Effects of homocysteine on the binding of extracellular-superoxide dismutase to the endothelial cell surface

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Abstract Homocysteine is known to be a risk factor for several vascular diseases. Previously, we found a significant association between plasma homocysteine and plasma extracellular-superoxide dismutase (EC-SOD) levels. The binding of EC-SOD to human and bovine aortic endothelial cell cultures showed significant decreases after incubation with 10 μM homocysteine, whereas the expression of EC-SOD in fibroblast cell cultures was inhibited with a high concentration (1 mM) of homocysteine. Furthermore, binding of EC-SOD to heparin immobilized on plates was decreased with homocysteine. These observations suggested that homocysteine decreases the binding of EC-SOD to vascular endothelial cell surfaces by degradation of endothelial heparan sulfate proteoglycan, which results in a loss of the ability to protect endothelial cell surfaces from oxidative stress.

Key words: Homocysteine; Extracellular-superoxide dismutase; Endothelial cell; Heparan sulfate proteoglycan; Atherosclerosis

1. Introduction

Modest elevation of plasma homocysteine, commonly referred to as hyperhomocysteinemia, is generally considered to be a risk factor for coronary, cerebral and peripheral vascular disease [1–4] and thrombosis [5]. While the mechanisms mediating vascular changes are still unclear, there is evidence that elevated plasma homocysteine may cause endothelial cell injury or dysfunction [6,7], inhibition of endothelium-dependent relaxation [8,9], vascular thrombosis [10,11], smooth muscle cell proliferation [12], oxidative stress [13–15] and may affect endothelial cell gene expression [14,16–18].

Extracellular-superoxide dismutase (EC-SOD) is a secretory glycoprotein with an affinity for heparin-like substances [19,20]. The plasma EC-SOD level might affect the physiological and pathological conditions of the vascular system since this enzyme is the principal enzymatic scavenger of superoxide in the extracellular space and is present in the circulation in equilibrium between the plasma phase and the glycosaminoglycans on the endothelium [21,22]. In previous studies, we showed that the plasma EC-SOD levels were elevated in patients with renal diseases [23] but reduced in patients with a history of myocardial infarction [24].

Recently, we reported that there was a significant positive relationship between plasma homocysteine and plasma EC-SOD levels in patients with homocysteinuria [25], but the mechanism responsible for this effect remains unclear. We hypothesized that homocysteine causes degradation of endothelial cell function, followed by detachment of EC-SOD from the endothelium and elevation of plasma EC-SOD. The present study was designed to elucidate the mechanism by which homocysteine increased the plasma EC-SOD level and modulated endothelial cell function.

2. Materials and methods

2.1. Reagents

DL-homocysteine and heparin–bovine serum albumin (BSA) complex were purchased from Sigma (St Louis, MO, USA). Human recombinant EC-SOD (r-EC-SOD), prepared as described previously [20], was kindly provided by Symbicom AB (Umeå, Sweden). Mouse anti-r-EC-SOD monoclonal antibody was prepared as described previously [26]. Alkaline phosphatase-coupled rabbit anti-mouse IgG antibody was purchased from Zymed Laboratories Inc. (San Francisco, CA, USA). Bradford reagent was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). Bovine aortic endothelial (BAE) cells, human aortic endothelial (HAE) cells and CS-C complete medium were purchased from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). Immunoplates were purchased from Nunc (Roskilde, Denmark).

2.2. EC-SOD production of fibroblast cells

Human skin fibroblast cell lines obtained from healthy volunteers were suspended in DMEM containing 10% (v/v) fetal calf serum (FCS) and cultured in 96-well plates, as described previously [27]. When the cells were grown to near confluence, the medium was replaced with fresh DMEM containing 0.5% (v/v) FCS followed by culture for 24 h. After the medium was replaced again, various concentrations of homocysteine were added and followed by incubation in a CO_2 incubator (37°C, 5% CO_2) for 24 h. Conditioned medium was harvested and EC-SOD in the medium was measured by ELISA [28].

2.3. Binding of EC-SOD to endothelial and fibroblast cells

BAE cells and fibroblast cells were suspended in DMEM containing 10% (v/v) FCS and cultured in 96-well plates or 35-mm tissue culture dishes as described previously [27]. HAE cells were cultured in 96-well plates containing CS-C complete medium supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. When endothelial cells and fibroblast cells were near confluent, the media were replaced with fresh DMEM containing 0.5% (v/v) FCS and cultured for 24 h. The medium was replaced again, and various concentrations of homocysteine were added followed by incubation in a CO_2 incubator for 24 h. The cell layers were rinsed with medium, and r-EC-SOD dissolved in the same medium was added followed by incubation at 4°C for 1 h. At the end of the incubation period, the unbound r-EC-SOD was collected and the cells were washed twice with phosphate-buffered

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saline (PBS). The cells were then incubated at 4°C for 30 min with 10 mg/ml of heparin dissolved in the same medium to remove the cell-surface-bound r-EC-SOD, which was then quantified by ELISA [28].

2.4. Binding of EC-SOD to immobilized heparin

Binding of r-EC-SOD to immobilized heparin was performed essentially by the method of Najjam, et al. [29] but with slight modifications. Briefly, immunoplate wells were coated with 100 μl of 50 mM Tris–HCl, pH 7.4, containing 12.7 mM EDTA and 0.4 μg/ml heparin–BSA complex and left to stand overnight at 4°C. Each well was washed three times with PBS containing 0.05% (v/v) Tween 20 (PBS–Tween), blocked with PBS–Tween containing 1% (w/v) BSA, and the plates were then left to stand overnight at 4°C.

A volume of 100 μl of the various concentrations of homocysteine diluted in DMEM containing 0.5% (v/v) FCS was added to each of the wells and the plates were kept for 24 h in a CO_2 incubator. The wells were then rinsed once with DMEM containing 0.5% (v/v) FCS and incubated for 3 h with r-EC-SOD diluted in the same medium. Wells were washed three times with PBS–Tween, and bound EC-SOD was then determined by ELISA [28].

2.5. Statistical analysis

The data shown are the means ± S.D. of four to six separate experiments. Statistical significance was estimated using the Student's *t*-test, and a value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effects of homocysteine on EC-SOD expression in human skin fibroblasts

EC-SOD is expressed in several human cell types, including fibroblast cells [30]. As shown in Fig. 1, EC-SOD release by human skin fibroblasts was significantly inhibited by the treatment with a high concentration of homocysteine (1 mM), but not with low concentrations of reagents (10 and 100 μM).

3.2. Effects of homocysteine on r-EC-SOD binding to the endothelial and fibroblast cells

Previous studies showed that homocysteine inhibited thymidine incorporation into human umbilical vein endothelial cells [12]. In the present study, treatment with homocysteine for 24 h did not show this cytotoxic effect on BAE or HAE cells (data not shown).

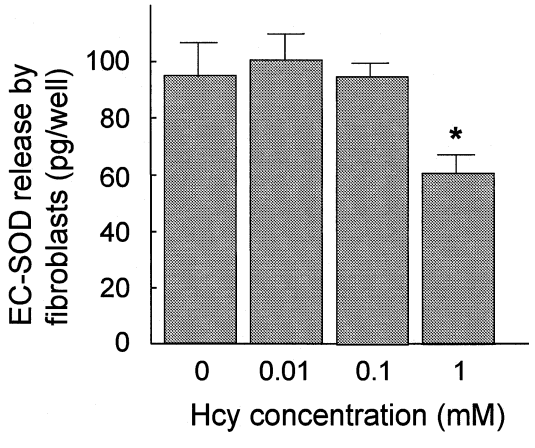

Fig. 1. Effects of homocysteine on EC-SOD expression. Human skin fibroblasts were incubated with various concentrations (0, 0.01, 0.1 and 1 mM) of homocysteine (Hcy) for 24 h. The amounts of EC-SOD in conditioned media were determined by ELISA. Data represent the means ± S.D. of four experiments. *$P < 0.05$ vs. control.

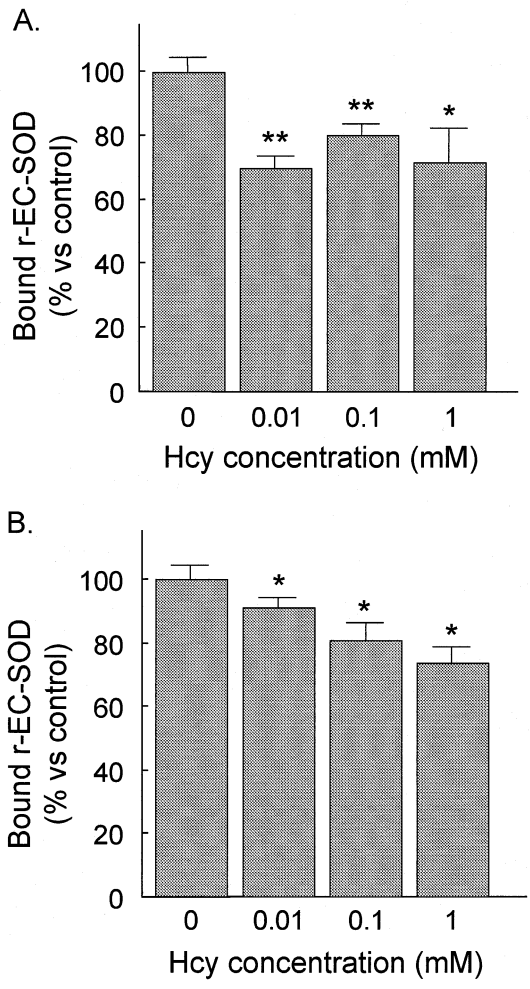

Fig. 2. Inhibition of EC-SOD binding to endothelial cells by homocysteine. HAE (A) and BAE (B) cells were incubated with various concentrations (0, 0.01, 0.1 and 1 mM) of homocysteine (Hcy) for 24 h, followed by addition of r-EC-SOD as described in Section 2. The amounts of bound r-EC-SOD were determined by ELISA and expressed as a percentage of control value. Data represent the means ± S.D. of six experiments. *$P < 0.05$, **$P < 0.001$ vs. control.

It is known that EC-SOD is mainly expressed by fibroblast cells and smooth muscle cells in vascular walls [30–32], and that this enzyme can bind on cultured anchorage-dependent cell lines including endothelial cells [22]. When 2.5 ng/well r-EC-SOD was added to a 96-well plate coated with HAE or BAE cells, the binding of the enzyme to endothelial cells (380 ± 26 ng/mg HAE cell protein and 267 ± 84 ng/mg BAE cell protein) was observed. We next assessed whether homocysteine affects the binding of EC-SOD to the endothelial cells. As shown in Fig. 2, the amount of r-EC-SOD bound to the endothelial cells surface was significantly ($P < 0.001$ for HAE and $P = 0.040$ for BAE cells) decreased by pretreatment with 10 μM homocysteine for 24 h. At 1 mM homocysteine, the binding of r-EC-SOD to HAE and BAE cell surfaces was reduced to 70.9% ($P = 0.0015$) and 73.8% ($P = 0.0017$), of the respective control values. On the other hand, r-EC-SOD slightly bound also on fibroblast cells (68 ± 20 ng/mg fibroblast cell protein). The extent of EC-SOD binding to fibroblasts was significantly ($P < 0.001$) less than that to endothelial cells. The amount of r-EC-SOD bound to the fibroblast

cells tended to decrease by pretreatment with 1 mM homocysteine, but not significantly (84.4% of control, $P = 0.0594$).

It has been reported that homocysteine induces endothelial cell injury due to copper-catalyzed generation of hydrogen peroxide [6,33]. To confirm whether this is due to a decrease of r-EC-SOD-binding to the endothelial cell surface, endothelial cells were incubated with or without copper ions. Addition of copper or homocysteine resulted in significant decreases in r-EC-SOD binding to HAE and BAE cell surfaces (Fig. 3). Furthermore, pretreatment of BAE cells with both homocysteine and copper significantly inhibited r-EC-SOD binding compared to those with homocysteine only ($P = 0.0092$) and copper only ($P = 0.0077$). In HAE cells, its effect was approximately the same as that of homocysteine or copper only.

3.3. *Effects of homocysteine on r-EC-SOD binding to immobilized heparin*

An immobilized heparin–BSA plate was incubated with various concentrations of homocysteine for 24 h. The amount of r-EC-SOD binding to immobilized heparin was significantly and concentration-dependently decreased by pretreatment with homocysteine (Fig. 4). At a concentration of 0.2 mM, the r-EC-SOD-binding was reduced to 84.1% of the control ($P = 0.0136$), and at 1 mM homocysteine, binding was reduced to 50.1% ($P < 0.0001$).

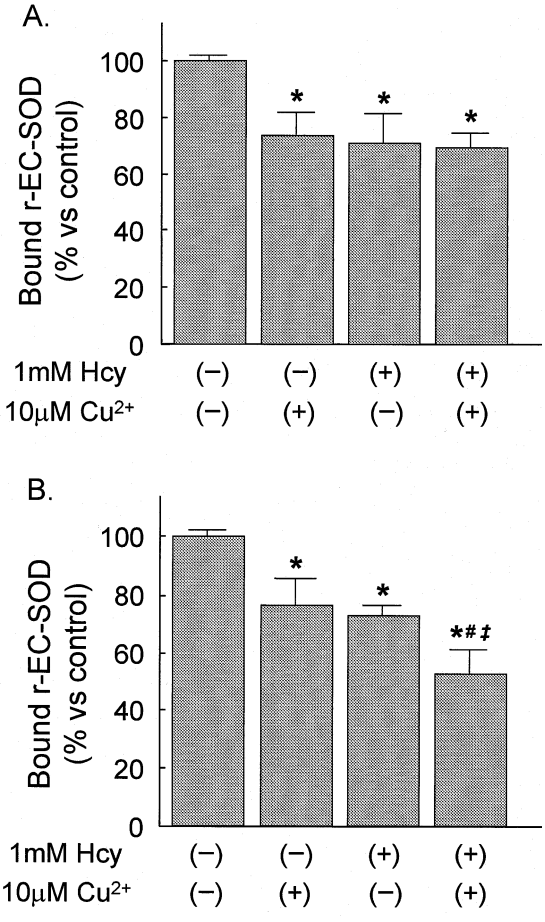

Fig. 3. Effects of copper on homocysteine-induced decrease of EC-SOD binding to endothelial cells. HAE (A) and BAE (B) cells were incubated in the absence or presence of 1 mM of homocysteine (Hcy) or 10 μM of copper ion ($CuCl_2$) for 24 h, followed by addition of r-EC-SOD. The amounts of bound r-EC-SOD were determined by ELISA and expressed as a percentage of control value. Data represent the means ± S.D. of six experiments. $*P < 0.05$ vs. control, $\#P < 0.01$ vs. Hcy (−) and copper ion (+), $\ddagger P < 0.01$ vs. Hcy (+) and copper ion (−).

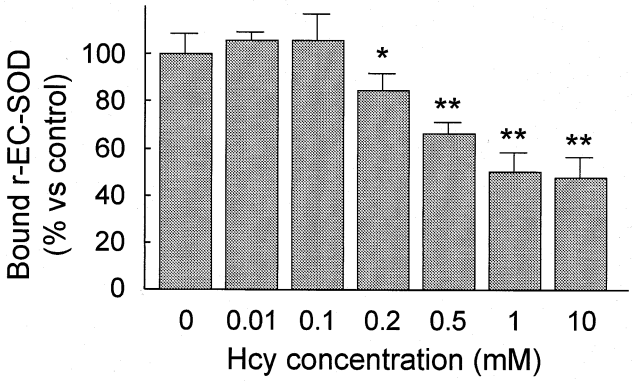

Fig. 4. Inhibition of EC-SOD binding to immobilized heparin by homocysteine. Heparin–BSA-coated plates were incubated with various concentrations of homocysteine (Hcy) for 24 h and r-EC-SOD was then added. The amounts of bound r-EC-SOD were determined as described in Section 2 and expressed as a percentage of control value. Data represent the means ± S.D. of five experiments. $*P < 0.05$, $**P < 0.001$ vs. control.

4. Discussion

It has been reported that homocysteine affects gene expression of antioxidant enzymes and protein in human vascular endothelial cells [16,17]. Recently, we reported that there was a significant correlation between plasma homocysteine and EC-SOD levels [25,34]. Therefore, we examined how homocysteine changes EC-SOD level in plasma. The aim of this study was two-fold: first, to determine the effect of homocysteine on EC-SOD release by fibroblast cells; second, the effect of homocysteine on EC-SOD-binding to endothelial cells. However, EC-SOD release by fibroblasts was not affected with 10 and 100 μM homocysteine, and was inhibited only at high concentration (1 mM). It has been reported that EC-SOD is mainly expressed by fibroblasts and smooth muscle cells and that it is located throughout the vascular wall, with significant amounts observed in all layers, adventitia, surrounding smooth muscle cells and endothelium [30–32]. This enzyme might slowly diffuse in the vascular wall and become distributed according to the affinity of glycosaminoglycans. On the other hand, EC-SOD in the vascular system apparently forms an equilibrium between the plasma phase and endothelial cell surface, and EC-SOD on the endothelial cell surface should provide an efficient protection for vessel walls. There is evidence that elevated plasma homocysteine may cause endothelial cell injury [6,7]. We hypothesized that homocysteine decreased the binding of EC-SOD to endothelial cell surfaces, and this result led to a significant correlation between plasma homocysteine and EC-SOD levels. In this report, we showed that homocysteine-treated endothelial cells lost r-EC-SOD binding activity, and a concentration-dependent decrease was seen in BAE cells. In several studies, the presence of both homocysteine and copper has been shown to cause cell injury, dysfunction, inhibition of cell growth [6,13,17] and mitochondrial damage of endothelial cells [14]. Furthermore, inhibition of antithrombin III binding to the endothelial cell surface by homocysteine has been suggested

to be due to the production of hydrogen peroxide [11]. We also observed the effects of homocysteine and copper on the binding of EC-SOD to BAE cells in this study. However, there were no morphological changes in BAE or HAE cells that were treated with homocysteine for 24 h (data not shown). Therefore, we speculated that homocysteine induces heparan sulfate proteoglycan dysfunction and decreases EC-SOD-binding to endothelial cells. It was found that binding of r-EC-SOD to immobilized heparin is markedly decreased by homocysteine treatment. The detailed mechanism by which homocysteine causes heparin dysfunction is not yet clear.

Nitric oxide (NO) is produced by a variety of cells in the vascular system. NO is produced constitutively and contributes to the modulation of vasomotor tone and to inhibition of platelet and leukocyte aggregation and adhesion to the endothelium, properties that have been shown to be anti-atherogenic. NO reacts extremely rapidly with superoxide to produce peroxynitrite, a potential mediator of oxidant-induced cellular injury. Peroxynitrite is a potent oxidant, capable of oxidizing thiols [35] and DNA bases [36], and of causing tyrosine nitration [37] and initiating lipid peroxidation [38]. It is possible that EC-SOD in the vascular system diffuses into the extracellular space and scavenges superoxide before it reacts with NO. Elimination of EC-SOD from the endothelium may result in the elevation of superoxide, which would then produce highly reactive peroxynitrite. Therefore, our results suggested that homocysteine decreases the ability to scavenge superoxide and indirectly causes injury of the vascular endothelium. Recently, it has been reported that homocysteine increases the superoxide production in porcine aortic endothelial cells [8], and vascular EC-SOD activity is substantially reduced in patients with coronary artery disease [39]. Increased superoxide levels caused by homocysteine and reduced EC-SOD activity may contribute to an imbalance in production and elimination of superoxide, which would result in oxidative stress in endothelial cells.

In conclusion, our study demonstrated that binding of EC-SOD to endothelial cell surfaces was significantly decreased by pretreatment with a low concentration of homocysteine, which might have been due to the dysfunction of heparan sulfate proteoglycan on the cells. Moreover, EC-SOD release by fibroblasts was also decreased by incubation with high concentration of homocysteine. While further studies are needed to explore these mechanisms, these findings suggest that homocysteine may decrease the protection of vascular endothelial cell surfaces from superoxide anions and result in the pathogenesis of atherosclerosis and cardiovascular disease.

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