Baicalin alleviates H_2O_2 -induced injury of H9c2 cardiomyocytes through suppression of the Wnt/ β -catenin signaling pathway

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Received April 5, 2017; Accepted September 6, 2017

DOI: 10.3892/mmr.2017.7748

Abstract. Baicalin is one of the active ingredients extracted from the dry root of Scutellaria baicalensis Georgi, which has been reported to be effective in preventing myocardial ischemia reperfusion injury. However, the mechanisms underlying its cardioprotective activities remain to be elucidated. In the present study, H₂O₂-treated cardiomyocyte H9c2 cell line served as an in vitro model of oxidation-damaged cardiomyocytes to evaluate the effects of baicalin on the cardiac injury, and to investigate the underlying molecular mechanism. The results of the TOPFlash/Renilla reporter gene assay indicated that baicalin significantly suppressed the activation of proto-oncogene Wnt-1 (Wnt)/β-catenin in 293 cells, in a dose dependent manner. In addition, baicalin significantly inhibited H₂O₂-induced loss of H9c2 cell viability in MTT assay. Furthermore, western blotting analysis demonstrated that baicalin markedly attenuated H₂O₂-induced cell apoptosis, as demonstrated by the down-regulation of cleaved caspase-3 and the increase in the apoptosis regulator Bcl-2 (Bcl-2)/apoptosis regulator BAX (Bax) ratio following baicalin treatment in H₂O₂-treated H9c2 cells. Furthermore, baicalin markedly decreased the expression of β -catenin and downstream Axin-2 and myc proto-oncogene protein in H₂O₂-treated H9c2 cells.

Abbreviations: APC, adenomatous polyposis coli; GSK-3β, glycogensynthase kinase-3β; LRP5/6, low-density lipoprotein receptor-related protein 5/6; Dvl, Dishevelled; NRX, nucleoredoxin; ROS, reactive oxygen species; IHD, ischemic heart disease

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Key words: baicalin, H9c2 cells, cardiac injury, Wnt/β-catenin signal pathway

Knockdown of β -catenin expression inhibited H₂O₂-induced cell apoptosis. Finally, LiCl (a β -catenin stabilizer) induced apoptosis of H9c2 cells by upregulating the expression of β -catenin, which was significantly neutralized by the treatment with baicalin. Taken together, it is hypothesized that baicalin exerts cardioprotective effects via suppression of the Wnt/ β -catenin signaling pathway.

Introduction

Proto-oncogene Wnt-1 (Wnt)/ β -catenin signaling is associated with many developmental and physiological processes during early stages of embryonic development (1-4). The deregulation of Wnt signaling often leads to abnormalities, including heart disease (5-8). In the absence of the Wnt ligand, intracellular β -catenin is constantly degraded by the adenomatous polyposis coli (APC)/Axin/glycogensynthase kinase-3 β (GSK-3 β) complex. However, once Wnt ligand binds to the membrane receptors Frizzled and low density lipoprotein receptor related protein 5/6 (LRP5/6), it leads to the activation of Dishevelled (Dvl), resulting in the suppression of the APC/Axin/GSK-3 β complex and activation of nuclear β -catenin, and associated downstream genes, including Axin2 and myc proto-oncogene protein (c-Myc) (9-11).

Baicalin (5,6-dihydroxyflavone-7-O-D-glucuronic) is an active ingredient extracted from dried roots of Scutellaria baicalensis Georgi. Baicalin has been used for the clinical treatment of certain acute and chronic inflammatory diseases. Baicalin attenuates oxidative damage and apoptosis of myocardial cells and protects against myocardial ischemia reperfusion injury (12,13). Previous in vivo studies suggested that cardioprotection mediated by baicalin was associated with the modulation of the mitogen-activated protein kinase 1 pathway (14,15). Baicalin may decrease the activity of the sympathetic nervous system by inhibiting the P2X3 receptor in rat superior cervical ganglia to protect the myocardium from the ischemic injury (16). Another study demonstrated that baicalin can protect cardiomyocytes from endoplasmic reticulum stress-induced apoptosis via the DNA-damage-inducible transcript/CCAAT/enhancer-binding protein/endothelia nitric oxide synthase/nitric oxide pathway (17). However, the Wnt/β-catenin signaling pathway-mediated molecular

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mechanism underlying the cardioprotective effect of baicalin remains to be elucidated.

Since the concentration of reactive oxygen species (ROS) is elevated in cardiac injury, including ischemic heart disease (IHD), H_2O_2 -treated cardiomyocyte cell lines have commonly been used as an *in vitro* model of cardiac injury to study the cardioprotective effects of drugs against oxidative damage (18-23). Therefore, in the present study, H_2O_2 -treated H9c2 cells were used to evaluate the effects of baicalin on cardiac injury and on the activation of Wnt/ β -catenin signal pathway.

Materials and methods

Cell culture, transfection and treatment. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Wisent Inc., St Bruno, QC, Canada) supplemented with 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), 100 U/ml penicillin and 100 mg/ml streptomycin (both, purchased from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and cultured in a 37°C humidified incubator supplemented with 5% CO₂. For the transfection assay, cells were plated at 1.0x10⁵ cells/well and were transfected with small interfering (si)RNA of β-catenin (Invitrogen; Thermo Fisher Scientific, Inc.) or Stealth RNAi® negative control duplexes (NC) (cat. no. 12935300, Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h using Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The following β -catenin siRNA oligo sequences were used: 5'-UGUAGC AGGAGAUUAUGCAGCGUGG-3' (forward) and 5'-CCA CGCUGCAUAAUCUCCUGCUACA-3' (reverse). H9c2 cells were stimulated with 200 μ M H₂O₂ for 2 h prior to an MTT assay and with 100 μ M H₂O₂ for 30 min prior to the western blotting assay, to establish the myocardial oxidative damage model. 293T cells were co-transfected with 1 ng/well V5-tagged Wnt3a (Wnt3a), 30 mM LiCl, 0.5 µM BIO or 40 ng/well β -catenin ΔN (β -catenin truncation mutant with N-terminal region deletion), as well as TOPFlash for 48 h before detecting RLU (relative light units) values in each experimental group. Negative control group was transfected with TOPFlash only and positive control group was transfected with wnt3a or activator of Wnt/β-catenin signaling pathway (LiCl, BIO or β -catenin ΔN), as well as TOPFlash but not treated with baicalin. Wnt3a was provided by X. He and C. Niehrs (German Cancer Research Center) (24-27). TOPFlash reporters were provided by RT. Moon (University of Washington, School of Medicine) (28). LiCl, BIO and NaCl were purchased from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany. Baicalin was purchased from Chengdu Must Bio-Technology Co., Ltd.; Chengdu, Sichuan, China.

Reporter gene assay. A TOPFlash/Renilla reporter gene assay was performed as previously described (24,29). Cells were seeded in 96-well plates at $4x10^3$ cells/well and transfected with 40 ng TOPFlash plasmids for Wnt and β -catenin and 10 ng Renilla luciferase control reporter vector. A total of 48 h following transfection, cells were lysed with cell lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% nadeoxycholale, 0.5 M EDTA, 1% Nonidet 40,5 mM DTT, 10 µg aprotinin, 10 µg leupeptin, 10 mM PMSF) and screened for luciferase activity using the Dual-Luciferase Reporter Assay system according to the manufacturer's protocol (Promega Corporation, Madison, WI, USA).

Cell isolation and protein extraction. For total cellular protein extraction, cells were lysed with a lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% nadeoxycholale, 0.5 M EDTA, 1% Nonidet 40, 5 mM DTT, 10 μ g aprotinin, 10 μ g leupeptin, 10 mM PMSF) on ice for 15 min and centrifuged at 4,000 x g for 15 min at 4°C, prior to the collection of the supernatant. Nuclear extracts were prepared as follows: Cells were resuspended in 400 μ l hypotonic buffer (20 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl₂; 10% Nonidet; 5 M DTT; 10 μ g Aprotinin; 10 μ g Leupeptin; 10 mM PMSF) and incubated on ice for 10 min, then centrifuged at 1,000 x g for 5 min at 4°C. Following separation, the pellet was re-suspended in 4,000 x g for 10 min at 4°C, the supernatant (nuclear extract) was collected.

Western blotting. Concentrations of proteins were quantified using the bicinchoninic acid method. Subsequently, 30 μ g proteins per lane were subjected in 10% sodium dodecyl polyacrylamide gel (Sangon Biotech Co., Ltd., Shanghai, China) using 1x SDS-PAGE running buffer (cat. no. P0014; Beyotime Institute of Biotechnology, Haimen, China), and then transferred onto a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA) using 1x western transfer buffer (cat. no. P0021A; Beyotime Institute of Biotechnology). Following blocking with 5% skimmed dry milk in TBS (20 mM Tris-HCl pH 7.4, 150 mM NaCl) with 0.1% Tween 20 (TBST) for 1 h at room temperature, membranes were incubated with the following polyclonal primary antibodies: Rabbit anti-Axin-2 (1:5,000; cat. no. ab32197; Abcam, Cambridge, UK), mouse anti-c-Myc (1:5,000; cat. no. ab32, Abcam), rabbit anti-β-catenin (1:1,000; cat. no. 8480; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-apoptosis regulator Bcl-2 (Bcl-2) (1:1,000; cat. no. 2870; Cell Signaling Technology, Inc.), rabbit anti-apoptosis regulator BAX (Bax) (1:1,000; cat. no. 2772; Cell Signaling Technology, Inc.), rabbit anti-cleaved caspase-3 (1:1,000; cat. no. 9664; Cell Signaling Technology, Inc.) and mouse anti-GAPDH (1:1,000; cat. no. 60004-1-Ig; ProteinTech Group, Inc., Chicago, IL, USA) at 4°C overnight. The membranes were subsequently washed with TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:25,000; cat. no. 31460; Thermo Fisher Scientific, Inc.) or rabbit anti-mouse IgG secondary antibody (1:25,000; cat. no. 27025; Thermo Fisher Scientific Inc.) at room temperature for 1 h. Following washing by TBST, the membranes were placed in enhanced chemifluorescence solution (EMD Millipore) for 1 min. Immunoreactive bands were visualized by an Image Quant LAS 500 Imager (GE Healthcare Life Sciences, Shanghai, China), and the densitometry of bands were calculated by Quantity One software (version 4.62; Bio-Rad laboratories Inc., Hercules, CA, USA).

MTT assay. H9c2 cells were seeded in 96-well plates at a density of $4x10^3$ cells/well. MTT (0.5 mg/ml; Beyotime Institute of Biotechnology) was added and cells were incubated



Figure 1. Baicalin inhibits Wnt/ β -catenin signaling pathway in 293 cells. RLU values for 293 cells co-transfected with (A) Wnt3a, (B) LiCl, (C) BIO or (D) β -catenin Δ N, as well as TOPFlash. All groups were treated with 0, 10, 50 or 100 μ M baicalin for 48 h. Negative control values were normalized to 1. *P<0.05 vs. the negative control group, *P<0.05 vs. positive control group. Wnt; proto-oncogene Wnt-1; β -catenin Δ N, β -catenin mutant with N-terminal region deletion; RLU, relative light units.

for 4 h at 37°C prior to the addition of dimethyl sulfoxide. The absorbance was measured at a wavelength of 490 nm according to the manufacturer's protocol (SpectraMax® M2/M2e Multiskan; Molecular Devices LLC, Sunnyvale, CA, USA). All experiments were performed in quintuplicate.

Statistical analysis. All data are expressed as the mean \pm standard deviation. All statistical analyses were performed using SPSS software (version 20.0; IBM Corp., Armonk, NY, USA). Statistical significance was concluded by one-way analysis of variance, followed by the Fisher post hoc test for multiple comparisons and unpaired t-test for between two groups comparisons. All experiments were replicated at least three times. P<0.05 was considered to indicate a statistically significant difference.

Results

Baicalin inhibits the activation of the Wnt/ β -catenin pathway in 293 cells. The TOPFlash/Renilla reporter gene assay was used to evaluate the effect of baicalin on the activation of the Wnt/ β -catenin pathway in 293 cells. As presented in Fig. 1, Wnt/ β -catenin signaling was significantly activated upon stimulation with Wnt3a, LiCl, BIO and β -catenin ΔN . However, baicalin induced a specific and significant inhibitory effect on the activation of TOPFlash with in a dose-dependent manner, demonstrating that baicalin efficiently inhibits the Wnt/ β -catenin signaling pathway.

Baicalin protects against H2O2-induced loss of H9c2 cell viability. To evaluate the cardioprotective effects of baicalin



Figure 2. Baicalin protects against H_2O_2 -induced loss of H9c2 cell viability. H9c2 cell viability was measured using an MTT assay. (A) H9c2 cells were pretreated with indicated concentrations of baicalin for 24 h prior to treatment with H_2O_2 or PBS with in the negative control group. (B) Cells were treated with baicalin alone for 24 h. In the negative control group the optical density at a wavelength of 490 nm was normalized to 1. *P<0.05 vs. the negative control group; *P<0.05 vs. positive control group.



Figure 3. Baicalin prevents H_2O_2 -induced apoptosis of H9c2 cells. Expression of Bcl-2, Bax, cleaved caspase-3 and Bcl-2/Bax ratio in H9c2 cells was detected following pre-treatment with different concentrations of baicalin for 24 h and subsequent H_2O_2 treatment. GAPDH served as a loading control. Bcl-2/Bax ratio in control cells were normalized to 1. *P<0.05 vs. the control group; *P<0.05 and vs. the H_2O_2 -stimulated alone group. Bcl-2, apoptosis regulator; Bax, apoptosis regulator BAX.

against H_2O_2 -induced cardiotoxicity, H_2O_2 -stimulated H9c2 cells were treated with different concentrations of baicalin and cell viability was assessed using an MTT assay. Stimulation with H_2O_2 led to a significant decrease in H9c2 cell viability, which was significantly reversed by the treatment with baicalin in a dose-dependent manner (Fig. 2A). Treatment with baicalin alone exhibited no effect on the viability of H9c2 cells even at increased concentrations (Fig. 2B). Therefore, baicalin can protect against oxidative damage of cardiomyocytes *in vitro*, without causing apparent cytotoxicity.

Baicalin prevents H2O2-induced apoptosis of H9c2 cells. The effect of baicalin on H_2O_2 -induced apoptosis of H9c2 cells was examined using western blot analysis. As presented in Fig. 3, H_2O_2 upregulated the expression of cleaved caspase-3



Figure 4. Decreased expression of β -catenin prevents H₂O₂-induced apoptosis of H9c2 cells. (A) Expression of Axin, c-Myc and β -catenin were detected following pre-treatment with different concentrations of baicalin for 24 h and subsequent H₂O₂ treatment in H9c2 cells. (B) Expression of β -catenin, Bcl-2, Bax, cleaved caspase-3 and Bcl-2/Bax ratio following siRNA knockdown of β -catenin for 48 h in H9c2 cells and subsequent treatment with 100 μ M of H₂O₂ at 30 min. GAPDH, loading control. *P<0.05 vs. the control group; *P<0.05 and vs. the H₂O₂-stimulated alone group). c-Myc, myc proto-oncogene protein; Bcl-2, apoptosis regulator; Bax, apoptosis regulator; BAX; siRNA, small interfering RNA.

and increased the pro-apoptotic Bax/Bcl-2 ratio in H9c2 cells, indicating pro-apoptotic property of H_2O_2 . However, baicalin significantly inhibited H_2O_2 -induced cardiomyocyte apoptosis, in a dose-dependent manner (Fig. 3).

Baicalin inhibits apoptosis by suppressing β -catenin expression in H9c2 cells. To investigate the mechanism underlying the anti-apoptotic and cardioprotective activities of baicalin, its effect on the expression levels of β -catenin and its downstream targets axin-2 and c-Myc in H9c2 cells was determined. As presented in Fig. 4A, baicalin treatment significantly suppressed H₂O₂-induced expression of β -catenin, axin-2 and c-Myc. Additionally, siRNA-mediated downregulation of β -catenin expression prevented apoptosis in H₂O₂-treated H9c2 cells (Fig. 4B). LiCl, a GSK-3 β



Figure 5. Baicalin attenuates LiCl-induced injury in H9c2 cells. Expression of Bcl-2, Bax, cleaved caspase-3 and β -catenin was detected in H9c2 cells following treatment with 10 mM LiCl and/or 10 μ M baicalin for 24 h. GAPDH served as a loading control. *P<0.05 vs. the control group; *P<0.05 vs. the LiCl-treated group. Bcl-2, apoptosis regulator; Bax, apoptosis regulator BAX.

inhibitor, commonly used to stabilize β -catenin, was used to further verify above-observed results (30-32). As presented in Fig. 5, LiCl increased β -catenin expression, which in turn induced apoptosis of H9c2 cells. However, LiCl-induced injury in H9c2 cells was significantly neutralized by the treatment with baicalin (Fig. 5). Taken together, the results of the present study indicate that baicalin exerts an anti-apoptotic activity, likely through the suppression of the Wnt/ β -catenin pathway.

Discussion

The present study demonstrated that baicalin attenuated H₂O₂-induced H9c2 cell injury through inhibition of the Wnt/β-catenin signaling pathway. It was demonstrated that β -catenin serves a crucial role in enhancing H₂O₂-induced injury of H9c2 cells, which is attenuated by pretreatment with baicalin. Previous studies have demonstrated that ROS can modulate the Wnt/ β -catenin signaling pathway via regulation of the redox-dependent interaction between Dvl and nucleoredoxin (NRX). NRX normally interacts with Dvl and inhibits activation of the Wnt/\beta-catenin pathway. However, under oxidative stress, NRX dissociates from Dvl and enables Dvl to activate Wnt/\beta-catenin signaling (33). An association between Dvl and NRX supports the results of the present study, and suggests that ROS can promote the activation of Wnt/β-catenin signaling, suggesting that baicalin can potentially regulate the expression of Dvl/NRX.

The present study indicates that the suppression of Wnt/ β -catenin signaling pathway can be a potential strategy for the treatment of IHD. It has been previously demonstrated that insulin-like growth factor binding protein 4 is a robust inhibitor of Wnt/ β -catenin signaling, which can effectively protect the heart from the ischemic injury (8,34), which further supports the results of the present study. Baicalin is commonly used in the clinical treatment of various diseases. Whether

baicalin can be applied for the treatment of IHD remains to be elucidated.

In conclusion, the present study is, to the best of the authors' knowledge, the first one to demonstrate the role of baicalin in the modulation of Wnt/ β -catenin signaling pathway. Baicalin suppresses the activation of Wnt/ β -catenin signaling pathway via regulation of β -catenin, leading to the protection against H₂O₂-induced apoptosis of H9c2 cells. The present study provides novel insights and theoretical basis for the potential use of baicalin in the treatment of IHD.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant no. 81302884) and the Developmental Fund of Chen Keji Integrative Medicine (grants no. CKJ 2016005, 2016003 and 2016002).

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