Taurine inhibits apoptosis by preventing formation of the Apaf-1/caspase-9 apoptosome

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Myocardial ischemia causes depressed myocardial function and associated deleterious morphological alterations that lead to heart failure. This injury is a pathological process that results in extensive cell death, a significant portion of which can be attributed to apoptosis (10). Myocyte apoptosis has been demonstrated in clinical cases of myocardial infarction as well as in rabbit, rat, and mouse models of continuous ischemia or ischemic reperfusion (7, 8, 18). It has been proposed that mitochondrial dysfunction is the primary cause of apoptosis in these animal models of ischemic myopathy (4, 14, 15, 26). A key step in the initiation of apoptosis by the mitochondrial pathway is the release of cytochrome c from the intermembrane space of the mitochondria into the cytoplasm. Because ischemia results in the disruption of the mitochondrial membrane potential (ΔΨm), the release of cytochrome c is likely triggered by the opening of the mitochondrial permeability transition pore (6, 11, 24). When cytochrome c enters the cytosol, it associates with the apoptotic protease activity factor-1 (Apaf-1) to form a large complex referred to as an apoptosome. The initiator caspase, caspase-9, is activated through a protein-protein interaction with the apoptosome (12). Effector caspases, such as caspase-3, are activated through cleavage by the initiator caspase.

The mitochondrial pathway of apoptosis is very tightly regulated. Although most cytoprotective agents prevent cytochrome c release, recent studies have revealed that other steps in the process are subject to regulation. In the present study, we have examined the mechanism underlying the cardioprotective activity of the β-amino acid, taurine. Taurine (2-aminoethanesulfonic acid) is a ubiquitous substance involved in osmoregulation, modulation of calcium transport, and regulation of oxidative stress, in particular through its ability to scavenge hypochlorous acid (16, 19). Moreover, taurine has been found to prevent high-glucose-mediated endothelial cell apoptosis through its antioxidant property and regulation of intracellular calcium homeostasis (27). Because osmotic stress, calcium overload, and oxidative stress adversely impact mitochondrial function (25), there is reason to suspect that taurine might benefit the cardiomyocyte through the mitochondrial-linked pathway.

In the present study, the interaction between taurine and mitochondria-mediated apoptosis is investigated in a newly developed simulated ischemia model utilizing isolated cardiomyocytes, which are incubated with medium containing and lacking taurine and then sealed within cultured flasks (21). Our findings show that taurine suppresses simulated ischemia-induced apoptosis in cardiomyocytes by targeting the Apaf-1/caspase-9 apoptosome.

METHODS

Cell cultures and the newly simulated ischemia model. Primary cardiomyocyte cultures from 1-day-old Wistar rats were prepared according to the procedure described previously (21). All experimental procedures were approved by the Animal Care Committee of Osaka University and conformed to international guidelines. The cardiomyocytes were plated onto 12.5-cm2 flasks (Falcon) at a density of 10,000 cells/cm2. The cultures were maintained in modified Eagle’s medium and Ham’s F-12 (containing 3,151 mg/l dextrose; ICN Biomedicals) supplemented with 5% newborn calf serum (ICN Biomedicals), 3 mM pyruvate, 100 µM ascorbic acid, 5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml selenium (Boehringer Mannheim). This procedure yielded cell prep-
arations containing 90–95% myocytes, as assessed by microscopic observation of cell beating (data not shown). These cells were maintained for 2 days with medium changed daily and were serum-starved for 24 h before the experiments. For seal-induced cardiac ischemia, the flask medium was first replaced with serum-free culture medium (2.5 ml containing 3,151 mg/ml dextrose) and filled with phosphate-buffered saline (PBS; 47.5 ml) containing the following constituents (in mM): 137 NaCl, 8.1 Na2HPO4·12 H2O, 2.7 KCl, 1.5 KH2PO4, 0.9 CaCl2·2H2O, and 0.3 MgCl2·6H2O. Next, the flasks were bubbled with 5% CO2–95% N2 for 2 min to fix the initial pH at 7.4 and to eliminate oxygen from the remaining air space. Subsequently, the lid was tightly sealed to prevent gas from entering the flask, and flasks were incubated at 37°C. For controls, the cells were replaced with 2.5 ml of serum-containing culture medium and incubated at 37°C in 95% air–5% CO2. The culture medium in the control group was changed daily. The simulated ischemia model mimics the clinical stresses of ischemia, including the stresses of hypoxia, acidosis, and stagnant incubation medium.

Detection of apoptotic cells. To visualize fragmented nuclei, we fixed cells with 1% paraformaldehyde for 30 min at room temperature. After being rinsed in PBS, the cells were permeabilized in 70% ethanol. The cells were rinsed twice in PBS and stained with a fluorescent dye, Hoechst 33258 (Sigma-Aldrich), for 15 min at room temperature. After a final rinse in PBS, the cells were mounted in the FlowFade antifade reagent (Molecular Probes) and visualized under ultraviolet light with the Olympus fluorescence microscopy system. More than 100 cardiomyocytes obtained from 3 different primary culture preparations were counted. The percentage of apoptotic nuclei was calculated as the ratio of fragmented nuclei to the total amount of nuclei. Further evaluation of apoptosis was performed with a commercially available cell death detection kit to find DNA strand breaks using the terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling (TUNEL) reagent according to the manufacturer’s protocol (Promega). Cells that showed positive TUNEL staining in the nuclei were identified as apoptotic.

Measurement of mitochondrial membrane potential. Loss of $\Delta V_m$ was assessed using a fluorescent dye, the lipophilic cationic probe JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine; Intergen). Cells were incubated with 5 μg/ml JC-1 for 15 min at 37°C and examined with the Olympus fluorescence microscopy system. The excitation wavelength was 488 nm, and the emission fluorescence for JC-1 was monitored at 530 and 590 nm. The red emission of the dye is attributable to a potential-dependent aggregation of the monomeric form of JC-1, appearing in the cytosol after mitochondrial membrane depolarization.

Preparation of mitochondrial and cytosolic fractions and total cell lysates. Mitochondrial and cytosolic fractions were prepared from nontreated or taurine-treated cells cultured under nons ischemic or ischemic conditions. Preparation of mitochondrial and cytosolic fractions was achieved using a commercially available mitochondria/cytosol fractionation kit according to the manufacturer’s protocol (BioVision). The cytosolic and mitochondrial fractions were stored at $-80^\circ$C. Total cellular proteins were extracted in lysis buffer containing 150 mM NaCl, 10 mM Tris (pH 7.4), and 1 mM EDTA, plus 1% Triton X-100, 1% deoxycholic acid and protease inhibitor mixture (Sigma-Aldrich), followed by centrifugation at 1,500 g for 20 min. Protein concentrations were determined by the method of Lowry et al. (13), using bovine serum albumin as a standard.

Western blot analysis. Proteins (12 μg) from the mitochondrial fraction, cytosolic fraction, or total cell lysate were analyzed by SDS-PAGE (12.5 or 14% gel). After blotting, the Immobilon-P membrane (Millipore) was blocked with 5% BSA in TWEEN 20 in PBS at room temperature for 1 h. Immunoblots were incubated at room temperature for 60 min with the specific primary antibody to cytochrome c (PharMingen) or Apaf-1 (BioVision). After further washing, the membranes were incubated for 1 h with the secondary antibody (horseradish peroxidase conjugated). The enhanced chemiluminescence procedure was used for detection of the bands (ECL; Santa Cruz Biotechnology). Blots were reprobed with antibody to actin (Sigma-Aldrich) as a loading control. Quantitative analysis of immunoblotted bands was performed by computer program (NIH Image, version 1.61).

Detection of caspase-3 and -9 immunoreactivity. Total cell lysate (12 μg) was separated by 14% SDS-PAGE and then subjected to Western blot analysis with the use of antibody against caspase-3 or -9 (Santa Cruz Biotechnology).

Immunoprecipitation. For determining the Apaf-1-caspase-9 interaction, the cell lysates were prepared in lysis buffer containing 150 mM NaCl, 10 mM Tris (pH 7.4), and 1 mM EDTA, plus 1% Triton X-100, 1% deoxycholic acid, and protease inhibitor mixture. After homogenization and centrifugation, the supernatants were immunoprecipitated with antibody against Apaf-1 (1:150 dilution; Santa Cruz Biotechnology) plus 10 μl of protein A-Sepharose (Santa Cruz Biotechnology) for 5 h. Immunoprecipitates were washed, separated by 12.5% SDS-PAGE, and then subjected to Western blot analysis with the use of antibody against either Apaf-1 or caspase-9 (Medical & Biological Laboratories).

**Fig. 1.** Taurine suppresses ischemia-induced apoptosis in cultured neonatal rat cardiomyocytes. A: percentage of cells undergoing apoptosis as measured by Hoechst 33258 stain. Samples ($n = 360–949$ cells) were obtained from 3 different primary culture preparations. $*P < 0.01$ vs. control group. $*P < 0.05$ vs. ischemia group at 24–72 h. B: photomicrographs show TUNEL staining ($a$–$c$: magnification ×800) and Hoechst 33258 staining ($d$–$f$: magnification ×600). Cells in $a$ and $d$ were exposed to normoxia for 72 h, cells in $b$ and $e$ were exposed to ischemia for 72 h in the absence of taurine, and cells in $c$ and $f$ were exposed to ischemia for 72 h in the presence of 20 mM taurine.
neonatal rat cardiomyocytes. In agreement with a previous

RESULTS

compare individual data points for a significance was determined using the Student statistical significance. Each value was expressed as a mean ± SE. Differences were considered significant when the calculated P value was <0.05.

RESULTS

Taurine suppresses ischemia-induced apoptosis in cultured neonatal rat cardiomyocytes. In agreement with a previous finding from our laboratory (22), we found that isolated neonatal cardiomyocytes showed resistance to ischemia-induced apoptosis when exposed to medium containing 20 mM taurine. Figure 1A shows a quantitative determination of apoptotic nuclei in each experimental group. Cells incubated for the 24- to 72-h ischemic period were stained by Hoechst 33258, and the apoptotic nuclei were identified by the characteristic condensed, fragmented nuclei. Exposure of the cells to medium containing 20 mM taurine reduced the frequency of apoptosis after 24, 48, and 72 h of ischemia from 16 to 6%, from 26 to 13%, and from 42 to 15%, respectively. Figure 1B shows representative photomicrographs of results of TUNEL assay and Hoechst 33258 staining after a 72-h ischemic insult. Similar results were shown by TUNEL indicating that taurine significantly decreased apoptosis by ~35% (from 29 to 10%, P < 0.01) after a 72-h ischemic insult.

Influence of taurine on ischemia-induced mitochondrial dysfunction. One of the mechanisms contributing to mitochondrial-mediated apoptosis is the activation of the mitochondrial permeability transition pore, an event associated with a decrease in membrane potential (ΔΨ) and the loss of cytochrome c from the mitochondria. To determine whether taurine affected the permeability pore, we assessed ΔΨ in ischemic cardiomyocytes using the potential-sensitive fluorescent probe JC-1. Whereas control cells exhibited punctate red staining (Fig. 2A, top) indicative of coupled mitochondria with a normal ΔΨ, ischemic myocytes developed a diffuse green staining pattern, representative of reduced ΔΨ. Although taurine treatment was cardioprotective, it had no effect on JC-1 staining (Fig. 2A, middle and bottom). Moreover, cells placed in medium containing 20 mM taurine showed no change in the levels of cytochrome c released into the cytosol. Figure 2B reveals that cytochrome c was primarily localized to the mitochondria of cardiomyocytes incubated under normoxic conditions. However, the mitochondrial cytochrome c content decreased ~75% in cardiomyocytes placed for 24 h in sealed containers, with the degree of cytochrome c release being the same for cells treated with or without taurine (Fig. 2C).

Taurine inhibits the activation of caspase-9 and -3 in ischemic cardiomyocytes. To examine the effects of taurine on the apoptotic machinery located downstream from the mitochondria, we initially examined the activation states of caspase-9 and -3 using Western blot analysis. No apparent activation of the two caspases was observed after a 24-h ischemic insult. However, after 30 h of ischemia, the active cleavage product of caspase-9 and -3 using Western blot analysis. No apparent activation of the two caspases was observed after a 24-h ischemic insult. However, after 30 h of ischemia, the active cleavage product of caspase-9 and -3 was detected in the untreated cells but not in cells treated with 20 mM taurine (Fig. 3A). Figure 3B shows that ischemia-mediated activation of caspase-3, the effector caspase located downstream from caspase-9, is also inhibited by inclu-
Fig. 4. Taurine inhibits formation of Apaf-1/caspase-9 complex in cultured cardiomyocytes. Cardiomyocytes were exposed to ischemia for 24 h in the absence or presence of 20 mM taurine. An equal volume of cell lysate was immunoprecipitated (IP) with antibody against Apaf-1, and the precipitate, as well as the cell lysate, was analyzed by immunoblot with antibody against either Apaf-1 or caspase-9. Membranes at bottom right were stripped and reprobed with an antibody directed against actin. Results shown are representative of 3 independent experiments.

Fig. 5. Effect of taurine on Apaf-1/caspase-9 apoptosome formation and intracellular ATP content. A: extent of caspase-9 binding to Apaf-1 after a 24-h ischemic insult. Cardiomyocytes were exposed to ischemia for 24 h in the absence or presence of 20 mM taurine. Cell lysates were immunoprecipitated with antibody against Apaf-1, and the precipitates were analyzed by immunoblot with antibody against either Apaf-1 or caspase-9. Apaf-1 and caspase-9 contents were quantified by computer software. **P < 0.01 vs. control group. *P < 0.05 vs. ischemia group (n = 3–6). B: Apaf-1 expression in cultured cardiomyocytes exposed to simulated ischemia for 24 h in the absence or presence of 20 mM taurine. C: intracellular ATP content in cultured cardiomyocytes exposed to simulated ischemia for 24 h in the absence or presence of 20 mM taurine.

DISCUSSION

In the present study, we demonstrated that taurine prevented the ischemia-induced apoptosis in cardiomyocytes, accompanied by the inactivation of caspase-9 and -3. Taurine treatment inhibited Apaf-1/caspase-9 apoptosome formation without preventing mitochondrial dysfunction under ischemic conditions. Thus we proposed that taurine mediates cardiomyocyte protection by regulating Apaf-1/caspase-9 apoptosome formation.

Despite the accumulating data concerning Apaf-1/caspase 9 apoptosome, it remains to be elucidated how taurine inhibits apoptosis formation. There was no difference in the level of intracellular ATP content, cytochrome c release, and Apaf-1 expression between taurine-treated and untreated myocytes after a 24-h ischemic insult. Recent reports (3, 5, 17) have documented that a heat shock protein, HSP70, interacts with Apaf-1 and blocks the assembly of functional apoptosome. To elucidate the involvement of HSP70 in taurine-mediated cytoprotection, the expression of HSP was analyzed by immunoblotting; however, HSP70 was not upregulated by taurine (data not shown). Recently, we have demonstrated that taurine prevents the ischemia-induced apoptosis in cardiomyocytes through Akt (23). Thus it is possible that taurine-mediated activation of Akt negatively regulates Apaf-1/caspase-9 interaction, although further studies are required.

Numerous studies (9, 12, 20, 28) have suggested that Apaf-1 plays a crucial role in mitochondria-mediated apoptosis. Mitochondria-mediated apoptosis is involved in the onset of cardiovascular diseases (4, 14, 15, 26), especially in ischemic heart disease. Taking these findings together with previous reports that the administration of taurine shows efficacy in the treatment of patients with congestive heart failure (1, 2), it could be proposed that analyses of cytoprotective mechanisms of taurine provide novel strategies for the treatment of ischemic heart disease.

In conclusion, our findings show that taurine suppresses ischemia-induced apoptosis in cardiomyocytes by preventing formation of the Apaf-1/caspase-9 apoptosome. This is the first demonstration of the molecular mechanisms for the antiapoptotic effects of taurine.
GRANTS

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REFERENCES