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Taurine protected myocardial mitochondria injury induced by hyperhomocysteinemia in rats

L. Chang[1,2,3], **J. Xu**[4], **F. Yu**[2], **J. Zhao**[2], **X. Tang**[2], and **C. Tang**[1,2,3]

[1] Institute of Cardiovascular Diseases Research, First Hospital, Peking University, Beijing, P.R. China
[2] Department of Physiology and Pathophysiology, Peking University, Beijing, P.R. China
[3] Key Lab for Cardiovascular Molecular Biology of Education Ministry, Beijing, P.R. China
[4] Institute of Biophysics, Science Academy of China, Beijing, P.R. China

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Summary. Taurine can protect against cardiovascular diseases, whereas elevated levels of plasma homocysteine are associated with atherosclerotic and thromboembolic cardiovascular diseases. To illustrate the effects of taurine on hyperhomocysteinemia, we observed the myocardial mitochondria dysfunction in the rats with hyperhomocysteinemia induced by diet methionine loading, and the therapeutic effect of taurine. A methionine diet increased plasma homocysteine concentration ($133.51 \pm 27.91\,\mu$mol/L vs $12.31 \pm 2.58\,\mu$mol/L in control, $P < 0.01$), stimulated the production of reactive oxygen species (ROS) in the myocardial mitochondria, and inhibited the activities of mitochondrial Mn-superoxide dismutase and catalase. The ^{45}Ca uptake and Ca^{2+}-ATPase activity in the myocardial mitochondria were significantly lowered in rats with hyperhomocysteinemia. Taurine supplements effectively attenuated the hyperhomocysteinemia-induced ROS production and inhibition of Mn-superoxide dismutase and catalase activities in the myocardial mitochondria, and increased its ^{45}Ca uptake and Ca^{2+}-ATPase activity. Thus, taurine antagonizes the oxidative stress injury in the myocardial mitochondria induced by the hyperhomocysteinemia.

Keywords: Hyperhomocysteine – Taurine – Heart – Mitochondria – Rat

Introduction

Homocysteine, a sulphur-containing amino acid, an intermediate metabolite of the methionine, is formed by the demethylation of methionine and is catabolised to cystathionine and cysteine by a pyridoxal phosphate (vitamin B_6)-dependent pathway or remethylated to methionine by a folate and cyanocobalamin (vitamin B_{12})-dependent reaction. Hyperhomocysteinemia is caused by an abnormality in either an enzyme (cystathionine β-synthetase or temperature-sensitive methylenetetrahydrofolate reductase) or a cofactor (folate, vitamin B_{12}, or vitamin B_6) required for homocysteine metabolism (Perna et al., 2003). In its most severe form, hyperhomocysteinemia confers a significant risk for thromboembolic complications that are often fatal (Carmel et al., 2003). In contrast, the less severe form of the disease is commonplace and indolent, not accompanied by clinical signs until the third or fourth decade of life. People with hyperhomocysteinemia have atherosclerosis as well as recurrent episodes of acute arterial and venous thrombosis (Carmel et al., 2003), with near normal levels of plasma homocysteine on fasting. Following a methionine challenge, however, levels rise significantly. Hyperhomocysteinemia is an independent risk factor for atherosclerosis, and almost 40% of patients with coronary or cerebrovascular atherosclerosis have hyperhomocysteinemia (Lawrence et al., 2003). Patients with myocardial infarction also have elevated homocysteine levels (Matetzky et al., 2003).

The mechanism by which hyperhomocysteinemia damages the vessels and myocardium is still unknown and may be multifactorial. Homocysteine is toxic to the vascular endothelium and impairs endothelial function by inhibiting the synthesis of the endothelium-derived relaxing factor-nitric oxide or by increasing its degradation via the generation of oxygen-derived radicals such as superoxide radical, peroxynitrite and hydrogen peroxide (Lang et al., 2000). These free radicals in turn can promote the growth of vascular smooth muscle cells (Nishio et al., 1997), modify proteins and peroxidize lipids (Besler et al., 2003). In addition to altering the nitric oxide pathway,

homocysteine may have other functions, such as inducing the expression of the inflammation factor (Zeng et al., 2003), thus, promoting platelet aggregation, which leads to atherothrombotic vascular disease (Guba et al., 1999; Li et al., 2002).

Taurine (α-amimethanesulfonate), another sulphur-containing amino acid derived from the metabolism of methionine, is the most abundant intracellular amino acid in humans and is implicated in numerous biological and physiological functions. Taurine is not used in protein synthesis but rather is found free or in simple peptides. The diet of healthy individuals is the major source of taurine; although in the presence of vitamin B6, it is also synthesized from methionine and cysteine. Taurine has been shown to be essential in certain aspects of mammalian development, and *in vitro* studies have demonstrated that low level of taurine is associated with various pathological lesions, including cardiomyopathy, retinal degeneration, and growth retardation, especially if deficiency occurs during development. The metabolic actions of taurine include bile acid conjugation, detoxification, membrane stabilization, osmoregulation, and modulation of cellular calcium levels. Taurine has an ameliorating effect on atherosclerosis together with a decreasing effect on the cholesterol and triglyceride levels in rabbits fed on a high-cholesterol diet. Supplemented with taurine diet caused a significant decrease in the malondialdehyde (MDA) and diene conjugate levels in the plasma and aorta of rabbits, indicating that taurine ameliorated oxidative stress and cholesterol accumulation in the aorta of rabbits fed on the high-cholesterol diet (Balkan et al., 2002; Anitha et al., 2002). However, in patients with atherosclerotic coronary artery disease with hypercholesterolmia, there was a significant elevation in the levels of plasma homocysteine and MDA, and there was a strong positive correlation between plasma homocysteine and MDA (Moselhy et al., 2003). So taurine and homocysteine exert different roles on oxidative stress in cardiovascular diseases. Taurine can protect against cardiovascular diseases, whereas an elevated level of plasma homocysteine is associated with atherosclerotic and thromboembolic cardiovascular diseases. In clinical, supplementation with folic acid seems to be effective in reducing hyperhomocysteinemia, but evidence is lacking that lowering homocysteine levels with vitamin supplementation reduces the risk of cardiovascular diseases. It is a new strategy to use of taurine supplements to reduce the cardiovascular risk of homocysteine for theirs adverse bio-effects in sulfur-containing amino acid system (SAAS). Our previous studies showed that taurine ameliorated *in vitro* homocysteine-induced vascular smooth muscle cell proliferation (Qinpin et al., 2000) and endothelial cell injury (Yaqing et al., 2000). Taurine also prevents the decrease in expression and secretion of extracellular superoxide dismutase induced by homocysteine, and ameliorate the homocysteine-induced endoplasmic reticulum stress (Nonaka et al., 2001). Furthermore, the incubation of human megakaryocytic cell line DAMI with homocysteine, similar to that with hydrogen peroxide, inhibits the respiratory rate and activities of cytochrome c oxidase III/ATPase 6, 8 and other respiratory chain enzymes in mitochondria (Austin et al., 1998).

Myocardial mitochondria is an important organelle for intracellular calcium homeostasis regulation and oxygen free radical generation. Myocardial mitochondria damage is considered an important trigger for the pathogenesis of heart disease (Marin-Garcia et al., 2002). To illustrate the protective effects of taurine on hyperhomocysteinemia, we observed the dysfunctional effect of hyperhomocysteinemia induced by methionine diet and the therapeutic effect of taurine on myocardial mitochondria in rats.

Materials and methods

Materials

Male Sprague-Dawley rats (body weight, 150–180 g) were obtained from the Experimental Animals Center at Peking University Health Science Center. Animals were treated following the Guidelines of Animal Experiments from the Committee of Medical Ethics, National Health Department of China. Horseradish peroxide, *L*-Taurine, *DL*-homocysteine, *DL*-methionine, β-alanine, 3-aminophthalhydrazide (luminol) and lucigenin were purchased from Sigma Co. (St. Louis, MO, USA). $^{45}CaCl_2$ and ^{3}H-taurine were products of NEN Life Science Products, Inc. (Boston, MA, USA). All other reagents were of analytical grade and obtained from various commercial suppliers.

Preparation of rat hyperhomocysteinemia
(Matthias et al., 1996)

Rats were randomly divided into following 4 groups ($n = 6$ in each group) according to feeding diet. The control group was routinely fed normal chow diet and tap water. The methionine alone group (Met) was fed 1% *DL*-methionine mixed in normal chow diet. The taurine alone group (Tau) was fed normal chow diet and 1% *L*-taurine tap water. The methionine + taurine group (Met + Tau) was fed 1% *DL*-methionine and 1% *L*-taurine tap water. All rats were raised for 6 wk.

Measurement of plasma homocysteine and taurine levels

Animal blood was collected at the end of 6 wk. Plasma was obtained by centrifugation at $2500 \times g$ for 10 min at 4°C and stored at −20°C for later use. Total homocysteine concentration was measured by high-performance liquid chromatography (HPLC, JASCO International Co. Ltd.,) with fluorometric detection as described (Minniti et al., 1998).

Heparinized blood samples were collected, centrifuged and treated with 10% 5-sulfosalicylic acid for plasma taurine measurement by amino acid analysis on an AminoTac amino acid analyser (Waters et al., 2001).

Preparation of myocardial mitochondria

Rat myocardial mitochondria were prepared as described, with modification (Tyler et al., 1967). Briefly, rats were anesthetized with 60 mg/kg sodium pentobarbital, and the hearts were harvested. The ventricular myocardium was immerged into isolation buffer (220 mmol/L mannitol, 70 mmol/L sucrose, 1.0 mmol/L EDTA, mmol/L Tris-HCl 20, pH 7.4, 4°C) containing 0.1 mg/mL snailase, and homogenized (10% w/v) on a glass Potter-Elvehjem homogenizer set at a standard velocity for 1 min. The homogenate was centrifuged at 800 × g for 10 min at 4°C. The obtained supernatant was centrifuged at 8000 × g for 10 min. The pellet was resuspended in isolation buffer and washed twice by recentrifugation at 8000 × g for 10 min. The final pellet was resuspended in isolation buffer as mitochondria preparation, and protein content was determined by Bradford's method. The respiration control ratio in prepared mitochondria was 5.37 ± 0.68 (n = 6), which indicates that the mitochondrial functional coupling was satisfactory.

Measurement of the respiration control ratio of mitochondria

The respiration control ratio was measured by use of a Gilson glass respirometer equipped with a Clark oxygen electrode (CYY, China) in 2 mL of solution (5 mmol/L KH_2PO_4–K_2HPO_4 [pH 7.4], 3 mmol/L $MgSO_4$, 1 mmol/L EDTA, 25 mmol/L sucrose, 2 mg mitochondrial protein). Then, 10 mmol/L succinic acid was added. The oxygen consumption was recorded as the absence (state 4) and presence (state 3) of 5 mmol/L adenosine diphosphate. The respiration control ratio was determined as the ratio of oxygen uptake in state 3 to state 4 (Xie et al., 1996).

Assay for succinate dehydrogenase activity

The succinate dehydrogenase activity was determined with iodonitrotetrozolium chloride violet to form formazan, which has an absorption of 490 nm (Gunter et al., 1994). An amount of 100 μg of mitochondrial protein was incubated with reaction buffer (5 mmol/L KH_2PO_4–K_2HPO_4, 25 mmol/L sucrose, 2 mmol/L iodonitrotetrozolium chloride violet, pH 7.4) for 3 min at 37°C; the reaction was then initiated with 10 mmol/L succinic acid and further incubated for 10 min at 37°C. The reaction was terminated by the addition of 10% trichloroacetic acid. The succinate dehydrogenase activity was calculated on the standard curve prepared with formazan, which was reduced to iodonitrotetrozolium chloride violet by vitamin C and NaOH. The succinate dehydrogenate activity was expressed as μmol · mg protein^{-1} · min^{-1}.

Assay of ^{45}Ca uptake

Mitochondria ^{45}Ca uptake was determined by the Millipore filtration method (Gunter et al., 1994). Mitochondria protein (0.2 mg) was added into 3 mL reaction buffer (30 mmol/L imidazole-HCl, 100 mmol/L KCl, 10 mmol/L $MgCl_2$, 2 mmol/L $CaCl_2$, 5 mmol/L KH_2PO_4, 0.01 mmol/L ruthenium red, 5 mmol/L succinic acid, pH 7.4). After preincubation for 2 min at 37°C, 37 kBq $^{45}CaCl_2$ was added. The reaction was initiated by adding 5 mmol/L Na_2-ATP and incubated at 37°C for 2, 5, 10 and 15 min. The reaction was terminated by rapid filtration with 1 mL ice-cold buffer (200 mmol/L $MgCl_2$, pH 7.4) through a Millipore filter (Type HA, pore size of 0.45 μm) under vacuum negative pressure. Each filter was immediately washed 3-times with 5 mL of ice-cold buffer (100 mmol/L $MgCl_2$, pH 7.4). After the addition of 5 mL of scintillation fluid, the radioactivity was determined by β-scintillation counter (Beckman LS 6500).

Measurement of Ca^{2+}-ATPase activity

The Ca^{2+}-ATPase activity was determined as the difference of Ca^{2+}–Mg^{2+}-ATPase and Mg^{2+}-ATPase activity (Anand et al., 1977). The Ca^{2+}–Mg^{2+}-ATPase activity was assayed in 5 mL reaction buffer (100 mmol/L imidazole-HCl, 100 mmol/L KCl, 0.1 mmol/L $MgCl_2$, 5.0 mmol/L $CaCl_2$, pH 7.4). The reaction was initiated by 5 mmol/L Na_2-ATP, incubated for 15 min at 37°C, then stopped by terminate buffer (a: 3% sodium bisulfite and 1% methylaminophenolsulfate, b: 2.5% molybdatoamine, c: 5.032 N sulfuric acid, and mixed at a:b:c:H_2O = 1:1:1:4 before use). The optical density value was measured by a spectrophotometer at 660 nm. The Mg^{2+}-ATPase activity was measured as above, except that 5.0 mmol/L $CaCl_2$ was replaced with 4 mmol/L EGTA in the reaction buffer.

Measurement of hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) production

H_2O_2 and O_2^- production in mitochondria was detected using luminol plus horseradish peroxidase-derived chemiluminescence (LHDCL) and lucigenin-derived chemiluminescence (LDCL), respectively, in a lighttight box with use of the BPCL Ultra-weak luminescence analyzer (BPCL, China) at 37°C as described (Allen et al., 1986). The photon counts were integrated over 1-s periods and shown on a computer monitor. The integral of the signal peak reflects the formation of H_2O_2 or O_2^-.

Determination for Mn-superoxide dismutase (Mn-SOD) and catalase (CAT) activity

Mn-SOD activity was determined by its inhibitory action on the O_2^--dependent reduction of xanthine–xanthine oxidase-stimulated ferricytochrome (Das et al., 1993). CAT activity was determined by its decomposing action on the H_2O_2-dependent optical density reduction at 240 nm.

Measurement of malondialdehyde (MDA) and plasma conjugated diene content

Lipid peroxidation was determined by the measurement of MDA in the isolated myocardial mitochondria with the thiobarbituric acid reaction (Takemura et al., 1994). Standard MDA was prepared by acid hydrolysis of 1,1,3,3-treaethyloxypropane. The amount of MDA was expressed as pmol · mg^{-1} protein. Plasma conjugated diene content was determined as described (Waller et al., 1977).

Electron microscopy of mitochondria

The samples of myocardium were fixed in 3% glutaraldehyde buffered to pH 7.4 with 90 mmol/L KH_2PO_4 for at least 2 h, washed in the buffer supplemented with 220 mmol/L sucrose and postfixed with 2% OsO_4 in 50 mmol/L veronal acetate buffer for 1 h. We followed standard procedures for dehydration in a graded series of alcohol and embedding in epoxy resin. Ultrathin sections were cut from each sample, counterstained with uranium acetate and lead citrate, and examined under a JEM-100CXII electron microscope.

Measurement of taurine uptake and release

Taurine uptake was measured as described (Shi et al., 2003). Briefly, 0.2 mg mitochondrial protein was incubated for 5 min in a rotary water bath at 37°C in 1 mL of incubation buffer (3 mmol/L KCl, 4 mmol/L $CaCl_2$, 5 mmol/L K_2HPO_4, 5 mmol/L KH_2PO_4, 4 mmol/L $MgSO_4$, 10 mmol/L $NaHCO_3$, pH 7.2) with 0.01 to 0.4 mmol/L of unlabeled taurine and 18.5 KBq ^3H-taurine and 0.1 to 1.0 mmol/L homocysteine or 0.1 to 1.0 mmol/L methionine. The uptake reaction was then terminated by rapid filtration with 1 mL cold phosphate buffered saline (PBS, pH 7.4) through a Millipore filter (type HA, pore size 0.45 μm) under vacuum negative pressure, and the filter was washed 3 times with cold PBS. After the addition of 5 mL of scintillation fluid, radioactivity was determined by calculating the Vmax and Km values of taurine uptake according to a Eadia Hofstee plot.

For measurement of taurine release, 1 mg mitochondria protein was incubated at 37°C in 1 mL of incubation buffer (3 mmol/L KCl, 4 mmol/L $CaCl_2$, 5 mmol/L K_2HPO_4, 5 mmol/L KH_2PO_4, 4 mmol/L $MgSO_4$, 10 mmol/L $NaHCO_3$, pH 7.2) with 0.1 mmol/L unlabeled taurine and 37 KBq ^{3}H-taurine for 20 min. After centrifugation at 8000 g at 4°C for 10 min, the free ^{3}H-taurine was removed, and 1 mL incubation buffer with 0.1 to 1.0 mmol/L homocysteine was added. The mitochondria was then incubated at 37°C, and aliquots of 0.1 mL incubation buffer were taken out at 1, 2, 5, 10 and 15 min. The radioactivity was determined by β-scintillation counter.

Statistical analyses

All data were expressed as mean ± S.E.M. A one-way ANOVA was first carried out to test for any differences between the mean values within the same study. When a significant F value was obtained, comparisons between individual means of groups were performed by use of the Student-Newman-Keuls test. A difference of $P<0.05$ was considered significant.

Results

Effect of methionine and taurine on plasma homocysteine and taurine levels (Table 1)

The Met group showed a significant 10.8-fold increase in plasma homocysteine concentration ($P<0.01$), and slight elevation of plasma taurine level (15%, $P<0.05$), compared with the control. The Tau and Met + Tau groups showed higher plasma taurine levels than the control and Met groups ($P<0.01$). Plasma taurine levels were increased by 88% ($P<0.01$) in the Met + Tau compared with the Met alone group, and by 104% in the Tau alone group compared with the control ($P<0.01$).

Table 1. Concentration of plasma homocysteine and taurine in different groups (n = 6 in each group)

Group	Plasma homocysteine (μM)	Plasma taurine (μM)
Control	12.31 ± 2.58	105 ± 12
Tau	11.74 ± 2.11$^{\#\#}$	214 ± 24$^{**\ \#\#}$
Met	133.51 ± 27.91**	123 ± 14*
Met + Tau	108.70 ± 19.07$^{**\ \#}$	231 ± 21$^{**\ \#\#}$

$^{*}P<0.05$, $^{**}P<0.01$ vs control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs Met group. *Tau*, taurine-alone group; *Met*, methionine-alone group; *Met + Tau*, methionine and taurine group

Effects of methionine and taurine on respiration control ratio of mitochondria

As shown in Table 2, the Met group showed a reduced state 3 respiratory rate and respiratory control ratio of about 14% and 13% ($P<0.05$), respectively, compared with the control. The ratio was not significantly different for the Tau group ($P>0.05$). However, the state 3 rate and respiratory control ratio in the Met + Tau group was higher, by about 17% and 12% ($P<0.05$), respectively, than that of the Met alone group. The state 4 respiratory rate did not differ among the groups.

Effects of methionine and taurine on succinate dehydrogenase activity

Succinate dehydrogenase is the marker enzyme of mitochondria. As shown in Table 3, the succinate dehydrogenase activity was obviously higher in isolated mitochondria than that in plasma membrane and cytoplasm. The succinate dehydrogenase activity in mitochondria was not affected by taurine alone administration ($P>0.05$). However, treatment with methionine significantly decreased succinate dehydrogenase activity ($P<0.01$). Co-administration with taurine and methionine ameliorated methionine-induced inhibition of succinate dehydrogenase activity, with a 26% increase compared with Met alone group ($P<0.01$).

Effects of methionine and taurine on ^{45}Ca uptake in mitochondria

The regulation of intracellular calcium homeostasis is an important function of mitochondria. As shown in Fig. 1,

Table 2. Effects of methionine and taurine on rat myocardial mitochondria respiratory control rate (n = 6 in each group)

Groups	State 3 (nmol $O_2 \cdot mg^{-1}$ protein)	State 4 (nmol $O_2 \cdot mg^{-1}$ protein)	RCR
Control	607 ± 54	108 ± 9	5.62 ± 0.67
Tau	612 ± 57	109 ± 7	5.61 ± 0.48
Met	502 ± 42*	106 ± 6	4.74 ± 0.39*
Met + Tau	589 ± 51$^{\#}$	111 ± 9	5.31 ± 0.27$^{\#}$

$^{*}P<0.05$ vs normal group; $^{\#}P<0.05$ vs Met alone group. *RCR*, respiratory control ratio; *Tau*, taurine-alone group; *Met*, methionine-alone group; *Met + Tau*, methionine and taurine group

Table 3. Effects of methionine and taurine on succinate dehydrogenase activity (μmol/mg protein) in rat mycocardial mitochondria (n = 6 in each group)

Groups	Mitochondria	Plasma membrane	Protein yield of mitochondria ($mg \cdot g^{-1}$ HW)
Control	2.09 ± 0.34	0.32 ± 0.02	18 ± 2.1
Tau	1.96 ± 0.33	0.33 ± 0.03	18 ± 2.6
Met	$1.40 \pm 0.27^{**}$	0.30 ± 0.02	$13 \pm 1.6^{**}$
Met + tau	$1.76 \pm 0.28^{**\ \#\#}$	0.29 ± 0.02	$16 \pm 1.8^{\#}$

$^{**}P<0.01$ vs control group; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs Met alone group; *HW*, heart weight; *Tau*, taurine-alone group; *Met*, methionine-alone group; *Met + Tau*, methionine and taurine group

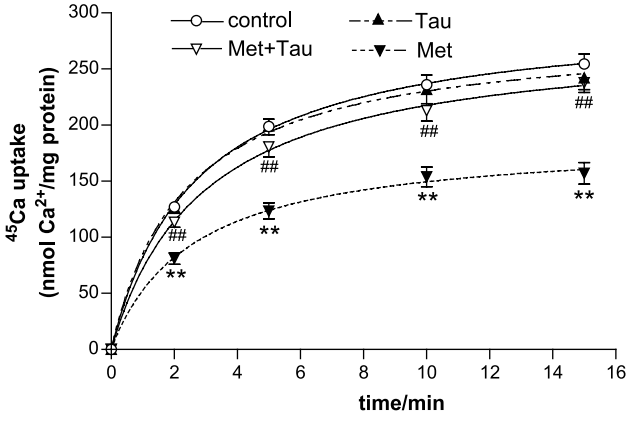


Fig. 1. Effects of methionine and taurine on ^{45}Ca uptake in myocardial mitochondria. $^{**}P<0.01$ vs control group; $^{\#\#}P<0.01$ vs methionine alone group. *Tau*, taurine-alone group; *Met*, methionine-alone group; *Met + Tau*, methionine and taurine group

^{45}Ca uptake in myocardial mitochondria reached the peak value at about 10 min (50% maximum ^{45}Ca uptake was at 2.7 min, and Vmax was 300 ± 3.2 nmol · mg protein^{-1} in the control group). The ^{45}Ca uptake was significantly inhibited (time-course curve shifted to right and down) by methionine diet (50% maximum ^{45}Ca uptake was at 2.6 min, and Vmax was 188 ± 5.2 nmol · mg protein^{-1}, $P<0.01$). Supplement with taurine in hyperhomocysteinemia rat increased the ^{45}Ca uptake (time-course curve shifted to left and up) compared with Met alone group, but taurine alone did not change the ^{45}Ca uptake (the Vmax was 284 ± 7.2 nmol · mg^{-1} protein vs 300 ± 3.2 nmol · mg^{-1} protein, $P>0.05$).

Effects of methionine and taurine on Ca^{2+}-ATPase activity in mitochondria

To investigate the mechanism of methionine and taurine on mitochondria calcium uptake, the Ca^{2+}-ATPase activity was determined. As shown in Fig. 2, methionine diet significantly decreased the activity of Ca^{2+}-ATPase in the Met group by 50% ($P<0.01$) compared with the control. Treatment with taurine alone did not affect the activity of Ca^{2+}-ATPase ($P>0.05$, compared with the control), but it did significantly attenuate the inhibition of Ca^{2+}-ATPase activity induced by the methionine diet in the Met + Tau group ($P<0.01$ vs the Met alone group).

Effects of methionine and taurine on H_2O_2 generation

Mitochondria is major source of intracellular oxygen free radicals. Luminol plus horseradish peroxidase were used as a chemiluminescent probe to monitor the H_2O_2 production. The signal curve composed by luminol plus horseradish peroxidase-derived chemiluminescence (LHDCL) photons represent H_2O_2 production, and the area under the signal curve represents the amount of H_2O_2 production in Fig. 3. The LHDCL photon signal peaked at 400 s and descended to a basal level at about 1200 s in the Met and Met + Tau groups after succinic acid was added (curve a and b), but the peak value was

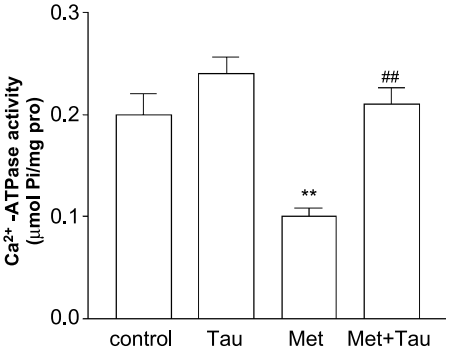


Fig. 2. Effects of methionine and taurine on calcium pump activity in rat myocardial mitochondria. $^{**}P<0.01$ vs control group; $^{\#\#}P<0.01$ vs Met group. *Tau*, taurine-alone group; *Met*, methionine-alone group; *Met + Tau*, methionine and taurine group

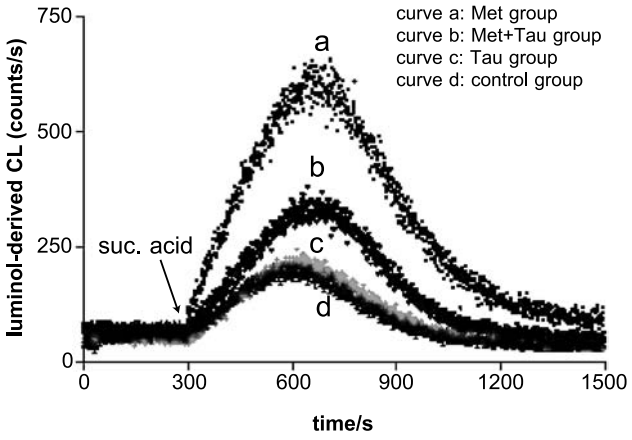


Fig. 3. Effects of methionine and taurine on H_2O_2 production in myocardial mitochondria. Hydrogen peroxide production was measured by the luminol chemiluminescence (*CL*) method. The photon counts were integrated once per second and shown on a computer monitor, and the photon count points shaped a curve (ordinate represent photon counts, and abscissa represent time[s]). The area under the curve represents the amount of H_2O_2 production. Methionine diet loading for 6 wk generated H_2O_2, and taurine antagonized methionine-stimulated H_2O_2 production, but administration of taurine alone did not affect H_2O_2 production

lower in the Met + Tau than that in the Met group. The LHDCL photon signal peaked at 350 s and descended to a basal level at about 1100 s in the control and Tau alone groups (curve c and d) and the peak values were similar between the groups. The amounts of H_2O_2 calculated with the area under curve were significantly increased by three-fold in the Met group compared with the control ($P<0.01$). Treatment with taurine plus

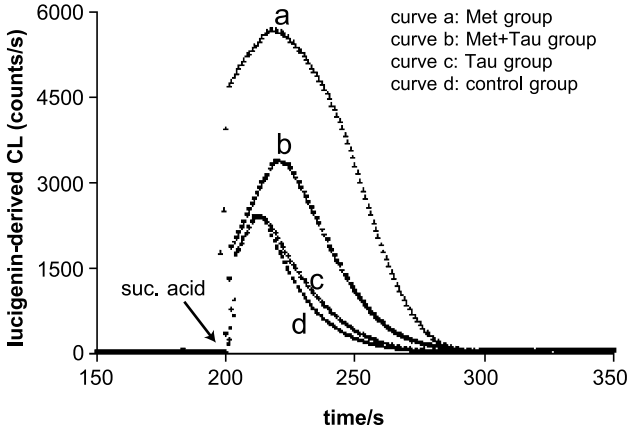


Fig. 4. Effects of methionine and taurine on O_2^- generation in myocardial mitochondria. The O_2^- production was measured by the lucigenin-derived chemiluminescence (*CL*) method. The photon counts were integrated once per second and shown on a computer monitor, and the photon signal points shaped a curve (ordinate represented photon counts, and abscissa represented time[s]). The area under the curve represents amounts of O_2^- production. Methionine diet loading for 6 wk generated O_2^-, and taurine antagonized methionine-stimulated O_2^- production, but administration of taurine alone did not affect O_2^- production

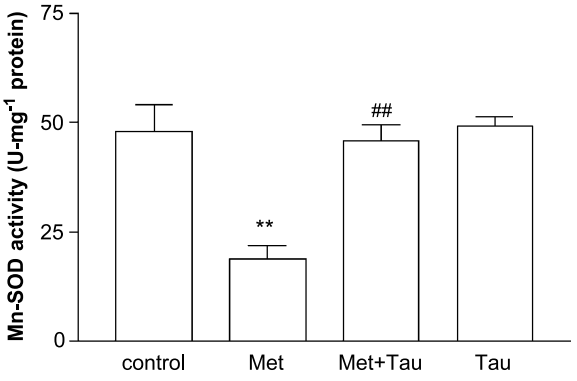


Fig. 5. Effects of methionine and taurine on Mn-superoxide dismutase activity in rat myocardial mitochondria. $^{**}P<0.01$ vs control group, $^{\#\#}P<0.01$ vs Met group. *Tau*, taurine-alone group; *Met*, methionine-alone group; *Met + Tau*, methionine and taurine group

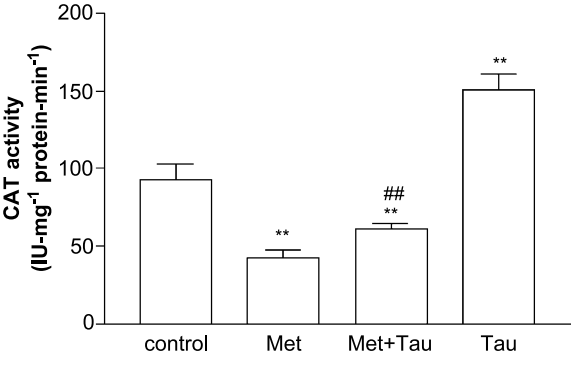


Fig. 6. Effects of methionine and taurine on catalase activity in rat myocardial mitochondria. $^{**}P<0.01$ vs control group, $^{\#\#}P<0.01$ vs Met group. *Tau*, taurine-alone group; *Met*, methionine-alone group; *Met + Tau*, methionine and taurine group

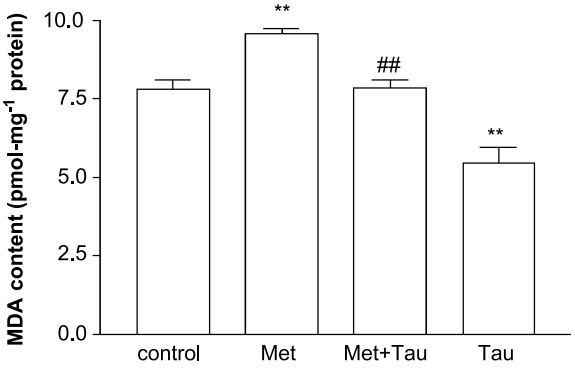


Fig. 7. Taurine prevented myocardial mitochondria malondialdehyde formation induced by methionine diet loading. $^{**}P<0.01$ vs control group, $^{\#\#}P<0.01$ vs Met group. *Tau*, taurine-alone group; *Met*, methionine-alone group; *Met + Tau*, methionine and taurine group

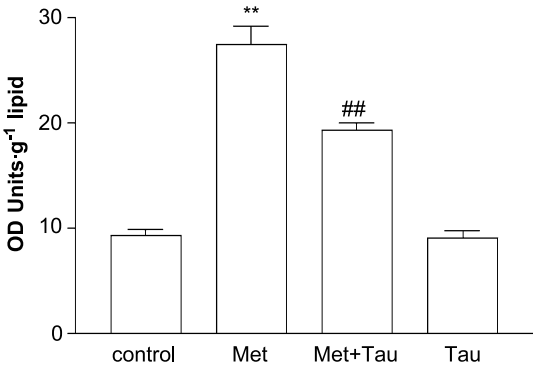


Fig. 8. Plasma diene levels in experimental animals. $^{**}P<0.01$ vs control group, $^{\#\#}P<0.01$ vs Met group. *Tau*, taurine-alone group; *Met*, methionine-alone group; *Met* + *Tau*, methionine and taurine group

methionine significantly attenuated the methionine-stimulated H_2O_2 production compared with the Met alone group ($P<0.01$).

Effects of methionine and taurine on O_2^- generation

Lucigenin was used as a chemiluminescent probe to monitor O_2^- production. As shown in Fig. 4, the signal quickly peaked at 25 s and descended to the basal level about 80 s after succinic acid was added. The area under the peak represents the amount of O_2^- production. The Met group showed significantly promoted LDCL photon production, and the area under curve was 4.9-fold higher than that of the control group ($P<0.01$). However, treatment with taurine alone had no effect on LDCL photon production. Interestingly, co-treatment with taurine plus methionine significantly prevented the methionine-stimulated LDCL photon production, and the amount of O_2^- was lower than that in the Met alone group ($P<0.01$).

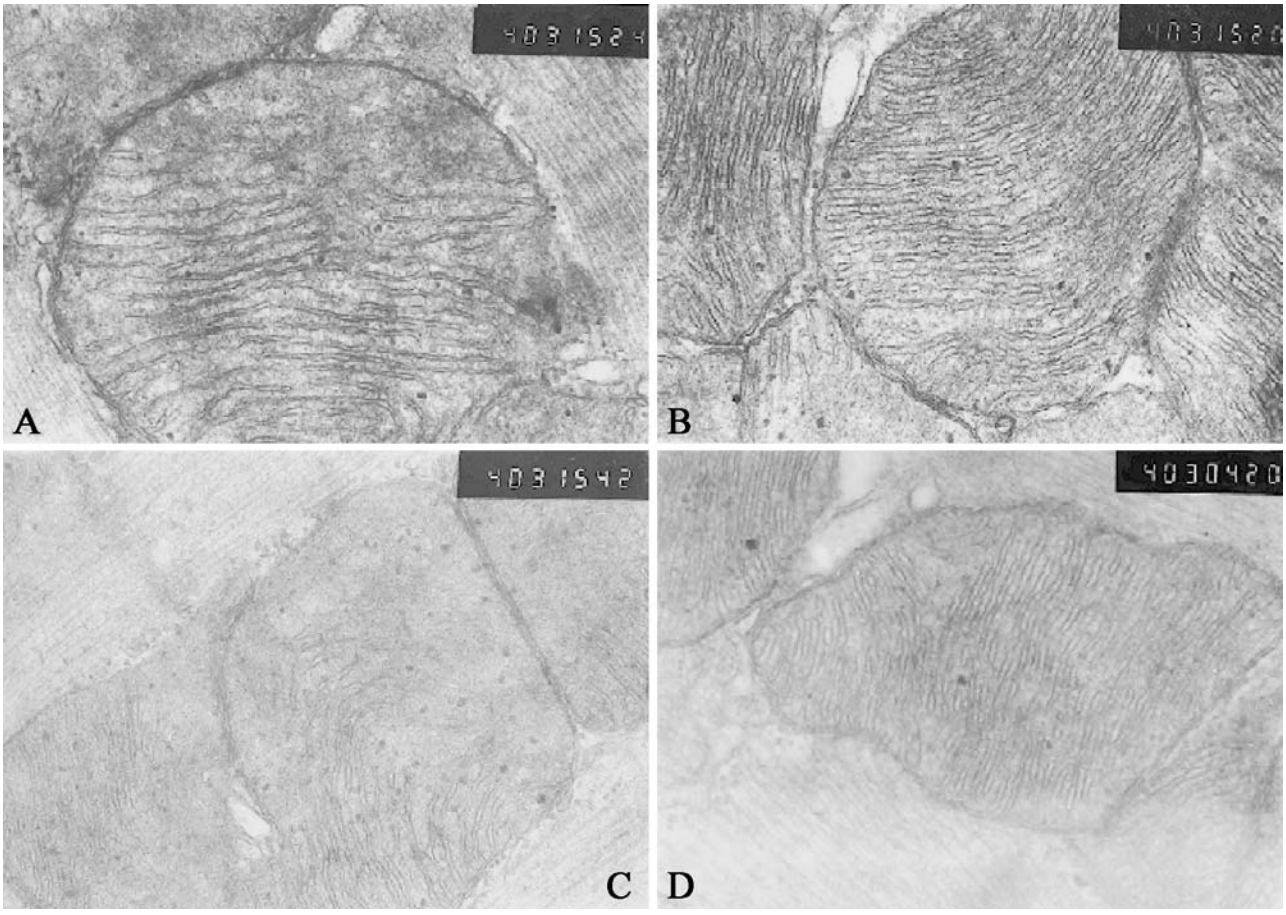
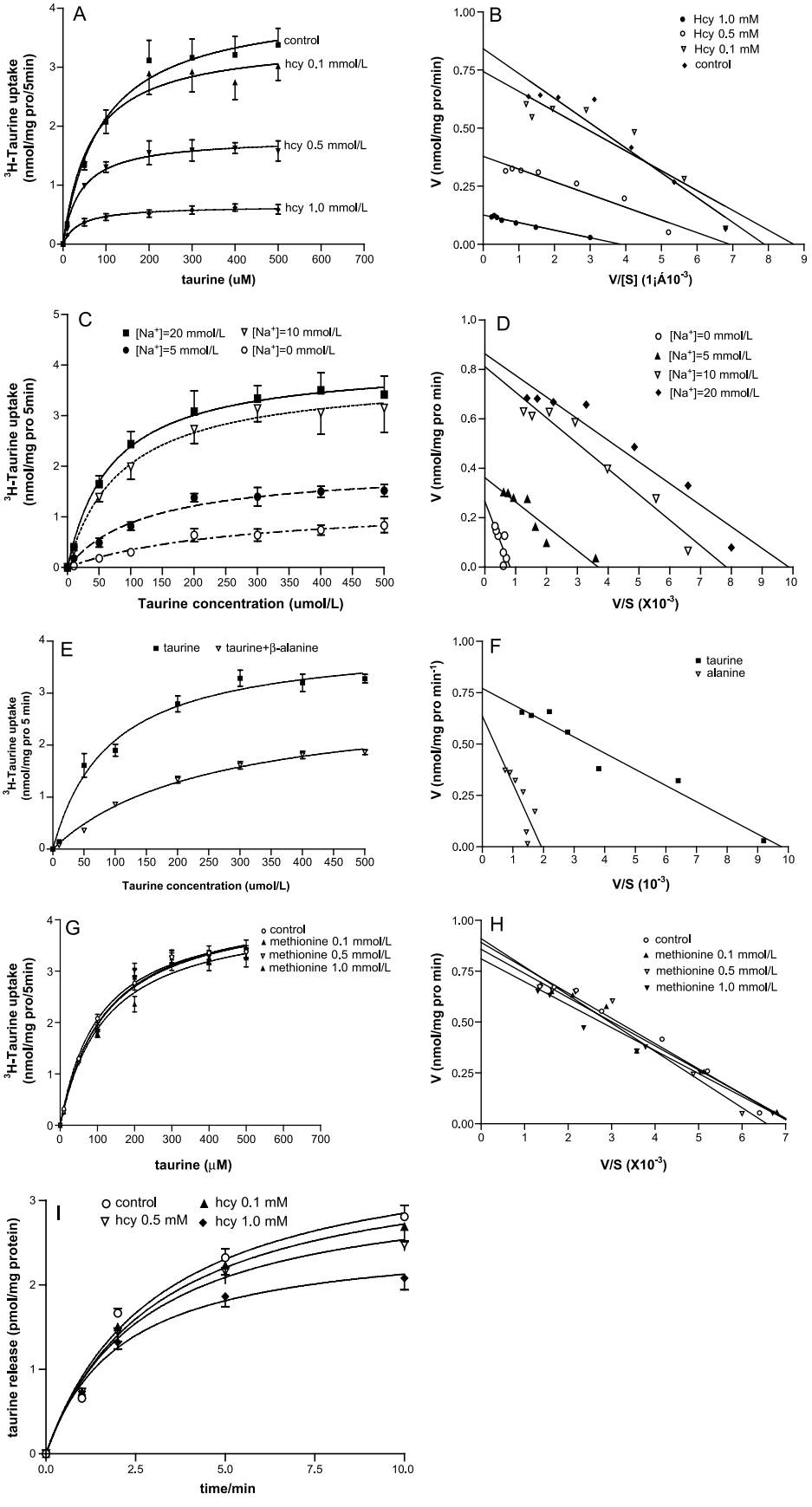


Fig. 9. Effects of methionine and taurine on myocardial mitochondria structure (×40 000). The transmitter electron microscope slides were prepared as described in Materials and methods. he mitochondrial cristae was normal in control group (**A**) and the Tau group (**B**). Methionine damaged the mitochondrial structure, and the cristae was almost gone in the Met group (**C**), whereas taurine blunted mitochondrial structure damage induced by methionine (**D**, Met + Tau group)


A
control
hcy 0.1 mmol/L
hcy 0.5 mmol/L
hcy 1.0 mmol/L
^{3}H-Taurine uptake (nmol/mg pro/5min)
taurine (uM)
B
Hcy 1.0 mM
Hcy 0.5 mM
Hcy 0.1 mM
control
V (nmol/mg pro/min)
V/[S] (1¡Á10^{-3})
C
[Na$^+$]=20 mmol/L
[Na$^+$]=10 mmol/L
[Na$^+$]=5 mmol/L
[Na$^+$]=0 mmol/L
^{3}H-Taurine uptake (nmol/mg pro 5min)
Taurine concentration (umol/L)
D
[Na$^+$]=0 mmol/L
[Na$^+$]=5 mmol/L
[Na$^+$]=10 mmol/L
[Na$^+$]=20 mmol/L
V (nmol/mg pro min)
V/S (X10^{-3})
E
taurine
taurine+β-alanine
^{3}H-Taurine uptake (nmol/mg pro 5 min)
Taurine concentration (umol/L)
F
taurine
alanine
V (nmol/mg pro min^{-1})
V/S (10^{-3})
G
control
methionine 0.1 mmol/L
methionine 0.5 mmol/L
methionine 1.0 mmol/L
^{3}H-Taurine uptake (nmol/mg pro/5min)
taurine (μM)
H
control
methionine 0.1 mmol/L
methionine 0.5 mmol/L
methionine 1.0 mmol/L
V (nmol/mg pro min)
V/S (X10^{-3})
I
control
hcy 0.1 mM
hcy 0.5 mM
hcy 1.0 mM
taurine release (pmol/mg protein)
time/min

Effects of methionine and taurine on Mn-SOD and CAT activity

Mitochondria Mn-SOD and CAT are crucial anti-oxidant enzymes to scavenge the oxygen free radicals produced by mitochondria *in vivo*. Mn-SOD is a major enzyme, which catalyzes the transformation of O_2^- into H_2O_2, and CAT catalyzes further H_2O_2 into H_2O. As shown in Fig. 5, the administration of taurine alone did not affect mitochondrial Mn-SOD activity ($P>0.05$). The Met group showed a decreased Mn-SOD activity, by 61% ($P<0.01$) compared with the control. However, co-administration of taurine and methionine markedly ameliorated the methionine-induced inhibition of Mn-SOD activity ($P<0.01$, compared with Met alone group), and its activity was close to the control level ($P>0.05$, compared with control group).

As shown in Fig. 6, the CAT activity in the Met group was only 33% of that of the control group ($P<0.01$). However, the CAT activity in the Tau group was significantly higher (61%) than that in control rats ($P<0.01$). Furthermore, the CAT activity in Met + Tau rats was significantly higher than that in the Met alone group ($P<0.01$) but significantly lower than that in Tau alone group ($P<0.01$).

Effects of methionine and taurine on mitochondrial MDA formation and plasma conjugated diene content

As Fig. 7 shows, the content of lipid peroxidation product MDA was significantly elevated in the Met group ($P<0.01$, compared with the control). The Tau group showed inhibited mitochondria MDA formation, by about 30% ($P<0.01$, compared with the control). Furthermore, in the Met + Tau group taurine effectively antagonized methionine-induced MDA formation ($P<0.01$ vs. Met alone group).

Plasma diene is another lipid peroxidation product, which is produced by tissues and secreted into the blood. As shown in Fig. 8, the plasma conjugated diene level was 78% higher in Met group than in the control ($P<0.01$). The administration of taurine had no affect on plasma conjugated diene levels ($P>0.05$, compared with the control). However, the co-treatment with Tau + Met showed significantly attenuated stimulatory effects of methionine on plasma diene production ($P<0.01$ vs Met group).

Taurine blunted mitochondrial structure damage induced by methionine

As shown in Fig. 9, feeding of methionine severely damaged the myocardial mitochondrial structure, and the cristae was vague in the Met group (Fig. 9C). Taurine alone did not alter the mitochondrial structure (Fig. 9B). However, co-administration of Tau + Met rats showed a slighter damage in mitochondrial cristae than methionine alone (Fig. 9D).

Homocysteine inhibited taurine uptake and release from in vitro *mitochondria*

The data in Fig. 10 showed that myocardial mitochodria exited a Na^+-dependent taurine transporter. A total of 5, 10 and 20 mmol/L Na^+ increased taurine uptake in a concentration-dependent manner. The uptake Vmax rate was increased 1.6, 3 and 3.2 fold than in controls (all $P<0.01$). Na^+ also increased the affinity of taurine to the transporter. The Km values were increased 53%, 68% and 74% (all $P<0.01$). The taurine uptake was inhibited by β-alanine (taurine transporter inhibiter). The uptake Vmax rate decreased by 28% and the Km increased 1.7 times more than that of controls (all $P<0.01$). Incubated mitochondria with methionine did not affect taurine uptake and the affinity of taurine to its transporter. Although homocysteine (0.1, 0.5 and 1.0 mmol/L) obviously increased the affinity of transporter for taurine by 29%, 55% and 60% of that of controls, its inhibition of taurine uptake was concentration dependent, and the uptake Vmax rates were lower, by 15% ($P<0.05$), 56% ($P<0.01$) and 84% ($P<0.01$) of that of controls. The taurine release from mitochondria was peaked at 5 min. A total of 0.1 and 0.5 mmol/L homocysteine did not affect the taurine release, but 1 mM homocysteine significantly inhibited it.

Discussion

Hyperhomocysteinemia is recognized as a significant risk factor for cardiovascular disease, including atherosclerosis, coronary artery disease, cerebrovascular disease, and myocardial infarction (Carmel et al., 2003). The pathogenic mechanism of homocysteine in cardiovascular dis-

Fig. 10. Taurine uptake and release from myocardial mitochondria. Homocysteine inhibited taurine uptake in a concentration dependent manner (**A**) and increased the affinity of taurine to its transporter (**B**); Na^+ increased taurine uptake in a concentration dependent manner (**C**) and increased the affinity of taurine to its transporter (**D**); taurine uptake of mitochondria was inhibited by alanine (**E**, **F**); methionine alone did not affect taurine uptake and the affinity of taurine to its transporter (**G**, **H**); homocysteine inhibited taurine release from mitochondria (**I**)

eases is not fully understood. The metabolite is considered to be involved in oxidative stress and the disturbance of cellular calcium homeostasis (Lawrence et al., 2003).

Mitochondria are important targets of cell damage and cytoprotection (Lemasters et al., 1998). Previous studies indicated that homocysteine induced oxidative stress by yielding superoxide anion and hydrogen peroxidase through its auto-oxidation in plasma (Lang et al., 2000). Our results showed that rats fed with 1% methionine for 6 wk had hyperhomocysteinemia, which was consistent with that reported by Matthias (Matthias et al., 1996). The obvious damage of myocardial mitochondria function and structure was found in these rats.

The mechanism of hyperhomocysteinemia-induced injury in mitochondria is not well defined. Our study showed that amounts of H_2O_2 and O_2^- were produced in the mitochondria of rats with hyperhomocysteinemia, which was related to the inhibition of Mn-SOD and CAT activity. Excessive reactive oxygen species (ROS) lead to mitochondrial membrane injury by lipid peroxidation. During the mitochondrial respiration process, as much as 1% to 2% of oxygen undergoes incomplete reduction to form superoxide anion by the electronic leakage pathway. Superoxide anion, produced in complexes I and III, is rapidly converted to hydrogen peroxide and hydroxyl radicals (Rodriguez et al., 2000). Experimental studies reported that homocysteine decreases the binding of extracellular-SOD (EC-SOD) to human and bovine aortic endothelial cells and platelets, and inhibits the expression of EC-SOD in cultured fibroblast cells (Nonaka et al., 2001). In addition, mitochondrial genome DNA (mtDNA) mutation is an important consequence due to ROS generation. About 30% of the subunits of enzymes in the mitochondria electron transport chain are coded by mtDNA (Bruce-Keller et al., 1999). Austin and colleagues found that the level of steady-state mRNA of cytochrome c oxidase III/ATPase 6,8, as well as other mitochondrial transcripts, were decreased in DAMI cells after treatment with homocysteine (Austin et al., 1998). In turn, superoxide anion can stimulate an auto-oxidation of homocysteine to induce oxidative stress damage.

Our results also showed that in rats with hyperhomocysteinemia fed methionine, Ca^{2+}-ATPase activity and Ca^{2+} uptake in myocardial mitochondria were significantly inhibited. Ca^{2+} uptake in mitochondria is a physiologically important mechanism for buffering or modulating cytosolic Ca^{2+}. In the mitochondrial matrix, Ca^{2+} is an important regulator of enzymes for components of electronic transfer chains. The decrease of calcium within mitochondria leads to an electronic transfer disturbance and stimulates the generation of ROS. An increase in the release of Ca^{2+} from mitochondria by oxidants also stimulates Ca^{2+}-dependent enzymes such as proteases, nucleases, and phospholipases, which could subsequently trigger apoptosis of the cells (Chakraborti et al., 1999). However, Sousa and associates recently reported that micromolar Ca^{2+} concentrations strongly stimulate the release of ROS in rotenone-treated mitochondria isolated from rat forebrains (Sousa et al., 2003). Ca^{2+}-stimulated mitochondrial ROS release was associated with membrane lipid peroxidation and was directly correlated with the degree of complex I inhibition by rotenone.

Taurine, another metabolite from methionine, was for a long time generally considered to be the inert waste product of sulfur metabolism in animals. Experimental and clinical data revealed that taurine had a membrane-stabilizing effect (e.g., regulating cellular calcium homeostasis, scavenging oxygen free radicals) (Lourenco et al., 2002). Taurine has been shown to be tissue protective in many models of oxidant-induced injury. One possibility is that taurine reacts with hypochlorous acid, produced by the myeloperoxidase pathway, to produce the more stable but less toxic taurine chloramine (Tau-Cl). Recent molecular studies on taurine bioaction provided an evidence that taurine is a constituent of biologic macromolecules. Specifically, two novel taurine-containing modified uridines have been found in both human and bovine mitochondria (Schuller-Levis et al., 2003). Moreover, it was found that taurine effectively rehabilitated the homocysteine-induced inhibition of the expression and secretion of cellular superoxide dismutase, and ameliorated homocysteine-induced proliferation of vascular smooth muscle cells and damage of vascular endothelial cells (Nonaka et al., 2001).

Taurine appears to have a biphasic biological effect on the myocardium, which depends on Ca^{2+} concentration. In the myocardium, taurine is positively inotropic at low Ca^{2+} concentrations and negatively inotropic at high ones (Satoh, 2003). In our study, the administration of taurine to rats with hyperhomocysteinemia significantly promoted $^{45}Ca^{2+}$ uptake in myocardial mitochondira. Cytosolic Ca^{2+} influx during cardiac ischemia is buffered by mitochondria, and mitochondria protects cardiomyocytes against cytosolic Ca^{2+} overload and, probably, against subsequent cell injury during ischemia and reperfusion (Amberger et al., 2001). Observations of rat liver mitochondria revealed that taurine-enhanced Ca^{2+} accumulation appeared to involve stimulating Ca^{2+} uptake via the uniport pathway rather than inhibiting Ca^{2+} release via ion exchangers (Na^+–Ca^{2+} and/or H^+–Ca^{2+}) or modu-

lating the permeability transition of the mitochondrial inner membrane (Palmi et al., 1999). In addition, we showed that taurine significantly increased Ca^{2+} ATPase activities in myocardial mitochondria, which suggests that taurine promotes calcium uptake through activating calcium pump activity in mitochondria membrane. In our study, taurine significantly inhibited the generation of ROS and increased Mn-SOD and CAT activities in rats with hyperhomocysteinemia. Taurine also inhibited lipid peroxidation products (MDA and conjugated diene) induced by hyperhomocysteinemia.

There are a number of possible pathways through which taurine may be generated, and these include 1) the oxidation of cysteine to cysteine sulfinic acid and subsequently to cysteic acid, which is decarboxylated to taurine, and 2) oxidation of cysteine to cysteine sulfinic acid with subsequent decarboxylation to hypotaurine, which is oxidized to taurine. The rate limiting step is the conversion of cysteine sulfinic acid to hypotaurine catalyzed by cysteine sulfinic acid decarboxylase (CSAD). This is a pyridoxal phosphate requiring enzyme, and therefore vitamin B6 deficiency can lead to a reduction in endogenous taurine. Generally humans have an inherent capacity to maintain endogenous taurine levels within a wide normal range from dietary methionine and cysteine (Redmond et al., 1998). However, in pathophysiological condition, the endogenous generated taurine did not match the body's need for possible taurine transporter dysfunction (Shi et al., 2003). Transport of taurine across cell membranes is one of the major steps to play its physiological roles. Taurine active transport in many types of cell membranes is Na^+ dependent. Taurine transporter knockout mice show reduced taurine, reduced fertility, and loss of vision due to severe apoptotic retinal degeneration (Heller-Stilb et al., 2002). However, the characteristic of the taurine transporter in the mitochondrial membrane is unknown. Our results showed that myocardial mitochondria exited a Na^+-dependent taurine uptake activity that saturated with an apparent Km of approximately 90 nmol/L for taurine and was inhibited by β-alanine, an antitransporter of the carrier-mediated taurine transport system. Withdrawal of Na^+ from the incubation buffer significantly inhibited the ^{3}H-taurine uptake. Interestingly, homocysteine's inhibition of ^{3}H-taurine uptake of isolated myocardial mitochondria was concentration dependent and blunted taurine's release from mitochondria. However, methionine alone did not affect the ^{3}H-taurine uptake of isolated myocardial mitochondria. These results suggest that homocysteine, but not methionine interferes with taurine transport in myocardial mitochondria.

In summary, supplementation with taurine ameliorated injury to the function and structure of myocardial mitochondria in methionine-loaded rats with hyperhomocysteinemia by decreasing the production of ROS and accelerating their clearance, and improving calcium regulation ability. This effect of taurine may be useful for cardioprotection in cardiovascular diseases accompanied by hyperhomocysteinemia. Researchers should pay more attention to the interactions and molecular mechanisms of SAAS under physiological and pathophysiological conditions.

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Authors' address: Prof. Chaoshu Tang, Institute of Cardiovascular Diseases Research, First Hospital, Peking University, 100034 Beijing, P.R. China,

Fax: +86-10-66176255, E-mail: changlin73@163.com