Homocysteine Down-regulates Cellular Glutathione Peroxidase (GPx1) by Decreasing Translation*

Received for publication, February 7, 2005
Published, JBC Papers in Press, February 7, 2005, DOI 10.1074/jbc.M501452200

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Hyperhomocysteinemia contributes to vascular dysfunction and an increase in the risk of cardiovascular disease. An elevated level of homocysteine in vivo and in cell culture systems results in a decrease in the activity of cellular glutathione peroxidase (GPx1), an intracellular antioxidant enzyme that reduces hydrogen peroxide and lipid peroxides. In this study, we show that homocysteine interferes with GPx1 protein expression without affecting transcript levels. Expression of the selenocysteine (SEC)-containing GPx1 protein requires special translational cofactors to “read-through” a UGA stop codon that specifies SEC incorporation at the active site of the enzyme. These factors include a selenocysteine incorporation sequence (SECIS) in the 3′-untranslated region of the GPx1 mRNA and cofactors involved in the biosynthesis and translational insertion of SEC. To monitor SEC incorporation, we used a reporter gene system that has a UGA codon within the protein-coding region of the luciferase mRNA. Addition of either the GPx1 or GPx3 SECIS element in the 3′-untranslated region of the luciferase gene stimulated read-through by 6–11-fold in selenium-replete cells; absence of selenium prevented translation. To alter cellular homocysteine production, we used methionine in the presence of aminopterin, a folate antagonist, co-administered with hypoxanthine and thymidine (HAT/Met). This treatment increased homocysteine levels in the media by 30% (p < 0.01) and decreased GPx1 enzyme activity by 45% (p = 0.0028). HAT/Met treatment decreased selenium-mediated read-through significantly (p < 0.001) in luciferase constructs containing the GPx1 or GPx3 SECIS element; most importantly, the suppression of selenium-dependent read-through was similar whether an SV40 promoter or the GPx1 promoter was used to drive transcription of the SECIS-containing constructs. Furthermore, HAT/Met had no effect on steady-state GPx1 mRNA levels but decreased GPx1 protein levels, suggesting that this effect is not transcriptionally mediated. These data support the conclusion that homocysteine decreases GPx1 activity by altering the translational mechanism essential for the synthesis of this selenocysteine-containing protein.

Hyperhomocysteinemia is a known risk factor for cardiovascular disease. Normal plasma homocysteine levels range from 5 to 15 μM (1, 2); however, clinical studies suggest that high normal levels may contribute to cardiovascular disease risk. Elevated levels of homocysteine contribute to endothelial dysfunction, an early marker of vascular injury in atherogenesis (3). In animal models of hyperhomocysteinemia, such as the heterozygous cystathionine β-synthase-deficient (CBS+/−) mouse, a modest doubling of homocysteine levels is sufficient to impair endothelium-dependent vasodilator responses in mesenteric arteries that have normal endothelium-independent vasodilator responses (4). CBS+/− mice have other detectable signs of oxidative stress, including a deficiency in acetylcysteine-stimulated cGMP accumulation in isolated aorta, an increase in immunodetectable aortic 3-nitrotyrosine, and an increase in the levels of plasma F2-isoprostanes (4–6). These findings suggest a lack of bioavailable nitric oxide in these mice. We have proposed that homocysteine contributes to the accumulation of reactive oxygen species and the subsequent inactivation of nitric oxide by decreasing the activity of cellular glutathione peroxidase (GPx1), a major intracellular antioxidant enzyme. In vivo, modest hyperhomocysteinemia decreases GPx1 activity (5, 7). Similarly, in cultured endothelial cells, micromolar concentrations of homocysteine are sufficient to decrease the activity of this important antioxidant enzyme (6, 8).

The molecular mechanism by which homocysteine decreases GPx1 activity is as yet unclear. GPx1 mRNA has been shown to be decreased in cells treated with supraphysiologic (5 mM) concentrations of homocysteine (8, 9), yet previous studies indicate that GPx1 activity is diminished by only physiologic or pathological concentrations of homocysteine (6, 8). GPx1 is one of several selenocysteine-containing proteins (10), the expression of which is known to be regulated post-transcriptionally by selenium, which increases GPx1 mRNA stability (11). Insertion of selenocysteine in the protein involves read-through of a UGA-stop codon that specifies selenocysteine incorporation. Many cofactors are involved in the translational incorporation of the amino acid selenocysteine, including cis-acting signals, such as a selenocysteine incorporation sequence (SECIS) in the 3′-untranslated region (UTR) of the GPx1 mRNA (12). Other cofactors for selenocysteine incorporation in eukaryotes include the following: selenium; enzymes involved in the synthesis of SEC; a tRNA specific for the selenocysteine amino acid (tRNAsec) that has an anticodon to recognize the UGA; an elongation factor for selenocysteine incorporation (eFsec); and a SECIS-binding protein (SBP2) (12–15).

1 The abbreviations used are: GPx1, cellular glutathione peroxidase; Hcy, homocysteine; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; RT, reverse transcriptase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; UTR, untranslated region; SEC, selenocysteine; SECIS, SEC incorporation sequence; BAEc, bovine aortic endothelial cells; NMD, nonsense-mediated decay; hSECIS, human SECIS; GPx3, plasma glutathione peroxidase; HAT, hypoxanthine, aminopterin and thymidine; CBS, cystathionine-β-synthase.

* This work was supported in part by National Institutes of Health Grants HL55993, HL58796, HL61795, and N01 HV28178. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.
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Because of the specialized mechanism of translation required for selenocysteine incorporation, we analyzed the potential interference of homocysteine with selenocysteine incorporation and GPx1 activity in cultured cells. Our data suggest that homocysteine interferes with GPx1 translation, resulting in a decrease in cellular production of this antioxidant enzyme.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**BAEC and COS7 cells were passaged in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Invitrogen) and 10% fetal calf serum (FCS). For selenium depletion, COS7 cells were grown in DMEM with 0.1% FCS for 5 days prior to treatment. To increase levels of homocysteine in culture, we added the following: HAT/Met, which includes aminopterin (800 μM), a dihydrofolic reductase inhibitor, to decrease cellular folate and homocysteine remethylation; hypoxanthine (200 μM) and thymidine (32 μM); to compensate for adverse effects of aminopterin on nucleotide biosynthesis; and excess (1 mM) methionine (Met), to increase homocysteine synthesis.

**Homocysteine Measurements—**Cell culture media were collected after 48 h of HAT/Met treatment and briefly centrifuged to remove any cellular debris. Total homocysteine (free homocysteine plus homocysteine derived from homocystine or mixed disulfides) levels were measured using the homocysteine kit (Cayman Chemical) with the addition of a protein photoinhibitor (Sigma). Protein concentrations were determined by the bicinchoninic assay using reagents from Pierce. Samples (50 μg) were separated by electrophoresis on 12% denaturing polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (Hybond, Amersham Biosciences) that were subsequently incubated overnight at 4 °C with a mouse anti-GPx1 monoclonal antibody (MBL, Woburn, MA) and a polyclonal rabbit anti-β-actin antibody (Sigma). Membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Sigma or Cell Signaling, Beverly, MA). Enhanced chemiluminescence (Amersham Biosciences) was used to detect immune complexes.

**RESULTS**

**GPx1 Enzyme Activity—**GPx1 activity in bovine aortic endothelial cells (BAEC) can be reduced by as little as 25 μM homocysteine (Fig. 1A). BAEC were treated with HAT, to inhibit dihydrofolic reductase and homocysteine remethylation, and Met, to increase intracellular levels of homocysteine. Both folate restriction (17) and methionine supplementation have been shown to increase production of homocysteine in a variety of cells (18). HAT/Met treatment resulted in a decrease in GPx1 activity similar to that achieved with 100 μM homocysteine. The GPx1 activity of cells treated with 25 μM homocysteine was further suppressed by the addition of adenosine (50 μM), a cofactor required for S-adenylation of homocysteine and methionine, in the presence of 10 μM erythro-9-(2-hydroxy-3-nonyl)adenine, an adenosine deaminase inhibitor. COS7 cells could be grown in media supplemented with only 0.1% FCS, allowing for depletion of cellular selenium, which is present in normal fetal calf serum. After 7 days of selenium depletion, GPx1 activity is decreased by ~75% compared with the activity in cells cultured in 0.1% FCS media supplemented with 10 ng/ml sodium selenite. The activity in cells cultured in 0.1% FCS media supplemented with 10 ng/ml sodium selenite is nearly the same as the activity of cells grown in 10% FCS media without additional selenium supplementation (data not shown). Cells supplemented with 1 ng/ml sodium selenite have no apparent increase in GPx1 activity over those grown with no added selenium, suggesting that there is a threshold level of selenium supplementation necessary to increase the production of GPx1. HAT/Met treatment decreased GPx1 activity by 42% in COS7 cells cultured in selenium-replete cells (Fig. 1B). Under these conditions, we found that HAT/Met treatment of COS7 cells increases the cellular release of homocysteine into the media by 30% (Fig. 2).

**GPx1 mRNA—**To determine whether the effects of low levels of homocysteine or HAT/Met treatment affected GPx1 mRNA levels, we used RT-PCR of RNA isolated from COS7 cells that were treated for 48 h with HAT/Met or 100 μM homocysteine.
As has been shown previously (11), selenium increases the levels of GPx1 mRNA by ~2-fold. Treatment with HAT/Met or 100 μM homocysteine had no effect on the steady-state levels of GPx1 mRNA.

Selenium-dependent UGA Read-through—GPx1 expression relies on a unique translational mechanism to incorporate selenocysteine at a UGA codon; thus, we next determined whether treatments that increase homocysteine production can affect read-through of the UGA codon during mRNA translation. To accomplish this end, we utilized a luciferase reporter gene with a substitution of a UGA codon for a glycine codon (GGA) in the protein-coding region of the luciferase mRNA (pGL2 UGA). Theoretically, in the absence of selenium and other necessary cofactors for selenocysteine incorporation, read-through of the UGA to produce a full-length functional luciferase protein would occur infrequently. Therefore, to promote read-through of the UGA as a site for selenocysteine incorporation rather than as a site for termination of translation, the human GPx1 SECIS element was inserted in the 3′-UTR of the luciferase gene in the sense (hSECIS/S) orientation (Fig. 4A). As a negative control for random effects of the SECIS element on luciferase expression, constructs were made with the SECIS element in the antisense orientation (hSECIS/AS). To assess any effect of the GPx1 promoter on luciferase expression, the SV40 promoter was replaced by the GPx1 promoter, resulting in the vectors GPx1/UGA, GPx1/hSECIS/S, and GPx1/aSECIS/AS (Fig. 4A).
or absence of selenium (Fig. 4, C and D). In the presence of selenium, HAT/Met treatment caused a significant reduction of read-through of both the GPx1/UGA/SECIS/S (Fig. 4C) and the UGA/SECIS/S (Fig. 4D) constructs. Similar reduction in read-through expression was obtained when cells were treated with 100 μM homocysteine (data not shown). No significant changes were found with the antisense constructs or in the absence of selenium. These data suggest that the HAT/Met-mediated reduction of selenium-dependent UGA read-through is primarily a translational event in this cell system, as the construct with the GPx1 promoter showed no additional suppression by this treatment.

To test whether the HAT/Met suppression of read-through was specific for the GPx1 SECIS element, we compared the function of the GPx3 SECIS element in this assay system. The GPx1 SECIS element has been defined as a form 1 SECIS element, whereas the GPx3 SECIS element has been defined as a form 2 SECIS element on the basis of the predicted secondary structure of the stem-loop region that directs selenocysteine incorporation (19). As expected, there was no read-through if the GPx3 SECIS element was in the antisense orientation (data not shown). Selenium stimulates read-through when the GPx3 SECIS element was in the sense orientation and, as with the GPx1 SECIS element, this read-through is significantly affected by HAT/Met treatment (p < 0.005) (Fig. 4E).

Immunodetection of GPx1—To confirm the effects of HAT/Met treatment on the expression of the GPx1 protein, we performed Western blots (Fig. 5). After adjusting for the slight lane-to-lane variation in immunodetectable actin levels, HAT/Met treatment leads to a maximum 50% reduction in immunodetectable GPx1, whereas 100 μM homocysteine reduces GPx1 by ~20%. Overall, these data suggest that an increase in cellular homocysteine results in a decrease in GPx1 activity because of a decrease in the translation of this protein.

**DISCUSSION**

Current views hold that homocysteine increases vascular oxidative stress (20), thereby contributing to a proatherogenic and prothrombotic state. Clinical studies suggest that modest increases in plasma homocysteine levels increase the risk of cardiovascular disease. *In vivo*, a mere doubling of homocysteine levels is sufficient to cause some proatherogenic changes, such as endothelial dysfunction, reduced bioavailable nitric oxide, increased lipid peroxidation, and increased adhesion molecule expression (4–6). In models of hyperlipidemia, moderate hyperhomocysteinemia (*i.e.* levels of total homocysteine of ~50 μM or ~5-fold higher than normal homocysteine levels) has been found to accelerate atherogenesis (21–23). In cell culture studies, although some detrimental effects of homocysteine are apparent at physiologically (10–50 μM) or pathophysiological (100–300 μM) relevant concentrations (24, 25), other adverse effects can only be measured when cells are treated with supraphysiological (1–5 mM) concentrations of homocysteine (9). In our previous studies, we have shown that micromolar increases in homocysteine correlate with decreased GPx1 activity *in vivo* and in cell culture systems; however, nonphysiological levels, in the millimolar range, are necessary to decrease the expression of GPx1 mRNA in endothelial cells (4–6, 8). The current study indicates that treatment with low micromolar concentrations of homocysteine does not affect GPx1 mRNA levels or GPx1 promoter activity, although this treatment reduces GPx1 enzyme activity and the levels of GPx1 protein. The reporter gene assays further establish that the unique mechanism of selenocysteine incorporation is reduced by homocysteine.

Homocysteine is produced by the hydrolysis of S-adenosyl-homocysteine, which is formed after a transfer of a methyl group from S-adenosylmethionine to a methyl acceptor (26). Excess methionine is known to augment homocysteine production *in vivo* and in cultured cell systems. All cells have the capacity to form methionine by the transfer of a methyl group from the folate-containing methyl donor, 5-methyltetrahydrofolate, to homocysteine by the enzyme methionine synthase. Restriction of folate inhibits this remethylation pathway and contributes to the accumulation of homocysteine, whereas dietary folate supplementation has been shown to lower homocysteine levels (20). It is known that dihydrofolate reductase inhibitors increase homocysteine production by limiting the production of 5-methyltetrahydrofolate and other cellular folate.
**FIG. 4. Selenium-dependent read-through expression.** A, vectors. The pGL2 UGA vector has a TGA mutation in the luciferase protein-coding region, a PvuII restriction endonuclease site in the 3'-untranslated region of the luciferase gene, and an SV40 promoter and enhancer. The GPx1/UGA vector has a GPx1 promoter substituted for the SV40 promoter. The human GPx1 selenocysteine incorporation sequence (hSECIS) was
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FIG. 5. Immunodetectable GPx1 is decreased in the presence of Hcy or HAT/Met. Cells were treated for 6 days with 100 μM Hcy, HAT, 0.5 mM Met or HAT, 1.0 mM Met. Samples (50 μg) were separated on 12% denaturing polyacrylamide gels and transferred to Hybond nitrocellulose membrane. Antibodies to the human GPx1 and actin were used in the Western blots. Horseradish peroxidase-conjugated second antibodies followed by ECL were used to detect proteins. Plots of densitometry units of this Western blot are shown. ADU, arbitrary densitometry units.

Collected from dietary folates (27). In our cell system, we used the dihydrofolate reductase inhibitor aminopterin to decrease homocysteine remethylation. Extra methionine was also added to the cultures to increase homocysteine production. Treatment with HAT/Met decreased GPx1 activity in BAEC to a similar extent as 100 μM homocysteine. We also found that in the presence of adenosine, the effects of 25 μM homocysteine are augmented. These data suggest conditions that increase S-adenosylhomocysteine production (i.e. intracellular production of homocysteine or homocysteine plus adenosine) may enhance the detrimental effects of homocysteine. We were only able to measure a modest increase in homocysteine release into media in treated COS7 cells. This 30% increase is comparable with that found in many other cell systems after short term treatment in the absence of folate (17) or in the presence of excess methionine (18). As we have shown in our system, this incremental change is sufficient to alter GPx1 activity, and the modest decrease in antioxidant enzyme activity is consistent with the changes in GPx1 activity levels that are associated with mild hyperhomocysteinemia in the CBS(+/−) mice (5, 6).

The expression of GPx1 is under complex regulation that includes transcriptional (28, 29), post-transcriptional (11), and translational mechanisms (12). Selenium has been shown previously to increase mRNA stability, and in the presence of selenium, we found a nearly 2-fold increase in the steady-state levels of GPx1 mRNA. Selenium is also necessary for the translation of GPx1 as it is a necessary precursor to selenocysteine formation. A specialized mechanism is involved in the incorporation of selenocysteine that involves decoding the UGA codon as a selenocysteine codon (12–15). This translational process requires other cis-elements in the mRNA, notably the SECIS element that forms a stem-loop structure in the 3′-UTR. Transfer of the SECIS element to the 3′-UTR of heterologous mRNAs has been shown previously to promote recognition of a UGA embedded in the protein-coding region of a transcript as a site for SEC incorporation (16, 30). An important requirement for read-through includes insertion of the SECIS element downstream of the protein-coding region in the proper orientation. In our system, selenium had no effect on the expression of constructs with the SECIS element in the antisense orientation. In fact, the activity of the antisense constructs was the same as the corresponding constructs that lacked the SECIS element, regardless of whether or not selenium was present. Selenium also had no effect on the activity of the UAA constructs and no effect on control constructs lacking nonsense mutations, indicating that the selenium effect is specific for the UGA codon.

In our studies, selenium increased the levels of GPx1 mRNA. Several studies have found that the stability of GPx1 mRNA is enhanced by selenium repletion (11, 31, 32); however, not all selenoprotein mRNAs are affected by selenium levels (32, 33). Transcript stability is often mediated by sequences such as “AU-rich” elements in the 3′-UTR of the mRNA (34). Although mRNA instability elements have been found in the 3′-UTR of some selenoprotein transcripts (35), these sequence elements are not found in the 3′-UTR of GPx1 mRNA. In studies that have swapped the protein-coding regions and 3′-UTRs among GPx2, GPx4, and GPx1, the presence of either the GPx1 protein-coding region or the GPx1 3′-UTR correlated with mRNA instability, suggesting that more than one, as of yet unidentified, region of the GPx1 mRNA is involved in regulating the stability of GPx1 transcripts. Other studies suggest nonsense-mediated decay (NMD) contributes to the degradation of transcripts with UGA codons that prematurely terminate translation. NMD appears to require splicing as intronless mRNAs with premature stop codons are exempt from NMD (36). NMD mechanisms play an important role in decreasing the levels of GPx1 transcripts when selenium is limiting (37); however, in selenium-replete conditions, NMD mechanisms do not influence GPx1 mRNA.

In selenium-replete conditions, HAT/Met or 100 μM homocysteine did not affect GPx1 mRNA. These treatments also did

cloned at the PvuII site in the sense (hSECIS/S) and antisense (hSECIS/AS) orientation. Bla refers to the ampicillin resistance gene. Additional constructs (not shown) have inserted the GPx3 SECIS element into pGL2 UGA vector at the PvuII restriction endonuclease site. B, luciferase levels were measured in cells transfected in the presence and absence of selenium. Values were normalized to the levels of the GPx1/hSECIS/S with no selenium in C and to the levels of the UGA/hSECIS/S with no selenium in D. E, comparison of UGA/hSECIS/S and GPx3/SECIS 500/S in the presence and absence of selenium, plus HAT/Met.
not affect the expression of wild type luciferase in constructs that lacked the elements for selenocysteine incorporation (data not shown). In pGL2 UGA constructs, however, both the GPx1 SECIS and the GPx3 SECIS element conferred sensitivity to treatments that increased homocysteine levels. GPx3 is the extracellular GPx, a selenoprotein produced primarily in the kidney. GPx1 and GPx3 differ in their primary sequence, in the location of the selenocysteine, and in the forms of the SECIS elements responsible for selenocysteine incorporation. The GPx1 SECIS element has been described as a form 1 SECIS element, whereas the GPx3 SECIS element is a form 2 element (19). According to Grundner-Culemann et al. (19), the form 1 elements have a 10–14-base loop at the apex of the SECIS stem, which contains three conserved adenosines. In the form 2 elements, there are two loops as follows: the conserved adenosines bulge forming a small loop proximal to the top of the stem, and there is a 3–6-base apical loop. Even though the SECIS elements differ between these transcripts, GPx1 mRNA and GPx3 mRNA are both sensitive to selenium depletion (38), and we found no statistically significant difference in the levels of selenium stimulation of read-through in constructs that used either the GPx1 or GPx3 SECIS elements. Minimal SECIS elements, like those studied in the context of a D1-selenoprotein reporter by Martin et al. (39), were not active in our heterologous reporter assay; thus, we cannot exclude the possibility that other elements present in the 3′-UTR may contribute to the sensitivity to HAT/Met-mediated suppression. Further analysis is necessary to determine whether a shared regulatory element or a common regulatory RNA-binding factor mediates the effects of homocysteine on selenium-dependent translation.

In the reporter gene system that we employed, the levels of expression of the TGA vectors do not approach the levels of activity of an unmutated control construct. Published reports suggest that incorporation of selenocysteine during translation is an inefficient event (40), as substitution of a cysteine codon for the selenocysteine codon results in a 20–400-fold increase in expression (41, 42). Others studies have found that location of the UGA in the context of the GPx1 transcript affects the activity and GPx1 immunodetectable protein levels are reduced though the SECIS elements differ between these transcripts, and we found no statistically significant difference in the levels of selenium stimulation of read-through in constructs that used either the GPx1 or GPx3 SECIS elements. Minimal SECIS elements, like those studied in the context of a D1-selenoprotein reporter by Martin et al. (39), were not active in our heterologous reporter assay; thus, we cannot exclude the possibility that other elements present in the 3′-UTR may contribute to the sensitivity to HAT/Met-mediated suppression. Further analysis is necessary to determine whether a shared regulatory element or a common regulatory RNA-binding factor mediates the effects of homocysteine on selenium-dependent translation.

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