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 gp91phox protein expression. Interestingly, administration of melatonin for one week significantly decreased LPS-induced BBB disruption, AMPK suppression, and gp91phox upregulation. Moreover, activation of AMPK with metformin significantly inhibited LPS-induced gp91phox upregulation in endothelial cells. Taken together, our findings demonstrate that melatonin alleviates LPS-induced BBB disruption through activating AMPK and inhibiting gp91phox 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 gp91phox-containing NADPH oxidase is highly expressed in the cerebral vascular endothelium (Kahles et al., 2007). Suppressing gp91phox 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
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 damage in old mice and found that LPS disrupted the BBB in old mice via downregulating tight junction protein expression via increasing gp91phox expression and inhibiting AMPK activation, and supplementation of melatonin could effectively inhibit the above changes.

**Results**

LPS has been shown to disrupt BBB integrity (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 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). 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 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 TJ degradation in old mice challenged by LPS. Given that ROS could disrupt the BBB and gp91phox-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 gp91phox, the catalytic unit of NADPH oxidase. Western blot analysis showed that LPS significantly increased gp91phox 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 gp91phox 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 μM metformin significantly activated AMPK (Fig. 5A, **P < 0.05 vs. the Veh group). Next, bEND3 cells were treated with or without metformin for 8 h before exposing to 1 μg ml⁻¹ LPS for additional 16 h (Zhao et al., 2014) before analyzing gp91phox expression by Western blot. As shown in Fig. 5B, LPS significantly upregulated gp91phox expression (**P < 0.01 vs. the Veh group) and metformin pretreatment significantly decreased LPS-induced gp91phox expression (**P < 0.05 vs. the Veh+LPS group), suggesting that LPS-induced gp91phox 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
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 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 gp91phox expression upregulation; and (3) activating AMPK by metformin significantly inhibited LPS-induced upregulation of gp91phox (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, TJs 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 (Zhau et al., 2014a). Of note, melatonin supplementation significantly inhibits LPS-induced TJ degradation in old mice.

LPS has been shown to enhance oxidative injuries via promoting enzymatic ROS generation (Zhao et al., 2014), and NAPD oxidase is a major source of ROS generation in the brain which contributes to BBB disruption under various conditions (Chrissohobis & Faraci, 2008). NAPD oxidase has several family members, of which gp91phox is the catalytic subunit. Gp91phox knockout mice showed significantly less BBB damage than wild-type mice after stroke (Kahles et al., 2007) and reduction of gp91phox 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 gp91phox protein levels are significantly elevated in LPS-challenged old mice, implicating a role of gp91phox-containing NAPD oxidase in LPS-induced BBB disruption. Our findings that

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**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 ± SEM.
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 TJs 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 occludin (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 < 0.01 vs. the Veh). Treatment with melatonin prevented the reduction in claudin-5 protein (#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 ± 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 μm.
pretreatment with melatonin inhibits LPS-induced gp91phox upregulation that indicates melatonin may protect the BBB in LPS-challenged old mice through reducing gp91phox 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 gp91phox in BBB disruption in LPS-treated old mice. In addition, besides decreasing ROS generation through inhibiting LPS-induced gp91phox 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 TJ 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. 4 Effect of melatonin on LPS-induced gp91phox expression upregulation and inhibition of AMPK activation in old mice. The mice were subjected to the indicated treatment before analyzing gp91phox with Western blot. (A) A representative immunoblot showed gp91phox protein expression (upper panel). The band intensity of gp91phox was quantitated after normalization to the beta actin (lower panel) and showed that LPS induced a significant increase in the protein level of gp91phox (**P < 0.01 vs. the Veh group). Pretreatment with melatonin inhibited LPS-induced gp91phox 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.

Fig. 5 Effect of metformin on LPS-induced gp91phox expression upregulation in bEND3 cells. bEND3 cells were subjected to the indicated treatment before analyzing AMPK and gp91phox 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 gp91phox (upper panel). The band intensity of gp91phox was quantitated after normalization to the beta actin and showed that LPS treatment significantly upregulated gp91phox 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.
inhibition and gp91phox upregulation, implying that the protective effects of melatonin on TJPs may result from AMPK activation-induced inhibition of gp91phox upregulation.

Lastly, our data that activation of AMPK by metformin inhibits LPS-induced gp91phox upregulation indicate that LPS increases gp91phox 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, AMPKx2 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 gp91phox upregulation in old mice, and melatonin supplementation alleviates LPS-induced BBB damage via activating AMPK and downregulating gp91phox, 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 (µg mL⁻¹) was measured at 620 nm on a microplate fluorescence reader (Infinite M200 Pro; TECAN, Grodig, Austria). The total Evan’s blue content (µg) in each sample was derived from concentrations of external standards (1–20 µg 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 (µg 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-µm-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-µm-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), anticleaved 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 incubator 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 L⁻¹ LPS (Zhao et al., 2014), and 16 h later, Western blot was used to detect gp91phox protein levels.

**Western blot analysis for p-AMPK, AMPK, occludin, claudin-5, and gp91phox**

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), gp91phox (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 conjugated anti-rabbit or anti-mouse antibodies (1:2000; Boster). 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.

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