Homocysteine Activates cAMP-response Element Binding Protein in HepG2 Through cAMP/PKA Signaling Pathway

Connie W.H. Woo, Yaw L. Siow, Karmin O

Objective—Hyperhomocysteinemia is an independent risk factor for cardiovascular disorders. Our previous studies demonstrated that hyperhomocysteinemia not only elicited inflammatory responses in the vascular endothelium but also induced fatty liver and hypercholesterolemia via transcriptional regulation. One of the transcription factors activated in the liver during hyperhomocysteinemia was cAMP-response element binding protein (CREB). CREB regulates the expression of many genes including those involved in lipid and glucose metabolism. In this study, we investigated the molecular mechanism by which Hcy activated CREB in rat liver and in hepatocytes (HepG2).

Method and Results—Hyperhomocysteinemia was induced in rats by feeding high-methionine diet for 4 weeks. There was a significant increase in hepatic cAMP levels, protein kinase A (PKA) activity and an activation of CREB. Incubation of HepG2 cells with Hcy (50 to 100 μmol/L) significantly enhanced CREB phosphorylation and subsequently increased CREB/DNA binding activity. PKA was activated in Hcy-treated cells as a result of increased cellular cAMP level. Inhibition of adenylyl cyclase not only reduced the intracellular cAMP levels elevated by Hcy treatment but also inhibited PKA activation and prevented Hcy-induced CREB phosphorylation.

Conclusion—These results suggest that the cAMP/PKA signaling pathway plays an important role in mediating Hcy-induced CREB activation in hepatocyte. (Arterioscler Thromb Vasc Biol. 2006;26:1043-1050.)

Key Words: cAMP ■ CREB ■ homocysteine ■ HMG-CoA reductase ■ PKA

Hyperhomocysteinemia is a condition of elevated homocysteine (Hcy) levels in the blood. Hcy is an intermediate amino acid formed during the metabolism of methionine. Hyperhomocysteinemia is recognized as an independent risk factor for cardiovascular and cerebrovascular disorders. Elevation of plasma Hcy levels is also associated with diseases that involve other organs. McCully observed signs of fatty liver in the necropsy of a pediatric patient died of hyperhomocysteinemia. In hyperhomocysteinemia mice, increased endoplasmic reticulum stress caused an excessive accumulation of lipid droplets in hepatocytes. It was reported that hyperhomocysteinemia caused by cystathionine β-synthase deficiency caused liver steatosis and fibrosis. Our previous studies demonstrated that hyperhomocysteinemia not only induced inflammatory responses in the vascular endothelium but also stimulated hepatic cholesterol biosynthesis. In diet-induced hyperhomocysteinemia rats, increased cholesterol biosynthesis caused a marked lipid accumulation in the liver and a significant elevation of cholesterol levels in the plasma. In another study, Hcy activated HMG-CoA reductase and increased cholesterol biosynthesis as well as secretion in human hepatocytes (HepG2). Activation of transcription factors was responsible for Hcy-induced HMG-CoA reductase gene expression in hepatocytes. One of the transcription factors activated in the liver during hyperhomocysteinemia was cAMP response element binding protein (CREB). CREB is a transcription factor that plays an important role in cell proliferation, hepatic lipid and glucose metabolism. CREB is a 43-kDa protein that belongs to the beta leucine zipper family of transcription factors. Several studies have suggested the importance of CREB activation in lipid metabolism in the liver. For example, CREB is an important coactivator of SREBPs for transcriptional regulation of HMG-CoA synthase and HMG-CoA reductase gene expression. It is generally believed that activation of CREB signaling pathway is the major mechanism responsible for the phosphorylation of CREB. Stimulation of adenylyl cyclase leads to an elevation of intracellular levels of cAMP which, in turn, activates PKA. Phosphorylation of CREB at serine-133 is essential for the activation of this transcription factor. Hormones such as glucagon or epinephrine can activate CREB through the cAMP/PKA signaling pathway. Although activation of the cAMP/PKA signaling pathway is regarded as an important mechanism for activation of CREB, recent studies indicate that PKA is not the only protein kinase that is responsible for phosphorylation and
activation of CREB.\textsuperscript{21} Other protein kinases such as extracellular signal regulating kinase (ERK) and p38-mitogen-activating protein (p38 MAP) kinase are also able to phosphorylate Ser-133 in CREB leading to activation of this transcription factor.\textsuperscript{21} Hcy, at pathologically high concentrations, was able to activate several protein kinase signaling pathways in mammalian cells.\textsuperscript{24,25} We observed that Hcy could activate p38 MAP kinase in vascular endothelial cells leading to increased chemokine expression.\textsuperscript{24} Another study showed that Hcy could activate the ERK2 cascade in vascular smooth muscle cell.\textsuperscript{23} Although CREB is found to be activated in rat hepatocytes during hyperhomocytostemia, it is not clear which protein kinase(s) signaling pathway is responsible for Hcy-induced activation of CREB.\textsuperscript{12} In the present study, we aimed to elucidate the mechanism by which Hcy induced activation of CREB in hyperhomocysteinemic rat liver and in cultured hepatocytes.

**Methods**

**Cell Culture**

HepG2 cells (American Type Culture Collection, Manassas, Va) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The HepG2, a cell line derived from human hepatoblastoma, is commonly used as a hepatocyte model to study metabolic regulation.\textsuperscript{16,27} Studies indicate that CREB regulates gene expression in HepG2 cells.\textsuperscript{19,28} L-Hcy was prepared by 3-Hcy thiolactone (Sigma-Alrich, St. Louis, Mo) to remove the thiolute group and then neutralized with HCl.\textsuperscript{12} Freshly prepared L-Hcy was used in all of the experiments.

**Animal Model**

Male Sprague-Dawley rats (Charles River Laborato ries, Wilmington, Mass) aged 8 weeks were divided into 2 groups and maintained for 4 weeks on the following diets: control diet (regular diet) consisting of Laboratory Diet Rodent Diet 5001 (PMI Nutrition International, St Louis, Mo) and high-methionine diet consisting of regular diet plus 1.7% (wt/wt) methionine. Each group consisted of 12 rats. The Hcy concentration in the serum was measured with the IMx Hcy assay (Abbott Diagnostics, Abbott Park, Ill).\textsuperscript{10–12} All procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals published by Canadian Council on Animal Care and approved by University of Manitoba Protocol Management and Review Committee.

**Electrophoretic Mobility Shift Assay and Supershift Assay**

Nuclear proteins were prepared from rat livers or HepG2 cells as described previously.\textsuperscript{11,12,28} Nuclear proteins were then incubated with \textsuperscript{32}P-end-labeled oligonucleotides containing the consensus sequence specific for the CREB/DNA binding site (5'-AGAGATGGCTGACGTAGAGACTAG-3') (Promega, Madison, Wis).\textsuperscript{12} The reaction mixture was separated in nondenaturing polyacrylamide gel (6%) followed by autoradiography. Supershift assay was performed by incubating nuclear proteins (2 \mu g) with anti-phospho-CREB (Ser 133) (Upstate USA Inc., Charlottesville, Va) antibodies for 1 hours at 4°C followed by 30 minutes at room temperature (21°C) before EMSA. To further confirm that the protein bound to the \textsuperscript{32}P-end-labeled oligonucleotides containing the consensus sequence specific for the CREB/DNA binding site was not other nuclear protein, nuclear proteins (2 \mu g) were incubated with monoclonal antibody specific against c-Rel (a subunit of NF-\kappa B) or polyclonal antibodies specific against c-Fos (a subunit of AP-1) (Santa Cruz Biotechnology, sc-6955X, sc-253X) before a supershift assay. In one set of experiment, the binding of labeled oligonucleotide to nuclear proteins was blocked by adding unlabeled oligonucleotide to the reaction mixture to confirm that binding of \textsuperscript{32}P end-labeled oligonucleotide to CREB was sequence-specific.

**Western Immunoblotting Analysis**

A portion of rat liver or HepG2 cells were homogenized in a lysis buffer.\textsuperscript{12} Equal amount of proteins prepared from different groups of samples were separated by electrophoresis on 10% SDS-polyacrylamide gel. Proteins in the gel were transferred to a nitrocellulose membrane. The membrane was probed with anti-CREB (Cell Signaling Technology), anti-phospho-CREB (Ser-133) (Cell Signaling Technology) or anti-HMG-CoA reductase (Upstate USA Inc) antibodies followed by incubation with peroxidase-conjugated secondary antibodies (Zymed, South San Francisco, Calif). Bands corresponding to the specific proteins were visualized using enhanced chemiluminescence reagent and exposed to Kodak X-Omat Blue XB-1 film. Films were analyzed with Bio-Rad Quantity One image analysis software (version 4.2.1). The same membranes were re-probed with anti-\beta-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, Calif) to confirm the equal loading of proteins for each sample.

**Determination of PKA Activity and cAMP Levels**

PKA activity was determined using a synthetic substrate, kemptide (Sigma-Aldrich).\textsuperscript{30} In brief, cells were lysed in the lysis buffer followed by centrifugation at 15 000 g for 10 minutes at 4°C.\textsuperscript{30} The supernatant (20 \mu g proteins) was incubated with 170 mmol/L kemptide, 0.1 mmol/L ATP (Cell Signaling Technology), and \textsuperscript{32}P-ATP (PerkinElmer, Boston, Mass) in a reaction buffer (pH 7.4) in a total volume of 25 \mu L for 15 minutes at 30°C.\textsuperscript{30} Samples were then spotted onto P-81 filter paper and washed 4 times with 0.4% phosphoric acid and once in 95% ethanol. Radioactivity of samples was measured by a scintillation counter. The cAMP levels in the liver or HepG2 cells were measured using DELFIA® cAMP kit (PerkinElmer).

**Determination of HMG-CoA Reductase Activity**

HMG-CoA reductase activity was measured by using [3-\textsuperscript{14}C]HMG-CoA as a substrate.\textsuperscript{12} The assay mixture contains liver homogenate (1 mg proteins) or HepG2 lysate (0.1 mg proteins), 20 mmol/L glucose 6-phosphate, 2.5 mmol/L NADP, 1 U glucose-6-phosphate dehydrogenase, 8 mmol/L dithiothreitol, 1.2 mmol/L EDTA, and 0.004 \mu Ci [3-\textsuperscript{14}C]HMG-CoA (PerkinElmer) in a phosphate buffer (pH 7.4). The reaction was performed at 37°C for 60 minutes followed by adding mevalonolactone and HCl. Radiolabeled HMG-CoA and mevalonolactone were separated by thin-layer chromatography in chloroform-acetone (2:1, vol/vol). The location of mevalonolactone on the chromatographic plate was visualized after staining with iodine vapor. The radioactivity associated mevalonolactone was measured with a scintillation counter.

**Determination of HMG-CoA Reductase mRNA**

Total RNAs were isolated from liver tissue with Trizol reagent (Invitrogen, Calsbad, Calif). The HMG-CoA reductase mRNA expression was determined by semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) analysis. In the RT reaction, 2 \mu g of total RNA was converted to cDNA. The cDNA with a sequence specific for HMG-CoA reductase was further multiplied by using Taq-DNA polymerase. The PCR product was separated by electrophoresis in a 1.5% agarose gel and visualized under UV light with a gel documentation system (Bio-Rad). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard to verify equal PCR product loading for each experiment. The values were expressed as a ratio of HMG-CoA reductase to GAPDH mRNA.

**Statistical Analysis**

The results were analyzed using 2-tailed independent Student t test. The level of statistical significance was set at \textit{P}<0.05.
Results

Phosphorylation and Activation of CREB in the Liver of Hyperhomocysteinemia Rat

Hyperhomocysteinemia was induced in rats fed a high-methionine diet for 4 weeks. A significant increase in serum Hcy concentrations was detected in this group of rats (25.32±2.85 μmol/L versus 4.06±0.49 μmol/L in control rats). To determine the levels of phosphorylated CREB protein in the liver, Western immunoblotting analysis was performed using specific antibodies that recognized phospho-CREB at Ser-133. There was a significant increase in the level of phosphorylated CREB in the livers isolated from hyperhomocysteinemia rats (Figure 1A). The level of the total CREB protein was not altered in these liver samples (Figure 1A). To confirm whether phosphorylation of CREB would lead to its activation, EMSA was performed. There was a significant increase in the binding activity of CREB to DNA (Figure 1B). Addition of anti-phospho-CREB antibodies resulted in a supershift of the CREB/DNA complex (Figure 1C). A stronger shift was obtained when more anti-phospho-CREB antibodies were used (8 μL versus 4 μL) (Figure 1C).

To further characterize the nuclear protein/DNA complex in the EMSA, antibodies specific against other nuclear proteins were added. Incubation of nuclear proteins with anti- c-Rel antibody or anti- c-Fos antibody did not induce a shift of nuclear protein/DNA complex in the gel (Figure 1C). These results indicated that CREB was activated in the liver during hyperhomocysteinemia. There was 46% increase in PKA activity in the livers of hyperhomocysteinemia rats as compared with the control group (Figure 1D). The hepatic levels of cAMP in hyperhomocysteinemia rats were markedly elevated (105.72±24.64 pmol/mg protein versus 39.23±8.31 pmol/mg protein in control rats).

Effect of Hcy on Phosphorylation and Activation of CREB in Hepatocytes

The effect of Hcy on CREB activation was further examined in HepG2 cells. Hcy treatment induced phosphorylation of CREB in a concentration-dependent manner (Figure 2A). However, such a treatment did not cause a significant change in total CREB protein levels (Figure 2B). The induction of CREB phosphorylation was observed in cells incubated with Hcy for 15 to 120 minutes (Figure 3A). Upon phosphorylation, there was a significant increase in the CREB/DNA binding activity in Hcy-treated cells (Figure 3B). These results suggested that Hcy was able to induce phosphorylation and subsequently activation of CREB in hepatocytes. Both Hcy and cysteine contain sulfhydryl group. Cysteine treatment did not alter the total CREB protein levels but caused an increase in the phosphorylated CREB protein level in HepG2 cells (Figure 2C). However, cysteine (100 μmol/L)
Mechanism of Hcy-Induced CREB Activation in Hepatocytes

To investigate whether the PKA signaling pathway was responsible for Hcy-induced CREB activation, a PKA inhibitor, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89, Calbiochem, San Diego, Calif), was added to cultured cells. Pretreatment of cells with H89 completely inhibited Hcy-induced CREB activation (Figure 3C), indicating that PKA signaling pathway might mediate Hcy-induced CREB activation. Next, to investigate whether other protein kinases might also be involved in CREB activation, cells were pretreated treatment did not increase CREB/DNA binding activity (data not shown).

Mechanism of Hcy-Induced CREB Activation in Hepatocytes

To investigate whether the PKA signaling pathway was responsible for Hcy-induced CREB activation, a PKA inhibitor, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89, Calbiochem, San Diego, Calif), was added to cultured cells. Pretreatment of cells with H89 completely inhibited Hcy-induced CREB activation (Figure 3C), indicating that PKA signaling pathway might mediate Hcy-induced CREB activation. Next, to investigate whether other protein kinases might also be involved in CREB activation, cells were pretreated
with PD98059 (Cell Signaling Technology), an inhibitor for extracellular signal regulating kinase 1/2 (ERK) or SB203580 (Calbiochem), an inhibitor for p38 MAP kinase before incubation with Hcy. Pretreatment of cells with PD98059 or SB203580 could not prevent Hcy-induced CREB activation in these cells (Figure 3C). These results suggested that ERK and p38 MAP kinase signaling pathways might not be involved in Hcy-induced CREB activation in hepatocytes.

To further investigate the mechanism by which the PKA signaling pathway was involved in Hcy-induced CREB activation, the activity of PKA was measured. There was a significant increase in PKA activity in cells incubated with Hcy (100 μmol/L) for 30 minutes (Figure 4A). Preincubation of cells with H89 completely abolished Hcy-stimulated PKA activity (Figure 4A). Cysteine treatment did not affect the PKA activity in HepG2 cells (Figure 4A). PKA is cAMP-dependent protein kinase and its activation depends on the elevation of intracellular cAMP levels.31 Therefore, the levels of cAMP were measured in cells before and after Hcy treatment. The cellular levels of cAMP were markedly elevated after incubation with Hcy for 10 to 15 minutes (Figure 4B). The classical mechanism to elevate intracellular cAMP concentrations is mediated via the activation of adenyl cyclase.32 To further demonstrate the involvement of the cAMP/PKA signaling pathway in Hcy-induced CREB activation in hepatocytes, cells were preincubated with adenyl cyclase toxin (ACT) (Calbiochem), a specific inhibitor for adenyl cyclase. Inhibition of adenyl cyclase effectively reduced the cellular cAMP content to the basal levels (Figure 5A) as well as inhibited Hcy-induced PKA activation (Figure 5B). Furthermore, inhibition of cAMP production by ACT completely abolished Hcy-induced phosphorylation of CREB in these cells (Figure 5C). Taken together, these results suggested that Hcy-stimulated CREB was mediated via activation of adenyl cyclase/PKA signaling pathway.

CREB is one of the important transcriptional factors for lipid metabolism.12,18–20 The HMG-CoA reductase activity was significantly increased in the livers of hyperhomocysteinemia rats (0.91±0.10 pmol/mg per minute versus 0.58±0.11 pmol/mg per minute in control rats). There was a significant elevation of HMG-CoA reductase mRNA and protein levels in the same livers (Figure 6A and 6B). The activity of HMG-CoA reductase was also increased in
Hcy-treated HepG2 cells (Figure 6C). Inhibition of adenylyl cyclase activity by ACT or inhibition of PKA activation by H89 completely abolished Hcy-induced HMG-CoA reductase activation (Figure 6C) and reduced the reductase protein to the control level (Figure 6D).

**Discussion**

The novel finding of this study is that Hcy-induced activation of CREB in hepatocytes is mediated via cAMP/PKA signaling pathway. The in vivo results have clearly demonstrated an elevation of cAMP levels and activation of CREB in the livers of hyperhomocysteinemia rats. Treatment of hepatocytes with Hcy elicits a significant elevation of intracellular cAMP levels followed by PKA activation. The activation of the cAMP/PKA signaling pathway, in turn, is responsible for enhanced CREB phosphorylation and subsequently its activation on Hcy treatment.

CREB is a transcription factor that has diverse functions in various tissues. In our previous study, activation of CREB along with other transcription factors in hepatocytes was found to contribute to increased gene expression of HMG-CoA reductase and cholesterol biosynthesis in the livers of hyperhomocysteinemia rats. Another study suggested that activation of CREB might contribute to insulin resistance in individuals susceptible to diabetes. CREB requires phosphorylation at its Ser-133 to enhance the transcription of its target genes that contain the cAMP response element in the promoter region. Several protein kinases are known to phosphorylate CREB such as PKA, ERK, and p38 MAP kinase. The type of protein kinases that phosphorylate as well as activate CREB depends on the type of stimuli and the type of cells. The PKA signaling pathway is thought to play a major role in CREB activation in many types of cells. Activation of PKA is regulated by changes in the intracellular cAMP levels. An elevation of intracellular cAMP levels can be caused by increased activity of adenylyl cyclase and/or decreased activity of phosphodiesterases. In the present study, several lines of evidence support the notion that the activation of adenylyl cyclase and cAMP/PKA signaling pathway play a major role in Hcy-induced activation of CREB in hepatocytes. First, Hcy treatment caused a significant elevation in the intracellular cAMP levels, which preceded the activation of PKA. Second, treatment of cells with an adenylyl cyclase inhibitor completely reversed Hcy-induced elevation of cellular cAMP levels and PKA activation. Inhibition of the cAMP/PKA signaling pathway also abolished Hcy-induced CREB phosphorylation. Third, inhibition of other protein kinase activities such as ERK or p38 MAP kinase could not block Hcy-induced CREB activation. Furthermore, a significant increase in hepatic cAMP levels, PKA activity and CREB activation was found in rats during hyperhomocysteinemia. Taken together, these results suggest that Hcy-induced elevation of cellular cAMP levels activates PKA that, in turn, phosphorylates and subsequently activates CREB. Hcy is a sulfhydryl-containing amino acid and some of its pathological effects may be mediated via a sulfhydryl-dependent mechanism.
treatment of HepG2 cells with another sulphhydril-containing amino acid (cysteine) failed to activate PKA as well as CREB. These results suggest that Hcy-induced CREB activation via PKA signaling pathway may be mediated through sulphhydril-independent process.

The cAMP is an important second messenger that involves a wide range of cellular processes and gene regulations. Studies have shown a consistently elevated basal cAMP level in the liver during diabetes. In the liver, the cAMP/CREB signaling pathway regulates the expression of key genes in glucose metabolism as well as in lipid metabolism. It is plausible that an alteration of intracellular cAMP levels may serve as one of the important mechanisms by which Hcy impairs lipid metabolism in the liver. We previously reported that activation of CREB was necessary for Hcy-induced HMG-CoA reductase expression and increased cholesterol biosynthesis in the liver.

In the present study, HMG-CoA reductase activity was elevated in the livers of hyperhomocysteinemia rats as well as in Hcy-treated HepG2 cells. Inhibition of adenyl cyclase or PKA activity completely abolished Hcy-induced HMG-CoA reductase activation. There results suggest that there is a direct link between Hcy-induced cAMP/PKA signaling and lipid metabolism via HMG-CoA reductase.

In summary, the present study for the first time to our knowledge clearly illustrates how CREB is activated in the livers of hyperhomocysteinemia rats and in hepatocytes. The in vivo and in vitro results suggest that activation of the cAMP/PKA signaling pathway is responsible for Hcy-induced CREB phosphorylation and subsequently its activation. Understanding the molecular mechanisms by which Hcy activates transcription factors may advance our knowledge on how to prevent and treat patients with hyperhomocysteinemia.

Acknowledgments

This study was supported by grants from the Heart & Stroke Foundation and the Natural Sciences & Engineering Research Council of Canada.

References

19. Roesler WJ, Graham JG, Koelen R, Klemm DJ, McFie PJ. The cAMP response element binding protein synergizes with other transcription


Homocysteine Activates cAMP-response Element Binding Protein in HepG2 Through cAMP/PKA Signaling Pathway
Connor W.H. Woo, Yaw L. Siow and Karmin O

Arterioscler Thromb Vasc Biol. 2006;26:1043-1050; originally published online February 23, 2006;
doi: 10.1161/01.ATV.0000214981.58499.32

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/26/5/1043

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/