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ORIGINAL PAPER

Mitochondria dependent pathway is involved in the protective effect of bestrophin-3 on hydrogen peroxide-induced apoptosis in basilar artery smooth muscle cells

Lei Jiang · Yun Liu · Ming-Ming Ma · Yong-Bo Tang · Jia-Guo Zhou · Yong-Yuan Guan

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Abstract Bestrophin 3 (Best-3) is expressed in a variety of tissues, such as cardiac, smooth muscle and renal tissues, and it is highly expressed in rat basilar arterial smooth muscle cells (BASMCs). Lee et al. (Biochim Biophys Acta 1823:1864–1876, 2012) reported that Best-3 prevented apoptotic cell death induced by endoplasmic reticulum stress. In the present study, we used small interference RNA (siRNA) and bestrophin 3 cDNA transfection strategy to investigate whether Best-3 can provide a protective effect on apoptosis induced by hydrogen peroxide (H_2O_2) in BASMCs and studied the underlying mechanisms. We found that silencing of Best-3 with siRNA resulted in an increased H_2O_2-induced apoptosis and a decreased cell viability, whereas overexpression of Best-3 significantly prevented the apoptotic cell death and increased the cell viability. Overexpression of Best-3 could stabilize the mitochondrial membrane potential, increase the ratio of Bcl-2/Bax, and decrease cytochrome *c* release and caspase-3 activation. In contrast, silencing of Best-3 produced the opposite effects. Our present data strongly suggest that Best-3 inhibits apoptosis induced by H_2O_2 in BASMCs through mitochondria dependent pathway.

Keywords Bestrophin 3 · Apoptosis · Hydrogen peroxide · Basilar artery · Vascular smooth muscle cell

Lei Jiang, Yun Liu, and Ming-Ming Ma contributed equally to this study.

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Introduction

Mutations in the bestrophin-1 gene are responsible for the Best's disease, a vitelliform macular dystrophy [1, 2]. Four Best's proteins are encoded in the mammalian genome, designated Bestrophin 1, 2, 3 (Best-1,-2,-3 and -4) [3]. Best-1 has been localized to the basolateral membrane of the retinal pigment epithelial [4], and autosomal dominant mutations in this gene cause several kinds of macular degeneration, including Best vitelliform macular dystrophy [2, 5]. Best-2 is expressed in nonpigmented epithelium [6] and plays a role in the generation of intraocular pressure by regulating the formation of aqueous [7]. Best-3 is widely expressed in a variety of tissues, such as cardiac and smooth muscle, and in renal tissues [8–10]. Best-4 is a pseudogene and not much is known about it. In our previous study, we found that Best-3 was highly expressed in rat basilar arterial smooth muscle cells (BASMCs) [11], however the exact function remained unclear. Although Best have been proposed to be the molecular candidates of Ca^{2+}-activated chloride channels (CaCC) in different kinds of cells [12–14], the topic is still controversial. We have shown that Best-3 does not contribute to CaCC in rat BASMCs [11]. Recently, it has been showed that the overexpression of Best-3 can inhibit endoplasmic reticulum stress-induced apoptotic cell death in renal epithelial cells [10]. However, the role of Best-3 in apoptosis of BASMCs remains to be clarified. In this study, the function of Best-3 in apoptotic process and the underlying mechanisms were investigated. We used small interference RNA (siRNA) and Best-3 cDNA (mBest3) transfection strategy to investigate Best-3 effect on apoptosis induced by H_2O_2 in BASMCs and the underlying molecular mechanisms. We found that silencing of Best-3 by siRNA could potentiate the H_2O_2-induced apoptosis and decrease the

cell viability, whereas overexpression of Best-3 could prevent the apoptosis and increased cell viability, through modulating mitochondrial apoptosis signal pathway.

Materials and methods

Cells and cell culture

Male Sprague–Dawley rats (100–120 g) were supplied by the Experimental Animal Center of Sun Yat-Sen University in Guangzhou, China. BASMCs were cultured from rat basilar arteries using a previously described method [15]. The cells were incubated in D-MEM/F-12 medium supplemented with 20 % fetal calf serum (GIBCO/Invitrogen, Carlsbad, CA, USA) and maintained 5 % CO_2 at 37 °C. BASMCs were confirmed as smooth muscle cells by morphology and immunostaining with monoclonal antibody specific for smooth muscle α-actin (Sigma, St Louis, MO, USA). Passages eight through 12 of cultured BASMCs were used for cell apoptosis assays.

Transfection with Best-3 siRNA and mBest3 plasmid

All Best-3 siRNA sequences were designed by and purchased from Qiagen (Valencia, CA, USA). The sequence of siRNA is: sense, r (ACGUUACUCUGGUAGUGAA) dTdT; antisense, r (UUCACUACCAGAGUAACGU) dAdA. These single-strand RNAs were annealed to produce duplexes. The Best-3 siRNA was transfected transiently with Hyperfect Transfection Reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and a negative siRNA (Qiagen, Valencia, CA, USA) sequence was used as negative control. The BASMC was transfected with 20 nM siRNA for 48 h. Best-3 plasmid (mBest3/pcDNA3.1 cDNA, kindly provided by Dr. Qu Zhiqiang, University of Qingdao, Shandong, China) was transfected with Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA). 48 h later, the cells transfected with 5 μg/ml cDNA were used for cell apoptotic assay or western blot analysis. A pcDNA3.1 vector was used as negative control.

Cell viability assay

Cell viability was measured by Cell Counting Assay Kit-8 (Dojindo Molecular Technologies, MD, Japan) according to the manufacturer's protocol. This colorimetric assay has higher detection sensitivity in comparison with the other tetrazolium salts assays, e.g. the widely used MTT assay. 100 μl of BASMCs were seeded in 96-well plates at a density of 1–2 $\times$ 10^4cells/ml for 24 h followed by a H_2O_2-treatment for 24 h. 10 μl of CCK-8 reagent was added to each well for 2 h, and the absorbance at 450 nm was measured using a microplate reader (Bio-Tek, Winooski, VT, USA).

Apoptosis detection

Cellular apoptosis ratio was determined by flow cytometry using Annexin V-FITC Apoptosis Detection Kit (KeyGEN Biotech, Nanjing, China) [16]. Briefly, cells were digested with trypsin and resuspended at a concentration of 1 $\times$ 10^6/ml. Following centrifugation, cells were washed with cold PBS twice and suspended in a binding buffer. Then the cells were incubated with annexin V-FITC and propidium iodide (PI) in the dark at room temperature for 15 min. Finally, the samples were analyzed using flow cytometry within 30 min.

Isolation of mitochondria

Intact mitochondria were isolated using Mitochondria Isolation Kit for Cultured Cells (Thermo Fisher Scientific Inc.,Rockford, USA), according to the manufacturer's protocol. Pellet containing 2 $\times$ 10^7 cells was obtained by centrifugation of the cell suspension for 2 min and the suspension was discarded. 800 μl of Mitochondria Isolation Reagent A were added and the tube was centrifuged at medium speed for 5 s, and then incubated for 2 min on ice. 10 μl of Mitochondria Isolation Reagent B were added and the tube was centrifuged at maximum speed for 5 s, and then incubated on ice for 5 min and during this time again vortexed each minute. 800 μl of Mitochondria Isolation Reagent C were added and the tube was centrifuged at 700$\times g$ for 10 min at 4 °C. The supernatant, i.e. the cytosol fraction, was transferred to a new tube and centrifuged at 12,000$\times g$ for 15 min at 4 °C. The pellet containing the isolated intact mitochondria was washed with 500 μl of Reagent C and centrifuged at 12,000$\times g$ for 15 min at 4 °C. Cytosol and mitochondria fractions were used for western blot. Cox IV (Beyotime Institute of Biotechnology, Nantong, China) was used as a loading control for the mitochondria fraction.

Mitochondrial membrane potential test

Mitochondrial membrane potential, which was measured using a membrane-permeant dye JC-1 (Beyotime Institute of Biotechnology, Nantong, China). JC-1 is an indicator of the loss of mitochondrial membrane potential. In healthy cells, JC-1 fluorescent dye accumulates in mitochondria in aggregates emitting red fluorescence, while the dye outflows into cytoplasm of apoptotic cells as a monomeric form emitting green fluorescence. The fluorescence was measured using confocal microscopy. The ratio of green

fluorescence to red fluorescence indicates the change of mitochondrial membrane potential.

Western blot

Whole cell protein extraction and concentration determination were performed as previously described [17]. Briefly, proteins from each group were subjected to SDS-PAGE and then transferred to nitrocellulose membranes (Millipore Corp, Bedford, MA, USA), after incubation with corresponding primary and secondary antibodies (Cell Signaling Technology, Boston, MA, USA). The target bands were developed using a chemiluminescence system and then visualized by exposure to Kodak X-ray film. Density of target bands was accurately determined using the image J software. β-Actin was used as the loading control. Primary antibody against caspase-3, caspase-9, cytochrome *c*, Bcl-2, Bax and PARP were all purchased from Cell Signaling Technology (Boston, MA, USA). The anti- Best-3 antibody (Fabgennix Inc, Frisco, USA) recognizes the sequence near the C-terminal end of rat Best-3 protein.

Statistical analyses

All data are expressed as mean ± SD, and n value represents the number of independent experiments. Statistical analyses including an unpaired two-tailed Student's t test, or one-way ANOVA followed by the Bonferroni multiple comparison post hoc test were carried out using the SPSS program. Values of $P < 0.05$ were considered significant.

Results

Effects of Best-3 siRNA and mBest-3 cDNA on H_2O_2-induced cell viability in BASMCs

Expression of Best-3 protein in BASMCs was determined by western blot using a polyclonal antibody directed

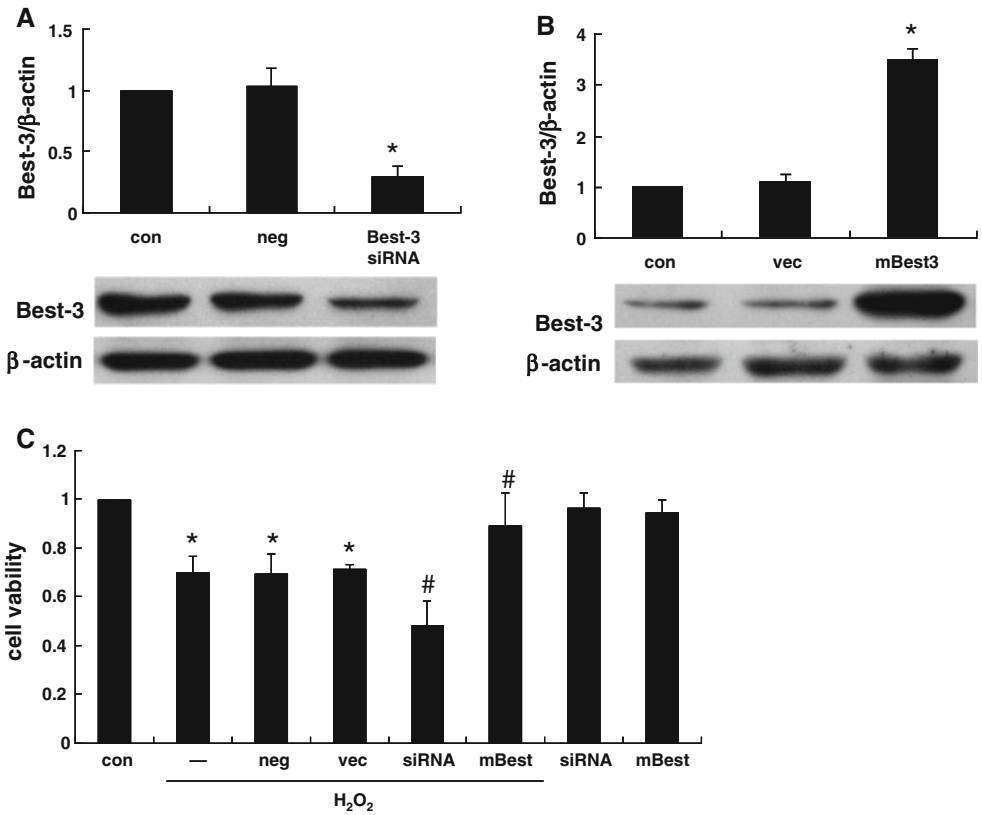


Fig. 1 Effects of Best-3 siRNA and mBest-3 cDNA transfection on H_2O_2-induced cell viability in BASMCs. **a** Western blot results show that transfection with 20 nM Best-3 siRNA for 48 h significantly decreased endogenous Best-3 expression, but transfection with negative siRNA control (neg) did not significantly change Best-3 expression. **b** Transfection with 5 μg/ml mBest3/pcDNA3.1 for 48 h significantly increased endogenous Best-3 expression, whereas transfection with pcDNA3.1 vector (vec) did not significantly change Best-3 expression. ($n = 6$; $^*P < 0.001$ versus control). **c** CCK-8 results show that treatment with H_2O_2 for 24 h significantly decreased cell viability. Best-3 siRNA accelerated H_2O_2-induced cell death and further decreased cell viability. Transfection with mBest3 cDNA defended against H_2O_2-induced cell death as documented by higher cell viability, whereas transfection with negative siRNA(neg) and pcDNA3.1 vector (vec) had no obvious effects. ($n = 6$; $^*P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. H_2O_2)

against Best-3. The anti-Best-3 antibody recognized a major band at 50–60 kDa. Figure 1a shows that Best-3 siRNA transfection with 20 nmol/L for 48 h significantly decreased endogenous expression of Best-3 by 70.5 ± 9.2 % ($n = 6$; $P < 0.001$ vs control), and negative siRNA transfection had no significant effect on Best-3 expression, which was consistent with the results from our previous study [11]. After transfection of 5 μg/ml mBest-3/pcDNA3.1 plasmid into cells for 48 h, western blot indicated an increase of 247.0 ± 25.0 % in protein expression compared with the control ($n = 6$; $P < 0.001$), and pcDNA3.1 alone had no effect on the expression of Best-3 (Fig. 1b).

Then, the effect of Best-3 on H_2O_2-treated cell viability was determined by CCK-8. As shown in Fig. 1c, after treatment with 250 μM H_2O_2 for 24 h, cell viability rate was significantly reduced to 70.1 ± 6.5 %, compared with untreated group ($n = 6$). Best-3 siRNA at 20 nM concentration further

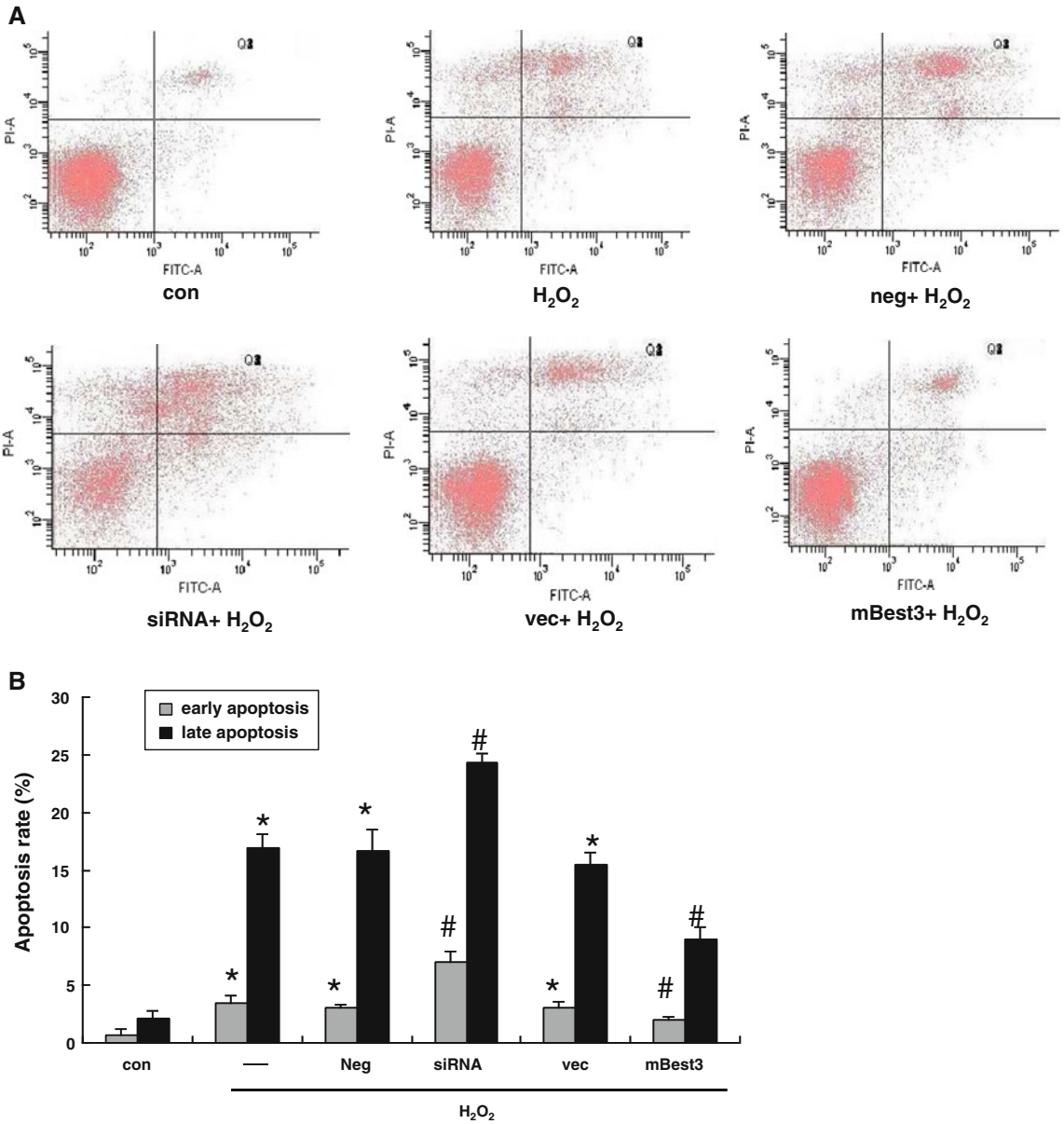


Fig. 2 Effects of Best-3 on H_2O_2-induced apoptosis in BASMCs. **a** Annexin V-FITC/PI flow cytometry analysis on the effects of Best-3 siRNA and mBest3 cDNA on H_2O_2-induced apoptosis. In each plot, viable cells are in the *lower left quadrant*, early apoptotic cells are in the *lower right quadrant*, and the *upper right* represents necrotic or late apoptotic cells. **b** Percentage of apoptotic cells was determined by quantitative analysis. Best-3 siRNA significantly enhanced H_2O_2 effect on cell apoptosis, whereas mBest3 cDNA significantly reversed the effect of H_2O_2 on apoptosis. Negative (neg) and pcDNA3.1 vector (vec) did not significantly affect apoptosis induced by H_2O_2. ($n = 6$; $^*P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. H_2O_2)

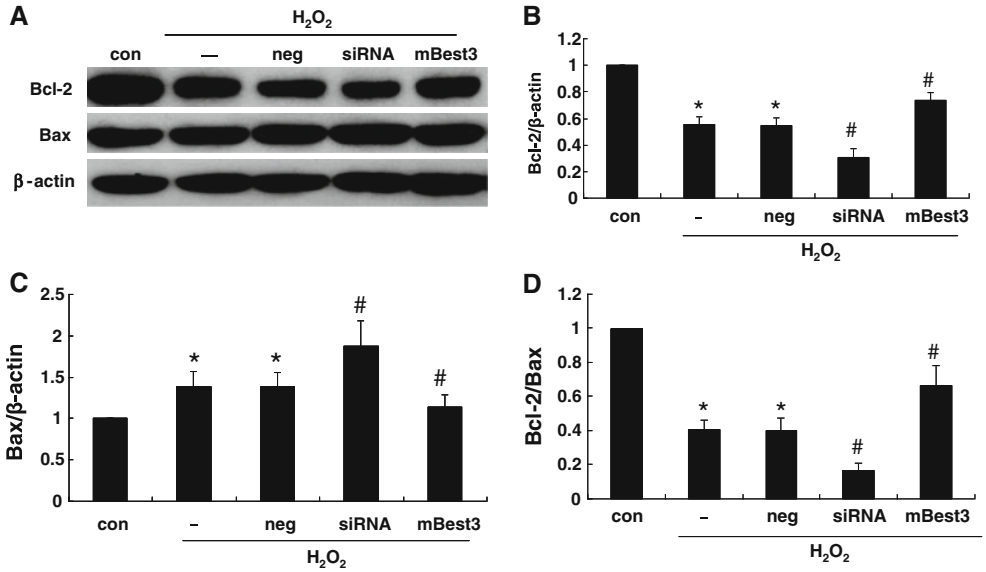


Fig. 3 Effects of Best-3 on H_2O_2-induced expression of Bcl-2 and Bax in BASMCs. **a** Representative Western blots on the effects of Best-3 on H_2O_2 -induced expression of Bcl-2 and Bax in BASMCs. **b** Densitometric analysis shows that treatment with H_2O_2 decreased the expression of Bcl-2, and Best-3 siRNA further decreased the Bcl-2 expression but transfection with Best-3 cDNA increased the Bcl-2 expression. **c** Densitometric analysis of Best-3 effect on Bax expression. Treatment with H_2O_2 increased Bax expression which was further increased by Best-3 siRNA and decreased by Best-3 cDNA. **d** Densitometric analysis shows that treatment with H_2O_2 reduced Bcl-2/Bax value, which was further decreased by Best-3 siRNA and increased by Best-3 cDNA. ($n = 8$; $^*P < 0.01$ vs. control, $^{\#}P < 0.05$ vs. H_2O_2)

reduced cell viability rate from 70.1 ± 6.5 to 48.2 ± 7.5 % ($n = 6$; $P < 0.05$, compared with H_2O_2-treated group), whereas an overexpression of Best-3 increased cell viability from 70.1 ± 6.5 to 89.1 ± 9.6 % ($n = 6$; $P < 0.05$, compared with H_2O_2-treated group). Negative siRNA and blank vector transfection did not significantly alter cell death rate induced by H_2O_2.

Effects of Best-3 on H_2O_2-induced apoptosis in BASMCs

H_2O_2-induced apoptosis was analyzed by Annexin V-FITC/PI flow cytomety. In Fig. 2, incubation with 250 μM H_2O_2 for 24 h could induce in BASMCs an apoptotic rate of 20.3 ± 2.2 %. Best-3 siRNA transfection further increased the apoptotic cell population to 31.3 ± 1.9 % ($n = 6$; $P < 0.01$; compared with H_2O_2 treatment), whereas overexpression of Best-3 significantly decreased apoptotic population from 20.3 ± 2.2 to 11.4 ± 1.0 % ($n = 6$; $P < 0.05$; compared with H_2O_2 treatment). The transfections with negative siRNA or vector did not change the apoptotic cell population in BASMCs.

Effects of Best-3 on H_2O_2-induced Bcl-2 and Bax expression in BASMCs

Bcl-2 and Bax are antiapoptotic and proapoptotic regulators, respectively, and the ratio of Bcl-2 to Bax determines a cell's survival or death fate. Here, the levels of the Bcl-2 and Bax were determined by western blots. Figure 3 shows that H_2O_2 treatment for 24 h obviously decreased Bcl-2 (Figs. 3a, b) and enhanced Bax expression (Figs. 3a, c), resulting in a decrease in Bcl-2/Bax ratio (Fig. 3d). Best-3 siRNA further increased Bax level, reduced Bcl-2 level and the Bcl/Bax ratio. In contrast, Best-3 cDNA increased the Bcl-2/Bax value, due to the increase in Bcl-2 and the decrease in Bax level. Negative siRNA and vector did not altered Bcl-2 and Bax expression modulation induced by H_2O_2.

Effect of Best-3 on H_2O_2-induced loss of mitochondrial membrane potential (MMP) and cytochrome *c* release in BASMCs

Mitochondrial membrane pathway is involved in H_2O_2-induced apoptosis in BASMCs [18]. To determine whether Best-3 protected H_2O_2-induced apoptosis in BASMCs through mitochondrial pathway, we further investigated the effects of Best-3 on MMP and cytochrome *c* release. When the MMP is decreased, the red fluorescence of JC-1 dye is decreased along an increase in the green fluorescence increase, resulting in an augmented ratio of green/red intensity in H_2O_2-treated cells, compared with control. As shown in Fig. 4, an increase in green/red fluorescence ratio induced by H_2O_2 was counteracted by Best-3 cDNA transfection, whereas it was enhanced by Best-3 siRNA. Cyclosporine A (CsA), an inhibitor of mitochondrial

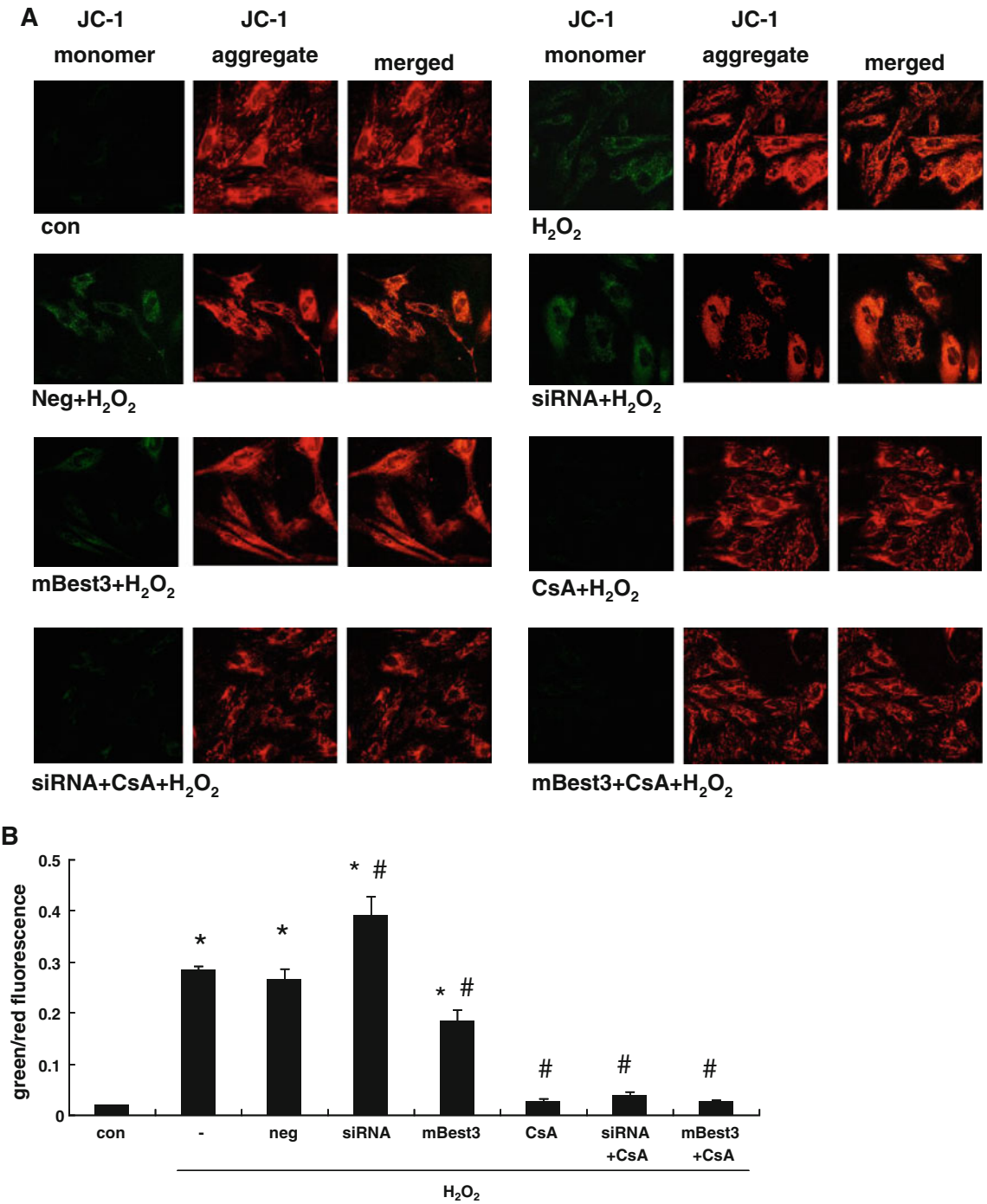


Fig. 4 Effects of Best-3 on H_2O_2-induced mitochondrial membrane potential (MMP) by live cell confocal microscopy.BASMCs were exposed to H_2O_2 for 6 h after different treatment, and MMP was measured using JC-1 staining. Cyclosporine A (CsA), an inhibitor of mitochondrial permeability transition, was used to determine if the apoptosis process is acting through mitochondria dependent pathway. **a** Representative images of JC-1 derived fluorescence in BASMCs with different treatment (400×). The JC-1 monomer was represented with *green* fluorescence; the JC-1 aggregate image was represented with *red* fluorescence; the merged images were the combined of the *green* and *red* images. The *topmost* line images represent the JC-1 as a monomer with *green* fluorescence; the *middle* line images represent the JC-1 aggregate with red fluorescence; the *bottom* line images are merged. Mitochondrial depolarization is indicated by an increase in the *green/red* fluorescence intensity ratio. **b** Quantitative analysis of the *green/red* fluorescence ratio shows that the Best-3 siRNA increased and mBest3 cDNA decreased *green/red* fluorescence ratio, meanwhile CsA inhibited H_2O_2-induced mitochondrial permeability transfected with Best-3 siRNA and mBest3 cDNA. ($n = 6$; $^{*}P < 0.01$ vs. control, $^{\#}P < 0.05$ vs. H_2O_2) (Color figure online)

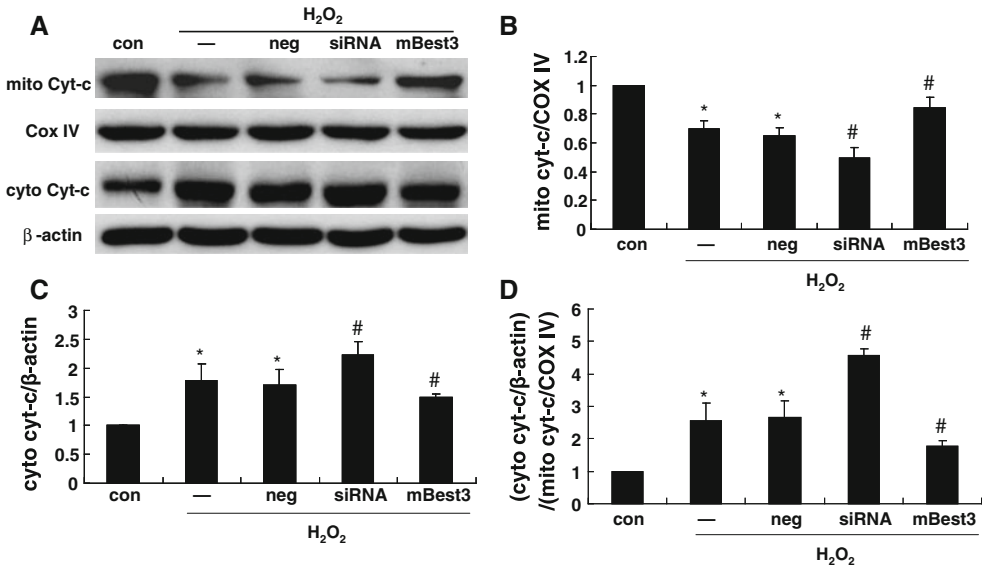


Fig. 5 Effects of Best-3 on H_2O_2-induced mitochondrial cytochrome *c* release in BASMCs. **a** Protein levels of cytochrome *c* in the cytoplasm (cyto) and mitochondria (mito). Cox IV is a loading control of mitochondrial protein. The mitochondrial cytochrome *c* level was decreased and the cytoplasm cytochrome *c* level was increased by treatment of H_2O_2, indicating that mitochondrial cytochrome *c* was released from mitochondria to cytoplasm. **b** The expression of mitochondrial cytochrome *c* of different treated cells by western blot, with densitometric analysis of the relative protein levels. ($n = 8$; $^*P < 0.01$ vs. control, $^{\#}P < 0.05$ vs. H_2O_2). **c**. The expression of cytoplasm cytochrome *c* of different treated cells by western blot, with densitometric analysis of the relative protein levels. ($n = 8$; $^*P < 0.01$ vs. control, $^{\#}P < 0.05$ vs. H_2O_2). **d**. Densitometric analysis of cytochrome *c* release from mitochondria to cytoplasm

permeability transition, inhibited the alteration in mitochondrial permeability induced by H_2O_2 and reversed the effect of Best-3 on MMP. All of these effects induced by H_2O_2-treatment were not significantly changed by transfection with negative siRNA or vector only.

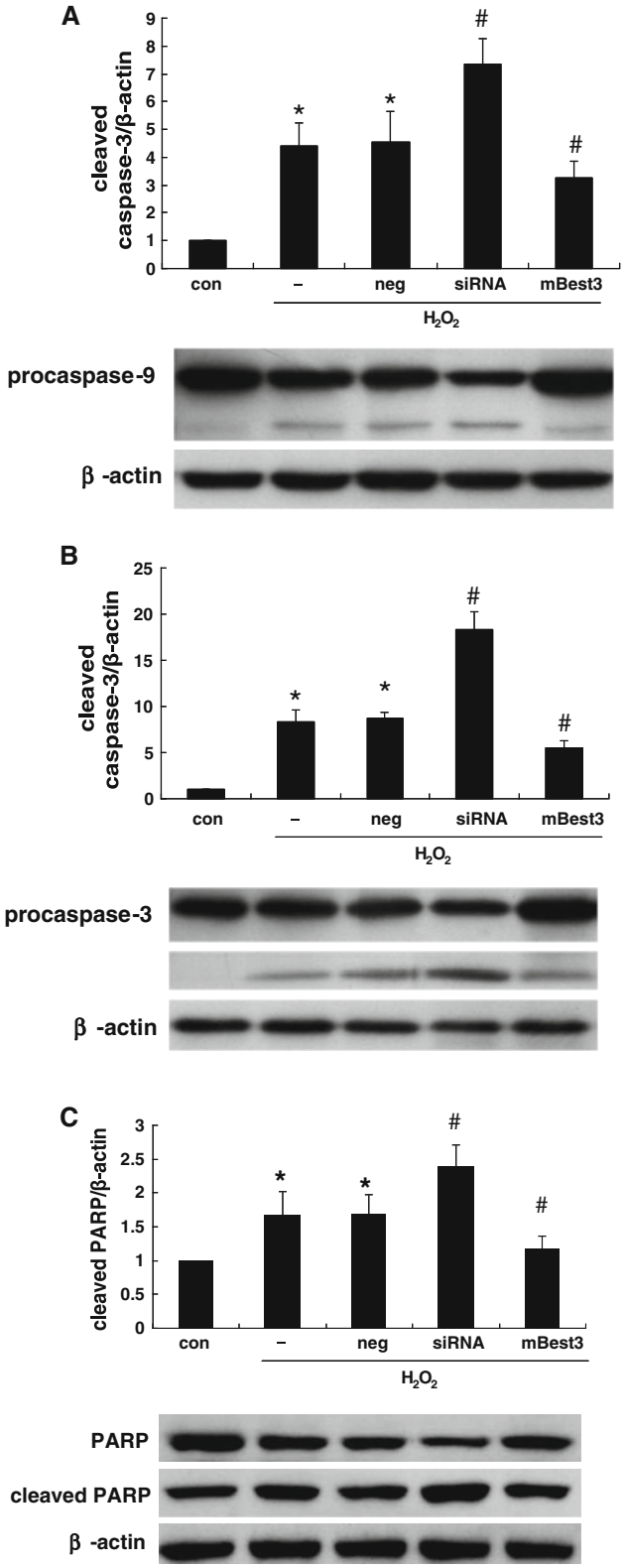
Treatment with H_2O_2 for 24 h could damage mitochondria, followed by the release of cytochrome *c* from mitochondria to cytoplasm. Figure 5 shows that cytoplasmic cytochrome *c* level was significantly elevated by H_2O_2-treatment and was further increased by Best-3 siRNA and inhibited by transfection of H_2O_2-treatment cells with Best-3 cDNA. The cytochrome *c* level was not significantly changed by negative siRNA and vector. These results indicate that Best-3 inhibits H_2O_2-induced mitochondrial cytochrome c release through the stabilization of MMP.

Effect of Best- 3 on H_2O_2-induced caspase activation

Cytochrome *c* release sequentially activates downstream apoptosis-associated proteins, such as caspase-9 and caspase-3. We used western blot to determine the effects of Best-3 on the activated caspase-9 and caspase-3, and PARP which is one of the main cleavage targets of caspase-3 induced apoptosis of BASMCs. Figure 6 shows that H_2O_2-treatment for 24 h significantly increased caspase-9, caspase-3 and PARP cleavage. The caspase activation was further increased by Best-3 siRNA and reduced by Best-3 cDNA transfection. The activation of caspase-9, caspase-3 and PARP was not significantly altered by negative siRNA and vector.

Discussion

Bestrophins, a recently discovered family of Cl^- channels, can also function as regulators of voltage-gated Ca^{2+} channels. Several bestrophins have been shown to produce a Ca^{2+}-activated chloride current. However, further studies have been shown difference in Ca^{2+}-sensitivity voltage dependence and current kinetics [19–22]. In RPE cells, Ca^{2+}-activated chloride currents were not abolished in mBest-1 knockout mice [20, 23], and Best-3 siRNA knockdown did not affect Ca^{2+}-actived Cl^- currents in rat BASMCs [11]. Matchdown et al. [9] has showed that Best-3 is essential for the cGMP-dependent Ca^{2+}-actived Cl^- current, which is different from classical Ca^{2+}-actived Cl^- current in vascular smooth muscle cells. In addition, it has been shown that best mediate cell proliferation [24, 25], cell death [10], vascular rhythmic movement [26], calcium movement through affecting L-type Ca^{2+} channels [27, 28] and Ca^{2+} release. However, in renal epithelial cells Best-3 can prevent apoptotic cell death induced by ER stress. It remained an question, whether Best-3 mediates apoptosis in vascular smooth muscle cells. In the present study, we first investigated Best-3 effects on apoptosis induced by



◀**Fig. 6** Effects of Best-3 on H_2O_2-induced caspases activation. **a** Densitometric analysis shows that treatment with H_2O_2 for 24 h increased the expression of cleaved caspase-9 that was further increased by Best-3 siRNA and decreased by Best-3 cDNA. Representative Western blots for cleaved caspase-9 expression is shown in the bottom. **b** Densitometric analysis shows that treatment with H_2O_2 increased the expression of cleaved caspase-3 that was further increased by Best-3 siRNA and decreased by Best-3 cDNA. Representative Western blots for cleaved caspase-3 expression is shown in the bottom. **c** PARP is cleaved from 116 to 89 kD, indicating apoptosis induction. Densitometric analysis shows that treatment with H_2O_2 increased the expression of cleaved PARP, and Best-3 siRNA further increased but transfection with Best-3 cDNA decreased the cleaved PARP expression. Representative western blots for PARP and cleaved PARP expression are shown in the *bottom*. ($n = 8$; $^{*}P < 0.01$ vs. control, $^{\#}P < 0.01$ vs. H_2O_2)

H_2O_2-treated rat BASMCs. Hydrogen peroxide belongs to the class of reactive oxygen species (ROS) and it is widely used to induce apoptosis in various cell types, including endothelial and vascular smooth muscle cells. Second, we investigated the underlying molecular mechanisms. First, we found that overexpression of Best-3 significantly inhibited H_2O_2-induced apoptosis in BASMCs and increased the cell viability, whereas Best-3 siRNA induced an opposite effect and enhanced the apoptotic rate and consequently, decreased the cell viability in BASMCs. Our data strongly indicate that Best-3 in BASMCs mediates H_2O_2-induced apoptotic process in an inhibitory manner.

In human renal epithelial cells, knockdown of Best-3 increased apoptotic cell death induced by ER stress, whereas overexpression of Best-3 significantly reduced cell death [10]. Our results are consistent with this report. Based upon above results, we further determined the mechanisms of Best-3 anti-apoptotic effect. It is known that there are two major signal pathways involved in the apoptotic process: the extrinsic death receptor-mediated pathway and the intrinsic mitochondrial pathway. Apoptosis process consists of a proteolytic cascade involving a family of proteases called caspase. It is usually associated with the alterations of both Bcl-2 and Bax levels. An increase in Bax level leads to a lower free concentration of Bcl-2 in the cell by combing with Bcl-2, driving the cell towards apoptosis. Upon apoptotic stimulation, Bax forms oligomers and translocates from the cytoplasm to the mitochondrial membrane. Through interactions with pore proteins on the mitochondrial membrane, Bax increases the membrane permeability, which leads to the release of cytochrome *c* from mitochondria to cytosol, and actives caspase activation pathway, leading to further deterioration of mitochondria electron transport and DNA fragmentation, triggering apoptosis [29, 30].

In our previous studies, H_2O_2-treatment of BASMCs for 24 h increased Bax and decreased Bcl-2 expression, induced the loss of MMP followed by the release of cytochrome *c*, indicating that H_2O_2-induced apoptosis in BASMCs through mitochondrial pathway [31]. In this study, it was demonstrated that Best-3 siRNA enhanced the decrease in Bcl-2/Bax ratio and MMP induced by H_2O_2, and increased cytochrome *c* release from mitochondria into

the cytoplasm. In contrast, an overexpression of Best-3 produced the reverse effects: elevations of Bcl-2/Bax value and MMP stabilization, and reduction of cytochrome *c* release. These conclusive results indicate that Best-3 effect on apoptosis is mitochondria -dependent.

During the apoptotic process, cytochrome *c* released from the mitochondria combines with caspase-9 and triggers sequentially caspase cascade characteristic of the apoptotic pathway, in which caspase-3 plays a dominant role. Upon DNA cleavage by activated caspases-3, PARP would deplete the ATP of a cell in an attempt to repair the damaged DNA. ATP depletion would then in a cell lead to lysis and cell death. In the present study, our data revealed that Best-3 siRNA promoted the cleavages of caspase-9, caspase-3 and PARP, resulting in cellular disassembly. An overexpression of Best-3, however, produced the opposite effect by inhibiting the cleavages of caspase-9, caspase-3 and PARP.

In conclusion, Best-3 prevents apoptosis induced by H_2O_2 in BASMCs through mitochondrial dependent pathway, including the followed steps: increases of Bcl-2/Bax ratio and stabilization of MMP, inhibition of mitochondrial cytochrome *c* release, and suppression of the activation of caspase-9, caspase-3 and PARP, participating in cell apoptosis execution.

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