Received: 11 January 2017 | Revised: 22 January 2017 | Accepted: 7 February 2017

DOI: 10.1111/cns.12686

ORIGINAL ARTICLE

WILEY CNS Neuroscience & Therapeutics

SIRT1-dependent AMPK pathway in the protection of estrogen against ischemic brain injury

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Funding information
National Natural Science Foundation of China, Grant/Award Number: 81402927 Natural Science Foundation of Shandong Province, Grant/Award Number: ZR2016HM12; China Postdoctoral Science Foundation, Grant/Award Number: 2015M582894

Summary

Aims: Stroke is a major cause of mortality and disability, especially for postmenopausal women. In view of the protective action of estrogen, hormone therapy remains the only effective way to limit this risk. The objective of this study was to investigate the efficiency and underlying mechanisms of estrogen neuroprotection.

Methods: Subcutaneous injection of 17β-estradiol in rats after ovariectomy (OVX) was used to manipulate estrogen level and explore the effects of estrogen in cerebral ischemic damage both in vivo and in vitro. Silent mating type information regulation 2 homolog 1 (SIRT1) knockout mice and adenosine monophosphate (AMP)-activated kinase (AMPK) inhibitor Compound C were also used to investigate the underlying pathway of estrogen.

Results: Estrogen deficiency induced by OVX aggravated brain infarction in experimentally induced cerebral ischemia rats, whereas estrogen pretreatment reduced ischemia-induced cerebral injuries. Neurons of estrogen deficiency models were susceptible to apoptosis under oxygen-glucose deprivation (OGD). In contrast, neurons with estrogen-supplemented serum exhibited restored resistance to cell apoptosis. In OGD neurons, estrogen promoted AMPK activation through estrogen receptor α, and neuroprotection of estrogen was prevented by AMPK inhibition. Estrogen increased SIRT1 expression and activation, and estrogen-induced AMPK activation disappeared in SIRT1 knockout neurons. Moreover, estrogen-induced neuroprotection was abolished in SIRT1 knockout mice and AMPK-inhibited rats.

Conclusion: Our data support that estrogen protects against ischemic stroke through preventing neuron death via the SIRT1-dependent AMPK pathway.

KEYWORDS
AMPK, estrogen, ischemic stroke, SIRT1

1 | INTRODUCTION

Stroke is one of the leading causes of death and disability worldwide.[1] Prevention is considered the most effective strategy to curb stroke.[2] Fully understanding the risk factors related to stroke is very important for disease prevention, such as hypertension and hyperlipidemia. Estrogen deficiency is believed as a new risk factor for female after menopause, which has drawn much attention.[3]

As to postmenopausal women, stroke has become a major health issue. Epidemiology studies showed that premenopausal women are less likely than male counterparts to suffer from cardio-cerebrovascular diseases, whereas elderly women suffer more frequently from severe strokes, poor recovery, and long-term disability compared with age-matched men.[4-10] The natural decrease in estrogen after menopause, a physiological state of estrogen deficiency, has been offered as a major explanation for such stroke-related gender difference.[11,12] In view of the protective action of estrogen, estrogen replacement therapy remains the only effective way to limit this risk.[13] However, estrogens'

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effect in stroke is still under debate. Numerous studies performed in humans and animals have shown beneficial action of estrogen therapy for stroke, whereas other clinical studies, such as the Women's Health Initiative, found that estrogen treatment failed to confer neuroprotection and even increased the susceptibility for stroke.[4,5] Although the window of opportunity hypothesis has been proposed to explain the conflicting results,[6,14,15] this discrepancy is still unclear. Moreover, given the extended life expectancy, contemporary women spend more time of their lives in a hypoestrogenic state, and it makes women more vulnerable to hormone-related diseases. Therefore, it becomes even more important to confirm the efficiency of estrogen and probe the potential mechanisms.

Ischemic stroke is the most common type of stroke.[16,17] In this pathology, brain energy exhaustion and metabolic stress aggravate the brain cell death under ischemic stress and determine the outcome of acute brain injury.[2] Therefore, the defense to energy exhaustion is an effective strategy to protect ischemic stroke. AMP-activated protein kinase (AMPK) is recognized as a major metabolic energy sensor, which is activated in response to decreased cellular energy charge (increased in AMP/ATP ratio) and further regulates energy metabolic homeostasis.[18–21] Silent mating type information regulation 2 homolog 1 (SIRT1) is also implicated in the regulation of energy metabolism, insulin sensitivity, and longevity.[22,23] The SIRT1-dependent AMPK pathway was reported to protect against ischemic stroke in the action of resveratrol and Nampt.[2,24,25] Estrogen was demonstrated to regulate SIRT1/AMPK/epigenetic modifications to prevent cardiovascular dysfunction in postmenopausal women with metabolic syndrome.[26] Despite the role of estrogen as a neuroprotectant in stroke is well established,[27–33] estrogen's contribution to energy exhaustion after ischemic injury is less studied.

This study aimed to validate the role of estrogen against stroke damage in vivo and in vitro, and discover the underlying pathway mediation of such neuroprotection effect.

2 | MATERIALS AND METHODS

2.1 | Animals

Female Sprague-Dawley (SD) rats and C57 mice were purchased from Sino-British SIPPR/BK Lab Animals (Shanghai, China). Female SIRT1 knockout mice and littermate wild-type (WT) mice were obtained from the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences. Controlled temperature (23-25°C) and lighting (8:00 AM-8:00 PM light, other time dark) were offered for the animals, as well as free access to standard food and drinking water.

2.2 | Ovariectomy (OVX) and estrogen replacement

To establish estrogen deficiency and estrogen replacement conditions, the rodents were divided into three groups: sham group (with vehicle), OVX group (with vehicle), and OVX+E (with 17β-estradiol). OVX was performed by dorsolateral incision, as previously described.[29] Briefly, after the animals were anesthetized, both the right and left horns of the uterus were exposed through two small lateral abdominal incisions. The ovaries were then carefully removed, leaving the uterus intact. Animals in the sham groups were subjected to the same operation, with the exception that the ovaries were left intact. Five days after OVX, the animals began receiving daily subcutaneous injections of estrogen (500 μg/kg in rats and mice, diluted in sesame oil solution) or vehicle at the back of the neck for 28 days.[29]

To confirm effectiveness of estrogen deficiency and replacement, the levels of serum estrogen were measured. Briefly, the animals were anesthetized with an overdose of pentobarbital sodium, and blood was collected from the ophthalmic artery. Serum levels of estrogen, progestin, and testosterone were measured using ELISA kit purchased from Xi-Tang BioTech (shanghai, China).

To observe the effect of estrogen deficiency and replacement on neurons, the serums of three groups were used to treat neurons. Blood samples were collected from the carotid artery using a polyethylene catheter which was inserted into the carotid artery under anesthesia, as previously described.[1] Then, serum was inactivated by heating in water bath for 30 minutes.

In addition, after OVX and estrogen treatment, some animals were subjected to middle cerebral artery occlusion (MCAO) and some were killed to examine morphometric parameters. At sacrifice, the heart, kidney, liver, and uterus were removed and weighed.

2.3 | Middle cerebral artery occlusion (MCAO)

The animals were anesthetized with chloral hydrate (300 mg/kg), and then, MCAO surgery in rats was performed as described.[16] Two hours after MCAO, the occluding filament was withdrawn to allow reperfusion. Twenty-four hours after MCAO, rats were killed for various examinations after neurological deficit scoring.

Permanent focal cerebral ischemia in mice was induced by cauterizing the left middle cerebral artery using methods described previously.[34] Focal ischemia was produced by permanent occlusion of the left middle cerebral artery by electrocoagulation.

Neurological deficits in MCAO rodents were examined using a 5-point scale as described.[16] The infarct size expressed as a percentage of the contralateral hemisphere was determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining as described.[16]

2.4 | Neuron culture

Primary rat and mouse neuronal cells were cultured from the cerebral cortex of neonatal animals within 6 hours after birth, as described previously.[16] One day after isolation, Neurobasal medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2% B27 (Invitrogen) was used to replenish the cultures. Uridine (10 μmol/L) was added to suppress glial growth. After 7-day culture in vitro, cultured cells were stained for NeuN (neuron marker, Millipore, Billerica, CA, USA) to access the proportion of neurons (>90%), and then, the neurons were treated with drugs, prior to oxygen-glucose deprivation.

2.5 | Oxygen-Glucose Deprivation (OGD) Model and cell injury assay

After 7-day culture in vitro, the neurons were exposed to OGD for 12 hours prior to apoptosis assay. Neurons' OGD condition was established by culturing in Dulbecco's modified Eagle's medium (DMEM) with no glucose and incubating for 12 hours in a hypoxic chamber (Thermo Fisher Scientific, Waltham, MA, USA) that was continuously flushed with 94% N2 and 5% CO2 at 37°C to obtain 1% O2, as described previously.[16] Control condition was established by culturing in DMEM containing glucose (25 mmol/L) and incubated under normal culture conditions.

Cell injury was assessed by four methods. Cell viability was measured by a nonradioactive cell counting kit (CCK-8) assay (WEIAO BioTech, Shanghai, China). Lactate dehydrogenase (LDH) release was analyzed with a colorimetric LDH cytotoxicity assay (WEIAO BioTech). Cell death was examined by manually counting the cells double-stained with Hoechst 33342 (1 μg/mL, Beyotime, Haimen, Jiangsu, China, staining the cell nuclei) and in situ cell death detection kit (Beyotime, labeling the dead or apoptotic cells), respectively. A fluorescent microscope (IX-71; Olympus, Tokyo, Japan) with 12.8 mol/L pixel recording digital color-cooled camera (DP72; Olympus) was used to acquire images. Then, death or apoptosis rate of cells was defined by the ratio labeled green vs blue. Annexin V/propidium iodide (PI) staining analysis (annexin V staining for early apoptotic cells, and PI staining for late apoptotic or necrotic cells) was also used to detect the cell death. According to the manufacturer's instruction, cells were stained and then measured by a BD FACSCalibur Flow Cytometer (BD FACSCalibur TM Flow Cytometer, San Jose, CA, USA).

2.6 | Real-time PCR

To analyze the level of the SIRT1 mRNAs in neurons, real-time PCR was performed as previously described.[16] Total RNA was extracted from neurons using TRIzol (Invitrogen) and reverse-transcribed into cDNA using the RevertAid™ First-Strand cDNA Synthesis Kit (Fermentas, Lithuania). Then, the mRNA levels were evaluated by appropriate specific primers and the SYBR Green RT-PCR Kit (Takara, Japan), with cDNA as the template. The following primer pairs (Takara, Japan) were used: SIRT1 sense (5′-GCTGACGACTTCGACGACG-3′) and antisense (5′-TCGGTCAACAGGAGGTTGTCT-3′); and GAPDH sense (5′-GTATGACTCCACTCACGGCAAA-3′) and antisense (5′-GGTCTC GCTCCTGGAAGATG-3′). The expression levels of the target-gene mRNA transcripts were normalized relative to GAPDH controls.

2.7 | Immunoblotting

The total protein from cells was prepared as described.[16] The following antibodies were used: anti-p-AMPK antibody (1:1000), anti-AMPK antibody (1:1000), anti-p-acetyl-coenzyme A (CoA)-carboxylase (p-ACC) antibody (1:1000), antiacetyl-coenzyme A (CoA)-carboxylase (ACC) antibody (1:2000), anti-SIRT1 antibody (1:1000), antiacetylated-histone 3 (Ace-H3) antibody (1:1000), antiestrogen receptor α (ERα) antibody (1:1000), and antiactin antibody (1:10 000). Then, IRDye 800CW-conjugated secondary antibody (Rockland, Gilbertsville, PA, USA) was incubated.

2.8 | Reagents and drugs

Anti-SIRT1, anti-Ace-H3, anti-p-AMPK, and anti-AMPK antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-p-ACC, anti-ACC, anti-ERα, and antiactin antibodies were purchased from Abcam (Cambridge, MA, USA).

17β-estradiol, ERα-specific inhibitor methyl-piperidino-pyrazole (MPP), and AMPK-inhibitor Compound C were purchased from Sigma-Aldrich (St Louis, MO, USA). Estrogen receptor β (ERβ)-specific inhibitor (R,R)-THC was purchased from Tocris Bioscience (Avonmouth, Bristol, UK).

After 7-day culture in vitro, the neurons were treated with estrogen (1 nmol/L-10 μmol/L), MPP (1 μmol/L), THC (1 μmol/L), Compound C (10 μmol/L), or vehicle for 12 hours, prior to OGD.

For Compound C administration in vivo, ALC-H motorized digital stereotaxic instrument (Shanghai Alcott Biotech Co. Ltd., Shanghai, China) was used to fix rats. Then, 5 μL of vehicle or Compound C (10 mg/mL) was injected into the left lateral ventricle for 15 minutes followed by MCAO. A temperature controller pad was used to maintain the core temperature (rectum) at 37°C, during the intracerebroventricular (i.c.v.) infusion.

2.9 | Statistical analysis

The investigators were blinded to the procedures, including assessing the infarct size and neurological deficit score of MCAO animals. The random permutation table was used to randomly assign the animals. Data are expressed as the mean±SD and analyzed with two-tailed Student's *t* test or one-way analysis of variance (ANOVA). $P<.05$ is considered statistically significant.

3 | RESULTS

3.1 | Estrogen supplement significantly increased serum estrogen levels and attenuated weight gain induced by OVX

Serum estrogen levels were measured to confirm the efficacy of estrogen supplement in SD rats. As shown in Figure 1A, the serum estrogen levels in the OVX rats were decreased by 50.6% compared with the sham-operated rats, suggesting estrogen deficiency. Serum estrogen levels in the OVX+E groups were 1.88 times greater than those in the OVX group, which was similar to those in the sham group, suggesting successful estrogen replacement therapy. We also measured other hormone levels. OVX significantly decreased the levels of progesterone and testosterone by 50.9% and 63.8%, respectively, and estrogen supplement did not change the levels of progesterone and testosterone (Figure 1B-C).

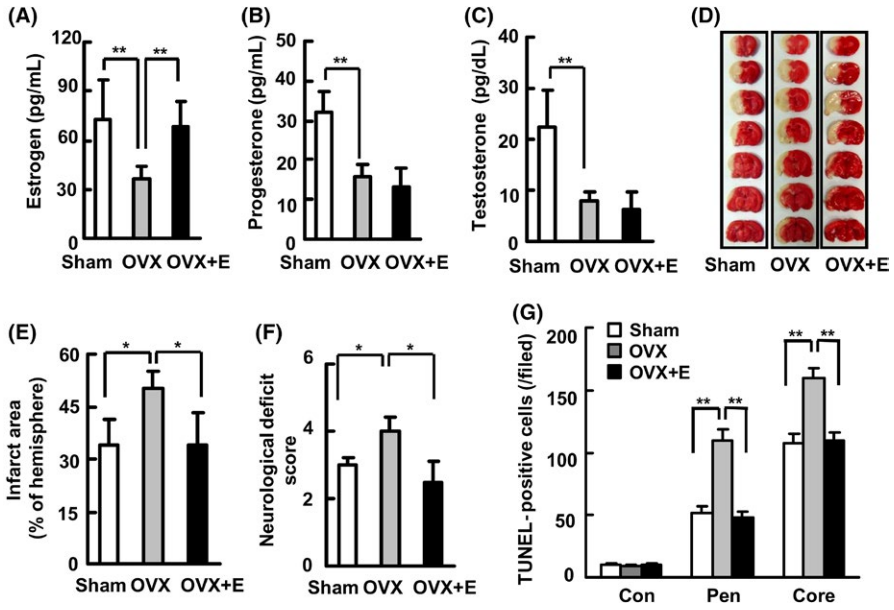

FIGURE 1 Estrogen supplement increased serum estrogen levels of OVX rats and protected against ischemic stroke in MCAO rats. (A) OVX led to a decrease in serum estrogen level, whereas estrogen supplement (500 μg/kg, daily subcutaneous injections) for 28 d rescued hormone level. (B-C) OVX significantly decreased the levels of progesterone and testosterone, whereas estrogen supplement did not change the levels of hormones. (D-F) Estrogen deficiency induced by OVX led to a substantial enlargement of MCAO-induced infarction volume and neurological deficits, compared with the sham group. This effect of OVX was significantly prevented by presupplementation with estrogen. D shows representative rostro-caudal series of TTC-stained coronal sections 24 h after MCAO. (G) TUNEL staining showing OVX increased brain cell death in both infarct core and peri-infarct penumbra after MCAO, which was totally prevented by supplementation with estrogen. E, estrogen; Con, unaffected contralateral region of brain in MCAO rats; Pen, peri-infarct penumbra after MCAO; Core, infarct core region after MCAO. Data were analyzed by one-way analysis of variance (ANOVA) followed by least significant difference *t* test, n=5-8 in each group. **P*<.05, ***P*<.01

Morphometric parameters were listed in Table 1. Rats had an increased body weight after OVX, and estrogen replacement therapy reversed this effect of body weight gain. Absolute weights of heart, kidney, and liver were comparable among the groups. The OVX-mediated increase in uterine weight was restored by estrogen treatment, consistent with its known effects.

3.2 | Estrogen supplement conferred neuroprotection

To determine the effect of estrogen supplement on ischemic stroke, experiments were performed using MCAO rats. Estrogen deficiency induced by OVX led to a substantial enlargement of MCAO-induced infarction volume (by 48.8%) and neurological deficits, compared with

TABLE 1 Tabular representation of morphometric parameters

	Sham	OVX	OVX+E
Initial BW (g)	157.82±3.50	160.09±5.00	158.45±4.25
Final BW (g)	229.11±6.02	259.50±8.00**	237.50±9.95##
Weight gain (g)	72.11±5.16	98.12±4.62**	78.40±8.98##
Heart weight (g)	0.75±0.06	0.81±0.03	0.77±0.06
HW/BW ($\times10^{-3}$)	3.28±0.21	3.12±0.05	3.22±0.16
Kidney weight (g)	1.34±0.10	1.46±0.07	1.38±0.09
KW/BW ($\times10^{-3}$)	5.82±0.31	5.61±0.12	5.82±0.24
Liver weight (g)	6.26±0.40	6.83±0.44	6.54±0.35
LW/BW ($\times10^{-3}$)	27.28±1.27	26.30±0.93	27.54±0.64
Uterus weight (g)	0.38±0.01	0.21±0.01**	0.39±0.02##
UW/BW ($\times10^{-3}$)	1.65±0.02	0.81±0.02**	1.66±0.33##

BW, body weight; HW, heart weight; KW, kidney weight; LW, liver weight; UW, uterus weight.
All the morphometric parameters were recorded after proper development of an estrogen-deficient condition and estrogen replacement.
Each data point represents the mean±SD, n=6-8 rats per group. **P*<.05; ***P*<.01; #*P*<.05; ##*P*<.01; *vs sham; #vs OVX.

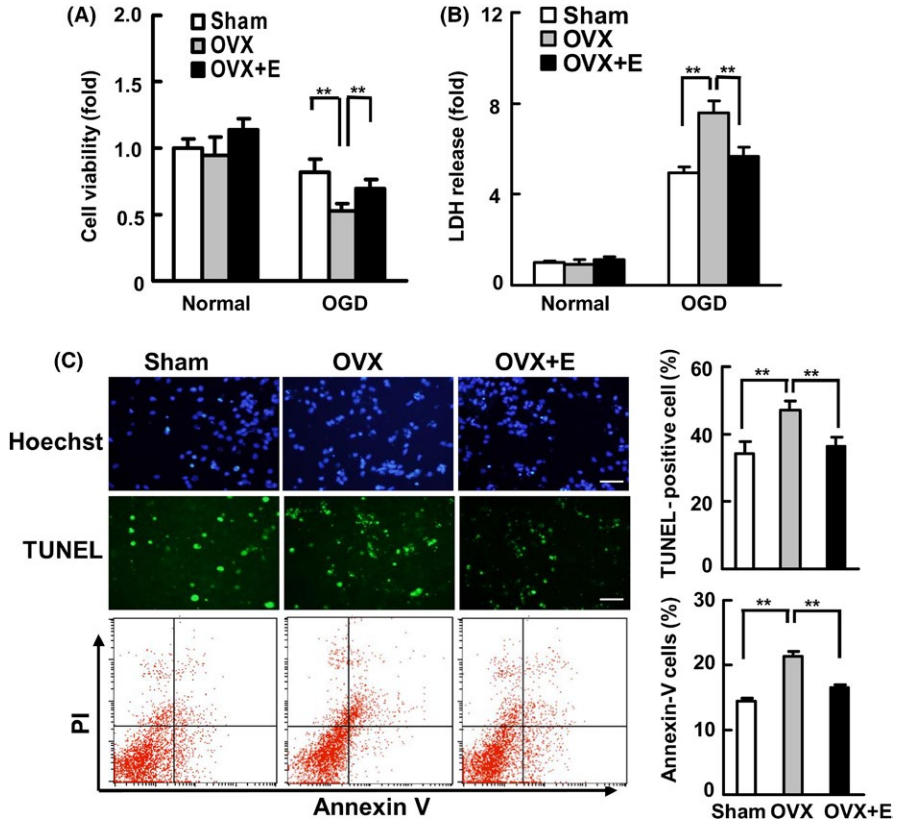

FIGURE 2 Estrogen is protective in OGD neurons. Cultured neurons were exposed to serum from sham, OVX, and OVX+E rats for 12 h prior to OGD. In normal conditions, estrogen deficiency and supplementation had no significant effect on neuronal survival. However, under OGD, pretreatment of OVX serum significantly aggravated neuronal injury by OGD for 12 h, as evidenced by a reduction in cell viability (A) and by an increased release of LDH (B), and this effect was partly prevented by supplementation with estrogen; n=6 in each group (A-B). (C) The cells were stained with TUNEL staining kit (green; for dead cells) and Hoechst 33342 to visualize the nuclei. The cell apoptosis (annexin V staining, R2 + R3) was also analyzed by flow cytometer. Scale bar, 20 μm; n=3 in each group. Data were analyzed by ANOVA followed by least significant difference *t* test. **P*<.05, ***P*<.01

the sham group. This effect of OVX was significantly prevented by presupplementation with estrogen (Figure 1D-F). TUNEL staining demonstrated that OVX increased brain cell death in both infarct core and peri-infarct penumbra, which was prevented by estrogen supplementation (Figure 1G). However, OVX and estrogen supplementation did not affect brain cell death in the unaffected contralateral region (Figure 1G).

Next, we studied the effect of estrogen in cultured primary neurons. No significant effect of estrogen on neuronal survival was observed in normal conditions (Figure 2). Under OGD, pretreatment of OVX serum significantly aggravated neuronal injury by OGD for 12 hours, as evidenced by a reduction in cell viability and by an increased release of LDH, and this effect was alleviated by supplementation with estrogen (Figure 2A,B). Using TUNEL analysis and flow cytometric analysis of annexin V-PI staining to identify cell apoptosis, similar results were observed (Figure 2C). Neurons of estrogen deficiency were susceptible to apoptosis under OGD. In contrast, neurons pretreated with estrogen-supplemented serum exhibited restored resistance to cell apoptosis.

3.3 | Estrogen-evoked neuroprotection is mediated by AMPK

AMPK is recognized as a metabolic master switch and maintains energy balance under energetic stress. The studies in vitro demonstrated that AMPK activation is regulated by estrogen.[34] Consistent with previous study, AMPK was significantly activated by estrogen in a dose-effect manner under OGD, and 1×10^{-6} mol/L estrogen promoted phosphorylation of AMPK and its substrate ACC to the peak level (Figure 3A-B). Therefore, we used 1×10^{-6} mol/L estrogen in the subsequent experiments. To explore the role of ERα and ERβ in this effect, intervention studies were performed in vitro using ERα-specific inhibitor MPP and ERβ-specific inhibitor THC. The action of estrogen on AMPK activity was reversed by antagonists of ERα, not ERβ (Figure 3C-D), suggesting that estrogen promoted phosphorylation of AMPK by ERα.

To determine the role of AMPK in estrogen-evoked neuron survival, we further examined the effect of AMPK-inhibitor Compound C. In cultured neurons, estrogen reduced OGD-induced neuronal injury, which was supported by an increase in cell viability, by a decreased release of LDH, and a reduced percentage of annexin V-positive cells. Neuroprotection of estrogen was abolished by AMPK inhibition induced by Compound C (Figure 3E-F,H-I). Similar results were obtained in TUNEL analysis (Figure 3E,G). These data indicate that the AMPK plays an important role in estrogen-evoked neuron survival.

The role of AMPK was further investigated in SD rats. The result was in agreement with that in vitro, AMPK inhibition attenuated neuroprotection of estrogen. In MCAO rats, inhibition of AMPK activity by Compound C attenuated the effect of decreasing infarction size and ameliorating-neurological deficit induced by estrogen, compared with OVX group (Figure 4).

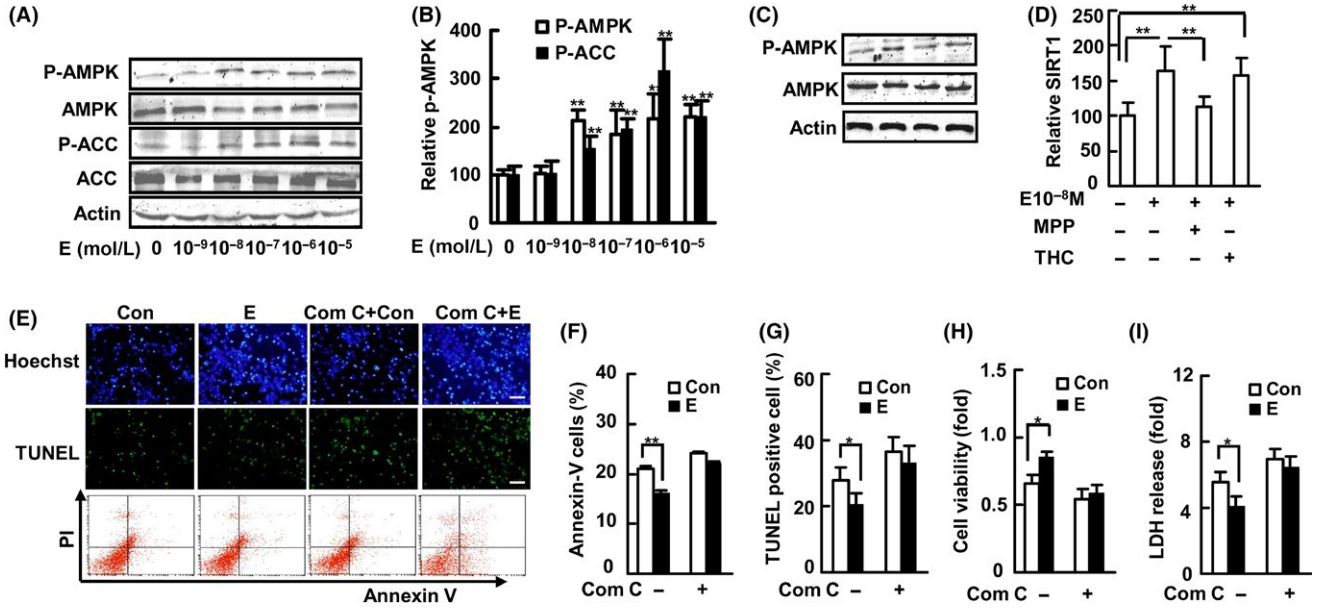

FIGURE 3 Estrogen-evoked neuron survival is mediated by AMPK pathway. (A-B) Under OGD, AMPK was significantly activated by estrogen in a dose-effect manner, and 1×10^{-6} mol/L estrogen promoted phosphorylation of AMPK and its substrate ACC to the peak level. (C-D) ERα-specific inhibitor MPP and ERβ-specific inhibitor THC were used. The action of estrogen on AMPK activity was reversed by antagonists of ERα, not ERβ. (E-I) In cultured neurons, estrogen reduced OGD-induced neuronal injury, which was supported by an increase in cell viability (H) and a decreased release of LDH (I), as well as a reduced percents of annexin V-positive cells and TUNEL-positive cells (E-G), whereas the neuroprotection of estrogen was abolished by AMPK inhibition induced by Compound C. Scale bar, 20 μm. Data were analyzed by ANOVA, n=3-6 in each group. *P<.05, **P<.01

3.4 | SIRT1 was essential for estrogen-induced neuroprotection

In cultured primary neurons, estrogen increased SIRT1 expression in a dose-effect manner under OGD, and 1×10^{-5} mol/L estrogen up-regulated SIRT1 expression to its peak level (Figure 5A). Apart from this, a significant decrease in Ace-H3, a known downstream target of SIRT1, was observed in neurons of estrogen treatment, indicating that estrogen increased SIRT1 deacetylase activity. Because studies of AMPK adopted the 1×10^{-6} mol/L estrogen, in which dosage SIRT1 expression and activity were significantly increased, we also used 1×10^{-6} mol/L estrogen in the subsequent experiments. In addition, the mRNA level of SIRT1 was evaluated using real-time PCR. As shown in Figure 5B, estrogen treatment increased the SIRT1 mRNA level in neuron, consistent with its results of protein profile. Moreover, the action of estrogen (10^{-6} mol/L) on SIRT1 was reversed by antagonist MPP of ERα, not THC of ERβ (Figure 5C). The results suggested that estrogen promotes the expression of SIRT1 by ERα.

Next, we compared neuron survival in WT and SIRT1$^{-/-}$ neurons. SIRT1$^{-/-}$ neurons exhibited exacerbation of OGD-induced cell death (Figure 5F-H), suggesting the key role of SIRT1 in neuron survival. Estrogen reduced OGD-induced neuronal injury in WT neurons, and neuroprotection of estrogen was abolished in SIRT1$^{-/-}$ neurons, which was supported by the results of cell viability, LDH release, percents

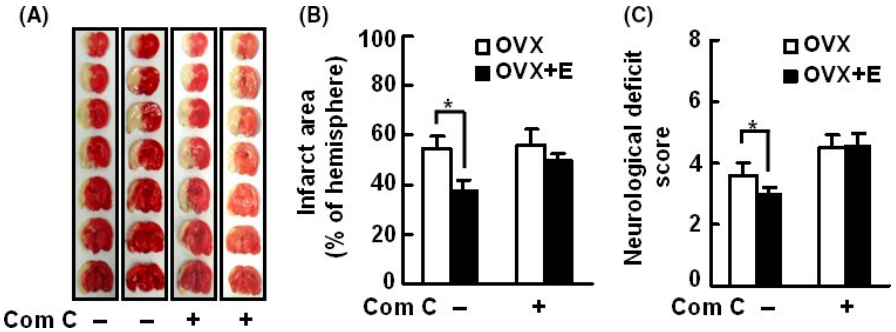

FIGURE 4 AMPK inhibition attenuated neuroprotection of estrogen. In MCAO rats, AMPK inhibition by Compound C attenuated the effect of decreasing infarct size and ameliorating-neurological deficit induced by estrogen, compared with OVX group. A shows representative rostro-caudal series of TTC-stained coronal sections 24 h after MCAO. Data were analyzed by ANOVA followed by least significant difference t test, n=5-7 in each group. *P<.05, **P<.01

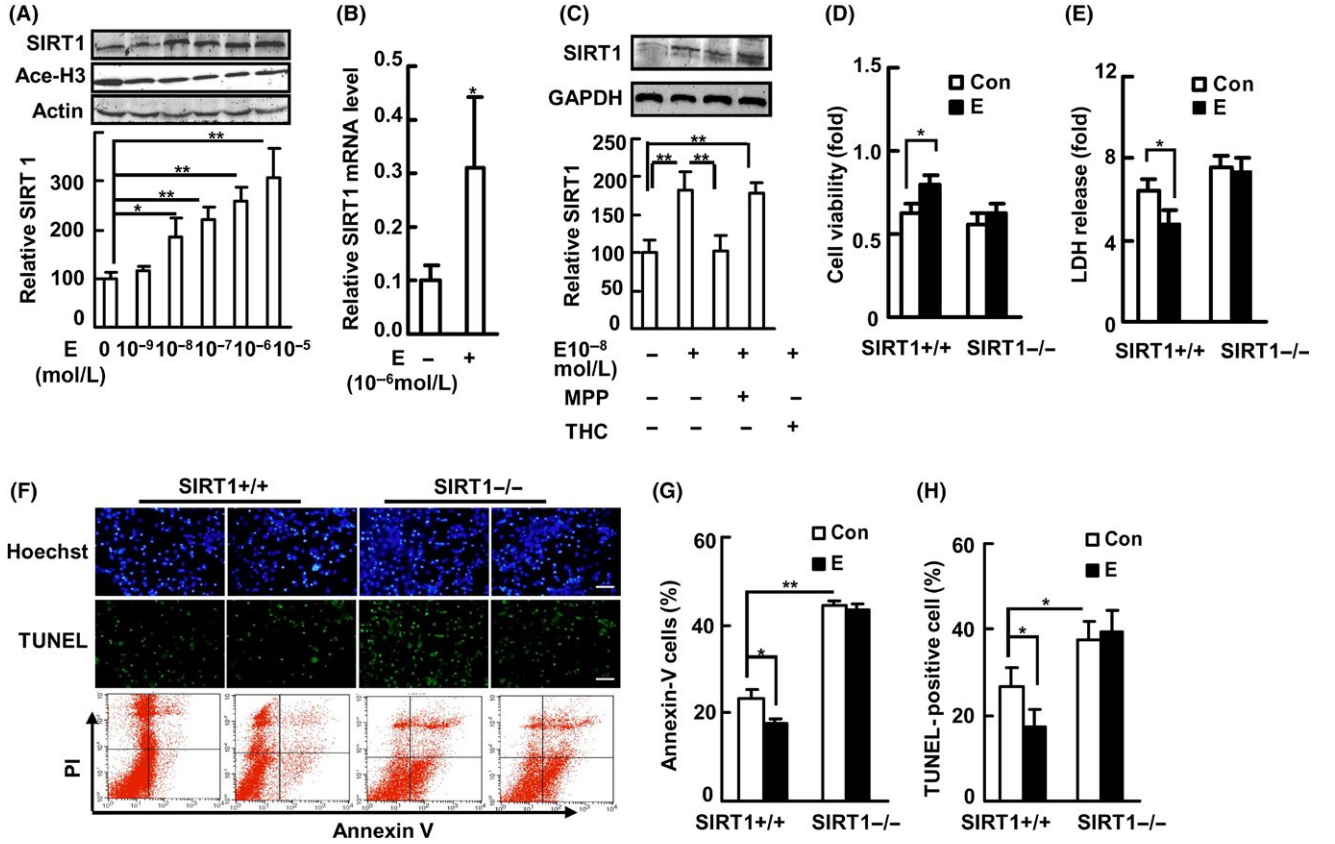

FIGURE 5 Estrogen-evoked neuron survival is mediated by SIRT1 pathway. (A) Under OGD, estrogen enhanced SIRT1 expression in a concentration-dependent manner, and 1×10^{-5} mol/L estrogen upregulated SIRT1 expression to its peak level. A significant decrease in acetylase-histone 3 (Ace-H3), a known downstream target of SIRT1, was observed in neurons of estrogen treatment. (B) Estrogen treatment increased the SIRT1 mRNA level in neuron. (C) ERα-specific inhibitor MPP and ERβ-specific inhibitor THC were used. The action of estrogen on SIRT1 expression was reversed by antagonists of ERα, not ERβ. (D-H) In $SIRT1^{+/+}$ neurons, estrogen reduced OGD-induced neuronal injury, which was supported by an increase in cell viability (D) and a decreased release of LDH (E), as well as a reduced percents of annexin V-positive cells and TUNEL-positive cells (F-H), whereas the neuroprotection of estrogen was abolished in $SIRT1^{-/-}$ neurons. Scale bar, 20 μm. Data in B were analyzed by two-tailed Student's *t* test, other data by ANOVA, n=3-6 in each group. **P*<.05, ***P*<.01

of annexin V-positive cells, and TUNEL-positive cells (Figure 5D-H). These data indicate that the SIRT1 plays an important role in estrogen-evoked neuron survival.

To further define the role of SIRT1, WT and $SIRT1^{-/-}$ mice were subjected to MCAO. In WT mice, estrogen supplement reduced infarction volume by 30.2%, compared with the OVX group. This effect of estrogen was significantly attenuated in $SIRT1^{-/-}$ mice (Figure 6). These data in vivo and vitro indicate that the SIRT1 is essential for estrogen-induced neuroprotection.

3.5 | SIRT1 was essential for estrogen-induced AMPK activation

Given that estrogen regulated AMPK activation by ERα, whether ERα can directly interact with AMPK was determined in neurons using an immunoprecipitation assay. Our data did not show a direct interaction between these two proteins (Figure 7A).

It is known that SIRT1 and AMPK are important mediators in energy expenditure. Our results have demonstrated that these two proteins were regulated by estrogen and both are important for neuroprotection of estrogen. Combining with previous studies, several biological effects of estrogen in cardiovascular system were mediated by SIRT1/AMPK/epigenetic modifications,[34] and the SIRT1-dependent

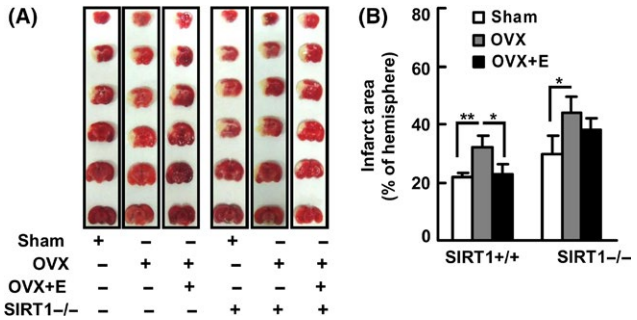

FIGURE 6 SIRT1 is essential for estrogen-induced neuroprotection. In WT mice, estrogen supplement reduced infarction volume, compared with the OVX group. This effect of estrogen was significantly attenuated in $SIRT1^{-/-}$ mice. A shows representative rostro-caudal series of TTC-stained coronal sections 24 h after MCAO. Data were analyzed by ANOVA followed by least significant difference *t* test, n=5-7 in each group. **P*<.05, ***P*<.01

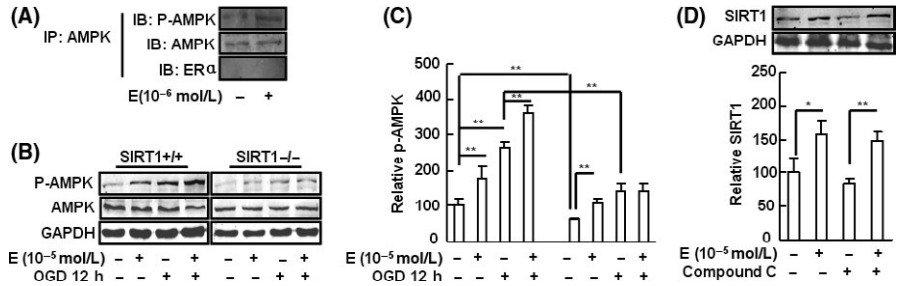

FIGURE 7 SIRT1 is essential for estrogen-induced AMPK activation. (A) Immunoprecipitation analysis did not show a direct interaction between ERα and AMPK after estrogen treatment in OGD neurons. IP, immunoprecipitation; IB, immunoblotting. (B-C) In WT neurons, estrogen upregulated AMPK phosphorylation in normal condition; OGD promoted AMPK activation, and estrogen treatment further enhanced AMPK activation in OGD neuron, whereas in SIRT1$^{-/-}$ neurons, AMPK activation was significantly weak in normal and OGD condition; moreover, estrogen-evoked AMPK activation was also abolished in SIRT1$^{-/-}$ neurons under OGD. (D) AMPK inhibition by Compound C did not hamper the upregulation of SIRT1 induced by estrogen. Data were analyzed by ANOVA, n=3-5 in each group. *P<.05, **P<.01

AMPK pathway was also demonstrated to be involved in neuroprotection of resveratrol and Nampt;[2,25,26] thus, we hypothesized that estrogen protects against ischemic injury through SIRT1-AMPK pathway, and SIRT1 is a key link between estrogen and AMPK.

Next, we compared estrogen-regulated AMPK activation in WT and SIRT1$^{-/-}$ neurons. AMPK activation was strong in WT neurons but weak in SIRT1$^{-/-}$ neurons (Figure 7B-C). In WT neurons, estrogen upregulated AMPK phosphorylation in normal condition; OGD promoted AMPK activation, and estrogen treatment further enhanced AMPK activation in OGD neuron. In SIRT1$^{-/-}$ neurons, the enhanced AMPK activation induced by estrogen was weakened in normal condition; furthermore, such effect was abolished under OGD (Figure 7B-C). These results indicated that the absence of SIRT1 impairs the estrogen activation of AMPK in neurons under OGD.

In contrast, AMPK inhibition by Compound C did not hamper the upregulation of SIRT1 induced by estrogen, suggesting that SIRT1 upregulation by estrogen is independent of AMPK activation (Figure 7D).

4 | DISCUSSION

Our data clearly showed that estrogen replacement protects against ischemic damage in MCAO rats and in OGD neurons. Furthermore, we demonstrated that SIRT1-AMPK signaling pathway is involved in the beneficial effect of estrogen under cerebral ischemic stress. Utilizing both pharmacological and genetic manipulations, this study showed that estrogen protects against ischemic stroke, through upregulation of SIRT1 expression and activity, thus promoting AMPK activation, further regulating energy exhaustion, and contributing to neuron survival under ischemic stress.

To our knowledge, discrepant results of experimental studies were reported as to the effect of estrogen on stroke.[27–33] Regarding the reason, except for the complex effect of estrogen in brain, the timing, duration, dosage, and route of administration are critical parameters in determining the effect. This dichotomized outcome has been analyzed in a recent systematic review and meta-analysis.[28] It suggested that subcutaneous injections, which generated estrogen concentrations of 10-110 pg/mL, showed consistent neuroprotection. Consistent with this analysis, in our study, the dosages of estrogen were within this range, and such neuroprotection was confirmed.

The brain consumes more energy per gram of tissue than any other organ.[34] Neuronal ischemia, the consequence of a stroke, confers energy exhaustion in brain cells. The defense to energy exhaustion promotes cell survival and improves the outcome of acute brain injury under ischemic stress. As cellular energy sensors, AMPK and SIRT1 show similarities in modulation of energy metabolic pathway, and activation of SIRT1-AMPK neuroprotective signaling pathway under cerebral ischemic stress was demonstrated in previous studies.[2,24,25] SIRT1 is a conserved protein NAD^+-dependent deacylase. It senses changes in energy levels in diverse cellular location, including nucleus, cytoplasm, and mitochondrion, and then deacetylates many target proteins to maintain energy balance.[22,23] In SIRT1-AMPK pathway, SIRT1 was reported to confer serine/threonine kinase 11 (LKB1) deacetylation, an upstream kinase of AMPK, and subsequent AMPK activation.[2]

Estrogen has well-documented neuroprotective effects in many clinical and experimental cerebral ischemia caused by stroke.[14–17,27–29] Previous mechanisms of estrogen as a neuroprotectant focus on oxidative stress, inflammation, apoptosis, and vascular modulation, much less on energy exhaustion after ischemic injury. Estrogen was previously reported to regulate SIRT1 and AMPK in many cells and rodents.[35-41] Moreover, estrogen prevents cardiovascular dysfunction in postmenopausal metabolic syndrome through SIRT1/AMPK pathway.[22] In view of the above studies, we hypothesized that "SIRT1-AMPK" signaling pathway may play a key role in neuroprotection of estrogen. As expected, we observed that estrogen upregulated the expression and activity of SIRT1 and promoted AMPK activation by ERα in OGD neurons. More importantly, experiments in vivo using SIRT1$^{-/-}$ mice and AMPK inhibitors confirmed that SIRT1 and AMPK are essential for the neuroprotection of estrogen in ischemic stroke, and AMPK activation induced by estrogen is dependent on SIRT1. These results demonstrated SIRT1-AMPK signaling pathway is involved in the neuroprotection of estrogen under cerebral ischemic stress.

Poly(ADP-ribose)polymerase (PARP)-1 plays an important role in neuronal survival following ischemic stress. Regarding PARP-1, it has been reported that NEDD4-1-mediated ubiquitin-proteasome degradation of PPAR-γ is required for the action of estrogen-upregulated

SIRT1.[42] According to our results, estrogen-induced neuroprotection was abolished in SIRT1$^{-/-}$ mice, indicative of a key role of SIRT1, consistent with previous study. However, as to the mechanism of estrogen-regulating SIRT1, we only observed mRNA and protein level of SIRT1. It is known that estrogens exert their effects via several different pathways, including the classical nuclear receptors ER-α and ER-β, membrane-linked receptors, and other direct molecular mechanisms. Thus, taking account of the diversity and complexity of estrogen pathway, as well as our inadequate study performed in this point, our result was not contradictory with previous study. We believe that PARP-1 may be an upstream target of SIRT1, which involves in the action of estrogen. It remains to be investigated in the future.

Regarding SIRT1 and AMPK pathway, some previous studies suggested that AMPK lies upstream of SIRT1,[43,44] which was different from our study. Auwerx J and colleagues reported that AMPK regulated energy expenditure by modulating SIRT1 activity in myocytes,[43] which is very different from the central nervous system. Pan TH and colleagues demonstrated that AMPK-SIRT1-autophagy pathway involved in the neuroprotection of resveratrol on Parkinson cellular models,[42] which is not ischemic stress pathology. The difference in pathology model and cell type may account for the dichotomized discrepancy of pathways. In our study, SIRT1 upregulation by estrogen in OGD neurons was not affected by AMPK inhibition, indicating that estrogen upregulating SIRT1 during ischemic stress is not driven by AMPK in neurons.

Although important discoveries were revealed in our study, there are also limitations. First, we did not assess estrogen level in brain. Estrogen acting on nervous tissue can be divided into two classes, those which are peripherally synthesized and then penetrate the blood-brain barrier and those which synthesized de novo within the brain. Peripherally, serum estrogen level was monitored in our study; however, it only represents a crude estimate of the concentrations in the brain, where the actual effects take place. Secondly, estrogens exert their effects via several different pathways, and the pathway beyond the classical nuclear receptors was not studied, such as membrane-linked receptors and direct molecular mechanisms. Thirdly, estrogen was measured on one occasion during the experiment, which provides little information about the serum concentrations fluctuation during the study. Fourthly, the concentrations of estrogens determined by in vitro experiments are several orders of magnitude higher than in vivo experiments and therefore hard to interpret to in vivo conditions.

In conclusion, this study demonstrated that estrogen protects against ischemic stroke, through rescuing neurons from death via the SIRT1-dependent AMPK pathway. Our results provide new sights into estrogen mechanisms in ischemic stroke and suggest that SIRT1-AMPK pathway is a therapeutic target for stroke prevention for postmenopausal women.

ACKNOWLEDGMENTS

This study was supported by National Natural Science Foundation of China (81402927), China Postdoctoral Science Foundation (2015M582894), and Shandong Provincial Natural Science Foundation (ZR2016HM12).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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How to cite this article: Guo J-M, Shu H, Wang L, Xu J-J, Niu X-C, Zhang L. SIRT1-dependent AMPK pathway in the protection of estrogen against ischemic brain injury. *CNS Neurosci Ther*. 2017;23:360-369. https://doi.org/10.1111/cns.12686