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Enhancement by Homocysteine of Plasminogen Activator Inhibitor-1 Gene Expression and Secretion from Vascular Endothelial and Smooth Muscle Cells

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In order to elucidate the relationship between homocysteine and the fibrinolytic system, we examined the effect of homocysteine on plasminogen activator inhibitor-1 (PAI-1) and tissue-type plasminogen activator (tPA) gene expression and protein secretion in cultured human vascular endothelial and smooth muscle cells *in vitro.* PAI-1 mRNA and secreted protein levels were both enhanced by homocysteine in a dose dependent manner, with significant stimulation of PAI-1 secretion observed at concentrations greater than 0.5 mM homocysteine. In contrast, secretion and mRNA expression of tPA were not significantly altered by homocysteine stimulation. Secretion of TGFβ (transforming growth factor β) and TNFα (tumor necrosis factor α), possible regulators of PAI-1 expression and secretion, were not stimulated by treatment with 1.0 mM homocysteine. These results suggests that hyperhomocysteinemia-induced atherosclerosis and/or thrombosis may be caused by homocysteine-induced stimulation of PAI-1 gene expression and secretion in the vasculatures by a mechanism independent from paracrine-autocrine activity of TGFβ and TNFα.

Key Words: **homocysteine; atherosclerosis; PAI-1; tPA; vascular endothelial cells; vascular smooth muscle cells; TGFβ; TNFα.**

Homocysteine, a sulfur-containing amino acid, is an intermediate of methionine metabolism and can be further catalyzed to cystathionine by cystathionine β-synthase, or remethylated by methylene tetrahydrofolate reductase (MTHFR). Patients homozygous for hereditary homocystinuria, a cystathionine β-synthase deficiency, exhibits extreme hyperhomocysteinemia which causes premature atherosclerosis and thrombosis. Recently, even mild or moderate hyperhomocysteinemia (0.01–0.1 mM) caused by vitamin deficiencies, drugs, cystathionine β-synthase deficiency heterozygosity or mutation of the MTHFR gene (C677T) has been reported to be an independent risk factor for atherosclerosis (1–6), and mild hyperhomocysteinemia has been found in 20–30% of patients with coronary and peripheral vascular disease (5–7). Hyperhomocysteinemia-induced atherosclerosis and/or thrombosis has been reported to be due to endothelial cell injury, platelet dysfunction, activation of the coagulation, stimulation of vascular smooth muscle cell growth or enhanced LDL deposition in the arterial wall (5–13).

PAI-1, a key regulator for fibrinolysis, is a 50-kDa glycoprotein of the serine protease inhibitor family (14) and is secreted by endothelial cells, hepatocytes, platelets and smooth muscle cells. Elevated plasma PAI-1 concentrations have been reported in individuals with angina pectoris, myocardial infarction, deep vein thrombosis, diabetes and insulin resistance (15–19), suggesting that PAI-1 may be significant in atherosclerosis and thrombosis. Direct effects of homocysteine on fibrinolytic system in the vasculatures remain to be clarified. T. Bienvenu *et al.* demonstrated that PAI-1 levels were correlated with plasma homocysteine concentration (21). Tissue type plasminogen activator secreted from endothelial cells, hepatocytes, platelets or smooth muscle cells, another regulator of fibrinolytic system, is elevated in patients in the acute phase of thrombosis. Van den Berg and colleagues showed that plasma concentration of tPA was not elevated in patients with hyperhomocysteinemia, in contrast to von Willebrand factor (vWF) and thrombomodulin (TM) that were elevated (20). Thus, it is possible that homocysteine may enhance secretion of PAI-1, but not that of tPA, in the vasculature, which may lead to abnormal fibrinolysis and thrombosis. However direct evidence

Abbreviations used: PAI-1, plasminogen activator inhibitor-1; tPA, tissue type plasminogen activator; TGFβ, transforming growth factor β; TNFα, tumor necrosis factor α; MTHFR, methylene tetrahydro-folate reductase; HUVEC, human umbilical vascular endothelial cells; HASMC, human aorta smooth muscle cells.

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and mechanisms of homocysteine regulation of that components of the fibrinolytic system in the vascular cells have not been reported.

In the present study, we investigated the effects of homocysteine treatment on secretion and mRNA expression of PAI-1 and tPA in cultured human endothelial and smooth muscle cells *in vitro.*

MATERIALS AND METHODS

Cell culture. Human umbilical vein endothelial cells (HUVEC), (KURABO Biomedical Business, Japan) were grown on fibronectin coated flasks (Falcon) in modified MCDB (Medium Complete with Trace Elements) 131 medium (KURABO Biomedical Business, Japan) supplemented with 2% fetal calf serum (FCS) (KURABO Biomedical Business, Japan), gentamicin (0.05 mg/ml), amphotericin B (0.05 mg/ml), endothelial cell growth factor (10 ng/ml) (KURABO Biomedical Business, Japan), hydrocortisone (1 g/ml), and heparin (10 g/ml). Human aorta smooth muscle cells (HASMC), (KURABO Biomedical Business, Japan) were grown in DMEM (Dulbecco's Modified Eagle's Medium) medium supplemented 5% FCS (KURABO Biomedical Business, Japan), gentamicin (0.05 mg/ml), amphotericin B (0.05 mg/ml), endothelial cell growth factor (0.5 ng/ml) (KURABO Biomedical Business, Japan), fibroblast growth factor-B (2 ng/ml) (KURABO Biomedical Business, Japan), and insulin (5 g/ml). Cells in the 5th-8th passage were used throughout the experiments. Cells were grown to near confluence prior to treatment with homocysteine. Cells were first washed twice with PBS and the medium replaced with fresh culture medium containing 2 or 0.4% FCS without EGF, hydrocortisone, FGF and insulin and incubated for 24 h. Homocysteine treatment was then performed at concentrations and durations indicated in the following section.

Measurement of PAI-1, tPA, TGFβ and TNFα. Cell culture-conditioned media were collected after 24 h culture periods by centrifugation at 1000 rpm, for 10 min to remove cell debrises, and then stored at −20°C until assayed. PAI-1 and tPA antigen concentrations were determined with a commercially available ELISA (TintElize PAI-1, TintElize tPA, respectively, Biopool, Umea, Sweden) using a double antibody technique in accordance with the manufacturer's instructions, as previously described (23). TGFβ and TNFα concentrations were measured with commercially available ELISA kits (TGFβ-1 KIT, TNFα- KIT, respectively, Genzyme Diagnostics, Cambridge, MA).

Northern blot analysis. Total cellular RNA was prepared from confluent cultures of HUVEC or HASMC by the acid-guanidium-phenol-chloroform (AGPC) method (Isogen, Nippon Gene Co., Japan). For Northern blot analysis, 10 μg of total RNA was separated on a 1.2% agarose gel containing 20 mM MOPS, 5 mM sodium acetate (pH 7.0), 1 mM EDTA, 0.76 g/ml ethidium bromide, and 0.67% formaldehyde, transferred to nylon filters, and then cross-linked by ultraviolet radiation. The membranes were prewashed in 5SSPE, 50% formamide, 5× Denhard's solution, 0.5% SDS containing heat-denatured salmon sperm DNA (500 g/ml) for 1 h at 65°C. Blots were hybridized for 24 h at 50°C with cDNA probes specific for PAI-1, tPA, and GAPDH labeled with [γ32P]ATP using T4 polynucleotide kinase. cDNA probes for PAI-1, tPA, GAPDH mRNA consisted 40 base-pair single-stranded synthetic oligonucleotide containing antisense orientation sequence derived from the N-terminal regions of translated sequences (Oncogene Research Products, Cambridge, MA) (24–26).

Statistical analysis. Results were expressed as the mean ± SE from 6 wells. Statistical analysis was performed using ANOVA test and significance was accepted at $P < 0.05$.

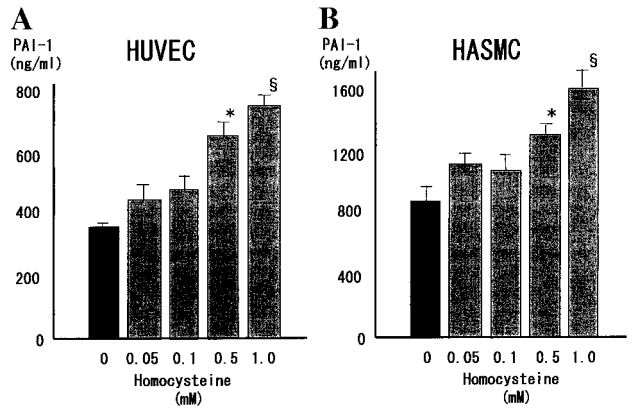

FIG. 1. The effect of homocysteine on the secretion of PAI-1 from HUVEC (A) and HASMC (B). Data represent the mean ± SE of six determinations. *$P < 0.05$ vs control, §$P < 0.01$ vs control.

RESULTS

Effect of Homocysteine on the Secretion of PAI-1 and tPA Antigen in the Culture Medium

Basal secretion levels of PAI-1 were found to be 380.5 ± 14.1 ng/ml from HUVEC and 854.5 ± 87.8 ng/ml from HASMC. Incubation for 24 h with varying concentrations of homocysteine stimulated the secretion of PAI-1 in a dose dependent manner, which reaches to two-fold increase at 1.0 mM, 782.4 ± 29.3 ng/ml from HUVEC and 1602.5 ± 172.4 ng/ml from HASMC (Figs. 1A and 1B). In contrast, basal secretion levels of tPA were 7.7 ± 1.4 ng/ml from HUVEC and 4.9 ± 1.4 ng/ml from HASMC, and secretion levels did not change after incubation with 1.0 mM homocysteine (Figs. 2A and 2B).

Measurement of TGFβ and TNFα in the Condition Medium

The concentrations of TGFβ in the culture medium was 0.28 ± 0.09 ng/ml in HUVEC and 0.48 ± 0.04 ng/ml in HASMC, and did not increase after incubation with 1.0 mM homocysteine (data not shown). Likewise, TNFα concentration in the culture medium was 1.7 ± 0.72 pg/ml in HUVEC and 4.9 ± 0.81 pg/ml, and also unaffected by stimulation with homocysteine (data not shown).

Effect of Homocysteine on mRNA Expression of PAI-1 and tPA in HUVEC and MASMC

As previously reported, two forms of PAI-1 mRNA have been detected. Homocysteine stimulation of cultured HUVEC for 24 h increased PAI-1 mRNA in a dose dependent manner as assessed by Northern blot analysis (Figs. 3A and 3B). In HASMC, homocysteine increased the expression of PAI-1 mRNA similar to HUVEC. Increased PAI-1 mRNA expression was found

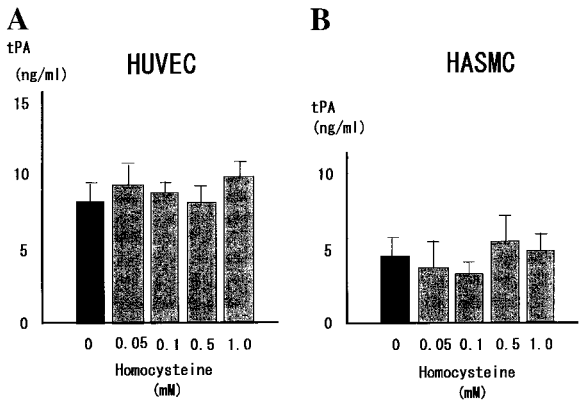

FIG. 2. The effect of homocysteine on the secretion of tPA from HUVEC (A) and HASMC (B). Data represent the mean ± SE of six determinations.

with more than 0.5 mM homocysteine same as the secretion of PAI-1. In contrast, t-PA mRNA expression was scarcely detectable and remained unchanged by treatment with 1.0 mM homocysteine (data not shown).

DISCUSSION

The present study has demonstrated that homocysteine-stimulated PAI-1 mRNA expression and secretion in human vascular endothelial and smooth muscle cells. The secretion of PAI-1 antigen and PAI-1 mRNA expression were stimulated at the same concentration of homocysteine, it is considered that homocysteine increased the production of PAI-1 by enhancing transcription. Recently, it was reported that plasma PAI-1 antigen levels were correlated with plasma homocysteine concentration (21–22), and that homocysteine decreased tissue plasminogen activator receptor activity (27). Our data present evidence that hyperhomocysteinemia impairs fibrinolytic system by directly affecting PAI-1 gene expression in vascular endothelial and/or smooth muscle cells.

Our data also revealed that homocysteine treatment did not affect tPA mRNA expression and secretion in HUVEC or HASMC. Hajjar reported that homocysteine treatment reduced the tPA binding sites in endothelial cells without changing the binding capacity of Lys-plasminogen (27). Thus, it is possible that homocysteine may reduce fibrinolytic activity by reduction of tPA binding to the endothelial cells, independent of tPA expression.

Other mechanisms for homocysteine-induced thrombosis have been proposed based on *in vitro* studies; including inhibition of the protein C anticoagulant pathway, competitive interference with heparan sulfate proteoglycans binding, inhibition of tPA receptor function, decreased endothelial ecto-ATPase activity and enhancement of fibrin binding to tissue factor. Therefore, homocysteine-induced enhanced of PAI-1 secretion and decreased tPA activity in combination predispose thrombosis in patients with hyperhomocysteinemia.

Regulatory factors of PAI-1 expression and secretion such as TGFβ, dexamethasone, LPS, angiotensin II and TNFα have been the focus of recent studies. For instance, TGFβ is a key regulator of atherosclerosis and TNFα is associated with insulin resistance. It has been hypothesized that stimulation of TGFβ and/or TNFα by homocysteine may induce over-expression of the PAI-1 gene and hypersecretion of PAI-1 protein. However, our study demonstrated that homocysteine did not affect TGFβ and TNFα secretion. Thus it is conceivable that the increase in PAI-1 secretion by homocysteine stimulation is not due to TGFβ and/or TNFα-mediated oversecretion, however, the mechanisms of PAI-1 gene regulation remain to be clarified.

In conclusion, the present study showed that homocysteine directly enhanced PAI-1 gene expression and antigen secretion in the vascular cells. Results suggested that the hypersecretion of PAI-1 might be one of the mechanisms underlying impaired fibrinolysis and tendency for thrombosis observed in patients with hyperhomocysteinemia.

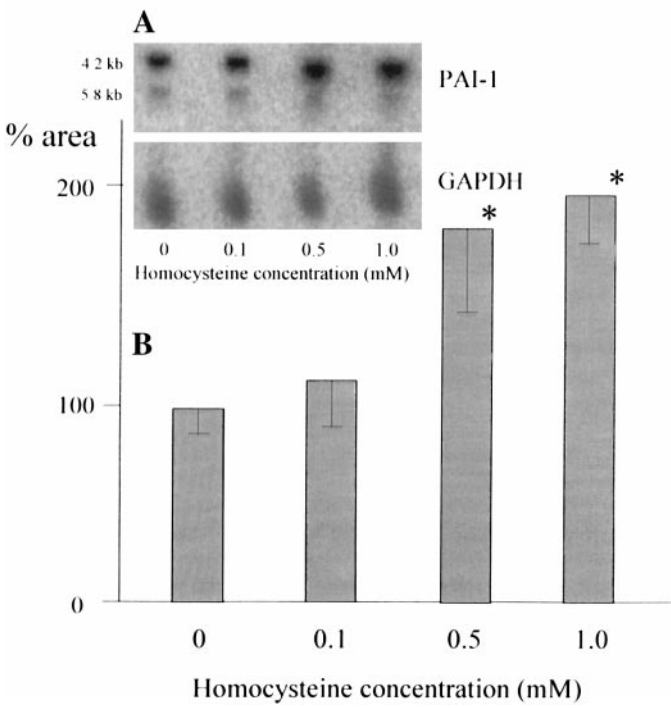

FIG. 3. The result of Northern blot analysis of PAI-1 mRNA expression in HUVEC. Northern blot pattern of PAI-1 mRNA is shown in the upper panel (A). Densitometric quantitation of the two PAI-1 mRNA bands is demonstrated in the lower panel ($n = 5$) (B). $^*P < 0.05$ vs control.

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