Taurine antagonized oxidative stress injury induced by homocysteine in rat vascular smooth muscle cells [1]

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ABSTRACT

AIM: To observe protective effects of taurine on reactive oxygen species generation induced by homocysteine in rat vascular smooth muscle cells (VSMC). **METHODS:** Rat VSMC was incubated with various concentrations of homocysteine and taurine. The lactate dehydrogenase (LDH) activity which released into culture medium was elevated as an indicator for VSMC injury. The reactive oxygen species (ROS) — hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{-\cdot}$)were measured with luminol or lucigenin chemiluminescences method, and the mitochondria Mn-superoxide dismutase (Mn-SOD) and catalase (CAT) were also measured in treated VSMC. **RESULTS:** LDH leakage from cultured VSMC treated with homocystenie, was increased (P<0.01 *vs* control), and it was markedly inhibited when co-incubated with taurine (P<0.01). Homocysteine induced H_2O_2 generation from VSMC in a concentration dependent manner (P<0.01 *vs* control). However, taurine (5, 10, and 20 mmol/L) significantly antagonized 0.5 mmol/L homocysteine-induced H_2O_2 generation in VSMC in a concentration dependent manner (P<0.01 *vs* homocysteine alone group), although taurine itself did not alter the H_2O_2 generation in VSMC (P>0.05 *vs* control). In this study, the superoxide anion in VSMC was not detectable by chemiluminent method. In addition, treatment of VSMC with taurine increased mitochondria Mn-SOD and CAT activity in a concentration dependent manner (P<0.05), but homocysteine decreased mitochondria Mn-SOD and CAT activity (P<0.01 *vs* control). In addition, co-administration of taurine markedly ameliorated homocysteine-induced inhibition of Mn-SOD and CAT activity in VSMC (P<0.01 *vs* homocysteine alone group). **CONCLUSION:** Taurine antagonized the effects of homocysteine on ROS generation and anti-oxidant enzyme activities in rat VSMC *in vitro*.

INTRODUCTION

Homocysteine is a thiol-containing amino acid that is formed when methionine was converted to cysteine. Plasma homocysteine concentration may increase in various pathophysiological conditions, including deficiency of vitamins such as folic acid, cyanocobalamin, and pyridoxal phosphate *etc*[1]. Incubation of human megakaryocytic cell line DAMI with homocysteine inhibited respiratory rate and activities of cytochrome c oxidase III/ATPase 6, 8 and other respiratory chain enzymes in mitochondria, and in which mitochondria was considered as a target of homocysteine attack[2]. VSMC mitochondria is an important organelle for regulation of

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intracellular calcium homeostasis[3] and generation of oxygen free radicals[4]. Mitochondria damage is considered as an important trigger for pathogenesis of cardiovascular diseases[5].

Taurine is another metabolite of methionine. Growing evidences show that taurine has strong cytoprotective effects by its antioxidant characteristics[6]. Our previous researches showed that taurine antagonized homocysteine-induced both proliferation in cultured rat VSMC[7] and injury in cultured human endothelial cells[8]. Nonaka *et al* reported that homocysteine induced endoplasmic reticulum stress and reduced the secretion and expression of extracellular superoxide dismutase (EC-SOD) in VSMC, leading to increased oxidative stress in the vascular wall. Taurine effectively antagonized homocysteine effects on the endoplasmic reticulum stress, restored the secretion and expression of EC-SOD[9]. Therefore, we supposed that taurine could be a natural antagonist against biological effects of homocysteine. In this study, we aimed to observe the injurious effects of homocysteine on rat VSMC mitochondria and the antagonistic effects of taurine.

MATERIALS AND METHODS

Materials Spague-Dawley (SD) rats (150 g) were obtained from the Experimental Animal Center, Health Science Center, Peking University (Clean grade, Certificate No SCXK 11-00-0008). Horseradish peroxide, *L*-Taurine, *D,L*-homocysteine, 3-aminophthalhydrazide (luminol) and lucigenin were purchased from Sigma Co [St Loius, MO]. All other reagents were of analytical grade and obtained from various commercial suppliers.

Culture of rat VSMC Rat VSMC were separated from aorta and cultured by an explant method originally described by Campbell JH[10]. The culture medium was DMEM containing 10 mmol/L sodium pyruvate supplemented with 15 % fetal bovine serum. The cells up to passage 8 were used. After confluence, the cells were inoculated on 6 well-plates (1×10^7 cells/ well). Different treatments were used to following groups: control group (CON), homocysteine groups (divided into 0.1, 0.5, and 1.0 mmol/L sub-groups, were simplified character as H1, H2, and H3, respectively), taurine groups (divided into 5, 10, and 20 mmol/L sub-groups, and were simplified character as T1, T2, and T3, respectively) and 0.5 mmol/L homocysteine plus taurine groups (divided into 5, 10, and 20 mmol/L sub-groups, and were simplified character as HT1, HT2 and HT3, respectively). VSMC were incubated with either homocysteine (0.1, 0.5, and 1.0 mmol/L), taurine (5, 10, and 20 mmol/L) or homocysteine 0.5 mmol/L+taurine (5, 10, and 20 mmol/L) for 12 h for determining ROS and antioxidant enzymes activities, and the culture medium was collected for measurement of lactate dehydrogenase (LDH) activity.

Measurement of LDH activity Loss of VSMC integrity was evaluated spectrophotometrically by measurement of the LDH activity into the culture medium at the end of the cell incubation. The measurement was based on the oxidation of lactate and the rate of increase in absorbance at 440 nm. The LDH activity was expressed as units per milliliter culture media.

Measurement for hydrogen peroxide(H_2O_2) and superoxide anion ($O_2^{\cdot-}$) production in VSMC Luminol and lucigenin chemiluminescences were respectively used to detect H_2O_2 and $O_2^{\cdot-}$ produced by rat mitochondria of VSMC in a light-tight box with BPCL Ultra-weak luminescence analyzer (Beijing, China) at 37 ºC according to Allen's method[11]. The photon counts were integrated over 1 s periods and shown on computer monitor. The integral of the signal peak reflects the formation of H_2O_2. In the absence of VSMC, interventions such as succinic acid and horseradish peroxidase did not cause change in luminol and lucigenin chemiluminescences. The tested sample for H_2O_2 production contained 1×10^7 cells/well, Krebs-Henseleit (K-H, pH 7.4) buffer, horseradish peroxides 0.01 g/L and luminol 5 mol/L by 1 mL of final reaction volume. After pre-incubation for 5 min, the reaction was initiated by adding 0.01 mL of succinic acid 200 mmol/L. The $O_2^{\cdot-}$ production assay was performed as method for H_2O_2 production assay, except for lucingenin 5 mol/L instead of luminol and free of horseradish peroxides.

Determination for mitochondria Mn-superoxide dismutase (Mn-SOD) and catalase (CAT) activity in VSMC Mitochondria Mn-SOD activity in VSMC was determined by its inhibitory action on the $O_2^{\cdot-}$-dependent reduction of xanthine–xanthine oxidase stimulated ferricytochrome[12]. The assay medium was composed of cytochrome c 100 mmol/L, hypoxanthine 100 mmol/L, Tris-HCl (pH 7.4) 10 mmol/L, and 1×10^7 cells (4.83 mL of final volume). The reaction was initiated by adding 8 mU xanthine oxidase. The inhibition rate of cytochrome c reduction was calculated. SOD activity was expressed as U/1×10^7 cells. CAT activity in VSMC was determined by its decomposing action on the H_2O_2-dependent optical density reduction at 240 nm.

Statistical analysis All data were expressed as

mean±SD. 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 Student-Newman-Keuls test. A difference of $P<0.05$ was considered significant.

RESULTS

Effects of homocysteine and taurine on LDH leakage from rat VSMC to culture media As shown in Fig 1, homocysteine 0.1 mmol/L had no obvious effect on LDH leakage from VSMC ($P>0.05$ *vs* control), whereas homocysteine 0.5 and 1.0 mmol/L increased LDH leakage by 28 % ($P<0.05$) and 54 % ($P<0.01$) respectively, when compared with control group. Taurine (5,10 and 20 mmol/L) alone did not obviously impact LDH leakage ($P>0.05$ *vs* control). However, after co-incubation with taurine (5, 10, and 20 mmol/L), the LDH leakage induced by homocystenie 0.5 mmol/L was markedly inhibited by 30 %, 26 %, and 29 % respectively, when compared with homocysteine 0.5 mmol/L alone group ($P<0.01$).

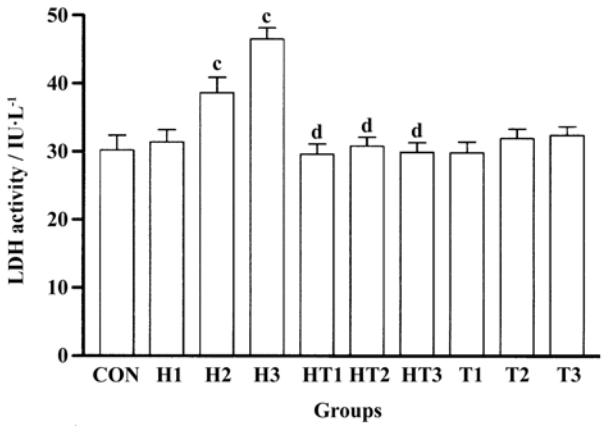

Fig 1. Effects of homocysteine and taurine on LDH leakage from rat VSMC. *n*=6. Mean±SD. LDH activity was determined as described in methods. H1, H2, H3, homocysteine 0.1, 0.5, 1.0 mmol/L, respectively; T1, T2, T3, taurine 5, 10, 20 mmol/L, respectively. HT1, HT2, HT3, 0.5 mmol/L homocysteine plus taurine 5, 10, 20 mmol/L, respectively. $^cP<0.01$ *vs* control group; $^dP<0.01$ *vs* homocysteine 0.5 mmol/L group.

Effects of homocysteine and taurine on H_2O_2 generation in VSMC Mitochondria is a major source of generation of intracellular oxygen free radicals. The homocysteine (0.1, 0.5, and 1.0 mmol/L) induced H_2O_2 generation from VSMC in a concentration-dependent manner, and increased by 10-, 20-, and 50-fold respectively, when compared with control group ($P<0.01$). Taurine (5, 10, and 20 mmol/L) did not alter the H_2O_2 generation in VSMC ($P>0.05$ *vs* control group). However, taurine (5, 10, and 20 mmol/L) significantly antagonized homocysteine 0.5 mmol/L induced H_2O_2 generation in VSMC in a concentration dependent manner, and the inhibition rate were 47 %, 60 %, and 71 %, respectively ($P<0.01$ *vs* homocysteine 0.5 mmol/L group). The results were shown in Fig 2. In this study, the superoxide anion in VSMC was not detected by chemiluminecent method.

Effects of homocysteine and taurine on Mn-SOD and CAT activity in VSMC 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 to catalyze $O_2^{\cdot-}$ into H_2O_2, while CAT further catalyzes H_2O_2 into H_2O. As shown in Fig 3A, treatment of VSMC with taurine (5, 10, and 20 mmol/L) increased Mn-SOD activity in a concentration-dependent manner when compared with control group ($P>0.05$). It was obvious that administration with 0.1, 0.5, and 1.0 mmol/L of homocysteine decreased Mn-SOD activity by 32 %, 41 % and 77 % respectively, when compared with control group ($P<0.01$). Co-administration of taurine (5, 10, and 20 mmol/L) with homocysteine 0.5 mmol/L significantly ameliorated inhibitory effects of Mn-SOD activity induced by homocysteine in VSMC ($P<0.01$ *vs* homocysteine 0.5 mmol/L group), and the activities were higher 2.1-, 2.2-, and 2.9-fold than that in homocysteine 0.5 mmol/L alone group ($P<0.01$, respectively).

As the Fig 3B shown, the CAT activities of VSMC were inhibited by homocysteine in a concentration-dependent manner, and were decreased by 17 %, 55 %, and 67 %, respectively, when compared with control group ($P<0.01$, respectively). Taurine significantly elevated the CAT activities of VSMC in a concentration-dependent manner by 2.1-, 3.4-, and 4.6-fold, respectively, than that in control group ($P<0.01$). Furthermore, taurine also significantly elevated the CAT activities in the VSMC co-incubated with 0.5 mmol/L of homocysteine in a concentration-dependent manner ($P<0.01$ *vs* homocysteine 0.5 mmol/L alone group).

DISCUSSION

Hyperhomocysteinemia is an independent risk factor for cardiovascular disease. It has been suggested that homocysteine-induced cell injury involves in oxida-

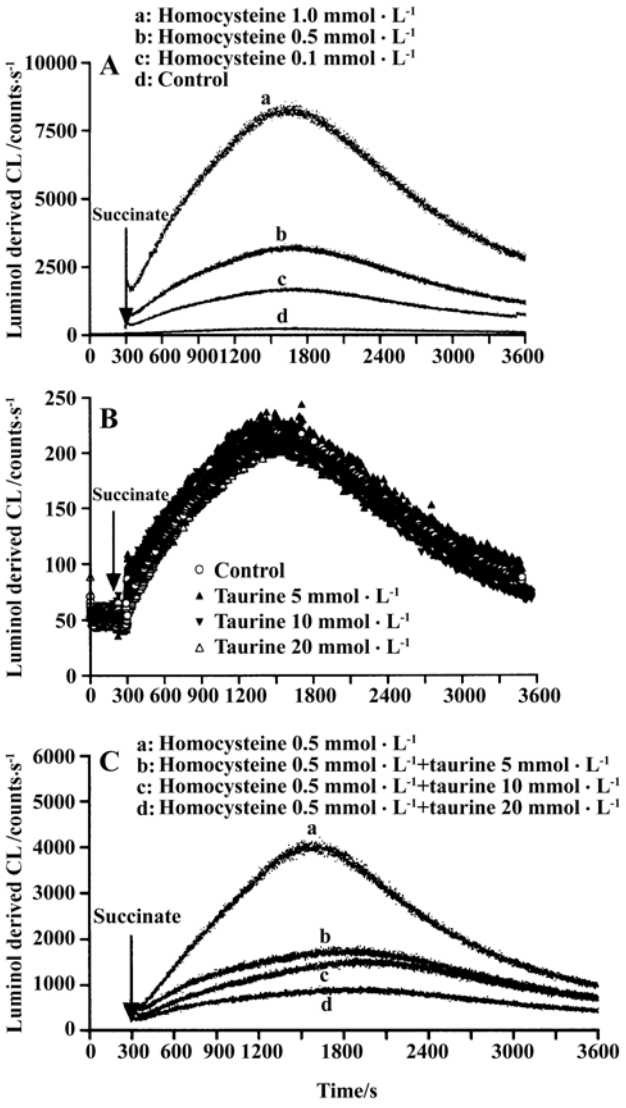

Fig 2. Effects of homocysteine and taurine on H_2O_2 generation in VSMC. There were similar results in 3 independent experiments, here only showed 1 result. H_2O_2 production in rat VSMC was measured by luminol chemiluminescence methods. The photon counts were integrated 1 time per second and shown on a computer monitor, and the photon count points shaped a curve (ordinate represent photon counts, and abscissa represent time/s). As shown in Fig 2A, homocysteine induced H_2O_2 production in VSMC in a concentration dependent manner, where taurine alone did not affect H_2O_2 production in VSMC (Fig 2B). Taurine antagonized hydrogen peroxide generation induced by homocysteine 0.5 mmol/L in a concentration-dependent manner (Fig 2C).

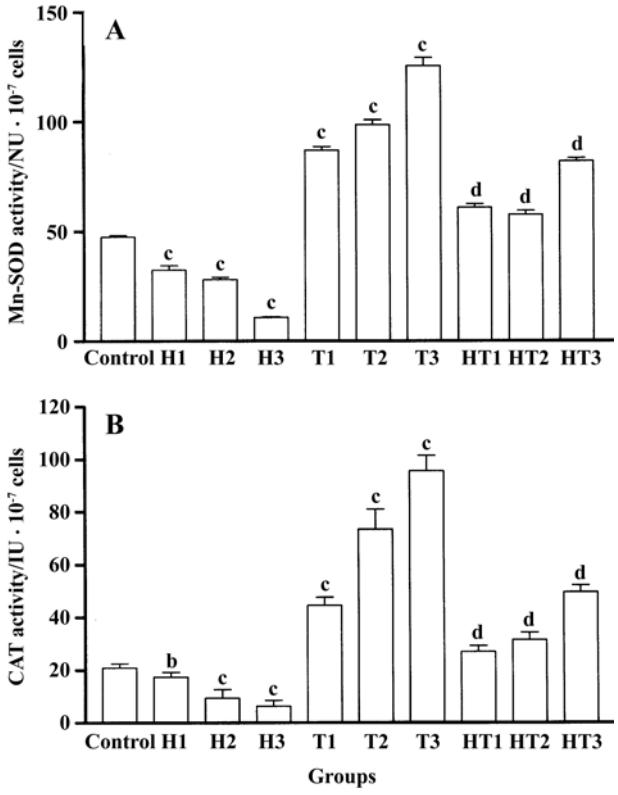

Fig 3. Effects of homocysteine and taurine on mitochondria Mn-SOD and CAT activity in rat VSMC. *n*=6. Mean±SD. H1, H2, H3, homocysteine 0.1, 0.5, 1.0 mmol/L, respectively; T1, T2, T3, taurine 5, 10, 20 mmol/L, respectively. HT1, HT2, HT3, 0.5 mmol/L homocysteine plus taurine 5, 10, 20 mmol/L, respectively. $^bP<0.05$, $^cP<0.01$ *vs* control; $^dP<0.01$ *vs* homocysteine 0.5 mmol/L group. Mitochondria Mn-SOD and CAT activities were determined as described in materials and methods.

tive damage, because homocysteine is readily oxidized in plasma to generate ROS, such as H_2O_2, and cell injury caused by homocysteine is blocked by catalase[13]. It has been evidenced that homocysteine decreases intracellular glutathione and glutathione peroxidase, which are responsible for the eliminating oxygen free radicals[14].

In this study, homocysteine 0.5 and 1.0 mmol/L increased LDH activity in culture medium released from VSMC, suggesting that VSMC membrane was injured. Homocysteine stimulated H_2O_2 production in a concentration-dependent manner, suggesting that it promoted generation of ROS in mitochondria of VSMC. These effects of homocysteine could result in ROS intracellular accumulation and injure VSMC. The detail mechanisms are still unclear by which homocysteine induces the production of ROS in VSMC. Mitochondria are considered as a major source for ROS production in eukaryotic cell. During the mitochondrial respiration process, as much as 1 %-2 % oxygen undergoes incomplete reduction to form $O_2^{\cdot-}$ by electronic leakage pathway. $O_2^{\cdot-}$ which produced at complex I and III is rapidly converted to H_2O_2 and the potent reactive species, hydroxyl radical spontaneously or by superoxide

dismutase[15]. About 30 % subunits of enzymes in mitochondria electron transport chain are coded by mitochondria genome DNA. Mitochondria genome DNA mutation or injury may affect ROS metabolism[16]. Austin *et al* reported that homocysteine inhibited mitochondrial respiration rates in a human megakaryocytic cell line DAMI. Steady-state mRNA levels of cytochrome c oxidase III/ATPase 6,8, as well as other mitochondrial transcripts, were increased in DAMI cells by treatment with homocysteine [2]. Inhibition of SOD activity may be another mechanism for oxidative stress injury induced by homocysteine. Three isozymes of SOD in mammal cells have been identified at the molecular level: intracellular Cu/Zn-SOD, mitochondrial Mn-SOD, and extracellular SOD (EC-SOD)[17]. In this study, mitochondrial Mn-SOD activity was inhibited by homocysteine in a concentration-dependent manner. It means a decrease in $O_2^{\cdot-}$ scavenging. Yamamoto *et al* reported that homocysteine decreased the binding of EC-SOD to human and bovine aortic endothelial cells and platelet, and inhibited the expression of EC-SOD in cultured fibroblast cell [16]. In this study, CAT activity in VSMC was also inhibited by homocysteine in a concentration-dependent manner. These effects of homocysteine on VSMC could result in intracellular ROS accumulation and injure VSMC.

Taurine, another metabolite from methionine, was generally considered to be the inert waste product of sulfur metabolism in animals for a long time. Experimental and clinical data showed that taurine had membrane-stabilizing effect, regulating cellular calcium homeostasis, scavenging oxygen free radicals, *etc*[6] and exert important regulatory roles in cardiovascular[18] and cerebral vascular disease[19]. Recently, it was found that taurine rehabilitated effectively homocysteine-induced inhibition of cellular SOD expression and secretion, ameliorated obviously homocysteine-induced proliferation of VSMC and damage of vascular endothelial cells[9].

In this study, LDH leakage from cultured VSMC induced by homocysteine were inhibited by taurine in a concentration-dependent manner. Taurine itself did not affect H_2O_2 production in VSMC, whereas taurine did antagonize H_2O_2 production induced by homocysteine in VSMC in a concentration-dependent manner. The mechanisms by which taurine antagonized H_2O_2 production in VSMC induced by homocysteine may be correlated with an increase of Mn-SOD activity. In this study, taurine itself increased mitochondria Mn-SOD activity, and it also antagonized homocysteine-induced inhibition of Mn-SOD activity. Taurine also increased CAT activity of VSMC in a concentration-dependent manner, and completely inversed the inhibitory effect of homocysteine on CAT activity. Thereby we inferred that taurine could activate mitochondria Mn-SOD and CAT in VSMC, and scavenge excessive ROS induced by homocysteine. Consistent with our findings, Nonaka *et al* reported that homocysteine induced endoplasmic reticulum stress and reduced the expression and secretion of EC-SOD in VSMC, leading to increased oxidative stress in the vascular wall. Taurine antagonized effectively homocysteine effects on the endoplasmic reticulum stress, restored the secretion and expression of EC-SOD[9]. In this study, $O_2^{\cdot-}$ produced by VSMC was not detectable by chemiluminescence methods. It was maybe correlated with the reasons that produced $O_2^{\cdot-}$ was promptly changed into H_2O_2 before it was transferred out of cell.

In summary, homocysteine injured VSMC by oxidative stress, and it also inhibited antioxidant enzyme activities which could weak the antioxidant effects in VSMC. Taurine antagonized effectively the effects of homocysteine on ROS production and antioxidant enzyme activities in VSMC. These data suggest that taurine is a biological antagonist against homocysteine. Replenishment with taurine could be an effective new strategy of prevention and therapy for cardiovascular diseases caused by hyperhomocysteinemia.

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