oxidative stress through enhanced energy production, which could be used as a potential therapy for PTSD patients.

Materials and Methods

Animals and Grouping

Male Wistar rats (aged 7 to 8 weeks; weighing 150–180 g at arrival) were acquired from the Experimental Animal Center of China Medical University. They were housed individually in an air-conditioned room at 23 ± 2 °C on a 12-h light/dark cycle with free access to food and water for 7 days to acclimate to the new environment. All procedures were carried out in compliance with the Guidance Suggestions for the Care and Use of Laboratory Animals, the Ministry of Science and Technology of the People's Republic of China. The number of animals and animal suffering during experiments were minimized.

SPS Model Constructing and Grouping

The rats ($n = 72$) were randomly divided into 12 groups ($n = 6$ per group). For the measurement of AMPK activation in the hippocampus, there were four groups, and rats in each group were sacrificed at 0, 1, 2 and 4 days after metformin (400 mg/kg/day; 1,1-dimethylbiguanide hydrochloride; cat no. D150959-5G; Sigma-Aldrich, St. Louis, MO, USA) subcutaneous injection. For the assessment of alterations of the AMPK pathway downstream with/without metformin treatment or SPS exposure, there were four groups of rats that were sacrificed at the same time, including the following: control group (CON), metformin group (MF), SPS group (SPS) and metformin with SPS group (MF + SPS). Thereinto, the CON rats were subcutaneously injected with an equal volume of phosphate-buffered saline (PBS) and were sacrificed at the same time as sacrifice occurred in the other groups. The PTSD model in our study was induced by the SPS, which has been established and recognized internationally (Eagle et al. 2013; Knox et al. 2012). SPS is carried out with the following consecutive steps: immobilization, 2 h; forced swimming, 20 min; and ether anesthesia (until consciousness was lost). Then, the rats remained undisturbed in their home cages until they were used for brain tissue sampling. The metformin (400 mg/kg/day) subcutaneous treatment was consistently given at 10:00 am everyday until the rats were sacrificed. The tissue collection after SPS exposure was executed 6 h after the procedure.

Western Blot Analysis

The fresh hippocampus tissues were respectively homogenized in ice-cold lysis buffer supplemented with 1 mM PMSF (Beyotime Biotechnology, China). The tissues were then ultrasonicated and high-speed centrifuged at a low temperature. The supernatant albumen was collected to be used in a protein assay with a BCA protein assay kit (Beyotime Biotechnology, China). Equal amounts of protein

(50 μg/lane) were subjected to 8–12% SDS-PAGE and then transferred onto PVDF membranes with electroblotting. After 2 h incubation and blocking with Tris-buffered saline (TBS) containing 5% skim milk and 0.1% Tween 20 and 1:2000 primary antibodies, including rabbit polyclonal antibodies against phospho-AMPKα (Thr172) (1:1000; Cell Signaling, USA), goat polyclonal antibodies against PGC1a (1:500; Abcam, UK) and rabbit polyclonal antibodies against PARP (1:1000; Cell Signaling, USA), they were then incubated overnight at 4 °C. The blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-mouse IgG-HRP and anti-rabbit IgG-HRP, 1:5000; Santa Cruz Biotechnology, Inc., USA) at room temperature for 2 h. The membranes were subjected to enhanced chemiluminescence (ECL; Thermo Scientific, USA). To confirm the amount of protein that was loaded, the same blot was incubated with mouse monoclonal antibodies against β-actin (1:5000; Abcam, UK). The optical density (OD) of the immunoreactive bands was analyzed and measured with a Gel Image Analysis System (Tanon 2500R, Shanghai, China).

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from the hippocampi of the rats of each group using TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. One microgram of total RNA was reverse-transcribed into complementary DNA (cDNA) using a Prime Script RT reagent kit (Takara Biotech, Otsu, Japan) according to the manufacturer's protocol. The cDNA was used as a template to determine the gene expression level with a SYBR Green Real-Time PCR master mix kit (Takara Biotech, Otsu, Japan). The primer sequences used for PCR amplification are the following:

PGC1a (upper: 5′-TGAAGTGGTGTAGTGACCAATC-3′, lower: 5′-GCAAGTTTGCCTCATTCTCTTC-3′); NRF1 (upper: 5′-CTATCCGAAAGAGACAGCAGAC-3′, lower: 5′-GGGTGAGATGCAGAGAACAA-3′); TFAM (upper: 5′-GCCTGTCAGCCTTATCTGTATT-3′, lower: 5′-TGCATCTGGGTGTTTAGCTTTA-3′); GADD45B (upper: 5′-CCTGGTCACGAACTGTCATAC-3′, lower: 5′-GGTTATTGCCTCTGCTCTCTT-3′); PARP14 (upper: 5′-AGGTGGAATTGATCCGAGATTT-3′, lower: 5′-GATTTGAGCGGTGGCTGATA-3′); TXNRD1 (upper: 5′-CGGGAGAAGAAGGTTGTCTATG-3′, lower: 5′-GAACCGCTCTGCTGAGTAAA-3′); COX2 (upper: 5′-GGCCATGGAGTGGACTTAAA-3′, lower: 5′-GTCTTTGACTGTGGGAGGATAC-3′); β-actin (upper: 5′-CGGAAAGAAGATGACGCAGATA-3′, lower: 5′-ACCAGAGTCCAAGACAATGC-3′); RPL19 (upper: 5′-CTTAGGCTACAGAAGAGGCTTG-3′, lower: 5′-GAGTTGGCATTGGCGATTTC-3′). Takara Biotech designed and synthesized all the primers. The data were analyzed and measured with a Rotor Gene PCR-3000 (Corbett Research, Australia). Relative mRNA expression levels were normalized against β-actin or RPL-19.

Statistical Analysis

The results are expressed as the mean ± standard deviation (SD). Statistical differences between the groups were analyzed with a one-way analysis of variance (ANOVA) using SPSS version 17.0 (SPSS, Inc., an IBM Company, Chicago, IL, USA), followed by Tukey's post-hoc multiple comparison tests. A result of $P < 0.05$ was considered to be statistically significant.

Results

Metformin Effectively Phosphorylated AMPK and Increased PGC1a in Hippocampal Tissue

By Western blotting analysis, as indicated in Fig. 1a, c, metformin (400 mg/kg) treatment by daily subcutaneous injection triggered the phosphorylation of AMPK (pAMPKa) in the hippocampi of rats in the 1-day, 2-day and 4-day groups compared with the 0-day group ($P < 0.05$). Not surprisingly, PGC1a, the important main downstream protein of AMPK, was also augmented remarkably in the 1-day, 2-day and 4-day groups compared with the 0-day rats (Fig. 1b, d; $P < 0.05$). One day after subcutaneous injection of metformin, pAMPKa and PGC-1a immunoreactivity increased up to twofold and then remained stable, indicating they reached a peak concentration in the hippocampus (Fig. 1c, d). These results revealed that metformin could go through the blood-brain barrier and react with hippocampal tissue. In addition, AMPK activation in the hippocampus demonstrated possible benefits to PTSD with metformin.

Metformin Pretreatment Activated the AMPK Pathway in the Hippocampus but not after SPS Stress

Since metformin treatment would sufficiently activate the AMPK/PGC1a pathway by increased immunoreactivity to pAMPKa and PGC1a after 1 day, it is important to understand the effective duration of metformin preconditioning. We further investigated the effects of SPS on the immunoreactivity of pAMPKa and PGC1a at 6 h and 1 day after metformin pretreatment to inspect the onset and early effects of metformin on SPS stress. We observed and compared the difference between four subgroups (CON group with vehicle injection; MF group with metformin pretreatment; SPS group with the SPS procedure only and the MF + SPS group with the SPS procedure following metformin pretreatment). Upon SPS, there was no distinction on pAMPKa and PGC1a
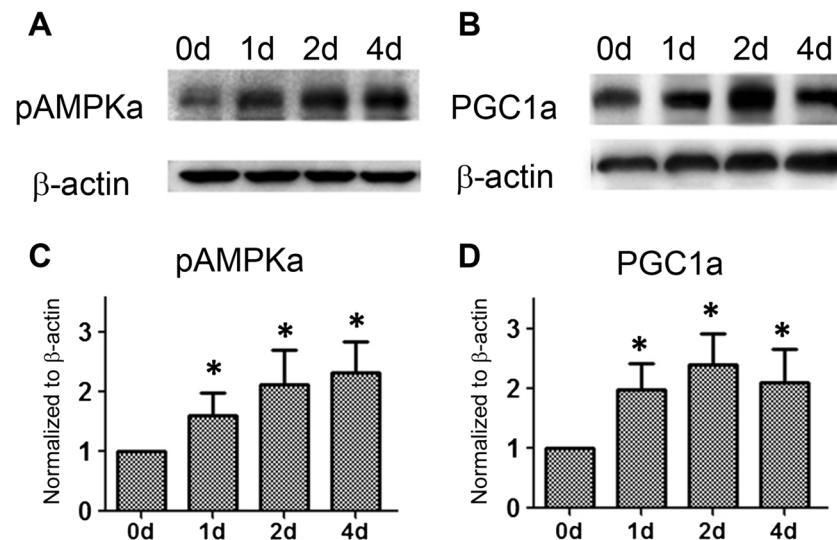
Fig. 1 Systemic injection of metformin activated AMPK pathway in the hippocampus. **a**, **b** Western blot analysis of pAMPKa and PGC1a in the hippocampus was performed at different days using antibodies as indicated. **c**, **d** Relative immunoreactive responses were evaluated by densitometry quantization of protein bands on the blots (data represent means ± SD; Tukey's test *$P < 0.05$, compared with the 0-day group, $n = 3$ for each group). β-actin protein served as loading controls

between those four subgroups with 6-h metformin treatment via Western blotting (Fig. 2). In contrast to the CON group or SPS group, significant increases of pAMPKa and PGC1a immunoreactivity, in both the MF and MF + SPS groups, supported metformin needing about 1 day to accumulate and activate the neurons in the hippocampus (Fig. 2). Interestingly, SPS stress did not show an influence on AMPK activity in the hippocampus at both time points (Fig. 2).

To explore the effects of metformin on transcription levels downstream of the AMPK pathway, mRNA expression was assessed via qRT-PCR. Consistent with immunoreactivity response, PGC1a mRNA expression was not altered by either metformin or SPS in the 6-h metformin treatment group. Yet, 1 day after metformin treatment, not only MF but also MF + SPS showed notable increases in mRNA expression of PGC1a, compared to the CON or SPS subgroups (Fig. 3a; *$P < 0.05$, #$P < 0.02$). Amazingly, SPS stress significantly downregulated PGC1a mRNA levels compared to the CON subgroup (Fig. 3a; @$P < 0.01$). Further, downstream of the AMPK pathway, two crucial transcription factors related to mitochondrial function, i.e., TFAM and NRF-1, showed uniform trends with PGC-1a (Fig. 3b, c). These results demonstrated that SPS stress suppressed the AMPK pathway in the hippocampus, while 1 day of metformin pretreatment could overturn the negative regulation of the AMPK pathway on mRNA levels in the hippocampus. Although we did not observe restraint of the AMPK pathway on immunoreactivity, this could be due to the short duration after SPS exposure or through another indirect mechanism, which need further investigation.

Metformin Prevented Hippocampus Apoptosis Induced by SPS and Promoted Mitochondrial Biogenesis in the Hippocampus

Our previous studies indicated that SPS could result in incremental caspase families and apoptosis in the hippocampus (Han et al. 2013). PARP could be activated in cells experiencing stress and/or DNA damage. Thus, the DNA damage and apoptosis marker, poly(ADP-ribose) polymerase (PARP), in the hippocampus was monitored via Western blotting. Four groups, based on 1-day metformin pretreatment with or without SPS exposure, were analyzed. As exhibited in Fig. 4a, b, SPS remarkably raised immunoreactivity of PARP (Fig. 4b; #$P < 0.01$, compared to the CON group; *$P < 0.05$, compared to the MF + SPS group). With metformin preconditioning for 1 day, SPS failed to increase the PARP immunoreactivity. In addition, metformin alone only slightly augmented PARP immunoreactivity. Together, our results suggested that metformin pretreatment protected against neuronal apoptosis in the hippocampus, although further morphologic observation needs to confirm and quantify the protective effects.

Our previous study illustrated that SPS-induced apoptosis is involved in multiple mechanisms, including mitochondrial dysfunction (Li et al. 2010). Yet, one of the most critical protective functions of metformin is to protect the mitochondria from oxidative stress through augmentation of the mitochondrial DNA (mtDNA) copy number to promote mitochondrial biogenesis. Our data illustrated that metformin increased TFAM mRNA expression in the hippocampus, which was reported to
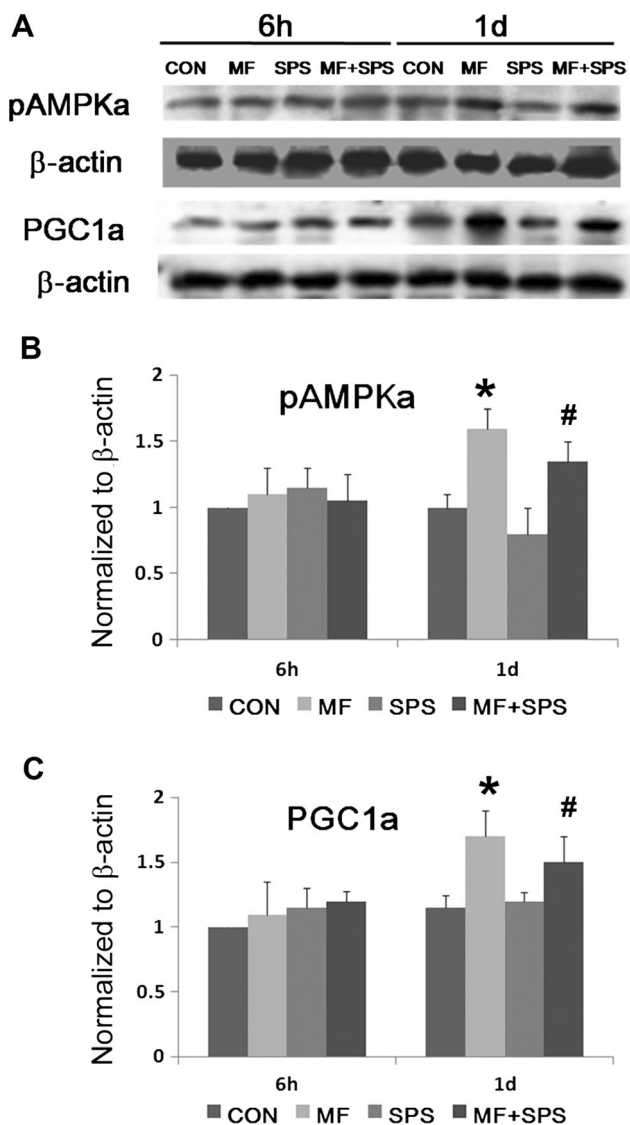
Fig. 2 Metformin activated AMPK pathway in the hippocampus following SPS after 1-day pretreatment. **a** Representative Western blot with 6 h and 1 day metformin pretreatment with or without SPS exposure. **b**, **c** Results of analysis of pAMPKa and PGC1a (data represent means ± SD; Tukey's test *$P < 0.05$ vs. the CON group; #$P < 0.05$ vs. the SPS group; $n = 3$ for each group). β-actin protein served as loading controls

Fig. 3 The mRNA level of PGC1a, TFAM and NRF1 was upregulated by metformin following SPS after 1-day pretreatment. qRT-PCR analysis comparing changes in expression of mRNA. Relative expression levels of PGC1a (**a**), TFAM (**b**) and NRF1 (**c**) were normalized with RPL19. Data represent means ± SD. $n = 3$ for each group. Tukey's test *$P < 0.05$ compared with the CON group; @$P < 0.02$ compared with the CON group; #$P < 0.02$ compared with the SPS group

upregulate the mtDNA copy number (Ekstrand et al. 2004; Picca and Lezza 2015). As expected, compared to the CON group, metformin increased the mtDNA copy number about twofold 1 day after metformin treatment and was stable for 4 days with the treatments (Fig. 4c; #$P < 0.05$).

Metformin Suppressed the Expression of Oxidative Stress-Related Genes in the Hippocampus Induced by SPS

It is revealed that oxidative stress in the hippocampus was elevated during PTSD in an animal model. Since
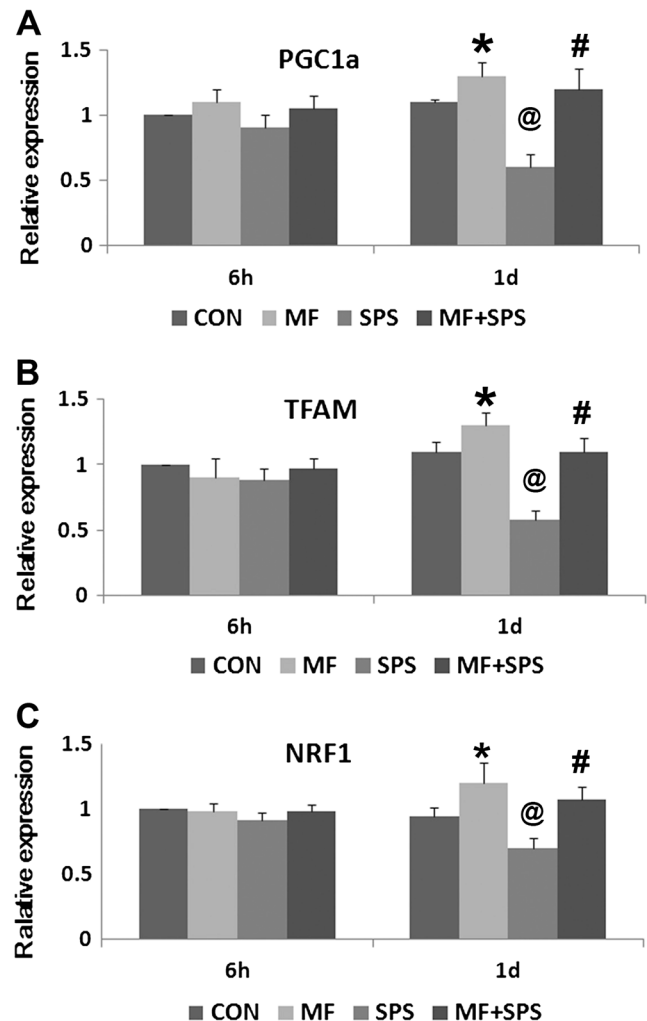
metformin manifested the effects of reduction on oxidative stress in the brain or blood vessels to prevent cell death (Rösen and Wiernsperger 2006; Zhao et al. 2014; Cahova et al. 2015), we measured three oxidative stress-related genes (GADD45B, TXNRD1, PARP14) to clarify the effects of metformin on oxidative stress in the hippocampus. Surprisingly, our results showed that SPS stimulated a two- to tenfold increase in mRNA levels, but metformin pretreatment totally abolished upregulation on all three genes by SPS exposure (Fig. 4d, e and f; #$P < 0.02$, compared to the CON group; *$P < 0.05$, compared to the MF + SPS group). Our results showed that SPS-induced oxidative stress in the hippocampus and metformin pretreatment could prevent this stress by SPS to protect the hippocampus from PTSD.

Fig. 4 Metformin prevented apoptosis and oxidative stress induced by SPS. **a** Western blot analysis comparing changes in expression of PARP. β-actin protein served as loading controls. **b** PARP bands were quantified by densitometry. **c** mtDNA copy number was measured by the ratio of COX2/β-actin DNA expression level ($\#P < 0.05$ compared with the 0-day group). **d–f** mRNA expression levels of PARP14, GADD45B and TXNRD1. RPL19 served as the housekeeping gene. Data represent means ± SD. $n = 3$ for each group. Tukey's test $\#P < 0.01$ compared with the CON group; $*P < 0.05$ compared with the MF + SPS group
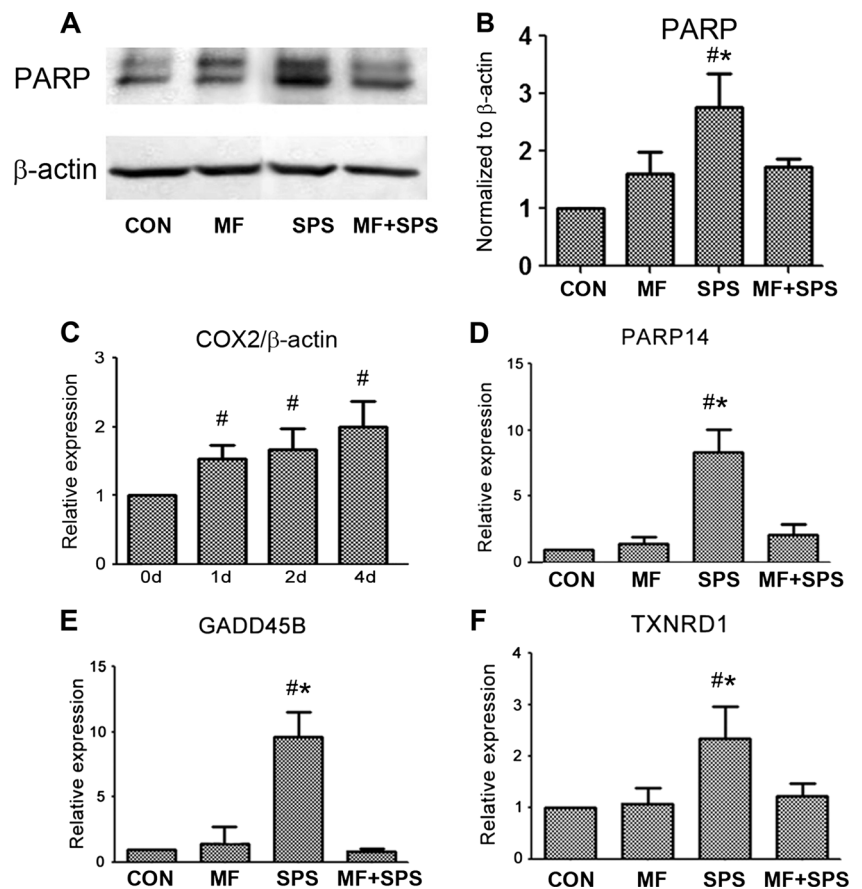
Discussion

PTSD is a complex mental disorder. It develops and is maintained in traumatized individuals as the result of impotent attempts to control unwanted feelings, thoughts and memories related to traumatic events. Functional neuroimaging studies showed that PTSD patients had excessive amygdala reactivity, smaller hippocampus volume and failed activation of the medial prefrontal/anterior cingulate cortex (Rauch and Shin 1997a; Rauch and Shin 1997b; Shin et al. 2006; Francati et al. 2007a, b; Hughes and Shin 2011). Our previous study showed that the mitochondrial pathway was involved in the process of SPS-induced apoptosis in the hippocampus. Li et al. demonstrated that there were an increased number of apoptotic cells in the hippocampus after SPS exposure, which was accompanied by release of cytochrome *c* from the mitochondria and increases in caspase-3 and -9 expressions (Li et al. 2010). Han et al. illustrated that the endoplasmic reticulum stress and impairment of glial cells in the hippocampus might be involved in PTSD-induced apoptosis (Han et al. 2013, 2015). In summary, protecting the hippocampus from stresses, including maintaining mitochondrial function, would be a promising protocol for the therapy of PTSD.

Recently, as described above, metformin, the AMPK pathway activator, is famous for its anti-obesity, anti-cancer and neuroprotective function (Rojas and Gomes 2013). An increasing number of studies indicated that metformin may be a novel method for treating brain damage (Wang et al. 2016). In this study, we are the first to investigate the possible role of metformin in the hippocampus of PTSD rats induced by SPS exposure. Multiple studies have demonstrated that metformin can activate the AMPK pathway in the brain. To date, no one ever measured AMPK activation in the hippocampus with metformin. Our results indicated that 1 day after the subcutaneous treatment, pAMPKa manifests in the hippocampus, followed by a PGC1a increase. It is promising that metformin could have the effect of protection of the hippocampus.

We further observed the effects of SPS on the AMPK pathway. Instead of activation, SPS suppressed the expression of PGC1a on mRNA expression, although it did not detect a change in phosphor-AMPKa on immunoreactivity. Meanwhile, the results indicated that metformin overturned the inhibited expression of PGC1a. The downstream transcription factors of PGC1a, NRF1 and TFAM are both important in mitochondrial biogenesis and normal functioning during stresses. As evidenced by our results, metformin pretreatment 1 day before SPS defended the downregulation of both

NRF1 and TFAM induced by SPS, which means metformin somehow prevented injury of the hippocampal neurons by SPS. We suspected that metformin preconditioning might enhance mitochondrial function against adverse stresses in the hippocampus. Further, as is known, TFAM is responsible for the mitochondrial copy number (Ekstrand et al. 2004). Our data indicated that metformin increased the mRNA expression of TFAM, which explained the increase in the mtDNA copy number in the hippocampus, which supported metformin promoting mitochondrial biogenesis, which might partially account for the neuroprotective mechanism of metformin. Additional evidence of metformin inducing protection of the hippocampus is the reduction of PARP immunoreactivity, the apoptosis marker, stimulated by SPS. Moreover, three oxidative related genes (PARP14, GADD45B and TXNRD1) that were affected by SPS all recovered to the normal level, which was the same as the control group. This indicated that the SPS-induced oxidative stress was diminished by metformin.

In summary, metformin can activate the AMPK pathway in the hippocampus, preserve mitochondrial function and promote its biogenesis in a rat model. This brings new light to potential therapies for PTSD patients, although more detailed research on the protective mechanism, as well as clinical trials, should be performed in the future.

Compliance with Ethical Standards

Conflicts of Interest The authors declare that they have no conflict of interest in this article.

Ethical Statement This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of the Laboratory Animals of the National Health and Medical Research Council of China. The experimental protocol was approved by the Department of Psychiatry, China Medical University Animal Ethics Committee, and was endorsed by the Research and Ethics Committee of the Liaoning Key Lab of Biological Psychiatry, China.

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