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Icariin enhances neuronal survival after oxygen and glucose deprivation by increasing SIRT1

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ABSTRACT

It has been reported that icariin protects neurons against ischemia/reperfusion injury. In this study, we found that icariin could enhance neuronal viability and suppress neuronal death after oxygen and glucose deprivation (OGD). Further study showed that neuroprotection by icariin was through the induction of Sirtuin type 1 (SIRT1), an effect that was reversed by SIRT1 inhibitor III and P38 inhibitor SB203580. SIRT1 is an endogenous gene of longevity, which increased neuronal viability and could be activated by stimulating the mitogen-activated protein kinase (MAPK) pathway. However, this study found that icariin activated the MAPK/P38 pathway, not the extracellular signal-regulated kinase (MAPK/ERK) or c-Jun N-terminal protein kinase (MAPK/JNK) to regulate SIRT1 expression. The results suggest that icariin may be developed into a neuroprotectant for ischemia-related brain injury.

1. Introduction

Cerebral ischemia is a common cause of neuronal injury. The mechanisms that trigger ischemic brain damage are complicated, including glutamate-mediated excitotoxicity, generation of reactive oxygen species, DNA damage, regulation of pro-apoptotic factors, and so on. Currently, there are less neuroprotectants for ischemic injury in clinic although a lot of drugs work well in basal research (Ford, 2008). To find natural products, which can activate endogenous survival signaling and repair mechanisms, is a critical strategy for stroke therapy.

Icariin, a flavonoid extracted from the traditional Chinese herb *Epimedium brevicornum* Maxim, has demonstrated a wide range of pharmacological and biological activities, including estrogenic activity, anti-tumor activity, antioxidant effect, immunoregulation and improved sexual function (Makarova et al., 2007; Wang et al., 2007; Ye and Lou, 2005; Liang et al., 1997). Recent studies suggest that icariin has protective effects against oxygen and glucose deprivation (OGD)-mediated injury in primary cultured neurons attributing to anti-apoptotic effect, anti-oxidative stress and induces stem cells' differentiation into neuronal cells (Li et al., 2005). However, the underlying mechanism of the neuroprotection of icariin remains unclear.

SIRT1 (Sirtuin type 1) is a member of a highly conserved gene family (Sirtuins) encoding nicotinamide adenine dinucleotide (NAD)$^+$-dependent deacetylases, increased DNA stability and prolonged survival in yeast and higher organisms, and mammals (Zschoernig and Mahlknecht, 2008). SIRT1 is expressed in the developing and the adult mammalian brain. SIRT1 may protect neurons undergoing ischemia/reperfusion insults from apoptosis by the inhibition of p53 and other transcription factors (Hasegawa and Yoshikawa, 2008). SIRT1 is activated by stimulating adenosine monophosphate-activated kinase (AMPK), mitogen-activated protein kinase (MAPK) and NF-κB pathways under different conditions (Chen et al., 2005; Dasgupta and Milbrandt, 2007; Longpré et al., 2006; Qin et al., 2006). Previous study indicated that estrogen protects neurons from damage after OGD through the activation of the extracellular signal-regulated kinase (MAPK/ERK) pathway (Xu et al., 2003, 2004). Since icariin shares a structural similarity to estrogen, we hypothesize that icariin may increase the neuronal viability after OGD by stimulating the MAPK pathway, which up-regulates the expression of SIRT1.

2. Materials and methods

2.1. Primary cerebral cortical neuron culture

All experimental procedures were carried out with the approval of the Animal Care Committee at Nanjing University, China. Primary

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cortical neurons were prepared from E16-17 mouse embryos as described (Xu et al., 2004). Neurons from each embryo cortex were isolated and seeded with equal cell amounts in 96 or 24-well plates. Cells were grown in Neurobasal media supplemented with B27 (Invitrogen, Carlsbad, CA) and 25 nM glutamine at 37 °C in a humidified 5% CO_2 incubator. The purity of neurons was over 95% after 8 days from initial seeding as examined by Neuronal Nuclei (NeuN) and glial fibrillary acidic protein (GFAP) immunostaining.

2.2. Oxygen–glucose deprivation (OGD) and drug treatments

At the 10th day of culture, neurons were subjected to OGD as described (Xu et al., 2004). Briefly, cells with OGD medium were incubated in a hypoxia chamber (Billups-–Rothenberg, Del Mar, CA) previously flushed for 15 min with 5%CO_2/95%N_2 at 2 psi (1 psi = 6.89 kPA). The chambers were sealed and incubated at 37 °C for 2 h. Then cells were returned to the normal feeding medium and incubated under normal conditions at 37 °C for 24 h for later experiments. Control cell cultures not deprived of oxygen and glucose were incubated under normal conditions in 10 mM glucose added OGD medium. Different concentrations of icariin were present during the OGD and reperfusion process. Medium was used as a vehicle. SIRT1 inhibitor III (100 nM, Calbiochem, USA) or P38 inhibitor SB203580 (10 μM, Calbiochem, USA) was added to the medium 15 min before and during the icariin treatment.

2.3. MTT assay

Following treatment as described above, neuronal viability was determined by MTT assay (Jia et al., 2008). Briefly, neurons were treated with 0.5 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 3 h at 37 °C. Then 100 μl DMSO was added. Two hours later, density was assessed at 570 nm using an ELISA plate reader (TECAN, Switzerland). Cell survival rates were expressed as percentages of the value of normal cells.

2.4. LDH (lactate dehydrogenase) assay

Lactate dehydragenase (LDH) is the most widely used marker in cytotoxicity study. At the end of incubation, the supernatant was collected from plates and the LDH content was determined using an LDH assay kit according to the manufacturer's instructions (Nanjing Institute of Jianchen Biological Engineering, China). LDH cytotoxicity was calculated by the following formula:

$$\text{LDH cytotoxicity} = (\text{sample OD} - \text{blank OD}) / (\text{standard solution OD} - \text{blank standard solution OD}) \times 2000.\ (\text{OD} = \text{the optical density}).$$

2.5. Flow cytometry assay

Neurons treated as above, were re-suspended in a staining buffer containing Propidium iodide (PI) (50 μg/ml; Sigma) at a concentration of 1 × 106 cells/ml. The cell suspension was placed in the dark for 10 min. The FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, USA) was used for single-cell light scattering and fluorescence measurements.

2.6. Real-time PCR

Total RNA was extracted by using Trizol reagent (Invitrogen, USA). RNA was reverse-transcribed into cDNA using AMV reverse transcriptase (Gibco, USA). Quantitative PCR (ABI 7500, USA) was performed in the presence of a fluorescent dye (Evagreen, BIOTIUM). Relative abundance of mRNA was calculated after normalization to GAPDH ribosomal RNA. The SIRT1 primer sequences used in this study were: F: 5-CAG CAA GGC GAG CAT AAA-3; R: 5-TTC AGA ACC ACC AAA GCG-3.

2.7. Western blotting

The cultured neurons were harvested according to procedures described previously (Xu et al., 2003, 2004). Membranes were probed with primary antibodies against SIRT1 (1:1000; Santa Cruz Biotechnology), phosphorylated ERK1/2, p38, c-Jun N-terminal protein kinase (JNK) (Cell Signaling, 1:1000) or total ERK1/2, p38, JNK (Cell signaling, 1:1000). The GAPDH, total ERK and P38 were used as loading controls.

2.8. Statistical analysis

Statistical tests to determine the differences between groups were performed by a *t*-test using SPSS 11.5. Data were assessed as mean ± S.D. Differences at $P<0.05$ were considered statistically significant.

3. Results

To study whether icariin could protect neurons from damage induced by OGD, neurons were treated with icariin in different concentrations (0, 0.1, 0.3, 0.6, 1.2, 2.4, 4.8, and 9.6 μM) during the OGD and reperfusion process, and then neuronal viability was measured. The results showed that cell viability in OGD was decreased by 52% of the control. And icariin increased the cell viability by 26%, 55%, 65%, 77%, 76%, 77% and 83% above concentrations in the vehicle, respectively, in a dose-dependent manner (Fig. 1). Next 1.2 μM icariin was used to treat the cells for different times after OGD (6, 12, 24, and 48 h) and the results indicated that icariin enhanced neuronal viability after OGD in a time-dependent manner (icariin *vs* vehicle: 6 h, 65 ± 1.6% *vs* 43 ± 1.7%; 12 h, 71 ± 4.4% *vs* 49 ± 2.4%; 24 h, 81 ± 2.4% *vs* 55 ± 2.4%; 48 h, 79 ± 4.0% *vs* 60 ± 2.4%). In order to further confirm the neuroprotection of icariin, cells were treated with 1.2 μM icariin during the OGD and reperfusion process, and 24 h later, the LDH cytotoxicity was determined. As expected, LDH cytotoxicity was increased by 32% in OGD neurons (1086.03 ± 58.7) compared to the control (824.59 ± 14.8, $P<0.05$), but decreased by 17% in the presence of icariin (897.46 ± 40.4, $P<0.05$) compared with the OGD group. Also, icariin decreased neuronal death induced by OGD in the flow cytometry assay. SIRT1 inhibitor III or P38 inhibitor SB203580 could reverse the effect of

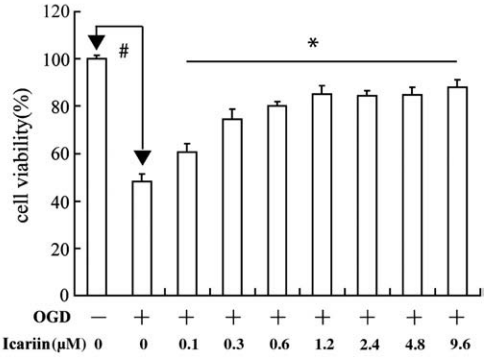

Fig. 1. Icariin enhanced neuronal viability in a dose-dependent manner. Icariin treated the neurons in different doses (0, 0.1, 0.3, 0.6, 1.2, 2.4, 4.8, and 9.6 μM) during the OGD and reperfusion process. At 24 h after OGD, the cell viability was assessed by MTT analysis. #$P<0.05$ *vs* normal control. *$P<0.05$ *vs* OGD. Data were presented as the mean ± S.E.M. from six independent experiments.

icariin (Fig. 2, the cell death: normal neurons 8.1 ± 0.14%; OGD 31.2 ± 0.58%; OGD + Icariin 16.9 ± 0.09%; OGD + Icariin + SB203580 27.9 ± 0.12%; OGD + Icariin + SIRT1 inhibitor III 28.5 ± 0.41%).

To examine whether the protective effect of icariin was mediated by up-regulating SIRT1, mRNA and protein of SIRT1 were determined. As Fig. 3A shows, mRNA of SIRT1 was induced by OGD, and was enhanced by icariin. But P38 inhibitor SB203580 suppressed the SIRT1 expression induced by icariin. The protein level of SIRT1 was consistent with mRNA (Fig. 3B,C). Furthermore, the study sought to determine whether SIRT1 was a critical contributor to neuroprotection by icariin, which was dependent on P38 activation. Neurons were pretreated with SIRT1 inhibitor III or P38 inhibitor before icariin for 15 min undergoing OGD. Flow cytometry and MTT showed that both SIRT1 inhibitor III and P38 inhibitor SB203580 could block the neuroprotection of icariin ($P<0.05$, Figs. 2E–G and 4).

Finally the study explored how icariin increased SIRT1. The activation states of MAPK/ERK1/2, MAPK/p38 and MAPK/JNK were determined (Fig. 3D,E). Phosphorylated ERK1/2, JNK and p38 were induced by OGD compared to the normal control (ERK1/2: 0.354 ± 0.016 *vs* 0.215 ± 0.010, JNK: 0.286 ± 0.014 *vs* 0.051 ± 0.003, P38: 0.334 ± 0.014 *vs* 0.154 ± 0.015, $P<0.05$) and icariin only significantly enhanced p38 activation (0.703 ± 0.023, $P<0.05$), not ERK1/2 or JNK.

4. Discussion

This study found: 1) Icariin protected neurons from damage induced by OGD. 2) Icariin could up-regulate the expression of SIRT1 and P38 inhibitor SB203580 suppressed the expression in neurons undergoing OGD. 3) Both SIRT1 inhibitor III and P38 inhibitor SB203580 at least partially blocked neuroprotection by icariin. 4) MAPK/p38 activation may contribute to the mechanism of neuroprotection by icariin.

Icariin has an anti-oxidative effect on various pathological processes such as stroke, ischemia reperfusion-induced cognitive impairments, congestive heart failure, endothelial cell damage induced by reactive oxygen species, DNA damage induced by radical, cardiovascular disease, and so on (Ho and Jie, 2007; Li et al., 2005; Song et al., 2008; Wang and Huang, 2005; Zhao et al., 2007; Zheng et al., 2008). However, the mechanism of neuroprotection by icariin remains unclear.

The present study shows that icariin facilitates neuronal viability after OGD by raising the expression of SIRT1 and SIRT1 inhibitor III could, in part, block the effect of icariin. SIRT1 is a kind of Sirtuins (SIRT1–SIRT7), which is a family of NAD^+-dependent histone/protein deacetylases and distributed across all kingdoms of life (Zschoernig and Mahlknecht, 2008). Accumulating evidence shows that SIRT1 has been implicated in various effects such as stress resistance, reduced apoptosis, and metabolic changes associated with calorie restriction. SIRT1 increases the NAD supply, which regulates a genetic program to effectively prevent axonal self-destruction (Araki et al., 2004). Activators of SIRT1 significantly attenuated retinal ganglion cell loss in optic neuritis and multiple sclerosis by increasing cellular stress resistance and survival. In addition, SIRT1 protects against microglia-dependent amyloid-β toxicity through inhibiting NF-κB signaling (Chen et al., 2005). This study exhibited that the expression of SIRT1 in cortical neurons was induced after OGD, and icariin amplified the up-regulation of SIRT1. SIRT1 inhibitor III suppressed the neuroprotection of icariin. It suggests that SIRT1 may at least partially contribute to mechanisms of neuroprotection of icariin.

This study also shows how icariin up-regulates SIRT1 in OGD neurons. Previous studies indicated that estrogen protects neurons from damage after OGD by activating the MAPK/ERK pathway (Xu et al., 2003, 2004). And EGb 761 reduced amyloid-β peptide-induced toxicity through increasing SIRT1 via the activation of ERK1/2 (Longpré et al., 2006). To test the hypothesis that icariin augments SIRT1 by activating the MAPK pathway, the expression of phosphorylated ERK1/2, P38 and JNK in the absence/presence of icariin was measured. The results indicated that ERK1/2, P38 and JNK were activated in OGD neurons. But icariin only enhanced the activation of P38, not ERK1/2 or JNK. Furthermore, P38 inhibitor SB203580 suppressed the expression of SIRT1 induced by icariin. This result agrees with another report, that icariin induces cardiomyocyte differentiation of murine embryonic stem cells through enhanced and prolonged phosphorylation of p38MAPK and this inducible effect of icariin was blunted by SB203580, a specific inhibitor of p38MAPK (Ding et al., 2008). The understanding of the role of p38

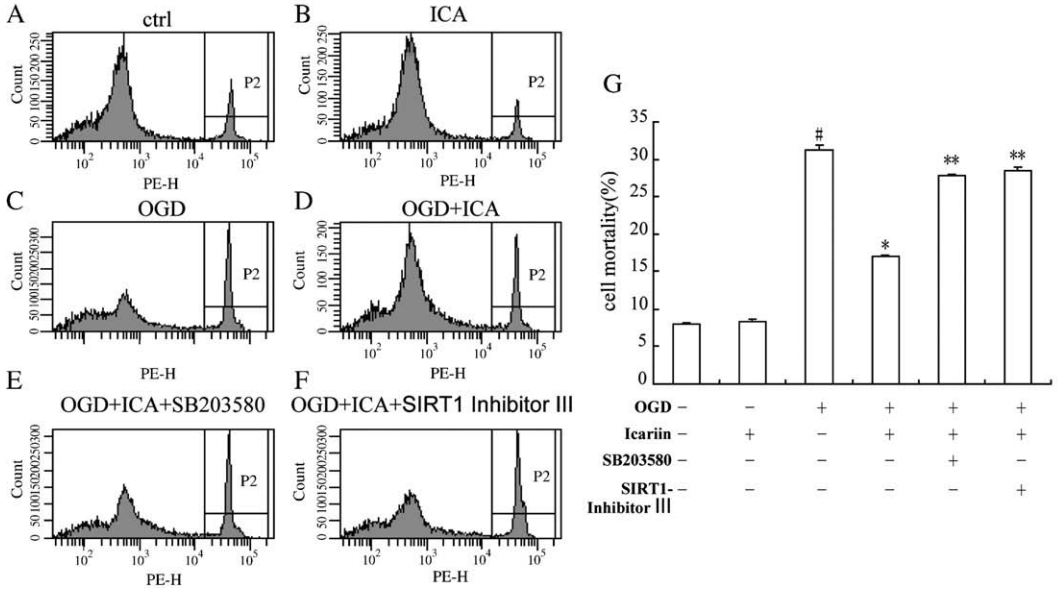

Fig. 2. Icariin suppressed OGD-mediated neuronal cell death by flow cytometry. SIRT1 inhibitor III (100 nM) and P38 inhibitor SB203580 (10 μM) pretreated the neurons before icariin (1.2 μM). At 24 h after OGD, cell death was measured by flow cytometry. (A) Normal neurons. (B) Normal neurons with icariin. (C) OGD neurons. (D) OGD neurons with icariin. (E) OGD neurons with icariin plus SIRT1 inhibitor III. (F) OGD neurons with icariin plus P38 inhibitor. (G) Cell mortality (%) by bar graph. #$P<0.05$ *vs* control group. *$P<0.05$ *vs* OGD group. **$P<0.05$ *vs* icariin treated OGD group. Data were presented as the mean ± S.E.M. from six independent experiments.

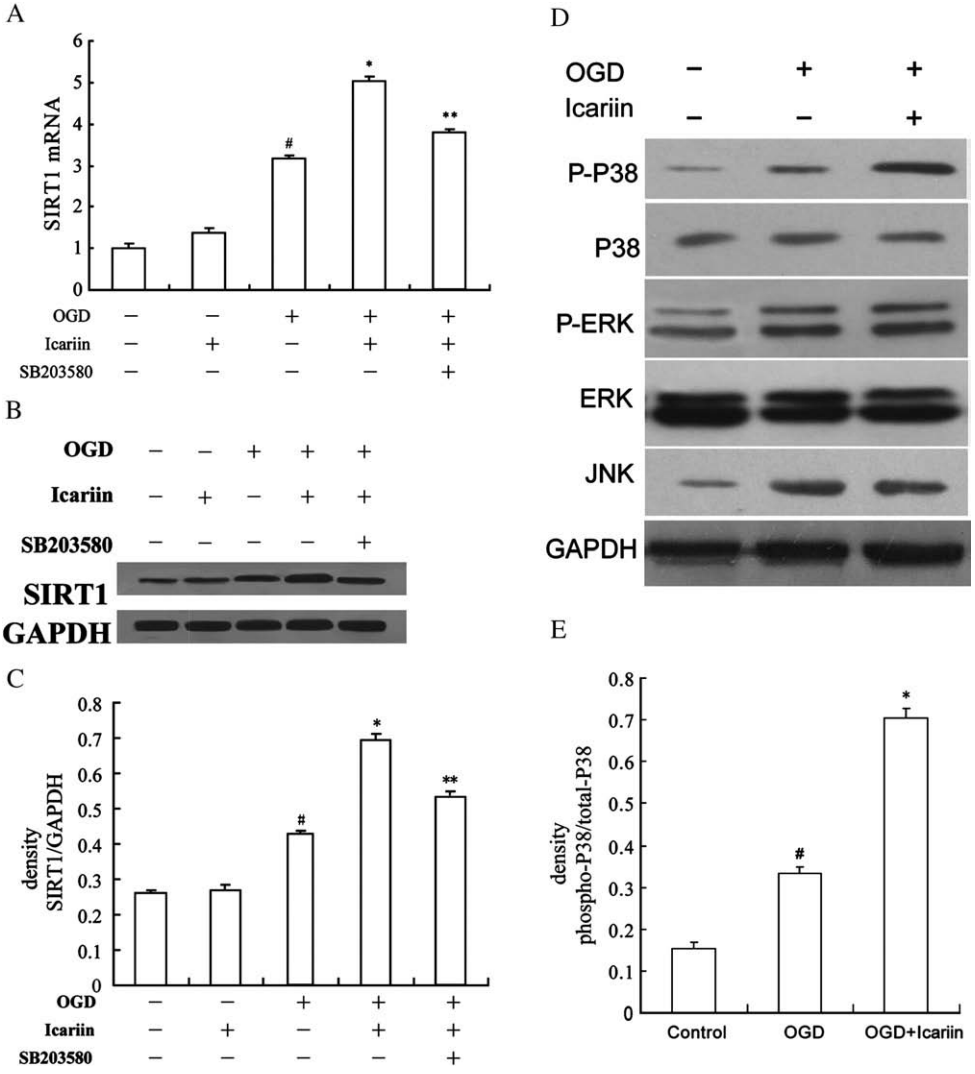

Fig. 3. Icariin enhanced the expression of SIRT1 and icariin activated P38 phosphorylation in OGD neurons. Neurons were treated with the same method as Fig. 2. At 24 h after OGD, cells were extracted for mRNA and protein measurements. The mRNA level of SIRT1 was measured by real-time PCR (A). The protein level of SIRT1 was measured by western blot (B). P38 inhibitor partially suppressed the activation of SIRT1 induced by icariin (A and C). Effects of icariin on the activation of P38, ERK and JNK by western blot (D). Icariin enhanced phosphorylation of P38 induced by OGD (E). #$P<0.05$ *vs* control group. *$P<0.05$ *vs* OGD group. **$P<0.05$ *vs* icariin treated OGD group. Data were presented as the mean ± S.E.M. from six independent experiments.

MAPKs in the nervous system is limited. Increasing evidence from mammalian cell culture systems and the strong genetic tool *C. elegans* suggests that neuronal p38 signaling has diverse functions beyond the control of cell death and survival (Takeda and Ichijo, 2002). This study indicated that p38 signaling enhanced neuronal survival after OGD.

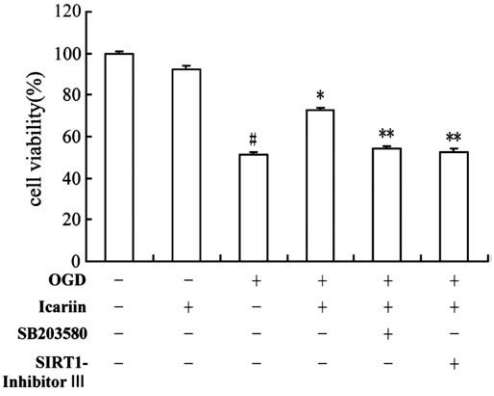

Fig. 4. SIRT1 inhibitor III and P38 inhibitor SB203580 blocked neuroprotection by icariin. SIRT1 inhibitor III (100 nM) and P38 inhibitor SB203580 (10 μM) pretreated the neurons before icariin (1.2 μM). At 24 h after OGD, cell viability was measured by MTT. #$P<0.05$ *vs* control group. *$P<0.05$ *vs* OGD group. **$P<0.05$ *vs* icariin treated OGD group. Data were presented as the mean ± S.E.M. from six independent experiments.

5. Conclusion

In conclusion, the results suggest that icariin has a protective effect on cortical neurons against OGD-induced damages, through enhancing the expression of SIRT1. The mechanism may be partially involved with the activation of MAPK/P38. It suggests that icariin may serve as a novel drug in the prevention of stroke-related brain damages.

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