Taurine Prevented Hypoxia Induced Chicken Cardiomyocyte Apoptosis Through the Inhibition of Mitochondrial Pathway Activated by Calpain-1

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Abstract **Objective** To determine whether taurine has protective effects on chicken myocardial apoptosis induced by hypoxic condition through inhibiting calpain-1 derived mitochondrial apoptotic pathway. **Methods** Chicken primary embryonic myocardial cells were isolated and cultured at 37 °C under a 5% CO_2 atmosphere. Firstly the optimum concentration of taurine or PD150606 was chosen by detecting the cell viability. Chicken cardiomyocytes were cultured in 95% N_2-5% CO_2 atmosphere for 12 h to produce hypoxic conditions. Before hypoxic treatment, 10 mM taurine and 10 uM PD150606 (a specific calpains inhibitor) were added separately or together. The cell apoptosis was detected by acridine orange/ethidium bromide (AO/EB) double staining. Western blotting was used to determine the protein expressions of calpain-1, cytochrome c, Bcl-2, procaspase-9 and procaspase-3 in the cardiomyocytes. **Results** Taurine administration effectively attenuated the myocardial apoptosis under hypoxic condition, reduced the calpain-1 protein level. In addition, pre-treated taurine could up-regulate the protein expressions of Bcl-2 and procaspase-3 in hypoxic myocardial cells, down-regulate protein expression levels of cytochrome c and procaspase-9. Moreover, taurine exhibited same inhibition effect as PD150606 on the cell apoptosis and proteins express under hypoxic condition. **Conclusions** Taurine could attenuate the chicken cardiomyocyte apoptosis impaired by hypoxia through inhibiting calpian-1-derived mitochondrial apoptotic pathway in vitro.

Keywords Taurine · Apoptosis · Calpain-1 · Mitochondrial apoptotic pathway · Chicken primary myocardial cells

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1 Introduction

Myocardial ischemia is the leading cause of myocardial cell injury in heart disease, such as coronary heart disease (CHD), hypertension and congestive heart failure (CHF). Many experimental studies have shown that cardiomyocyte injuries induced by ischemic/hypoxia are closely related to the apoptosis (Kositprapa et al. 2000; Inserte et al. 2005; Bajaj and Sharma 2006; Li et al. 2009a; Zheng et al. 2015).

Calpains are a family of calcium-dependent cysteine proteases, which participate in cardiac patho-physiology. Among them, calpain-1 is one of the ubiquitous calpains and was studied extensively. Studies have reported calpain-1 plays an important role in promoting myocardial cells apoptosis under hypoxic conditions (Kositprapa et al. 2000; Zhang et al. 2015). The up-regulation of calpain-1 has also been observed in ischemic hearts (Inserte et al. 2005; Li et al. 2009b). Furthermore, transgenic mice with over-expression of calpian-1 are sufficient to heart failure (Galvez et al. 2007). Inhibitions of calpains could reduce ischemic cardiac injury in animal models of ischemia/reperfusion or myocardial infarction (MI) (Mani et al. 2009; Hernando et al. 2010; Ma et al. 2012; Zheng et al. 2015).

Taurine, 2-aminoethylsulfonic acid, presents a high concentration in mammalian heart and accounts for more than 50% of the total amino acid pool of heart tissues (Lombardini 1996). Studies have reported that taurine has myocardial protective effects, such as tissue structure and function maintenance, osmoregulation, membrane stabilization, antioxidant, modulation of ion movement and anti-apoptosis (Ito et al. 2008; Schaffer et al. 2010; Wojcik et al. 2013; Wang et al. 2018). Exogenous administration of taurine could inhibit myocardial cell apoptosis under hypoxia/ischemic conditions (Takahashi et al. 2003; Rivard et al. 2007; Yang et al. 2013; Setyarani et al. 2014; Wang et al. 2018).

However, the relationship between taurine and calpain-1 in the hypoxic cardiomyocytes is unknown. In this study, taurine and PD150606 were pre-incubation in the chicken primary myocardial cells and hypoxia was used to induced cell apoptosis. The protective effect of taurine was evaluated by detection the cell apoptosis and protein expressions of calpain-1, cytochrome c, Bcl-2, procaspase-9 and procaspase-3 to explore whether taurine could inhibit calpian-1-derived mitochondrial apoptotic pathway against myocardial hypoxic injury.

2 Material and Methods

2.1 Isolation of Chicken Primary Embryonic Cardiomyocytes

Firstly, AA broiler eggs were obtained from Shenyang Huamei Livestock and Poultry CO., LTD (Shenyang, China). Animal handling and experimental procedures followed the guidelines of the Animal Care and Use Committee of Shenyang

Agricultural University. Chicken embryos at 12 d were used to get the hearts under sterile conditions. The ventricular tissues were cut into pieces after being washed five times in pre-cooled phosphate buffered saline (PBS) solution and then were digested with 0.1% trypsin solution at 4 °C for 14–16 h to obtain a cell suspension. The reaction was terminated by addition of same volume Dulbecco's modified Eagle's medium (DMEM/F12; Hycloe, USA) containing 10% fetal bovine serum (FBS; Life Technologies), 100 units/mL penicillin and 100 units/mL streptomycin at 37 °C for 5 min. After discarding the supernatant, the cardiac tissues were washed by double volume DMEM/F12 medium containing 0.08% collagenase type II, 0.05% bovine serum albumin (BSA; Hycloe, USA), 100 units/mL penicillin and 100 units/mL streptomycin and then resuspended with three times volume of same medium at 37 °C for 15–20 min. Repeat this step 4–5 times. All the supernatant was filtered through a 200 μm nylon mesh cell strainer into a new tube, and then centrifuged at 1000 × g for 5 min. Discard the supernatant and resuspend the cells in DMEM/F12 medium containing 10% FBS, 100 units/mL penicillin and 100 units/mL streptomycin. Cell density was adjusted at 5×10^5/mL and cells were cultured in cell culture bottle at 37 °C under a 5% CO_2 atmosphere. Cells were transferred to new cell culture bottle after 1 h Repeat this step again. Cell density was adjusted at 4×10^5/mL and cells were cultured in DMEM/F12 medium containing 10% FBS, 0.1 mM 5-Brdu (Sigma, St. Louis, MO, USA), 100 units/mL penicillin and 100 units/mL streptomycin for 72 h.

2.2 Measurement the Myocardial Cell Activity by MTS

Cardiomyocytes viability was measured by using MTS. Briefly, isolated cells were plated in 96-well plates at a density of 4×10^4 cells per well. When the cell fusion got 80%, the cells were pre-treated with taurine (0, 5, 10, 20 mM; Sigma) or PD150606 (0, 10, 20 mM; Sigma; dissolved in 0.1% DMSO) for 12 h, 24 h. The cell viability was detected by adding MTS solution following the kit manufacture instruction (Cell Titer 96® AQ_{ueous} One Solution Cell Proliferation Assay kit, Promega, USA) and optical density was determined at 490 nm by a spectrophotometer (Infinite M200PRO; Tecan, Switzerland). Each administration repeated at least three times.

2.3 Experimental Treatment

Isolated chicken primary myocardial cells were plated in 24-well plates at a density of 2×10^5 cells per well. After cultured 60–72 h at 37 °C in the CO_2 incubator, the cells were divided into eight groups and treated as Table 1, each treatment repeated three times. Group 1 served as the normal control group (C), the cells were cultured

Table 1 The experiment design

Group	Taurine (10 mM)	PD150606 (10 μM)	Hypoxia (5% CO_2 + 95% N_2, 12 h)
Normal control (C)	–	–	–
Taurine control (CT)	+	–	–
Taurine +PD150606 control (CTP)	+	+	–
PD150606 control (CP)	–	+	–
Model (M)	–	–	+
Taurine +Model (MT)	+	–	+
Taurine +PD150606+Model (MTP)	+	+	+
PD150606+Model (MP)	–	+	+

at 37 °C for 24 h without any treatment. Group 2 was the taurine control group (CT), taurine was supplemented to the cell cultural medium with the final concentration at 10 mM after dividing group. Group 3 was taurine +PD150606 control group (CTP), PD150606 was added to the culture solution with 10 μM final concentration at 11.5 h after taurine supplement. Group 4 was the PD150606 control (CP), PD150606 was added to the culture solution at 11.5 h, and then the cells culture continued 12.5 h till the end. The cells in group 5 were cultured in 5% CO_2 and 95% N_2 at 37 °C for 12 h from 12 to 24 h after gouping, as the hypoxia model group (M). In group 6 (MT), 10 mM taurine was added to the cell culture medium at 12 h before hypoxia treatment. In group 7 (MTP), taurine and PD150606 was added respectively at 12 h and 0.5 h before hypoxia administration. In group 8 (MP), PD150606 was added at 0.5 h before hypoxia treatment.

2.4 Detection of Myocardial Cell Apoptosis by AO/EB Double Staining

After cells were cultured at 37 °C, 5% CO_2 and 95% N_2 for 12 h (the time was decided by previous experiment), removing culture medium completely, cells were washed three times with pre-cooled PBS and then fixed with neutral 4% paraformaldehyde buffered for 3 h at room temperature. The cells were washed three times by PBS again after discarding the fixed solutions. 0.2 mL 0.5% Triton X – 100 was added to each well. After 10 min, washing the cells with PBS for three times, dual fluorescent staining solution (AO/EB double staining kit, Beijing Solarbio Science & Technology Co., Ltd.) was added to each well. The apoptotic cells and the total cells were counted at 5 view in each well using a Leica DM4000B inverted fluorescent microscope (Leica, Germany). The percentage of apoptosis was calculated by the ration of the number of apoptotic cells to the number of total cells.

2.5 Western Blotting Analysis

The proteins in chicken primary myocardial cells were extracted according to kit instructions (Beyotime, China). After detection the protein concentration with a BCA protein assay kit (Beyotime, China, P0012S), the proteins were separated with SDS-PAGE electrophoresis and then transferred onto polyvinylidenedifluoride (PVDF) membranes (Beyotime, China). After blocking with 5% (W/V) non-fat milk in Tris buffer solution tween (TBST) (Applygen, China) for 3 h at room temperature, the membranes were incubated with antibodies against β-actin (anti rabbit β-actin, Abcam, Cambridge, UK, ab8227, dilution: 1:5000), calpain-1 (anti goat calpain-1, Abcam, Cambridge, UK, ab174683, dilution: 1:1000), cytochrome c (anti rabbit cytochrome c, BBI, UK, D220521; dilution: 1:1000), Bcl-2 (anti rabbit Bcl-2, Abcam, Cambridge, UK, ab174683, dilution: 1:1000), caspase-9 (anti rabbit caspase-9 P10, Bioss, UK, bs-8502R, dilution: 1:1000), and caspase-3 (anti rabbit caspase-3, ABclonal, USA, A11953, dilution: 1:1000) overnight at 4 °C. After being washed in TBST for three times, the membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000, ZSGB-Bio, China, ZB-2305) for 45 min at room temperature. Signals were visualized by a Super ECL kit (New Cell & Molecular Biotech Co., Ltd. (Suzhou, China, P0018). The optical densities of protein bands were recorded by DNR bio-imaging system (Microchemi 4.2, Isreal) and quantified by Image Quant 5.0 software, the relative expressions were normalized to β-actin.

2.6 Statistical Analysis

All the data were expressed as means ± standard error and significant differences were determined by one-way ANOVA and Duncan's multiple -range test using SPSS 16.0 software. A difference was considered significant at the $p < 0.05$ level. A highly significant was considered at the $p < 0.01$ level.

3 Results

3.1 Optimum Dose of Taurine or PD150606

The effect of different concentrations of taurine on the myocardial cell viability at 12 h and 24 h were shown in Fig. 1a. Cell viability was not influenced by different taurine treatment at 12 h. While at 24 h, all taurine treatment increased the cell viability, especially when taurine was added at 10 mM, cell viability was elevated

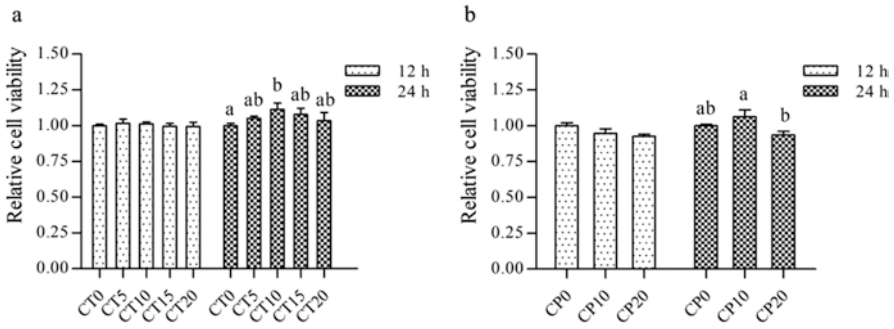

Fig. 1 The cell viability of myocardial cells at 12 h and 24 h. (**a**) Effect of taruine on cell viability. (**b**) Effect of PD150606 on cell viability. Data are the mean ± SEM (n = 3). The column with different lowercase letters on the top represent significant difference ($p < 0.05$)

significantly compared with no taurine treatment, but there wasn't significant difference between the taurine treatment groups. From these results, 10 mM taurine was chosen for subsequent experiment.

The effect of different concentrations of PD150606 on the cell viability cells at 12 h and 24 h were shown in Fig. 1b. At 12 h and 24 h, no matter 10 μM PD150606 or 20 μM PD150606 had no effect on the cell viability compared with no adding group. However, the cell viability treated with 20 μM PD150606 were reduced significantly than that treated with 10 μM PD150606 ($p < 0.05$). In the following experiment, 10 μM PD150606 was used.

3.2 The Effect of Taurine on the Cardiomyocyte Apoptosis

The effect of taurine on the cardiomyocyte apoptosis was shown in Figs. 2 and 3. Under the normal culture condition, cell apoptosis also could be detected (Fig. 2a, b, c, d). Hypoxia treatment 12 h induced obvious apoptosis, not only the early-stage apoptotic cells but also the late-stage apoptotic cells were also increased (Fig. 2e). Pre-treated taurine, PD150606, taurine + PD150606 respectively could reduce the myocardial cell apoptosis induced by hypoxia (Fig. 2f, g, h). As shown in Fig. 3, compared with C group, the percentage of apoptosis was decreased by 23.42% ($P < 0.01$) (group CT), 20.49 ($P < 0.01$) (group CTP) and 23.42% ($P < 0.01$) (group CP) respectively. However there wasn't significant difference between the different treatment groups under normal culture. These outcomes indicated that taurine or PD150606 was contributed the anti-apoptosis at normal condition. The percentage of apoptosis in M group was increased significantly compared with the C group ($P < 0.01$). As compared with the M group, the percentage of apoptosis was decreased by 16.56% ($P < 0.05$) (group MT), 39.11% ($P < 0.01$) (group MTP) and

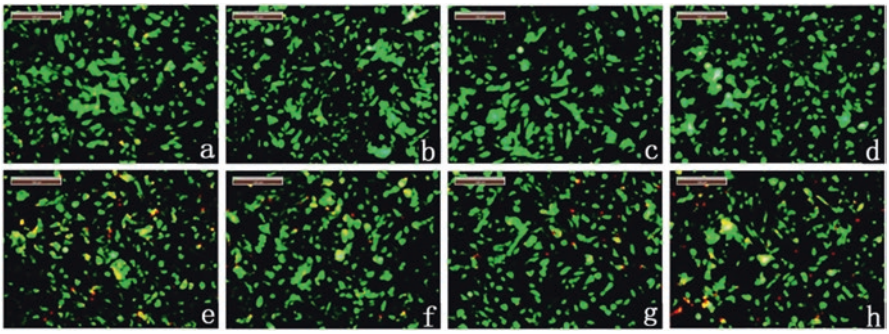

Fig. 2 The fluorescence micrographs of myocardial cells at 12 h after hypoxia treatment (100× magnification). (**a**) C group. (**b**) CT group. (**c**) CTP group. (**d**) CP group. (**e**) M group. (**f**) MT group. (**g**) MTP group. (**h**) MP group. Normal cells: the cells showed green. Early apoptotic cells: nucleus showed yellow-green fluorescence by AO staining and concentrated into a crescent or granular that located in 1 side of cells. Late apoptotic cells: the nucleus showed orange-green or red fluorescence and gathered in concentration and located in bias

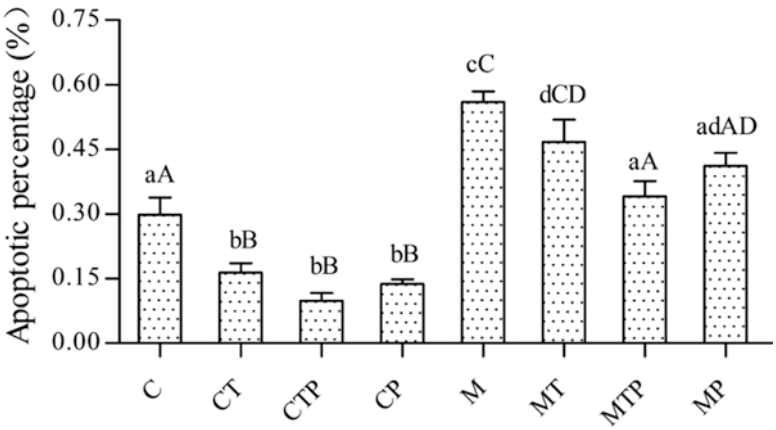

Fig. 3 Effect of taurine on the percentage of apoptosis in myocardial cells (%) Data are the mean ± SEM (n = 3). The column with different lowercase letters on the top represent significant difference ($p < 0.05$), with different capitals represent significant difference ($P < 0.01$)

26.47% ($P < 0.01$) (group MP) respectively. Pre-treated taurine + PD150606 was significantly inhibited the cell apoptosis by 27.03% ($P < 0.01$) compared with taurine administration (group MT) and 17.21% ($P > 0.05$) compared with PD150606 (group MP) administration in hypoxia conditions. However, there was no significant difference between group MT and MP. These results implied taurine had the same effect as PD150606 in inhibiting myocardial cell apoptosis induced by hypoxia.

3.3 *The Effect of Taurine on the Protein Expression Levels of Calpain-1, Cytochrome c, Bcl-2, Procaspase-9 and Procaspase-3*

Figure 4 illustrated the protein level of calpain-1 under different treatments. Compared with the control group, the expression levels of calpain-1 in CT, CTP and CP groups were increased significantly ($P < 0.01$), but there was no significant difference between the treatment groups under normal conditions. The expression level of calpain-1 in M group were elevated significantly ($P < 0.01$) compared with the group C. Pre-treated taurine or PD150606 could significantly decrease the protein level of calpain-1 ($P < 0.01$), and there was no difference between group MT and group MP. The results indicated that calpain-1 was involved in the myocardial cell injury induced by hypoxia and taurine could down-regulate the calpain-1 protein expression against the injury. Besides, the inhibited effect of taurine was similar with the PD150606.

As shown in Fig. 5, compared with the control group, the expression levels of cytochrome c and procaspase-9 in model group (M) were increased significantly ($P < 0.01$), but the expression levels of Bcl-2 and procaspase-3 were decreased ($P < 0.01$). In contrast with the M group, the values in the group MT showed a significant decline in protein expressions of cytochrome c ($P < 0.01$), procaspase-9 ($P < 0.01$) and a notable elevation in the protein expression levels of Bcl-2 and

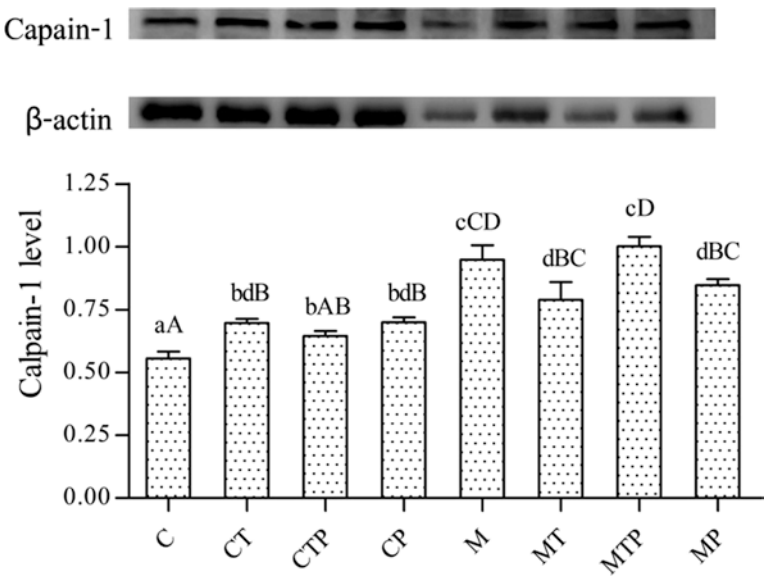

Fig. 4 The effect of taurine on the expression levels of calpain-1 in myocardial cells. Data are the mean ± SEM (n = 3). The column with different lowercase letters on the top represent significant difference ($p < 0.05$), with different capitals represent significant difference ($P < 0.01$)

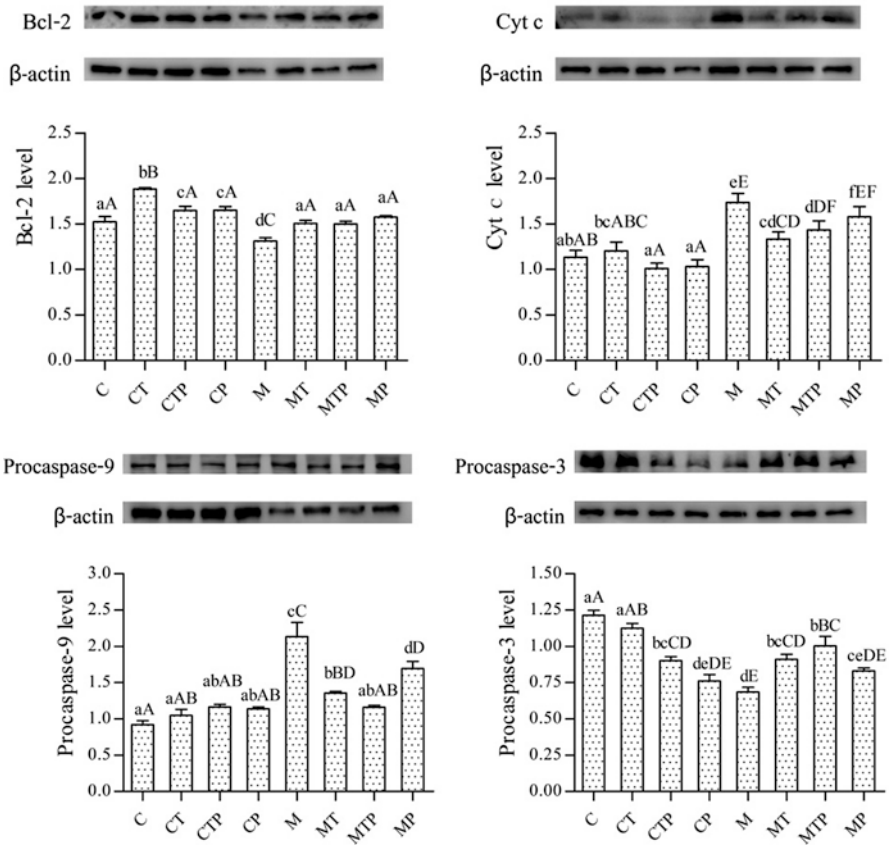

Fig. 5 The effect of taurine on the expression levels of cytochrome c, Bcl-2, procaspase-9 and procaspase-3 in myocardial cells. Data are the mean ± SEM (n = 3). The column with different lowercase letters on the top represent significant difference ($p < 0.05$), with different capitals represent significant difference ($P < 0.01$)

procaspase-3 ($P < 0.01$). The expression levels of the four proteins had similar changes in the MP group, while the values showed the regulation effect of pre-administrated PD150606 was lower than that of pre-administrated taurine on the cytochrome c ($P < 0.01$) and procaspase-9 ($P < 0.05$) expression. No significant difference was observed between MT and MTP groups on the four proteins, but obvious difference was found between MTP and MP groups. Compared with the MP group, the values in group MT showed significant decrease of the protein expressions of cytochrome c ($P < 0.05$) and procaspase-9 ($P < 0.01$) and a further increase of the protein level of procaspase-3 ($P < 0.01$). The results indicated that taurine had the similar regulation as PD150606 in hypoxia conditions.

4 Discussion

The major findings of the present study are as follows: (1) Taurine attenuated the myocardial cells apoptosis induced by hypoxia and exhibited the same inhibitory effect as PD150606. (2) Taurine inhibited the protein express of calpain-1 in hypoxic myocardial cells and the effect of inhibition was equal to the PD150606. (3) Pretreated taurine and/or PD150606 up-regulated the protein expressions of Bcl-2 and procaspase-3 and down-regulated protein levels of cytochrome c and procaspase-9 in hypoxic myocardial cells, the regulating effect of taurine on cytochrome c and procaspase-9 is more effective than that of PD150606.

Apoptosis, or programmed cell death, is an important way to maintain the cellular homeostasis between cell division and cell death. Myocardial cell is terminally differentiated cell, apoptosis could lead to a progressive reduction in overall heart function and possible heart failure (Gaballa and Goldman 2002). In modern broiler flocks, fast-growing broiler chickens are susceptible to heart disease, the right ventricular and heart failure was found easily in broilers with sudden death syndrome or ascites syndrome (Olkowski 2007). In this experiment, the apoptosis was significantly increased in chicken primary myocardial cells impaired by 12 h hypoxia. Taurine administration attenuated the myocardial cells apoptosis and reduced the calpain-1protein expression level under hypoxic condition. It also showed that taurine played the same effect of inhibition as PD150606. These results indicated calpain-1 involved the chicken primary myocardial cell injury induced by hypoxia, taurine played anti-apoptosis through inhibiting the protein express of calpain-1.

In this study, calpain-1 involved the chicken primary myocardial cell injury induced by hypoxia, which is consist with previous reports that activation and/or up-regulation expression of calpain-1 contributes to the cardiomyocytes injury under hypoxia/ischemic conditions (Kositprapa et al. 2000; Inserte et al. 2005; Li et al. 2009b; Zhang et al. 2015; Zheng et al. 2015). Taurine played the similar inhibition effect as PD150606 on the protein express of calpain-1. PD 150606 is a selective, cell-permeable, non-peptide and uncompetitive calpains inhibitor. It inhibits calpains by binding to the calcium-binding domain of the enzyme (Waters et al. 1997). As a calcium-dependent cysteine protease, the accumulation of intracellular Ca^{2+} could significantly increase apoptosis susceptibility by the activation of calpain-1 and unregulated over-activation of calpains led to the loss of Ca^{2+} homeostasis (Zatz and Starling 2005). Another researches reported calpains inhibits Na^{+}/K^{+} ATPase activity in the heart (Inserte et al. 2006) which may induce apoptosis via Ca^{2+} overload (Ramirez-Ortega et al. 2007). Numerous studies have demonstrated taurine could inhibit the increase of myocardial intracellular Ca^{2+} (Yang et al. 2013; Wang et al. 2018) and prevent the Ca^{2+} overload in cardiomyocytes under stress conditions (Xu et al. 2006).

It has been reported that mitochondria apoptosis pathway plays an important roles in the apoptosis of myocardial cells (Yang et al. 2013). Cytochrome c leads to caspase-3 activation which is an important mitochondrial apoptotic marker (Hu et al. 2018). Once cytochrome c was released into cytosol, cytochrome c, apoptosis activating factor-1 (Apaf-1) and procaspase-9 could form apoptosome complex in

the presence of dATP, and this complex could drive the activation of caspase-3. In the present study, we demonstrated that taurine prevented the hypoxia-induced apoptosis in cardiomyocytes, accompanied by the up-regulation of Bcl-2 and procaspase-3 protein expressions and down-regulation of cytochrome c and procaspase-9 protein levels. Taurine could effectively prevent myocardial ischemia-induced apoptosis by inhibiting the assembly of the Apaf-1/caspase-9 apoptosome and suppressing cleavage of caspase-9 and caspase-3 (Takatani et al. 2004). Our results also showed that taurine administration decreased the protein levels of cytochrome c and procaspase-9 under hypoxia-induced myocardial injury, which maybe reduce the form of cytochrome c/Apaf-1/procaspase-9 and inhibit the mitochondrial mediated apoptotic pathway. Bcl-2 family members could regulate apoptosis by modulating the release of cytochrome c from mitochondria to cytosol. In the study, taurine increased the Bcl-2 protein level under hypoxia. The increase of the Bcl-2 protein express could decrease the release of cytochrome c which could inhibit mitochondrial mediated apoptotic pathway.

5 Conclusion

In summary, this study shows that preventive administration of taurine effectively decreased hypoxia-induced cardiomyocyte apoptosis by inhibiting mitochondrial pathway activated by calpain-1 in chicken primary myocardial cells.

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