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Baicalin Suppresses Hypoxia-Reoxygenation-Induced Arterial Endothelial Cell Apoptosis via Suppressing PKCδ/p53 Signaling

Authors' Contribution:
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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Funds Collection G

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Background: This study was aimed to investigate the protective role of baicalin on vascular endothelium exposed to ischemia reperfusion injury and the involved molecular mechanisms.

Material/Methods: Cultured human arterial endothelial cells (HAECs) were exposed to hypoxia/deoxygenation (H/R). Cells were also treated with baicalin at serially diluted concentrations. Cells were also treated with PKC activator PEP005 or specific siRNA against protein kinase Cδ (PKCδ). MTT assay was used to evaluate the cell viabilities. Flow cytometry was used to detect cell apoptosis. The protein phosphorylation and expression levels were determined by Western blotting.

Results: PKCδ-siRNA transfection increased cell viabilities and reduced cell apoptosis in HAECs exposed to H/R. Baicalin treatment preserved cell viabilities and reduced apoptosis of H/R-exposed HAECs in a concentration- dependent manner. Baicalin treatment reduced phosphorylation levels of PKCδ and p53, as well as the expression levels of active caspase3 and bax in HAECs exposed to H/R. The treatment of PKC activator PEP005 impaired the protective effects of baicalin in increasing cell viabilities and reducing apoptosis in HAECs exposed to H/R.

Conclusions: Baicalin exerts vascular a protective effect on HAECs exposed to H/R by reducing cell apoptosis. The PKCδ/p53 apoptotic signaling pathway was the pharmacological target of baicalin.

MeSH Keywords: Apoptosis • Endothelial Cells • Protein Kinase C-delta • Reperfusion Injury

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Background

Ischemia reperfusion (I/R) injury occurs after restoration of blood flow following ischemia, which exacerbates the cell damage [1]. It is accepted that I/R injury is one of the most prominent and characterized pathological processes involved in the onset of acute coronary syndrome (ACS) [2]. Arterial endothelial cells (ECs) are an important component of the vessel wall, and are responsible for the integrity of the inner lumen of vessels. Ischemia can lead to injury of ECs, which receive further perpetuated damages from reperfusion. I/R injury impairs the endothelial barrier function [3]. The increased vascular permeability, discontinuity, and inflammation will eventually result in pathological changes such as thrombosis, myocardial hematoma, and myocardium apoptosis [4].

The molecular mechanism of I/R injury is very complicated: oxidative stress, mitochondrial dysfunction, calcium overload, inflammation, and apoptosis were suggested previously [5]. ECs are extremely susceptible to I/R injury according to several previous reports [6]. Apoptosis was identified in ECs exposed to I/R injury within just a few minutes [7]. The endothelial barrier dysfunction and apoptosis have been linked to the activation of protein kinase Cs (PKCs) [8]. One of the novel PKC isoforms, the PKC delta (PKCδ), was indicated to be associated with the responses to DNA damage [9]. The tumor-suppressor p53 was identified as one of the substrates of PKCδ [10]. The activation of PKCδ can lead to modification of p53, which is essential for initiation of activation of the caspase cascade, leading to apoptosis [10].

Baicalin is one of the bio-active components extracted from a Chinese Medical herb, *Scutellaria baicalensis* Georgi (Lamiaceae), which has been used from ancient times in treatments of ischemic diseases such as stroke [11]. Investigations indicated baicalin has various bioactivities, including anti-virus, anti-cancer, anti-inflammatory, and anti-apoptotic properties [12–15]. Several previous studies showed that baicalin was protective against I/R injury in multiple organs such as the brain, kidney, and heart [16–18]. However, the protective effects of baicalin on arterial endothelial cells and related molecular mechanisms were rarely studied. In the current study, cultured human aortic endothelial cells (HAECs) were subjected to hypoxia/reoxygenation (H/R). The protective role of baicalin was investigated. Using the PKC activator and specific siRNA, the involvement of PKCδ/p53 signaling was also evaluated. We believe that the results of this study not only deepen our understanding of the mechanism of I/R vascular injury, but also provide a theoretical basis for the potential clinical application of baicalin in treating cardiovascular diseases.

Material and Methods

Cell culture and H/R exposure

HAECs were purchased from the Type Culture Collection of the Chinese Academy of Sciences. Cells were cultured with endothelial basal medium (EBM-2) supplement with 5% fetal bovine serum (FBS, Invitrogen) and an antibiotic mix (containing 100U/ml penicillin and 0.1 mg/ml streptomycin, Invitrogen) in a humidified normoxia environment with 5%CO_2/95% fresh air at 37°C. Cells at confluence of 80–90% were used for the subsequent experiments. The H/R exposure was performed in accordance with previous descriptions [19]. Briefly, cultured HAECs were washed with phosphate-buffered saline (PBS) 3 times. The original medium was changed with a modified ischemia-mimetic solution [mM, 135 NaCl; 0.33 NaH_2PO_4; 8 KCl; 0.5 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); 5 $CaCl_2$; 20 lactate; pH=6.8]. Cells were then transferred to a hypoxic environment with 5%CO_2/1%O_2/94%N_2 to incubate for 8 h. After that, the medium was changed to the original medium and the cells were incubated under normoxia condition for 2 h. Cells were treated with baicalin at serially diluted concentrations of 0, 10, 20, and 40 μmol/l, respectively. Several cells were also treated with PKC activator PEP005 (Sigma-Aldrich) at a final concentration of 0.1 μmol/l for 12 h. Several cells were also infected with specific small interfering RNA (siRNA) against PKCδ.

siRNA transfection

In the current study, the expression of PKCδ was silenced by using siRNA technique. PKCδ siRNA (Cat# sc-36246, Santa Cruz) was used to knock down PKCδ. Scramble siRNA (Cat# sc-37007, Santa Cruz) was used as control. siRNAs were transfected into cultured HAECs by using Mirus TransIT-TKO reagent (Mirus) for 48 h according to the protocol provided by the manufacturer.

Cell viability assessment

The cell viability was evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay in this study. Briefly, cultured HAECs were seeded in a 96-well plate, and 5 mg/ml MTT (Sigma-Aldrich) was added into each well to incubate the cells for 4 h at 37°C. Dimethyl sulphoxide (DMSO) was then added into each well to dissolve the formazan crystals that formed. The absorbance at 490 nm (A490) was measured with a plate reader. Cell viability was calculated with the formation: cell viability=(A490 of treated cells/A490 of control cells)×100%.

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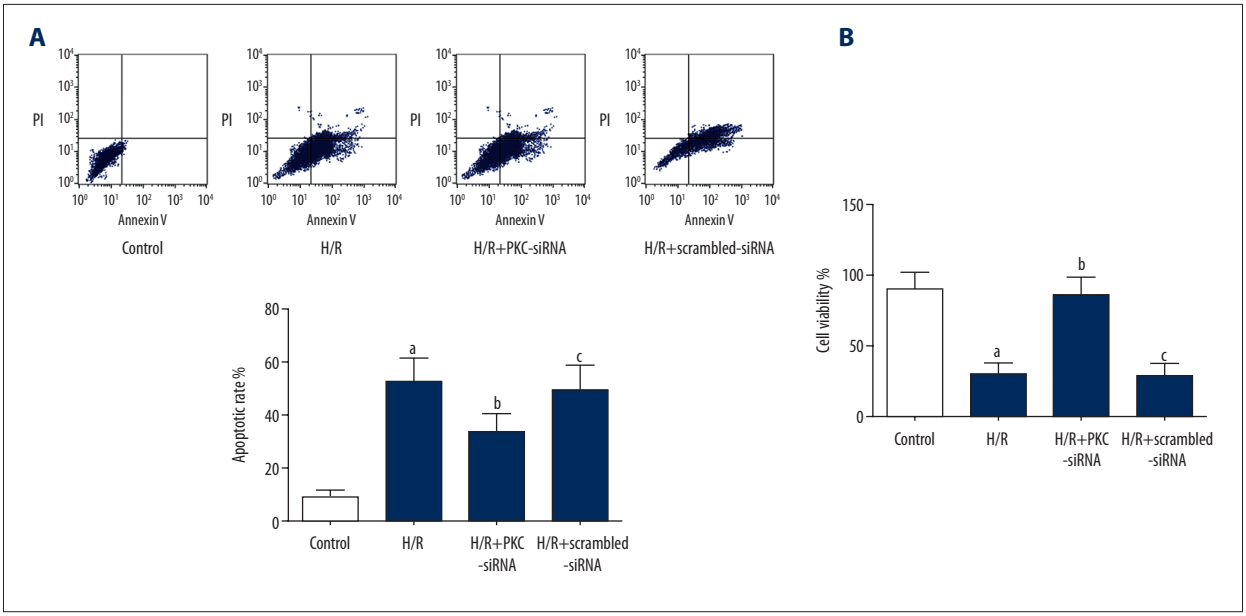

Figure 1. (**A**) The upper part of this figure demonstrates the charts of flow cytometry, which detected cell apoptosis of cultured HAECs treated with H/R, siRNA silencing PKCδ, and scrambled siRNA, respectively. The lower part shows the columns indicating the apoptotic rate of cultured HAECs. (**B**) Columns in this figure indicated the cell viabilities of cultured HAECs treated with H/R, siRNA silencing PKCδ, and scrambled siRNA, respectively. [[a]] differences were significant when compared with Control ($p<0.05$); [[b]] differences were significant when compared with H/R; [[c]] differences were significant when compared with H/R+PKC-siRNA].

Cell apoptosis evaluation

Cell apoptosis of cultured HAECs was detected by flow cytometry in this study. Briefly, after washing in PBS, HAECs cell suspension was incubated with Annexin V-FITC (BD) in a dark chamber at room temperature for 10 min. Then, cells were washed by PBS and resuspended in deliquated binding buffer, which was then incubated with PI (BD) in a dark chamber at room temperature for 15 min. The apoptosis was detected and measured by using a flow cytometer (FACS Calibur, BD).

Western blotting

RIPA lysis system buffer (Santa Cruz) with PSMF (Santa Cruz) was used to lyse the HAECs. The total protein was extracted with a Protein Extraction Kit (Beyotime) according to the protocol provided by the manufacturer. A bicinchoninic acid (BCA) protein assay kit (Pierce) was used to determine the protein concentration before electrophoresis. Proteins samples were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes electronically. Membranes were incubated with blocking buffer (Abcam). Primary antibodies against PKCδ (Cat# ab182126, Abcam), phospho-PKCδ (Cat# ab76181, Abcam), p53 (Cat# ab26, Abcam), phospho-p53 (Cat# ab1101, Abcam), active caspase3 (Cat# ab2302, Abcam), bax (Cat#ab32503, Abcam), and GAPDH (Cat#ab8245, Abcam) were used to incubate the membranes for 8 h at 4°C. Then, the membranes were washed by Tris-buffered Saline with Tween 20 (TBST) and then incubated with secondary antibodies conjugated to horseradish peroxidase HRP (Abcam) for 20 min at room temperature. Signal West Pico reagent (Pierce) was used to develop the membranes which were exposed to X-ray films in a dark room. The densities of immunoblots were then measured and analyzed by the software Image J (version 1.38e, NIH).

Statistics

Data collected in this study are presented as mean±SEM. SPSS software (ver.16.0, SPSS) was used to analyze the data. Differences between groups were assessed by the *t* test and ANOVA. Post hoc tests were carried out with LSD or SNK tests. Differences were considered statistically significant when $p<0.05$.

Results

H/R exposure reduced cell viability by inducing apoptosis in HAECs, which was reversed by siRNA knocking down PKCδ

The results are demonstrated in Figure 1. After H/R exposure, the cell viability of HAECs was significantly inhibited. Moreover, the H/R exposure dramatically induced cell apoptosis. However,

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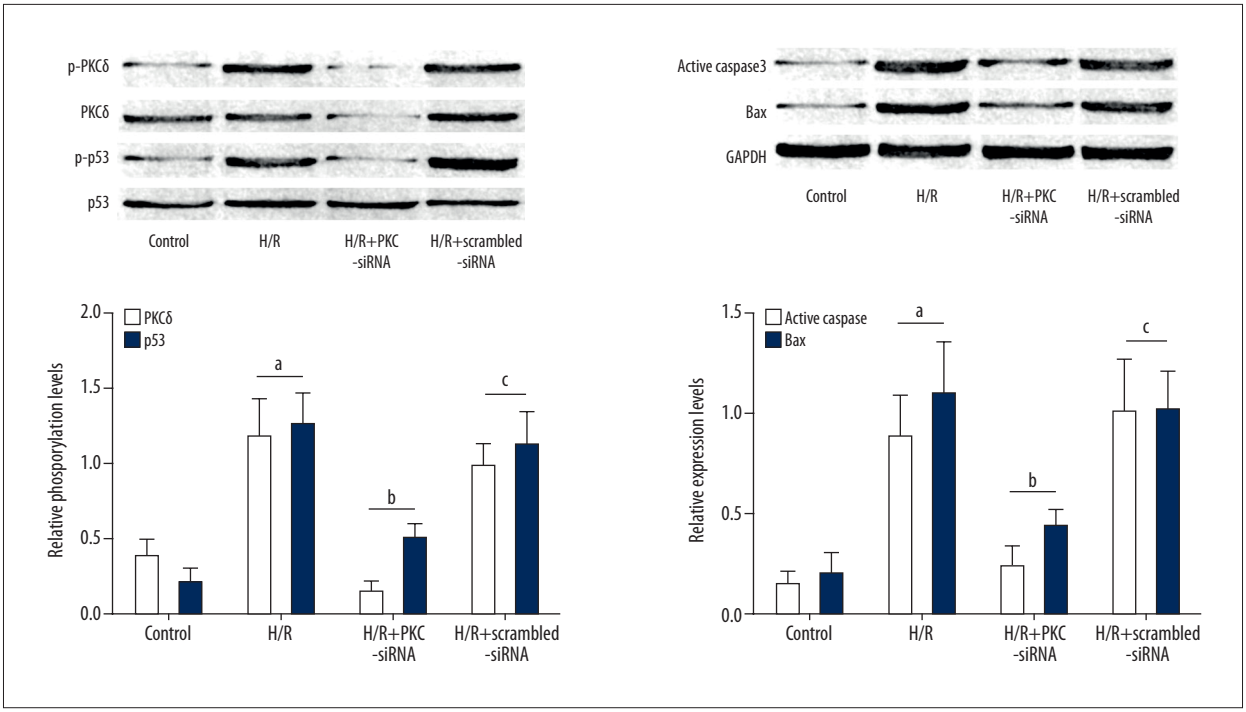

Figure 2. The upper panel of this figure demonstrates the immunoblots of p-PKCδ, PKCδ, p-p53, p53, active caspase3, bax, and GAPDH in cultured HAECs treated with H/R, siRNA silencing PKCδ, and scrambled siRNA, respectively. Columns on the left part of lower panel indicate the relative phosphorylation levels of PKCδ (white) and p53 (blue) in HAECs, respectively. Columns on the right part of lower panel indicate the relative expression levels of active caspase3 (white) and bax (blue) in HAECs, respectively. [[a] differences were significant when compared with Control ($p<0.05$); [b] differences were significant when compared with H/R; [c] differences were significant when compared with H/R+PKC-siRNA]

siRNA against PKCδ significantly reserved cell viability in H/R exposed HAECs. Moreover, siRNA silencing PKCδ also increased the resistance of HAECs to apoptosis induced by H/R exposure.

H/R exposure activated the PKCδ/p53 apoptotic signaling pathway, which was suppressed by PKCδ-siRNA transfection in HAECs. The results are demonstrated in Figure 2. In cultured HAECs exposed to H/R, the phosphorylation levels of PKCδ and p53, along with the expression levels of active caspase3 and bax, were elevated significantly. However, PKCδ- siRNA transfection dramatically decreased the phosphorylated levels of PKCδ and p53. The expression levels of active caspase3 and bax in HAECs were also reduced by PKCδ-siRNA transfection.

Baicalin attenuated H/R- induced cell viability reduction and apoptosis elevation, which was reversed by PKCδ activator PEP005 in HAECs.

The results are demonstrated in Figure 3. Baicalin incubation dramatically increased cell viabilities and decreased apoptotic rate of HAECs exposed to H/R exposure in a concentration-dependent manner. However, in HAECs treated with PEP005, the ability of baicalin to increase cell viability and its anti-apoptotic effects were dramatically impaired.

Baicalin inhibited activation of the PKCδ/p53 apoptotic signaling pathway in HAECs exposed to H/R, which was reversed by PEP005 administration. The results are shown in Figure 4. Baicalin incubation significantly decreased the phosphorylation levels of PKCδ and p53. Baicalin incubation also reduced the expression levels of active caspase3 and bax in HAECs exposed to H/R. The inhibitory effect was in a concentration-dependent manner. However, the PEP005 treatment dramatically increased phosphorylation levels of PKCδ and p53, as well as the expression levels of active caspase3, acting as a counterweight to baicalin treatment in HAECs.

Discussion

I/R injury is thought to be one of the most critical causes of many cardiovascular diseases, acting by undermining the normal function of the blood vessel barrier. *In vivo* and *in vitro* models show that the normal biological functions of tissue or cells were not improved, but instead deteriorated, with the restoration of blood flow or oxygenation [20]. H/R is an accepted and ideal *in vitro* model mimicking I/R injury [21]. It was reported that endothelial cells are very vulnerable to I/R and H/R injury [7,22]. In the present study, we found that H/R

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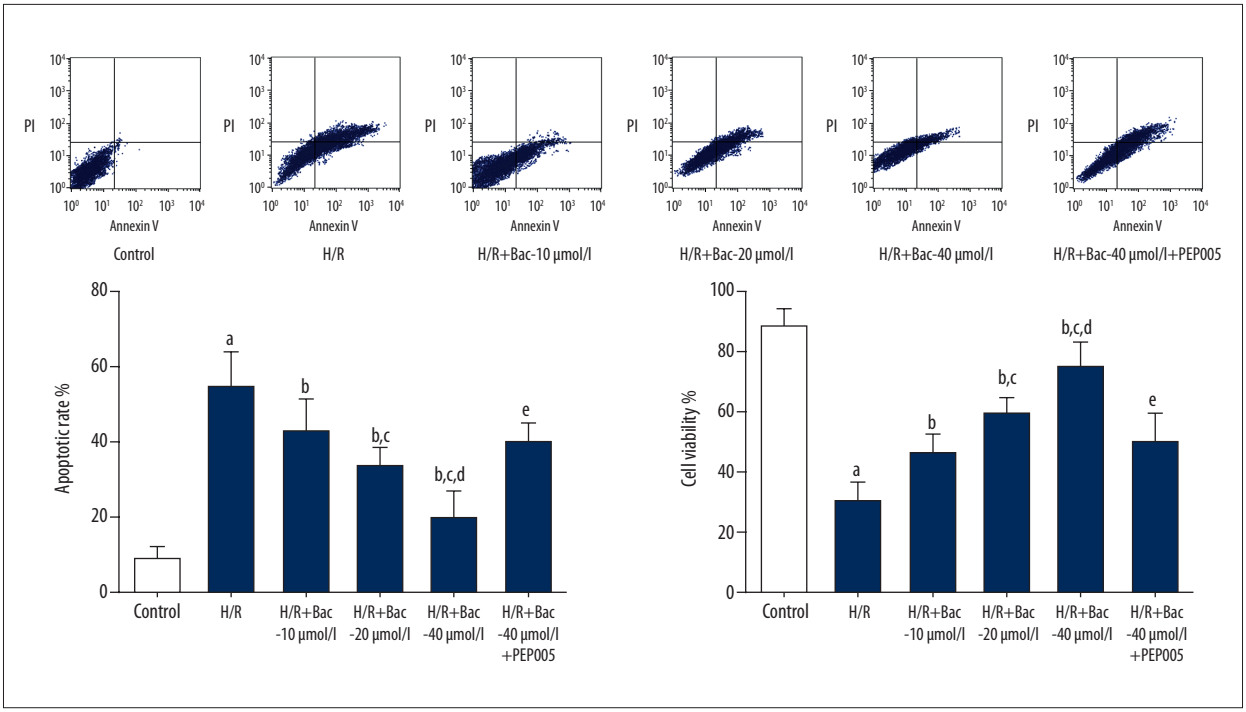

Figure 3. The upper part of this figure demonstrates the charts of flow cytometry detecting apoptosis of cultured HAECs treated with H/R, baicalin (10, 20, and 40 μmol/l), and PEP005. Columns on the left side of the lower panel indicate the apoptotic rate of cultured HAECs. Columns on the right side of the lower panel indicate the cell viabilities of cultured HAECs. [[a]] differences were significant when compared with Control ($p<0.05$); [[b]] differences were significant when compared with H/R ($p<0.05$); [[c]] differences were significant when compared with H/R+Bac-10μmol/l ($p<0.05$); [[d]] differences were significant when compared with H/R+Bac-20 μmol/l ($p<0.05$); [[e]] differences were significant when compared with H/R+Bac-40 μmol/l ($p<0.05$)].

exposure significantly induced apoptosis of cultured HAECs, resulting in the dramatic inhibition of cell viability. However, until now, the molecular mechanisms of I/R-induced cell apoptosis have not been completely elucidated.

Consisting of 10 isoforms, PKCs belong to the AGC family of serine/threonine protein kinases, which is encoded by 9 different genes. These isoforms can be divided into classic, atypical, and novel subfamilies [23]. PKCδ is one of the novel isoforms, playing important roles in regulating multiple cellular biological functions such as proliferation, differentiation, and apoptosis [24–26]. p53 was identified as an important substrate of PKCδ, participating in regulating cell survival, cell cycle checkpoint control, and DNA repair [27,28]. When encountering harmful stimuli, PKCδ is activated by autophosphorylation and initiates the phosphorylation-dependent activation of p53. This PKCδ/p53 signaling is considered proapoptotic because it initiates activation of the caspase cascade by cleaving caspase3 [29]. In the present study, we found that H/R exposure significantly up-regulated the phosphorylation levels of both PKCδ and p53 in HAECs, indicating that H/R exposure activates the PKCδ/p53 signaling pathway. This conclusion was further confirmed by using siRNA technique. The expression of PKCδ was silenced by siRNA in HAECs exposed to H/R. The results show that PKCδ silencing inhibited activation of p53 and thus attenuated H/R-induced apoptosis of HAECs.

The clinical application of the medical herb *Scutellaria baicalensis Georgi* has a long history in Traditional Chinese Medicine in treatment of stroke, heart diseases, and peripheral vascular diseases [30]. Baicalin is one of the most effective bio-active components extracted from this medical herb. Previous studies indicated that baicalin exerted cardiac protective effects against I/R injury [31]. Our results in this study indicated the endothelial protective effects of baicalin against I/R injury. We found that in H/R-exposed HAECs, baicalin treatment dramatically increased the cell viability by reducing apoptosis. Moreover, baicalin treatment suppressed the phosphorylation of PKCδ and p53 in H/R-exposed HAECs. As a result, the caspase cascade activation was blocked. These results suggest that baicalin inhibits H/R-induced apoptosis by deactivating PKCδ/p53 signaling in H/R-exposed HAECs. To further support this conclusion, the activator of PKCδ PEP005 was used. The results showed that the treatment of PEP005 impaired the anti-apoptotic effect of baicalin on H/R-exposed HAECs. The treatment of PEP005 dramatically activated the PKCδ/p53 pathway by increasing phosphorylation of PKCδ. These results further indicate that PKCδ is one of the pharmacological targets of baicalin.

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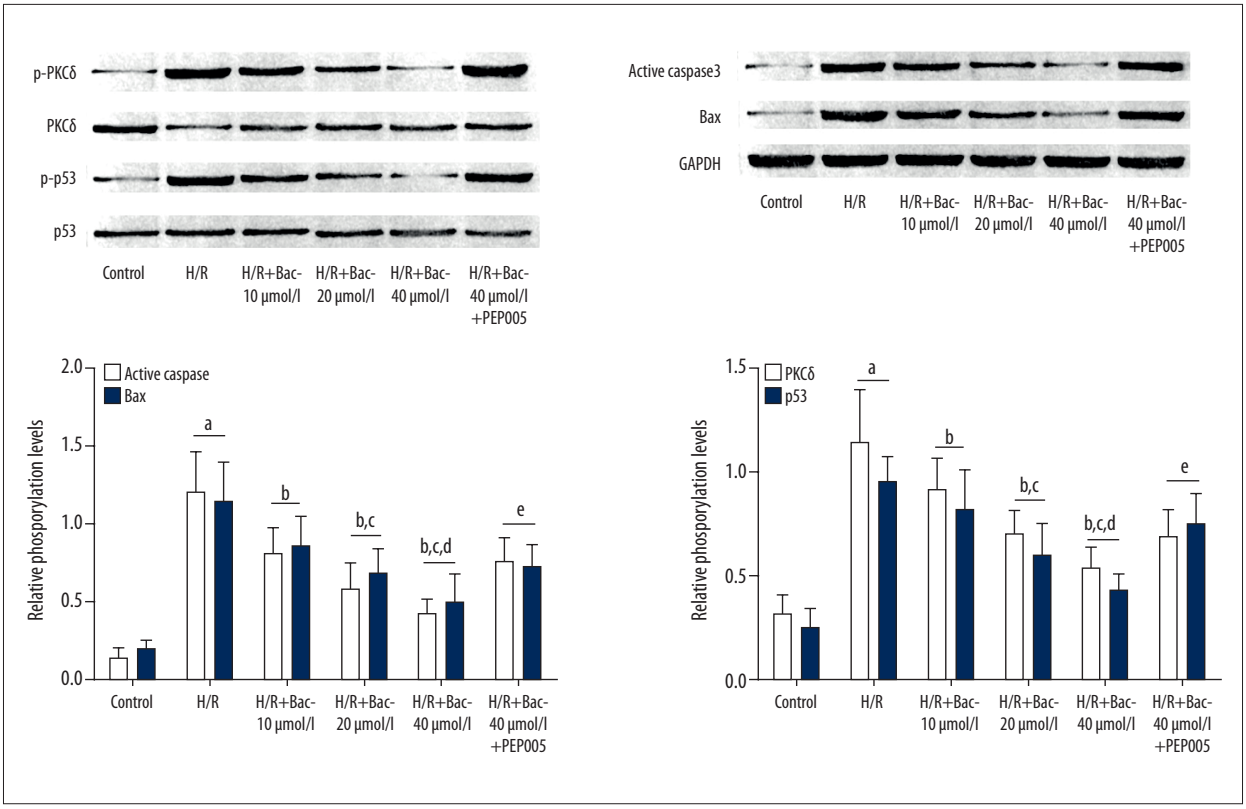

Figure 4. The upper part of this figure shows the immunoblots of p-PKCδ, PKCδ, p-p53, p53, active caspase3, bax, and GAPDH in cultured HAECs treated with H/R, baicalin (10, 20, and 40 μmol/l) and PEP005. Columns on the left part of lower panel indicate the relative phosphorylation levels of PKCδ (white) and p53 (blue) in HAECs, respectively. Columns on the right part of lower panel indicate the relative expression levels of active caspase3 (white) and bax (blue) in HAECs, respectively. [[a] differences were significant when compared with Control ($p<0.05$); [b] differences were significant when compared with H/R ($p<0.05$); [c] differences were significant when compared with H/R+Bac-10μmol/l ($p<0.05$); [d] differences were significant when compared with H/R+Bac-20 μmol/l ($p<0.05$); [e] differences were significant when compared with H/R+Bac-40 μmol/l ($p<0.05$)].

Conclusions

The results of the present study show the potential therapeutic value of baicalin in protecting vascular endothelium against I/R injury, which has rarely been described in previous investigations. Our study shows that baicalin exerts protective effects by preserving cell viability in cultured HAECs exposed to H/R by reducing cell apoptosis. Moreover, baicalin was proved to exert this protective effect by inhibiting activation of the PKCδ/p53 apoptotic signaling pathway. Our findings not only add our current understanding of the mechanism of I/R vascular injury, but also provide novel evidence for possible clinical application of baicalin or baicalin-containing drugs.

Our study has certain limitations. Although the H/R *in vitro* model can simulate the I/R *in vivo* model, there are still difference between them. It would be better to utilize an I/R *in vivo* model. Moreover, HAECs do not represent all of the many types of arterial endothelial cells, including coronary/microcirculatory arterial endothelial cells. More rigorous and comprehensive investigations should be conducted in the future.

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