**Taurine Protects Primary Neonatal Cardiomyocytes Against Apoptosis Induced by Hydrogen Peroxide**

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**Summary**

The aim of this study was to investigate the effects of taurine (Tau) on primary cultured neonatal myocardial cells treated with hydrogen peroxide (H$_2$O$_2$) and the underlying mechanism. Primary cardiac myocytes from neonatal Wistar rats were pre-incubated with Tau, and its effects on cell viability and expression of CaM, CaMKII, p53, Bcl-2, and Bax were examined. Tau enhanced the viability of myocardial cells, decreased apoptosis, and alleviated the intracellular calcium overload, especially at dosages of 40 or 80 mM ($P < 0.01$ or $P < 0.001$, respectively). Moreover, Tau could inhibit the H$_2$O$_2$-induced decrease in CamKII and CaM expression at both the mRNA and protein levels. The pattern of CaMKII expression was consistent with that of the anti-apoptotic protein Bcl-2, but contrasted the pattern of the pro-apoptotic proteins p53 and Bax. Thus, our results show that Tau protects myocardial cells against damage caused by H$_2$O$_2$ exposure, suggesting that it might play a role in the mitochondrial apoptotic pathway by upregulating the expression of CaMKII to rescue myocardial cells. However, the underlying mechanism still needs to be investigated. In addition, we tested the protective effect of taurine on cardiac myocytes, and the effect of taurine on another model, specifically an animal model.

**Key words:** Myocytes, Ca$^{2+}$/CaM/CaMKII

**Oxidative stress has been implicated in the pathogenesis of myocardial ischemia-reperfusion injury.** Under the pathological conditions of ischemia-reperfusion, the levels of reactive oxygen species (ROS) are elevated, causing oxidative stress. ROS, including hydrogen peroxide (H$_2$O$_2$), have been reported to induce apoptosis at higher concentrations via the regulation of mitochondrial Ca$^{2+}$ levels. Intracellular Ca$^{2+}$ is a quintessential intracellular messenger and many of its cellular effects are transduced via calmodulin (CaM). ROS modify a number of cardiac ion channels and transporters either through direct oxidation or through the activation of signaling molecules, such as Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII).

CaMKIIδ is the predominant CaMKII isoform in the heart and plays an important role in the maintenance of Ca$^{2+}$ homeostasis and regulation of apoptosis. Normally, CaMKIIδ phosphorylates myocardial proteins involved in Ca$^{2+}$ transport, including ryanodine receptors, sarcoplasmic reticular Ca$^{2+}$-ATPase, and phospholamban; it also modulates L-type Ca$^{2+}$ channels and excitation-contraction coupling in cardiac myocytes. In addition, CaMKIIδ has also been implicated in apoptotic signaling in the heart. CaMKII is activated by ROS-induced oxidation of methionine residues at positions 281 and 282. ROS-dependent modification of CaMKII activity is responsible for cardiac myocyte apoptosis. Furthermore, the role of CaMKII in promoting apoptosis is associated with Fas and mitochondrial apoptotic pathways. These findings imply that CaMKII is involved in ROS-induced apoptosis via the regulation of Ca$^{2+}$ homeostasis.

Taurine (2-aminoethanesulfonic acid, Tau) is a natural amino acid that is expressed widely in all mammalian tissues but most abundantly in the heart. Previous studies have suggested that Tau can act as an antioxidant and protect the heart against various pathophysiological stimulators. Tau attenuates myocardial apoptosis induced by Ca$^{2+}$ overload or ischemia-reperfusion injury. The mechanism underlying the anti-apoptotic action of Tau is related to the regulation of L-type Ca$^{2+}$ channels and Ca$^{2+}$-ATPase-mediated Ca$^{2+}$ uptake, which is associated with the apoptotic pathway. However, the mechanisms underlying cardiac protection by Tau are not understood completely.
In this study, we exogenously applied H$_2$O$_2$, which has been used to mimic ROS exposure in reperfused myocardium in vivo, to rat cardiomyocytes and examined whether Tau played a role in protecting myocardial cells from H$_2$O$_2$-induced oxidative damage. To determine the underlying mechanism, we also investigated the effect of Tau on the Ca$^{2+}$/CaM/CaMKII signaling pathway.

**Methods**

**Isolation and culture of rat cardiac myocytes:** Primary cultures of ventricular cardiac myocytes from neonatal Wistar rats were prepared by gentle serial trypsinization, as described previously. Briefly, the rat ventricular myocardium was surgically removed, minced into 1-3 mm pieces, and then washed twice in phosphate-buffered saline (PBS). The minced tissues were dissociated by 4-6 repeated exposures to 0.25% (wt/vol) trypsin/1 mM EDTA solution at 37°C, along with gentle agitation. The supernatant was collected and centrifuged at 500 g for 5 minutes at room temperature. Cell pellets were resuspended in IMEM (10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin), and plated onto a culture dish. Isolation and culture of rat cardiac myocytes: Rat cardiac myocytes (3 × 10$^5$ cells/mL) were seeded onto each well of 96-well plates, which were randomly divided into the following 6 groups: a control group, an H$_2$O$_2$ damaged group, a Tau alone group (40 mM), and 3 groups pretreated with various concentrations of Tau for 12 hours (80, 40, 20 mM) before H$_2$O$_2$ exposure (incubated with a final concentration of 0.3 mM/L H$_2$O$_2$ for 6 hours). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) test was used to perform cardiac myocyte viability on an enzyme-linked immunosorbent assay plate reader (TECAN, Austria) at 490 nm wavelength.

**Determination of LDH, SOD, and MDA concentrations:** Rat cardiac myocytes (3 × 10$^5$ cells/mL) were seeded onto each well of 96-well plates and incubated with H$_2$O$_2$ at a final concentration of 0.3 mM/L for 6 hours. The concentration of lactate dehydrogenase (LDH) in the culture solution was then measured using a colorimetric LDH assay kit. After flushing the adherent cardiac myocytes with PBS 3 times, 0.5 mL PBS was added to each well, and the plates were placed in -80°C 3 times, 0.5 mL PBS was added to each well, and the plates were placed in -80°C 3 times, 0.5 mL PBS was added to each well, and the plates were placed in -80°C 3 times to break the cells open. The suspension was then centrifuged and the concentrations of superoxide dismutase (SOD) and malondialdehyde (MDA) in the supernatant were measured using SOD and MDA assay kits, respectively. Results have been expressed as the mean ± standard deviation (SD).

**Measurement of intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$):** Rat cardiac myocytes (3 × 10$^5$ cells/well) were seeded onto each well of 96-well plates and treated as described above (in section 2.3). Changes in [Ca$^{2+}$]$_i$ were measured using the fluorescent dye Fura-3. Briefly, after the indicated treatment, the cells grown on cover slips were incubated for 40 minutes with 10 μM Fura-3/AM at 37°C. The cells were then flushed 3 times with HEPES, and 200 μL PBS containing different concentrations of Tau was added. After observation under a confocal laser scanning microscope (1 scan per second; observed for 60 seconds), H$_2$O$_2$ was added followed by observing the changes in [Ca$^{2+}$]$_i$ for 10 minutes. Quantitative fluorescence intensity data were acquired at the excitation wavelength of 506 nm and emission wavelength of 526 nm at the sampling rate of 1 Hz; 7 cells per coverslip were selected for observation. The fluorescence ratio at 506 and 526 nm (F506/F526) was used to reflect the changes in [Ca$^{2+}$]$_i$.

**RNA extraction and RT-PCR:** Total RNA was extracted using TRIzol Reagent (Invitrogen, #15596-026), according to the manufacturer’s protocol, and the synthesis of cDNA was carried out using RevertAid™ HMinus M-MulV reverse transcriptase (Trangen, Beijing, China, AT301-03). The expressions of CaM, CaMKII, Bcl-2, Bax, and the housekeeping gene β-actin were quantified using a PCR kit. The reaction mixture for PCR contained 2 μL cDNA, 10 μL SYBR Premix Ex Taq™, 0.4 μL of each primer (10 μM), and 7.2 μL of ultra-pure water. The thermal cycler conditions were as follows: Taq activation at 95°C for 30 seconds, 40 cycles of amplification at 95°C for 5 seconds, and final extension at 60°C for 30 seconds. The following primers were used for PCR: CaM forward, 5'-AC AACAAAGGACCTGGGGAC-3'; CaM reverse, 5'-TCTG CTGCACCTGTAGTTGCC-3'; CaMKII forward, 5'-TCAG CAGCAAAGAGTTGTCTG-3'; CaMKII reverse, 5'-AAT ACAAGGGTGCTTGTAGTG-3'; Bcl-2 forward, 5'-TGGGAT GCCCTCTGTGGAGAT-3'; Bcl-2 reverse, 5'-CGATGTTGTC ACTCTCTGCAAGG-3'; Bax forward, 5'-ATCCAGGATCG AGCAGGGGAGATGG-3'; Bax reverse, 5'-AGATGTTGTC ACTGTCGGCATGTCGAG-3'; β-actin forward, 5'-AGATTCGAGCGAGGGGAGATGG-3'; β-actin reverse, 5'-GAGGGCTTGGATGGCGAGG-3'.

**Protein extraction and Western blot analysis:** Cell lysate was prepared using Passive Lysis Buffer (Biottium, catalog no. 99912). The protein content in each fraction was analyzed using the Bradford assay; 30 μg of protein from each fraction was separated on 12% SDS-PAGE gel and transferred onto PVDF membrane. The membranes were blocked in 5% non-fat milk/PBST and blotted overnight at 4°C with the following primary antibodies individually, according to the manufacturer’s protocol: anti-CaMKII (1:500; sc-5306 Santa Cruz Biotechnology), anti-Bcl-2 (1:500; sc-7382 Santa Cruz Biotechnology), anti-Bax (1:500; sc-7480 Santa Cruz Biotechnology), anti-β-actin (1:500; sc-47778 Santa Cruz Biotechnology). The next day, membranes were incubated with peroxidase-conjugated anti-mouse secondary antibody (1:1000; sc-2005 Santa Cruz Biotechnology) for 1 hour at room temperature. SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, USA) was used according to the manufacturer’s protocol, to detect bound antibodies.
Figure 1. Effect of Tau on the survival of myocardial cells damaged by H2O2. Rat cardiac myocytes (5 x 105 cells/mL) were seeded onto each well of 96-well plates, which were randomly divided into 5 groups: control group, H2O2 group, and 3 Tau pre-treatment groups (80, 40, and 20 mM) before H2O2 damage. The cells were pre-incubated with various concentrations of Tau for 12 hours and then incubated with a final concentration of 0.3 mmol/L H2O2 for another 6 hours. Cell viability was tested by MTT assay. *P < 0.001 or **P < 0.001, compared with the control group, ***P < 0.001, compared with the H2O2-damaged group.

Statistical analysis: Data are reported as the mean ± SD and median for general characteristics of subjects. All statistical analyses were performed using SPSS 16.0 software. Differences between different groups were assessed using one-way ANOVA. Values of P < 0.05 were considered to indicate statistical significance.

Results

Effects of Tau on myocardial cell viability after exposure to H2O2: To analyze the protective effects of Tau against H2O2-induced injury, neonatal rat myocardial cells were pre-treated with various concentrations of Tau (20, 40, or 80 mM) for 12 hours and then treated with H2O2 for 6 hours. Cell viability was determined using the MTT assay. As shown in Figure 1, the viability of cells treated with H2O2 decreased significantly, and pre-treatment of the cells with 40 mM Tau alone did not affect viability. However, cells pre-treated with 20, 40, and 80 mM Tau for 12 hours were dose-dependently protected against H2O2-induced damage (P < 0.001 or P < 0.01).

Effects of Tau on the expressions of LDH, SOD, and MDA in myocardial cells damaged by H2O2 exposure: The level of LDH is an important parameter that reflects cell membrane injury induced by exogenous compounds.28 We tested the effect of Tau on LDH expression. As shown in Figure 2A, a significant increase in LDH levels was observed following exposure to H2O2. Myocardial cells pre-treated with Tau (at 40 and 80 mM) secreted significantly lower levels of LDH following H2O2 exposure (P < 0.05 and P < 0.01, respectively) than did the cells in the H2O2 group.

MDA and SOD activity in the cell lysates was measured to evaluate the lipid peroxidation and oxidant stress. As shown in Figure 2B, myocardial cells pre-treated with Tau (at 20, 40 and 80 mM; P < 0.01 or P < 0.05) showed significantly higher SOD concentrations than did the cells in the H2O2 group. In addition, compared with the H2O2 group, the groups treated with Tau (at 40 and 80 mM)

Figure 2. Effects of Tau on the expression of LDH, SOD, and MDA in myocardial cells damaged by H2O2 exposure. Rat cardiac myocytes were randomly divided into 6 groups: control group, H2O2 group, Tau alone (40 mM), and 3 Tau pre-treatment groups (80, 40, 20 mM) before H2O2 damage. The cells were pre-incubated with various concentrations of Tau for 12 hours and then incubated with a final concentration of 0.3 mmol/L H2O2 for another 6 hours. A: The concentration of LDH was measured to evaluate cell membrane injury. B: MDA in the cell lysates was measured to evaluate lipid peroxidation. C: SOD activity in the cell lysates was measured to evaluate oxidative stress. *P with control group, **P < 0.05, ***P < 0.01 compared with H2O2-damaged group. (n = 6, x ± s).
showed significantly \((P < 0.01)\) lower MDA production (Figure 2C).

**Effects of Tau on \([\text{Ca}^{2+}]_i\):** Oxidative stress induces apoptosis, which is associated with increasing \([\text{Ca}^{2+}]_i\). In this study, changes in \([\text{Ca}^{2+}]_i\) were measured using a confocal laser scanning microscope. Taking 10 μM fura-3/AM-marked cells as the baseline, we added H2O2 and observed changes in \([\text{Ca}^{2+}]_i\) for the following 10 minutes. The fluorescence (F506/F526) measured between 0 and 5 minutes was taken to reflect the \([\text{Ca}^{2+}]_i\) changes. There was no difference between the control and 40 mM Tau groups, indicating that Tau had no effect on normal \([\text{Ca}^{2+}]_i\). Compared to cells treated with H2O2 alone, cells pre-incubated with 20, 40, and 80 mM Tau for 12 hours before H2O2 treatment showed a dose-dependently lower \([\text{Ca}^{2+}]_i\) \((P < 0.001\) or \(P < 0.01)\) (Table).

**Effects of Tau on expression of CaM and CaMKII:** Both CaM and CaMKII play an important role in the regulation of Ca2+ homeostasis and cell apoptosis induced by oxidative damage.\(^20\) In response to stress, \(p53\) regulates apoptosis via the regulation of Bcl-2 family members, including Bcl-2 itself, as well as pro-apoptotic proteins, such as Bax.\(^20\) In this study, we investigated the expression of \(p53\), Bcl-2, Bax, and caspase-3 to explore the role of CaMKII in myocardial cell apoptosis. Damage by H2O2 stimulates \(p53\), Bax, and caspase-3 expression significantly; however, it inhibits the expression of Bcl-2 (versus control). Upon pre-incubating myocardial cells with Tau for 12 hours, the downregulation of Bcl-2 expression and upregulation of \(p53\), Bax, and caspase-3 expression were attenuated in a dose-dependent manner (versus H2O2 group, \(P < 0.05\)) (Figure 4).

**Discussion**

Consistent with other findings,\(^{13,14,16}\) our data reveals that Tau protects cardiomyocytes against H2O2-induced apoptosis. The production of LDH is an important parameter that indicates cell membrane injury. In this study, we found that H2O2 significantly increased LDH secretion, and that Tau can inhibit this increase. The mitochondrion plays a pivotal role in the induction of apoptotic cell death after oxidative stress. Mitochondria-related apoptosis

### Table

| Group       | Dose (mmol/L) | Change in fluorescence intensity |
|-------------|---------------|---------------------------------|
| Control     | 0.0           | 10.6 ± 4.5                      |
| Tau         | 40.0          | 28.4 ± 6.4                      |
| H2O2        | 0.3           | 202.5 ± 23.4*                   |
| Tau + H2O2  | 80.0          | 103.7 ± 10.1**                  |
|             | 40.0          | 156.0 ± 16.3*                   |
|             | 20.0          | 186.4 ± 19.2                    |

\(^*P < 0.001, \text{compared with control group}\), \(^*P < 0.05\), \(^**P < 0.01\) \text{compared with the H}_2\text{O}_2\text{-damaged group}\). \((n = 6, x ± s)\)

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**Figure 3.** Effects of Tau on the expression of CaM and CaMKII. A: Effect of Tau on the level of CaM mRNA in cultured neonatal rat cardiomyocytes damaged by H2O2; \(^*P < 0.001\), versus control group. \(^*P < 0.05\). B: Effect of Tau on the level of CaMKII mRNA in cultured neonatal rat cardiomyocytes damaged by H2O2; \(^*P < 0.05\) versus H2O2 group. C: Effect of Tau on CaMKII protein expression in cultured neonatal rat cardiomyocytes damaged by H2O2; \(^*P < 0.001\), versus control group. \(^**P < 0.01\), \(^*P < 0.05\) versus H2O2 group.
is associated with an abnormal increase in \([\text{Ca}^{2+}]_i\), followed by mitochondrial \(\text{Ca}^{2+}\) overload.\(^{21}\) In the mammalian heart, heteromultimer CaMKII is composed of many \(\delta\)-isoforms and \(\gamma\)-subunits,\(^{22}\) of which the CaMKII \(\delta\)-class is expressed in higher amounts and therefore is the best-characterized cardiac CaMKII isoform.\(^{22,23}\) In this study, we found that Tau at different dosages increased the mRNA and protein levels of isoform \(\delta\), compared to the \(\text{H}_2\text{O}_2\) damage group. Tau also inhibited the decrease in \(\text{CaM}\) mRNA levels induced by \(\text{H}_2\text{O}_2\). The pattern of variation of the CaMKII levels is consistent with the observed cell viability. In this study, we found that Tau mediated cell protection via regulation of the \(\text{CaM}/\text{CaMKII}\) mediated pathway. Similarly, Tau was found to exert a neuroprotective effect via regulation of CaMKII activity and a CaMKII inhibitor partially reversed the protective effect of taurine.\(^{32}\) Another report showed that the changes in \(\text{Cam}\) and CaMKII induced by taurine treatment could be attributed to differences in the levels of calcium or alterations in calcium signaling pathways.\(^{33}\) Taurine affects the opening of chloride channels by interactions with \(\gamma\)-aminobutyric acid receptors, glycine receptors, or taurine receptors, thereby preventing the increase in calcium influx and other downstream events.\(^{34}\)

CaMKII/p53 signaling cascade plays an important role in apoptosis.\(^{31}\) In this study, we found that Tau upregulates CaMKII and p53 protein expression, compared to the \(\text{H}_2\text{O}_2\) damage group. The CaMKII-specific inhibitor KN-93 has been found to significantly increase p53 expression to regulate the cell cycle in myofibroblastic hepatic stellate cells (HSCs).\(^{28}\) One of the key contributions of p53 to apoptosis is the upregulation of pro-apoptotic Bax gene expression and the downregulation of anti-apoptotic Bcl-2 gene expression.\(^{26}\) Bax and p53 directly initiate the translocation of pro-apoptotic members to mitochondria and activate them to trigger mitochondrial leakage.\(^{27,28}\) Mitochondrial outer membrane permeabilization (MOMP) results in the release of mitochondrial proteins, dissipation of the mitochondrial membrane potential (\(\Delta\Psi_m\)), and cessation of ATP production. MOMP might be associated with the pore-forming ability of some of the BCL-2 family proteins.\(^{29}\) The Bcl-2 family proteins Bcl2 and Bax are involved in the regulation of apoptosis and mitochondrial membrane pore formation. Compared to the \(\text{H}_2\text{O}_2\) damage group, Tau at 40 and 80 mM inhibited mitochondrial membrane potential (\(\Delta\Psi_m\)) decrease and apoptosis by upregulating Bcl-2 protein expression and downregulating Bax protein expression. The pattern of variation in the expression of anti-apoptotic Bcl-2 expression is consistent with that of the CaMKII protein. Moreover, the above findings are consistent with the results obtained using HK-2 cells.\(^{30}\)

It was reported that inflammatory cytokine or hypertrophic stimuli also could induce oxidative stress and mitochondrial dysfunction in cardiac myocytes and other type cells.\(^{34,35}\) Also, taurine exerted a protective effect against apoptosis induced by inflammatory cytokine or hypertrophic stimuli. For example, an inflammatory stimuli, lipopolysaccharide (LPS), was found to increase oxide stress in myocardium and lung cells.\(^{35}\) Taurine could reduce LPS-mediated inflammation and ROS formation, enhance SOD concentration, inhibit MDA production, and attenuate up-regulation of caspase-3 expression to greater extents than did the cells in the LPS group.\(^{35}\) Furthermore, a hypertrophic stimulus, angiotensin II, was also found to promote apoptosis of cardiac myocytes and Tau
could reduce the angiotensin II-mediated oxidative stress. Consistently, we found that taurine protected cardiomyocytes against apoptosis induced by H₂O₂. Taurine enhanced the SOD concentration and inhibited MDA production to a greater extent than did the cells in the H₂O₂ group. In addition, Tau attenuated the down-regulation of Bcl-2 expression and up-regulated the expressions of p53, Bax, and caspase-3 caused by H₂O₂ in a dose-dependent manner.

In conclusion, we have demonstrated the myocardial protective effect of Tau and the molecular mechanism by which Tau prevents H₂O₂-induced apoptosis in myocardial cells, suggesting that it may be involved in the mitochondrial apoptotic signaling pathway by upregulating CaMKII expression.

Limitations
We studied the effect of taurine on primary cultured neonatal myocardial cells treated with H₂O₂ and found that taurine-mediated protection was associated with regulation of Cam/CaMKII. However, the underlying mechanism still needs to be investigated. In addition, we only examined the protective effect of taurine on cardiac myocytes. The effects of taurine on other models, particularly animal models, should be investigated.

Disclosures
Conflicts of interest: The authors claim that none of the material in the paper has been published or is under consideration for publication elsewhere. We also confirm that all the listed authors have participated actively in the study, and have seen and approved the submitted manuscript. The authors have no conflicting financial interests.

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