Inhibition of Endothelial Cell Thromboresistance by Homocysteine

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ABSTRACT  Homocysteine (HC) is a highly reactive thiol intermediate in amino acid metabolism, which can modify the function of endothelial cells in a myriad of ways. In vitro, homocysteine can inhibit the thromboresistance properties of the endothelial cell by induction of procoagulant factors, inactivation of natural anticoagulant systems, and suppression of vasodilatory and platelet-modulating factors. HC also inhibits the fibrinolytic system by impairing the ability of the endothelial cell to bind tissue plasminogen activator (t-PA), by interacting directly with the t-PA binding “tail” domain of its endothelial cell receptor, annexin II. Moreover, HC influences endothelial cell gene expression as exemplified by induction of the elongation factor-1 family of polypeptides, which promote polypeptide chain elongation during mRNA translation. Induction of EF-1 subunits α, β, γ and δ by homocysteine is associated with increased turnover of at least one free thiol-containing protein, suggesting that up-regulation of these subunits may represent a mechanism for replacement of damaged or modified proteins. A more complete understanding of the diverse effects of homocysteine on endothelial cell function may provide important clues to the precise role homocysteine may play in the initiation and progression of vascular disease. J. Nutr. 130: 373S–376S, 2000.

KEY WORDS: • homocysteine elongation factors • endothelial cell • tissue plasminogen activator • plasmin

Homocysteine impairs endothelial cell thromboresistance

Endothelial cells line the vasculature and function to maintain the fluidity of blood. Several studies indicate that exposure of cultured endothelial cells to micromolar or millimolar concentrations of homocysteine (HC) poses a threat to this system (Table 1). For example, there is an association between exogenously applied HC and inhibition of prostacyclin synthesis (Wang et al. 1993), activation of procoagulant factor V (Rodgers and Kane 1986), impaired activation of the natural anticoagulant protein C (Rodgers and Conn 1990) and down-regulation of cell surface thrombomodulin (Hayashi et al. 1992, Lentz and Sadler 1991). Additional studies have shown impaired regulation of nitric oxide (Stamler et al. 1993), induction of tissue factor (Fryer et al. 1993) and suppression of heparin sulfate expression (Nishinaga et al. 1993). Many of these studies, however, employed supraphysiologic levels of HC; thus, they must be interpreted with caution. In one salient in vivo study, monkeys made mildly hyperhomocysteinemic (10 μmol/L) by dietary manipulation showed abnormal vasoconstriction and reduced thrombomodulin activity in the aorta (Lentz et al. 1996). The mechanisms by which HC may exert these diverse effects are largely unclear, as is the question of whether functional modifications observed in vitro also apply in vivo.

Homocysteine inhibits endothelial cell surface fibrinolytic assembly

The fibrinolytic system represents the principal means by which unnecessary fibrin is eliminated from the body (Hajjar 1998). This system consists of a cascade of proteolytic reactions oriented largely on fibrin-containing surfaces, i.e., thrombi, or on the surfaces of cells. Plasminogen is a 93-kDa glycoprotein that circulates in plasma at a concentration of ~1.5 μmol/L. Itsself inactive, plasminogen is converted to the functional serine protease plasmin upon cleavage of a single peptide bond at position 560–561 (Arg-Val). This hydrolysis can be carried out by either of two major plasminogen activators, tissue plasminogen activator (t-PA), a product of some endothelial cells, or urokinase (u-PA), a product of renal epithelial cells. Fibrin, the principal substrate of plasmin, is also the most potent known cofactor for t-PA-dependent activation of plasminogen, accelerating its catalytic efficiency ~500-fold. Further regulation of the system is provided by an array of circulating inhibitors such as plasminogen activator inhibitor-1 or α2-antiplasmin, which immediately neutralize freely circulating t-PA and u-PA or plasmin, respectively. Fibrinolytic activity is localized to cell surfaces through the interaction of plasminogen and its activators with specific cell surface receptors.

Annexin II is a widely distributed, highly conserved, 36-kDa peripheral membrane protein expressed abundantly on endothelial cells, macrophages, myeloid cells and some tumor

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3 Abbreviations used: EF, elongation factor; HC, homocysteine; t-PA, tissue plasminogen activator; u-PA, urokinase plasminogen activator; u-PA, urokinase plasminogen activator.
All 20 known annexins consist of a conserved "core" domain (30–40 kDa), which imparts phospholipid-binding capacity, and a variable amino terminal "tail" domain (3–6 kDa) through which specialized functions are acquired (Raynal and Pollard 1994, Swairjo and Seaton 1994). The human annexin II gene consists of 13 exons distributed over 40 kb of genomic DNA on chromosome 15 (15q21) (Spano et al. 1990).

Annexin II possesses the unique property of binding both plasminogen ($K_d = 5.114$ nmol/L) and tPA ($K_d = 30$ nmol/L) (Hajjar and Hamel 1990, Hajjar 1991, Hajjar et al. 1994), thereby stimulating the catalytic efficiency of t-PA–dependent plasminogen activation 60-fold (Cesarman et al. 1994) (Fig. 1). This "fibrin-like" cofactor activity is inhibited completely in the presence of lysine analogs or upon removal of its basic carboxyl-terminal amino acids. Although it lacks a classical signal peptide, annexin II is translocated constitutively to the endothelial cell surface within 16 h of its biosynthesis. It binds to cell surface phospholipid via core repeat 2 containing the linear sequence KGLGT and downstream aspartate residue (Asp161) (Hajjar et al. 1996). Annexin II heterotetramer, composed of two annexin monomers and two p11 subunits, may have even greater stimulatory effects on t-PA–dependent plasmin generation (Kassam et al. 1998).

HC appears to impair profoundly the endothelial cell's ability to generate plasmin in vitro (Hajjar and Jacovina 1998). Treatment of cultured endothelial cells with DL-homocysteine is associated with a 65% reduction in functional binding sites for t-PA, whereas no effect on plasminogen binding is observed (Hajjar et al. 1994). This finding was associated with a 60% reduction in cell-associated t-PA activity even though the intrinsic catalytic capability of t-PA is not altered by HC. Binding of t-PA, but not plasminogen, to annexin II monomer is also markedly reduced by ligand blot analysis. These studies suggest that HC might perturb the intrinsic fibrinolytic potential of the endothelial cell by selectively blocking the t-PA binding domain of annexin II (Fig. 1).

### TABLE 1

<table>
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<th>Process</th>
<th>Endothelial cell effect</th>
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<td>Stamler et al. (1993)</td>
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<td>Impaired NO production in vivo</td>
<td>Lentz et al. (1996)</td>
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<td>Induction of procoagulants</td>
<td>Activation of factor V</td>
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<td>Inhibition of natural anticoagulants</td>
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<td>Induction of acute translational response genes</td>
<td>Elongation factor-1α, β and δ</td>
<td>Chacko et al. (1998)</td>
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1 Abbreviations: NO, nitric oxide; t-PA, tissue plasminogen activator.

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**FIGURE 1** Working model illustrating the effect of homocysteine on annexin II-mediated assembly of fibrinolytic proteins. **Left panel:** annexin II is expressed on endothelial cells as a peripheral membrane protein that binds plasminogen (PLG) via its core domain. The tail domain of annexin II contains a linear sequence that appears to be crucial for binding of tissue plasminogen activator (t-PA). Upon successful assembly, plasmin is generated efficiently. **Right panel:** in the presence of homocysteine (HC), the tail domain of annexin II is derivatized at cysteine 9 (C), inhibiting binding of t-PA. As result, plasmin generation is sharply curtailed.
main has been cleaved, fails to compete with immobilized intact annexin II for t-PA binding. In addition, two overlapping dodecapeptides that mimic the extreme amino terminal portions of the tail domain of annexin II compete specifically and effectively with the intact protein for t-PA binding. Similarly, the hexapeptide LCKLSL, but not the mutated peptide LGKLSL, representing the region of overlap, blocks binding of t-PA to both intact recombinant annexin II and endothelial cell monolayers. These studies serve to pinpoint the tPA binding domain of annexin II to residues 8–13 (LCKLSL) of the tail domain.

The effect of HC on the t-PA-binding annexin II tail domain has been examined in detail (Hajjar et al. 1998). Electrospray ionization mass spectrometric analysis of recombinant annexin II indicated that HC alters the receptor physically, increasing its mass by 135 ± 4 Da. Tandem mass spectrometric analysis of a tryptic digest of HC-treated annexin II revealed derivatization of a single cysteine residue (C9) within the amino terminal t-PA binding domain. A disulfide-mediated complex between HC and annexin II was also demonstrated in cultured endothelial cells that were metabolically labeled with [35S]-HC. Modification of annexin II by micromolar concentrations of HC was associated with a dose- and time-related reduction in t-PA binding. Interestingly, the IC50 for inhibition of t-PA binding to annexin II was ~11 μM/L, a value close to the upper limit of normal for HC in plasma (~14 μM/L). Thus, inhibition of t-PA-annexin II assembly on the endothelial cell, and subsequent reduction in plasmin generation, could underlie the prothrombotic/proatherogenic activity associated with HC in vivo.

**Homocysteine induces expression of redox-adaptive genes**

To determine whether HC might influence the cell’s program of gene expression, we used differential display to analyze mRNA transcripts from vascular endothelial cells treated for 6–42 h with HC (Chacko et al. 1998). We found a 3.7-fold, time-dependent induction of a 220-bp fragment that demonstrated complete sequence identity with elongation factor-1β (EF-1β), a member of a multimeric complex regulating mRNA translation. HC-treated endothelial cells exhibited significantly increased steady-state mRNA levels for EF-1α, β and δ, as well as increased protein levels for EF-1α, β, γ and δ. These results parallel recent studies in which tunicamycin and reducing agent-responsive protein, the stress protein GRP78/BiP, activating transcription factor 4 and a methylenetetrahydrolate dehydrogenase/cyclohydrolase were all induced adaptively in endothelial cells exposed to high dose HC (Kokame et al. 1996). In vascular smooth muscle cells, the cyclin A gene also appears to be activated transcriptionally after exposure to HC (Tsi et al. 1994 and 1996).

In most eukaryotes, the two primary elongation factors, multifunctional EF-1 and monomeric EF-2, are primary sites of regulation of protein translation (Proud 1994), and EF-1 appears to play a crucial role in cell growth, proliferation and differentiation (Merrick 1992, Morris 1995, Proud 1994). EF-1 is a complex of polypeptides that regulate the efficiency and fidelity of mRNA translation in eukaryotic cells. EF-1 consists of five subunits (α2, β, γ and δ) that promote GTP-driven delivery of aminoacyl tRNAs to the ribosome. The EF-1α-GTP complex is converted to active EF-1αGDP by the nucleotide exchange activities of EF-1β and EF-1γ. The EF-1γ moiety is known to enhance the nucleotide exchange activity of EF-1β and may also serve to anchor the complex to membrane structures. Interestingly, EF-1δ, which is homologous to EF-1β in the C-terminal nucleotide exchange region, is unique among these factors in that it contains a leucine zipper motif of unknown function.

Importantly, fibroblasts from cystathionine β-synthetase –/– individuals also show a 1.5- to 3.0-fold increase in levels of mRNA for EF-1α, β and δ, compared with normal cells (Chacko et al. 1998). Treatment of normal cells with the HC precursor, methionine, induced a 1.5- to 2.0-fold increase in EF-1α, β and δ mRNA. These experiments demonstrate that EF-1 subunits are also stimulated under conditions in which HC is generated intracellularly. As judged by nuclear run-on studies, up-regulation of EF-1 subunits by HC reflects enhanced transcriptional activity in settings in which HC is elevated. This induction, furthermore, was completely inhibited by cycloheximide, suggesting a pathway by which HC may stimulate synthesis of an intermediate protein, which then induces gene transcription.

Finally, additional experiments suggest that free thiol–containing proteins, such as annexin II, which may be susceptible to derivatization upon exposure to HC (Hajjar et al. 1998), may experience increased rates of turnover compared with “cysteineless” proteins (Chacko et al. 1998). Pulse-chase studies in HC-treated endothelial cells revealed a doubling in the rate of appearance and disappearance of annexin II, but no change in synthesis or degradation of plasminogen activator inhibitor-1. These changes may represent an adaptive response to HC-induced oxidative stress and protein degradation.

The precise role played by EF-1α, β, γ and δ in cells with elevated levels of HC is unclear. On the one hand, HC may elicit an increase in synthesis of a select population of polypeptide(s) with which it forms mixed disulfides. This hypothesis might require the presence of a protein thiol-sensing mechanism that can recruit newly synthesized translational cofactors and stimulate polypeptide elongation. Alternatively, increased levels of EF-1 subunits might also play a role in protein degradation because EF-1α has recently been reported to have isopeptidase activity to promote the degradation of N-acetylated proteins via the ubiquitin pathway (Gonen et al. 1994). Further studies in this area are clearly required to resolve these issues.

**LITERATURE CITED**


