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Involvement of NF-κB and AP-1 activation in icariin promoted cardiac differentiation of mouse embryonic stem cells

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ABSTRACT

Icariin has been reported to facilitate the differentiation of mouse embryonic stem (ES) cells into cardiomyocytes; however, the mechanism on cardiomyogenic cell lineage differentiation has not been fully elucidated yet. In the present studies, an underlying signaling network including p38, extracellular signal-regulated kinase 1, 2 (ERK1, 2), nuclear factor-κB (NF-κB), activator protein-1 (AP-1) transcription factors c-*jun* and c-*fos* was assumed in icariin induced cardiomyogenesis. Icariin rapidly activated p38 and ERK1, 2 in embryoid bodies, treatment with p38 antagonist 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole (SB203580) or ERK1, 2 inhibitor 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene (U0126) significantly abolished icariin induced cardiac commitment, MEF2C gene expression and nuclear translocation, as well as cardiac-specific protein α-actinin expression, indicating that p38 and ERK1, 2 are specifically involved in icariin stimulated cardiomyogenic cell lineage differentiation of ES cells. Further, IκBα phosphorylation and NF-κB p65 translocation to the nucleus appeared rapidly when embryoid bodies exposed to icariin, and the expression of IκBα or NF-κB p65 in cytoplasm was decreased concomitantly. Moreover, icariin increased c-*jun* and c-*fos* mRNA and protein expression. Either SB203580 or U0126 displayed inhibitory effect on icariin induced NF-κB and AP-1 activation. It could be concluded that p38 and ERK1, 2 are activated in a coordinated manner, which in turn contribute to NF-κB and AP-1 activation in icariin induced cardiomyogenic cell lineage differentiation of mouse ES cells.

1. Introduction

Embryonic stem (ES) cells are promising source in cardiomyocytes therapeutic potential. However, obstacles such as control of stem cell fate, allergenic rejection and limited cell availability should be overcome before their therapeutic potential can be performed (Bettiol et al., 2006a,b; Capi and Gepstein, 2006; Goh et al., 2005), thus understanding of signaling pathways that affect stem cell fate and differentiation was extremely required. Recently, cell-based phenotypic and pathway-specific screens of natural products and synthetic compounds have made some achievement, and small molecules used to selectively control cardiac differentiation of ES cells have been attracting great attention (Sato et al., 2006; Takahashi et al., 2003; Wu et al., 2004). Such molecules will likely provide new insights into molecular mechanism of cardiac differentiation, and may ultimately contribute to therapeutic use of ES cells in cardiomyocytes therapeutic potential.

In former work, we have confirmed that icariin at a concentration of 100 nM facilitated cardiomyogenesis via regulation of cell cycle, as well as induction of apoptosis during the differentiation of mouse ES cells into cardiomyocytes *in vitro*, indicating that icariin influence cardiac differentiation partly by inducing apoptosis in cells not committed to cardiac differentiation (Zhu et al., 2005). However, the underlying mechanism involved in cardiomyogenic cell lineage differentiation promoted by icariin has not been fully elucidated yet.

The mitogen-activated protein kinase (MAPK) pathways are major signaling systems by which cells transduce extracellular signals into intracellular responses (Chang and Karin, 2001). At present, three conserved MAPKs have been identified in detail: extracellular signal-regulated kinases (ERK), stress-activated c-*jun* NH2-terminal kinases (JNK), and p38 kinases. The biological effects of MAPKs are mediated by downstream phosphorylation substrates, which in the nucleus are often transcription factors. Nuclear factor-kappaB (NF-κB) and activator protein-1 (AP-1) have been previously reported to function as key regulators of cardiac gene expression programs downstream of multiple signal transduction cascades in a variety of physiological and pathophysiological states (Jones et al., 2003; Jochum et al., 2001). NF-κB is a heterodimer comprising p50 and p65 subunits. It is sequestered in the cytoplasm by association with an inhibitory protein named IκB. Phosphorylation targets IκB for protein ubiquitination and subsequent degradation through a proteasome-dependent pathway, resulting in dissociation of NF-κB:I-κB complex followed by

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translocation of the released NF-κB into nucleus (Van Waes, 2007). AP-1 is a heterodimer of Fos and Jun oncoproteins. Typical AP-1 is composed of two subunits c-Jun and c-Fos, which constitute leucine zipper (Herbein et al., 2006).

Herein, the signaling networks of MAPKs, NF-κB and AP-1 in cardiomyogenic cell lineage differentiation of ES cells induced by icariin were investigated.

2. Materials and methods

2.1. ES cell culture and differentiation

ES-D3 cell lines (American Type Culture Collection, CRL-1934) were cultured in an undifferentiated state on primary cultures of mouse embryonic fibroblasts in Dulbecco's modified Eagle's minimal essential medium (DMEM; Gibco) supplemented with 10% fetal calf serum (Hyclone), 0.1 mM β-mercaptoethanol (Sigma), non-essential amino acids (Hyclone) and 10^6 units/l recombinant mouse leukemia inhibitory factor (Chemicon). Cultures of differentiating ES cells were established by formation of embryoid bodies in hanging drop cultures (Zhu and Lou, 2006; Zhu et al., 2005) with differentiation medium. On day 0, 30 μl of drops containing approximately 600 ES cells were placed on the lids of Petri dishes filled with D-Hanks' solution, and cultivated in hanging drops for 3 d followed by another 2 d in the Petri dishes. On day 5, embryoid bodies were plated separately onto gelatin-coated 24-well culture plates, and at this time, 100 nM icariin (Zhu and Lou, 2005) (Drug Biology Product Examination Bureau, Beijing, China, batch no 0737-200011, purity 99%), 1 μM p38 antagonist 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole (SB203580, Sigma) or 10 μM ERK1, 2 inhibitor 1,4-diamino-2,3-dicyano-1,4-bis [2-aminophenylthio] butadiene (U0126, Sigma) were added. Fresh medium containing inhibitors were changed every 24 h. In the experiment, day 5+*x* referred to as *x* day after embryoid bodies were plated onto gelatin-coated culture plates. Rhythmically beating embryoid bodies were considered to be spontaneously beating cardiomyocytes in embryoid body outgrowths, and were defined as the phenotypic landmark of successful differentiation (Fig. 1).

2.2. Western blot analysis

Whole cell lysates was prepared as described previously (Zhu et al., 2005). In brief, cells were washed with PBS, collected in RIPA buffer (containing 150 mM Sodium Chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 20 mM EGTA, 50 mM Tris (hydroxymethyl) aminomethane hydrochloride, 1 mM dithiothreitol, 20 mM sodium fluoride, 1 mM sodium orthovanadate, 0.1 mg/ml phenylmethanesulfonyl fluoride, 10 μg/ml leupeptin and 2.0 μg/ml aprotinin) and lysed for 30 min on ice. Cytoplasmic and nuclear extracts were prepared according to the manufacturer's instructions (Applygen Technologies Inc, China). After the cell samples were lysed, the protein concentration was estimated by Dc protein assay kit (Bio-Rad, USA). Proteins were loaded onto 10% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes (Pall Corporation, NY, US). The sheets were analyzed with antibodies according to the suppliers' protocol (antibodies to p-ERK, p-JNK, p-IκBα, IκBα, c-*fos* and c-*jun* were from Santa Cruz Biotechnology, antibodies to ERK, p-p38, p38, JNK, NF-κB p65 were from Cell Signaling Technology and antibodies to GAPDH were from KangChen Bio-tech) and visualized autoradiographically with an enhanced chemiluminescent substrate (ECL, Pierce, USA), and scanned using a bio-imaging analyzer (Bio-Rad, USA). The density of the products was quantitated using Quantity One version 4.2.2 software (Bio-Rad, USA).

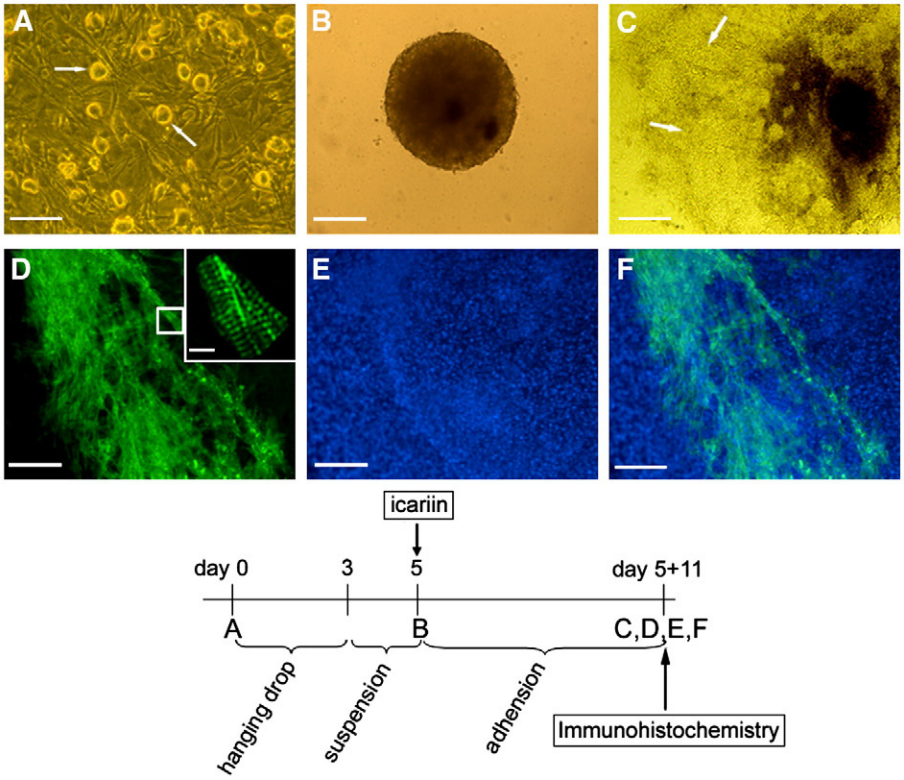

Fig. 1. Cardiac differentiation of mouse ES cells *in vitro*. (A) Colonies of ES cells on a feeder layer of mouse embryonic fibroblasts (arrows: colonies of ES cells). (B) Embryoid bodies before plating onto gelatin-coated 24-well culture plates. (C) Synchronously contracting, functional syncytium of cardiac clusters derived from embryoid bodies (arrows: contraction foci). (D) Embryoid bodies was stained for anti-α-actinin antibody (insert: higher magnification of rectangular frames, bar=25 μm). (E) Nuclear blue stain with DAPI (4′, 6-diamino-2-phenylindole). (F) D and E were merged. Note that ES cells have differentiated into α-actinin positive cardiomyocytes. Scale bar=400 μm (A, C), 200 μm (B, D, E, F).

Table 1
Primer sequence information for RT-PCR

Gene	Primer sequence (5'–3')	Annealing temperature (°C)	Product size (bp)	Cycle
MEF2C	Forward: agatacccacaacacaccacgcgcc Reverse: atccttcagagagtcgcatgcgctt	60	197	38
c-*fos*	Forward: gagtgatgccgaagggataa Reverse: gagaagcattccggtcagag	56	150	32
c-*jun*	Forward: aaaaccttgaaagcgcaaaa Reverse: cgcaaccagtcaagttctca	56	220	32
GAPDH	Forward: aactttggcattgtggaagg Reverse: acacattgggggtaggaaca	58	223	25

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR analysis was performed as described previously (Zhu and Lou, 2006; Zhu et al., 2005). In brief, total RNA from the outgrowths of embryoid bodies was extracted using Trizol reagent (Shanghai Sangon Biological Engineering Technological and Service Company) according to the manufacturer's instructions. Reverse transcription was performed on 1 μg RNA. Following reverse transcription, the cDNAs were used for semi-quantitative PCR using sets of specific primers designed by Primer 3.0 software and published sequences (Table 1). The PCR products were separated by 1.5% agarose gel electrophoresis, visualized with ethidium bromide staining, and quantified using a bio-imaging analyzer (Bio-Rad, CA, USA), and the density of the products were quantitated using Quantity One (version 4.2.2) software (Bio-Rad). The intensity of each band was normalized to that of the corresponding band of GAPDH and calculated as the ratio to the value in the control.

2.4. Immunohistochemistry

Immunohistochemistry was performed with whole mount embryoid bodies. As primary antibodies, the mouse monoclonal anti-sarcomeric α-actinin (dilution 1:200; Sigma) and the goat polyclonal anti-MEF2C (dilution 1:200; Santa Cruz) were used. The tissues were fixed in cold acetone for 10 min, and subsequently incubated at 4 °C overnight together with the primary antibodies. FITC-conjugated goat anti-mouse IgG (α-actinin) or FITC-conjugated rabbit anti goat IgG (MEF2C) (dilution 1:100; Santa Cruz) were used as the secondary antibody. For MEF2C, fluorescent signals were obtained with a fluorescence conversion microscope (Leica DMIL, German). For α-actinin, fluorescence recordings were performed by means of a confocal laser scanning setup (Leica TCS SP2, Bensheim, Germany) connected to an inverted microscope. The 488-nm band of the argon ion laser of the confocal setup was used as fluorescence excitation. Emission was recorded using a longpass LP505-nm filter set.

2.5. Flow cytometry (FACS analysis)

Embryoid bodies on day 5+11 were harvested and disrupted with 0.05% trypsin and 0.53 mM EDTA for 3 min at room temperature with frequent shaking, then gently passed through a 21G needle on a 3 ml syringe three times to generate a single cell suspension (Li et al., 2005). The experiments were performed at the condition of cell viability more than 90%. For detection of α-actinin, cells were stained as described above, then suspended in 0.5 ml PBS/1% BSA and assayed in a flow cytometer (FACSCalibur; Becton Dickinson, Heidelberg, Germany). Each plot represents 10,000 viable cells (non-viable cells were excluded from FACS analysis by appropriate gating). Untreated cells and cells lacking primary antibody were used as controls. In addition, isotype controls were used to assess the level of non-specific antibody binding. All data analysis was carried out using CellQuest software (Becton Dickinson). Differentiation was determined by comparing the fluorescence intensity of the treated cells to that of untreated cells obtained from a solvent control plate, and the results were expressed as the percentage of the fluorescence intensity of the control group.

2.6. Statistical analysis

All data are expressed as means ± standard deviation (S.D.). At least three independent experiments were done. Statistical analysis was

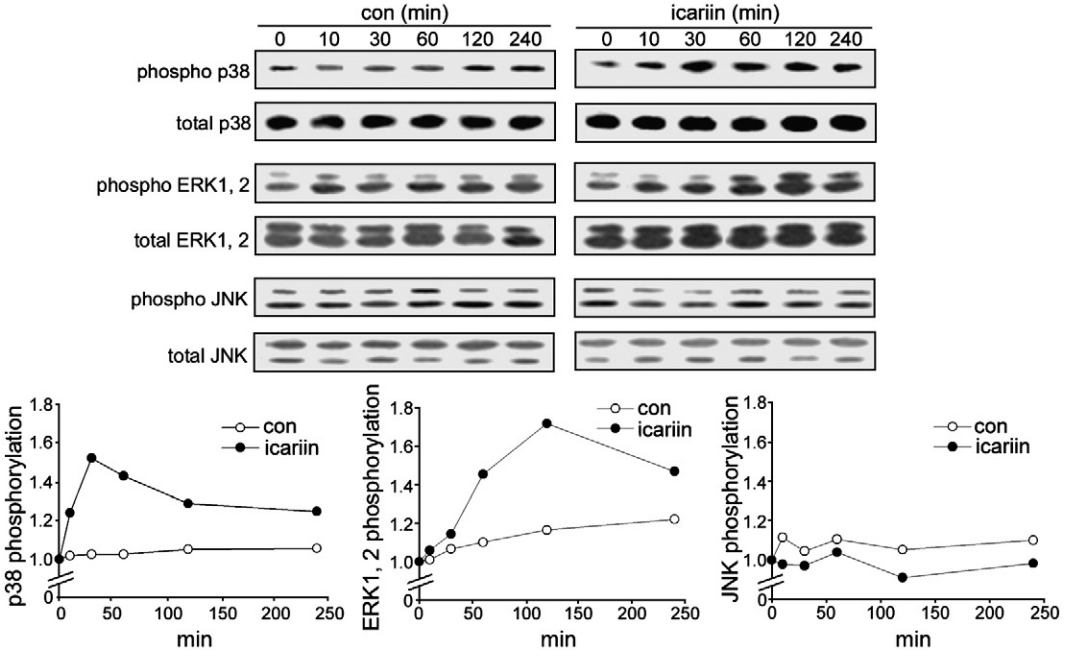

Fig. 2. Involvement of MAPKs in icariin stimulated cardiac differentiation. Embryoid bodies on day 5 were treated with 100 nM icariin from 0 to 240 min. The phosphorylated p38, ERK1, 2, and JNK were evaluated.

performed by one way ANOVA, followed by adjusted *t* tests with *P* values corrected by the Bonferroni method. A value of $P<0.05$ was considered to be significant.

3. Results

3.1. Differentiation of ES cells into cardiomyocytes after icariin treatment

ES cells grew aggregates with clear boundaries and appeared ovoid or nodule shaped on mouse embryonic fibroblast feeder cells (Fig. 1A). The attached culture was established by plating a single, day 5 embryoid bodies (Fig. 1B) onto a 24-well plate and allowing continued cellular proliferation and differentiation, and at this time, 100 nM icariin were added. Within this multicellular arrangement in embryoid body outgrowth, cardiomyocytes appeared as spontaneously contracting, round cell clusters. Each embryoid body contained 1 or more beating areas (Fig. 1C). The synchronously contracting cardiomyocytes were positive for the anti-α-actinin antibody (Fig. 1D, E, F).

3.2. Involvement of MAPKs in icariin stimulated cardiomyogenic cell lineage differentiation

Icariin induced rapid and sustained activation of p38 in embryoid bodies on day 5, which peaked at 30 min and remain activation until 240 min (Fig. 2). The maximum level of phosphorylated ERK1, 2 appeared at 120 min after icariin administration (Fig. 2).

The number of beating embryoid bodies induced by icariin was significantly decreased to approximately 54% or 48% respectively on day 5+11 when incubation embryoid bodies together with 1 μM p38 antagonist SB203580 or 10 μM ERK1, 2 inhibitor U0126 from day 5 to day 5+11(Fig. 3A). Comparably, SB203580 or U0126 down-regulated icariin increased MEF2C expression 24 h following icariin treatment 329±75% to 54±64% or 118±29% respectively (Fig. 3B), without icariin treatment, no significant change in MEF2C expression was observed in SB203580 or U0126 treated embryoid bodies (Fig. 3B). In embryoid bodies treated with icariin 48 h, nuclei were strongly stained by the anti-MEF2C antibody (compared with the case in the control) (Fig. 3C),

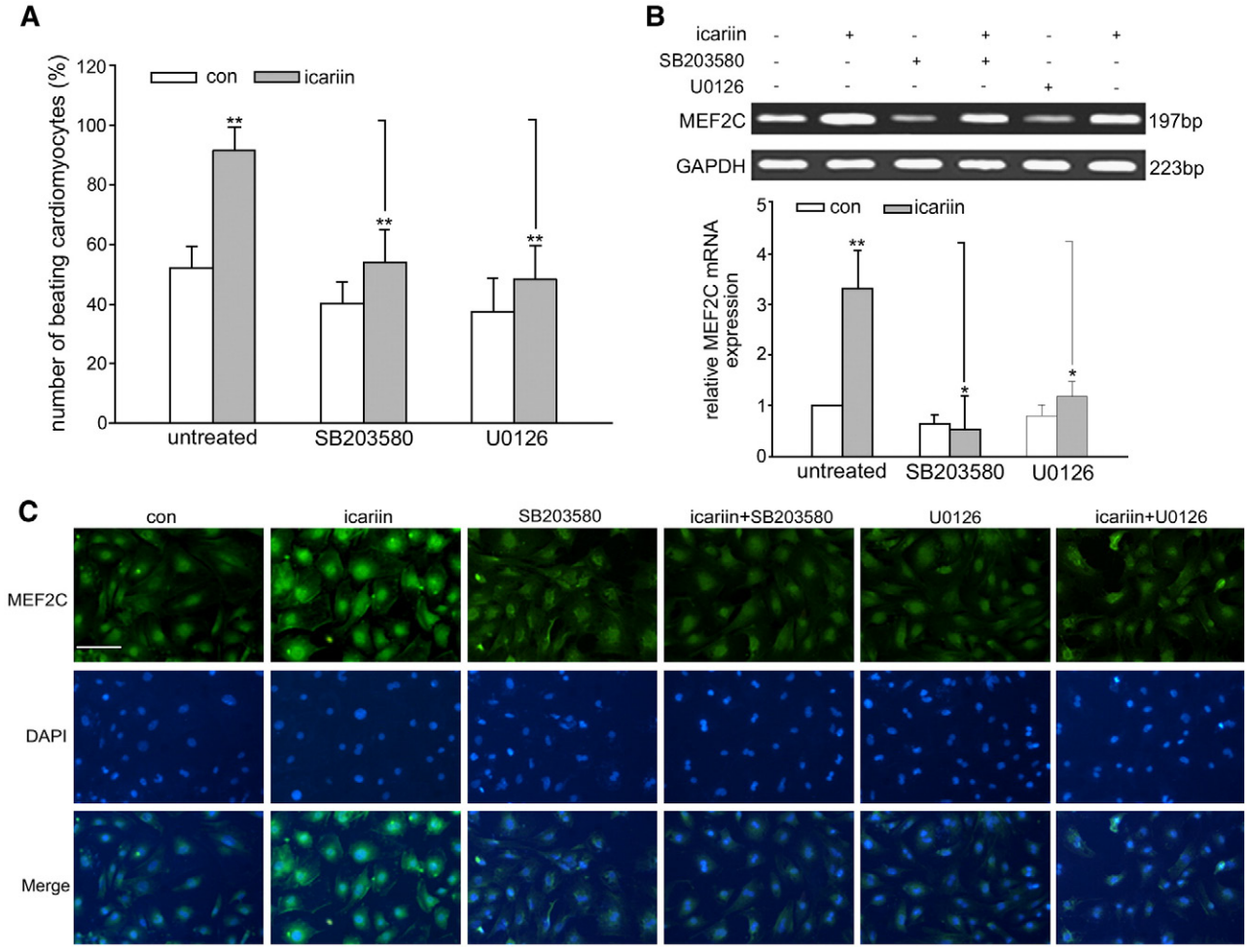

Fig. 3. Effects of inhibition of p38 or ERK1, 2 pathways on icariin induced cardiomyogenesis. (A) Effects of inhibition of p38 or ERK1, 2 pathways on icariin induced cardiac commitment. Embryoid bodies were treated from day 5 to day 5+11 with either 1 μM p38 antagonist SB203580, or 10 μM ERK1, 2 inhibitor U0126, cardiomyogenesis was assessed by counting the number of spontaneously contracting cardiomyocyte foci on day 5+11. (B) Effects of inhibition of p38 or ERK1, 2 pathways on icariin induced MEF2C gene expression. Embryoid bodies on day 5 were treated with or without icariin in the absence and presence of SB203580 or U0126, MEF2C gene expression was evaluated 24 h following icariin administration. (C) Effects of inhibition of p38 or ERK1, 2 pathways on icariin induced MEF2C intranuclear distribution. Embryoid bodies on day 5 were treated with or without icariin in the absence and presence of SB203580 or U0126, MEF2C intranuclear distribution was assessed by immunohistochemistry 48 h after icariin treatment. Upper panel: Embryoid bodies labeled with antibodies for MEF2C; middle panel: Nuclear blue stain with DAPI (4′, 6-diamino-2-phenylindole); below panel: upper and middle panels were merged. Scale bar=100 μm. (D) Effects of inhibition of p38 or ERK1, 2 pathways on icariin stimulated α-actinin expression. Embryoid bodies were treated with or without icariin in the absence and presence of SB203580 or U0126 from day 5 to day 5+11. A typical FACS profile of embryoid bodies staining with anti-sarcomeric α-actinin-mAb on day 5+11 is shown. The *X*-axis corresponds to the fluorescence intensity and the *Y*-axis to the number of cells per channel (events). Bar chart shows quantification of α-actinin expression by FACS analysis. (E) Effects of inhibition of p38 or ERK1, 2 pathways on icariin induced p38 and ERK1, 2 phosphorylation. Embryoid bodies on day 5 were treated with or without icariin in the absence and presence of SB203580 or U0126, p38 and ERK1, 2 activation was evaluated either 30 min (p38) or 2 h (ERK1, 2) following icariin administration. $n=3$. *$P<0.05$ and **$P<0.01$.

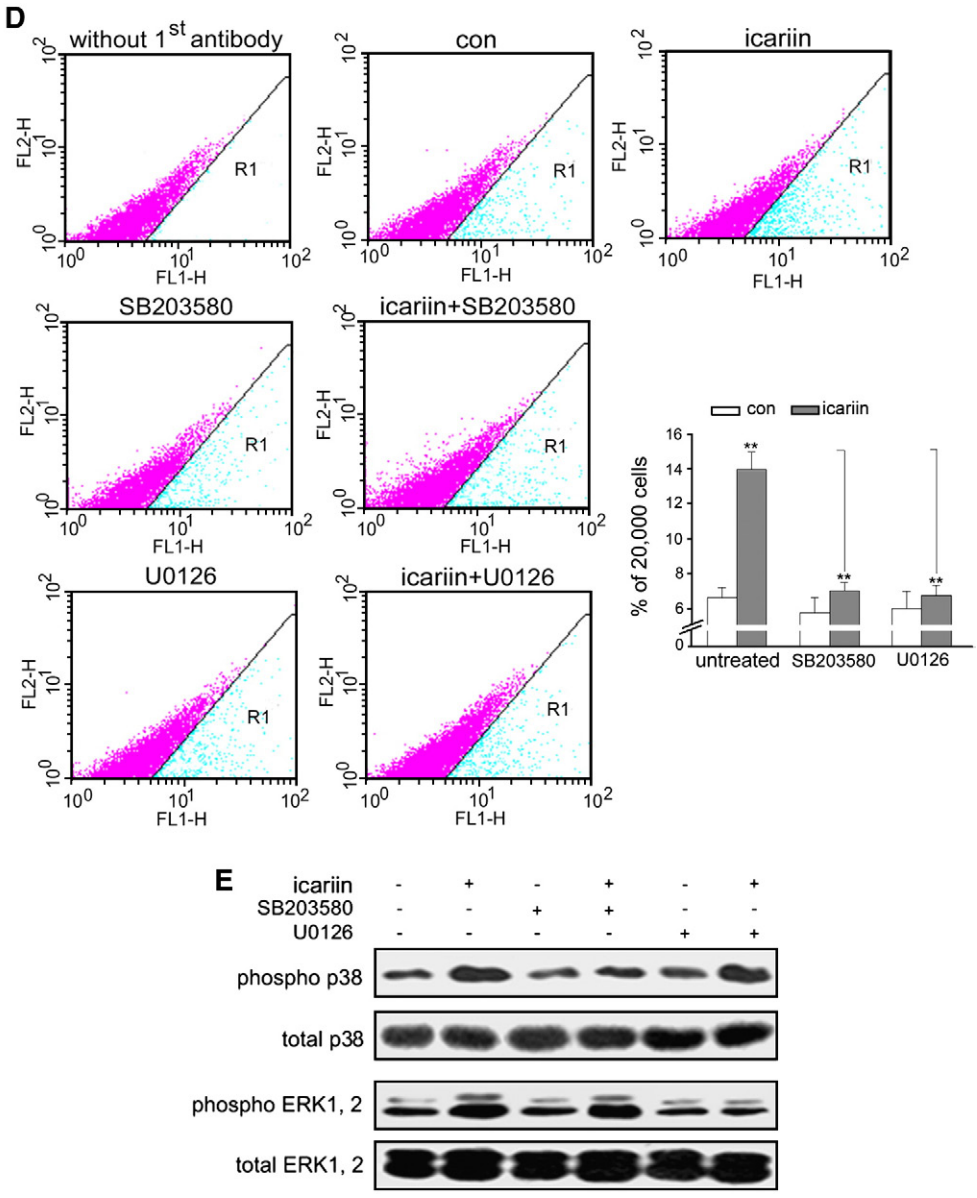

Fig. 3 (*continued*).

which was completely abolished in the presence of SB203580 or U0126 (Fig. 3C). SB203580 or U0126 treated only displayed no significant influence on MEF2C nuclear translocation.

Further, cardiac-specific protein α-actinin expression was investigated by flow cytometry. On day 5+11, only 6.6±0.6% of cells stained positively for sarcomeric α-actinin in control, while about 14.0±1.0% of cells treated with icariin stained for α-actinin (Fig. 3D). The proportion of cells induced by icariin that contained sarcomeric α-actinin was significantly decreased to 7.0±0.4% or 6.8±0.6% when incubation embryoid bodies together with SB203580 or U0126 (Fig. 3D).

In addition, our results demonstrated that the use of SB203580 is not affecting ERK1, 2 phosphorylation and conversely, that U0126 is not blocking the p38 pathway (Fig. 3E), which discarded unspecific effects of the antagonists.

3.3. Effects of icariin on transcription factors NF-κB and AP-1

Embryoid bodies on day 5 treated with icariin for 30 min resulted in a significant increase of p-IκBα expression to 182±21% and a decrease of IκBα expression to 47±16% (Fig. 4A). Icariin administrated for 2 h up-regulated NF-κB p65 expression to 189±17% in nucleus fraction, meanwhile, its expression in cytoplasm fraction was down-regulated to 41±14% compared with the case in the control (Fig. 4B), indicating the nuclear translocation of NF-κB p65 after icariin treatment.

c-*fos* and c-*jun* gene expression in embryoid bodies was increased to 152±7% and 184±24% respectively 30 min after icariin administration (Fig. 4C); at 2 h following icariin treatment, up-regulation of the protein expression was observed (Fig. 4D).

Further studies shown that treatment with SB203580 or U0126 prominently inhibited the IκBα phosphorylation and degradation, NF-κB p65 nuclear translocation, as well as c-*fos* and c-*jun* up-regulation in embryoid bodies after icariin administration (Fig. 4A, B, D), pointing toward that NF-κB and AP-1 activation were p38- and ERK-dependent.

4. Discussion

In contrast to the embryonic heart, cardiomyocytes in adult heart lose their capability of cell division for so far unknown reasons. After

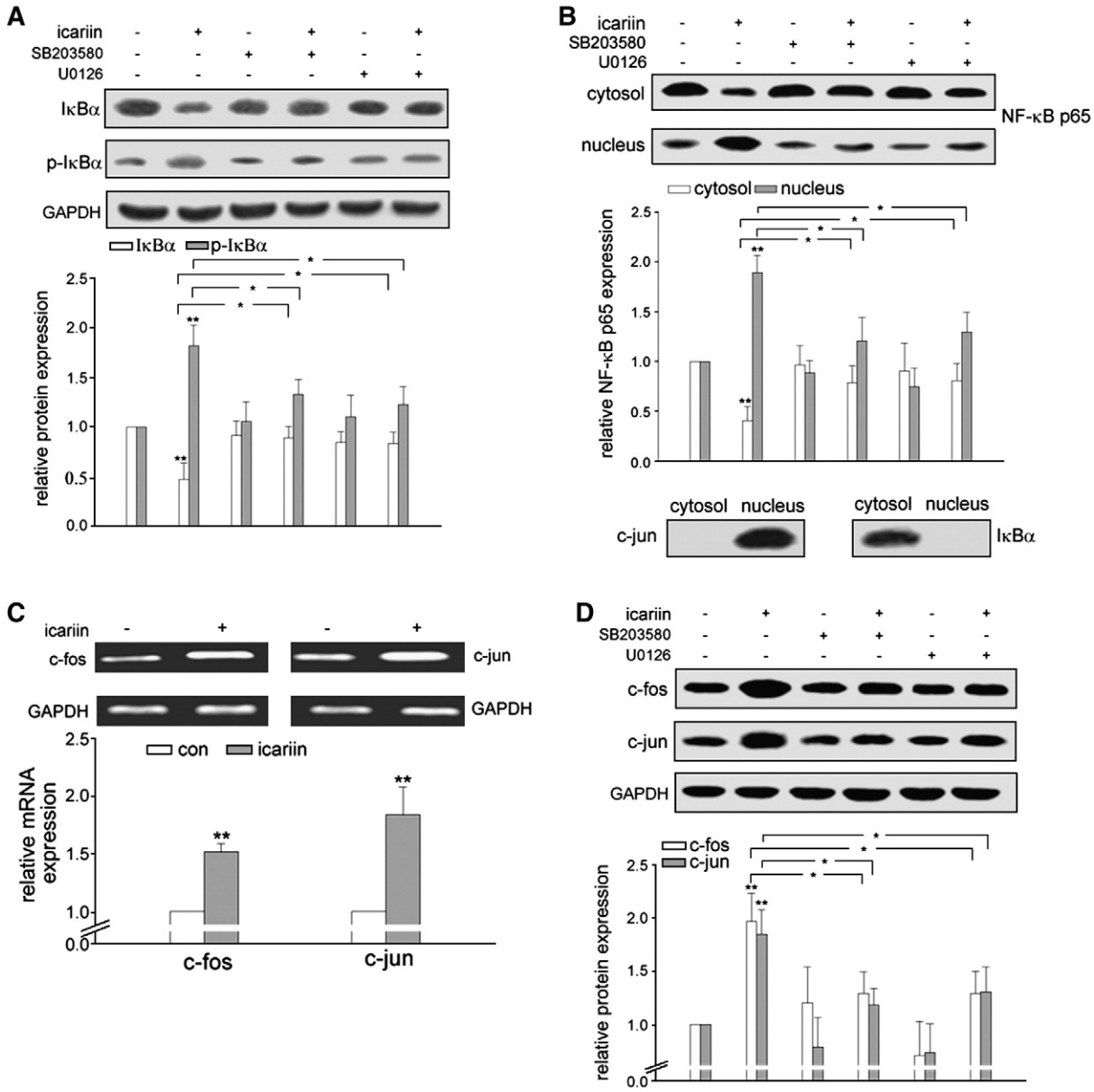

Fig. 4. Involvement of NF-κB and AP-1 in icariin induced cardiac differentiation. (A) Embryoid bodies on day 5 were treated with or without 100 nM icariin in the absence and presence of either 1 μM p38 antagonist SB203580 or10 μM ERK1, 2 inhibitor U0126, IκBα and p-IκBα were evaluated 30 min following icariin administration. (B) Embryoid bodies on day 5 were treated with or without icariin in the absence and presence of either SB203580 or U0126, and harvested to separate the nuclear cellular fraction from the cytoplasmic fraction, NF-κB p65 translocation were evaluated 2 h following icariin treatment, c-*jun* and IκBα expression in cytosol or nucleus was used to control the purity of the sub-cellular fractions studied. (C) Embryoid bodies on day 5 were treated with 100 nM icariin, c-*fos* and c-*jun* gene expression were evaluated 30 min following icariin administration. (D) Embryoid bodies on day 5 were treated with or without icariin in the absence and presence of either SB203580 or U0126, c-*fos* and c-*jun* protein expression were evaluated 2 h after icariin treatment. $n=3$. $^{*}P<0.05$ and $^{**}P<0.01$.

injuries such as myocardial infarctions or heart failure, cardiomyocytes have only limited regenerative capacity, thus, transplantation of functional cardiomyocytes into damaged myocardium would have therapeutic potential. Recent studies have demonstrated that ES cells can differentiate into cardiomyocytes *in vitro* with structural and functional properties (Harding et al., 2007; Bettiol et al., 2006a,b), which suggests that the property can be used in a new therapeutic approach and will have considerable potential in treating cardiovascular diseases. However, at present, obstacles such as control of stem cell fate, allergenic rejection and limited cell availability must be overcome before their therapeutic potential can be realized. This requires an improved understanding of signaling pathways that affect stem cell fate and differentiation. From this observation, the knowledge of signal transduction cascades that regulate cardiac differentiation will provide new insights into stem cell biology, and may ultimately contribute to the therapeutic use of ES cells. Small molecules have been found to have effects on stem cell differentiation have provided new tools to study signaling pathways that affect stem cell fate and differentiation (Emre et al., 2007).

Our previous data suggested that icariin influence cardiac differentiation partly by inducing apoptosis in cells not committed to cardiac differentiation (Zhu et al., 2005). However, which mediators are involved in icariin induced signaling cascades that result in the transcription of genes directing differentiation toward the cardiomyogenic cell lineage has not been fully elucidated yet.

MAPKs pathways are major signaling systems by which cells transduce extracellular signals into intracellular responses (Chang and Karin, 2001). It has been shown that p38 was critical for

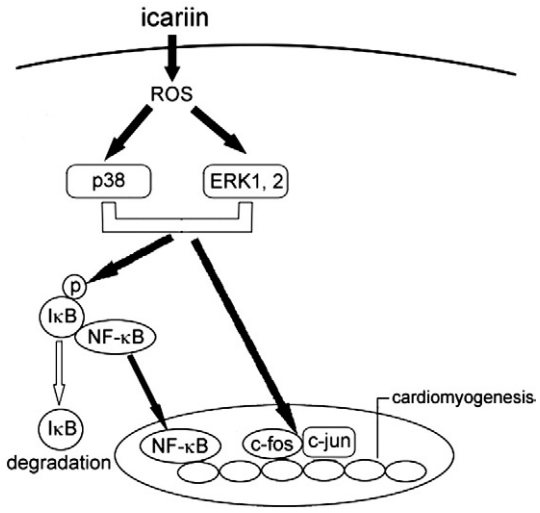

Fig. 5. Model depicting NF-κB and AP-1 signaling pathways involved in icariin induced cardiac differentiation of mouse ES cells. Icariin rapidly activated p38 and ERK1, 2 in cardiomyogenic cell lineage differentiation of ES cells, which subsequently phosphorylated IκB followed by IκB degradation, resulting in NF-κB:IκB complex dissociation and thereafter translocation of the released NF-κB into the nucleus. Furthermore, c-*fos* and c-*jun* expression were up-regulated by the activated p38 and ERK1, 2.

cardiomyogenic cell lineage (Li et al., 2006; Schmelter et al., 2006). Inhibition of p38 prevented the differentiation program in myogenic cell lines and human primary myocytes (Wu et al., 2000). Moreover, activation of ERK was inhibitory toward myogenic transcription in myoblasts but contributed to the activation of myogenic transcription and regulated postmitotic responses (i.e., hypertrophic growth) in myotubes (Wu et al., 2000).

Icariin rapidly activated p38 and ERK1, 2, treatment with p38 antagonist SB203580 or ERK1, 2 inhibitor U0126 significantly abolished icariin induced cardiac commitment, MEF2C gene expression and nuclear translocation, as well as cardiac-specific protein α-actinin expression, pointing toward the notion that p38 and ERK1, 2 are specifically involved in icariin stimulated cardiomyogenic cell lineage differentiation of ES cells. What is more, the results of MEF2C nuclear translocation suggested an icariin induced increase in the expression of cardiomyocyte-specific MEF2C marker. MAPK inhibition is not reducing the number of cardiomyocytes in each microscopic field but the intensity of the cardiac-specific marker, indicating that the inducible effects of icariin were partly related to increases in the expression of cardiac developmental-dependent MEF2C on the early phase of cardiomyogenic cell lineage differentiation of mouse ES cells.

The biological effects of MAPKs are mediated by downstream phosphorylation substrates, which in nucleus are often transcription factors. Transcription factors such as NF-κB and AP-1 have been implicated in the inducible expression of a variety of genes directing differentiation toward cardiomyocytes (Jones et al., 2003; Jochum et al., 2001). NF-κB is normally present in cytoplasm in an inactive form through its association with inhibitory subunit IκB. It has been shown that the principal NF-κB subunits p65, p50, IκBα, and IκBβ are present throughout development, suggesting that this transcription complex may participate in myocardial gene regulation throughout development (Norman et al., 1998). The crucial role of AP-1 transcription factors during differentiation and development is well established (Jochum et al., 2001). Transcriptional induction of certain AP-1 encoding genes, c-*jun* and c-*fos*, are required for AP-1 activation. Previous report has found the induced AP-1 activity in P19 embryonal carcinoma cells undergoing cardiac differentiation after treatment with DMSO (Eriksson and Leppä, 2002). Our data demonstrated a rapid activation of NF-κB in embryoid bodies upon icariin treatment, as up-regulation of phosphorylated IκBα expression, down regulation of IκBα expression, as well as translocation of p65 subunit of the transcription factor into the nucleus were detected. In addition, proto-oncogenes c-*jun* and c-*fos*, as well as their protein expression were up-regulated by icariin, indicating the involvement of AP-1 in icariin stimulated cardiomyogenic cell lineage differentiation. Treatment with SB203580 or U0126 prominently inhibited the NF-κB p65 translocation to the nucleus, and AP-1 expression, which implied that NF-κB and AP-1 activation were p38- and ERK-dependent in icariin induced cardiomyogenic cell lineage differentiation of ES cells.

In conclusion, icariin rapidly activated p38 and ERK1, 2 during cardiomyogenic cell lineage differentiation from mouse ES cells, which subsequently phosphorylated IκB followed by IκB degradation, resulting in NF-κB:IκB complex dissociation and thereafter translocation of the released NF-κB into the nucleus. Furthermore, the activated p38 and ERK1, 2 up-regulated c-*fos* and c-*jun* expression (Fig. 5). The findings provide a new insight into icariin induced cardiac differentiation of ES cells and may ultimately contribute to the future therapeutic use of ES cells.

Acknowledgments

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