



Melatonin alleviates lipopolysaccharide-compromised integrity of blood–brain barrier through activating AMP-activated protein kinase in old mice

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Summary

Blood–brain barrier (BBB) dysfunction is considered to be an early event in the pathogenesis of a variety of neurological diseases in old patients, and this could occur in old people even when facing common stress. However, the mechanism remains to be defined. In this study, we tested the hypothesis that decreased melatonin levels may account for the BBB disruption in old mice challenged with lipopolysaccharide (LPS), which mimicked the common stress of sepsis. Mice (24–28 months of age) received melatonin (10 mg kg⁻¹ day⁻¹, intraperitoneally, i.p.) or saline for one week before exposing to LPS (1 mg kg⁻¹, i.p.). Evan's blue dye (EB) and immunoglobulin G (IgG) leakage were used to assess BBB permeability. Immunostaining and Western blot were used to detect protein expression and distribution. Our results showed

that LPS significantly increased BBB permeability in old mice accompanied by the degradation of tight junction proteins occludin and claudin-5, suppressed AMP-activated protein kinase (AMPK) activation, and elevated gp91^{phox} protein expression. Interestingly, administration of melatonin for one week significantly decreased LPS-induced BBB disruption, AMPK suppression, and gp91^{phox} upregulation. Moreover, activation of AMPK with metformin significantly inhibited LPS-induced gp91^{phox} upregulation in endothelial cells. Taken together, our findings demonstrate that melatonin alleviates LPS-induced BBB disruption through activating AMPK and inhibiting gp91^{phox} upregulation in old mice.

Key words: AMPK; blood–brain barrier; lipopolysaccharide; Melatonin; old mice; tight junction protein.

Introduction

Blood–brain barrier (BBB) dysfunction is an early event in the pathogenesis of a variety of neurological diseases (Rosenberg, 2012), and this could occur when facing various extrinsic or intrinsic stimuli (Weiss *et al.*, 2009). Sepsis is a common stress that old people often face (Martin *et al.*, 2006), in which lipopolysaccharide (LPS) is released into circulation (Shukla *et al.*, 2014), promoting the generation of reactive oxygen species (ROS) in cerebral microvascular endothelial cells and BBB disruption (Seok *et al.*, 2013).

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases is a major source of ROS generation in the brain, which critically contributes to BBB disruption under various CNS disorders (Kahles *et al.*, 2007). There are several NADPH oxidase family members, among which gp91^{phox}-containing NADPH oxidase is highly expressed in the cerebral vascular endothelium (Kahles *et al.*, 2007). Suppressing gp91^{phox} has been shown to protect mice from a variety of stimuli that promote cerebrovascular dysfunction (Liu *et al.*, 2008; Tang *et al.*, 2010).

LPS has been shown to induce BBB dysfunction via NADPH oxidase-derived ROS (Liu *et al.*, 2012; Zhao *et al.*, 2014). In addition, it could also inhibit the activation of AMP-activated protein kinase (AMPK), a serine/threonine protein kinase regulating cellular and organismal metabolism (Zhou *et al.*, 2014b). When AMPK is phosphorylated at Thr172 (p-AMPK), the kinase activity of the α subunit increases >100-fold (Hawley *et al.*, 1996). Interestingly, AMPK activation (p-AMPK) has been shown to attenuate LPS-induced BBB disruption *in vitro* (Zhao *et al.*, 2014) and protect the BBB in diabetes by inhibiting LPS-enhanced NADPH oxidase expression in brain capillary endothelial cells (Liu *et al.*, 2012).

Melatonin, mainly secreted by the pineal gland (Manchester *et al.*, 2015), exerts many physiological and biochemical functions when it is released into blood and cerebrospinal fluid (Reiter *et al.*, 2014), such as acting as a circadian rhythm regulator, an anti-inflammatory and immunoregulating molecule, and an oncostatic agent (Manchester *et al.*, 2015). Of note, melatonin and its metabolites are known to scavenge a variety of free radicals and reactive oxygen intermediates *in vivo* and *in vitro* (Galano *et al.*, 2014; Manchester *et al.*, 2015), which

Abbreviations

AMPK, AMP-activated protein kinase; BBB, blood–brain barrier; EB, Evan's blue dye; IgG, immunoglobulin G; i.p., intraperitoneally; LPS, lipopolysaccharide; NADPH, nicotinamide adenine dinucleotide phosphate; p-AMPK, phosphorylated AMP-activated protein kinase; ROS, reactive oxygen species; TJPs, tight junction proteins.

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may account for its protective effects against LPS toxicity to the brain (Wong *et al.*, 2014; Carloni *et al.*, 2016), liver (Wang *et al.*, 2007), and heart (Lu *et al.*, 2015). Moreover, melatonin also shows protective effects against BBB damage resulting from excitotoxicity in neonatal rats (Moretti *et al.*, 2015) and transient focal cerebral ischemia in young mice (Chen *et al.*, 2006a,b). However, it is not clear whether low melatonin levels contribute to the BBB disruption in old mice whose melatonin levels in serum and pineal gland decline as a result of aging (Hill *et al.*, 2013).

In this study, we investigated the effect of melatonin supplementation on LPS-induced BBB disruption in old mice and found that LPS disrupted the BBB in old mice via downregulating tight junction protein expression and increasing gp91^{phox} expression and inhibiting AMPK activation, and supplementation of melatonin could effectively inhibit the above changes.

Results

LPS has been shown to disrupt BBB *in vitro* (Zhao *et al.*, 2014) and *in vivo* (young mice) (Zhou *et al.*, 2014a). However, its impact on the BBB of old mice remains unknown. To address this question, mice were randomly divided into four groups receiving 0, 0.25, 0.5, and 1 mg kg⁻¹ LPS, respectively (Nonaka *et al.*, 2004). As shown in Fig. 1, LPS at a dose of 1 mg kg⁻¹, but not 0.25 or 0.5 mg kg⁻¹, significantly increased the EB leakage in old mice ($***P < 0.001$ vs. the Veh group). Therefore, we chose 1 mg kg⁻¹ LPS as the treatment for the rest of the study.

We next examined the effect of melatonin treatment on LPS-induced BBB damage in old mice. As shown in Fig. 2A, LPS significantly increased EB leakage (Fig. 2B, $**P < 0.01$ vs. the Veh group), and pretreatment with melatonin for one week significantly alleviated LPS-induced EB leakage in old mice (Fig. 2B, $^{\#}P < 0.05$ vs. the Veh+LPS group). As a good complimentary analysis of the BBB permeability, IgG immunostaining was performed to detect IgG extravasation. Consistent with the results of EB leakage, LPS significantly increased IgG leakage (Fig. 2D, $**P < 0.01$ vs. the Veh group), which was significantly decreased by pretreatment with melatonin (Fig. 2D, $^{\#}P < 0.05$ vs. the Veh+LPS

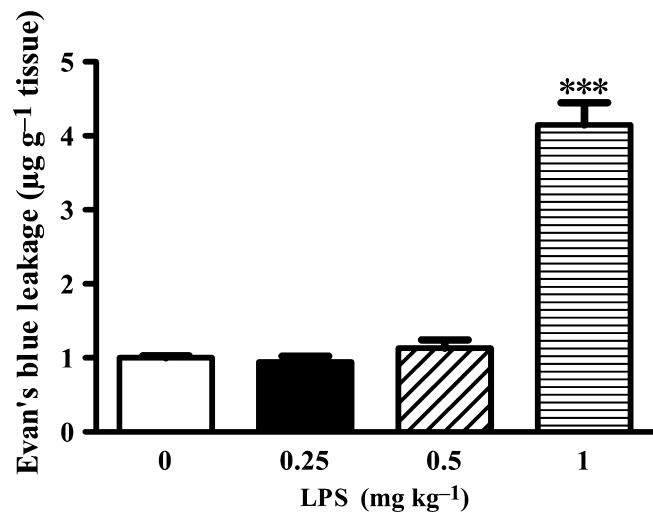


Fig. 1 Effect of LPS treatment on BBB integrity in old mice. Quantification of EB leakage showed that LPS at 1 mg kg⁻¹, but not at 0.25 or 0.5 mg kg⁻¹, significantly increased BBB permeability ($***P < 0.001$). $n = 5-6$ for each group. Data were expressed as mean \pm SEM.

group), further supporting that melatonin alleviated LPS-induced BBB disruption in old mice.

Loss or altered distribution of tight junction proteins (TJPs), particularly occludin and claudins, were seen in the compromised BBB following LPS treatment (Zhao *et al.*, 2014). Here using immunostaining, our results showed that melatonin decreased LPS-induced degradation of claudin-5 (Fig. 3A) and occludin (Fig. 3B) in old mice. The results were further confirmed by Western blot showing that LPS induced a dramatic reduction in total protein levels of claudin-5 (Fig. 3C, $**P < 0.01$ vs. the Veh group) and occludin (Fig. 3D, $**P < 0.01$ vs. the Veh group), and these changes were significantly inhibited by melatonin (Fig. 3, $^{\#}P < 0.05$ vs. the Veh+LPS group).

Death of endothelial cells of microvessels is a major contributor to the disruption of BBB integrity (Simard *et al.*, 2007). In order to determine whether endothelial cell death contributes to LPS-induced BBB disruption in old mice, double immunostaining of RECA-1 (marker of endothelial cells) and cleaved caspase-3 (marker of cell death) was performed. As shown in Fig. 3E, there were limited colocalizations of RECA-1 with cleaved caspase-3, and pretreatment with melatonin did not significantly affect this colocalization, suggesting that under our experimental conditions, endothelial cell death was not a major contributor to LPS-induced BBB damage in old mice.

We next examined the underlying mechanisms of TJP degradation in old mice challenged by LPS. Given that ROS could disrupt the BBB and gp91^{phox}-containing NADPH oxidase is an important source of ROS in the brain (Liu *et al.*, 2008; Tang *et al.*, 2010), we examined the expression of gp91^{phox}, the catalytic unit of NADPH oxidase. Western blot analysis showed that LPS significantly increased gp91^{phox} protein levels compared with the Veh group (Fig. 4B, $**P < 0.01$ vs. the Veh group), and this change was significantly inhibited by melatonin treatment (Fig. 4B, $^{\#}P < 0.05$ vs. the Veh+LPS group).

AMPK has been shown to play an important role in maintaining BBB integrity (Liu *et al.*, 2012), and LPS has shown an inhibitory effect on AMPK activation (p-AMPK) (Zhou *et al.*, 2014b). Therefore, we examined the role of p-AMPK in LPS-induced BBB damage. As shown in Fig. 4, LPS significantly decreased p-AMPK (Fig. 4D, $**P < 0.01$ vs. the Veh group) and melatonin treatment significantly alleviated this effect (Fig. 4D, $^{\#}P < 0.05$ vs. the Veh+LPS group).

To investigate the relationship between AMPK activation (p-AMPK) and gp91^{phox} expression after LPS treatment, we performed *in vitro* experiments using cultured brain microvascular endothelial cells (bEND3 cells) and metformin, the AMPK activator (Takata *et al.*, 2013). Firstly, we conducted experiments to identify the optimal dose of metformin for the activation of AMPK in endothelial cells and found that incubation of endothelial cells with 1 mM metformin significantly activated AMPK (Fig. 5A, $*P < 0.05$, $**P < 0.01$ vs. the Veh group). Next, bEND3 cells were treated with or without metformin for 8 h before exposing to 1 μ g mL⁻¹ LPS for additional 16 h (Zhao *et al.*, 2014) before analyzing gp91^{phox} expression by Western blot. As shown in Fig. 5B, LPS significantly upregulated gp91^{phox} expression ($**P < 0.01$ vs. the Veh group) and metformin pretreatment significantly decreased LPS-induced gp91^{phox} expression ($^{\#}P < 0.05$ vs. the Veh+LPS group), suggesting that LPS-induced gp91^{phox} expression was through inhibition of AMPK activation (p-AMPK).

Discussion

BBB damage induced by extrinsic or intrinsic stimuli plays an important role in neurological diseases (Rosenberg, 2012), and BBB disruption could occur in old people even when facing common stress. It is well known

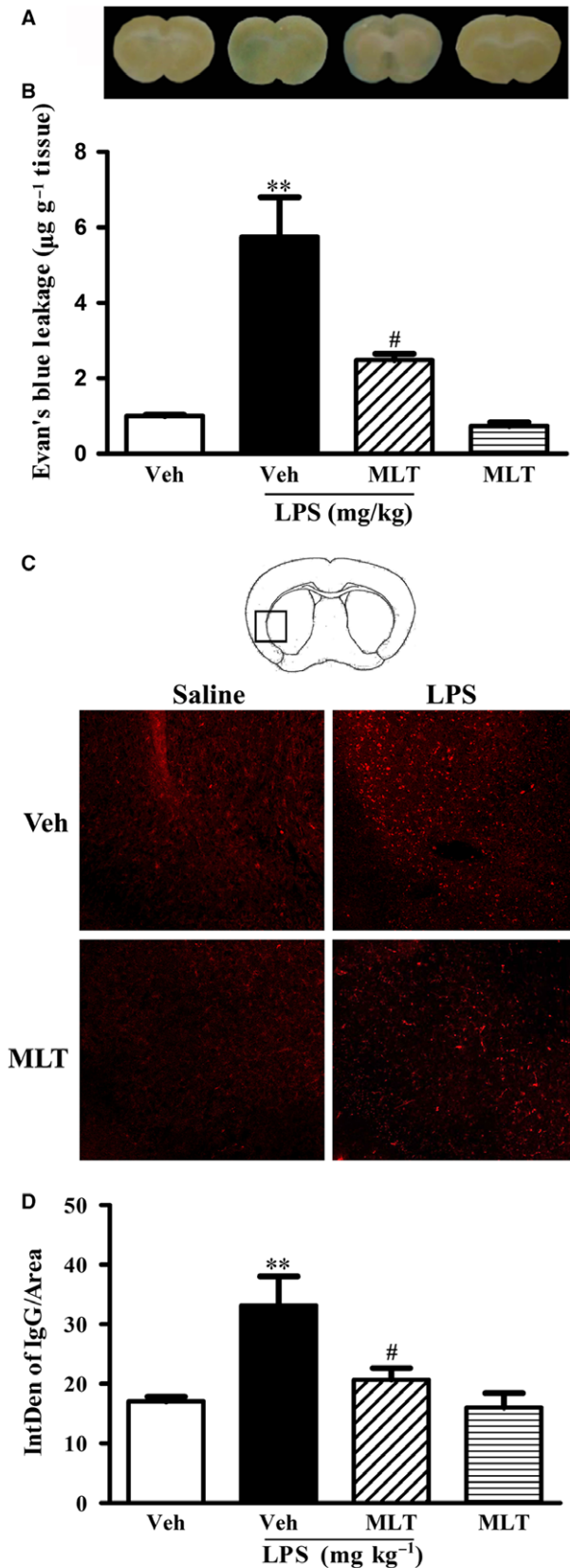


Fig. 2 Effect of melatonin on LPS-induced BBB damage in old mice. (A) Representative brain coronal sections showed EB leakage from vehicle, LPS-treated, or melatonin-treated group. (B) Quantification of EB leakage showed that BBB was disrupted in LPS-treated animals and pretreatment with melatonin significantly alleviated the change (** $P < 0.01$ vs. the Veh control, # $P < 0.05$ vs. the Veh+LPS, $n = 5$ for each group). (C) Representative confocal micrographs showed IgG leakage (red fluorescence) in the brain. (D) IgG leakage was quantitated and showed that pretreatment of mice with melatonin reduced LPS-induced IgG leakage (** $P < 0.01$ vs. the Veh control, # $P < 0.05$ vs. the Veh+LPS), $n = 3$ for each group. Data were expressed as mean \pm SEM.

mice, we investigated the effect of melatonin on LPS-induced BBB damage. Our important findings include the following: (1) Melatonin alleviates LPS-induced BBB damage in old mice, which is accompanied by decreased tight junction protein (TJP) degradation; (2) melatonin decreased LPS-induced BBB damage by activating AMPK and inhibiting gp91^{phox} expression upregulation; and (3) activating AMPK by metformin significantly inhibited LPS-induced upregulation of gp91^{phox} (Fig. 6).

LPS has been shown to dose-dependently increase BBB permeability (Nonaka *et al.*, 2004) and 1 mg kg⁻¹ LPS compromised BBB integrity in young mice (Ruiz-Valdepenas *et al.*, 2011; Zhou *et al.*, 2014a). Unexpectedly, we found that only 1 mg kg⁻¹ LPS significantly increased the extravasation of EB and IgG in old mice, suggesting that the BBB of old mice does not appear to be more vulnerable in response to LPS than young mice.

Oxygen radical detoxification processes decrease during aging (Reiter, 1995) and there was a marked drop in pineal biosynthetic activity in aging hamster (Reiter *et al.*, 1980) as well as extrapineal melatonin decreases in aging mice (Hill *et al.*, 2013). Interestingly, LPS not only induces ROS production, but also induces inflammatory response and chronic melatonin treatment has shown reduction of age-dependent inflammatory process in senescence-accelerated mice (Rodriguez *et al.*, 2007). Moreover, melatonin protects against LPS-induced toxicity not only to the brain (Wong *et al.*, 2014; Carloni *et al.*, 2016), but also to other organs including myocardial hypertrophy (Lu *et al.*, 2015), liver damage (Wang *et al.*, 2007), and acute lung inflammation (Lee *et al.*, 2009). Of note, melatonin has been shown to be protective against BBB damage induced by various stimuli, including excitotoxic injury in neonatal rats (Moretti *et al.*, 2015) and transient focal cerebral ischemia in mice (Chen *et al.*, 2006a,b). However, it is not clear whether melatonin decreases LPS-induced BBB damage in old mice. Our data here clearly show that LPS-induced BBB dysfunction is attenuated by melatonin, suggesting that pretreatment with melatonin might render the BBB of old mice more resistant to LPS stimuli. Along with BBB disruption, TJPs claudin-5 and occludin are also degraded in LPS-challenged old mice which is consistent with previous results obtained from *in vitro* cultured endothelial cells (Zhao *et al.*, 2014) as well as in young mice (Zhou *et al.*, 2014a). Of note, melatonin supplementation significantly inhibits LPS-induced TJP degradation in old mice.

LPS has been shown to enhance oxidative injuries via promoting enzymatic ROS generation (Zhao *et al.*, 2014), and NADPH oxidase is a major source of ROS generation in the brain which contributes to BBB disruption under various conditions (Chrissobolis & Faraci, 2008). NADPH oxidase has several family members, of which gp91^{phox} is the catalytic subunit. Gp91^{phox} knockout mice showed significantly less BBB damage than wild-type mice after stroke (Kahles *et al.*, 2007) and reduction of gp91^{phox} expression has shown protective effect on ischemia-induced brain injury and BBB damage (Liu *et al.*, 2008; Tang *et al.*, 2010). Here, our data also show that gp91^{phox} protein levels are significantly elevated in LPS-challenged old mice, implicating a role of gp91^{phox}-containing NADPH oxidase in LPS-induced BBB disruption. Our findings that

that the old people have decreased levels of melatonin in the brain and circulation (Karasek, 2004); however, whether there is a link between BBB disruption and decreased melatonin remains unknown. Using old

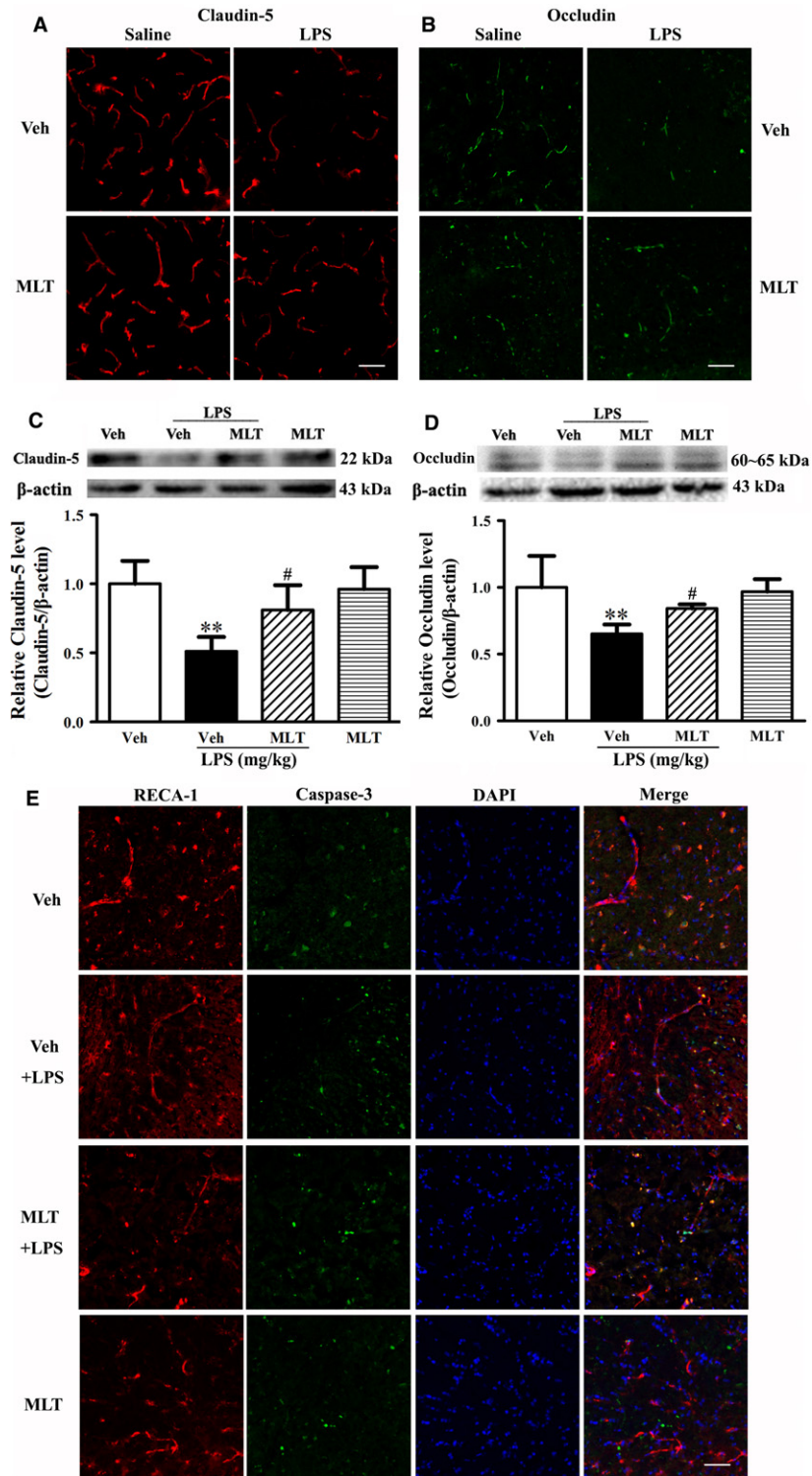


Fig. 3 Effect of melatonin on LPS-induced tight junction protein (TJP) degradation in old mice. Old mice were subjected to the indicated treatment and TJPs claudin-5 and occludin were analyzed by immunostaining and Western blot. Representative confocal micrographs showed that LPS decreased immunostaining for claudin-5 (A) and occludin (B), and pretreatment with melatonin ameliorated this change. Experiments were repeated three times with similar results. Western blot analysis for claudin-5 (C) and occluding (D) confirmed the finding of immunostaining. Representative immunoblots showed the bands of claudin-5 and occludin (upper panel). The band intensities of occludin and claudin-5 were quantitated after normalization to the beta actin. LPS induced a significant reduction in the protein levels of claudin-5 (** $P < 0.01$ vs. the Veh) and occludin (* $P < 0.05$ vs. the Veh). Treatment with melatonin prevented the reduction in claudin-5 protein (C, # $P < 0.05$ vs. the Veh+LPS group) and occludin protein (D, # $P < 0.05$ vs. the Veh+LPS group) in LPS-treated mice. $n = 4$ for each group. Data were expressed as mean \pm SEM. Double immunostaining of RECA-1 and cleaved caspase-3 showed limited co localization, and melatonin supplementation did not affect this colocalization (E). Scale bar = 50 μ m.

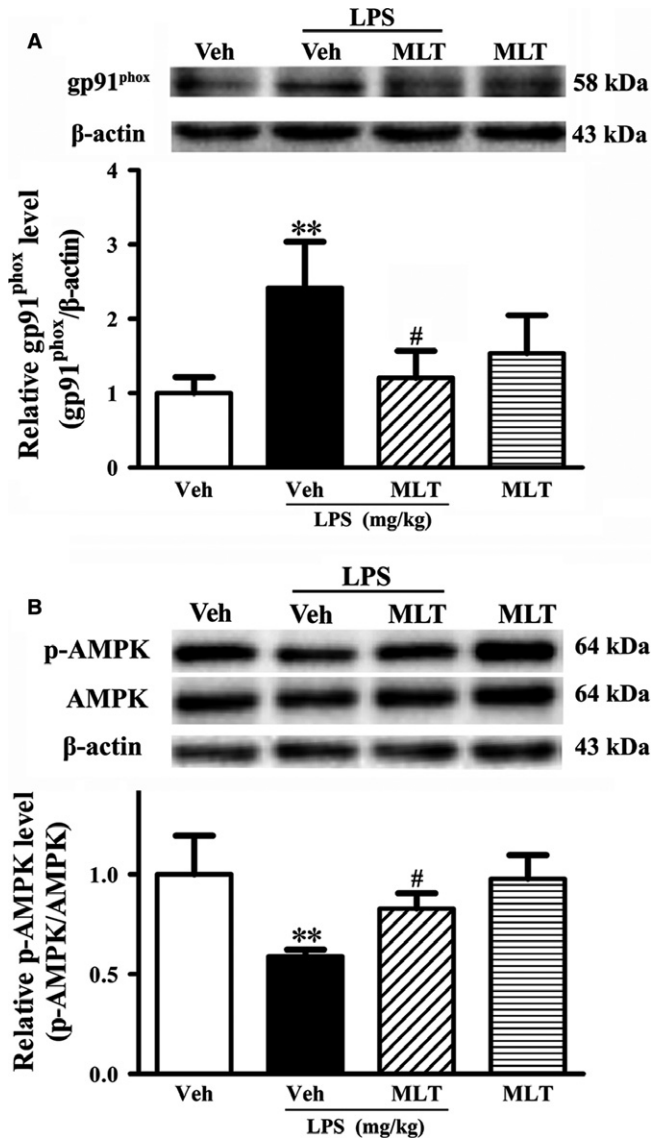


Fig. 4 Effect of melatonin on LPS-induced gp91^{phox} expression upregulation and inhibition of AMPK activation in old mice. The mice were subjected to the indicated treatment before analyzing gp91^{phox} with Western blot. (A) A representative immunoblot showed gp91^{phox} protein expression (upper panel). The band intensity of gp91^{phox} was quantitated after normalization to the beta actin (lower panel) and showed that LPS induced a significant increase in the protein level of gp91^{phox} (***P* < 0.01 vs. the Veh group). Pretreatment with melatonin inhibited LPS-induced gp91^{phox} upregulation (#*P* < 0.05 vs. the Veh+LPS group). *n* = 4 for each group. Data were expressed as mean ± SEM. (B) Representative immunoblots showed the protein bands of p-AMPK and AMPK (upper panel). The band intensities of p-AMPK and AMPK were quantitated after normalization to the beta actin (lower panel) and showed that LPS significantly inhibited AMPK activation (***P* < 0.01 vs. the Veh group). Pretreatment with melatonin prevented this inhibition induced by LPS (#*P* < 0.05 vs. the Veh+LPS group). *n* = 4 for each group. Data were expressed as mean ± SEM.

pretreatment with melatonin inhibits LPS-induced gp91^{phox} upregulation indicate that melatonin may protect the BBB in LPS-challenged old mice through reducing gp91^{phox} expression. It is worth pointing out that as the role of NADPH oxidase in BBB disruption has been well established in previous studies, here we did not use pharmacological NADPH oxidase inhibitors or genetic approach (knockout) to identify the contribution of gp91^{phox} in BBB disruption in LPS-treated old mice. In addition, besides

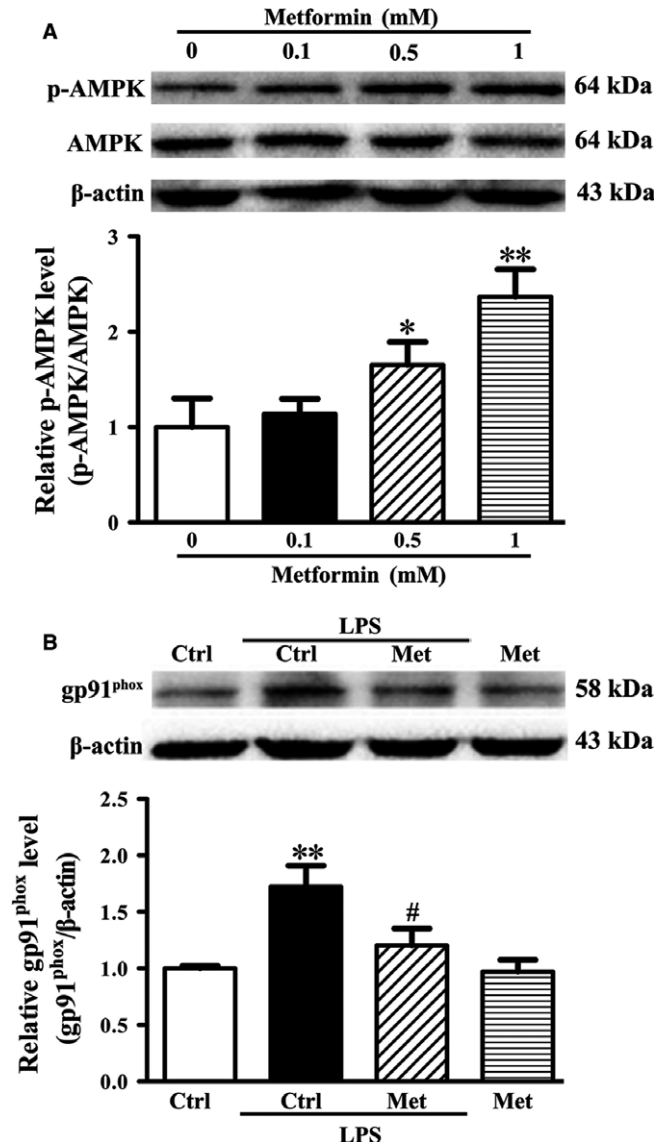


Fig. 5 Effect of metformin on LPS-induced gp91^{phox} expression upregulation in bEND3 cells. bEND3 cells were subjected to the indicated treatment before analyzing AMPK and gp91^{phox} with Western blot. (A) Representative immunoblots revealed the protein band of p-AMPK and AMPK and metformin at 1 mM significantly activated AMPK (**P* < 0.05, ***P* < 0.01 vs. the Veh group). (B) A representative immunoblot showed the protein band of gp91^{phox} (upper panel). The band intensity of gp91^{phox} was quantitated after normalization to the beta actin and showed that LPS treatment significantly upregulated gp91^{phox} expression (***P* < 0.01 vs. the Veh group). Treatment with metformin significantly inhibited this change (#*P* < 0.05 vs. the Veh+LPS group). *n* = 6 for each group. Data were expressed as mean ± SEM.

decreasing ROS generation through inhibiting LPS-induced gp91^{phox} upregulation, melatonin may also function as a ROS scavenger to protect the BBB from impairment induced by LPS

AMPK is involved in melatonin-induced modulation of endoplasmic reticulum stress and autophagy modulation after fatty liver graft preservation (Zaouali *et al.*, 2013). Moreover, activation of AMPK has been shown to alleviate LPS-induced BBB disruption (Zhao *et al.*, 2014), high glucose-induced TJP dysfunction in brain endothelial cells (Liu *et al.*, 2012), and LPS-induced ROS generation. Here, our data show that melatonin supplementation concurrently suppresses LPS-induced AMPK

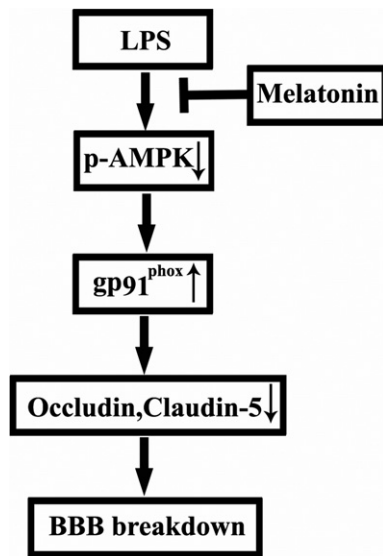


Fig. 6 A schema for proposed molecular mechanism underlying melatonin's protective effect on LPS-induced BBB damage in old mice. LPS-induced BBB disruption accompanied by tight junction protein occludin and claudin-5 degradation through decreasing AMPK activation (p-AMPK) and upregulating gp91^{phox} protein levels. Melatonin supplementation alleviates LPS-induced BBB damage via activating AMPK and downregulating gp91^{phox}.

inhibition and gp91^{phox} upregulation, implying that the protective effects of melatonin on TJPs may result from AMPK activation-induced inhibition of gp91^{phox} upregulation.

Lastly, our data that activation of AMPK by metformin inhibits LPS-induced gp91^{phox} upregulation indicate that LPS increases gp91^{phox} through regulating AMPK. This result is consistent with previous reports in which AMPK has been shown to regulate NADPH oxidase (Song & Zou, 2011; Ou *et al.*, 2013; Tang *et al.*, 2014). As an example, AMPK α 2 has been reported to function as a physiological suppressor of NADPH oxidase and ROS production in endothelial cells, and its deletion causes aberrant expression and activation of NADPH oxidase and consequent endothelial dysfunction *in vivo* (Wang *et al.*, 2010). In addition, AMPK activation ameliorates oxidative stress by suppressing NADPH oxidase-derived ROS production in endothelial cells (Wang *et al.*, 2010; Song *et al.*, 2011).

In summary, our results demonstrate that LPS induces BBB disruption, AMPK inhibition and gp91^{phox} upregulation in old mice, and melatonin supplementation alleviates LPS-induced BBB damage via activating AMPK and downregulating gp91^{phox}, supporting that decreased levels of melatonin may contribute to common stress-induced BBB disruption in old people.

Experimental procedures

Animals

Twenty-four to 28-month-old C57BL/6 mice were purchased from Tongji University Experimental Animal Center (Shanghai, China). They were housed 4–5 per cage under constant temperature (23 ± 1°C) and light-controlled vivarium (12-h light/12-h dark cycle). Mice that housed in the same cage underwent the same treatment. Food and water were available *ad libitum*. All animal procedures were approved by the University Committee on Animal Care of Soochow University and performed according to the NIH Guide for the Care and Use of

Laboratory Animals. All efforts were made to reduce the number of animals and to minimize animal suffering. Detailed number of animal use for each experiment was listed in Figure legends.

Tissue processing and drug administration

Mice were anesthetized with an overdose of chloral hydrate (60 mg kg⁻¹, i.p.), followed by transcardially perfusion under deep anesthesia with cold PBS to remove intravascular blood. Brain was quickly removed and stored at -80°C until further use. The brain perfused 4% paraformaldehyde (PFA) was postfixed in 4% PFA at 4°C for 2 h and then stored in a 10% sucrose solution at 4°C until further experiment.

Melatonin (Sigma, St Louis, MO, USA) was dissolved in 2% ethanol (diluted in saline) (Rennie *et al.*, 2008), given intraperitoneally (i.p.) at 9:00 every 24 h at a dose of 10 mg kg⁻¹ day⁻¹ for 1 week (Chern *et al.*, 2012; Zhou *et al.*, 2014a). LPS (Sigma) was dissolved in saline and administered i.p. at a dose of 1 mg kg⁻¹ (Ruiz-Valdepenas *et al.*, 2011; Zhou *et al.*, 2014a) at 24 h after the last melatonin injection (Ortiz *et al.*, 1999).

Assessment of Evan's blue (EB) dye leakage

BBB damage was determined by assessing the extravasation of EB (Sigma). Twenty-four hours after LPS treatment, EB (2% wt/vol in sterile PBS) was administered (3 mL kg⁻¹) through the tail vein, and 20 min later, mice were transcardially perfused with ice-cold PBS and then the brain was quickly taken out and BBB disruption was quantitatively assessed by measuring EB contents as we have reported (Liu *et al.*, 2016). In brief, the brain tissue was harvested as described above and homogenized in 50% wt/vol trichloroacetic acid (Sigma). After centrifugation (14 000 g for 15 min) at 4°C, the supernatant was collected, and fluorescence intensity (μ g mL⁻¹) was measured at 620 nm on a microplate fluorescence reader (Infinite M200 Pro; TECAN, Grodig, Austria). The total Evan's blue content (μ g) in each sample was derived from concentrations of external standards (1–20 μ g mL⁻¹). The difference of dye content between each group was calculated as Evan's blue leakage and expressed as per gram of brain tissue (μ g g⁻¹).

Evaluation of BBB permeability by detection of immunoglobulin G (IgG) leakage

IgG leakage was used to evaluate BBB permeability as we described previously (Wang *et al.*, 2016). In brief, the 20- μ m-thick section was fixed with 4% PFA for 20 min at room temperature, followed by staining with Cy-3-conjugated affinity pure goat anti-mouse IgG (1:200; KPL, Gaithersburg, MD, USA) for 2 h. After mounted with a glass coverslip, the slide was scanned in a LSM 700 microscope (Carl Zeiss) and the coronal image was reconstructed using Adobe Photoshop.

Immunostaining for RECA-1, cleaved caspase-3, occludin, and claudin-5

The 20- μ m-thick cryosection was fixed with 4% PFA for further analysis as we described previously (Wang *et al.*, 2016). In brief, blocking buffer containing 0.3% Triton X-100, 1% BSA, and 5% goat serum was used to block nonspecific binding. Sections were then incubated overnight with anti-RECA-1 (1:200 dilution; Abcam, Cambridge, UK), anticlaved caspase-3 (1:300 dilution; R&D System Inc., Minneapolis, MN, USA), anti-occludin (1:100 dilution; Invitrogen, Carlsbad, MA, USA), anti-

claudin-5 (1:200 dilution; Invitrogen) primary antibody at 4°C. After 24 h, sections were incubated with mouse (1:1000 dilution; Boster, Wuhan, Hubei, China) secondary antibody for 2 h at room temperature. Confocal images were obtained using a laser scanning confocal microscope (Zeiss LSM 700, Carl Zeiss).

Cell culture

Mouse brain microvascular endothelial cells bEND3 (American Type Culture Collection, Rockville, MD, USA) were grown as a monolayer in DMEM with 15% fetal bovine serum (FBS), 100 U mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin at 37°C in a humidified incubators with 5% CO₂ and 95% room air. Cells were subcultured into 35-mm dishes and allowed to grow to confluence before incubating with metformin.

The confluent endothelial cells were first treated with metformin (Beyotime, Shanghai, China) at a dose of 0, 0.1, 0.5, and 1 mM for 24 h to define the optimal dose in activating AMPK. Then, the cells were treated with metformin at the optimal dose for 8 h before adding 1 µg mL⁻¹ LPS (Zhao *et al.*, 2014), and 16 h later, Western blot was used to detect gp91^{phox} protein levels.

Western blot analysis for p-AMPK, AMPK, occludin, claudin-5, and gp91^{phox}

Homogenate aliquots (50 µg of total protein) were boiled and then electrophoresed in 12% SDS-PAGE acrylamide gels, transferred onto PVDF membrane (Millipore, Billerica, MA, USA), and incubated for 1 h in TBS-T (Tris-buffered saline and 0.1% Tween-20) containing 5% nonfat milk. Membranes were then incubated with primary antibodies against p-AMPK (1:1000; Cell Signaling Technology, Danvers, MA, USA), AMPK (1:1000; Cell Signaling Technology), gp91^{phox} (1:1000; BD Transduction Laboratories, Lexington, KY, USA), occludin (1:300; Invitrogen), or claudin-5 (1:500; Invitrogen) overnight at 4°C, washed in TBS-T, and then incubated for 1 h at room temperature with corresponding HRP-conjugated anti-rabbit or anti-mouse antibodies (1:2000; Boster). The membranes were developed with the SuperSignal West Pico HRP substrate kit (Pierce, Rockford, IL, USA) and photographed. Protein band intensities were quantitated after normalization to beta actin.

Statistical analysis

The data are presented as mean ± SEM. Statistical analysis was carried out using ANOVA (SPSS software, version 17.0). Significant effects were probed using Newman–Keuls *post hoc* comparison. A value of $P < 0.05$ was considered statistically significant.

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Conflicts of interest

The authors declare that they have no conflict of interests.

Author contributions

JL, WL, and XJ are the principal investigators at the three collaborating institutions and are responsible for project design, supervision of technical personnel, interpretation of results, and preparation of manuscript drafts. YES and CFL provided advice on experimental design and interpretation, and comments on the manuscript. XW, GX, WCL, HS, MW, YS, and XL performed experiments, analyzed the data, made the figures, and drafted the manuscript.

References

- Carlioni S, Favrais G, Saliba E, Albertini MC, Chalou S, Longini M, Gressens P, Buonocore G, Balduini W (2016) Melatonin modulates neonatal brain inflammation through endoplasmic reticulum stress, autophagy, and miR-34a/silent information regulator 1 pathway. *J. Pineal Res.* **61**, 370–380.
- Chen HY, Chen TY, Lee MY, Chen ST, Hsu YS, Kuo YL, Chang GL, Wu TS, Lee EJ (2006a) Melatonin decreases neurovascular oxidative/nitrosative damage and protects against early increases in the blood-brain barrier permeability after transient focal cerebral ischemia in mice. *J. Pineal Res.* **41**, 175–182.
- Chen TY, Lee MY, Chen HY, Kuo YL, Lin SC, Wu TS, Lee EJ (2006b) Melatonin attenuates the postischemic increase in blood-brain barrier permeability and decreases hemorrhagic transformation of tissue-plasminogen activator therapy following ischemic stroke in mice. *J. Pineal Res.* **40**, 242–250.
- Chern CM, Liao JF, Wang YH, Shen YC (2012) Melatonin ameliorates neural function by promoting endogenous neurogenesis through the MT2 melatonin receptor in ischemic-stroke mice. *Free Radic. Biol. Med.* **52**, 1634–1647.
- Chrissobolis S, Faraci FM (2008) The role of oxidative stress and NADPH oxidase in cerebrovascular disease. *Trends Mol. Med.* **14**, 495–502.
- Galano A, Medina ME, Tan DX, Reiter RJ (2014) Melatonin and its metabolites as copper chelating agents and their role in inhibiting oxidative stress: a physicochemical analysis. *J. Pineal Res.* **58**, 107–116.
- Hawley SA, Davison M, Woods A, Davies SP, Beri RK, Carling D, Hardie DG (1996) Characterization of the AMP-activated protein kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. *J. Biol. Chem.* **271**, 27879–27887.
- Hill SM, Cheng C, Yuan L, Mao L, Jockers R, Dauchy B, Blask DE (2013) Age-related decline in melatonin and its MT1 receptor are associated with decreased sensitivity to melatonin and enhanced mammary tumor growth. *Curr Aging Sci.* **6**, 125–133.
- Kahles T, Luedike P, Endres M, Galla HJ, Steinmetz H, Busse R, Neumann-Haefelin T, Brandes RP (2007) NADPH oxidase plays a central role in blood-brain barrier damage in experimental stroke. *Stroke* **38**, 3000–3006.
- Karasek M (2004) Melatonin, human aging, and age-related diseases. *Exp. Gerontol.* **39**, 1723–1729.
- Lee YD, Kim JY, Lee KH, Kwak YJ, Lee SK, Kim OS, Song DY, Lee JH, Baik TK, Kim BJ, Baik HW (2009) Melatonin attenuates lipopolysaccharide-induced acute lung inflammation in sleep-deprived mice. *J. Pineal Res.* **46**, 53–57.
- Liu W, Sood R, Chen Q, Sakoglu U, Hendren J, Cetin O, Miyake M, Liu KJ (2008) Normobaric hyperoxia inhibits NADPH oxidase-mediated matrix metalloproteinase-9 induction in cerebral microvessels in experimental stroke. *J. Neurochem.* **107**, 1196–1205.
- Liu C, Wu J, Zou MH (2012) Activation of AMP-activated protein kinase alleviates high-glucose-induced dysfunction of brain microvascular endothelial cell tight-junction dynamics. *Free Radic. Biol. Med.* **53**, 1213–1221.
- Liu Y, Liu WC, Sun Y, Shen X, Wang X, Shu H, Pan R, Liu CF, Liu W, Liu KJ, Jin X (2016) Normobaric Hyperoxia Extends Neuro- and Vaso-Protection of N-Acetylcysteine in Transient Focal Ischemia. *Mol. Neurobiol.* doi:10.1007/s12035-016-9932-0.
- Lu Q, Yi X, Cheng X, Sun X, Yang X (2015) Melatonin protects against myocardial hypertrophy induced by lipopolysaccharide. *In Vitro Cell. Dev. Biol. Anim.* **51**, 353–360.
- Manchester LC, Coto-Montes A, Boga JA, Andersen LP, Zhou Z, Galano A, Vriend J, Tan DX, Reiter RJ (2015) Melatonin: an ancient molecule that makes oxygen metabolically tolerable. *J. Pineal Res.* **59**, 403–419.
- Martin GS, Mannino DM, Moss M (2006) The effect of age on the development and outcome of adult sepsis. *Crit. Care Med.* **34**, 15–21.
- Moretti R, Zanin A, Pansiot J, Spiri D, Manganozzi L, Kratzer I, Favero G, Vasiljevic A, Rinaldi VE, Pic I, Massano D, D'Agostino I, Baburamani A, La Rocca MA, Rodella LF, Rezzani R, Ek J, Strazielle N, Ghersi-Egea JF, Gressens P, Titomanlio L

- (2015) Melatonin reduces excitotoxic blood-brain barrier breakdown in neonatal rats. *Neuroscience* **311**, 382–397.
- Nonaka N, Hileman SM, Shioda S, Vo TQ, Banks WA (2004) Effects of lipopolysaccharide on leptin transport across the blood-brain barrier. *Brain Res.* **1016**, 58–65.
- Ortiz GG, Coto-Montes A, Bitzer-Quintero OK, Falcon-Franco MA, Ruiz-Rizo L, Bravo-Cuellar A, Reiter RJ, Feria-Velasco A (1999) Effects of melatonin on the Harderian gland of lipopolysaccharide-treated rats: morphological observations. *Biomed. Pharmacother.* **53**, 432–437.
- Ou HC, Hsieh YL, Yang NC, Tsai KL, Chen KL, Tsai CS, Chen JJ, Wu BT, Lee SD (2013) Ginkgo biloba extract attenuates oxLDL-induced endothelial dysfunction via an AMPK-dependent mechanism. *J. Appl. Physiol.* (1985) **114**, 274–285.
- Reiter RJ (1995) Oxygen radical detoxification processes during aging: the functional importance of melatonin. *Aging (Milano)*. **7**, 340–351.
- Reiter RJ, Richardson BA, Johnson LY, Ferguson BN, Dinh DT (1980) Pineal melatonin rhythm: reduction in aging Syrian hamsters. *Science* **210**, 1372–1373.
- Reiter RJ, Tan DX, Kim SJ, Cruz MH (2014) Delivery of pineal melatonin to the brain and SCN: role of canaliculi, cerebrospinal fluid, tanycytes and Virchow-Robin perivascular spaces. *Brain Struct. Funct.* **219**, 1873–1887.
- Rennie K, de Butte M, Frechette M, Pappas BA (2008) Chronic and acute melatonin effects in gerbil global forebrain ischemia: long-term neural and behavioral outcome. *J. Pineal Res.* **44**, 149–156.
- Rodriguez MI, Escames G, Lopez LC, Lopez A, Garcia JA, Ortiz F, Acuna-Castroviejo D (2007) Chronic melatonin treatment reduces the age-dependent inflammatory process in senescence-accelerated mice. *J. Pineal Res.* **42**, 272–279.
- Rosenberg GA (2012) Neurological diseases in relation to the blood-brain barrier. *J. Cereb. Blood Flow Metab.* **32**, 1139–1151.
- Ruiz-Valdepenas L, Martinez-Orgado JA, Benito C, Millan A, Tolon RM, Romero J (2011) Cannabidiol reduces lipopolysaccharide-induced vascular changes and inflammation in the mouse brain: an intravital microscopy study. *J. Neuroinflammation* **8**, 5.
- Seok SM, Kim JM, Park TY, Baik EJ, Lee SH (2013) Fructose-1,6-bisphosphate ameliorates lipopolysaccharide-induced dysfunction of blood-brain barrier. *Arch Pharm Res.* **36**, 1149–1159.
- Shukla P, Rao GM, Pandey G, Sharma S, Mittapelly N, Shegokar R, Mishra PR (2014) Therapeutic interventions in sepsis: current and anticipated pharmacological agents. *Br. J. Pharmacol.* **171**, 5011–5031.
- Simard JM, Kent TA, Chen M, Tarasov KV, Gerzanich V (2007) Brain oedema in focal ischaemia: molecular pathophysiology and theoretical implications. *Lancet Neurol.* **6**, 258–268.
- Song P, Zou MH (2011) Regulation of NAD(P)H oxidases by AMPK in cardiovascular systems. *Free Radic. Biol. Med.* **52**, 1607–1619.
- Song P, Wang S, He C, Liang B, Viollet B, Zou MH (2011) AMPK α 2 deletion exacerbates neointima formation by upregulating Skp2 in vascular smooth muscle cells. *Circ. Res.* **109**, 1230–1239.
- Takata F, Dohgu S, Matsumoto J, Machida T, Kaneshima S, Matsuo M, Sakaguchi S, Takeshige Y, Yamauchi A, Kataoka Y (2013) Metformin induces up-regulation of blood-brain barrier functions by activating AMP-activated protein kinase in rat brain microvascular endothelial cells. *Biochem. Biophys. Res. Commun.* **433**, 586–590.
- Tang X, Liu KJ, Ramu J, Chen Q, Li T, Liu W (2010) Inhibition of gp91(phox) contributes towards normobaric hyperoxia afforded neuroprotection in focal cerebral ischemia. *Brain Res.* **1348**, 174–180.
- Tang H, Pan CS, Mao XW, Liu YY, Yan L, Zhou CM, Fan JY, Zhang SY, Han JY (2014) Role of NADPH oxidase in total salvianolic acid injection attenuating ischemia-reperfusion impaired cerebral microcirculation and neurons: implication of AMPK/Akt/PKC. *Microcirculation* **21**, 615–627.
- Wang H, Xu DX, Lv JW, Ning H, Wei W (2007) Melatonin attenuates lipopolysaccharide (LPS)-induced apoptotic liver damage in D-galactosamine-sensitized mice. *Toxicology* **237**, 49–57.
- Wang S, Zhang M, Liang B, Xu J, Xie Z, Liu C, Viollet B, Yan D, Zou MH (2010) AMPK α 2 deletion causes aberrant expression and activation of NAD(P)H oxidase and consequent endothelial dysfunction in vivo: role of 26S proteasomes. *Circ. Res.* **106**, 1117–1128.
- Wang X, Liu Y, Sun Y, Liu W, Jin X (2016) Blood brain barrier breakdown was found in non-infarcted area after 2-h MCAO. *J. Neurol. Sci.* **363**, 63–68.
- Weiss N, Miller F, Cazaubon S, Couraud PO (2009) The blood-brain barrier in brain homeostasis and neurological diseases. *Biochim. Biophys. Acta* **1788**, 842–857.
- Wong CS, Jow GM, Kaizaki A, Fan LW, Tien LT (2014) Melatonin ameliorates brain injury induced by systemic lipopolysaccharide in neonatal rats. *Neuroscience* **267**, 147–156.
- Zaouali MA, Boncompagni E, Reiter RJ, Bejaoui M, Freitas I, Pantazi E, Folch-Puy E, Abdennebi HB, Garcia-Gil FA, Rosello-Catafau J (2013) AMPK involvement in endoplasmic reticulum stress and autophagy modulation after fatty liver graft preservation: a role for melatonin and trimetazidine cocktail. *J. Pineal Res.* **55**, 65–78.
- Zhao Z, Hu J, Gao X, Liang H, Liu Z (2014) Activation of AMPK attenuates lipopolysaccharide-impaired integrity and function of blood-brain barrier in human brain microvascular endothelial cells. *Exp. Mol. Pathol.* **97**, 386–392.
- Zhou T, Zhao L, Zhan R, He Q, Tong Y, Tian X, Wang H, Zhang T, Fu Y, Sun Y, Xu F, Guo X, Fan D, Han H, Chui D (2014a) Blood-brain barrier dysfunction in mice induced by lipopolysaccharide is attenuated by dapsone. *Biochem. Biophys. Res. Commun.* **453**, 419–424.
- Zhou X, Cao Y, Ao G, Hu L, Liu H, Wu J, Wang X, Jin M, Zheng S, Zhen X, Alkayed NJ, Jia J, Cheng J (2014b) CaMKK β -dependent activation of AMP-activated protein kinase is critical to suppressive effects of hydrogen sulfide on neuroinflammation. *Antioxid. Redox Signal.* **21**, 1741–1758.