Journal of Cellular Biochemistry 103:1536–1550 (2008)

Reactive Oxygen Species Involved in Prenylflavonoids, Icariin and Icaritin, Initiating Cardiac Differentiation of Mouse Embryonic Stem Cells

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Abstract The significant promoting effects of some prenylflavonoids on cardiac differentiation of mouse embryonic stem (ES) cells via reactive oxygen species (ROS) signaling pathway were investigated. The most effective differentiation was facilitated by icariin (ICA), followed by icaritin (ICT), while desmethylicaritin (DICT) displayed the weakest but still significant inducible effect. Contrarily, DICT demonstrated the strongest anti-oxidative activity while ICA displayed only little in vitro, which was well matched with the hydroxyl (OH) numbers and the positions in the molecular structures. Therefore, ROS signaling cascades were assumed to be involved in prenylflavonoids induced cardiomyogenesis. Treatment with ICA, intracellular ROS in embryoid bodies was rapidly elevated, which was abolished by the NADPH-oxidase inhibitor apocynin; elimination of intracellular ROS by vitamin E or pyrrolidine dithiocarbamate (PDTC) inhibited ICA induced cardiomyogenesis; ROS-sensitive extracellular-regulated kinase 1, 2 (ERK1, 2) and p38 activation were further observed, the cardiomyogenesis was significantly inhibited in the presence of ERK1, 2 or p38 inhibitor U0126 or SB203580, indicating the roles of NADPH-ROS-MAPKs signaling cascades in prenylflavonoids induced cardiac differentiation. There was no difference in Nox4 NADPH oxidase expression between ICA and ICT treatments, however, ROS concentration in EBs after ICT administration was lower than that after ICA treatment, followed by less activation of ERK1, 2, and p38. These results revealed that the significant promoting effects of prenylflavonoids on cardiac differentiation was at least partly via ROS signaling cascades, and the facilitating abilities preferentially based on the nature of prenylflavonoids themselves, but anti-oxidative activity determined by the OH numbers and the positions in the structures do influence the cardiomyogenesis in vitro. J. Cell. Biochem. 103: 1536–1550, 2008.

Key words: prenylflavonoid; icariin; icaritin; reactive oxygen species; cardiac differentiation; embryonic stem cell

Embryonic stem (ES) cells will become a source of all adult differentiated cells once reliable protocols for directed differentiation have been established. This resource will revolutionize laboratory cell biology and will provide much improved cell culture models for discovery and development of drugs, and fundamental studies of the genetic basis of disease [McNeish, 2004; Pouton and Haynes, 2005]. It is well accepted that the microenvironment of ES cells, such as pH, dissolved oxygen and nutrient concentrations, can influence ES cells differentiation and phenotypic expression. From the point of view, ES cell fate is determined by both intrinsic regulators and the extra-cellular niche, and their expansion and differentiation ex vivo are generally controlled by growing them in a specific configuration with "cocktails" of growth factors and signaling molecules [Chen et al., 2006; Thomson, 2007]. To supply physiologically relevant cells for use in drug discovery, movement towards controllable,

Yan-bo Wo and Dan-yan Zhu contributed equally to this work.

Grant sponsor: National Natural Sciences Foundation of China; Grant numbers: 30672564, 30600762, 30472112, 30171121; Grant sponsor: Key Grant of the Chinese Ministry of Education; Grant number: 03088; Grant sponsor: International Joint Key Grant of Zhejiang Province; Grant number: 2003C24005.

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Received 8 June 2007; Accepted 18 July 2007

DOI 10.1002/jcb.21541

scalable culture environments is an important step. Essential to these pursuits is the identification of renewable sources of engraftable functional cells, an improved ability to manipulate ES cell proliferation and differentiation, and a better understanding of the signaling pathways that control ES cell fate [Chen et al., 2006; Thomson, 2007].

Increasing research have been demonstrated that embryoid bodies (EBs) grown from ES cells actively generated reactive oxygen species (ROS) presumably through the activity of ROS-generating NADPH oxidase [Li et al., 2006]. Small amounts of ROS could be function as intracellular second messengers and utilized within signaling cascades that result in the transcription of genes directing differentiation toward the cardiomyogenic as well as endothelial cell lineage [Sauer et al., 2000, 2004, 2005]. Notably, ROS generation in EBs was induced at times when the cells were still undifferentiated (i.e., at day 4 of cell culture) whereas differentiation of the cardiovascular cell lineage occurs between day 6 and day 9 of cell culture [Hescheler et al., 1999]. Recently, it has been reported that the initiation of the cardiovascular differentiation program of ES cells is largely depend on the ROS sensitive p38 MAPKs signaling cascades [Schmelter et al., 2006], which implies that ROS generated at a suitable concentration exerts a role as signaling molecules in the initiation of cardiovascular differentiation of ES cells.

Our previous studies shown that prenylflavonoids, icariin (ICA), icaritin (ICT), and desmethylicaritin (DICT), facilitated the directional differentiation of mouse ES cells into cardiomyocytes by enhancing and accelerating differentiation into beating cardiomyocytes, as well as increasing in the expression of cardiac developmental-dependent genes, indicating the roles of prenylflavonoids on the early phase of cardiac differentiation [Zhu and Lou, 2005, 2006]. However, the underlying mechanisms involved in were still not revealed yet.

The structural distinctions among ICA, ICT, and DICT are the hydroxyl (OH) numbers and the positions in the molecular structures (Fig. 1), the prenylflavonoid structures might be related to the intracellular ROS concentration in EBs. Therefore, ROS signaling cascades were assumed to be involved in prenylflavonoids induced cardiomyogenesis. In the present studies, the relationship between the chemical structure and the inducible effects of ICA, ICT, and DICT was investigated; subsequently, NADPH-ROS-MAPKs signaling cascades in the initiation of cardiac differentiation of mouse ES cells induced by ICA and ICT were further assessed.

MATERIALS AND METHODS

Cultivation of EBs

The ES-D3 cells (American Type Culture Collection, CRL-1934) were differentiated into beating cardiomyocytes as described previously [Zhu and Lou, 2005, 2006; Zhu et al., 2005]. In brief, the cells were cultivated in 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 (FCS; Hyclone), 0.1 mM β-mercaptoethanol (Sigma), non-essential amino acids (Hyclone) and 10^6 U/L recombinant mouse leukemia inhibitory factor (Chemicon). Cultures of differentiating ES cells were established by the formation of EBs in hanging drop cultures with differentiation medium, which consisted of DMEM, 20% FCS, 0.1 mM β-mercaptoethanol, non-essential amino acids, without recombinant mouse leukemia inhibitory factor. Drops (30 μl) containing about 600 ES cells were placed on the lids of Petri dishes filled with D-Hanks solution, and then cultivated in hanging drops for 3 days and another 2 days in Petri dishes. At this time (day 5), ICA (Drug Biology Product Examination Bureau, Beijing, China, batch no 0737-200011, purity 99%), ICT, DICT (prepared as described previously [Wang and Lou, 2004])

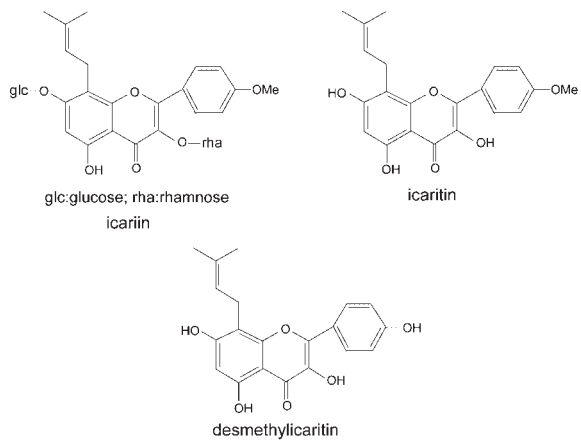


Fig. 1. Molecular structures of ICA, ICT, and DICT.

TABLE I. Primer Sequence Information for RT-PCR

Gene	Primer sequence (5′-3′)	Annealing temperature (°C)	Product size (bp)	Cycle
MEF2C	Forward: gatacccacaacacaccacgcgcc Reverse: atccttcagagagtcgcatgcgctt	60	197	38
GATA-4	Forward: tctcactatgggcacagcag Reverse: gcgatgtctgagtgacagga	54	135	33
α-MHC	Forward: ctgctggagaggttattcctcg Reverse: ggaagagtgagcggcgcatcaagg	66	301	38
MLC-2v	Forward: tgtgggtcacctgaggctgtggttcag Reverse: aaggctgactatgtccgggagatgc	61	189	43
NOX4	Forward: ccagaatgaggatcccagaa Reverse: accacctgaaacatgcaaca	58	196	45
GAPDH	Forward: aactttggcattgtggaagg Reverse: acacattgggggtaggaaca	58	223	25

MEF2C, myocyte enhancer factor 2C; *α-MHC*, α-myosin heavy chain; *MLC-2v*, ventricular myosin light chain; GAPDH, lyceraldehyde-3-phosphate Dehydrogease.

at a final concentration of 100 nM [Zhu and Lou, 2005], or various inhibitors were added to the differentiation medium. ES cells treated with retinoic acid (RA; Sigma) or with dimethyl sulfoxide (DMSO; Sigma) solvent were used as positive or negative controls. Fresh medium containing inhibitors were changed every 24 h. In the experiment, day 5 + x referred to x day after EBs were plated onto gelatin-coated culture plates. Rhythmically beating EBs were considered to be spontaneously beating cardiomyocytes in EBs outgrowths, and were defined as the phenotypic landmark of successful differentiation.

PCL Assessment of ICA, ICT, and DICT

Measurements of anti-oxidative activity were performed on the PHOTOCHEM® (FAT, Berlin, Germany) by a photochemiluminescence (PCL) method in an aqueous system. The key feature of this method is the speed of oxidative reactions that is up to 1,000 times higher compared to normal conditions. This increase is achieved by photochemical stimulation of the reacting molecules, which makes very fast measuring processes possible. Defined free radicals (superoxide anion radical) are generated photo chemically by UV irradiation of photosensitizer compound. The free radicals are detected by their reaction with a chemiluminogenic substance (e.g., luminol) and the measurement of the produced light. In the presence of substances acting as "radical traps", the intensity of the photochemiluminescence is attenuated as a function of concentration. That way, the antiradical properties of the analyzed substances can reliably be quantified. The results are presented in equivalent concentration units of ascorbic acid (Sigma). In the aqueous system, the presence of antioxidants leads to a temporary inhibition of PCL. The resulting lag-phase was used to calculate the anti-oxidative activity by comparison with the lag-phase time of ascorbic acid standards. Antioxidants cause a decrease in the PCL intensity. The percentage of inhibition was used as a parameter for quantification, expressed as ascorbic acid equivalents. To measure the anti-oxidative activities of prenylflavonoids, 6 nmol ICA, 0.6 nmol ICT and DICT were added to the assay mixture, respectively. The quantification and comparison of antioxidant properties of three substances was made using standard curve for ascorbic acid. The results were transformed to the anti-oxidative activities of ascorbic acid equal to 1 nmol sample. Analyses were carried out in triplicate.

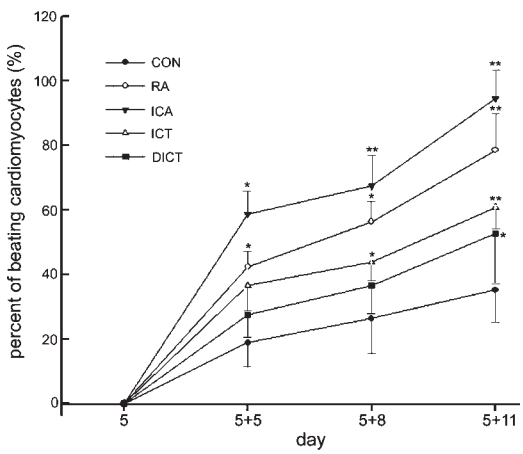


Fig. 2. Effects of ICA, ICT, and DICT on cardiac differentiation. EBs were treated with 100 nM ICA, ICT, DICT or10 nM RA. Percentage of beating EBs was monitored on day 5 + 5, day 5 + 8 and day 5 + 11. n = 3. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control.

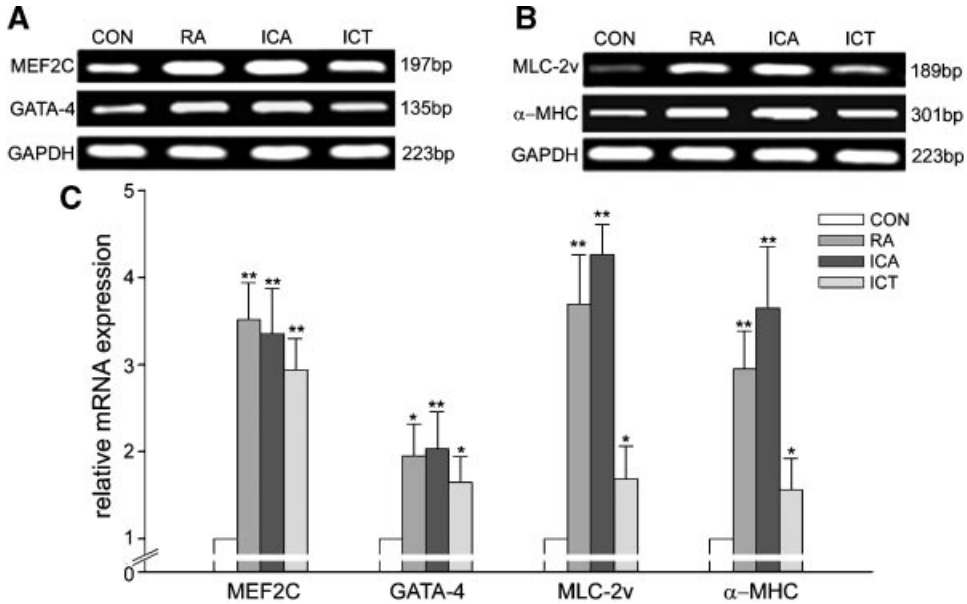


Fig. 3. The mRNA expression of *MEF2C, GATA-4, α-MHC* and *MLC-2v* following treatment with ICA and ICT. **A**,**B**: A representative of mRNA expressions of *MEF2C, GATA-4, α-MHC,* and *MLC-2v, MEF2C* and *GATA-4* were analyzed on day 5 + 1, *α-MHC,* and *MLC-2v* were analyzed on day 5 + 3. **C**: Densitometric analysis of PCR products of *MEF2C, GATA-4, α-MHC,* and *MLC-2v*. 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; n = 3. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control.

Measurement of ROS Generation in EBs

ROS was assessed by the ROS-specific probe 2′, 7′-dichlorofluorescein diacetate (DCF-DA; Sigma) [Zhu et al., 1994; Luo et al., 2001]. For the single cell experiments, EBs on day 5 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 21 G 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%. Single cell was incubated for 30 min with 5 μM DCF-DA dissolved in DMSO, then 100 nM ICA, 100 nM ICT, 10 nM RA, 100 μM vitamin E (Sigma) or 100 μM apocynin (Sigma) were added. The fluorescence intensity was then measured with a fluorescence microplate reader (Beckman) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Untreated cells showed no fluorescence and were used to determine background fluorescence, which was subtracted from the treated samples. The final fluorescence intensity was normalized to the protein content in each group and was expressed as the percentage of the fluorescence intensity of the control group. For whole mount EBs, fluorescent signals were obtained with a fluorescence conversion microscope (Leica DMIL, German).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Reverse transcription polymerase chain reaction (RT-PCR) analysis was performed as described previously [Zhu and Lou, 2005, 2006; Zhu et al., 2005]. In brief, total RNA from the outgrowths of the EBs 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 I). 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), 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.

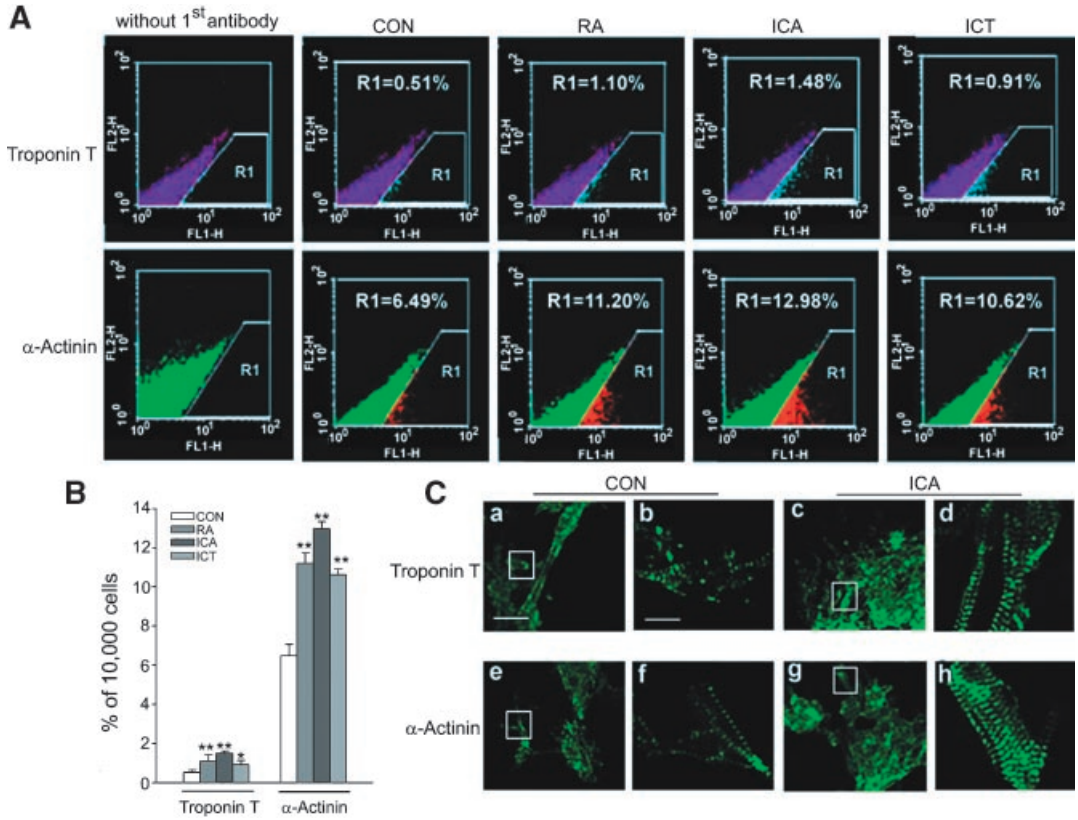


Fig. 4. The expression of troponin T and α-actinin following treatment with ICA and ICT. **A**: A typical FACS profiles of EBs staining with anti-sarcomeric troponin T mAb and anti-sarcomeric α-actinin mAb on day 5 + 11. The X-axis corresponds to the fluorescence intensity and the Y-axis to the number of cells per channel (events). **B**: Quantification of troponin T and α-actinin expression by FACS analysis. **C**: Positive immunoassaying of beating EBs treated with ICA on day 5 + 11. **a**,**c**: Positive staining with anti-cardiactroponin T mAb. The rectangular frames indicate the part magnified in panel (**b**,**d**). b,d: Higher magnification of sarcomeric troponin T staining. **e**,**g**: Positive staining with antisarcomeric α-actinin mAb. The rectangular frames indicate the part magnified in panel (**f**,**h**). f,h: Higher magnification of sarcomeric α-actinin staining. Scale bar = 50 μm (a,c,e,g), 10 μm (b,d,f,h); n = 3. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control.

Immunohistochemistry

Immunohistochemistry was performed with whole mount EBs. As primary antibodies, the mouse monoclonal anti-troponin T (dilution 1:100; Sigma), the mouse monoclonal anti-sarcomeric α-actinin (dilution 1:200; Sigma), the goat polyclonal anti-NOX4 (dilution 1:200; Santa Cruz), the mouse monoclonal anti-p-ERK1, 2 (dilution 1:50; Santa Cruz), the rabbit polyclonal anti-p-p38 (dilution 1:50; Cell Signaling Technology) and the mouse monoclonal anti-p-JNK (dilution 1:50; Santa Cruz) were used. For troponin T, α-actinin and NOX4 staining the respective tissues were fixed in cold acetone for 10 min and washed with PBS. For ERK1, 2, p38, and JNK, the tissues were fixed for 60 min at 4°C in 4% formaldehyde in PBS. Blocking against unspecific binding was performed for 60 min with 10% FCS dissolved in 0.01% PBST. The tissues were subsequently incubated at 4°C overnight together with the primary antibodies dissolved in PBS supplemented with 10% FCS in 0.1% PBST. The tissues were thereafter washed three times with PBST (0.01% Triton) and reincubated with a FITC-conjugated goat anti-mouse IgG (troponin T, α-actinin, ERK1, 2), a FITC-conjugated rabbit anti goat IgG (NOX4) or a Rhodamine-conjugated goat anti-rabbit IgG (p38) (dilution 1:1,000; Santa Cruz) in PBS containing 10% FCS in 0.01% PBST. After washing three times in PBST (0.01% Triton), the tissues were stored in PBS until inspection. For NOX4, ERK1, 2, p38, and JNK, fluorescent signals were obtained with a fluorescence conversion microscope (Leica DMIL) and assayed by its image processing and analysis system, the results were expressed as the percentage of the fluorescence intensity of the control group.

TABLE II. Anti-Oxidative Activity of ICA, ICT, and DICT Evaluated by the PCL Method in Aqueous System

Samples	nmol ascorbic acid/nmol sample
ICA	0.051 ± 0.003
ICT	0.289 ± 0.009
DICT	1.400 ± 0.146

Confocal Imaging

For troponin T and α-actinin, fluorescence recordings were performed by means of a confocal laser scanning setup (Leica TCS SP2) 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.

Flow Cytometry (FACS Analysis)

EBs were washed with PBS/1 mM EDTA and dissociated to a single-cell suspension by EDTA/trypsin treatment (same as above). Cells were suspended in 0.5 ml PBS/1% BSA and assayed in a flow cytometer (FACSCalibur; Becton Dickinson). For detection of troponin T and α-actinin, cells were stained as described above. 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. The results were expressed as the percentage of the fluorescence intensity of the control group.

Western Blot Analysis

Whole Cell lysates were prepared as described previously [Zhu et al., 2005]. In brief, EBs were washed with PBS, collected in RIPA buffer (containing 150 mM NaCl, 1% NP-40, 0.5% SDC, 0.1%SDS, 20 mM EGTA, 50 mM Tris-HCl, 1 mM DTT, 20 mM NaF, 1 mM Na_3VO_4, 0.1 mg/ml PMSF, 10 μg/ml leupeptin and 2.0 μg/ml aprotinin) and lysed 30 min on ice. After the cell samples were lysed, the protein concentration was estimated by Dc protein assay kit (Bio-Rad Laboratories). Proteins (30 μg) were loaded onto 10% SDS–polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes (Pall Corporation, NY). The sheets were analyzed with antibodies according to the suppliers' protocol and visualized autoradiographically with an enhanced chemiluminescent substrate (ECL, Pierce), and scanned using a bio-imaging analyzer (Bio-Rad). The density of the products was quantitated using Quantity One version 4.2.2 software (Bio-Rad).

Statistical Analysis

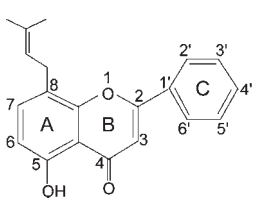
Data were given as mean values ± SD. Statistical analysis was performed by one way ANOVA, followed by adjusted t tests with P values corrected by the Bonferroni method. A value of $P < 0.05$ was considered to be significant.

RESULTS

Inducible Effects of ICA, ICT, and DICT on the Cardiac Differentiation

ICA, ICT, and DICT displayed different influence on cardiac differentiation of ES cells. On day 5 + 5 after the 100 nM ICA treatment, the percentage of EBs containing beating areas

TABLE III. Relationships Between the Structure of Anti-Oxidative Activity and the Inducible Effects on Cardiomyocytes Differentiation of ICA, ICT, and DICT

Compounds	Molecular skeletor	3-OH	7-OH	4′-OH	Anti-oxidative activity	Differentiation percentage(%)
ICA		−	−	−	−	+++
ICT		+	+	−	+	++
DICT		+	+	+	++	+

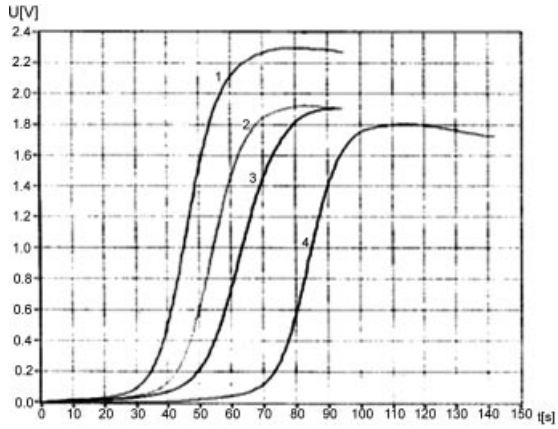


Fig. 5. A standard light emission curve of the photo-induced chemiluminescence of luminal (1) and effects of addition of ICT 0.6 nmol, ICA 6.0 nmol, and DICT 0.6 nmol: curves 2–4. Original recordings with the PHOTOCHEM®.

showed a remarkable increase compared with the control, on days $5+8$ and $5+11$, this percentage remained significantly higher than that in the untreated cells (Fig. 2). Treatment of EBs with 100 nM ICT resulted in a remarkable increase in the number of EBs with spontaneously beating cardiomyocytes on days $5+8$ and $5+11$, whereas the differentiation effect of EBs induced by 100 nM DICT was only prominent on day $5+11$ (Fig. 2). Upregulation of the cardiac transcription factors *MEF2C* and *GATA-4*, as well as the cardiac-specific genes *α-MHC* and *MLC-2v* were observed as indicated times following ICA and ICT application (Fig. 3). Compared to ICA, ICT displayed lower effects on the upregulation of these cardiac marker genes (Fig. 3). Furthermore, cardiac-specific proteins troponin T and α-actinin expression were investigated by flow cytometry and indirect immunofluorescence. On day $5+11$, only $0.51 \pm 0.13\%$ of cells stained positively for troponin T in spontaneous differentiation population, while $1.48 \pm 0.12\%$ of cells treated with ICA or $0.91 \pm 0.22\%$ of cells treated with ICT stained for troponin T (Fig. 4A,B). The proportion of cells induced by ICA or ICT that contained positive sarcomeric α-actinin protein labeled sarcomeres was significantly increased to$12.98 \pm 0.34\%$ or $10.62 \pm 0.28\%$ in contrast with the case of $6.49 \pm 0.57\%$ in the control (Fig. 4A,B). Figure 4C shows the representative staining results for the cardiac-specific troponin T and sarcomeric a-actinin proteins after ICA treatment.

Anti-Oxidative Activity of ICA, ICT, and DICT

A standard light emission curve was shown as curve 1, and curve 2–4 demonstrated the effect of adding 0.6 nmol ICT, 6 nmol ICA, and 0.6 nmol DICT to the assay mixture (Fig. 5). The anti-oxidative activities of 1 nmol ICA, ICT, and DICT were found to be 0.05 nmol, 0.28 nmol, and 1.40 nmol equivalents of ascorbic acid, respectively (Table II). DICT showed a much higher anti-oxidative activity than either ICA or ICT. A relationship between the structures and anti-oxidative activities was demonstrated among prenylflavonoids, which was in turn well matched with the effects of ICA, ICT, and DICT on cardiac differentiation (Table III).

Effects of Free Radical Scavengers on ICA-Induced Cardiac Differentiation

EBs on day 5 were incubated with or without 100 nM ICA in the absence and presence of 100 μM vitamin E or 1 μM pyrrolidine dithiocarbamate (PDTC) from day 5 to day $5+11$, the number of beating EBs was counted on day $5+11$. Comparably, the effects of vitamin E or PDTC on transcription factor *MEF2C* mRNA expression were investigated. Treatment of EBs with vitamin E or PDTC resulted in a significant decrease in the number of ICA-induced beating EBs by approximately 51% or 45% respectively (Fig. 6A). ICA increased *MEF2C* expression 24 h following ICA treatment to $329 \pm 75\%$, which was abolished in the presence of the free radical scavenger vitamin E ($167 \pm 45\%$) or PDTC ($118 \pm 40\%$; Fig. 6B). Without ICA treatment, no significant change in *MEF2C* expression was observed in vitamin E or PDTC treated EBs.

Effects of ICA on Generation of ROS

To evaluate ROS generation, single cells from EBs were labeled with the ROS sensitive indicator DCF-DA and the generation of oxidized fluorescent DCF was monitored either directly or at different times as indicated after ICA treatment (Fig. 7A). Already after ~30 min a significant increase in ROS generation was observed, ROS levels remained elevated until 120 min as assessed by determination of DCF fluorescence at 15, 30, 60, 120, 240, and 480 min after ICA treatment (Fig. 7A). At 120 min after ICA treatment, the fluorescence was increased to $189 \pm 11\%$, which was totally inhibited in the presence of the free radical scavenger vitamin E

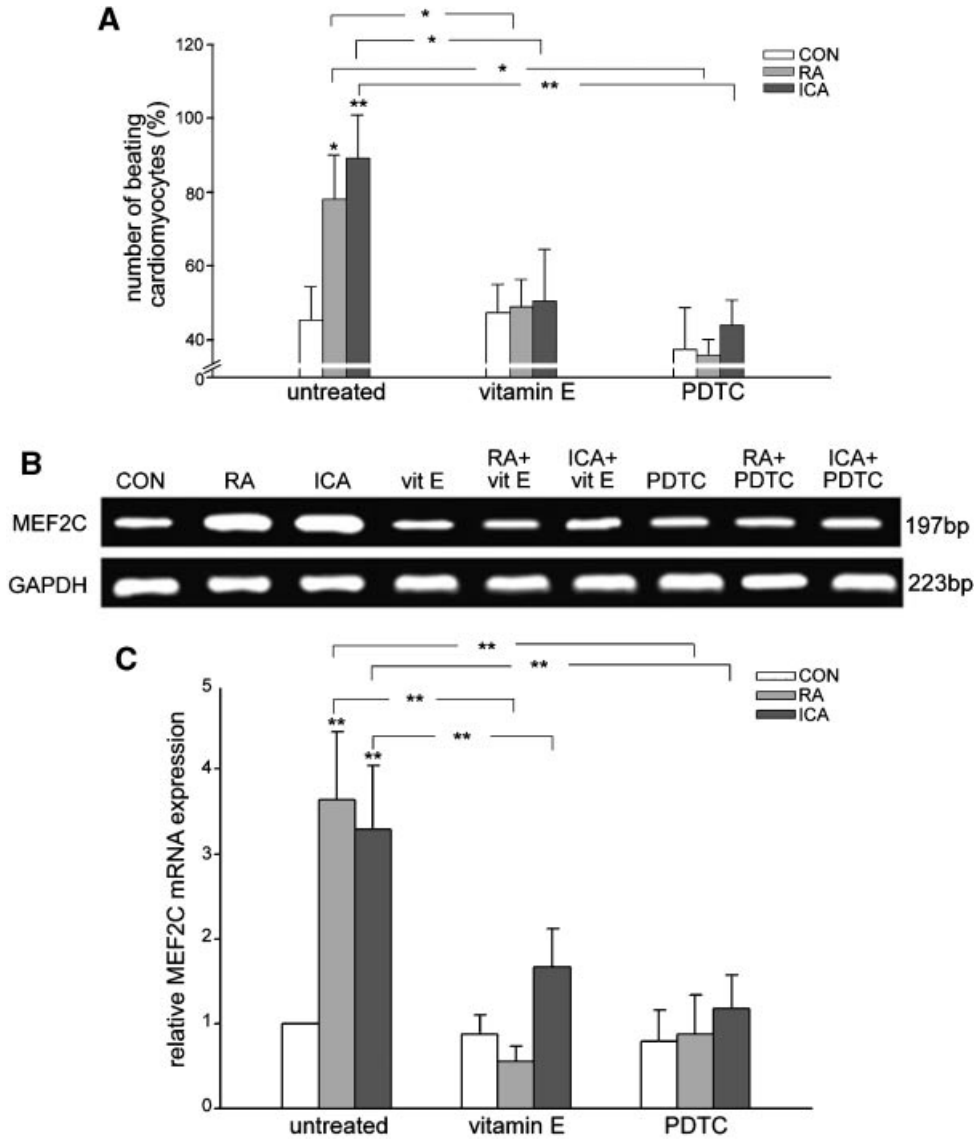


Fig. 6. Effects of inhibition of ROS pathway on ICA induced cardiomyogenesis. **A**: Inhibition of ICA-stimulated cardiomyocyte differentiation by the free radical scavengers vitamin E or PDTC. EBs were treated from day 5 to day 5 + 11 with vitamin E (100 μM) or PDTC (1 μM), Cardiomyogenesis was assessed by counting the number of spontaneously contracting cardiomyocytes foci on day 5 + 11; **B**,**C**: Inhibition of ICA-stimulated *MEF2C* expression by vitamin E or PDTC. EBs on day 5 were either untreated or were treated with ICA in the absence and presence of vitamin E or PDTC. *MEF2C* expression was evaluated 24 h following ICA application; n = 3. $^{*}P < 0.05$ and $^{**}P < 0.01$.

(112 ± 25%; Fig. 7C). Figure 7B shows the representative images of oxidized fluorescent DCF in the whole EBs analyzed by fluorescence conversion microscope.

To evaluate the role of a NADPH oxidase-like enzyme in the endogenous ROS generation in EBs during ICA induced cardiac differentiation, single cell from EBs on day 5 were incubated with the NADPH oxidase inhibitor apocynin, subsequently DCF oxidation was monitored. We observed that upon apocynin treatment, fluorescence in cells treatment with ICA 120 min was reduced to 86 ± 12% (Fig. 7C).

Involvement of MAPKs in ICA Stimulated Cardiac Differentiation

Phosphorylation of p38 (maximal after 30 min; Fig. 8A–C) and ERK1, 2 (maximum after 2 h; Fig. 8A,B,D), but not of JNK (data not shown) were induced by ICA administration. Treatment with vitamin E significantly inhibited the effects of ICA on phosphorylation of

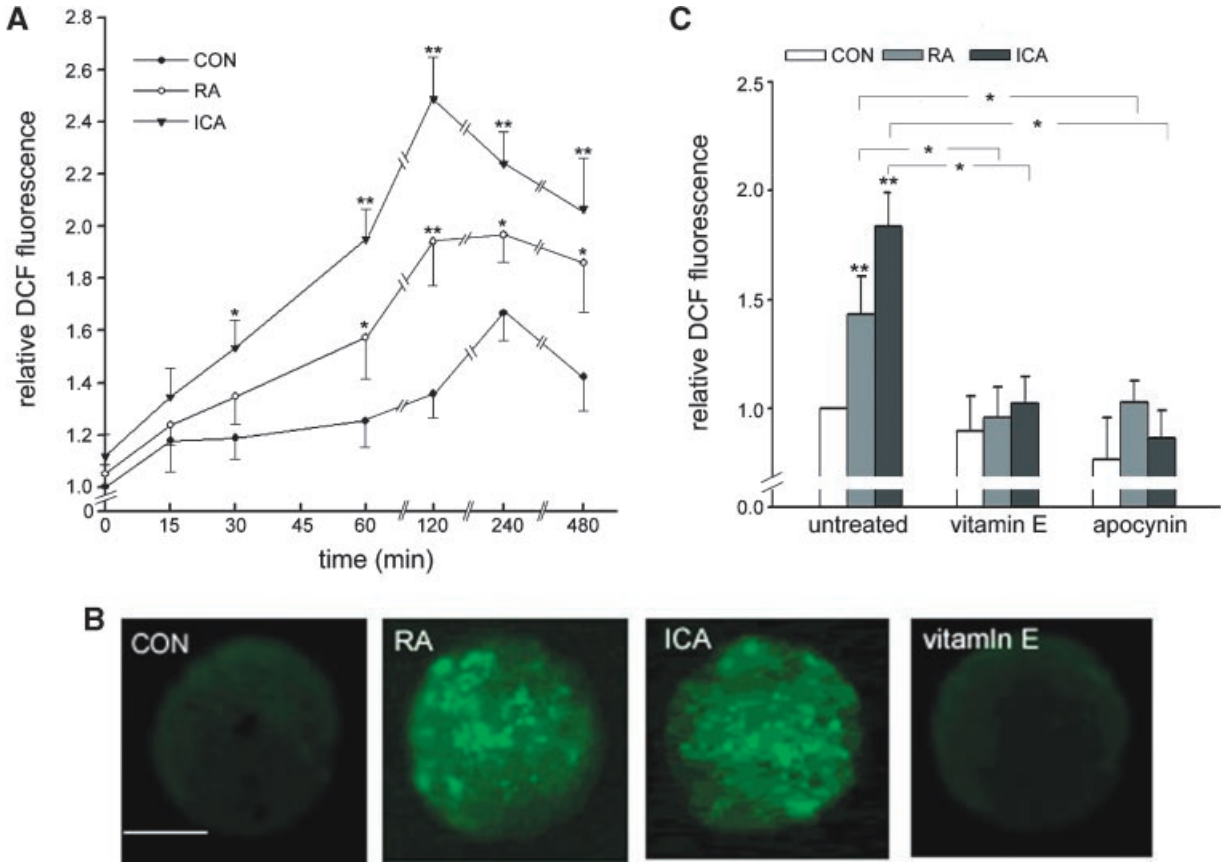


Fig. 7. Generation of ROS following treatment of EBs with ICA. **A**: Intracellular ROS generation was assessed at 15, 30, 60, 120, 240, and 480 min by use of the redoxsensitive dye DCF-DA. **B**: Representative images of intracellular ROS generation (evaluated at 120 min) analyzed by the fluorescence conversion microscope (the bar represents 200 μm). **C**: Intracellular ROS generation (evaluated at 120 min) was significantly inhibited in the presence of the vitamin E (100 μM) or the NADPH oxidase inhibitor apocynin (100 μM); n = 3. $^{*}P < 0.05$ and $^{**}P < 0.01$.

ERK1, 2, and p38, indicating regulation of ERK1, 2, and p38 activities by ROS. To investigate the impact of ERK1, 2, and p38 activities on cardiomyogenic differentiation, EBs were incubated with either 10 μM the ERK1, 2 inhibitor U0126, or 1 μM the p38 inhibitor SB203580 with ICA. Subsequently, the expression of the transcription factor *MEF2C* (Fig. 9A) and the number of spontaneously beating EBs were assessed (Fig. 9B). It was shown that the transcription factor *MEF2C* expression and the spontaneously beating EB number were significantly inhibited by U0126 or SB203580, indicating the involvement of p38 and ERK1, 2 in the signaling cascades.

Effects of ICA and ICT on the NADPH-ROS-MAPKs Signaling Cascades

It has been reported that NOX4 is the major NOX isoform present during early stages of cardiomyocyte differentiation of ES cells [Li et al., 2006]. In the present studies, upregulation of NOX4 protein expression was observed 2 h after ICA and ICT treatment (Fig. 10A–C), no significant difference was found between ICA and ICT treatment (Fig. 10A–C). Comparably, ICA and ICT administrated for 30 min resulted in a significant increase of Nox4 gene expression to $170.5 \pm 20.6\%$ or $168.9 \pm 21.9\%$ respectively (Fig. 10D,E). At 2 h after ICT application, DCF fluorescence in single cells from EBs was increased to $141 \pm 4.3\%$, while at this time, the fluorescence in cells treatment with ICA was significantly increased to $189 \pm 11\%$ (Fig. 11A). Similar to ICA, ICT subsequently activation the p38 and ERK1, 2, however, its effect was lower compared to that in ICA treatment (Fig. 11B–D). These results indicated that ICA and ICT posses the similar effects on the generation of intracellular ROS catalyzed by NOX4, however, the anti-oxidative activity of ICT eliminated parts of ROS, therefore, resulting in its lower

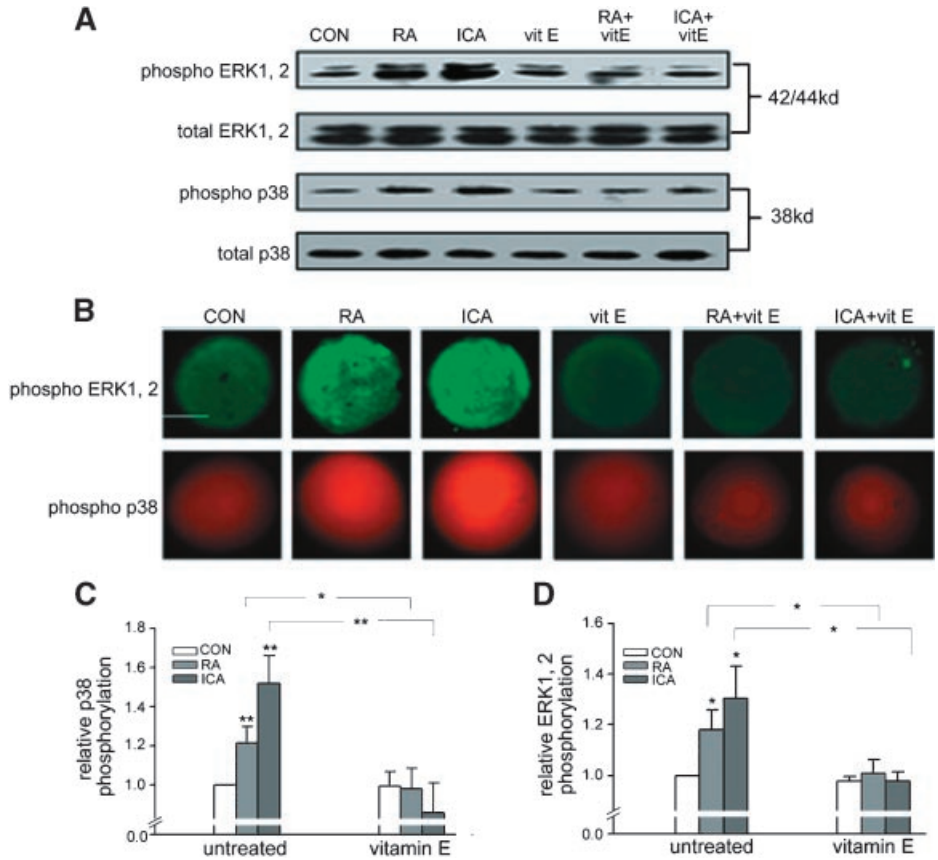


Fig. 8. Inhibition of ICA-stimulated ERK1, 2 and p38 activation by the free radical scavenger vitamin E. EBs on day 5 were either untreated or were treated with ICA in the absence and presence of vitamin E (100 μM). ERK1, 2, and p38 activation was evaluated after either 2 h (ERK1, 2) or 30 min (p38). **A**: Representative levels of phosphorylated ERK1, 2, and p38 MAPK protein analyzed by Western blot as described in Materials and Methods section. **B**: Representative images of EBs (the bar represents 200 μm) labeled with antibodies for active ERK1, 2 and p38 analyzed by immunohistochemistry. **C**,**D**: Plots of semiquantitative analysis of ERK1, 2, and p38 fluorescent signals after ICA treatment in the absence and presence of vitamin E; $n = 3$. $^{*}P < 0.05$ and $^{**}P < 0.01$.

effects on the phosphorylation of ERK1, 2, and p38 and lower inducible effect on cardiac differentiation of ES cells consequently.

These results revealed that the significant promoting effects of prenylflavonoids on cardiac differentiation was at least partly via ROS signaling cascades, and the facilitating abilities preferentially based on the nature of prenylflavonoids themselves, but anti-oxidative activity determined by the OH numbers and the positions in the structures do influence the cardiomyogenesis in vitro.

DISCUSSION

Although numerous studies in the past years have been reported on cardiomyogenesis of ES cells [Hescheler et al., 2002; Xu et al., 2002; Sachinidis et al., 2003; Fukuda, 2005], a specific protocol for the generation of cardiomyocytes from ES cells has not been established yet; besides, the underlying molecular mechanisms of cardiac differentiation are still largely unknown. It has been reported that embryonic cardiomyogenesis involves a dynamic interaction of genetic and epi-genetic (environmental) factors [Taber, 2001], the latter including ROS as a key stimulus during early development of the heart [Sauer et al., 2000, 2004, 2005].

Present results revealed that treatment of EBs with ICA resulted in increased and accelerated differentiation into beating cardiomyocytes. The facilitating activity of ICT was less than that of ICA, furthermore, DICT had the least but still significant promoting effect on the directional cardiac differentiation of ES cells.

ICT and DICT, but not ICA have been found to exert strong binding to estrogen receptors and

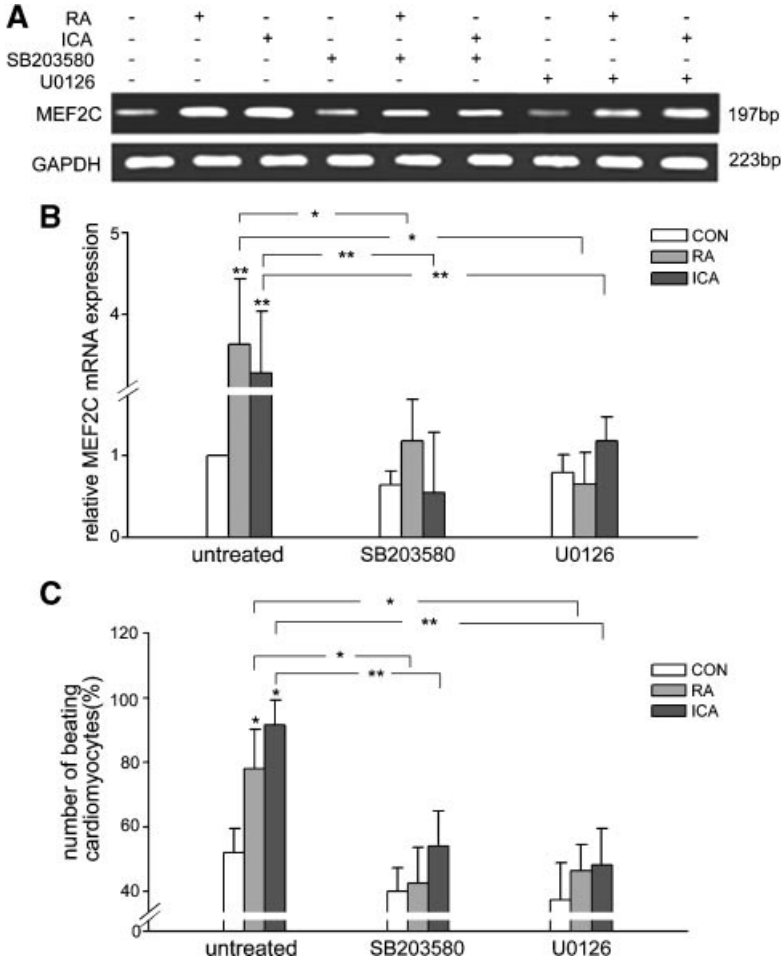


Fig. 9. Effects of inhibition of ERK1, 2, or p38 pathways on ICA induced cardiomyogenesis. **A**,**B**: Effects of inhibition of ERK1, 2, or p38 pathways on *MEF2C* expression 24 h following ICA application. **C**: Effects of inhibition of ERK1, 2, or p38 pathways on ICA induced cardiomyocyte differentiation. EBs were treated from day 5 to day 5 + 11 with either the ERK1, 2 inhibitor U0126 (10 μM), or the p38 inhibitor SB203580 (1 μM), cardiomyogenesis was assessed by counting the number of spontaneously contracting cardiomyocytes foci on day 5 + 11; n = 3. $^{*}P < 0.05$ and $^{**}P < 0.01$.

the resulting estrogenic activity in our previous works [Wang and Lou, 2004; Wang et al., 2006], however, it seems that the estrogenic activity might not involved in the prenylflavonoids induced cardiac differentiation, since the inducible effects were inverse ratio with the estrogenic activity.

The different effects of ICA and ICT on the NADPH-ROS-MAPKs signaling cascades were investigated subsequently. Our data revealed that ICA and ICT share the equal effects on the activation of Nox4 NADPH oxidase which has been reported to be the major NOX isoform present during early stages of cardiac differentiation of ES cells [Li et al., 2006]. Therefore, the question whether it is the difference in the regulation of intracellular ROS concentration and activation of ERK1, 2, and p38 downstream resulted in the different promoting effects of ICA and ICT on cardiac differentiation was raised.

Several studies have shown the potential use of free radicals and ROS to facilitate ES cells differentiation in vitro [Moreno-Lopez et al., 2000; Sauer et al., 2000; Mody et al., 2001; Oravecz et al., 2002]. ROS generated at low concentrations exert a role as signaling molecules that are involved in signal transduction cascades of numerous growth factor-, cytokine-, and hormone-mediated pathways, and regulate biological effects such as apoptosis, cell proliferation, and differentiation [Sauer et al., 2001]. Several researches have shown that cardiomyogenic differentiation of ES cells within EBs was enhanced in the presence of hydrogen peroxide [Sauer et al., 2000]. On the other hand,

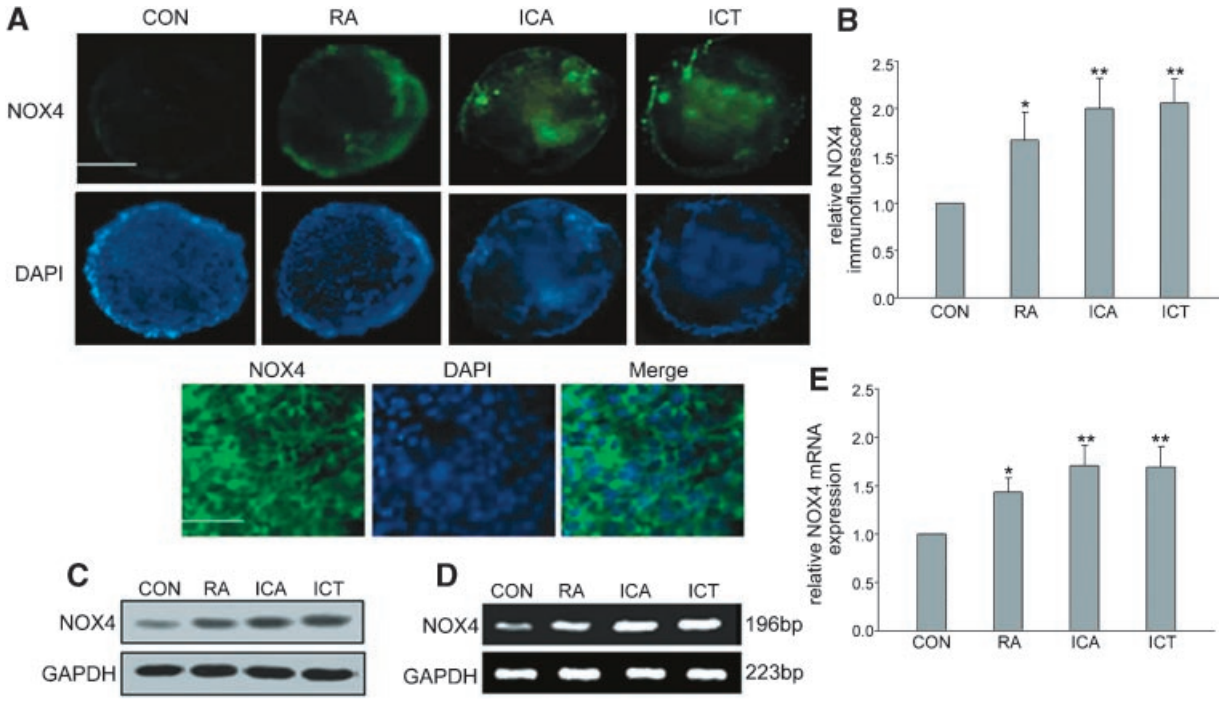


Fig. 10. Effects of ICA and ICT on the expression of NOX4 during cardiomyocyte differentiation. **A**: Representative images of EBs labeled with antibodies for NOX4 analyzed by immunohistochemistry 2 h after ICA or ICT application; **upper panel**: EBs labeled with antibodies for NOX4; **middle panel**: Nuclear blue stain with DAPI (4′, 6-diamino-2-phenylindole); **below panel**: higher magnification images. Scale bar = 200 μm (upper and middle panels), 100 μm (below panel). **B**: Plots of semiquantitative analysis of NOX4 fluorescent signals. **C**: NOX4 expression evaluated by Western blot 2 h after ICA or ICT application. **D**: NOX4 mRNA expression 30 min after treatment with ICA or ICT. **E**: Plots of semiquantitative analysis of NOX4 mRNA expression. n = 3; $^{*}P < 0.05$ and $^{**}P < 0.01$.

incubation with free radical scavengers and antioxidants had an inhibitory effect on cardiomyogenic differentiation.

Further evidence demonstrated that cardiac differentiation induced by ICA was parallel with rapidly ROS generation in EBs. ROS production could be abolished by either NADPH-oxidase inhibitor apocynin or free radical scavengers vitamin E or PDTC. Meanwhile, the beating cardiomyocytes in EBs outgrowths induced by ICA were inhibited when treated with free radical scavengers. In addition to the phenomenological investigation of ICA stimulated cardiomyogenesis by quantification of beating EBs, we assessed the expression of transcription factors known to be involved in cardiomyogenesis of ES cells and embryonic heart development. In the present studies, we found that ICA robustly increased the expression of *MEF2C*, which was inhibited in the presence of either vitamin E or PDTC. These data clearly indicated that cardiac differentiation induced by ICA is critically dependent on the intracellular ROS via NADPH-oxidase activation.

Using PCL method, it was demonstrated that DICT displayed the strongest anti-oxidant activity, while ICA displayed the weakest in vitro. It has been implicated that flavonoids exhibit an anti-oxidative effect [Bors et al., 1990], which may play an important role in preventing or delaying disease process, such as cardiovascular disease [Devasagayam et al., 2004; Manach et al., 2005; Hill et al., 2006; Riccioni et al., 2007]. The anti-oxidative activity of flavonoids depend on the OH numbers and the positions in the structure [Cao et al., 1997; Heim et al., 2002]. Among the three prenylflavonoids, ICT has two OH groups more bound to C-3 and C-7 compared with ICA. C-7 OH is prone to form complex with transition metal and displays stronger acidity, which has the tendency to exhibit anti-oxidative activity. Comparatively, DICT possesses one further OH group bound to C-4′ than ICT, the formation of P-p conjugation between C-7 OH and C-4′ OH

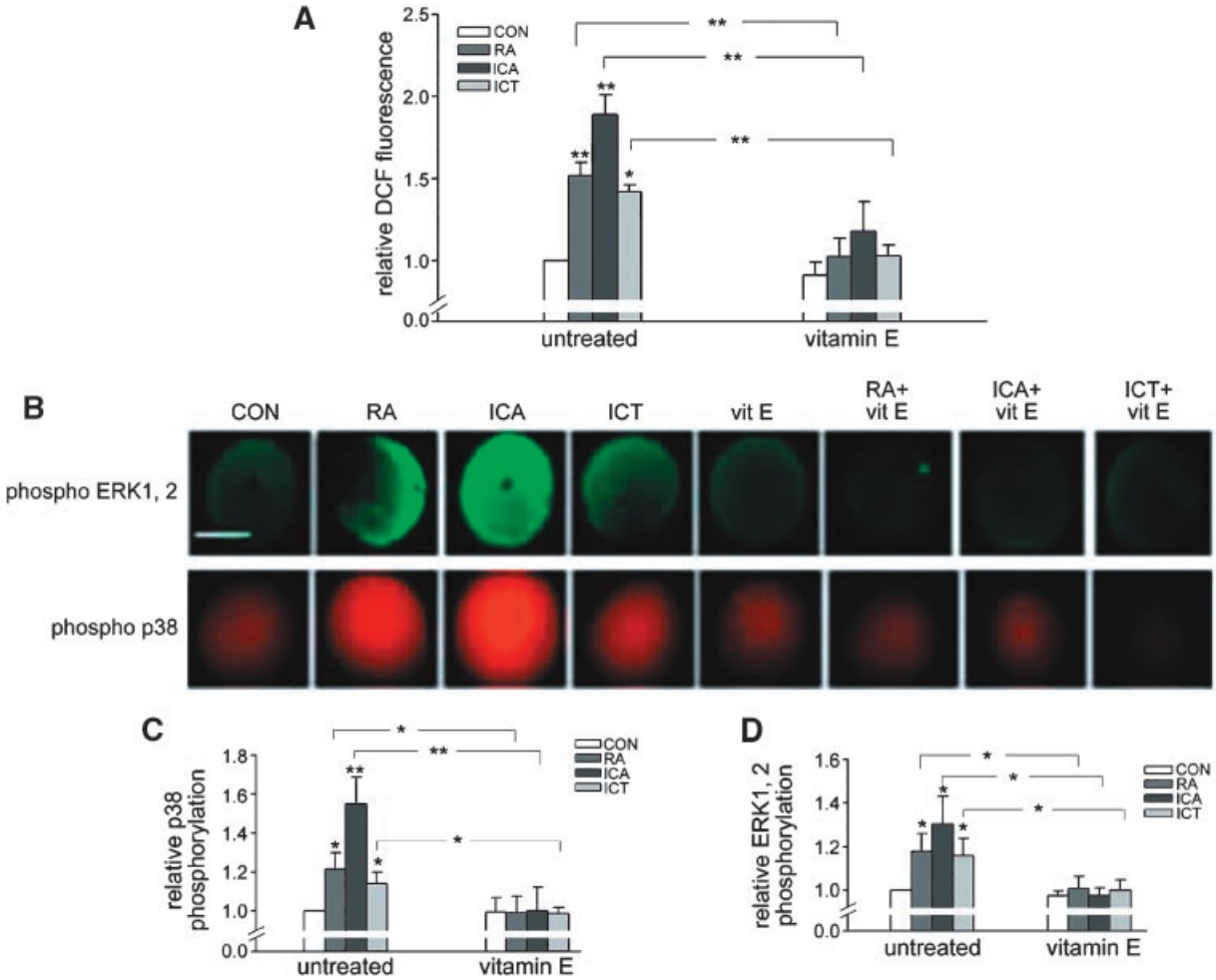


Fig. 11. Effects of ICA and ICT on the generation of ROS, and the activation of ERK1, 2, and p38. **A**: Intracellular ROS generation was assessed at 2 h by use of the redox-sensitive dye DCF-DA. **B**: Representative images of EBs labeled with antibodies for active ERK1, 2, and p38 analyzed by immunohistochemistry after either 2 h (ERK1, 2) or 30 min (p38) of ICA or ICT application (the bar represents 200 μm). **C**,**D**: Plots of semiquantitative analysis of ERK1, 2, and p38 fluorescent signals; n = 3. $^{*}P < 0.05$ and $^{**}P < 0.01$.

extends the conjugative system and accelerates the electron delocalization of the prenylflavonoids, redounding to form the relatively stabilized radical intermediates which further enhances the anti-oxidative activity.

MAPKs have been reported to be regulated by ROS in cardiovascular differentiation of ES cells [Schmelter et al., 2006]. In the present studies, phosphorylation of ERK1, 2, and p38 were observed following application of ICA, obviously, it is clearly ROS-dependent, since incubation with free radical scavenger vitamin E abolishes the observed effects; treatment with the ERK1, 2 inhibitor U0126 or the p38 inhibitor SB203580 notably inhibits the cardiomyogenesis. This point toward the notion that ROS-dependent ERK1, 2 and p38 signaling are involved in ICA induced cardiac differentiation, indicating NADPH-ROS-MAPKs signaling cascades required for ICA induced cardiac differentiation.

Our data point toward that there is no difference of ICA and ICT on the activation of intracellular ROS, for their effects on the activation of NOX4 were similar; the ROS eliminated by ICT on account of its anti-oxidative activity resulted in its weaker effects on the activation of ERK1, 2, and p38, and leading to lower enhancing effect on cardiomyogenesis consequently. In conclusion, present data uncovered the underlying mechanisms that result in the different inducible effects of prenylflavonoids, ICA, ICT, and DICT, on cardiac differentiation and deciphered why

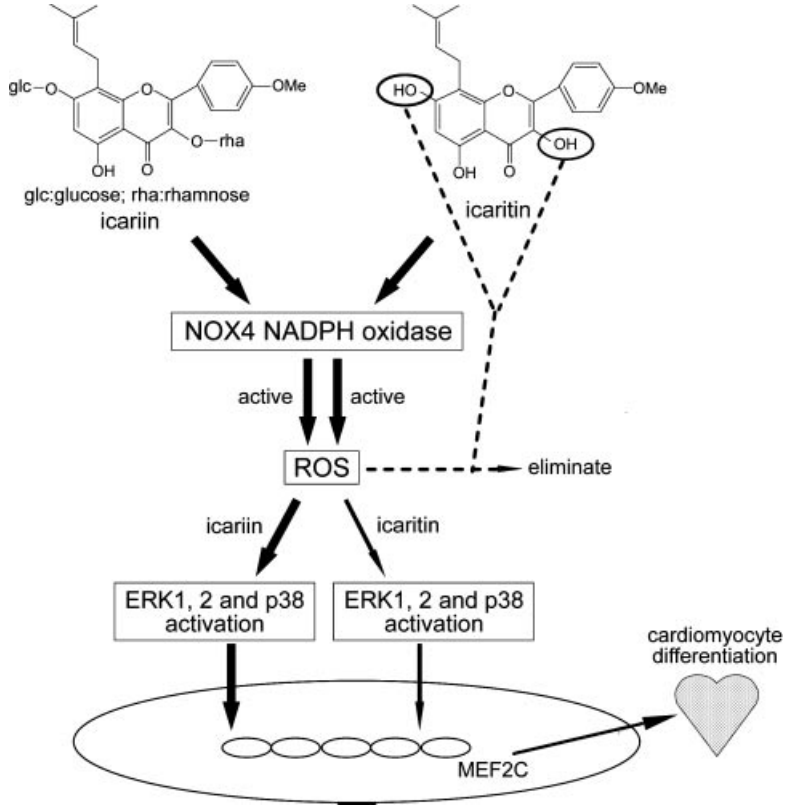


Fig. 12. Model depicting ROS signaling pathways involved in ICA and ICT induced cardiomyocyte differentiation of ES cells. The real line represents activation, while the broken line represents elimination; the thick or thin arrows represent the strong or weak effect, respectively. ROS generated by the NOX4 NADPH oxidase act through ERK1, 2, and p38, resulting in *MEF2C* expression and thereafter cardiomyocyte differentiation. ICT eliminates parts of ROS generated by itself, therefore, displays lower inducible effect on cardiomyocyte differentiation.

ICT elevated intracellular ROS in EBs paradoxically in spite of its anti-oxidative activity (Fig. 12). At upstream, all the three prenylflavonoids activated Nox4 NADPH oxidase, which stimulated intracellular ROS and followed by MAPKs signaling cascades activation. It is the NADPH-ROS-MAPKs signaling cascades that ICA, ICT and probably DICT regulated a microenvironment suitable for inducing cardiomyogenesis differentiation programs, but the anti-oxidative activities due to the OH numbers and the positions in the structure do influence the effects of differentiation initiation in vitro. The findings provide a new insight into the prenylflavonoids induced cardiac differentiation of ES cells and may ultimately contribute to the future therapeutic use of ES cells.

ACKNOWLEDGMENTS

The authors thank Bo Li for generously supplying PHOTOCHEM®, and Yan-fen Fang in 985 platform, Zhejiang University for FACS analysis.

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