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Research Report

Neuroprotective effects of icariin on corticosterone-induced apoptosis in primary cultured rat hippocampal neurons

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ABSTRACT

Neurons are damaged following prolonged exposure to high concentrations of corticosterone, particularly during chronic inflammatory and immune diseases. One of the main mechanisms underlying neuronal injury is apoptosis. In the present study the neuroprotective effects of icariin, an active natural ingredient from the Chinese plant *Epimedium sagittatum maxim* against corticosterone-induced apoptosis were examined in primary cultured rat hippocampal neuronal cells. Pre-treatment of neuronal cells with icariin suppressed corticosterone-induced cytotoxicity in a dose-dependent manner. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick-end-labeling assay (TUNEL) labeling demonstrated that icariin significantly reduced TUNEL-positive cell numbers induced by exposure of cultured neurons to corticosterone. Moreover, icariin markedly inhibited corticosterone-induced mitochondrial dysfunction, including improved mitochondrial membrane potential and inhibition of caspase-3 activation. Using western blot analysis, corticosterone activated p38MAPK, extracellular regulated kinase 1/2 (ERK1/2) ,and c-*jun* N-terminal protein kinase 1(JNK1) ,while icariin blocked p38 MAPK, but not JNK1 or ERK1/2. Pharmacological approaches showed that the activation of p38MAPK plays a critical role in corticosterone-induced mitochondrial dysfunction and apoptosis. Taken together, the present results suggest that the protective effects of icariin on apoptosis in hippocampal neuronal cells are potentially mediated through blockade of p38 MAPK phosphorylation.

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Abbreviations: DAPI, 4′,6′-diamidino-2-phenylindole dihydrochloride; ERK1/2, extracellular regulated kinase 1/2; GR, glucocorticoid receptor; IBD, inflammatory bowel disease; ICT, 3,5,7-Trihydroxy-4′-methoxy-8-(3-hydroxy-3-methylbutyl)-flavone; JNK1, c-*jun* N-terminal protein kinase 1; LDH, lactate dehydrogenase; LPS, lipopolysaccharides; MAPK, mitogen-activated protein kinases; MMP, mitochondrial membrane potential; MR, mineralocorticoid receptor; MTT, 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide; NF-κB, nuclear factor-κB; PI3-K, phosphoinositide 3-kinase; RA, rheumatoid arthritis; ROS, reactive oxygen species; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole; SLE, systemic lupus erythematosis; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick-end-labeling

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1. Introduction

Corticosteroids are the most potent and effective therapy for many chronic inflammatory and immune diseases, including asthma, rheumatoid arthritis (RA), inflammatory bowel disease (IBD), and systemic lupus erythematosis (SLE) (Barnes and Adcock, 2009). However, long-term corticosteroid therapy causes systemic side effects, including diabetes, osteoporosis, infection, glaucoma, and cataracts, which are well documented. Additionally, the effects of these medications on the brain have not been extensively described (Brown, 2009). Many studies have shown that the hippocampus is one of the most vulnerable brain regions to various neurobiological insults (Reagan and McEwen, 1997). Prolonged or excessive exposure to corticosteroids leads to neuronal damage, particularly in the hippocampus, which is enriched with corticosteroid receptors (de Quervain et al., 2009; Murray et al., 2008).

However, the precise cellular mechanisms underlying corticosteroid-induced neuronal cell damage have not been fully elucidated at present. Studies have suggested that these mechanisms are potentially involved in inhibition of glucose transport (Jr Virgin et al., 1991), impairment of energy metabolism (de Leon et al., 1997) and energy-dependent disruption of neuronal Ca^{2+} regulation (Grammer and Shaughnessy, 1993). A key mechanism underlying neuronal injury is apoptosis, which is a key focus of present research. It is well known that corticosterone can cause apoptosis in hippocampal neurons (Zhu et al., 2006). Thus, it is believed that molecular signaling mechanisms involved in corticosteroid-induced apoptotic cell death can be helpful in clinical practice in order to find a molecular agent which has the ability to protect hippocampal neurons from corticosteroid-induced neuronal damage.

Icariin, a biologically active component purified from the Chinese herbal plant *Epimedium*, has been shown to improve cardiovascular function, induce tumor cell differentiation and increase bone formation (He et al., 1995). Recently, we reported that icariin and its derivative, 3,5,7-trihydroxy-4′-methoxy-8-(3-hydroxy-3-methylbutyl)-flavone (ICT), attenuates lipopolysaccharides (LPS)-induced acute inflammatory responses *in vitro* and *in vivo*, which involve activation of the phosphatidylinositide 3-kinase(PI3K)/Akt pathway and inhibition of nuclear factor-κB (NF-κB) (Wu et al., 2010; Xu et al., 2010). Previous studies have suggested that icariin protects against oxygen and glucose deprivation-mediated injury in primary cultured neurons, which is attributable to its potent antioxidative ability. Icariin also reduces oxidative stress and induces stem cells differentiation in neuronal cells (Li et al., 2005). However, the mechanisms of the neuroprotective effects of icariin have not been fully elucidated. The present study investigated the protective effects of icariin on corticosterone-induced apoptosis in primary cultured rat hippocampal neurons and the potential signal transduction pathways involved. The current results demonstrate that icariin can suppresses corticosterone-induced primary cultured rat hippocampal neuron apoptosis via the blockade of p38 MAPK phosphorylation.

2. Results

2.1. *Establishment of the corticosterone-insulted primary culture rat hippocampal neuronal cell model*

To determine the property modeling concentration of corticosterone, hippocampal neurons were exposed to an increasing concentration of corticosterone (0.01–5 μM) for 24 h or exposed to corticosterone (1 μM) for 12–72 h. As shown in Fig. 1A, a significant effect of exposure of cultured hippocampal neurons to corticosterone was observed. 0.5 μM and 1 μM corticosterone significantly decreased 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-diphenytetrazoliumromide (MTT) levels by 34.90% ($p<0.01$) and 54.45% ($p<0.01$) in 24 h, respectively, whereas 0.05 μM and 0.01 μM of corticosterone did not induce a significant change in cell viability, indicating a concentration dependent neurotoxicity of corticosterone on cultured hippocampal neurons grown in Neurobasal+B27 medium. As shown in Fig. 1B, 24 h and 48 h treatment with 1 μM corticosterone significantly decreased neuronal viability by 54.29% ($p<0.01$) and 70.46% ($p<0.01$), respectively, whereas 12 h treatment had no effect, indicating a time dependent neurotoxicity of corticosterone on cultured hippocampal neurons. According to these results, 24-h treatment with 1 μM corticosterone was selected for further experiments in the present study.

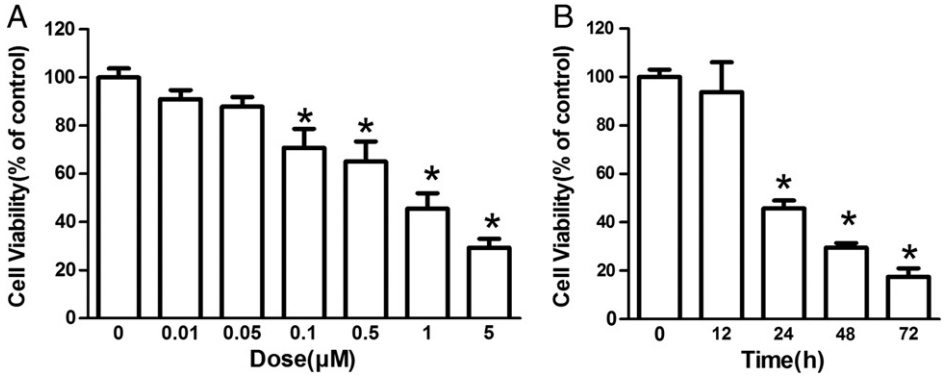


Fig. 1 – Corticosterone induced reduced viability of primary cultured neuronal cells in time- and dose-dependent manners as measured using the MTT assay. The ordinate represents percentage of cell survival compared with control as measured by the MTT assay. Data are presented as mean±S.D. *P<0.01 vs. control group.

2.2. *Neuroprotective effects of icariin on corticosterone-induced cytotoxicity*

To investigate the possible neuroprotective effects of icariin on corticosterone-induced neuronal damage, hippocampal neurons were pretreated with icariin at 0.1 μM, 1 μM, and 10 μM for 2 h, followed by the challenge with corticosterone (1 μM) for 24 h. As shown in Fig. 2A, viability of the culture exposed to corticosterone (1 μM) for 24 h was reduced compared with vehicle-treated control. Pretreatment with icariin significantly reduced corticosterone-induced cell death in a dose-dependent manner, with a maximal effect obtained at 1 μM. 0.1 μM, 1 μM, and 10 μM of icariin had no cytotoxicity, except at 100 μM, compared with untreated control hippocampal neurons (data not shown). Lactate dehydrogenase (LDH), an enzyme that catalyzes the conversion of lactate to pyruvate which is an important step in energy production in cell, is released from the cleaved cell membrane following insult. As shown in Fig. 2B, exposure of hippocampal neurons to 1 μM corticosterone for 24 h caused a neuronal injury, as indexed by a significant increase of LDH release, whereas pretreatment with 10 μM and 1 μM icariin caused a dramatic decrease in LDH release in a dose-dependent manner. Using LDH assay, icariin at the concentrations of up to 10 μM was tested alone for possible intrinsic cytotoxicity activity and showed no significant differences in cell survival compared with untreated control cells.

2.3. *Neuronal morphological changes*

Cell morphological changes were assessed by inverted microscope (Leica DFIL) (Fig. 4E). Cultured hippocampal cells were pretreated with or without 1 μM icariin for 2 h, followed by a challenge with corticosterone (1 μM) for 24 h. As shown in Fig. 3A, hippocampal cells treated with vehicle (control) or icariin (1 μM) alone exhibited large, vacuole-free cell bodies

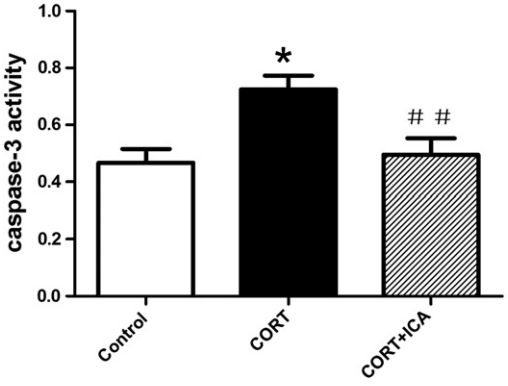


Fig. 3 – Icariin inhibits corticosterone-induced activation of caspase-3 in cultured hippocampal neurons. Data are expressed as the absorbance from each sample mean ±± S.D. of six measurements. *P<0.01 vs. vehicle control, ##P<0.05 vs. model. ICA,icariin. CORT, corticosterone.

with elaborate networks of neurites. Exposure to 1 μM corticosterone for 24 h resulted in obvious cell loss, with the disappearance of neurites, appearance of disrupted membranes and shrinkage of cell bodies. This neuronal loss was prevented by the addition of 1 μM icariin to the cultures. By quantifying neurite outgrowth (Fig. 4F), results indicated that there were no significant differences ($P>0.05$) between neurites from the vehicle-control group and 1 μM icariin single treatment group without corticosterone challenge. Corticosterone treatment induced a significant neurite loss or cleavage. Compared to the model group, proportions of long neurites in good health were significantly increased following treatment of 1 μM icariin, indicating the protective effects of icariin on neurites. Icariin alone did not markedly affect the morphology of hippocampal neurons compared with those exposed to corticosterone.

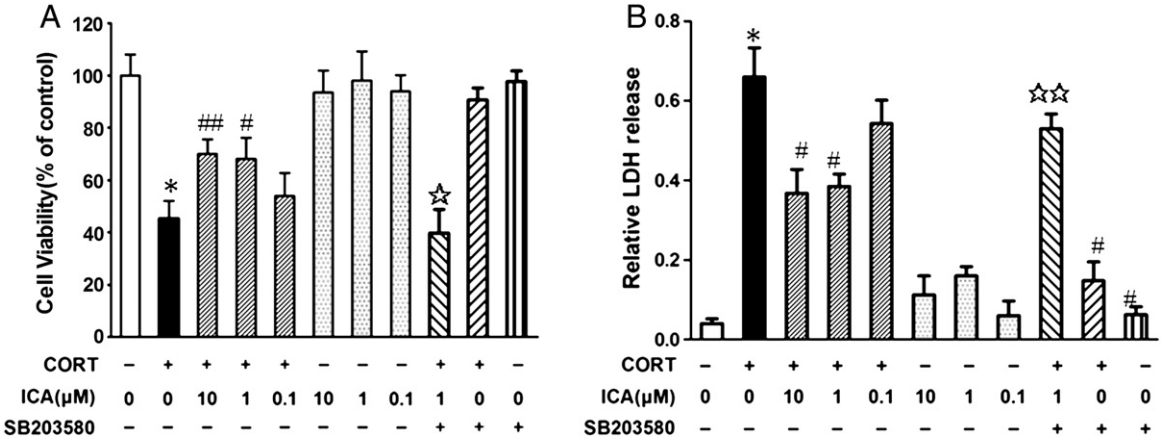
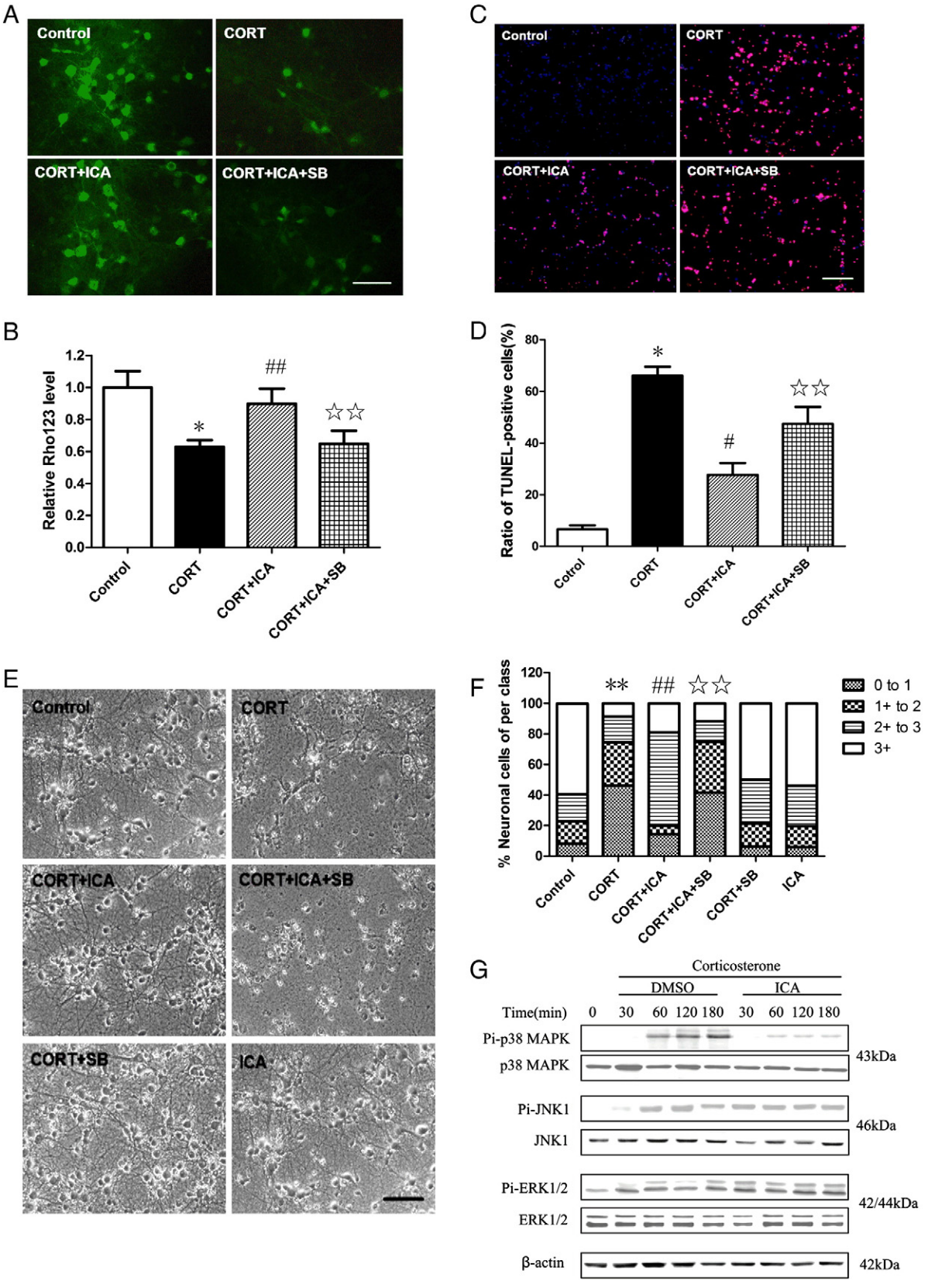


Fig. 2 – Icariin protects primary cultured neuronal cells from corticosterone-induced neurotoxicity. Eight-day-old primary hippocampal neurons were treated with icariin for 2 h prior to a 24-h exposure to 1 μM corticosterone, after which, (A) cell viability and (B) LDH release were measured. Data represent means ± S.D. of three independent experiments, each performed in triplicate. Statistical significance using a t-test was set as *P<0.01 vs. vehicle control, ##P<0.05, #P<0.01 vs. model, ☆☆P<0.05, ☆P<0.01 vs. icariin group. ICA, icariin.CORT, corticosterone.

2.4. *Icariin attenuates apoptosis induced by corticosterone*

We investigated the effect of icariin on corticosterone-induced apoptosis in cultured hippocampal neurons using DNA strand breakage by deoxyuridine triphosphate (dUTP) nick-end-labeling assay (TUNEL) staining. As shown in Fig. 4C, sparse numbers of TUNEL labeled cells that exhibited inter-nucleosomal DNA fragmentation were found in normal hippocampal cultures. In



contrast, a large number of TUNEL-positive cells (Red) were observed in corticosterone-treated cultures. Icariin pretreatment for 2 h before and during the 24 h corticosterone incubation, TUNEL-labeled cells were markedly reduced. Quantification of TUNEL staining (Fig. 4D) indicated that corticosterone treatment yielded 66.06%±7.83% of TUNEL-positive cells relative to total neurons, higher than the 6.59%±3.54% observed in the normal untreated group. Moreover, when compared with corticosterone exposure alone, pretreatment of cells with icariin significantly decreased the ratio of TUNEL-positive cells to 27.73%±10.08%.

2.5. *Icariin improves impaired mitochondrial function*

The mitochondrial fluorescent probe Rhodamine 123 was used to evaluate mitochondrial transmembrane potential (Emaus et al., 1986). The mitochondria are known to be involved in apoptosis. Permeability changes can lead to caspase-dependent cytotoxicity and downstream apoptotic signaling, while a loss of mitochondrial transmembrane potential, denoted as mitochondrial dysfunction, leads to cytochrome *c* release from the mitochondria and triggers other apoptotic factors. Rhodamine 123 distributes across the mitochondrial membrane with respect to transmembrane potential. As shown in Fig. 4A, Rhodamine 123 was strongly accumulated in untreated neuronal cells. Weak Rhodamine 123 accumulation was observed in corticosterone-treated neurons, while icariin inhibited these changes. 1 μM corticosterone treatment for 24 h induced Rhodamine 123 fluorescence intensities up to 0.63-fold compared with normal levels (Fig. 4B). When compared with the corticosterone injury group, 1 μM icariin pre-treatment for 2 h before and during the 24 h corticosterone incubation enhanced Rhodamine 123 fluorescence intensity to 0.90-fold of control levels, similar to the control group. This suggests that icariin treatment can completely prevent neuronal mitochondrial dysfunction during corticosterone-induced neuronal injury.

2.6. *Icariin inhibits caspase-3 activation*

We examined caspase-3 activity as a specific apoptotic marker in hipppcampal neuronal cultures exposed to corticosterone alone or in combination with icariin. As shown in Fig. 3, there was a significant effect of corticosterone treatment on caspase-3 activity. Results revealed that 1 μM corticosterone increased caspase-3 activity compared to control. In cultured hippocampal neurons exposed to 1 μM corticosterone plus 1 μM icariin, caspase-3 activity was similar to that of control.

2.7. *Icariin suppresses p38 MAPK, but not JNK1 and ERK1/2 phosphorylation*

Activation of the p38 MAPK and JNK1 signaling cascades is considered to play a crucial role as the regulator of apoptotic signaling pathways (Gomez-Lazaro et al., 2008; Luo et al., 1998), therefore we attempted to determine whether icariin suppresses p38 MAPK and/or JNK1 activation, as well as that of another member of the MAPK family, ERK1/2. Neurons were pretreated with icariin or control vehicle for 30 min followed by stimulation with corticosterone for 30, 60, 120 or 180 min. The cytosolic proteins obtained were subjected to western blot analysis using specific antibodies. As shown in Fig. 4G, corticosterone induced both p38MAPK and ERK1/2 activation within 60 min and JNK1 within 30 min, whereas icariin blocked p38 MAPK, but not JNK1 or ERK1/2, activation. The expression levels of the inactive forms of each protein kinase remained constant.

2.8. *SB203580 blocks the neuroprotective effects of icariin*

To determine whether p38MAPK is involved in the neuroprotective effects of icariin, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole (SB203580), a p38MAPK inhibitor, was used. Pre-treatment with 10 μM SB203580 significantly blocked the neuroprotective effects of icariin against corticosterone-induced cytotoxicity. These effects were confirmed using cell viability (Fig. 2), mitochondrial membrane potential loss (Figs. 4A, B), cell apoptosis detected by TUNEL fluorescence staining (Figs. 4C, D) and cell morphological changes (Figs. 4E, F). SB203580 at the concentrations of 10 μM was tested alone for possible intrinsic cytotoxicity activity and showed no significant differences in cell survival compared with untreated control cells (Fig. 2). Results suggest that 10 μM SB203580 significantly inhibited the neuroprotective effects of icariin in cellular and molecular levels.

3. Discussion

The present study evaluated icariin, an active component purified from the Chinese herbal plant *Epimedium*, as a protective agent against corticosterone-induced neuronal

Fig. 4 – Icariin attenuates apoptosis induced by corticosterone through blockade of p38 MAPK, but not JNK1 or ERK1/2, phosphorylation in cultured hippocampal neurons. (A) Mitochondrial function was determined using Rho123 fluorescence intensity. Scale bar = 1 mm for all images. (B) Rho123 fluorescence intensity were quantitatively analyzed. Data are expressed as fold over control group in five to six coverslips from separate cultures. (C) Morphological apoptosis was determined using TUNEL staining. TUNEL-negative (non-apoptotic) cells appear blue, while TUNEL-positive (apoptotic) cells appear red. Scale bar = 1 mm for all images. (D) The percentage of TUNEL-positive cells in total cultured hippocampal neurons. (E) Photomicroscopic pictures of morphology. Scale bar = 50 μM. (F) Histograms of neurite measurements. Neurite preference was analyzed from five images per condition. Results were statistically analyzed using a non-parametric ANOVA method with post hoc comparisons using a Dunnett's test. (G) Neurons were incubated with icarrin (1 μM) or vehicle (0.5% DMSO,v/v) for 30 min and stimulated with corticosterone (1 μM) for the indicated times. The intensity of bands for p38MAPK, JNK1, or ERK1/2 phosphorylation were analyzed using western blot. The experiments were repeated three times independently, with one representative result shown. Statistical significance was set as $^{**}P<0.05$ vs. vehicle control, $^{*}P<0.01$ vs. vehicle control, $^{\#\#}P<0.05$ vs. model, $^{☆☆}P<0.05$ vs. icariin group. ICA, icariin; SB,SB203580; CORT, corticosterone.

cell apoptosis. Icariin attenuated neuronal apoptosis, increased cell viability, improved mitochondrial membrane potential and inhibited activation of caspase-3. More importantly, we found that the protective effects of icariin on apoptosis in hippocampal neuronal cells could be attributed to blockade of p38 MAPK activation.

Glucocorticoids are adrenal steroid hormones secreted during stress and are necessary for survival. Meanwhile, corticosteroids are the most potent and effective therapies for many chronic inflammatory and immune diseases, including asthma, RA, IBD, and SLE. However, sustained elevations of endogenous glucocorticoids (as observed in depression, Cushing's disease or human aging) and prolonged glucocorticoid therapy are associated with numerous deleterious effects, including neuronal survival (Coluccia et al., 2008). The actions of glucocorticoid on target tissues are mediated by interactions with the glucocorticoid receptor (GR) or mineralocorticoid receptor (MR). The hippocampus is a primary target in the brain, which abundantly expresses both types of receptors (Reagan and McEwen, 1997). Considerable clinical evidence suggests that patients receiving chronic corticosteroid therapy have a smaller hippocampal volume and declarative memory deficits compared with controls (Brunner et al., 2005; Brown et al., 2004).

The precise cellular mechanisms underlying corticosteroid-induced neuronal damage have not been fully elucidated. Increasing evidence suggests that corticosterone-induced apoptosis plays a key role in the induction of hippocampal neuronal damage *in vivo* and *in vitro* (Reagan and McEwen, 1997; Crochemore et al., 2005; Lu et al., 2003; Woolley et al., 1990). Mitochondria produce energy through the Krebs tricarboxylic-acid cycle and oxidative phosphorylation cycles. They also play a pivotal role in apoptosis signaling. Permeability changes lead to caspase-dependent cytotoxicity and downstream apoptotic signaling (Desagher and Martinou, 2000; Huttemann et al., 2007). In the present study, we examined primary cultured rat hippocampal neurons as a cellular model to understand the molecular basis of corticosterone-induced neuronal apoptosis. Firstly, we observed morphological changes in hippocampal neuronal cells following corticosterone treatment, where the cells displayed increased TUNEL-positive cell numbers and apparent morphological features typical of apoptosis, including obvious cell loss, neurite injury, destructive neuronal networks and cell body shrinkage. Furthermore, in view of the critical role of mitochondrial function in regulating neuronal viability/resilience, we also measured mitochondrial membrane potential using Rho123 staining in cultured hippocampal neurons following corticosterone treatment. We found that mitochondrial membrane potential was significantly decreased following corticosterone treatment. Moreover, caspase-3 is a critical downstream protease in the apoptotic cascade, and we examined caspase-3 activity as a specific apoptotic marker in hipppocampal neuronal cultures exposed to corticosterone treatment. Consistent with previous reports (Zhu et al., 2006), our data suggest that exposure of corticosterone increased caspase-3 activity. In the present study, we confirm that apoptosis plays a primary role in the induction of hippocampal neuronal damage.

It was previously reported that elevated corticosterone protects against neurotoxic injury, however high concentrations of corticosterone increased the vulnerability of cultured cells to neurotoxic insults (Du et al., 2009). Cortisol concentrations in normal human serum vary from 137 nM to 283 nM (Davis et al., 1981), pathophysiological states have been associated with elevated levels in the 420–779 nM range (Pirich and Vierhapper, 1988). Therefore, we chose various concentrations (10 nM, 50 nM, 100 nM, 500 nM, 1 μM and 5 μM) of corticosterone to screen for proper therapeutic concentrations of corticosterone. In our study, relatively high concentrations (1 μM) of corticosterone were used. However, this result appears to disagree with previous reports that this concentration of corticosterone does not cause cell damage (Roy and Sapolsky, 2003). One possibility for this disparity could be the different experimental conditions. For example, the previous study used growth media which facilitate the growth of glia and produced hippocampal cultures with a mixture of 30–40% neurons and largely GFAP positive glia (Roy and Sapolsky, 2003). The present study used neurobasal medium with a supplement of B-27, which produced an almost pure neuronal culture with less than 1% glia.

Given that corticosterone-induced neuronal death in the hippocampus has been implicated in depression, aging and prolonged glucocorticoid therapy due to many chronic inflammatory and immune diseases, this could have significance in the clinic if we could find a molecular agent which has the ability to protect the hippocampal neurons from corticosterone-induced neuronal damage. There is a growing interest in the treatment of corticosterone-induced neurotoxicity with plant-based therapies including traditional Chinese medicine (TCM), which extensive experience has been documented over thousands of years (Harvey, 1999). Icariin is a major constituent of flavonoids from the Chinese medical herb *Epimedium brevicornum* Maxim. Much of the current work on the neuroprotective effects of icariin suggest that icariin can attenuate amyloid β-induced neurotoxicity involved in Alzheimer's disease (Li et al., 2010; Urano and Tohda, 2010; Zeng et al., 2010). Our previous study reported that icariin and its derivative, ICT, can attenuate LPS-induced acute inflammatory responses *in vitro* and *in vivo* which involve activation of the PI3K/Akt pathway and inhibition of NF-κB (Wu et al., 2010; Xu et al., 2010). Perhaps the most significant finding in the present study was that both increased TUNEL-positive cell numbers and increased caspase-3 activity produced by exposure to corticosterone were blocked by the addition of icariin into the medium, suggesting that icariin may exert a neuroprotective effect through blockade of apoptosis.

The signaling pathways that mediate the neuroprotective actions of icariin against corticosterone damage remain elusive. MAPKs control many cellular events, including differentiation, proliferation, and apoptosis and until now at least three major MAPK subfamilies, ERKs, JNKs, and p38 MAPKs, have been described. The JNK1/2 and p38 MAPK pathways are preferentially activated by inflammatory cytokines and extracellular stress including UV light, heat, and ROS and are known as potent effectors of apoptosis (Lewis et al., 1998). The molecular mechanisms by which icariin suppresses corticosterone-induced apoptosis are not fully understood. The current study investigated whether MAPKs are involved in the protective effects of icarrin on corticosterone-induced apoptosis. The results obtained using western

blot and pharmacological blockade (Fig. 4) suggest that icariin protected against corticosterone-induced apoptosis via inactivation of the p38 MAPK pathway but not by ERK1/2 and JNK1/2. Inhibition of p38 MAPK using the inhibitor SB203580 blocked phosphorylation of p38 MAPK in response to corticosterone, as well as prevented the neuroprotective effects of icariin. These effects could be confirmed using cell viability (Fig. 2), cell morphological changes (Figs. 4E, F), mitochondrial membrane potential loss (Figs. 4A, B) and cell apoptosis determined using TUNEL fluorescence staining (Figs. 4C, D). These results indicate that p38 MAPK plays a key role in mediating the neuroprotective effects of icariin. In support of these findings, p38 MAPK has been reported as necessary for typical apoptosis in corticosterone-stimulated primary cultured rat hippocampal neurons. To our knowledge, we report for the first time that the protective effects of icariin against corticosterone-induced cytotoxicity in primary cultured rat hippocampal neurons are mediated through MAPK signaling pathway.

In conclusion, the present results suggest that icariin markedly suppresses corticosterone-induced apoptosis in primary cultured rat hippocampal neurons, presumably through blockade of p38MAPK activation and mitochondrial dysfunction including improving mitochondrial membrane potential and inhibiting caspase-3 activation. More attention should be paid to further clarify the mechanisms of icariin, which may help to elucidate potential applications for this potent neuroprotective candidate. The possibility that neuroprotective agents may be useful in countering the deleterious effects of excessive glucocorticoid secretion or prolonged glucocorticoid therapy is an exciting prospect for future investigation.

4. Experimental procedures

4.1. *Primary cultures of hippocampal neurons*

Newborn Sprague–Dawley (SD) rats, less than 24 h old, were purchased from the Shanghai Institute of the Chinese Academy of Science. All experimental procedures were performed in accordance with Fudan University experimental standards as well as international guidelines on the ethical treatment of experimental animals. Primary hippocampal neuronal cultures were prepared as described previously (Banker and Cowan, 1977) with some modifications. In brief, hippocampi were dissected from neonatal SD rats, stripped of meninges and blood vessels and minced in Hanks' balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+}. The tissue was dissociated using 0.125% trypsin (solarbio, USA) digestion for 15 min at 37 °C and gentle titration through a series of fire-polished constricted pasteur pipettes. The cells were then plated on poly-L-lysine (molecular weight 30,000–70,000, 0.1 mg/ml; Sigma, St. Louis, MO, USA) coated-glass coverslips, 96-well plates, or 100 mm dishes at a density of 1×10^6 cell/ml and maintained at 37 °C in a humidified 5% CO_2 incubator. Neurons were cultured in Neurobasal-A medium (Gibco BRL, Grand Island, NY) and supplemented with 2% B27 supplement (Gibco BRL, Grand Island, NY), 10 μl/ml Penicillin–streptomycin, 2 mM glutamine (solarbio, USA), 5 ng/ml bovine fibroblast growth factor 2 (FGF2) (R&D Systems, Minneapolis, MN, USA) and 1% FBS. After a 24-hour culture period, cultures grown in serum-free Neurobasal medium yield approximately more than 80% neurons, as estimated using immunocytochemical staining with antibodies against neurofilament proteins (data not shown). The culture neurons were used for *in vitro* studies at day 8 (DIV 8).

4.2. *Drug exposures*

Icariin (purity > 99%) was purchased from Shanghai Ronghe Bio-Pharmaceutical company. The extract was dissolved in culture-grade DMSO (Sigma, St. Louis, MO, USA) (final concentration <0.1%) in serum-free media. Corticosterone (Sigma, St. Louis, MO, USA) was dissolved initially in ethanol as a stock solution and then in culture media (final concentration of ethanol was 0.1%). Eight-day primary hippocampal neurons were used in all experiments in this study for drug exposure. The neuronal cultures were washed twice with Mg^{2+}-free, HEPES-buffered saline (HBS, 146 mM NaCl, 10 mM HEPES, 2 mM CaCl2, 5 mM KCl, 10 mM D-glucose, pH 7.4) and pre-treated with different concentrations of icariin without or with 10 μM SB203580 (Sigma, St. Louis, MO, USA) for 2 h at 37 °C, then exposed to corticosterone for 24 h. Control condition was treated with the appropriate amount of vehicle, DMSO, at a final concentration of 0.1%, which had no effect on cell viability. Following drug exposure, cell viability assessment and lactate dehydrogenase (LDH) release were performed.

4.3. *Cell viability assays*

After exposure to various concentrations of corticosterone, icariin and/or SB203580, the viability of cells was determined using the MTT assay system (Beyotime Institute of Biotechnology, Shanghai, China). In brief, the cells were plated on 96-well culture plates at a density of 1×10^6 cell/ml. After the specific timeframe, we added 10 μl MTT reagent to each well and then cultured the cells for 4 h. Afterwards, 100 μl formazan solution was added to each well. The 96-well culture plate was agitated for 10 min on a shaker. Finally, the OD value of each well was detected at 490 nm using a MK3ELISA Reader (Thermo fisher scientific, USA).Cell viability was calculated as follows: treated group OD/control group OD × 100%.

4.4. *Cell injury assay*

LDH, the most widely used cytotoxicity marker, was determined using a LDH cell proliferation and toxicity of Quantitative Detection Kit (GENMED Scientifics Inc. USA). In brief, at various time points, media (50 μl) were transferred from the culture wells to 96-well plates and mixed with 50 μl reaction solution provided by the kit. The mixtures were incubated at room temperature in the dark for 30 min, and then 50 μl of stopping solution was added to each well. After 30 min, the absorbance was read at 492 nm using a microplate reader. Maximal LDH release was obtained in each well at the end of each experiment following repeated freezing and thawing. Each experimental condition was repeated in triplicate, with each experiment containing eight readings. Results are expressed as a percentage of maximal LDH release, after the subtraction of background levels determined from medium alone.

4.5. *Cell morphological evaluation and neurite measurement*

To evaluate morphological changes in neuronal cells, cultures were observed using an Leica DFIL inverted microscope with a phase-contrast optic lens. Images were captured using Leica QWin plus 3 Image Processing Software (Media Cybernetics, Silver Spring, MD, USA) through a Leica DFC300 FX camera device. We analyzed neurite preference from five images per condition. The neurite lengths were sequentially and manually assessed as an index. Neurite length was divided into four types (0–3+) by comparison with cell body (0, no neurites or neurites shorter than diameters of cell bodies, 1+, occasional short neurites with lengths equivalent to or more than the diameters of the cell bodies, 2+ or 3+, increasing neurite length equivalent to or more than twice or triple the diameters of the cell bodies)(Wang et al., 2007). Results were statistically analyzed using a non-parametric ANOVA with post hoc comparisons using Dunn's test. Data are presented as means ± S.D. of at least three independent experiments.

4.6. *Apoptosis detection using TUNEL staining*

To confirm the presence of cell death by an apoptotic-like mechanism following exposure to corticosterone and icariin, we performed *in situ* labeling of TUNEL-positive nuclei using the *In Situ Cell Death Detection Kit, TMR red* (Roche,USA) according to the manufacturer's protocol. Red apoptotic cells were visualized on a microscope, counted (6 fields per sample), and photographed using a digital fluoresence camera, and counterstained with DAPI to visualize the nucleus. In brief, culture media in the wells was removed after treatments, and the cells were rinsed three times with PBS followed by addition of freshly prepared 4% paraformaldehyde in PBS for 20 min at room tempreture and 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice , after which the cells were incubated with 50 μl of TUNEL reaction mixture, incubated for 60 min in a humidified atmosphere for 60 min at 37 °C in the dark, and rinsed three times with PBS followed by fluorescent counterstaining with DAPI. Negative controls were performed by substituting distilled water for TdT in the working solution. Inverted fluorescence microscopy was used to observe TUNEL staining. The number of TUNEL-positive (apoptotic) cells, which appeared red, and the total number of cells, appearing as a mixture of red and blue, were determined in eight randomly chosen microscopic fields, each at 200 × magnification. Data are expressed as the ratio of apoptotic neurons to total neurons.

4.7. *Caspase-3 activity detection*

Following exposure to various concentrations of corticosterone and icariin, the effects of icariin against corticosterone-induced neuronal apoptosis were determined using a Chemicon caspase colorimetric activity assay kit. The assay is based on spectophotometric detection of the chromophore *p*-nitroaniline (pNA) after cleavage from the labeled substrate LEHD-pNA. In brief, cells treated were resuspended in 50 μl of cell lysis buffer and incubated on ice for 30 min. After centrifugation for 5 min at 10 000 × *g*, the supernatant was transferred to a fresh tube followed by addition of Reaction Buffer and Caspase-3 Substrate for 4 h at 37 °C in the dark. The free pNA can be quantified using a microtiter plate reader at 405 nm. The fold-increase in caspase 3 activity was determined by comparing the absorbance from each sample with untreated neurons.

4.8. *Determination of mitochondrial membrane potential*

Mitochondrial membrane potential (MMP) was monitored using the fluorescent cationic dye Rhodamine 123 as previously described (Zamzami et al., 2001) with minor modifications. Briefly, following various treatments, hippocampal cell cultures grown on 96-well plates were loaded with Rhodamine 123 (10 μM) (Beyotime Institute of Biotechnology, Shanghai, China) at 37 °C in the dark for 15 min and then washed in triplicate with PBS. Cellular fluorescence intensity of Rhodamine 123 was quantified using a fluorescence microplate reader (TECAN Infinite 200 microplate reader, Tecan Trading AG, Switzerland) with excitation at 485 nm and emission at 530 nm. Background fluorescence signals of Rhodamine 123 were determined without cells and subtracted from those obtained in hippocampal neurons. Cellular MMP was expressed as fold over control levels. Each group had eight samples, and each experiment was repeated in triplicate.

4.9. *Protein expression detection by Western blot analysis*

Following treatment, cells in each of the six-well plates were rinsed twice with cold PBS, followed by the addition of cell lysis buffer containing 150 mM NaCl, 50 mM Tris–HCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 1% SDS with proteinase inhibitor cocktail (Sigma, St. Louis, MO, USA) on ice for 15 min, and then centrifuged for 20 min at 12,000 g. Supernatant was collected and the protein concentration was measured using the Bradford method. Fifty milligrams of total protein were dissolved in sample buffer and boiled for 5 min prior to loading onto polyacrylamide gels. Proteins were then transferred to poly (vinylidine difluoride) filter membranes, and blocked with 5% non-fat dry milk in Tris-buffered saline/0.05% Tween 20. The membrane was incubated with a monoclonal antibody against (phospho-ERK1/2 (Thr202/Tyr204), ERK, phospho-p38MAPK, p38 MAPK, phospho-JNK1 (Thr183), JNK1, (Santa Cruz Biotechnology Inc, USA), followed by incubation with horseradish peroxidase-conjugated(HRP) secondary antibodies and visualized using an enhanced chemiluminescence ECL kit.

4.10. *Statistical analysis*

All data are expressed as mean ± S.D. Differences between groups were examined for statistical significance using a one-way ANOVA with Dunnett's test as a post hoc analysis. A *P* value less than 0.05 denoted statistical significance.

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