

Taurine Prevents the Decrease in Expression and Secretion of Extracellular Superoxide Dismutase Induced by Homocysteine

Amelioration of Homocysteine-Induced Endoplasmic Reticulum Stress by Taurine

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Background—Hyperhomocysteinemia is an independent risk factor for atherosclerosis. Homocysteine has been shown to induce endoplasmic reticulum (ER) stress in vascular endothelial cells. ER stress is a condition in which glycoprotein trafficking is disrupted and unfolded proteins accumulate in the ER. ER molecular chaperons, such as GRP78, are induced and an ER resident kinase, PERK, is activated when cells are subjected to ER stress. Conversely, taurine is reported to have antiatherogenic effects by unknown mechanisms. To elucidate the mechanisms by which homocysteine induces atherosclerosis and taurine prevents it, we examined whether homocysteine and taurine affect the expression and secretion of extracellular superoxide dismutase (EC-SOD), a glycoprotein secreted from vascular smooth muscle cells (VSMCs) that protects the vascular wall from oxidative stress.

Methods and Results—We assessed the expression of EC-SOD and GRP78 mRNA in cultured rat VSMCs by Northern blot analysis. The EC-SOD protein secreted into the culture medium was examined by Western blot analysis. Homocysteine (5 mmol/L) and other ER stress inducers, including A23187, were found to decrease EC-SOD mRNA expression and protein secretion. Furthermore, they upregulated GRP78 mRNA expression and activated PERK. Taurine (0.5 to 10 mmol/L), conversely, prevented these actions induced by homocysteine.

Conclusions—Homocysteine induces ER stress and reduces the secretion and expression of EC-SOD in VSMCs, leading to increased oxidative stress in the vascular wall. Taurine restores the secretion and expression of EC-SOD by ameliorating ER stress induced by homocysteine. (*Circulation*. 2001;104:1165-1170.)

Key Words: atherosclerosis ■ antioxidants ■ vasculature ■ risk factors

Homocysteine and taurine (2-aminoethanesulfonate), both sulfur-containing amino acids sharing the same biosynthetic pathway, have been shown to have opposite effects in the development of atherogenic vascular diseases. Homocysteinemia, caused by genetic deficiencies in enzymes related to homocysteine metabolism, is associated with increased incidence of vascular thrombosis and increased development of atherosclerosis.^{1,2} Recent epidemiological studies have demonstrated that even a mildly elevated plasma homocysteine level is considered an independent risk factor for the development of premature atherosclerosis and thrombosis. In contrast, taurine, one of the metabolites of methionine and cysteine, has been shown to have antihypertensive^{3,4} and antiatherogenic effects^{3,5} in animal models. Epidemiological studies also revealed that taurine intake correlates inversely with the incidence of coronary heart disease.⁶

Earlier studies suggested that atherothrombosis associated with hyperhomocysteinemia reflects endothelial cell injury.^{7,8} Oxidative stress induced by homocysteine was widely noticed to account for endothelial cell injury and/or dysfunction.^{8,9} Another mechanism, however, the induction of endoplasmic reticulum (ER) stress by homocysteine, is now attracting considerable attention.^{10,11} ER stress is a condition in which misfolded proteins accumulate in the ER lumen.¹² When cells are subjected to ER stress, an ER resident kinase, PKR-like ER kinase (PERK), is activated to suppress protein synthesis.¹³ Transcription of ER resident chaperones (eg, GRP78, GRP94, protein disulfide isomerase, etc) is then upregulated to restore proper protein folding. Previous studies suggested that homocysteine is one of the ER stress inducers on endothelial cells, on the basis of observations that it could cause the induction of ER chaperones and interfere with the

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transport of anticoagulant factors like thrombomodulin without disturbing their synthesis.¹⁴

Increased oxidative stress plays a crucial role in cardiovascular diseases, such as atherosclerosis and hypertension.¹⁵ Superoxide dismutase (SOD), essential to catalyze the dismutation of superoxide, has been shown to protect cells from oxygen free radicals.¹⁶ Three isozymes of SOD have been identified at the molecular level in mammals: intracellular Cu,Zn-SOD, mitochondrial Mn-SOD, and extracellular (EC)-SOD. The most abundant among these in vascular tissues is EC-SOD, a copper- and zinc-containing glycoprotein secreted from vascular smooth muscle cells (VSMCs).^{17,18} Several studies indicated that EC-SOD is an important mediator in modulating vascular tone and inhibiting atherogenesis. Thus, decreased secretion of EC-SOD could eventually weaken the defense against oxidative stress in the vascular wall.

In the present study, we examined the role of homocysteine in the expression and secretion of EC-SOD in VSMCs. We demonstrated that homocysteine worked as an ER stress inducer on VSMCs, resulting in decreased expression and secretion of EC-SOD. Furthermore, we showed that taurine has a protective effect against homocysteine-induced ER stress.

Methods

Materials

A polyclonal anti-rat EC-SOD antibody and a rat EC-SOD cDNA probe were gifts from Dr Jean Willems (Interdisciplinary Research Center, Belgium).¹⁹ The rat GRP78 cDNA probe was a gift from Dr Sean Munro (Laboratory of Molecular Biology, Cambridge University, Cambridge, England).²⁰ The rabbit anti-rat PERK serum was given by Dr David Ron (New York University Medical School, New York, NY).¹³ DL-Homocysteine was purchased from Sigma-Aldrich Japan. Taurine (2-aminoethanesulfonate) was from Taisho Pharmaceutical. Other materials and chemicals used were obtained commercially.

Cell Culture

VSMCs were isolated from rat thoracic aorta by enzymatic dissociation as described previously.²¹ The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, and 100 μ g/mL streptomycin. Cells between passages 8 and 17 were made quiescent by incubation with serum-free DMEM for 24 hours before use.

Northern Blot Analysis

Total RNA was extracted from VSMCs with Isogen (Nippon Gene). Total RNA (15 μ g per lane) was electrophoresed on a formaldehyde-containing 1.2% agarose gel, transferred to a nylon membrane (Hybond-N⁺; Amersham), and hybridized to random-primed, ³²P-labeled probes. The membrane was washed and exposed on an imaging plate, and the fragments were visualized with a Bio-imaging analyzer, BAS 2000 (FUJIX). mRNA was densitometrically quantified with software supplied by FUJIX BAS 2000. Loading variations were standardized by scanning the ethidium bromide staining of 28S ribosomal RNA and were performed with National Institutes of Health Image software (NIH Image).

Western Blot Analysis

VSMCs were cultured in 6-cm dishes with 2 mL culture medium. To detect the cumulative amount of secreted EC-SOD in the culture medium, 40 μ L of medium was separated on 12% SDS-PAGE under reduced conditions, transferred to a polyvinylidene fluoride mem-

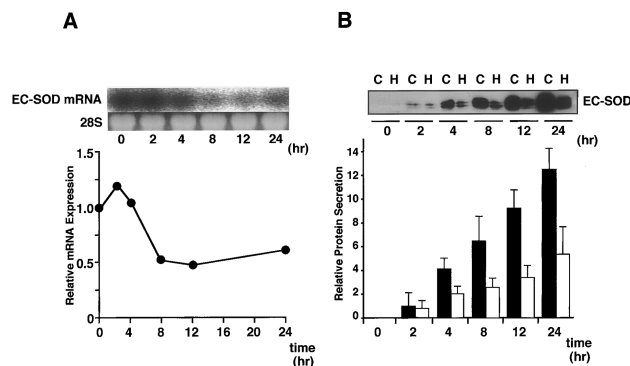


Figure 1. Time course of EC-SOD mRNA expression (A) and protein secretion (B) in rat VSMCs treated with 5 mmol/L homocysteine for indicated time periods. A, Results are relative ratio to control (time 0). B, Results are relative ratio to control at 2 hours. Data are mean of results from 2 (A) and 5 (B) separate experiments. Top, Autoradiographs from representative Northern blot (A) or Western blot (B). Rat EC-SOD typically shows 3 bands, with lower 2 representing C-terminal truncations. C and closed bars indicate control; H and open bars, homocysteine.

brane (Millipore), and probed with rat anti-EC-SOD antiserum. The immunoreactive bands were visualized by use of horseradish peroxidase-conjugated anti-rabbit IgG with the ECL Plus detection kit (Amersham). Stained protein bands were quantified by NIH Image.

Detection of PERK Activation

VSMCs were lysed in a buffer described by Harding et al.¹³ The lysate was then centrifuged at 15 000 rpm for 10 minutes at 4°C and was immunoprecipitated with 1 μ L of anti-PERK bound to 30 μ L protein A sepharose. Bound proteins were resolved by 7% SDS-PAGE under reducing conditions and subjected to immunoblotting with the anti-PERK antibody. Activated PERK was detected as an upward-shifted band as a result of its autophosphorylation, which reduced its mobility.²²

Statistical Analyses

Values in the figures are mean \pm SD, and statistical analysis was carried out with Student's unpaired *t* test or ANOVA when appropriate. Differences were considered significant when the probability value was *P* < 0.05.

Results

Homocysteine Decreases EC-SOD mRNA Expression and Protein Secretion

We first examined the effect of homocysteine on EC-SOD mRNA expression and protein secretion in VSMCs. Treatment of VSMCs with homocysteine decreased EC-SOD mRNA expression (Figure 1A) and protein secretion (Figure 1B), followed by decreased EC-SOD activities (data not shown). We found, however, that the decrease in EC-SOD protein secretion, apparent from 4 hours, was detected earlier than its mRNA expression (8 hours). Figure 2 shows that both EC-SOD mRNA and protein secretion were reduced by homocysteine treatment in a dose-dependent manner. EC-SOD mRNA was reduced by 50% compared with the control (0.52 ± 0.06 -fold versus control, *n* = 3, *P* < 0.05) when treated with 5 mmol/L homocysteine (Figure 2A). In contrast, 0.1 mmol/L homocysteine was sufficient to decrease EC-SOD protein secretion (0.55 ± 0.14 -fold versus control, *n* = 3, *P* < 0.05) (Figure 2B), indicating that protein secretion is more sensitive to homocysteine than mRNA expression.

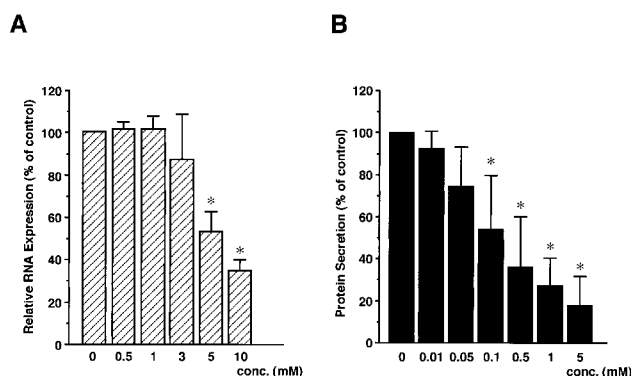


Figure 2. Dose-response effects of homocysteine on EC-SOD mRNA expression in rat VSMCs (A) and EC-SOD protein secretion into culture medium (B). VSMCs were treated with indicated concentrations (conc) of homocysteine for 12 hours. Results are percent of control. Data are mean \pm SD of 3 independent experiments, each performed in triplicate. * P <0.05.

VSMCs treated with 5 mmol/L homocysteine for 24 hours did not show any evidence of cell injury, which was assessed by trypan blue exclusion and total lactate dehydrogenase activity in the culture medium. We also confirmed that 10 mmol/L cysteine had no effect on EC-SOD mRNA expression and protein secretion, suggesting that the effect of homocysteine is specific (data not shown). Taken together, these observations suggested that homocysteine disturbs EC-SOD secretion, followed by a decrease of EC-SOD mRNA expression.

Homocysteine Is an ER Stress Inducer in VSMCs and EC-SOD Is Decreased by Classic ER Stress Inducers

These findings prompted us to speculate that homocysteine may induce ER stress and that decreased expression and secretion of EC-SOD are due to ER stress. To test this hypothesis, we treated VSMCs with known ER stress inducers (A23187, tunicamycin, and dithiothreitol [DTT]) and assessed the EC-SOD mRNA expression and GRP78 mRNA expression by Northern blot analysis. Previous studies reported that treatment of vascular endothelial cells and fibro-

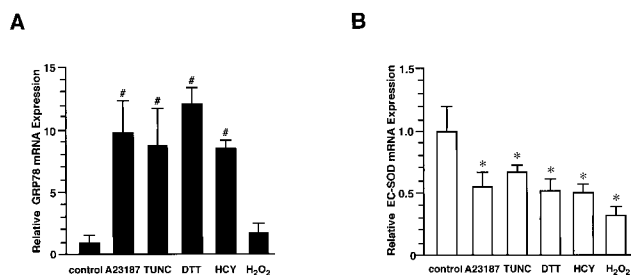


Figure 3. Effects of homocysteine (Hcy) and other ER stress inducers on GRP78 mRNA expression (A) and on EC-SOD mRNA expression (B) in rat VSMCs. VSMCs were treated with each agent (A23187 1 mmol/L, tunicamycin [TUNC] 1.5 μ g/mL, DTT 2 mmol/L, HCY 5 mmol/L, H₂O₂ 100 μ mol/L) for 12 hours. mRNA levels were determined by Northern blot analysis. Results are relative ratio to control. Data are mean \pm SD of 3 independent experiments, each performed in triplicate. # P <0.01, * P <0.05.

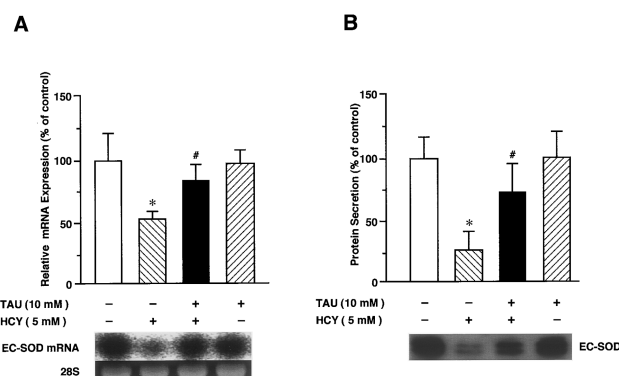


Figure 4. Effects of taurine (TAU) on decrease of EC-SOD mRNA expression (A) and protein secretion (B) induced by homocysteine (Hcy). VSMCs were pretreated with 10 mmol/L taurine for 30 minutes and treated with 5 mmol/L HCY for 12 hours. Results are percent of control. Data are mean \pm SD of 3 independent experiments, each performed in triplicate. * P <0.01 vs control, # P <0.05 vs HCY. Bottom, Representative blots.

blasts with ER stress inducers caused a marked increase in GRP78 mRNA expression.¹² As shown in Figure 3A, homocysteine, as well as other ER stress inducers, markedly induced GRP78 mRNA expression (8.41 ± 0.63 -fold versus control, $n=3$, P <0.05) in VSMCs, suggesting that homocysteine works as an ER stress inducer. In addition, we found that ER stress inducers were able to reduce EC-SOD mRNA expression, thus indicating that ER stress results in a decrease of EC-SOD expression (Figure 3B).

Next, we examined whether H₂O₂, which has accounted for the cytotoxic effects of homocysteine, could reduce EC-SOD expression and induce ER stress. As shown in Figure 3B, H₂O₂ (100 μ mol/L) reduced EC-SOD expression (0.34 ± 0.10 -fold versus control, $n=3$, P <0.05) to the same extent as homocysteine (5 mmol/L), thereby indicating that oxidative stress could decrease the expression of EC-SOD. Importantly, however, H₂O₂ failed to induce GRP78 mRNA expression, suggesting that oxidative stress caused by homocysteine is unable to induce ER stress. In addition, we observed that the antioxidants catalase and ebselen did not effect homocysteine-induced EC-SOD expression (data not shown). Thus, induction of ER stress by homocysteine does not reflect oxidative stress.

These observations suggest that the decrease in EC-SOD expression is due to not only oxidative stress but also ER stress induced by homocysteine.

Taurine Ameliorates the Decrease of EC-SOD mRNA Expression and Protein Secretion and the Induction of GRP78 Caused by Homocysteine

We examined the effect of taurine on decreased EC-SOD expression and secretion caused by homocysteine. As shown in Figure 4, thirty minutes of preincubation with taurine (10 mmol/L) ameliorated the decrease of EC-SOD mRNA expression (control versus homocysteine versus taurine plus homocysteine versus taurine, percent of control: 100 ± 22 versus 52 ± 6 versus 81 ± 13 versus 94 ± 8) (Figure 4A) and protein secretion into culture medium (control versus homocysteine versus taurine plus homocysteine versus taurine:

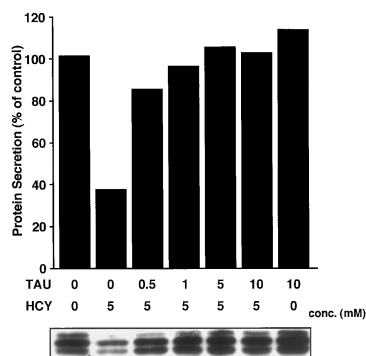


Figure 5. Dose-response effects of taurine (TAU) on decrease in EC-SOD secretion induced by homocysteine (HCY). VSMCs were preincubated with medium containing indicated concentrations (conc) of taurine for 30 minutes and then incubated with HCY to final concentration of 5 mmol/L. Relative amount of EC-SOD secretion was determined by Western blot analysis. Results are percent of control. Data are mean of 2 independent experiments, performed in duplicate. Bottom, Representative blot.

100±19 versus 26±15 versus 75±20 versus 101±24) (Figure 4B) caused by homocysteine (5 mmol/L). Taurine prevented the decrease of EC-SOD protein secretion caused by homocysteine in a dose-dependent manner (Figure 5). As little as 0.5 mmol/L taurine prevented the decrease of EC-SOD secretion by homocysteine (5 mmol/L).

Furthermore, we found that taurine (10 mmol/L) abolished the induction of GRP78 mRNA caused by homocysteine (5 mmol/L), as shown in Figure 6A, indicating that taurine prevents the induction of ER stress by homocysteine. In contrast, taurine had no effect on the induction of GRP78 mRNA caused by one of the classic ER stress inducers, A23187 (1 μmol/L) (Figure 6B), suggesting that the prevention of ER stress by taurine is specific for that elicited by homocysteine.

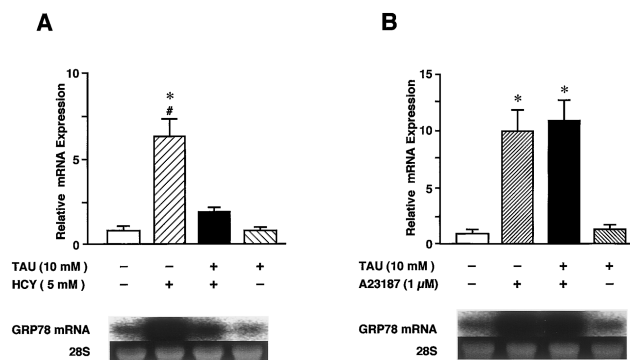


Figure 6. Effects of taurine (TAU) on increased GRP78 mRNA expression induced by homocysteine (HCY) (A) or by A23187 (B). VSMCs were incubated in medium in absence (–) or presence (+) of 10 mmol/L taurine for 30 minutes and incubated with 5 mmol/L HCY (A) or 1 μmol/L A23187 (B) for 12 hours. Results are relative expression of GRP78 mRNA to control. Data are mean±SD of 3 independent experiments, each performed in triplicate. **P*<0.001 vs control, #*P*<0.005 vs HCY plus taurine. Bottom, Representative blots.

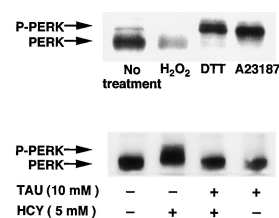


Figure 7. Effects of various agents on PERK activation. Upper species indicated as P-PERK represent autophosphorylated and activated PERK. Top, ER stress inducers DTT and A23187 activate PERK. VSMCs are incubated with each agent (A23187 1 μmol/L, DTT 2 mmol/L, H₂O₂ 100 μmol/L) for 12 hours. Bottom, Homocysteine (HCY) activates PERK, and preincubation of taurine (TAU) ameliorates activation of PERK by HCY. VSMCs were pretreated with 10 mmol/L taurine for 30 minutes and treated with HCY to final concentration of 5 mmol/L for 12 hours.

Homocysteine Activates PERK, and Taurine Inhibits Its Activation

Although the upregulation of GRP78 suggests the induction of ER stress, it does not provide definite evidence that cells are subjected to ER stress. To demonstrate that homocysteine induces ER stress, we investigated whether PERK is activated by homocysteine in VSMCs. ER stress inducers such as DTT (2 mmol/L) and A23187 (1 μmol/L) activated PERK, as shown by the upward mobility shifts (Figure 7). Homocysteine treatment of VSMCs activated PERK, indicating that homocysteine does induce ER stress in VSMCs. Conversely, preincubation of VSMCs with taurine (10 mmol/L) prevented the mobility shift of PERK caused by homocysteine, indicating that taurine prevents ER stress induced by homocysteine.

Discussion

Homocysteine is now recognized as an independent risk factor for atherosclerosis in the coronary, cerebral, and peripheral vasculature.^{1,2} It is thought to cause oxidative stress to blood vessels, because it yields superoxide and hydrogen peroxide by auto-oxidation.²³ In addition, homocysteine was shown to reduce the expression of glutathione peroxidase, an antioxidant enzyme expressed in endothelial cells, thereby causing the accumulation of its oxidative byproducts.²⁴ In the present study, we demonstrated that homocysteine reduces the secretion and expression of EC-SOD, the most abundant isozyme of SOD in the vascular wall.¹⁸ Thus, in addition to causing endothelial injury directly by oxidative stress, homocysteine also reduces the superoxide anion scavenging capacity and thereby potentiates endothelial cell injury.

The findings presented in this study indicated that homocysteine works as an ER stress inducer on VSMCs. Although previous studies indicated that homocysteine is one of the ER stress inducers on vascular endothelial cells, the conclusions derived were mainly from observations that homocysteine treatment of endothelial cells resulted in a disturbance in the transport of anticoagulant factors from the ER and/or the induction of molecular chaperones, including GRP78.¹⁴ Indeed, we have shown that homocysteine interfered with the secretion of EC-SOD and induced the expression of GRP78 in VSMCs, suggesting that homocysteine elicits ER stress in VSMCs. In addition, we have shown that homocysteine

increases autophosphorylation of PERK in VSMCs. PERK is a type I transmembrane ER-resident protein composed of an ER stress-sensing domain in its luminal portion, a transmembrane helix, and a serine/threonine kinase domain in its cytoplasmic portion. Because PERK is selectively activated on ER stress to prevent further translation of the proteins, activation of PERK is now regarded as the most definitive marker for ER stress.^{13,22} Taken together, our findings provide direct evidence that homocysteine could induce ER stress in VSMCs to accumulate unfolded proteins, including EC-SOD, in the ER lumen.

The molecular and cellular mechanisms explaining how homocysteine induces ER stress have not been defined yet. Lentz and Sadler^{14,25} reported that homocysteine inhibits cell surface expression of thrombomodulin and secretion of the von Willebrand factor from the endothelial cell by preventing their exit from the ER. They suggested that homocysteine disturbs the formation of disulfide bonds in the molecules by its reactive sulfhydryl residue or disturbs its glycosylation and subsequently inhibits their proper folding and multimer formation. In addition, they mentioned the possibility that homocysteine may cause ER retention by altering the intracellular redox potential through its free thiol group. Thus, the mechanisms by which homocysteine induces ER stress may be multifactorial. Because EC-SOD is one of the glycoproteins and also contains an intramolecule disulfide bond and forms a dimer with another disulfide bond,¹⁷ it is tempting to speculate that homocysteine decreases the secretion of EC-SOD by disturbing the formation of its disulfide bond and/or inhibiting the glycosylation, resulting in the incorrect assembly of the EC-SOD protein. From the data presented in this article, however, we cannot rule out other mechanisms. Further studies are necessary to dissect the precise molecular mechanisms.

We have shown that taurine ameliorated the decrease of EC-SOD secretion, the induction of GRP78 mRNA expression, and the activation of PERK caused by homocysteine. These findings indicated that taurine reduced ER stress elicited by homocysteine and restored correct EC-SOD folding and assembly. Although our data clearly demonstrated that taurine prevents ER stress elicited by homocysteine, the exact site at which taurine acts in the course of homocysteine-induced ER stress still remains unknown. ER stress is found to be induced by several conditions, as we discussed above. Taurine was unable to antagonize ER stress elicited by other ER stress inducers, including A23187 (Figure 6), DTT, and tunicamycin (H.N. and T.T., unpublished data, 2000), indicating that taurine is not able to restore the correct protein folding directly. The effect of taurine on ER stress thus seems specific for that induced by homocysteine. Obviously, further studies are necessary to elucidate the precise mechanisms. Our findings, nonetheless, suggest that increased taurine intake may prevent progression of atherosclerosis and ischemic heart disease by antagonizing the atherogenicity due to homocysteine.

In this context, it is interesting to note that increased homocysteine and decreased taurine content in plasma have been reported in hemodialysis and end-stage renal disease patients.^{26,27} Because uremic patients are known to develop a

variety of atherogenic diseases, a decrease in taurine concentration could thereby exacerbate the atherogenicity due to homocysteine in these patients. Hyperhomocysteinemia in such patients has proved to be quite refractory to pharmacological doses of folic acid and vitamin B supplementation.²⁸ Although the mechanisms against the effects of homocysteine are different between folic acid, vitamin B, and taurine, supplementation of taurine could be an alternative therapeutic approach to prevent atherogenic diseases in these patients. A well-controlled clinical study would therefore allow us to evaluate the efficacy of taurine supplementation for such patients.

The concentrations of homocysteine used in our studies are an order of a magnitude higher than the serum homocysteine level in patients with homocystinuria (0.1 to 0.5 mmol/L).^{1,2} One may argue that our observations of decreased secretion and expression of EC-SOD with homocysteine treatment were due to pharmacological toxic effects. We observed, however, that as little as 0.1 mmol/L homocysteine caused a 45% decrease in the secretion of EC-SOD. Outinen et al¹¹ reported that 1 to 5 mmol/L homocysteine in culture medium is necessary to significantly increase the intracellular homocysteine concentration and subsequently alter gene expression in human umbilical vein endothelial cells. These observations are supported by the fact that <1% of homocysteine exogenously added to the culture medium is actually taken up intracellularly.²⁹ On the basis of these observations, we believe that the intracellular homocysteine concentration achieved by 5 mmol/L extracellular homocysteine may be compatible for patients with hyperhomocysteinemia.

In summary, homocysteine induces ER stress in VSMCs and decreases the secretion and expression of EC-SOD. This may be one mechanism by which homocysteine induces atherosclerosis. We have also shown that taurine antagonizes the effect of homocysteine. Increased taurine intake may therefore be beneficial for the prevention of atherogenic diseases.

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