An Atherogenic Stimulus Homocysteine Inhibits Cofactor Activity of Thrombomodulin and Enhances Thrombomodulin Expression in Human Umbilical Vein Endothelial Cells

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Thrombomodulin plays a role as a cofactor for thrombin-catalyzed activation of protein C on endothelial cells. We examined the effect of homocysteine, a stimulant of atherosclerosis and thrombotic disease, on the cofactor activity and protein level of thrombomodulin and also on the expression of thrombomodulin in endothelial cells. Homocysteine inhibited the cofactor activity of thrombomodulin both on the surface of endothelial cells and in the whole cells dose- and time-dependently, and maximal inhibition of the cofactor activity occurred after a 3- to 6-hour incubation with 10 mmol/L homocysteine (10% of initial activity). Homocysteine also decreased the amount of intact (unreduced) thrombomodulin in endothelial cells. In this study, we examined the effect of homocysteine on the cofactor activity, protein level of thrombomodulin, and expression of thrombomodulin mRNA in endothelial cells.

MATERIALS AND METHODS

Material. Protein C,17 thrombin,18 and antithrombin III19 were purified from human materials. Thrombomodulin was purified from human placenta.20 The M, and absorption coefficient used for the respective proteins were as follows: protein C, 62,000, Acnm = 13.5; thrombin, 37,000, A14,0 nm = 20.0; placental thrombomodulin, 78,000, A280 nm = 14.0.21 Homocysteine, cysteine, and methionine were purchased from Wako Pure Chemical, Osaka. Bovine serum albumin (BSA) was obtained from Sigma Chemical Co (St Louis, MO). Triton X-100 and Tween-20 were purchased from Nacalai Tesque (Kyoto, Japan). Boc-Leu-Ser-Thr-Arg-MCA, a fluorogenic substrate for activated protein C, was obtained from the Protein Research Foundation (Osaka, Japan). A chromogenic substrate for thrombin, H-D-Phe-Pip-Arg-pNA (S-2238) was obtained from Kabi (Stockholm, Sweden). Nitrocellulose membranes for dot immunoblotting and Northern blotting were purchased from Schleicher & Schuell (Dassau, Germany). The ECL Western blot detection kit was obtained from Amersham International plc (Buckinghamshire, UK). Maxisorp II microwell plates were purchased from Nunc (Roskilde, Denmark). Gelatin and peroxidase-conjugated antimonous IgG-goat IgG were purchased from Bio-Rad Laboratories (Richmond, CA). All other chemicals and reagents obtained were of the best grade commercially available.

Cell culture techniques. Human umbilical vein endothelial cells (HUVECs) were prepared by the method of Jaffe et al22 and cultured in modified MCD131 (Chlorrella Industry, Tokyo, Japan).
supplemented with 10% fetal calf serum (GIBCO, Grand Island, NY), 50 µg/mL gentamycin (Flow Laboratories, Irvine, Scotland), 10 ng/mL mouse epidermal growth factor (EGF) (Takara Shuzo, Kyoto, Japan), 10 µg/mL endothelial cell growth supplement (Collaborative Research, MA), and heparin (Sigma) in a 5% CO₂ atmosphere at 37°C using 90-mm diameter collagen-coated Petri dishes or 48-well collagen-coated plate (Sumitomo Bakelite Co, Ltd). HUVECs were passaged using trypsin-EDTA and were used for assay between three or four passages.

Homocysteine treatment to HUVECs. Homocysteine was dissolved in serum-containing medium at the concentration of 250 mmol/L. This solution was sterilized by filtration and added to the medium at the desired concentration (0.25 to 10.0 mmol/L). Media containing homocysteine at various concentrations were added to cell monolayers at 1 to 2 days postconfluence and incubated for various time.

Extraction of thrombomodulin from HUVECs. HUVECs washed three times with serum-free medium, scraped off with a rubber policeman, and washed were collected by centrifugation at 1,800 rpm for 10 minutes. The supernatant was discarded and 1 mL of TBS (0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, pH 7.5) containing 0.5% Triton X-100 was added to the cell pellet. After 1 hour of incubation at room temperature, the cells were centrifuged at 15,000 rpm for 10 minutes and the supernatant was collected.

Determination of cofactor activity of thrombomodulin on the surface of HUVECs. Cofactor activity of thrombomodulin on the surface of HUVECs was assayed for its ability to accelerate the thrombin-catalyzed activation of protein C using a 48-well collagen-coated plate. To HUVECs washed three times with serum-free medium were added 50 µL of TBS containing 0.5% bovine serum albumin (BSA) and 5 mmol/L CaCl₂, 25 µL of thrombin (0.5 µg/mL), and 25 µL of protein C (50 µg/mL). After 1 hour of incubation at 37°C, antithrombin III (5 µg/mL final) and heparin (2 µg/mL final) were added to the culture wells. The amount of activated protein C in 100 µL of the reaction mixture in the well was assayed by incubating with 2 mL of 200 µmol/L Boc-Leu-Ser-Thr-Arg-MCA in a buffer consisting of 0.05 mol/L Tris-HCl and 0.1 mol/L CsCl, pH 8.0. The fluorescence of the released aminomethylcoumarin was determined by using a fluorospectrophotometer with excitation at 380 nm and emission at 440 nm.

Determination of the cofactor activity of thrombomodulin in HUVECs. Cofactor activity of thrombomodulin in extract from HUVECs was assayed as follows. Thirty microliters of TBS containing 0.2% BSA and 10 mmol/L CaCl₂, 10 µL of thrombin (1 µg/mL), and 20 µL of protein C (100 µg/mL) were mixed with 60 µL of extract from HUVECs and incubated at 37°C for 1 hour. The reaction was stopped by the addition of antithrombin III (2.0 µg/mL final) and heparin (1 U/mL final). The amount of activated protein C formed in the incubation mixture was assayed as described before.

Determination of thrombomodulin proteins. (1) Intact ( unreduced) thrombomodulin was determined by enzyme-linked immunosorbent assay (ELISA) using two monoclonal antibodies (MoAbs) as reported previously. The antibody that recognized both the reduced and unreduced form of the fifth EGF-like structure of thrombomodulin was fixed to a microwell plate, and the other antibody that recognized only the unreduced form of the third to the fourth EGF-like structure of thrombomodulin was used as a peroxidase-conjugated antibody.

(2) The amount of total protein of thrombomodulin was determined by a Dot-Blot system (Bio-Rad) using an MoAb recognizing both reduced and unreduced thrombomodulin. Twenty microliters of the endothelial cell extract was fixed to a nitrocellulose filter using Dot-Blot apparatus (Bio-Rad). The filter was blocked with TBS containing 3% gelatin and 1% BSA for 2 hours, and washed three times with TBS containing 3% Tween-20 to prevent nonspecific binding of the antibody. Then the filter was treated with an MoAb that recognized both reduced and unreduced thrombomodulin. After washing with TBS containing 3% Tween-20 three times, the filter was treated with peroxidase-conjugated antigoat IgG goat IgG and incubated for 2 hours. After washing with the same buffer three times, thrombomodulin on the filter was quantitated using an ECL Western blotting detection kit and densitometer (LKB Ultrascan).

Effect of homocysteine on cofactor activity of thrombomodulin fixed to microwell plate. Wells of microwell plates were coated with 100 µL of thrombomodulin (3 µg/mL) in 0.1 mol/L sodium bicarbonate buffer, pH 9.2, for 2 hours. To the wells blocked with 150 µL of 10% BSA in TBS for 1 hour was added 100 µL of various concentrations of homocysteine solution diluted appropriately with TBS containing 0.5% BSA. The plates were then incubated for 2 hours. After three washings with TBS containing 0.5% BSA, 50 µL of TBS containing 0.5% BSA and 5 mmol/L CaCl₂, 25 µL of thrombin (0.5 µg/mL), and 25 µL of protein C (50 µg/mL) were added to the wells, which were then incubated at 37°C for 90 minutes. The reaction was stopped by the addition of antithrombin III (5 µg/mL final) and heparin (2 U/mL final). The amount of activated protein C formed in 100 µL reaction mixture was assayed using Boc-Leu-Ser-Thr-Arg-MCA as described before.

Effect of homocysteine on thrombin binding to thrombomodulin fixed to microwell plate. Wells of microwell plates were coated with 100 µL of thrombomodulin (3 µg/mL) in 0.1 mol/L sodium bicarbonate buffer, pH 9.2, for 2 hours. To the wells blocked with 150 µL of TBS containing 10% BSA for 1 hour was added 100 µL of various concentrations of homocysteine diluted appropriately with TBS containing 0.5% BSA. The plates were then incubated for 2 hours. After three washings with TBS containing 0.5% BSA, 100 µL of thrombin in TBS containing 0.5% BSA was added to each well. The plates were then incubated for 1 hour. After three washings with the same buffer, 100 µL of a 200 µmol/L peptide substrate S-2238 solution was added to the wells, which were then incubated at 37°C for 4 hours. The absorbance at 410 nm of each well was determined by a Dynatech MR-600 Microplate Reader.

Effect of homocysteine on expression of thrombomodulin mRNA in HUVECs. After three washes with serum-free medium, the cells were harvested by a rubber policeman with washing. Total RNA was isolated from cell pellets by the cesium chloride method. Slot blot was performed using a slot-blot apparatus (Bio-Rad). Five or 10 µg of formamide denatured RNA per lane was spotted onto a nitrocellulose filter. Then the filter was dried and hybridized with a random prime 32P-labeled cDNA probe for thrombomodulin or β-actin as a control. After hybridization, the radioactivity of each band was quantitated by a bio-image analyzer (Fuji Film). Thrombomodulin mRNA levels were normalized to the concentration of β-actin mRNA.

RESULTS

Effect of homocysteine on protein C-activating cofactor activity of thrombomodulin on the surface of HUVECs. By the treatment of the cells with various concentrations of homocysteine for 6 hours, the cofactor activity of thrombomodulin on the surface of the cells was inhibited dose-dependently (Fig 1). Only 5% of the initial activity remained by incubating with 10 mmol/L homocysteine. When the cells were incubated with 10 mmol/L homocysteine, the cofactor activity of thrombomodulin on the cells was decreased time-dependently to approximately 5% of the initial activity after 6 hours of incubation (data not shown).
Effect of homocysteine on cofactor activity of thrombomodulin in HUVECs. In the cells, treated with various concentration of homocysteine, the cofactor activity of thrombomodulin was inhibited dose-dependently and maximal inhibition was observed at 10 mmol/L homocysteine (10% of initial activity). In addition, this inhibition curve was quite similar to that of cofactor activity of thrombomodulin on the cells by homocysteine treatment (Fig 2A). Incubation with 10 mmol/L homocysteine at 37°C showed maximal inhibition at 3 hours of incubation (10% of initial activity), and the inhibition was reduced to show 60% of the initial activity after 24 hours of incubation (Fig 2B). In the cells incubated with cysteine or methionine instead of homocysteine, the cofactor activity of thrombomodulin was not reduced (data not shown).

Effect of homocysteine on intact thrombomodulin level in HUVECs. The effect of homocysteine on the amount of intact (unreduced) thrombomodulin in the cells was determined using ELISA specific for the unreduced form of thrombomodulin. Homocysteine dose-dependently decreased the level of the intact form of thrombomodulin in the cells, and at 10 mmol/L homocysteine the level was decreased to 20% of the initial value after 6 hours of incubation (Fig 3A). In the cells treated with 10 mmol/L homocysteine, the level of the intact form of thrombomodulin showed the greatest decrease after 6 hours of incubation (20% of the initial value), and gradually increased to 180% of the initial value after 24 hours of incubation (Fig 3B).

Effect of homocysteine on total thrombomodulin level in HUVECs. To determine the effect of homocysteine on the total protein level of thrombomodulin, in both reduced and unreduced forms, in the cells, the same samples used in the examination in Fig 3 were subjected to dot immunoblot analysis using an MoAb recognizing both reduced and unreduced thrombomodulin. Unlike the case of intact (unreduced) form of thrombomodulin, total protein level of thrombomodulin was not decreased markedly (Fig 4A). In the cells treated with 10 mmol/L homocysteine, the level decreased after 3 hours of incubation (70% of the initial value), and then gradually increased to 165% of the initial value after 24 hours of incubation (Fig 4B). When the other MoAb that recognized only intact (unreduced) thrombomodulin was used as the first antibody on dot immunoblot analysis, the level of the total protein level of thrombomodulin was not decreased markedly (Fig 4C).
HOMOCYSTEINE INHIBITS THROMBOMODULIN

Fig 3. Dose-response (A) and time-course (B) curves showing homocysteine-induced decrease of intact thrombomodulin level in HUVECs. (A) After incubation of the cells with various concentrations of homocysteine for 6 hours, the intact (unreduced) thrombomodulin level was determined by ELISA using two MoAbs, as described in Materials and Methods. (B) After incubation of the cells with 10 mmol/L homocysteine for various lengths of time, intact thrombomodulin level was determined by ELISA.

Analysis, the level of thrombomodulin was decreased to 30% of initial value after 3 to 6 hours of incubation (data not shown).

Effect of homocysteine on cofactor activity of thrombomodulin fixed to microwell plate. To determine the effect of homocysteine on the cofactor activity of thrombomodulin in a cell-free system, purified thrombomodulin fixed to a microwell plate was treated with homocysteine at various concentrations. As shown in Fig 5, cofactor activity of fixed thrombomodulin was also inhibited by homocysteine treatment in a dose-dependent manner. By treatment with 10 mmol/L homocysteine, the activity of thrombomodulin was

Fig 4. Dose-response (A) and time-course (B) curves of total thrombomodulin level in HUVECs treated with homocysteine. (A) After incubation of the cells with various concentrations of homocysteine for 6 hours, the total thrombomodulin level was determined by dot blot using an MoAb that recognized both intact and reduced thrombomodulin, as described in Materials and Methods. (B) After incubation of the cells with 10 mmol/L homocysteine for various lengths of time, the total thrombomodulin level was determined by dot immunoblotting.
reduced to 5% of the initial activity. Half maximum inhibition concentration of homocysteine was approximately 1.6 mmol/L. The activity was not reduced in fixed thrombomodulin treated with methionine and cysteine (data not shown).

**Effect of homocysteine on the binding ability of thrombomodulin fixed to microwell plate with thrombin.** To determine whether the ability of thrombomodulin to bind with thrombin is affected by homocysteine, fixed thrombomodulin was treated beforehand with various concentrations of homocysteine and then the binding ability of thrombin to the thrombomodulin was determined. As shown in Fig 6, thrombin binding to fixed thrombomodulin was inhibited dose-dependently. By treatment with 10 mmol/L homocysteine, the binding ability of thrombin to thrombomodulin was reduced to approximately 23% of the initial value. In this case, half maximum inhibition concentration of homocysteine was approximately 2.2 mmol/L.

**Effect of homocysteine on expression of thrombomodulin mRNA in HUVECs.** The level of thrombomodulin mRNA in the cells was determined after treatment with varying concentrations of homocysteine in comparison with the level of β-actin mRNA. As shown in Fig 7A, the ratio of the amount of thrombomodulin mRNA to that of β-actin mRNA appeared to increase in proportion to the concentration of homocysteine. Even if the amount of β-actin mRNA was ignored, the amount of thrombomodulin mRNA in the cells significantly increased during the homocysteine treatment. When treated with 10 mmol/L homocysteine, the level of thrombomodulin mRNA appeared to increase to twofold to threefold after 6 hours of incubation (Fig 7B). Even after 24 hours of incubation, thrombomodulin mRNA level appeared to be kept at the level of twofold of the initial value.

**DISCUSSION**

Recently, Rodgers and Conn reported that an atherogenic stimulus, homocysteine, inhibited thrombin-catalyzed protein C activation on HUVECs, and suggested that this inhibition is mainly caused by a decrease in the Km of thrombin on the cells for protein C. In the present study, we examined the effect of homocysteine on the protein C-activating cofactor activity and protein level of thrombomodulin and also on the expression of its mRNA in HUVECs to elucidate the mechanism of homocysteine inhibition against the protein C-activating cofactor activity on the cells. The level of homocysteine and/or homocysteine in plasma of homozygous patients with homocystinuria was reported to be 0.01 to 0.24 mmol/L. However, in the present study we used a much higher concentration of homocysteine (this high concentration was examined in the study of Rodgers and Conn) because HUVECs used probably have normal levels of cystathionine β-synthase and we would also like to know the critical effect of homocysteine on thrombomodulin in the cells and on the purified thrombomodulin. Microscopic observation did not show any significant cell detachment or cell morphologic change after homocysteine treatment. We also measured the level of soluble thrombomodulin in the cultured medium to determine whether cellular thrombomodulin was released from the HUVECs by the treatment with homocysteine by using ELISA, consisting of polyclonal antibody, which could detect both reduced and unreduced thrombomodulin. However, the level of thrombomodulin in the culture medium did not change during the homocysteine treatment (data not shown). This means that homocysteine did not stimulate the release of thrombomodulin from the cells. Then we determined the cofactor activity of thrombomodulin on the surface of HUVECs and also that of the extract of whole cells after
Homocysteine inhibits thrombomodulin.

Fig 7. Effect of homocysteine on expression of thrombomodulin mRNA in HUVECs. (A) Dose-response curve of the ratio of thrombomodulin mRNA/β-actin mRNA in the cells treated with homocysteine. (B) Time-course curve of the ratio of thrombomodulin mRNA/β-actin mRNA in the cells treated with 10 mmol/L homocysteine. Details are described in Materials and Methods.

Treatment with homocysteine. The cofactor activity of thrombomodulin both on the surface of the cells and in whole cells was decreased in the same manner in proportion to the concentration of homocysteine, although these phenomena were not observed by the treatment with cysteine or methionine. Therefore, in the following experiments we used the cell extract as a source for thrombomodulin. In HUVECs treated with 10 mmol/L homocysteine, the cofactor activity of thrombomodulin was decreased maximally after a 3- to 6-hour incubation (10% of the initial activity), but 60% of the initial activity was recovered after 24 hours of incubation. Similar phenomena were observed in the level of the intact (unreduced) form of thrombomodulin when determined using ELISA specific for unreduced thrombomodulin. However, after 24 hours of incubation at 10 mmol/L homocysteine, the unreduced form of thrombomodulin showed an obviously higher level than the level of the cofactor activity. This discrepancy between the protein level and the activity level may be caused by the synthesis of the functionally abnormal thrombomodulin in the cells treated with homocysteine, eg, abnormal glycosylation, incomplete folding of the EGF-like structures, or other abnormal processing in the formation of mature thrombomodulin. Precise chemical analysis of thrombomodulin synthesized in HUVECs after 24-hour incubation with homocysteine is now under investigation.

On the other hand, the level of total protein of thrombomodulin (both reduced and unreduced form) appeared to be decreased slightly in comparison with that of unreduced form of thrombomodulin. Thus, we assumed that homocysteine inhibited the cofactor activity of thrombomodulin on the cells by reduction, but did not essentially decrease the protein level of thrombomodulin. To confirm this assumption, we examined the effect of homocysteine on thrombomodulin in a cell-free system using microwell plates. The cofactor activity and thrombin-binding ability of thrombomodulin fixed to microwell plate were also inhibited by homocysteine treatment dose-dependently, and these inhibition behaviors were similar to those of the cofactor activity on the cells. This means that homocysteine immediately modifies the thrombomodulin molecule by chemical reduction or replacement with half-cystine residues.

The cofactor activity of thrombomodulin is stable against acidic or alkaline pH treatment and detergents such as urea or sodium dodecyl sulfate, but is very unstable against treatments with reducing reagents such as β-mercaptoethanol and dithiothreitol. We reported previously that thrombin binds to the fifth EGF-like structure of thrombomodulin and protein C probably to the fourth EGF-like structure. These EGF-like structures were easily reduced by reducing agents and reduced thrombomodulin no longer showed the cofactor activity. Like β-mercaptoethanol, homocysteine must reduce or replace half-cystines of the EGF-like structures of thrombomodulin to inactivate the cofactor activity.

On the other hand, homocysteine stimulated the expression of thrombomodulin mRNA in HUVECs. The increase of this mRNA appeared to be responsible for the increased synthesis of thrombomodulin. The precise mechanism of stimulation of mRNA synthesis by homocysteine in the cells is unknown. The destruction of thrombomodulin and the resulting decrease of antithrombotic properties of the cells presumably induce the expression of thrombomodulin mRNA by some unknown mechanisms. The present findings also suggest that HUVECs are capable of self-restoration, which is important in the expression of anticoagulant activity on the cells. This is the first report that a simple amino acid influences thrombomodulin expression.
REFERENCES


