# Hyperhomocysteinemia in Liver Cirrhosis : Mechanisms and Role in Vascular and Hepatic Fibrosis

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# Hyperhomocysteinemia in Liver Cirrhosis
## Mechanisms and Role in Vascular and Hepatic Fibrosis

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***Abstract***—Numerous clinical and epidemiological studies have identified elevated homocysteine levels in plasma as a risk factor for atherosclerotic vascular disease and thromboembolism. Hyperhomocysteinemia may develop as a consequence of defects in homocysteine-metabolizing genes; nutritional conditions leading to vitamin $B_6$, $B_{12}$, or folate deficiencies; or chronic alcohol consumption. Homocysteine is an intermediate in methionine metabolism, which takes place mainly in the liver. Impaired liver function leads to altered methionine and homocysteine metabolism; however, the molecular basis for such alterations is not completely understood. In addition, the mechanisms behind homocysteine-induced cellular toxicity are not fully defined. In the present work, we have examined the expression of the main enzymes involved in methionine and homocysteine metabolism, along with the plasma levels of methionine and homocysteine, in the liver of 26 cirrhotic patients and 10 control subjects. To gain more insight into the cellular effects of elevated homocysteine levels, we have searched for changes in gene expression induced by this amino acid in cultured human vascular smooth muscle cells. We have observed a marked reduction in the expression of the main genes involved in homocysteine metabolism in liver cirrhosis. In addition, we have identified the tissue inhibitor of metalloproteinases-1 and $\alpha$1(I)procollagen to be upregulated in vascular smooth muscle cells and liver stellate cells exposed to pathological concentrations of homocysteine. Taken together, our observations suggest (1) impaired liver function could be a novel determinant in the development of hyperhomocysteinemia and (2) a role for elevated homocysteine levels in the development of liver fibrosis. **(*Hypertension*. 2001;38:1217-1221.)**

**Key Words:** homocysteine ■ methionine ■ muscle, smooth, vascular ■ liver ■ cirrhosis ■ fibrosis ■ gene expression

Homocysteine (Hcy) is a sulfur containing amino acid that is formed as an intermediary in methionine metabolism (Figure 1).[1] Extensive evidence shows that elevated plasma Hcy concentration, a reflection of impaired cellular metabolism, can be considered as an independent risk factor for atherothrombotic vascular disease (reviewed in Refsum et al[2]). This condition has been observed in 20% to 30% of patients with premature arteriosclerosis and in 21% of the general population above a certain age.[3,4] Three enzymes utilize Hcy as a substrate: methionine synthase (MS) and betaine-homocysteine methyltransferase (BHMT), which convert homocysteine back to methionine, and cystathionine $\beta$-synthase (CBS), the first enzyme in the transsulfuration pathway.[1] The distribution of Hcy among them depends on metabolic conditions: when methionine is relatively deficient remethylation of Hcy is favored, whereas in situations of methionine excess, the transsulfuration pathway prevails (Figure 1).[1,2] *S*-Adenosylmethionine (AdoMet), the first metabolite of methionine, modulates the flow of Hcy through these metabolic pathways: increased levels of AdoMet activate CBS and inhibit the activity of MS and BHMT.[1,5] Impairment of Hcy remethylation or transsulfuration leads to hyperhomocystinemia. Such situations may develop as a consequence of genetic defects in the enzymes MS, CBS, or methylenetetrahydrofolate reductase (the enzyme that synthesizes the MS cosubstrate 5-methyltetrahydrofolate).[2,3] Nutritional deficiencies in vitamin $B_6$, the cofactor of CBS, or folates and vitamin $B_{12}$, cosubstrate and cofactor of MS, can also lead, along with impaired renal function, to hyperhomocystinemia.[2–4]

The liver plays a central role in the synthesis and metabolism of homocysteine, given the fact that the majority of dietary methionine is metabolized in this organ, where ≈85% of the whole body capacity for transmethylation resides.[1,5] Accordingly, the liver displays a specific pattern of expression of genes involved in methionine and homocysteine metabolism. There are 2 genes coding for methionine adenosyltransferase (MAT), the enzyme that converts methionine

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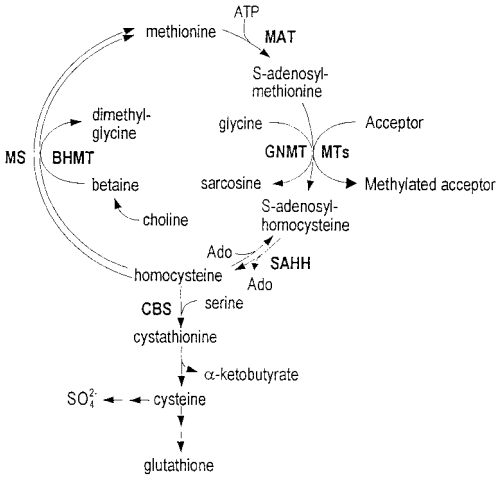


**Figure 1.** Liver methionine cycle and transsulfuration pathway. *GNMT* indicates glycine *N*-methyltransferase; *MTs*, methyltransferases; *SAHH*, and *S*-adenosylhomocysteine hydrolase, Reproduced from Avila M et al[20] with permission from Elsevier Science.

into AdoMet, one (MAT1A) is expressed exclusively in the liver and a second gene (MAT2A) is expressed in all tissues.[5] BHMT and CBS expression is confined mainly to the liver, whereas MS is widely expressed (Figure 1).[1] Thus, it is conceivable that in situations of liver damage, alterations in Hcy may occur. In fact, hyperhomocysteinemia has been reported in chronic alcoholics and in patients with alcoholic cirrhosis, as well as in experimental models of liver damage.[6–10] Although there is extensive evidence about the above-mentioned genetic and nutritional determinants for hyperhomocystinemia, knowledge of the molecular basis of the alteration of Hcy metabolism in liver injury is still limited.

The pathological mechanisms by which elevated Hcy promotes atherothrombotic vascular diseases are not completely known.[2,3] Endothelial injury, which can lead to altered NO production and impaired platelet modulating activity, has been demonstrated.[11–13] In addition, Hcy promotes DNA synthesis and collagen production in vascular smooth muscle cells (VSMCs),[14,15] cholesterol production by hepatic cells,[16] and lymphocyte DNA hypomethylation.[17] These observations suggest a multifactorial mechanism of action for Hcy that may take place not only at the vascular level but on a variety of cellular backgrounds.

In the present report, we describe our attempt to gain further insight into the mechanisms behind the hyperhomocysteinemia associated with liver damage and into the molecular basis of Hcy interference with normal cell function.

## Methods

DL-Hcy was from Sigma Chemical Co.. Cell culture media, fetal bovine serum, and antibiotics were from GIBCO-BRL. Anti-tissue inhibitor of metalloproteinases-1 (TIMP-1) monoclonal antibody was from Calbiochem. Anti-proliferating cell nuclear antigen (PCNA) monoclonal antibody was from Santa Cruz Biotechnology. Radioactive isotopes were purchased from Amersham. Restriction enzymes were from Boehringer Mannheim, M-MLV reverse transcriptase was from GIBCO-BRL, and Bio Taq DNA polymerase was from Bioline.

### Patients

We have studied a group of 26 patients (17 males and 9 females; mean age, 54±8.5 years) with liver cirrhosis of different etiology (13 hepatitus C virus cirrhosis, 10 alcoholic cirrhosis, 1 hepatitis B virus cirrhosis, 1 cryptogenetic cirrhosis, and 1 primary biliary cirrhosis). The control group consisted of 10 subjects in whom a cholecystectomy was performed for the treatment of symptomatic cholelithiasis and who consented to have a liver biopsy during the surgical procedure. Liver samples were immediately frozen and kept at −80°C until processed. In the subjects from the control group, both the liver function tests and the liver biopsy were normal. The study was approved by the human research committee of the University of Navarra.

### Animals

In vivo effects of Hcy were studied in male C57BL/6 mice (Harlan, Barcelona, Spain) of 30 g of weight. Animals received a daily intraperitoneal injection of Hcy (0.1 mg/g of weight)[18] or saline for 4 days, and then were euthanized by cervical dislocation. Aorta was removed, immediately frozen, and stored at −80°C. Four animals were used per group. Studies were approved by the University of Navarra animal research review committee.

### Cell Culture

The hepatic stellate cell (HSC) clone CFSC-2G, derived from $CCl_4$-treated rats, was kindly provided by Dr Marcos Rojkind (Albert Einstein College of Medicine, New York). Human and porcine (Yucatan minipigs) VSMCs were isolated from mammary artery and aorta, respectively, and cultured as reported.[19] Cells were made quiescent before Hcy treatment. Rat hepatocytes (from male Wistar rats) were isolated by collagenase perfusion and cultured as reported.[19] The HepG2 human hepatoma cell line was from the American type culture collection and was propagated as described.[19] All treatments were performed in serum-free medium.

### Differential Display Analysis by Means of Polymerase Chain Reaction

Differential display analysis by means of polymerase chain reaction (DDPCR) was performed using the Hyeroglyph mRNA Profile Kit (Genomyx, Beckman Instruments) as described.[19] RNA isolation, by the guanidinium thiocyanate method, and Northern blot analysis were performed as reported previously.[19] The probes used ($\alpha$1(I)procollagen and $\beta$-actin) have been described elsewhere.[19] Quantitation of gene expression in human liver samples was performed by reverse transcriptase-PCR; the primers employed have been reported previously.[20] The Alternate 18S Internal Standards primer set (Ambion) was used to amplify the 18S rRNA that served as internal control.

### Immunoblot Analysis

Tissue and cell samples were homogenized as described in the presence of protease inhibitors at 4°C.[19] Conditioned media from HepG2 cells was concentrated 10-fold as reported.[19] Samples were subjected to 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis. Proteins were electroblotted onto nitrocellulose membranes and probed with the corresponding antibodies. Blots were developed by enhanced chemiluminescence according to manufacturer's instructions (Dupont).

### Serum Methionine and Total Hcy Determination

Serum methionine and total serum Hcy (tHcy=protein-bound and free Hcy) determinations were as described.[20]

### Statistics

Data are mean±SEM. In the human studies, the Mann-Whitney unpaired test was used to compare groups. Otherwise, statistical significance was estimated with Student's *t* test.

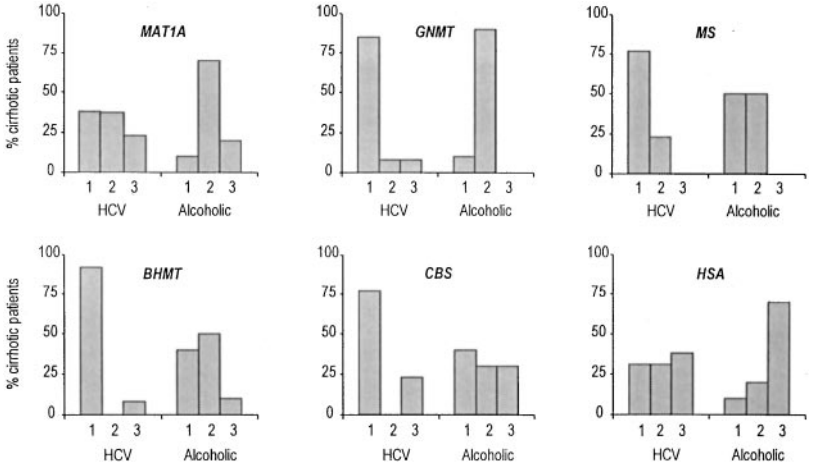


**Figure 2.** Expression of liver methionine metabolizing genes and serum albumin (HSA) in cirrhotic patients according to the etiology of the disease. Patient groups are defined in the text, and gene names are defined in the text or in the legend for Figure 1. The percentage of patients in each group is shown. Reproduced from Avila M et al[20] with permission from Elsevier Science.

## Results

We have first measured the expression of liver genes involved in methionine metabolism. According to the level of expression, compared with that of control livers, cirrhotics were divided into 3 groups: group 1, patients with very low or non-detectable expression; group 2, patients with a level of expression lower than that of controls (50% of the expression found in controls); and group 3, patients with a level of expression similar to that of controls. Human serum albumin (HSA) expression was also measured. The distribution of patients among these groups, and according to the etiology of the disease (hepatitis C virus or alcoholism), is shown in Figure 2. Individual patients with a marked reduction in the expression of a given gene (group 1) tended to be in this same group for all genes tested. To evaluate if the reduced expression of the various genes involved in methionine metabolism was related to the severity of the disease, expressed as the Child-Pugh score,[21] patients were divided into 2 groups. One group (group A) included patients showing normal or only reduced levels of mRNA for at least 3 of the 5 genes analyzed that were involved in methionine metabolism. The second group (group B) included those patients with very low or undetectable levels of mRNA for all or 4 of the 5 genes analyzed involved in methionine metabolism. The mean value for the Child-Pugh score was significantly higher in the cirrhotic patients in group B (9.0±0.7) than in the cirrhotic patients in group A (7.1±0.4, $P<0.03$).

The mean value for serum Hcy was significantly higher in the cirrhotic patients in group B than in group A (Figure 3). Accordingly, mean Hcy concentration was significantly higher for all cirrhotics (14.1±1.3 μmol/L) than for the control group (8.1±0.9 μmol/L, $P<0.03$). In agreement with previous publications,[5,22] fasting serum methionine was higher in cirrhotics (106.3±34.7 μmol/L) than in the control group (30.8±4.8 μmol/L, $P<0.01$). Differences in methionine concentration in cirrhotic patients in groups A and B were also statistically significant (Figure 3).

The second aim of this work was to improve our knowledge on the molecular basis of cellular Hcy effects. We observed that Hcy treatment of quiescent human VSMCs induces the expression of PCNA, a marker of cellular proliferation (Figure 4A). This effect was also observed when Hcy was administered to mice, and PCNA expression was determined in aortic tissue (Figure 4B). We have searched for other genes with expression that could be altered by Hcy in cultured human VSMCs by DDPCR. Cells were made quiescent by serum deprivation and then treated with 100 μmol/L of Hcy, a concentration compatible with intermediate hyperhomocystinemia,[2] for 24 hours. By DDPCR analysis, we have identified TIMP-1 to be upregulated in response to Hcy treatment (2-fold induction) (Figure 5A and 5B). This effect was also observed in pig aorta–cultured VSMCs (Figure 5C). Moreover, intraperitoneal administration of Hcy to mice also resulted in the induction of TIMP-1 expression in

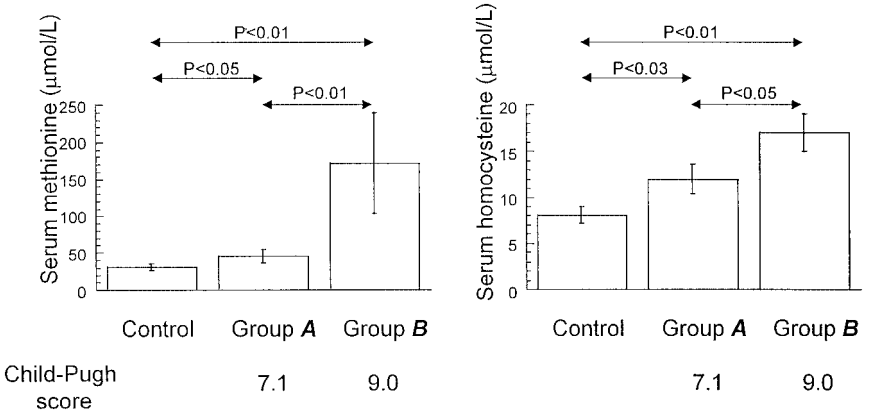


**Figure 3.** Serum levels of methionine and Hcy in control and cirrhotic patients (all 26 patients). Groups A and B are defined in the text.

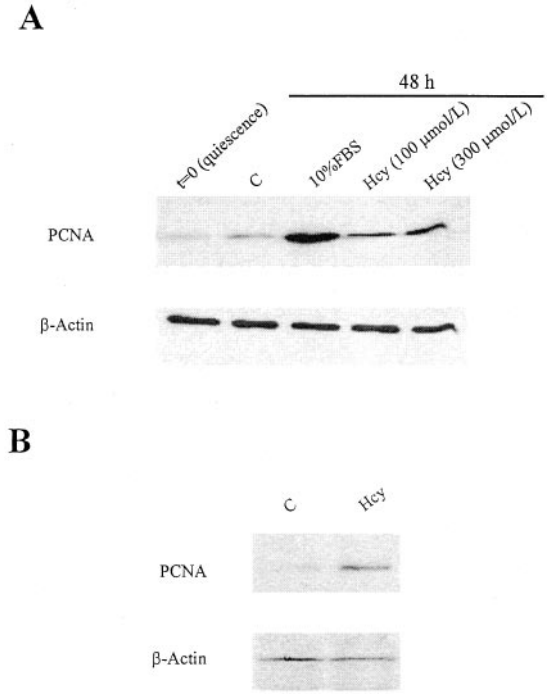


**Figure 4.** Western blot analysis of PCNA expression in quiescent human VSMCs treated with Hcy (100 to 300 $\mu$mol/L) or FBS for 48 hours (A) and aortic tissue from control (saline) and Hcy-treated mice (as described in Methods) (B). Representative blots are shown (n=4).

aorta (Figure 5D). TIMP-1 plays an important role in the regulation of extracellular matrix (ECM) homoeostasis,[23] which is essential not only in the vessel wall but also in the liver.[24] Consequently, we have studied TIMP-1 and $\alpha$1(I)procollagen expression in rat HSCs, observing that both genes are time and dose dependently induced by Hcy in this cell type (Figure 6A and 6B). This effect of Hcy on TIMP-1

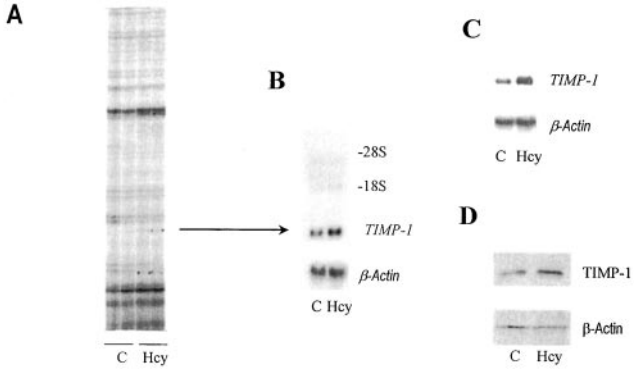


**Figure 5.** A, Detection of differential gene expression induced by Hcy in quiescent human VSMCs (24-hour treatment with 100 $\mu$mol/L Hcy) by DDPCR. B, Representative Northern blot analysis performed with cloned band from DDPCR gel, identified as TIMP-1, on control and Hcy-treated human VSMCs (24 hours, 100 $\mu$mol/L Hcy). C, Northern blot analysis of TIMP-1 in pig VSMCs treated as in panel B. D, Western blot analysis of TIMP-1 expression in aortic tissue from control (saline) and Hcy-treated mice (as described in Methods). Representative blots are shown (n=4). Taken in part from Torres et al[19] with permission from Elsevier Science.

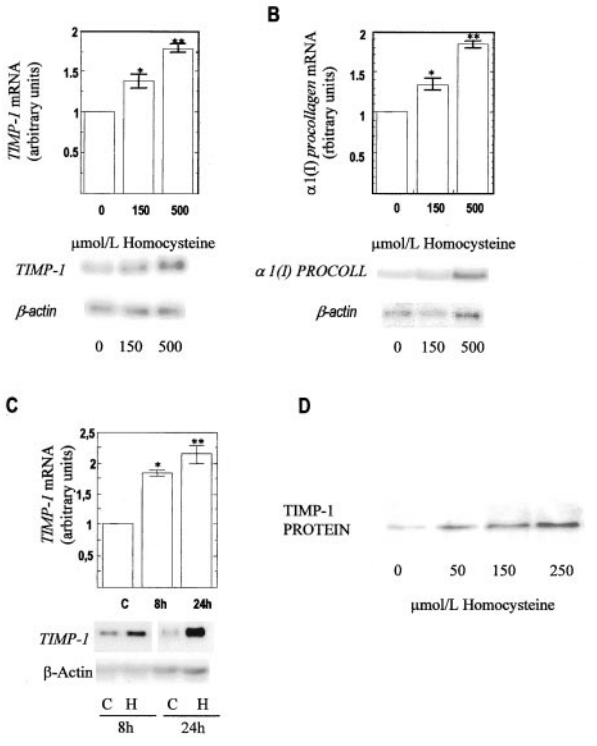


**Figure 6.** A, Northern blot analysis of TIMP-1 expression in rat HSCs treated for 4 hours with Hcy. B, Northern blot analysis of $\alpha$1(I)procollagen expression in rat HSCs treated for 4 hours with Hcy. C, Northern blot analysis of TIMP-1 expression in rat hepatocytes treated with 500 $\mu$mol/L Hcy. D, Western blot analysis of TIMP-1 in conditioned medium from Hcy-treated (24-hour treatment) HepG2 cells. Reproduced from Torres et al[19] with permission from Elsevier Science.

expression was also extended to rat hepatocytes and HepG2 cells (Figure 6C and 6D).

## Discussion

Our results show that alterations in Hcy metabolism in human liver cirrhosis can be ascribed in part to a marked reduction in the expression of the main genes involved in its metabolism, namely MS, BHMT, and CBS. The expression of these genes was always more compromised than that of *HSA* and was related to the severity of the disease, expressed as the Child-Pugh score. We observe reduced expression of Hcy-metabolizing genes, both in alcoholism and hepatitis C virus cirrhosis. It has been suggested that impairment of Hcy metabolism in cirrhosis can be also related to decreased availability or utilization of vitamins $B_6$, $B_{12}$, or folates,[8] which is possible. However, our present data on hyperhomocysteinemia in cirrhosis has been confirmed in other human and experimental studies in which hyperhomocysteinemia was not associated with altered plasma levels of the above-mentioned vitamins.[25,26] We also observed a decrease in MAT1A expression in cirrhotic liver, which contributes to the reported hypermethioninemia and impairment of AdoMet synthesis in this condition.[5] We have previously shown that AdoMet treatment of cirrhotic rats reduces elevated plasma Hcy.[10] Reduced AdoMet levels plus impaired CBS expression in cirrhotics may result in decreased flow of Hcy through

the transsulfuration pathway and contributes to the hyperhomocysteinemia associated with this condition.

We have also addressed the cellular consequences of elevated Hcy levels. Our observations of increased PCNA expression in cultured human VSMCs and in aortic mouse tissue contribute to explain the growth promoting effects described for Hcy in the arterial tissue.[3,11,14] Our DDPCR study identified TIMP-1 as a gene upregulated by Hcy in human and pig cultured VSMCs. This observation was also made in vivo, when administration of Hcy to mice increased TIMP-1 protein in aortic tissue. TIMP-1 is an essential component of the ECM regulating system, acting as an inhibitor of matrix metalloproteinases involved in collagen degradation.[23] Hcy-promoted cell growth and TIMP-1 expression, together with the reported induction of collagen synthesis in VSMCs treated with Hcy,[13,15] may lead to altered ECM remodeling, intimal fibrosis, and cardiovascular dysfunction. The control of ECM homeostasis is important in most tissues, but it is central to preserve liver function. Liver fibrosis occurs at the onset of most situations of chronic liver damage.[24] Hcy-induced $\alpha$1(I)procollagen expression in HSCs and TIMP-1 induction in HSCs and hepatocytes suggest that the previously mentioned profibrogenic effects of Hcy in the vascular bed could be extended to the liver tissue. Interestingly, in $CCl_4$-treated rats hyperhomocysteinemia develops before liver fibrosis,[10] and AdoMet treatment downregulates plasma Hcy levels and diminishes collagen deposition.[5] Hcy may thus cooperate in the onset of liver fibrosis potentiating the effect of other agents, such as ethanol and cytokines. In support of this hypothesis is the observation that the administration of a vitamin $B_{12}$/cobalt-defficient diet to lambs results in hyperhomocystinemia, which is accompanied by the development of liver steatosis and periportal fibrosis.[27]

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