Homocysteine Thiolactone and Protein Homocysteinylation in Human Endothelial Cells
Implications for Atherosclerosis
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Abstract—Editing of the nonprotein amino acid homocysteine by certain aminoacyl-tRNA synthetases results in the formation of the thioester homocysteine thiolactone. Here we show that in the presence of physiological concentrations of homocysteine, methionine, and folic acid, human umbilical vein endothelial cells efficiently convert homocysteine to thiolactone. The extent of this conversion is directly proportional to homocysteine concentration and inversely proportional to methionine concentration, suggesting involvement of methionyl-tRNA synthetase. Folic acid inhibits the synthesis of thiolactone by lowering homocysteine and increasing methionine concentrations in endothelial cells. We also show that the extent of post-translational protein homocysteinylation increases with increasing homocysteine levels but decreases with increasing folic acid and HDL levels in endothelial cell cultures. These data support a hypothesis that metabolic conversion of homocysteine to thiolactone and protein homocysteinylation by thiolactone may play a role in homocysteine-induced vascular damage. (Circ Res. 2000;87:45-51.)

Key Words: homocysteine ■ proteins ■ HDL lipoproteins ■ endothelial cells ■ atherosclerosis

Elevated levels of the nonprotein amino acid homocysteine (Hcy)1 are associated with vascular disease in humans.1 However, it is not known why Hcy can be harmful. The conversion of Hcy to thiolactone as a result of an error-editing function of some aminoacyl-tRNA synthetases (AARS in Equation 1)2,3 is one feature of Hcy metabolism that may account for detrimental effects of elevated Hcy levels.

\[
\text{AARS + Hcy + ATP} \leftrightarrow \text{AARS-Hcy-AMP + PP}_1
\]

Hcy thiolactone

This conversion involves reaction of Hcy with ATP to form an AARS-bound homocysteinyl adenylate (Hcy-AMP) and inorganic pyrophosphate, PP1. Misactivated Hcy is not transferred to tRNA by any of these synthetases.3–7 Instead, Hcy-AMP undergoes a reaction in which the side-chain thiol group (SH in Equation 2) of Hcy displaces the AMP group from the carbonylate of the activated Hcy, forming Hcy thiolactone as a product (Equation 2).5,7 The energy of the anhydride bond of Hcy-AMP is conserved in an intramolecular thioester bond of thiolactone. Consequently, Hcy thiolactone is chemically reactive and acylates free amino groups, such as side-chain lysine groups in proteins.8–10 Homocysteinylated proteins lose their biological activity.10

Hcy thiolactone, originally discovered in cultures of microbial cells,2–4,11–13 is also synthesized by cultured mammalian nonvascular cells.8,14 Whereas methionyl-tRNA synthetase is involved in synthesis of thiolactone in all cell types examined,11–14 isoleucyl- and leucyl-tRNA synthetases can also convert Hcy to thiolactone, at least in bacteria.13 Because of its mostly neutral character at physiological pH (pK5 = 7.1; Reference 15), thiolactone accumulates in culture media.8,11–14 Small amounts of Hcy are present in proteins.2,8,9

It is not known whether Hcy thiolactone synthesis occurs in human vascular endothelial cells. If it did, this could provide a plausible chemical mechanism explaining Hcy toxicity to the human vascular endothelium, the damage of which plays a central role in atherosclerosis.16 Human umbilical vein endothelial cells (HUVECs), used frequently as a model of vascular cells, have been reported to have Hcy thiolactone–hydrolyzing activity17 and therefore are thought...
not to be able to support thiolactone synthesis. However, in this work, we found that cultured HUVECs efficiently metabolize Hcy to thiolactone and provide evidence of post-translational incorporation of Hcy into proteins in these cultures. We also examined factors affecting thiolactone synthesis and protein homocysteinylnation in HUVECs.

**Materials and Methods**

**Cell Culture**

HUVECs were obtained from umbilical cords of 6 healthy newborns. Each cord was separately harvested in a solution containing (in mmol/L) HEPES (pH 7.3) 10, NaCl 140, KCl 4, and glucose 11; 50 μg/ml penicillin; and 100 μg/ml streptomycin (Sigma). Endothelial cells were isolated by collagenase treatment within 4 to 5 hours after delivery. Cells were seeded into 75-cm² culture flasks (Kodak). 8,11,12 Total Hcy was determined by carboxymethylation of DTT-treated samples followed by 1-dimensional TLC on cellulose plates. Determinations of [35S]Hcy and [35S]Met in cellular and extracellular proteins, treated with 10 mmol/L DTT (5 minutes, 25°C), were carried out as described. DTT-labile Hcy represented up to 60% of total Hcy incorporated into protein.

**Preparation of 1-[35S]Hcy Thiolactone and 1-[35S]Hcy**

The method of Baernstein was used to convert 5 mCi l-[35S]Met (Amersham) to l-[35S]Hcy thiolactone. l-[35S]Hcy thiolactone was purified by 2-dimensional thin-layer chromatography (TLC). Fresh [35S]Hcy was prepared for each experiment by hydrolysis of l-[35S]Hcy thiolactone (1 mmol/L, 10 000 Ci/mol) with NaOH in the presence of DTT. 8

**Preparation of HDL**

HDLs were prepared from human serum by ultracentrifugation in potassium bromide (density of 1.225 g/mL) and gel filtration on a Sephacryl HR S-300 (Pharmacia) column in the presence of 1 mmol/L CaCl₂.


HUVECs were grown to confluence on 3.5-cm dishes. The medium was then replaced with 0.5 mL of methionine (Met)-free M199 supplemented with 15% dialyzed FBS, heparin, and bovine endothelial cell growth factor; 5 μmol/L [35S]Met (0.05 mg/mL); or 5 to 100 μmol/L [35S]Hcy (0.05 mg/mL), and the cultures were maintained at 37°C/5% CO₂ for up to 48 hours. Because streptomycin destroys Hcy thiolactone (see Results), we thoroughly rinsed the cells with streptomycin-free medium before adding fresh Met-free and streptomycin-free medium containing [35S]-labeled amino acids. Even though fresh [35S]Hcy was added to the medium at the beginning of an experiment, it oxidized within 2 to 4 hours to [35S]Hcy and mixed protein-S-[35S]Hcy and Cys-S-[35S]Hcy disulfides.

**Determination of Hcy Thiolactone, Hcy, and Hcy-Protein**

Hcy thiolactone was determined by 2-dimensional TLC on cellulose plates (Kodak). Total Hcy was determined by carboxymethylation of DTT-treated samples followed by 1-dimensional TLC on cellulose plates. Determinations of [35S]Hcy and [35S]Met in cellular and extracellular proteins, treated with 10 mmol/L DTT (5 minutes, 25°C), were carried out as described. DTT-labile Hcy represented up to 60% of total Hcy incorporated into protein.

**Edman Degradation**

Edman degradation of DTT-treated and carboxymethylated [35S]Hcy-labeled protein was carried out as described by Chang. Standard of phenylthiohydantoin (PTH)–(S-carboxymethyl)-Hcy was prepared by Edman degradation of in vitro homocysteinylated human serum proteins.

**Results**

**Synthesis of Hcy Thiolactone From Endogenous Hcy**

In human cells, endogenous Hcy is formed from Met in enzymatic reactions that start with the formation of S-adenosyl (Ado)Met. As a result of subsequent methylation reactions, AdoMet is converted into AdoHcy. AdoHcy is then hydrolyzed to Hcy, which can be converted into thiolactone, particularly when the conversion to Met through the folate- and vitamin B₁₂-dependent transmethylation, or to cysteine through the vitamin B₁₂-dependent trans-sulfuration pathway, is compromised. To examine thiolactone synthesis from the endogenous Hcy in HUVECs, we incubated the cells with 5 μmol/L [35S]Met for up to 48 hours. Most (>95%) [35S]Met was metabolized within 20 hours. The 3% of [35S]Met that remained in the media after 20 hours (Table 1) is due, most likely, to protein turnover. Eighty-three percent of the metabolized [35S]Met was incorporated into protein as determined by trichloroacetic acid precipitation of cell ex-
Conversion of Hcy to thiolactone represents a major pathway under these conditions. About 4% of the metabolized [35S]Met was converted into [35S]Hcy thiolactone (Figure 1A), most of which accumulated in the culture medium (Table 1). In agreement with previous studies, 13% of [35S]Met was metabolized to [35S]Hcy, most of which accumulated in the culture medium (Figure 1B). In addition, folic acid inhibited thiolactone synthesis in HUVECs, most likely by facilitating transmethylation of Hcy to Met by Met synthase. This was shown by the addition of 10 μmol/L folic acid to HUVEC cultures. Such an addition prevented accumulation of both thiolactone (Figure 1A) and Hcy (Figure 1B) in the culture medium and in cells (Table 1). These data demonstrate that endogenous Hcy is efficiently metabolized to thiolactone by HUVECs and that the metabolism is inhibited by supplementation with folic acid.

### Level of Hcy Thiolactone Is Proportional to the Concentration of Exogenous Hcy

The kinetics of thiolactone synthesis from exogenous Hcy were examined at physiological concentrations of Hcy (10 to 80 μmol/L), folic acid (26 nmol/L; Reference 22), and vitamin B12 (180 pmol/L from serum; Reference 22). Thiolacltone accumulation positively correlated with the incubation time and Hcy concentration (Figure 2A). Up to 4 μmol/L thiolactone accumulated in culture media under these conditions, representing up to 5% exogenous Hcy. There was very little (0.1%) incorporation of H2S label from exogenous Hcy into cellular protein, as determined by trichloroacetic acid precipitation of cell extracts (not shown). Thus, metabolic conversion of Hcy to thiolactone represents a major pathway under these conditions.

### Met Inhibits Synthesis of Hcy Thiolactone

We next examined the effects of physiological concentrations of Met on the kinetics of thiolactone synthesis from [35S]Hcy. At low (10 μmol/L) and high (80 μmol/L) concentrations of [35S]Hcy, 5 to 20 μmol/L Met progressively inhibited the synthesis of [35S]Hcy thiolactone (Figures 2B and 2C). At incubation times <20 hours, a greater degree of inhibition by Met was found than at later times. Even in the presence of 20 μmol/L Met, up to 1 μmol/L [35S]Hcy thiolactone was formed from 80 μmol/L [35S]Hcy (Figure 2C). With 40 μmol/L [35S]Hcy, Met progressively inhibited the synthesis of [35S]Hcy thiolactone, reaching as high as 80% inhibition at 40 μmol/L Met after 48 hours (Figure 4D). These results suggest involvement of methionyl-tRNA synthetase in thiolactone synthesis in HUVECs.

### Supplementation With Folic Acid or HDL Inhibits Synthesis of Hcy Thiolactone

Effects of supplementation with folic acid, methylcobalamine, and HDL on kinetics of thiolactone synthesis from exogenous Hcy in HUVEC cultures are shown in Figure 2D. At low [35S]Hcy (10 μmol/L), supplementation with 10 μmol/L folic acid resulted in >90% inhibition of thiolactone synthesis (Figure 2D). At high [35S]Hcy (80 μmol/L), inhibition of thiolactone synthesis by folic acid was less pronounced (75%), and significant (1 μmol/L) thiolactone levels still formed (not shown). Methylcobalamine (1.5 μmol/L) had only a minor effect (10% to 20% inhibition) on thiolactone synthesis (Figure 2D). About 5% of 35S label from exogenous [35S]Hcy was incorporated into cellular protein from folic acid–supplemented cultures (not shown). More Met and less Hcy was present in cells maintained in folic acid–supplemented medium than in unsupplemented medium (Table 1). This finding supports the role of folate in transmethylation of Hcy to Met also in HUVECs.

Because it contains tightly bound Hcy thiolactonase as one of its components, HDL should inhibit accumulation of Hcy thiolactone. Indeed, supplementation of HUVEC cultures with HDL led to 80% inhibition of thiolactone synthesis (Figure 2D). The half-life of thiolactone decreased from 3.5 hours in unsupplemented cultures to 10 minutes in HDL-supplemented cultures (not shown).

### Turnover of Hcy Thiolactone

In M199 without antibiotics, thiolactone was hydrolyzed to Hcy with a half-life of 3.5 hours in the absence and presence of HUVECs (Figure 3A), indicating that the turnover of thiolactone in HUVECs.
thiolactone in HUVEC cultures was due to hydrolysis by the medium, not by cells. However, in antibiotic-containing medium, thiolactone was turned over much faster, with a half-life of 10 minutes (Figure 3B). This was due to a second-order reaction of thiolactone with streptomycin (k = 2000 mol L⁻¹ h⁻¹; Reference 10). Possible chemistry may involve the a-amino group of thiolactone.

Incorporation of Hcy Into Protein

To determine whether Hcy can be incorporated into protein, we prepared cellular and extracellular [³⁵S]proteins from [³⁵S]Hcy-labeled HUVECs and acid-hydrolyzed them to liberate their [³⁵S]amino acids, which were then separated by 2-dimensional TLC. In addition to [³⁵S]Met, substantial amounts of [³⁵S]Hcy were present in proteins. The amount of Hcy incorporated into protein was proportional to Hcy concentration in HUVEC cultures in the range of 3 to 100 μmol/L (Figures 4A and 4B). Hcy incorporation into intracellular proteins ranged from 20% (at low Hcy) to 95% (at high Hcy) of Met incorporation (Figure 4A). Extracellular proteins incorporated 2- to 4-fold more Hcy than Met (Figure 4B). Hcy incorporation represented ~20% of Hcy thiolactone present in HUVEC cultures (Figure 4C).

We also examined the effects of supplementation with folic acid, Met, and HDL, each of which inhibits indirectly or directly Hcy thiolactone formation, on protein homocysteinyl-ylation. Folic acid abolished incorporation of Hcy into cellular and extracellular protein (Table 2), most likely by facilitating transmethylation of Hcy to Met, thereby indirectly preventing synthesis of Hcy thiolactone (Table 1). As expected, ~10 times more Met was incorporated into protein in the presence of 10 μmol/L folic acid than in its absence (Table 2). About 70% inhibition of Hcy incorporation into protein was observed in the presence of 20 μmol/L Met, possibly because of Met inhibition of thiolactone synthesis (Figure 4D). HDL, apparently because of its tightly associated Hcy thiolactonase, which hydrolyzes thiolactone²³ (Figure 2D), inhibited incorporation of Hcy into extracellular protein. However, HDL did not affect incorporation of Hcy into intracellular protein (Table 2). Incorporation of Met into cellular and extracellular protein was not affected by HDL, as expected.

**Hcy Is Recovered From Proteins Subjected to Edman Degradation**

To determine whether incorporation of Hcy into protein is post-translational or translational, [³⁵S]proteins from HUVEC cultures were subjected to Edman degradation. Amino acids having a free α-amino group are released from protein after 1 cycle of Edman degradation. Thus, Hcy incorporated posttranslationally as a result of homocysteinyl-ylation by thiolactone of side-chain amino groups of protein lysine residues would be released by Edman degradation.¹⁰

As shown in Figure 5, Edman degradation liberated PTH-(S-carboxymethyl)-[³⁵S]Hcy from [³⁵S]proteins obtained from HUVEC cultures incubated with (in μmol/L) 10 (lane 1), 40 (lane 2), and 80 (lane 3) [³⁵S]Hcy. Quantification of the radiolabeled spots by scintillation counting indicated that PTH-(S-carboxymethyl)–[³⁵S]Hcy represented ~30% of total [³⁵S]protein (not shown). Less PTH-(S-carboxymethyl)–[³⁵S]Hcy was liberated from [³⁵S]proteins obtained from cultures supplemented with 20 μmol/L Met (lane 4) or 1 mg/mL HDL (lane 6). Much less PTH-(S-carboxymethyl)–[³⁵S]Hcy was liberated from [³⁵S]proteins obtained from cultures supplemented with 10 μmol/L folic acid (lane 5) or 10 μmol/L folic acid and 1.5 μmol/L methylcobalamine (lane

**TABLE 2. Levels of Protein-Hcy and Protein-Met in HUVEC Cultures**

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>Intracellular Protein-Hcy</th>
<th>Intracellular Protein-Met</th>
<th>Extracellular Protein-Hcy</th>
<th>Extracellular Protein-Met</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μmol/L Hcy, control</td>
<td>0.72</td>
<td>10±5</td>
<td>5.5±0.3</td>
<td>5.4±1.7</td>
</tr>
<tr>
<td>+10 μmol/L folic acid</td>
<td>&lt;0.1</td>
<td>106</td>
<td>&lt;0.1</td>
<td>89</td>
</tr>
<tr>
<td>+1 mg/mL HDL</td>
<td>0.7</td>
<td>10.1</td>
<td>1.9</td>
<td>6.2</td>
</tr>
<tr>
<td>+20 μmol/L Met</td>
<td>0.25</td>
<td>5.8</td>
<td>1.5</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Data are presented as pmol/10⁵ cells.
of albumin, 5 distinct protein bands (of molecular weights ~45, ~50, and ~180, with 2 of >200 kDa) were visible on SDS-PAGE patterns obtained with protein samples (containing up to 80% [35S]Hcy–protein; see Figure 4B) from HUVEC cultures maintained on (in μmol/L) 2 (lane 2), 10 (lane 6), 40 (lane 5), and 80 (lane 1) [35S]Hcy. These distinct proteins became more heavily labeled with [35S]Met (Table 2) in cultures supplemented with folic acid (lane 4). This suggests that newly synthesized secreted HUVEC proteins, the identity of which remains to be established, are targets for protein homocysteinylation.

**Discussion**

Our data demonstrate that (1) Hcy thiolactone is an important component of Hcy metabolism in human vascular endothelial cells; (2) Hcy is incorporated into proteins; and (3) the extent of thiolactone formation and protein homocysteinylation depends on the extracellular concentrations of Hcy, folic acid, and HDL, factors that have been implicated in susceptibility to vascular disease in humans. Under deregulated metabolic conditions (low folate, high Hcy), protein homocysteinylation was increased relative to that under normal metabolic conditions (high folate, low Hcy). The ability to support thiolactone synthesis and protein homocysteinylation may underlie susceptibility of human endothelial cells to Hcy-induced damage in atherosclerosis.

Dudman et al17 reported that HUVECs exhibit thiolactone-hydrlyzing activity, which we could not find in the present work. Instead, we detected exceptionally efficient synthesis of thiolactone in these cells (Figures 1 and 2). A possible explanation for the finding by Dudman et al17 is that incomplete removal of streptomycin (present in endothelial cell culture media used by Dudman et al17 ) would lead to a false thiolactonase activity in an indirect thiol release assay. However, Lineweaver-Burk plots of the activity observed by Dudman et al17 make this explanation unlikely. Another possible explanation for the discrepancy could be differences between HUVEC donors used in the 2 studies.

HUVECs excrete large amounts of Hcy when they are cultured in standard Met-containing medium that contains 26 nmol/L folic acid (Reference 22 and this work). However, in addition to Hcy, HUVECs also synthesized large amounts of thiolactone. Although 26 nmol/L folic acid is apparently sufficient to support growth of HUVECs on Met, it is not sufficient for transmethylation of Hcy to Met in these cells.22 Supplementation of culture media with 10 μmol/L folic acid restored transmethylation of Hcy to Met and prevented accumulation of both Hcy and thiolactone.

Because Hcy thiolactone forms in the active site of methionyl-tRNA synthetase,3,5 the synthesis of thiolactone is expected to increase with an increase in Hcy/Met ratio.3–5 Indeed, the synthesis of thiolactone by HUVECs increased with an increase in Hcy concentration and decreased with an increase in Met concentration. Factors such as folic acid that affect Hcy/Met ratios in human plasma1,22 also affected thiolactone synthesis in vascular cells (this work).

Large amounts of Hcy were also found to be incorporated into cellular and extracellular proteins in HUVEC cultures, with up to 4-fold more Hcy than Met being incorporated in
the presence of high Hcy and low folate levels. The exact mechanism of Hcy incorporation into protein is unknown. However, the recovery of Hcy from these proteins by Edman degradation suggests that Hcy incorporation is most likely due to homocysteinylination of protein amino groups by thiolactone.10 Not only thiolactone synthesis but also protein homocysteinylination was affected by the levels of Hcy, folate, and HDL, all of which have been linked to vascular diseases.16

Elevated levels of Hcy are an independent risk factor for vascular diseases.1 However, it is not clear why excess Hcy is harmful. The following hypotheses have been proposed to explain the effects of Hcy. Hcy may induce oxidative damage of endothelial cells,24 promote vascular smooth muscle growth, and inhibit regeneration of endothelial cells.25 Inhibition of endothelial cells growth may result from inhibition of methylation by Hcy.25 In addition, Hcy may affect the blood-clotting mechanisms, thereby enhancing a prothrombotic state.26 Alterations in the expression of multiple genes induced by Hcy in endothelial cells could also contribute to atherosclerosis.27 However, in most experiments that led to these hypotheses, nonphysiological concentrations of Hcy (1 to 10 mmol/L) were used, and, where reported, similar effects were observed with cysteine or 2-mercaptopethanol.24,26,27 Although effects of Hcy on growth and methylation were found at physiological Hcy concentrations, these effects could only be observed in the presence of high levels of adenosine,25 which are not present in human cells.

Our findings that thiolactone and Hcy-protein are present in HUVEC cultures support an alternative hypothesis, namely, that metabolic conversion of Hcy to thiolactone, protein homocysteinylination, and resulting protein damage10 cause Hcy toxicity to human endothelium. Protein homocysteinylination is specific for Hcy because thiolactone—the metabolite generating the modification—can only arise from Hcy.5,8–14 The synthesis of thiolactone and protein homocysteinylination occur at physiological concentrations of Hcy and depend on the Hcy/Met ratio, which in turn depends on folate. By using folate-limited media, we were able to obtain the levels of thiolactone and protein-Hcy in HUVEC cultures at least 100 times higher than the levels observed in previous experiments with fibroblast cultures,8 thus demonstrating that the lack of folate is a major determinant of efficient thiolactone synthesis and protein homocysteinylination.

Hcy thiolactone is known to be toxic to endothelial cells. For example, chronic infusions of baboons with Hcy thiolactone cause endothelial cell injury.28 Thiolactone, but not Hcy, was found to induce gross changes in endothelial cell morphology and to induce cell death.29 It should be noted, however, that it is not known whether these effects were due to protein homocysteinylination.

That protein homocysteinylination may be physiologically detrimental is suggested by several studies. For instance, enzymes, such as MetRS and trypsin,10 are inactivated by homocysteinylation. Lysyl oxidase, an important enzyme responsible for post-translational modification essential for the biogenesis of connective tissue matrices, is inactivated by Hcy thiolactone, which modifies the active-site tyrosinequinone cofactor.30 In addition, homocysteinylated cytochrome c is particularly prone to aggregation,10 and protein homocysteinylination can be physiologically detrimental by eliciting immune response, as shown in rabbits immunized with homocysteinylated LDL.31

In conclusion, this work shows that in human vascular endothelial cells Hcy is incorporated into proteins and that this incorporation, as well as thiolactone formation, is dependent on extracellular Hcy, folic acid, and HDL. At this stage, our findings and observations by others, suggesting that protein homocysteinylation impairs physiological function, do not establish causality of Hcy in atherosclerosis. However, they underscore the importance of examining protein homocysteinylation in the context of Hcy-induced pathologies in humans.

Acknowledgments
This research was supported in part by grants from the National Science Foundation (MCB-972-4929 to H.J.) and the NIH (HL47906 to A.A.).

References


