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A role of AMPK and connexin 43 in the suppression of $CoCl_2$-induced apoptosis of spiral modiolar artery smooth muscle cells by adiponectin

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ABSTRACT

Aims: Adiponectin (APN) is a protein hormone secreted mainly by adipose tissue that exhibits biological functions such as anti-inflammatory, anti-atherosclerotic, anti-apoptotic, hearing-protective and microcirculation-regulating functions. In this study, we explored whether APN could attenuate damage caused by $CoCl_2$-induced hypoxic conditions in smooth muscle cells (SMCs) of the spiral modiolar artery (SMA).

Main methods: We first cultured and identified primary SMCs of the SMA. Afterward, the SMCs were pre-treated with APN and then stimulated with $CoCl_2$.

Key findings: Compared with the control group, the group treated with $CoCl_2$ for 24 h exhibited significantly decreased cell viability, significantly increased apoptosis rates and Malondialdehyde (MDA) levels, and decreased Superoxide Dismutase (SOD) activity. In addition, the expression levels of Bax and cleaved caspase-3 were upregulated, while those of Bcl2 were downregulated evidently. Compared with the $CoCl_2$ group, the group pre-treated with APN before receiving $CoCl_2$ treatment had increased cell viability and SOD activity but decreased MDA levels and apoptosis rates. The expression levels of Bcl2, p-AMPKα and Cx43 were evidently increased, while those of Bax and cleaved caspase-3 were decreased, in the group pre-treated with APN compared to the $CoCl_2$ group. The protective effect of APN was blocked by the AMPK inhibitor Compound C and the Cx43 inhibitor Gap19.

Significance: Our study demonstrated that APN protected SMCs against $CoCl_2$-induced hypoxic injury via the AMPK signalling pathway and regulated the expression of Cx43 in cells. Therefore, APN might be a promising treatment for diseases related to circulation disturbances of the inner ear.

1. Introduction

Numerous studies have shown that disorders of the circulatory system in the inner ear are closely linked to sudden deafness, Meniere's disease, senile hearing loss and ototoxicity-induced hearing loss [1,2]. Moreover, the blood supply of the spiral modiolar artery (SMA) is vital in maintaining the function of the auditory apparatus because of the high energy demands of auditory conduction, and the SMA is the only artery responsible for supplying blood to the cochlea, and its upstream arteries are the anterior inferior cerebellar and basilar arteries [3,4]. Therefore, the blood supply of the SMA may have a decisive influence on the maintenance of normal hearing [5].

Pathophysiological development processes, such as proliferation and apoptosis of cells in various systems and tissues, depend on close communication between cells [6]. Communication at the intercellular level is mainly mediated by gap junction channels (GJCs) and hemichannels (HCs, also called connexons), which are made up of six connexin (Cx) proteins [7]. It is well recognized that GJCs are open in normal physiological conditions for facilitating cell/cell communication and are programmed to close in response to specific physiological and/or pathophysiological conditions [8]. Connexin 43 (Cx43) is the most widely distributed Cx and is expressed at higher levels than other Cxs in SMCs, and recent data suggest that, in addition to the role of GJCs in the passage of molecules through gap junctions and/or hemichannels, Cx protein independently may control cell death [9,10]. Suggested by the findings that a wide spectrum of apoptotic genes

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coding for BcL-xL, Bok, Bax, caspase-3, caspase-6 and caspase-9 proteins are altered in the heart of Cx43 knockout mice [11].

Oxidative stress in cells is generated by the persistent imbalance between the generation of toxic reactive oxygen species (ROS) and the antioxidative defences of aerobic metabolism [12]. The massive production of ROS can lead to the significant destruction of cell structure and function, which leads to the progression of apoptosis. Malondialdehyde (MDA) is a marker of lipid peroxidation. When the MDA content is higher, the degree of oxidative stress is more severe; antioxidative enzymes, which are responsible for eliminating free radicals such as superoxide and hydrogen peroxide, play a defensive role against oxidation. Superoxide Dismutase (SOD), an antioxidative enzyme, has a strong antioxidative ability [13]. Approximately 90% of ROS are generated by the mitochondria, and Oxidative stress can trigger apoptosis through the mitochondrial pathwa. Increasing the ratio of Bax/Bcl2 expression in the mitochondrion leads to a decrease in mitochondrial membrane potential, the release of cytochrome c, and the activation of caspase-induced apoptosis [14].

Adiponectin (APN) is a protein hormone secreted mainly by adipose tissue that exhibits biological functions such as anti-inflammatory, antiatherosclerotic, anti-apoptotic, hearing-protective and microcirculation-regulating functions [15]. In general, the biological effects of APN depend on two types of signalling pathways: AMP-activated protein kinase (AMPK) and non-AMPK pathways [16]. Related research on the effects of APN on smooth muscle cell (SMC) apoptosis in the SMA under hypoxic conditions has not been reported. Given the existing knowledge, we hypothesised that APN might exert anti-apoptotic effects on SMCs by adjusting SOD activity and MDA levels and regulating Bax, Bcl2 and cleaved caspase-3 expression via the AMPK signalling pathway and by regulating Cx43 expression in cells.

2. Materials and methods

2.1. Animals

Guinea pigs (2 weeks old and weighing 150–200 g) were purchased from the Animal Experimental Centre of Xinjiang Medical University, China. All animals (licence lot number CXK New (2012-0001)) met the highest standards. The feeding and experimental procedures were performed with the approval of the Institutional Ethics Review Board of Shihezi University.

2.2. Primary culture

Highly purified SMCs were obtained from the SMAs of guinea pigs, as described previously [17]. SMCs in passage 4 (P4) were used for all experiments. The SMCs were cultured in DMEM/F12 culture medium (Gibco, Carlsbad, CA, USA) containing 10% foetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Experimental design

The SMCs were treated with 0, 25, 50, 100, 200 and 400 μM $CoCl_2$ (Cat. No. C8661, Sigma-Aldrich, St. Louis, MO, USA) for 0, 3, 6, 12, 24 and 48 h to determine the optimal APN treatment conditions [18]. Untreated cells cultured in DMEM/F12 served as the control group. Cells were pre-treated with APN (Cat. No. 0911545, PeproTech, Rocky, NJ, USA) at different concentrations (0.5, 1 and 2 μg/ml) for 2 h before being treated with $CoCl_2$ (100 μM, 24 h) [19-21]. Also, Cells were pre-treated with AMPK inhibitor Compound C (Cat. No. B3252, APExBIO TechnologyLLC, USA, 40 μM, 30 min) [22] or Cx43 inhibitor Gap19 (Cat. No. B4919, APExBIO TechnologyLLC, USA, 50 μM, 1 h) [23] before being treated with $CoCl_2$. The concentration with maximal protective effects was determined.

2.4. Cell counting Kit-8 (CCK-8) assay

The viability of cells was measured using a CCK-8 (Cat. No. 70-CCK805, MultiSciences Lianke Biotech Co., Ltd. Hangzhou, China) following the manufacturer's instructions.

2.5. Immunofluorescence analysis

As described previously [17], cells were uniformly seeded on slides in a 6-well plate and allowed to adhere. The medium was discarded after treatment, and the cells were washed with PBS and fixed in paraformaldehyde (40 g/L) for 15 min. The cells were then rewashed with PBS and permeabilised with Triton X-100 (2 g/L) for 3 min. Thereafter, the cells were rewashed with PBS and incubated with BSA (Sigma-Aldrich, USA) (50 g/L) at room temperature for 30 min. Primary antibodies (monoclonal rabbit anti-α-SM-actin (1:300; Cat. No. ab124964; Abcam, UK), polyclonal rabbit anti-SM22α (1:100; Cat. No. ab14106; Abcam, UK), monoclonal rabbit anti-calponin (1:300; Cat. No. ab46794; Abcam, UK), monoclonal mouse anti-desmin (1:100; Cat. No. ab8470; Abcam, UK), polyclonal rabbit anti-Cx43 (1:100; Cat. No. ab11370, Abcam, UK), monoclonal rabbit anti-AMPK (1:200; Cat. No. ab32047, Abcam, UK) and monoclonal rabbit anti-p-AMPKα (Thr172) (1:200; Cat. No. 2532, CST, USA)) were added, and the wet box was kept at 4 °C overnight. The next day, the cells were rewarmed for 30 min at 37 °C and washed with PBS. Goat anti-rabbit or goat anti-mouse secondary antibodies (1:50; Beijing Fir Jinqiao Biotechnology Co., Beijing, China) were added in a dark room, and the cells were incubated at 37 °C for 1 h. Next, the cells were washed with PBS, and the nuclei were stained with DAPI (Solarbio Science and Technology Co., Beijing, China). Then, confocal microscopy (Zeiss LSM 510 META, Carl Zeiss AG, Germany) was performed to analyse the results. Open the target image with AimImage Examiner (Zeiss LSM 510 META, Carl Zeiss AG, Germany), select the Histogram mode, turn off the bright field and DAPI. Then select these cells to be measured by Threshold. Finally, select Statistics to get statistics, including average optical density and area, and analyse the data.

2.6. Elisa

SOD (Cat. No. A001-3-2, Jiancheng Bioengineering Institute, Nanjing, China) and MDA (Cat. No. A003-1-2, Jiancheng Bioengineering Institute, Nanjing, China) enzyme immunoassay assay kits were used to determine the activity of SOD and the levels of MDA following the manufacturer's instructions.

2.7. Flow cytometry

Cells from each group were digested with 0.25% trypsin, collected and washed twice by PBS after treatment. Then, the culture medium was removed and 500 μL pre-cooled 1 × Bingding Buffer working solution was added for cell suspension, and the cell density was adjusted to 1×10^6 cells/ml. Thereafter, Annexin V-FITC (5 μl) and propidium iodide (PI, 10 μl) were added in accordance with the instructions of the manufacturer (Cat. No. 70-AP101-100, MultiSciences Lianke Biotech Co., Ltd. Hangzhou, China). It was worth noting that the single standard tubes were only added Annexin V-FITC (5 μl) or PI (10 μl), and the negative control tube does not need to be added any Annexin V-FITC or PI. The cells were then shaken gently and incubated with mixing at 4 °C for 30 min in the dark. Quantitative analysis of the proportion of apoptotic cells was conducted by flow cytometry (BD FACSAria™ III Cell Sorter No.648282, Becton Dickinson, USA).

2.8. Western blot analysis

Cells were uniformly plated in a 6-well plate, treated, and lysed, and the protein levels were estimated by BCA protein assay. Samples with

equal amounts of protein were separated by 10% or 12% SDS–PAGE. Thereafter, the resolved proteins were transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with non-fat milk (50 g/L) in TBST buffer for 2 h and then probed with various primary antibodies (monoclonal mouse anti-β-actin (1:1000; Cat. No. TA-09, Beijing Fir Jinqiao Biotechnology Co., China), monoclonal mouse anti-GAPDH (1:1000; Cat. No. TA-08, Beijing Fir Jinqiao Biotechnology Co., China), polyclonal rabbit anti-Cx43 (1:1000; Cat. No. ab11370, Abcam, UK), monoclonal rabbit anti-AMPK (1:1000; Cat. No. ab32047, Abcam, UK), monoclonal rabbit anti-p-AMPKα (Thr172) (1:500; Cat. No. 2532, CST, USA), polyclonal rabbit anti-Bax (1:1000; Cat. No. ab199677, Abcam, UK), polyclonal rabbit anti-Bcl2 (1:1000; Cat. No. ab196495, Abcam, UK) and polyclonal rabbit anti-caspase 3 (1:500; Cat. No. ab13847, Abcam, UK)) overnight at 4 °C. After primary antibody incubation, the blots were washed with TBST and incubated with the goat anti-rabbit or goat anti-mouse secondary antibodies (Beijing Fir Jinqiao Biotechnology Co., Beijing, China) at room temperature for 2 h. Next, the blots were washed with TBST and visualised on X-ray film using an enhanced chemiluminescence (ECL) reagent (GE Healthcare Life Sciences, UK). The optical density of each target protein band was assessed using Quantity One (Bio-Rad, Hercules, CA, USA) and normalised to the density of the corresponding GAPDH or β-actin bands in the same sample.

2.9. Statistical analysis

All values are presented as the mean ± SE. Differences between groups were assessed using one-way ANOVA and *t* tests, as appropriate. *P* values less than 0.05 ($P < 0.05$) indicated significant differences.

3. Results

3.1. $CoCl_2$ reduced cell viability, but the effects were attenuated by APN

In SMCs, $CoCl_2$ treatment for 24 h induced cell injury in a concentration-dependent manner (Fig. 1A). Moreover, 100 μM $CoCl_2$ treatment induced injury in cells in a time-dependent manner, and $CoCl_2$ treatment for 24 and 48 h significantly reduced the viability of cells (Fig. 1B). Therefore, SMCs were pre-treated with different APN concentrations (0.5, 1 and 2 μg/ml) for 2 h to observe the protective effects of APN. As shown in Fig. 1 and 2 μg/ml APN showed significant protective effects. Thereafter, 24 h of treatment with 100 μM $CoCl_2$ and 2 h of pre-treatment with 2 μg/ml APN were used in the following experiments to study the signalling pathway involved in the protective effects of APN.

3.2. $CoCl_2$ reduced SOD activity, increased MDA levels and apoptosis rates, upregulated bax and cleaved caspase-3 expression and downregulated Bcl2 expression in SMCs, but these effects were attenuated by APN

SMCs were pre-treated with different APN concentrations for 2 h to observe the protective effects of APN. Compared with that in the control group, the activity of SOD was significantly decreased in the $CoCl_2$-treated model group (Fig. 2A). The MDA levels in SMCs were significantly increased after treatment with $CoCl_2$ (Fig. 2B). Moreover, the expression levels of related proteins in the mitochondrial apoptotic signalling pathway were significantly altered. Specifically, $CoCl_2$ upregulated the expression levels of Bax and cleaved caspase-3 and downregulated those of Bcl2 (Fig. 2C–F). However, these effects were reversed by APN. As shown in Fig. 2G, the Q1 region was characterised by necrotic cells mixed with a small amount of late apoptotic cells or cells with mechanical damage. Similarly, the cells in the Q2 region showed late apoptosis, whereas the cells in the Q3 region showed early apoptosis. Some cells in the Q4 region were living cells. We found that 100 μM $CoCl_2$ treatment induced apoptosis in a time-dependent manner (Fig. 2H). Among the SMCs pre-treated with APN, the rate of apoptosis increased after treatment with $CoCl_2$ (Fig. 2I), whereas the apoptosis of each group decreased after pre-treatment with APN (Fig. 2J).

3.3. APN upregulated Cx43 expression and activated p-AMPKα

As shown in Fig. 3A and B, $CoCl_2$ (100 μM, 24 h) and APN (2 μg/ml, 2 h) did not significantly affect the expression of AMPK in cells, but APN activated p-AMPKα. Moreover, $CoCl_2$ downregulated the expression of Cx43 in a time-dependent manner (Fig. 3E and F). These effects were reversed by APN (Fig. 3G and H). The immunofluorescence results in SMCs showed that AMPK was mainly distributed in the cytoplasm (Fig. 3C), while p-AMPKα and Cx43 were mainly distributed in the cytoplasm and nucleus (Fig. 3D and I). The expression changes in AMPK and Cx43 in the cells of each group revealed by immunofluorescence were consistent with the results of Western blot analysis.

3.4. APN modulated $CoCl_2$-induced changes in MDA, SOD, and apoptosis and in bax, cleaved caspase-3 and Bcl2 expression levels, and these effects could be attenuated by the AMPK inhibitor Compound C and the Cx43 inhibitor Gap19

SMCs were pre-treated with the AMPK inhibitor Compound C (40 μM, 30 min), the Cx43 inhibitor Gap19 (50 μM, 1 h) and APN (2 μg/ml, 2 h) separately in the following experiment. As shown in Fig. 4A and B, APN modulated the $CoCl_2$-induced changes in MDA and SOD, and these effects could be modulated by Compound C and Gap19. The rate of apoptosis increased after treatment with $CoCl_2$ compared to the control regimen, and the effect was reversed by APN (Fig. 4C and D). The anti-apoptotic effect of APN was also reversed by Compound C and

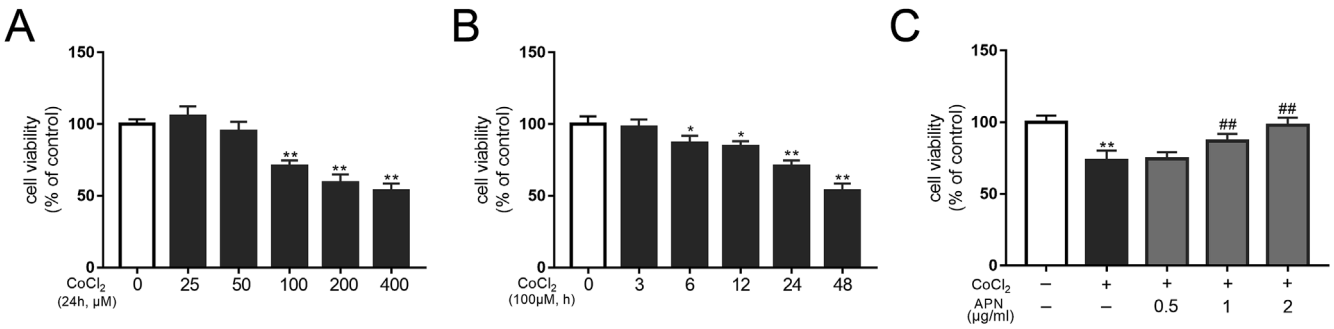

Fig. 1. The effects of $CoCl_2$ and APN on the activity of SMCs. (A) $CoCl_2$ treatment for 24 h reduced the activity of SMCs in a concentration-dependent manner. (B) Treatment with 100 μM $CoCl_2$ reduced the activity of SMCs in a time-dependent manner. (C) APN at 1 and 2 μg/ml significantly increased cell viability, which was decreased by $CoCl_2$. (*$P < 0.05$ *vs.* control, **$P < 0.01$ *vs.* control, $^{\#\#}P < 0.01$ *vs.* $CoCl_2$ treatment, n = 5, data shown as the mean ± SE).

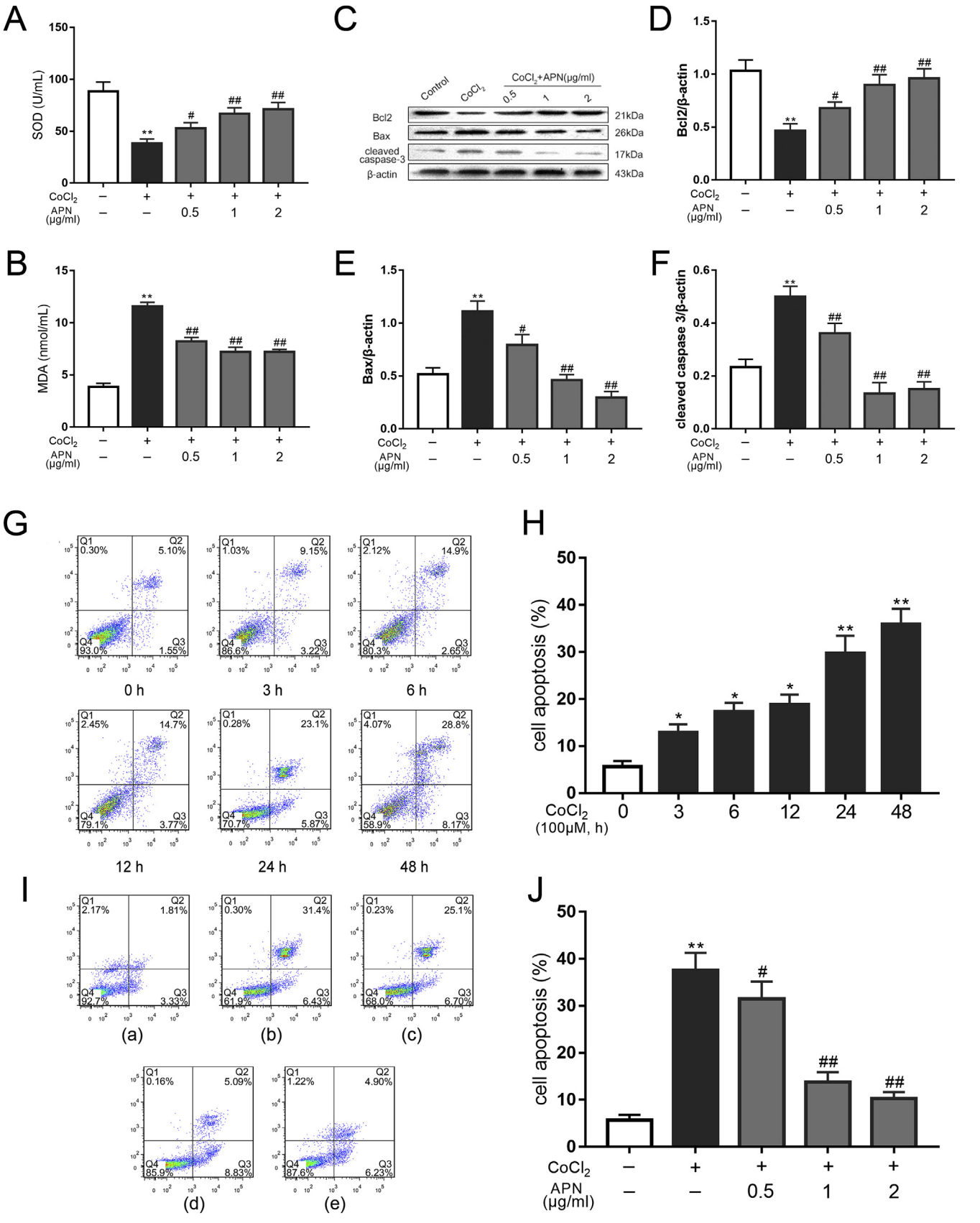

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Fig. 2. Effects of APN on $CoCl_2$-induced changes in SOD, MDA, and apoptosis and on Bax, Bcl2 and cleaved caspase-3 expression levels in SMCs. (A) The $CoCl_2$-induced reduction in SOD activity was attenuated by APN. (B) The $CoCl_2$-induced increase in MDA levels was attenuated by APN. (C) The $CoCl_2$-induced Bax and cleaved caspase-3 upregulation and Bcl2 downregulation were reversed by APN. (D, E and F) Statistical analysis of the expression levels of Bcl2, Bax and cleaved caspase-3. (G) Treatment with 100 μM $CoCl_2$ induced apoptosis in a time-dependent manner. (H) Statistical analysis of the rate of apoptosis in each group. (I) Apoptosis of cells after treatment with $CoCl_2$ and APN. APN (1 and 2 μg/ml) significantly reduced $CoCl_2$-induced apoptosis. (J) Statistical analysis of the rate of apoptosis in each group ((a) control, (b) treatment with $CoCl_2$, (c) pre-treatment with APN (0.5 μg/ml) and treatment with $CoCl_2$, (d) pre-treatment with APN (1 μg/ml) and treatment with $CoCl_2$ and (e) pre-treatment with APN (2 μg/ml) and treatment with $CoCl_2$.) ($^{*}P < 0.05$ *vs.* control, $^{**}P < 0.01$ *vs.* control, $^{\#}P < 0.05$ *vs.* $CoCl_2$ treatment $^{\#\#}P < 0.01$ *vs.* $CoCl_2$ treatment, n = 5, data shown as the mean ± SE).

Gap19. Moreover, $CoCl_2$ upregulated the expression levels of Bax and cleaved caspase-3 and downregulated those of Bcl2. These effects were reversed by APN. Again, the effects of APN were reversed by Compound C and Gap19 (Fig. 4E–L). Compared with APN pre-treatment, treatment with the AMPK inhibitor Compound C downregulated the expression of Cx43, whereas treatment with the Cx43 inhibitor Gap19 did not significantly affect the expression levels of AMPK and p-AMPKα (Fig. 4M–P).

4. Discussion

To the best of our knowledge, the SMA is the only artery that supplies blood to the cochlea. The SMA is a small-calibre vessel with a single layer of smooth muscle that lacks tightly attached connective tissue [4]. Given that the SMA is the only cochlea-supplying artery and has low collateral circulation, compensatory cochlear blood flow is likely difficult once vasospasm or hypoxia occurs in the SMA. This condition can result in disturbances and pathological damage to the

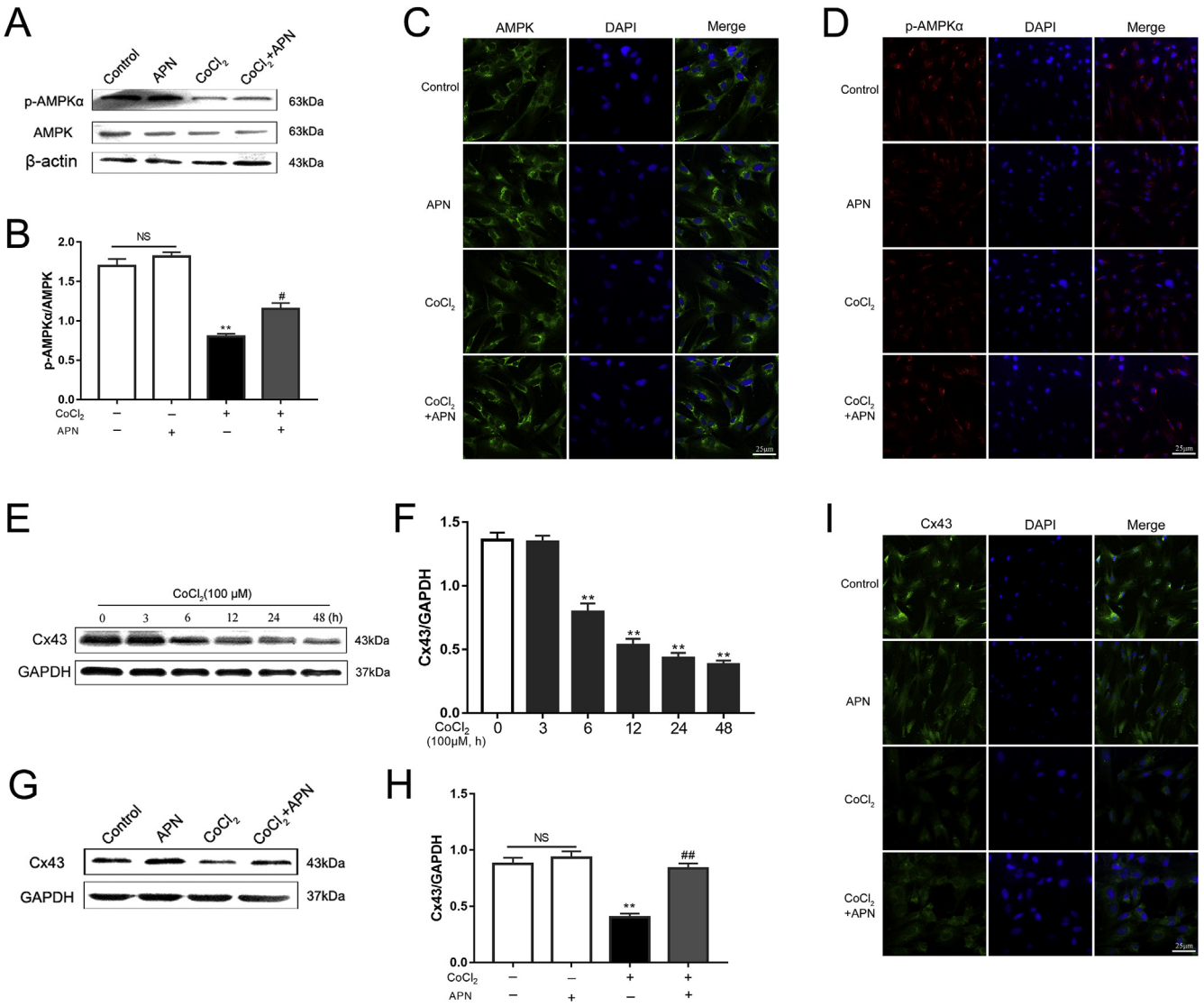

Fig. 3. Effects of APN on $CoCl_2$-induced expression changes in AMPK and Cx43 in SMCs. (A) APN (2 μg/ml) activated p-AMPKα. (B) Statistical analysis of the expression of p-AMPKα. (C, D) Expression and localization of AMPK (green) and p-AMPKα (red) in SMCs. Nuclei were counterstained with DAPI (blue). (E) $CoCl_2$ (100 μM) downregulated the expression of Cx43 in a time-dependent manner. (F) Statistical analysis of the expression of Cx43 in each group. (G) APN upregulated the expression of Cx43. (H) Statistical analysis of the expression of Cx43. (I) Expression and localization of Cx43 (green) in SMCs. Nuclei were counterstained with DAPI (blue). Scale bar: 25 μm ($^{**}P < 0.01$ *vs.* control, $^{\#}P < 0.05$ *vs.* $CoCl_2$ treatment, $^{\#\#}P < 0.01$ *vs.* $CoCl_2$ treatment, n = 5, data shown as the mean ± SE). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

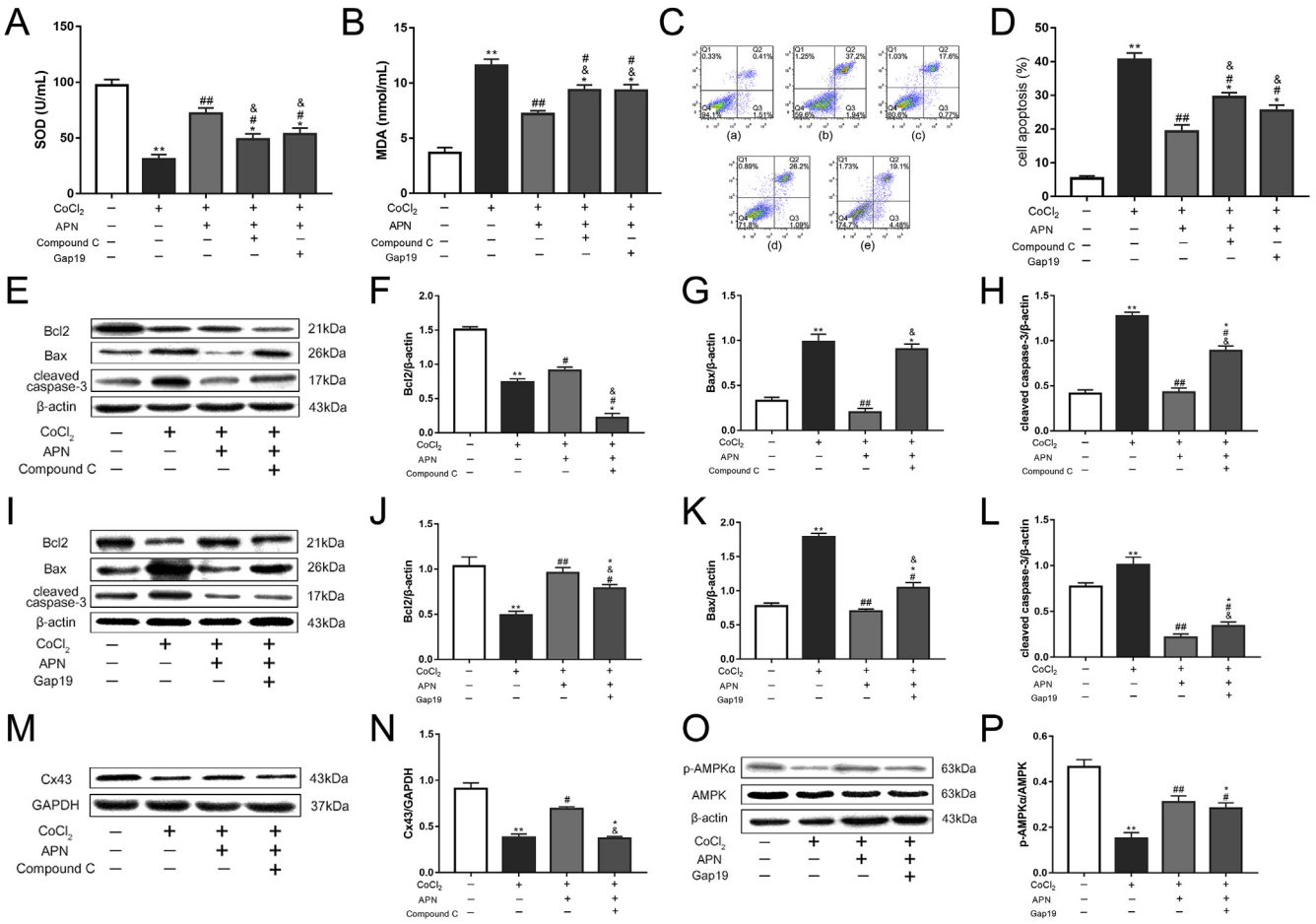

Fig. 4. Effects of APN, Compound C and Gap19 on $CoCl_2$-induced changes in SOD, MDA, and apoptosis and in Bax, Bcl2, cleaved caspase-3, Cx43 and AMPK/p-AMPKα expression levels in SMCs. (A) The $CoCl_2$-induced reduction in SOD activity was attenuated by APN. (B) The $CoCl_2$-induced increase in MDA levels was attenuated by APN, and the effects of APN were reversed by Compound C and Gap19. (C) Apoptosis of cells after treatment with $CoCl_2$, APN, Compound C and Gap19. (D) Statistical analysis of the rate of apoptosis in each group. (E, I) $CoCl_2$-induced Bax and cleaved caspase-3 upregulation and Bcl2 downregulation were reversed by APN. The effects of APN were reversed by Compound C and Gap19. (F–H, J–L) Statistical analysis of the expression levels of Bcl2, Bax and cleaved caspase-3. (M) Effects of $CoCl_2$, APN and Compound C on the expression of Cx43. (N) Statistical analysis of the expression of Cx43. (O) Effects of $CoCl_2$, APN and Gap19 on the expression levels of AMPK and p-AMPKα. (P) Statistical analysis of the expression levels of AMPK and p-AMPKα. ((a) control, (b) $CoCl_2$, (c) pre-treatment with APN and $CoCl_2$, (d) pre-treatment with APN, Compound C and $CoCl_2$ and (e) pre-treatment with APN, Gap19 and $CoCl_2$) ($^{*}P < 0.05$ *vs.* control, $^{**}P < 0.01$ *vs.* control, $^{\#}P < 0.05$ *vs.* $CoCl_2$ treatment $^{\#\#}P < 0.01$ *vs.* $CoCl_2$ treatment, $^{\&}P < 0.05$ *vs.* $CoCl_2$ + APN treatment, n = 5, data shown as the mean ± SE).

cochlear microcirculation [24]. Therefore, SMA blood supply is crucial in maintaining normal hearing [1,2].

APN has received increasing attention in recent years. APN, a fat-derived cytokine that was first discovered in 1996, is abundant in the blood circulation and has various functions [15]. Studies have shown that APN can inhibit apoptosis associated with myocardial ischaemia–reperfusion injury by regulating the ratio of Bax to Bcl2 and the expression of cleaved caspase-3 [25]. Notably, AMPK, which is known as the 'energy detector' in cells, closely monitors the state of cellular energy metabolism. In other words, AMPK maintains the balance of cell energy supply and demand by affecting multiple aspects of cellular material metabolism. APN can also activate the AMPK pathway and affect cell proliferation and apoptosis by binding to its receptors AdipoR1 and AdipoR2. Furthermore, APN suppressed the proliferation of pulmonary artery smooth muscle cells (PASMCs) isolated from the pulmonary arterial hypertension (PAH) rats by regulating the AMPK/BMP/Smad pathway [26], full-length APN (f-Ad) could be a potential anti-inflammatory reagent for cancer therapy [27,28], and APN inhibits inflammatory response of microglia to amyloid-β oligomer (AβO) via AdipoR1-AMPK-NF-κB signaling [21]. Our study revealed no significant change in AMPK expression after SMCs were treated with $CoCl_2$. However, apoptosis was significantly increased. Therefore, AMPK phosphorylation is involved in $CoCl_2$-induced cellular oxidative stress and apoptosis. Our further study found that the AMPK inhibitor Compound C attenuated the protective effect of APN. These results were consistent with the results of liu [29]. Therefore, the AMPK signalling pathway plays a key role in the protection of SMCs by APN.

Hypoxia can cause accumulation of large amounts of ROS in body tissues or cells and production of large amounts of oxidation products, such as MDA, which cause varying degrees of damage to the body [30]. ROS include free radical intermediates, such as singlet oxygen (·O), superoxide (·O2−) and hydroxyl free radical (·OH−), as well as non-radical molecules, such as hydrogen peroxide (H2O2) and hypochlorous acid (HOCl) [31]. Among oxidation-related molecules, SOD is an important transcription factor widely found in mammals and humans that plays an important role in maintaining the ability of the body to adapt to hypoxia [32]. Apoptosis can be divided into either the intrinsic apoptotic pathway or the receptor-mediated extrinsic pathway. The intrinsic apoptotic pathway is caused by internal stimuli such as DNA injury, oxidative stress, hypoxia, cytoplasmic Ca^{2+} overload and endoplasmic reticulum stress [33]. Thus, the oxidation and the changes in the apoptotic indexes, together with the expression of Bax, Bcl2,

caspase-9, caspase-3 and cleaved caspase-3 were thoroughly investigated here [34]. Notably, $CoCl_2$ is commonly used to induce hypoxic environments by replacing Fe^{2+} in haemoglobin to form deoxygenated haemoglobin. $CoCl_2$ has been used for hypoxic preconditioning in many cell models and has been found to induce cellular damage, decrease mitochondrial membrane potential and induce apoptosis in many cell types [35]. We found that the activity of SOD in $CoCl_2$-treated SMCs was significantly lower than that in control SMCs. However, in SMCs pre-treated with APN, SOD activity was increased and MDA levels were decreased. As oxidative stress injury caused by ROS is a key factor leading to apoptosis, the increased SOD activities and decreased MDA with APN pre-treatment further demonstrated that APN has strong antioxidative activity and the ability to scavenge free radicals.

Cxs are protein subunits that form GJCs and intercellular connections [36]. Such connections permit direct cell-to-cell communication via passive diffusion of signalling molecules smaller than 1.5 kDa, similar to ions or second messengers [37]. In addition to be present at the plasma membrane, Cx, particularly Cx43, has been found in mitochondria of several tissues including human umbilical vein endothelial cells, cardiomyocytes, and primary hepatocytes [38]. The functionality of cardiomyocyte mitochondrial Cx43 hemichannels has been verified by the use of channel blockers, such as carbenoxolone (CBX), Gap19 and heptanol [39]. Many factors, such as endothelin, AngII, TNF-α, b-FGF and VEGF, can affect the expression of Cxs in cells through different signalling pathways, further affecting the associated biological functions. In an oxygen–glucose deprivation model, the expression level of activated caspase-3 and the rate of neuronal apoptosis have been found to be significantly decreased after pre-treatment with neuronal CBX [40]. Moreover, studies have reported that there is evidence that Cx associates with apoptotic factors such as Bcl2 proteins Bak, Bcl-xL and Bax that colocalize with Cx43 in human tumor cells [41]. Consistently, Sun [42] demonstrated that the Cx43 effect on the caspase pathway is GJIC-independent and mediated through association of the Cx43 COOH-terminus with Bax. In our study, after treatment with $CoCl_2$, the expression of Cx43 in SMCs decreased in a time-dependent manner, and apoptosis increased gradually. Different concentrations of APN not only reduced apoptosis but also upregulated the expression of Cx43. Similarly, the Cx43 inhibitor Gap19 attenuated the protective effect of APN on SMCs. Therefore, Cx43 in cells also played a key role in the protection of SMCs by APN.

APN protected the SMCs of the SMA from $CoCl_2$-induced injury not only by adjusting SOD activity and MDA levels but also by regulating Bcl2, Bax and cleaved caspase-3 expression via activation of the AMPK signalling pathway and regulation of Cx43 expression in cells. Therefore, APN might be a promising treatment for diseases related to circulatory disturbances in the inner ear.

5. Conclusion

The present study demonstrates that APN protects SMCs of the SMA from $CoCl_2$-induced injury via activation of the AMPK signalling pathway and regulation of Cx43 expression in cells. Therefore, APN might be a promising treatment for diseases related to circulatory disturbances in the inner ear.

Declaration of competing interest

The authors report no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116876.

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Author contributions

Jingjie Xiao, Rui Yang and Ketao Ma performed study design; Jingjie Xiao, Xuqing Qin, Zhiping Zhang, and Rui Yang performed data collection and statistical analysis; Xinzhi Li, Li Li and Junqiang Si performed data interpretation; Jingjie Xiao, Rui Yang, Xinzhi Li and Ketao Ma performed manuscript preparation; Jingjie Xiao, Rui Yang, and Li Li performed literature search; Li Li, Xinzhi Li and Ketao Ma performed funds collection.

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