Homocysteine Induces Monocyte Chemoattractant Protein-1 Expression in Hepatocytes Mediated via Activator Protein-1 Activation*

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Hyperhomocysteinemia is characterized by abnormally high concentrations of homocysteine (Hcy) in the plasma. It is a metabolic disorder associated with dysfunction of several organs such as atherosclerosis, altered lipid metabolism, and liver injury. In this study we investigated the effect of Hcy on transcriptional regulation of monocyte chemoattractant protein-1 (MCP-1), a potent chemokine, expression in hepatocytes. Hyperhomocysteinemia was induced in rats by a high-methionine diet for 4 weeks. MCP-1 mRNA and protein levels were significantly elevated in the liver tissue homogenate and in hepatocytes of hyperhomocysteinemic rats. The role of transcription factors in MCP-1 expression was examined by electrophoretic mobility shift assay. Activation of activator protein (AP)-1 but not nuclear factor-κB was detected in the liver tissue of those rats. Incubation of rat hepatocytes with Hcy (50–200 μM) caused a significant increase in AP-1 activation followed by an increase in intracellular MCP-1 mRNA expression and an elevation of MCP-1 protein secreted into the culture medium. Hcy markedly increased the DNA binding activity of human recombinant AP-1 (c-Fos and c-Jun proteins). The presence of a sulphydryl group in Hcy was essential for Hcy-induced AP-1 activation. When hepatocytes were transfected with decoy AP-1 oligodeoxynucleotide to inhibit AP-1 activation, Hcy-induced MCP-1 mRNA expression was abolished. Further analysis revealed that increased hepatic MCP-1 expression was positively correlated with the serum MCP-1 level. These results suggest that Hcy-induced MCP-1 expression in the liver is mediated via AP-1 activation, which may contribute to chronic inflammation associated with hyperhomocysteinemia.

Homocysteine (Hcy)2 is a sulphydryl group containing amino acid formed during the metabolism of methionine. The metabolism of methionine to Hcy generates a methyl group that serves as an important methyl donor in cell function (1). The cellular homeostasis of Hcy is tightly regulated under physiological conditions. Factors that perturb the step(s) in the Hcy metabolic pathway(s) cause an increase in its cellular levels and lead to hyperhomocysteinemia (plasma Hcy level above 16 μM) (2). Hyperhomocysteinemia is a metabolic disorder that is regarded as an independent risk factor for cardiovascular and cerebral vascular disorders (3, 4). A significant proportion of patients (up to 30%) with coronary artery disease have displayed elevated blood Hcy levels (5). Increasing evidence suggests that hyperhomocysteinemia is also associated with dysfunction of other organs (6–10). Apart from atherosclerosis, moderately fatty liver (lipid accumulation) has been observed in the autopsy of pediatric patients with hyperhomocysteinemia (11, 12). Subsequent studies indicate that hyperhomocysteinemia can induce oxidative stress and fatty liver in experimental animal models (7–9, 13). It is increasingly recognized that abnormal lipid metabolism and inflammatory responses in the liver can elicit systemic inflammation, which in turn contributes to the development of cardiovascular disease (14–16). Nonalcoholic fatty liver disease is characterized by fat accumulation and inflammation in the liver, which is not caused by alcohol consumption (17). It is accompanied by increased serum levels of inflammatory markers and chemokines (14). An elevation of monocyte chemoattractant protein-1 (MCP-1) has been detected in the serum and the liver tissue in patients with nonalcoholic fatty liver disease (14). It is postulated that elevation of MCP-1 production in the liver may contribute to systemic inflammation, which in turn affects cardiovascular function. MCP-1 is a potent chemokine for monocyte recruitment into the intima of the arterial wall or the other inflamed tissues (18, 19). Higher plasma MCP-1 levels (20–35% increase) have been found in patients with stable angina pectoris, vasospasm, and acute coronary syndrome (20). It is suggested that an increase in the plasma MCP-1 level would increase the risk of myocardial infarction and death during acute coronary syndrome (21). In our recent study the expression of MCP-1 and several adhesion molecules are found to be elevated in the aorta of hyperhomocysteinemic rat (22). Two transcription factors, namely, nuclear factor-κB (NF-κB) and activator protein (AP-1), play important roles in regulating MCP-1 expression in mammalian cells (23, 24). AP-1 and NF-κB are the redox-sensitive transcription factors regulating the expression of genes that are involved in apoptosis and cell proliferation in the liver (25, 26). In human and...
rat genomes, the binding site for NF-κB is present in the enhancer region of MCP-1 gene, whereas the binding site for AP-1 is present in the promoter region (23, 24). Activation of both or either one of these transcription factors can elicit MCP-1 gene expression (27–29). Both AP-1 and NF-κB are present in hepatocytes and can be activated in response to various stimuli (30, 31). Hcy-induced MCP-1 expression in vascular smooth cells and monocytes/macrophages appears to be mediated via NF-κB activation (29, 32). The effect of Hcy on AP-1 activation varies in different types of cells. For examples, Hcy inhibits AP-1 activation in endothelial cells and fibroblasts (33, 34), whereas induces AP-1 activation in macrophages (35). Results from our previous study demonstrate that Hcy, at elevated concentrations, is able to stimulate cholesterol biosynthesis in hepatocytes leading to lipid accumulation in the liver (8). However, it is not known if Hcy can induce inflammatory responses such as MCP-1 expression in hepatocytes.

The aim of this study was to investigate the effect of Hcy on transcriptional regulation of MCP-1 expression in the liver tissue of hyperhomocysteinemic rat. Our data revealed that Hcy could induce AP-1 activation and enhance MCP-1 production in hepatocytes. Inhibition of AP-1 binding to DNA by decoy oligodeoxynucleotide abolished Hcy-induced MCP-1 expression in hepatocytes. The in vitro protein-oligonucleotide binding assay showed that Hcy was able to directly increase the DNA binding of recombinant AP-1 (c-Fos and c-Jun proteins). Furthermore, a positive correlation between the liver MCP-1 expression and the serum MCP-1 level was observed in hyperhomocysteinemic rats. Our results suggest that Hcy-induced MCP-1 expression in the liver is mediated via AP-1 activation.

**EXPERIMENTAL PROCEDURES**

Reagents—The wild type AP-1 and scrambled decoy oligodeoxynucleotides were synthesized by Sigma-Genosys. The forward and reverse primers for amplification of MCP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were synthesized by Invitrogen. Human recombinant c-Fos, c-Jun, p50, and p65 were purchased from Active Motif (Carlsbad, CA).

Animal Model—Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) aged 8 weeks were fed the following types of diet for 4 weeks; 1) a control diet consisting of Lab Diet Rodent Diet 5001 (regular diet containing 0.43% methionine and 0.32% cysteine, wt/wt) (PMI Nutrition International, St Louis, MO), 2) a high methionine diet consisting of regular diet plus 1.7% (wt/wt) methionine, and 3) a high cysteine diet consisting of regular diet plus 1.2% (wt/wt) cysteine as described previously (8, 9, 22, 36, 37). Each group consisted of 12 rats. Our previous studies demonstrated that hyperhomocysteinemia could be induced in rats by feeding a high methionine diet for 4 weeks (8, 9, 22, 36, 37). Hcy and cysteine concentrations in serum and livers were measured by using the IMx Hcy assay and high performance liquid chromatography, respectively (Abbott Diagnostics, Abbott Park, IL) (8, 9, 22, 36, 37). 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 the University of Manitoba Protocol Management and Review Committee.

Cell Culture—Primary hepatocytes were isolated from rat liver as described previously (8). Briefly, liver was perfused with phosphate-buffered saline via the portal vein at 3 ml/min for 15 min to remove blood cells followed by disassociation with 0.05% type I collagenase (Sigma-Aldrich) in Hanks’ buffered salt solution. Hepatocytes were harvested, filtered aseptically, and collected using differential centrifugation at low speed, 50 × g. The trypan blue test showed a typical >95% viability of hepatocytes isolated by this method. Hepatocytes were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Invitrogen). Hepatocytes were incubated for 24 h post-isolation before experiments (8, 31).

Preparation of l-Hcy—l-Hcy was prepared from l-Hcy thiolactone (Sigma-Aldrich) by a method described previously (8, 37). In brief, l-Hcy thiolactone was dissolved and hydrolyzed in NaOH (5 M) to remove the thiolactone group (8, 37). The preparation was then neutralized with HCl. The concentration of l-Hcy in the preparation was quantified with the IMx Hcy assay. Freshly prepared l-Hcy was used in all the experiments.

Decoy Oligodeoxynucleotide (ODN) Transfection—Double-stranded ODNs (Sigma-Genosys) were prepared from complementary single-stranded phosphorothioate-bonded ODNs by melting at 95 °C for 5 min followed by a cool-down phase of 3 h to room temperature (38). The sequence of the single-stranded ODNs were 5′-CGGTGATGACTCAAGCCGGAA-3′ for wild type AP-1 and 5′-CATCTGTCACTGCGCTC-3′ for scrambled ODN (underlined letters denote phosphorothioate-bonded bases, and bold letters denote the consensus binding site) (39). In brief, after the attachment of hepatocytes onto culture plates, cells were treated with 100 nM ODN with 4 μl of Oligofectamine® (Invitrogen) in fetal bovine serum-free medium for 4 h. After the delivery of ODN into hepatocytes, the medium containing the decoy ODN was replaced with fresh medium with 10% fetal bovine serum. Hepatocytes were incubated for another 20 h before experiments.

Reporter Plasmid Transfection and Luciferase Reporter Assays—A 144-bp rat MCP-1 promoter 5′-flanking region (~90 to +55) (GenBank™/EMBL accession number AF079313) with either a wild type (pGL3-AP-1, TGA TCCAC) or a mutated (pGL3-AP-1-Mutant, TGG TACCAC) AP-1 element (Integrated DNA Technology, Inc., Coralville, IA) was inserted into pGL3 luciferase reporter vectors (Promega, Madison, WI) (24). Hepatocytes were transfected with pGL3-AP-1 mutant, pGL3-AP-1 (wild type), or pGL3 vector (control) using Lipofectamine 2000 (Invitrogen) for 18 h. After transfection, cells were incubated with Hcy for 5 h followed by measuring the luciferase activity in the cell lysate using a luciferase assay kit (Promega) and a luminometer.

Chromatin Immunoprecipitation Assay (ChIP)—ChIP was performed as previously described (40). In brief, cross-linking of hepatic DNA was performed by treating a portion of liver with 1% formaldehyde. Nuclei were isolated, and sheared chromatin was prepared by sonication. The sheared chromatin was immunoprecipitated with anti-c-Fos and anti-c-Jun antibodies. Another aliquot of sheared chromatin without incubation with antibodies was prepared to normalize the amount of total DNA
subjected to ChIP assay (termed mock IP). DNA was then isolated from the immunoprecipitate, and PCR was performed using primers specific for the MCP-1 promoter region with AP-1 binding consensus sequence. The sequences of the primers (GenBank\textsuperscript{TM}/EBI accession number AF079313) were 5’-CCCAGTAGTGGCTGGAAAAA-3’ (forward) and 5’-TTGGTAAGCCAGAGGTGGAG-3’ (reverse).

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear proteins were prepared from rat livers or hepatocytes as previously described, and activation of AP-1 or NF-κB was determined by EMSA (8, 37). Nuclear proteins (10 μg) were incubated with excess 32P-end-labeled oligonucleotides containing the consensus sequence specific for the AP-1/DNA binding site or NF-κB/DNA binding site (Promega) in a binding buffer (8, 36, 37, 41). The reaction mixture was then separated in nondenaturing polyacrylamide gel (6%) and dried on a piece of filter paper followed by autoradiography. The specificity of binding of 32P-labeled AP-1 or NF-κB consensus sequence was examined by competition with 100-fold excessive unlabeled oligonucleotide or 32P-labeled mutant AP-1 consensus sequence (Santa Cruz Biotechnology, Santa Cruz, CA) (8, 36, 37). The supershift assay was performed by preincubating 2 μg of nuclear protein with 12 μg of c-Fos and/or c-Jun antibodies (Santa Cruz Biotechnology, sc-253X and sc-45X) followed by nondenaturing polyacrylamide gel (4%) electrophoresis (37). For the in vitro binding assay, recombinant c-Fos and/or c-Jun or p50 and/or p65 (Active Motif) were incubated in the presence or absence of Hcy or cysteine in the binding buffer for 15 min at 25 °C before the addition of 32P-labeled oligonucleotide (41). The autoradiograph was analyzed with UN-SCAN-IT gel (Silk Scientific Inc.). For the analysis of kinetic parameters of c-Fos/c-Jun binding to DNA, the area of the AP-1 bands and free probe on the filter paper was cut out according to the autoradiograph image, and radioactivity corresponding to each band was measured using a liquid scintillation counter. The equilibrium dissociation constant (K\textsubscript{D}) of c-Fos/c-Jun dimer binding to DNA was calculated by Lineweaver-Burk plot analysis (42). Preliminary data showed that c-Fos/c-Jun dissociation followed first order kinetics. To calculate the dissociation constant (k\textsubscript{off}), a single-exponential dissociation rate equation [AP-1] = [AP-1]\textsubscript{eq}e^{-k\textsubscript{cat}t} was applied. The slope of the semi-log plot of the natural log of [AP-1] /[AP-1]\textsubscript{eq} versus time (t) represented the dissociation constant of c-Fos/c-Jun dimer. Association constant (k\textsubscript{on}) was calculated based on the equation K\textsubscript{D} = k\textsubscript{off}/k\textsubscript{on}.

Western Immuno blot Analysis—An aliquot of nuclear proteins (10 μg) prepared for the EMSA assay was subjected to Western immunoblot analysis (8, 37). In brief, the nuclear proteins were separated by electrophoresis in a 15% SDS-polyacrylamide gel. Proteins in the gel were transferred to a nitrocellulose membrane that was probed with anti-histone antibodies (Santa Cruz Biotechnology).

Reverse Transcription (RT)-PCR and Ribonuclease Protection Assay—The MCP-1 mRNA expression was determined by semiquantitative RT-PCR analysis and ribonuclease protection assay (37, 43). In brief, total RNAs were isolated from liver tissue or hepatocytes with TriZol reagent (Invitrogen). For RT-PCR analysis, 2 μg of total RNA was converted to cDNA. The cDNA with a sequence specific for MCP-1 was further amplified by using Taq-DNA polymerase. The sequences of the primers specific for rat MCP-1 (GenBank\textsuperscript{TM}/EBI accession number NM_031530) were: 5’-ATGCAGGTCTCTGTACGCTT-3’ (forward) and 5’-CTCTGTACGCTTACCTTC-3’ (reverse). The PCR product was separated by electrophoresis in a 1.5% agarose gel and visualized using ethidium bromide under UV light with a gel documentation system (Quantity One analysis software, Bio-Rad). GAPDH was used as an internal standard to verify equal RT product loading for each experiment. Ribonuclease protection assay was performed with the BD Ribonquant\textsuperscript{TM} Multi-Probe RNase Protection Assay System (BD Biosciences). Briefly, total RNA (10 μg) was hybridized with a 32P-end-labeled MCP-1 oligonucleotide probe (171 bp) overnight at 56 °C followed by ribonuclease digestion to remove nonhybridized probe. After digestion, the protected fragments (142 bp) were resolved on a denaturing 4.75% polyacrylamide gel containing 8 M urea followed by autoradiography. GAPDH oligonucleotide probe (126 bp, protected fragment 97 bp) was used as an internal control. The values were expressed as a ratio of MCP-1 to GAPDH mRNA.

Determination of MCP-1 Protein Concentration—MCP-1 protein expression in the liver, hepatocytes, culture medium, or serum was quantified using a commercial solid-phase enzyme-linked immunosorbent assay kit (BIOSOURCE, Carmarillo, CA) according to the manufacturer’s instructions. In brief, a portion of liver or hepatocytes were first homogenized in Laemmli buffer. Supernatant was collected after centrifugation at 3000 × g for 10 min at 4 °C. Samples (homogenate, culture medium, or serum) were placed in a 96-well plate coated with anti-MCP-1 antibodies followed by incubation first with biotinylated anti-MCP-1 antibodies and, second, with streptavidin-conjugated horseradish peroxidase. Tetramethylbenzidine was then added to react with peroxidase to elicit colorimetric signal. It was confirmed that Laemmli buffer alone did not elicit any colorimetric signal.

Statistical Analysis—Results were analyzed by a two-tailed Student\textapos;s t test or using one-way analysis of variance followed by Pearson correlation test. p values less than 0.05 were considered as statistically significant.

RESULTS

Elevation of MCP-1 Protein Levels in the Liver and Serum during Hyperhomocysteinemia—Hyperhomocysteinemia was induced in rats fed a high methionine diet for 4 weeks as described in our previous studies (8, 9, 22, 36, 37). The serum Hcy concentration was significantly elevated in this group of rats (Table 1). The Hcy concentration in the liver tissue of

### TABLE 1

|               | Control | HHcy |
|---------------|---------|------|
| Serum Hcy (μM) | 4.59 ± 0.37 | 35.05 ± 3.65$^a$ |
| Liver Hcy (nmol/g) | 3.74 ± 0.21 | 12.23 ± 0.97$^a$ |
| Serum MCP-1 protein (ng/ml) | 67.27 ± 6.13 | 84.00 ± 2.72$^a$ |
| Liver MCP-1 protein (ng/mg) | 502.19 ± 33.22 | 625.83 ± 48.34$^a$ |

$^a$ p < 0.05 when compared with the control value.
Increased MCP-1 mRNA Level and AP-1 Activation in the Liver—The level of MCP-1 mRNA was significantly elevated in the livers of hyperhomocysteinemic rats (Fig. 1A). To determine whether NF-κB and/or AP-1 were activated in the liver during hyperhomocysteinemia, nuclear proteins were prepared from the liver tissue, and EMSA was performed. The binding activity of AP-1 to the 32P-labeled consensus oligonucleotide was significantly increased in the liver tissue of hyperhomocysteinemic rats (Fig. 1B). However, the binding activity of NF-κB to the 32P-labeled consensus oligonucleotide was not significantly changed (Fig. 1B). To further confirm the specificity of AP-1 binding to the 32P-labeled consensus oligonucleotide, a 100-fold excess of unlabeled AP-1 consensus oligonucleotides were added to the nuclear extracts. Unlabeled AP-1 consensus oligonucleotides at an excessive amount competed with 32P-labeled AP-1 probe leading to an absence of 32P-labeled AP-1-DNA complex in the EMSA gel (Fig. 1C, lane 1 and 2). In another set of samples, nuclear proteins isolated from the liver tissue were incubated with 32P-labeled AP-1 mutant oligonucleotides. No 32P-labeled AP-1-DNA complex was detected in the EMSA gel (Fig. 1C, lane 3 and 4). Furthermore, to identify the proteins in the AP-1-DNA complex in the EMSA, a supershift assay was performed using either anti-c-Fos or anti-c-Jun antibodies. The nuclear extract of hyperhomocysteinemic rat liver preincubated with anti-c-Fos and anti-c-Jun antibodies resulted in a slower migration of the protein-DNA complex (Fig. 1D). These results suggested that MCP-1 expression and AP-1-DNA binding activity were increased in the liver during hyperhomocysteinemia.

Increased MCP-1 Expression in Hepatocytes—To further elucidate the mechanism by which MCP-1 expression was increased in the liver during hyperhomocysteinemia, experiments were performed in rat hepatocytes. A significantly higher level of MCP-1 mRNA was found in hepatocytes isolated from hyperhomocysteinemic rats (Fig. 2A). Next, hepatocytes were isolated from the control rats (fed a regular diet) and then incubated with Hcy (10–200 μM), stimulated MCP-1 mRNA expression in hepatocytes (Fig. 2B). Such a stimulatory effect reached a peak at 8 h of incubation (Fig. 2C). Enzyme-linked immunosorbent

(HHcy) (n = 12 for each group). A, MCP-1 mRNA levels were measured by ribonuclease protection assay and expressed as a ratio of MCP-1 to GAPDH mRNA. B, the activation of AP-1 and NF-κB was determined by EMSA. Histone in the nuclear content detected by Western immunoblotting analysis served as a sample loading control for EMSA. Results were expressed as mean ± S.E. *, p < 0.05 when compared with the control value. C, nuclear proteins prepared from the liver tissue were incubated with 32P-labeled wild type or mutant AP-1 oligonucleotide or unlabeled wild type AP-1 oligonucleotide (Lanes 1, 3, and 5) control liver; (lanes 2, 4, and 6, HHcy liver). D, nuclear proteins prepared from the liver tissue were incubated with c-Fos or and c-Jun antibodies (Ab) for supershift assay. As a negative control, supershift assay was performed using nonspecific rat IgG.
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![Graphs and images]

assay detected a significantly higher amount of MCP-1 protein secreted into the culture medium by hepatocytes incubated with Hcy (50 μM) for 12 h (1.88 ± 0.16 ng/mg of cell protein produced by Hcy-treated cells versus 1.43 ± 0.069 ng/mg of cell protein produced by control cells). These results suggested that Hcy was able to stimulate MCP-1 production in hepatocytes.

AP-1 Activation in Hepatocytes—There was a significant increase in AP-1 activation in hepatocytes incubated with Hcy for 15–60 min (Fig. 3A), but no change in NF-κB activation (Fig. 3B). Hcy is a sulfhydryl group-containing amino acid. To examine whether the stimulatory effect of Hcy on AP-1 activation and, subsequently, MCP-1 expression was associated with its sulfhydryl group, another amino acid-containing sulfhydryl group (cysteine) was added to cultured hepatocytes. Cysteine treatment caused AP-1 activation (Fig. 4A) and an increased MCP-1 mRNA expression in hepatocytes (Fig. 4B). Cysteine treatment had no effect on NF-κB activation in hepatocytes (Fig. 4A). Interestingly, there was no significant increase in AP-1 or NF-κB activation in the liver tissue of rats fed a high cysteine diet (Fig. 5A). Furthermore, there was no change in MCP-1 protein (Fig. 5B) and mRNA levels (Fig. 5C) in the livers of rats fed a high cysteine diet. This might be due to the fact that there was no significant elevation of cysteine level in the serum (194.54 ± 9.12 μM in cysteine-fed rats versus 177.98 ± 5.78 μM in control rats) and in the liver tissue (258.97 ± 27.01 nmol/g in...
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expression in hepatocytes (Fig. 6, A and B). To confirm that Hcy was able to directly stimulate AP-1 binding to the promoter region of MCP-1 gene, hepatocytes were transfected with the following plasmids (Fig. 7A); 1) pGL3-AP-1 containing wild type AP-1 binding site in MCP-1 promoter region (TGACTC-CAC), 2) pGL3-AP-1 mutant containing a point mutation (TGGTACCAC) in AP-1 binding site in the MCP-1 promoter region, and 3) pGL3 luciferase reporter vector. The transfected cells were incubated with Hcy, and the luciferase activity was determined. Hcy treatment resulted in a 25-fold increase in luciferase activity in hepatocytes transfected with pGL3-AP-1 (Fig. 7B). The luciferase activity was unchanged in hepatocytes transfected with either pGL3-AP-1 mutant or pGL3 vector upon Hcy treatment (Fig. 7B). To further investigate whether Hcy stimulated the interaction between AP-1 and MCP-1 promoter, a ChIP assay was performed in the liver tissue. There was a significant increase in the expression of the MCP-1 promoter region associated with c-Fos/c-Jun proteins in hyperhomocysteinemic rat livers as compared with the control (Fig. 7C).

Effect of Hcy on DNA Binding Activity of Recombinant c-Fos and c-Jun Proteins—AP-1 can exist as a homodimer or a heterodimer that consists of Fos, Jun, or activating transcription factor (44, 45). Among those protein dimers, Fos and Jun form a thermostable heterodimer that most efficiently interacts with DNA binding sites (44, 45). To further explore the mechanism by which Hcy-induced AP-1 activation, the direct effect of Hcy on DNA binding of AP-1 was examined. Human recombinant c-Fos and c-Jun proteins were incubated with Hcy, and the EMSA was performed. As shown in Fig. 8A, incubation with Hcy markedly increased the DNA binding activity of the c-Fos and c-Jun proteins. Similar effect was observed when cysteine was added (Fig. 8A). Interestingly, such a stimulatory effect was lost when oxidized Hcy was used (Fig. 8A). To further investigate the mechanism by which Hcy affects the binding of Fos-Jun to DNA motif, Hcy was incubated with the recombinant c-Fos or c-Jun followed by EMSA. Results showed that in contrast to the Fos-Jun heterodimer, the binding activity of c-Fos homodimer or c-Jun homodimer to DNA was not stimulated by Hcy (Fig. 8B). Next, the kinetic parameters of the interaction between the c-Fos/c-Jun dimer and DNA were determined. In brief, nuclear proteins were incubated with 32P-labeled AP-1 consensus oligonucleotides (probe) for 15 min at 37 °C in the presence of excess unlabeled AP-1 probe (70–350 fmol) (Fig. 9A). The amount of AP-1-DNA complex was separated from free DNA oligonucleotides by a gel shift assay. Lineweaver-Burk plot (Fig. 9B) was made based on the data obtained from Fig. 9A. Results indicated that Hcy decreased the equilibrium dissociation constant from 3.3004 nM (of the control) to 1.5192 nM (Table 2). Furthermore, the dissociation constant was determined in another set of experiments in which nuclear proteins were incubated with 12 fmol of 32P-labeled AP-1 consensus oligonucleotides (probe) and 1.75 pmol of unlabeled AP-1 probe for various time periods (5–15 min) in the absence or presence of Hcy (Fig. 9C). The radioactivity of each band corresponding to AP-1-DNA complex was quantified. A semi-log plot was made based on the data obtained from the gel shift assay (Fig. 9D). To calculate the dissociation constant (\(k_{off}\)), a single-exponential dissociation rate equation
AP-1$e^{-k_{\text{off}}t}$ was applied. The slope of the semi-log plot of the natural log of [AP-1],/[AP-1]$_0$ versus time ($t$) represented the dissociation constant of c-Fos/c-Jun dimer. There was an approximate 40% increase in the dissociation of dimer from DNA in the presence of Hcy ($k_{\text{off}}/H11005$ 0.0032 s$^{-1}$ versus 0.0023 s$^{-1}$ of the control) (Table 2). Association constant ($k_{\text{on}}$) was calculated based on the equation $K_D = k_{\text{off}}/k_{\text{on}}$. Results demonstrated that Hcy accelerates the binding of the dimer to DNA in

$^{(\text{HHcy})}$ or a high cysteine diet ($n = 12$ for each group). AP-1 and NF-$\kappa$B activation (A) and MCP-1 protein (B) were determined by EMSA and enzyme-linked immunosorbent assay, respectively. C, MCP-1 mRNA levels were measured by ribonuclease protection assay and expressed as a ratio of MCP-1 to GAPDH mRNA. Results were expressed as mean $\pm$ S.E. from five individual experiments. $^*$, $p < 0.05$ when compared with the control value.
3-fold ($k_{on} = 2.1064 \text{ pm}^{-1} \text{s}^{-1}$ versus $0.6969 \text{ pm}^{-1} \text{s}^{-1}$ of the control) (Table 2). In addition, Hcy was unable to directly affect the binding of p50/p65 to DNA (Fig. 10). Taken together, these results suggested that Hcy directly affected the binding of c-Jun/c-Fos heterodimer to DNA.

**DISCUSSION**

Increasing evidence suggests that production and secretion of proinflammatory factors in the liver plays an important role in systemic inflammation. It is suggested that abnormal lipid and lipoprotein metabolism or production of proinflammatory factors contribute to cardiovascular disease (6, 14, 15). The novel findings of the present study are 1) in the absence of other risk factors, hyperhomocysteinemia stimulates MCP-1 production in the liver, 2) Hcy-induced MCP-1 expression in hepatocytes is mediated via AP-1 activation, 3) Hcy can directly increase the DNA binding activity of recombinant AP-1, and 4) increased MCP-1 production in the liver correlates to the serum MCP-1 level in hyperhomocysteinemic rats.

MCP-1 is a potent chemokine that recruits monocyte/macrophage to the inflamed tissues. Increased hepatic expression of MCP-1 has been detected in patients with chronic liver diseases or acute liver injury (46, 47). A recent study showed that MCP-1 levels were elevated in the liver and serum of patients with nonalcoholic fatty liver disease or nonalcoholic steatohepatitis, suggesting an occurrence of systemic inflammation (14). Our previous study demonstrated that hyperhomocysteinemia caused lipid accumulation in the liver (steatosis) in rats (8). It has been suggested that hepatic inflammation secondary to liver steatosis may contribute to the low-grade systemic inflammation and metabolic syndrome (48). The ability of Hcy to
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Stimulate MCP-1 production in hepatocytes suggests that increased hepatic MCP-1 expression may contribute to the elevation of this chemokine in the circulation during hyperhomocysteinemia. In the present study increased hepatic MCP-1 production was found to be positively correlated with the serum MCP-1 level. We investigated the mechanism by which

stimulate MCP-1 production in hepatocytes suggests that increased hepatic MCP-1 expression may contribute to the elevation of this chemokine in the circulation during hyperhomocysteinemia. In the present study increased hepatic MCP-1 production was found to be positively correlated with the serum MCP-1 level. We investigated the mechanism by which

and unlabeled AP-1 consensus oligonucleotide (150-fold, 1.75 pmol) in the absence or presence of Hcy (1 μM) for various time periods (0–15 min) followed by EMSA. The radioactivity of each band corresponding to AP-1-DNA complex was measured, and a semi-log plot of the natural log of [AP-1]/[AP-1]0 versus time was shown. The calculated kinetic parameters of c-Fos/c-Jun-DNA binding are summarized in Table 2.

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TABLE 2
Effect of Hcy on the kinetic parameters of c-Fos/c-Jun-DNA binding

|                  | Control | Hcy  |
|------------------|---------|------|
| $K_D$ (nM)       | 3.3004  | 1.5192 |
| $k_{on}$(s$^{-1}$)| 0.0023  | 0.0032 |
| $k_{off}$(pm$^{-1}$s$^{-1}$)| 0.6969  | 2.1064|

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FIGURE 9. Determination of c-Fos/c-Jun-DNA binding kinetics. A, c-Fos/c-Jun were incubated with $^{32}$P-labeled (12 fmol) consensus oligonucleotide and unlabeled AP-1 consensus oligonucleotide (0–375 fmol) in the absence or presence of Hcy (1 μM) for 15 min followed by EMSA. The radioactivity of each band corresponding to AP-1-DNA complex was measured. B, Lineweaver-Burk plot was made based on the data shown in panel A. C, c-Fos/c-Jun were incubated with $^{32}$P-labeled consensus oligonucleotide (12 fmol) and unlabeled probe (fmmol) Hcy and $^{32}$P-labeled consensus oligonucleotide (0–375 fmol) in the absence or presence of Hcy (1 μM). EMSA was performed to determine the binding of p50 and/or p65 proteins to the oligonucleotide. Results were expressed as the mean ± S.E. from two individual experiments.

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FIGURE 10. Binding of recombinant p50 and p65 proteins to DNA oligonucleotide. Human recombinant p50 (10 ng) and/or p65 (10 ng) were incubated with $^{32}$P-labeled NF-κB consensus oligonucleotide in the absence or presence of Hcy (1 μM). EMSA was performed to determine the binding of p50 and/or p65 proteins to the oligonucleotide. Results were expressed as the mean ± S.E. from two individual experiments.
Hcy stimulated MCP-1 expression in the liver. Several lines of evidence suggested that Hcy-induced MCP-1 expression in hepatocytes was mediated via AP-1 activation. First, a significant increase in MCP-1 mRNA and protein levels was detected in the liver tissue of hyperhomocysteinemic rats. The effect of Hcy on MCP-1 expression was further examined in the in vitro system. Adding Hcy to the culture medium stimulated MCP-1 production in hepatocytes. These results suggested that Hcy was able to stimulate MCP-1 expression in the liver during hyperhomocysteinemia. Second, activation of AP-1 was detected in the liver tissue of hyperhomocysteinemic rat. Results from a chromatin immunoprecipitation assay demonstrated that there was an increase in the binding of AP-1 to the promoter region of MCP-1 gene in the liver tissue isolated from hyperhomocysteinemic rats. Hcy-induced AP-1 activation was also detected in cultured hepatocytes, which preceded the elevation of MCP-1 mRNA expression. Third, the involvement of AP-1 activation in Hcy-induced MCP-1 production in hepatocytes was further demonstrated in cells transiently transfected with AP-1 decoy oligodeoxynucleotide. Inhibition of AP-1 activation completely abolished Hcy-induced MCP-1 expression in hepatocytes. In addition, Hcy was unable to stimulate the transcription in hepatocytes transfected with pGL3-AP-1-mutant containing MCP-1 promoter region with a mutation in the AP-1 binding site.

Apart from AP-1, activation of NF-κB, another transcription factor, could potentially up-regulate MCP-1 expression in mammalian cells. NF-κB was shown to participate in Hcy-induced MCP-1 expression in extrahepatic cells, such as vascular smooth muscle cells and macrophages (29, 32). However, Hcy did not seem to stimulate NF-κB activation in hepatocytes or in the liver of hyperhomocysteinemic rat. In an in vitro assay, Hcy treatment did not stimulate the binding of NF-κB subunits (p50 and/or p65) to the κB consensus sequence. It has been reported that activation of either one of those transcription factors (AP-1, NF-κB) is sufficient to enhance MCP-1 gene expression (27, 28, 49). A recent study demonstrated a differential activation of NF-κB in liver cells upon normothermic ischemia-reperfusion in the mouse liver (50). NF-κB activation was only observed in Kupffer cells (liver-residing macrophages) but not in hepatocytes after ischemia-reperfusion, suggesting regulation of NF-κB activation might be influenced by the type of stimuli as well as the type of cells (50). Selective activation of transcription factors in different types of cells indicates that Hcy-elicited inflammatory responses in hepatocytes and in other types of cells may be regulated by different signaling mechanisms. Taken together, results from the present study suggest that Hcy-induced MCP-1 expression in hepatocytes is mediated via AP-1 activation.

Evidence obtained from the present study also indicates that Hcy can directly stimulate AP-1 binding to the consensus DNA sequence of the target genes. Among many AP-1 protein dimers, the c-Fos and c-Jun protein complex is a heterodimer that most efficiently interacts with the AP-1 binding site in the target genes (51). Both c-Fos and c-Jun interact with DNA directly (52). However, the homodimers of c-Jun and c-Fos bind weakly to DNA. Our in vitro experiment suggested that Hcy could directly increase the DNA binding of recombinant c-Fos/c-Jun heterodimer. Kinetic analysis suggested that Hcy accelerated the association of c-Jun/c-Fos to the DNA.

The leucine zipper and the adjacent basic region of Fos and Jun are required for DNA binding of AP-1 (41). Two cysteine residues in the leucine zipper and in its adjacent basic region are highly conserved in Fos and in Jun (41). It has been shown that the free sulfhydryl groups of these cysteine residues are essential for the DNA binding activity of Fos and Jun complex (41, 52). The DNA binding of Fos and Jun complex can be abolished by an alkylating agent, whereas reduction of cysteine residues with a reducing agent such as dithiothreitol can restore the DNA binding activity of Fos and Jun complex (52). Hcy as a reduced form with the -SH moiety appears to be essential in its stimulatory effect on AP-1 binding to DNA. Once Hcy was oxidized (with -S-S- moiety), it lost such a stimulatory effect. In circulation, majority of Hcy is either bound to proteins (70–80%) or in a disulfide moiety (-S-S-) with cysteine or another Hcy molecule (20–30%) and only 2% Hcy remains in the reduced sulfhydryl form. However, the intracellular Hcy is predominantly in the reduced sulfhydryl form in the liver due to the presence of glutathione and glutathione-homocysteine transsulfhydrase (53). Cysteine is another sulfhydryl group-containing amino acid. Our in vitro data showed that cysteine could also induced AP-1 activation and MCP-1 expression in hepatocytes. However, increased AP-1 activation and MCP-1 expression were only detected in hyperhomocysteinemic rats but not in rats fed a high cysteine diet. This might be due to the fact that a high cysteine diet did not cause a significant elevation of cysteine levels in rat serum and liver. When the supply of cysteine exceeds the requirement, cysteine can be efficiently catabolized to pyruvate and sulfate by cysteine desulphhydrase in the liver (54). This may explain why there was no elevation of cysteine levels in the serum and liver tissue of rats fed a high cysteine diet.

In summary, the present study for the first time demonstrates an increased expression of MCP-1 in the liver and in the circulation during hyperhomocysteinemia. The in vivo and in vitro results suggest that AP-1 activation is responsible for Hcy-induced MCP-1 production in hepatocytes. This study has identified a unique molecular mechanism of Hcy-induced AP-1 activation and MCP-1 expression in hepatocytes. Hyperhomocysteinemia is a metabolic disease associated with multiple dysfunctions. The contribution of increased hepatic chemokine production to chronic systemic inflammation remains to be examined in future studies.

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