Induction of Hepatic Cyclooxygenase-2 by Hyperhomocysteinemia via Nuclear Factor kappa-B Activation

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Running head: Activation of hepatic COX-2 during hyperhomocysteinemia

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Total Figures: 9
Abstract

Hyperhomocysteinemia, an elevation of blood homocysteine (tHcy), is a metabolic disorder associated with dysfunction of multiple organs. Apart from endothelial dysfunction, Hcy can cause hepatic lipid accumulation and liver injury. However, the mechanism responsible for Hcy-induced liver injury is poorly understood. The aim of this study was to investigate the regulation of cyclooxygenase-2 (COX-2), a proinflammatory factor, expression in the liver during the initial phase of hyperhomocysteinemia. Sprague-Dawley rats were fed a high-methionine diet for 1 week or 4 weeks. Serum and liver concentrations of Hcy were significantly elevated after 1 week or 4 weeks of dietary treatment. COX-2 mRNA and protein levels were significantly elevated in the liver of hyperhomocysteinemic rats. The induction of COX-2 expression was more prominent in 1-week hyperhomocysteinemic rats than that in 4-week group. Electrophoretic mobility shift assay revealed an activation of NF-κB in the same liver tissue in which COX-2 was induced. Administration of a NF-κB inhibitor to hyperhomocysteinemic rats effectively abolished hepatic COX-2 expression, inhibited the formation of inflammatory foci and improved liver function. Further investigation revealed that oxidative stress due to increased superoxide generation was responsible for increased phosphorylation and degradation of IκB-α leading to NF-κB activation in the liver. Administration of 4-hydroxy-tetramethyl-piperidine-1-oxyl (TEMPO), a superoxide dismutase mimetic, to hyperhomocysteinemic rats not only inhibited NF-κB activation but also prevented hepatic COX-2 induction and improved liver function. These results suggest that hyperhomocysteinemia-induced COX-2 expression is mediated via NF-κB activation. Increased oxidative stress and inflammatory response may contribute to liver injury associated with hyperhomocysteinemia.

Key words: homocysteine, cyclooxygenase-2, NF-κB, oxidative stress, inflammatory response
INTRODUCTION

Hyperhomocysteinemia is a metabolic disorder characterized by an elevation of total plasma homocysteine (tHcy) levels (26). Epidemiological studies indicate that hyperhomocysteinemia is associated with dysfunction of multiple systems including cardiovascular and cerebrovascular disease, osteoporosis, glomerulosclerosis and fatty liver (15, 23, 33, 41, 42, 50). Hcy is an intermediate amino acid produced in cells through metabolism of methionine to cysteine. The cellular homeostasis of Hcy is tightly regulated and any perturbation of Hcy metabolism causes an increase in its cellular concentrations, leading to hyperhomocysteinemia. Moderately fatty liver (lipid accumulation) has been observed in the autopsy of pediatric patients with hyperhomocysteinemia (25). Hcy, at elevated levels, causes abnormal lipid metabolism, endoplasmic reticulum stress and oxidative stress in hyperhomocysteinemic rodents (45, 46, 49). It is increasingly recognized that oxidative stress can elicit an inflammatory response in tissues. Hcy-induced oxidative stress is regarded as one of important mechanisms for the expression of proinflammatory factors in vascular cells (10, 36). However, the mechanism by which hyperhomocysteinemia promotes inflammatory response in the liver remains poorly understood.

Induction of cyclooxygenase-2 (COX-2), a proinflammatory factor, is involved in hepatic inflammatory response in alcoholic and non-alcoholic liver disease (28, 52). Cyclooxygenase (COX), also known as prostaglandin (PG) G/H synthase, is the rate-limiting enzyme that catalyzes the conversion of arachidonic acid to prostanoids (38). Two major COX isoforms (COX-1 and COX-2) are identified in mammalian cells. COX-1 is expressed constitutively in most kind of cells and is responsible for the synthesis of prostaglandins at low levels while COX-2 expression is inducible. In the liver, COX-2 can be rapidly induced by proinflammatory stimuli, hormones or growth factors. Induction of COX-2 has been detected in kupffer cells (28),
hepatocytes (17) and stellate cells (12). COX-2 mediated prostanoid generation is involved in early inflammatory response (9, 24). It converts arachidonic acid into PGG₂ and subsequently PGH₂ that serves as a precursor for the synthesis of prostanoids including PGs, thromboxanes (TXs) and prostacyclins (38). COX-2 elicits the onset of inflammation mainly through the production of proinflammatory prostanoids such as PGE₂ and TXB₂ (19, 28). In murine models, activation of COX-2 contributes to liver injury by enhancing the synthesis of proinflammatory prostanoids (28, 51). The promoter region of COX-2 gene contains sequences that are responsive to regulation by transcription factors including nuclear factor kappa-B (NF-kB) (8, 32). NF-kB is a protein complex present as a dimer (p50/p65, (p65)₂ or c-rel/p65) which is responsible for the expression of many genes including those of proinflammatory factors. Apart from its key role in regulating the immune response to infection, aberrant regulation of NF-kB is linked to many diseases such as cancer, inflammatory and autoimmune diseases, neurodegenerative and cardiovascular disorders (2). NF-kB is sequestered in the cytoplasm by a family of inhibitor proteins called IkB. Upon stimulation, there is a rapid phosphorylation and subsequently degradation of IkB by proteasomes, leading to the release of NF-kB followed by its translocation into the nucleus (30). NF-kB then binds to the κB binding motifs in the promoter or enhancer of the genes. We have recently observed that NF-kB is activated in the aorta of hyperhomocysteinemic rat (4), promoting the expression of chemokines and cytokines in vascular cells (43, 44).

In the last decade, substantial progress has been made in identifying molecular and pathological changes associated with hyperhomocysteinemia. However, most of reported studies conducted in animal models were focused on its chronic effects, in which, induction of hyperhomocysteinemia ranges from a few weeks to months (4, 5, 20, 40, 44, 48). Little
information is available regarding the pathophysiological impact during the initial phase of hyperhomocysteinemia. It is plausible that the early response of liver to hyperhomocysteinemia may affect the subsequent local and systemic pathological processes. In the present study, we aimed to elucidate the mechanism by which Hcy induced COX-2 expression in rats during the initial phase of hyperhomocysteinemia. The involvement of oxidative stress and NF-κB activation in COX-2 induction and liver injury were investigated.
MATERIALS AND METHODS

Animal model. Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) aged 8 weeks were fed the following types of diet for 1 week or 4 weeks: (i) control diet consisting of Lab Diet Rodent Diet 5001 (PMI Nutrition International, St Louis, MO) which contained 0.43% (wt/wt) methionine; (ii) high-methionine diet consisting of regular diet plus 1.7% (wt/wt) methionine (6, 46). In one set of experiments, an NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC) were administered intraperitoneally (100 mg/kg, i.p.) 16 h before euthanasia to rats fed a control or a high-methionine diet (20, 53). In another set of experiments, a membrane-permeable superoxide dismutase mimetic, 4-hydroxy-tetramethyl-piperidine-1-oxyl (TEMPO), was injected intraperitoneally (1.5 mmol/kg, i.p.) into rats fed a control or a high-methionine diet 6 h before euthanasia (18). Results from our previous studies demonstrated that hyperhomocysteinemia could be induced in rats by feeding a high-methionine diet (20, 48, 49). Serum and liver Hcy concentrations were measured by using the IMx Hcy assay based on the fluorescence polarization immunoassay technology (Abbott Diagnostics Division, Abbott Park, IL) (47, 48). Liver function was examined by measuring the activity of alanine aminotransferase (ALT) in serum samples with an enzymatic kit (Diagnostic Chemical Limited, Charlottetown, PE). 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.

Real-time polymerase chain reaction (Real-time PCR). Total RNAs were isolated from liver tissue with TRIzol reagent (Invitrogen, Carlsbad, CA). COX-2 mRNA was determined by a real-time polymerase chain reaction (PCR) analysis using the iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA). In brief, 2 μg of total RNA was converted to cDNA by reverse
transcriptase. The reaction mixture of real-time PCR contained 0.4 μM of 5’ and 3’ primer and 2 μl of cDNA product in iQ-SYBR green supermix reagent (Bio-Rad, Hercules, CA). Crossing threshold values were normalized to GAPDH. The mRNA expression was expressed as percentage change relative to control. The primers used in this study were rat COX-2, 5’-AATCGCTGTACAAGCAGTGG -3’ (Forward), 5’- GCAGCCATTCTTTTCTCTCC -3’ (Reverse) (GenBank accession No. NM_017232); rat GAPDH, 5’-TCAAGAAGGTGGTGAAGCAG -3’ (Forward), 5’- AGGTGGAAGAATGGGAGTTG -3’ (Reverse) (GenBank accession No. NM_017008). All primers were purchased from Invitrogen.

**Western immunoblotting analysis.** COX-2, IκBα and phospho-IκBα protein levels were determined by Western immunoblotting analysis. In brief, liver proteins (60 μg) were separated by electrophoresis on a 10% SDS polyacrylamide gel. Proteins in the gel were transferred to a nitrocellulose membrane. The membrane was probed with rabbit anti-IκBα polyclonal antibodies and anti-phospho-IκBα (Ser32) polyclonal antibodies (New England Biolabs, Beverly, MA) or rabbit anti-COX-2 polyclonal antibodies (Lab Vision, Fremont, CA). HRP-conjugated anti-biotin antibodies (Zymed, South San Francisco, CA) were used as secondary antibodies. The corresponding protein bands were visualized using enhanced chemiluminescence reagents and analyzed with a gel documentation system (Bio-Rad Gel Doc1000).

**Electrophoretic mobility shift assay (EMSA).** Nuclear proteins were prepared from rat liver as previously described (4, 48). Nuclear proteins (10 μg) were incubated with excess 32P-end-labeled oligonucleotides containing a consensus sequence specific for NF-κB/DNA binding site (5’-AGTTGAGGGGACTTTCCCAGGC-3’) (Promega, Madison, WI). The reaction mixture was then separated in 6% non-denaturing polyacrylamide gel and dried on a piece of filter paper followed by autoradiography.
**Determination of COX-2 activity.** COX-2 activity was detected by using an assay kit (Cayman Chemical, Ann Arbor, MI). In brief, a portion of liver tissue was homogenized in a cold buffer containing 0.1 M Tris-HCl (pH 7.8) and 1 mM EDTA. After centrifugation at 10,000×g for 15 min at 4°C, the supernatant was collected and assayed according to the manufacturer’s instruction.

**Histology analysis.** The liver was excised and a portion of it was frozen immediately in liquid nitrogen. Cryosections of the liver tissue were prepared and stained with hematoxylin and eosin (H&E) to evaluate the morphological changes. The image of H&E sections (5 per liver) were captured by using an Axioskop2 MOT microscope (Carl Zeiss Microimaging, Thornwood, NY) and Axiocam camera. The number of inflammatory foci was counted in a blinded manner in 10 microscopic fields per section under a light microscope at a magnification of ×100, ×200 and ×400.

**Measurement of superoxide anion, NADPH oxidase activity and antioxidant enzyme activities.** The superoxide anion level in the liver tissue was measured by lucigenin chemiluminescence assay (46). A portion of the liver was homogenized in a 50 mM phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM PMSF. After centrifugation at 3000×g for 10 min, an aliquot of the supernatant (100 μg proteins) was incubated with lucigenin (5 μM) in a phosphate buffer (50 mM, pH 7.0) for 2 min followed by initiation of the reaction by adding 100 μM NADPH, or xanthine substrate. Chemiluminescent signal (photon emission) was measured every 15 s for 3 min using a luminometer (Lumet LB9507, Berthold Technologies GmbH and Co. KG, BadWildbad, Germany). The NADPH oxidase activity was calculated based on the amount of superoxide anion produced in the reaction mixture when NADPH substrate was used. Hepatic superoxide dismutase (SOD) and catalase activities were determined as previously described (7,
Statistical analysis. Results were analyzed by a two-tailed Student's t-test or using one-way analysis of variance followed by Newman-Keuls post test or using Prism 4 (GraphPad Software, Inc., La Jolla, CA). $P$ values less than 0.05 were considered statistically significant.
RESULTS

*Induction of hepatic COX-2 expression in hyperhomocysteinemic rats.* Rats were fed a high-methionine diet for 1 week or for 4 weeks to induce hyperhomocysteinemia (46, 49). The high-methionine diet caused a significant increase in serum concentrations of Hcy (Fig. 1A). Simultaneously, there was a significant elevation of Hcy levels in the liver of hyperhomocysteinemic rats (Fig. 1B). The level of hepatic COX-2 mRNA was markedly elevated in rats fed a high-methionine diet for 1 week or for 4 weeks (Fig. 2A). Such a stimulatory effect on COX-2 mRNA expression in the liver was more prominent in rats fed a high-methionine diet for 1 week (3.5-fold increase) than that in 4-week hyperhomocysteinemic rats (1.8-fold increase).

To determine whether increased COX-2 gene expression would lead to an elevation of COX-2 protein level in the liver, Western immunoblotting analysis was carried out. There was a significant increase in COX-2 protein level in the liver tissue of hyperhomocysteinemic rats as compared to that in control rats (Fig. 2B). In view that 1-week hyperhomocysteinemia had a stronger stimulatory effect on the expression of hepatic COX-2, the subsequent investigation was mainly conducted in rats fed a high-methionine diet for 1 week. To identify whether NF-κB was involved in COX-2 up-regulation, a known inhibitor for NF-κB activation named pyrrolidine dithiocarbamate (PDTC) was injected intraperitoneally (100 mg/kg, i.p.) into rats fed a high-methionine diet (20, 53). Administration of PDTC to hyperhomocysteinemic rats reduced COX-2 mRNA expression (Fig. 3A), protein level (Fig. 3B) and enzyme activity (Fig. 3C) when compared to that found in control rats. Injection of PDTC into rats fed a regular diet did not affect the basal expression of COX-2 in the liver (Fig. 3). These results suggested that induction of COX-2 expression in the liver of hyperhomocysteinemic rats might be mediated via NF-κB activation.
Activation of hepatic NF-κB during hyperhomocysteinemia. To further substantiate the role of NF-κB activation in the liver during hyperhomocysteinemia, the binding activity of NF-κB with $^{32}$P-labeled consensus oligonucleotide was determined by EMSA. Nuclear proteins in the liver were isolated from different groups of rats. The binding activity of NF-κB to DNA was significantly increased in the liver tissue of hyperhomocysteinemic rats (Fig. 4A), indicating that hepatic NF-κB was activated. Administration of an NF-κB inhibitor (PDTC) effectively blocked the activation of NF-κB/DNA binding activity in hyperhomocysteinemic rats (Fig. 4A). Inhibition of NF-κB activation also improved liver function as indicated by a significant reduction of serum ALT levels in hyperhomocysteinemic rats receiving PDTC injection (Fig. 4B). Moreover, hepatic morphological changes were observed in hyperhomocysteinemic rats by H&E staining. Hepatic inflammatory foci characterized by dense aggregates of mononuclear cells (31, 34) were visible in hyperhomocysteinemic rats. Few foci were observed in control rats as well as in hyperhomocysteinemic rats treated with PDTC (Fig. 5). To further investigate the mechanism of NF-κB activation, protein levels of total IκBα and phosphorylated IκBα were determined by Western immunoblotting analysis. The level of total IκBα protein was significantly decreased in the liver tissue of hyperhomocysteinemic rats (Fig. 6A). Moreover, the level of phosphorylated IκBα protein was markedly increased (Fig. 6B). These results suggested that activation of NF-κB in the liver during hyperhomocysteinemia might be due to the increased phosphorylation and subsequent degradation of its inhibitor protein IκBα.

Involvement of oxidative stress in NF-κB activation and COX-2 expression. To investigate the involvement of oxidative stress in NF-κB activation and COX-2 expression, superoxide anions were measured in the liver. The level of superoxide anions was significantly increased in the liver of hyperhomocysteinemic rats as compared to that in the control group (Fig. 7A). To
further confirm the role of oxidative stress, a membrane-permeable superoxide dismutase mimetic, 4-hydroxy-tetramethyl-piperidine-1-oxyl (TEMPO), was injected intraperitoneally (1.5 mmol/kg, i.p.) into hyperhomocysteinemic rats. TEMPO treatment effectively reduced the hepatic level of superoxide anions in hyperhomocysteinemic rats (Fig. 7A). Inhibition of oxidative stress by TEMPO treatment also completely abolished NF-κB activation (Fig. 7B) and COX-2 expression (Fig. 7C) induced by hyperhomocysteinemia. Furthermore, TEMPO treatment restored liver function in hyperhomocysteinemic rats (Fig. 7D). Administration of TEMPO to rats fed a control diet did not affect the basal levels of COX-2 expression, NF-κB activation and ALT activity (Fig. 7). These results suggested that oxidative stress activated NF-κB, which, in turn, induced COX-2 expression in the liver. To further investigate the mechanism of increased hepatic superoxide anion level in hyperhomocysteinemic rats, activities of enzymes that are involved in superoxide production (NADPH oxidase) and metabolism (superoxide dismutase, catalase) were determined. The hepatic NADPH oxidase activity was remarkably increased while superoxide dismutase and catalase activities were significantly decreased in hyperhomocysteinemic rats (Fig. 8). Collectively, these results suggested that oxidative stress and the inflammatory response might contribute to liver injury during hyperhomocysteinemia.
DISCUSSION

Hyperhomocysteinemia is a chronic disease affecting multiple organs. In recent years, increasing evidence suggests that an elevation of blood Hcy level is linked to liver dysfunction (1, 25, 34, 46). Based on the results obtained from this study, a novel mechanism by which hyperhomocysteinemia promotes liver injury is presented here (Fig. 9): (1) hyperhomocysteinemia induces COX-2 expression in rat liver; (2) COX-2 expression is mediated by NF-κB activation; (3) oxidative stress contribute to liver injury through activation of NF-κB and subsequent induction of proinflammatory factor COX-2 during the initial phase of hyperhomocysteinemia.

COX-2 is a proinflammatory factor that plays an important role in tissue damage during the early inflammatory response (9, 24). Transgenic expression of COX-2 has been shown to exacerbate liver injury in murine models (17, 51). In the present study, the induction of hepatic COX-2 expression was more prominent in rats that were hyperhomocysteinemic for 1 week than those were hyperhomocysteinemic for 4 weeks, indicating that inflammatory response started to occur early in hyperhomocysteinemia. During this early stage, NF-κB activity was increased in the liver tissue. Activation of NF-κB is regarded as one of the critical steps in the initiation of liver injury (21, 27, 39), which is thought to precede the appearance of morphological and functional changes in the liver. NF-κB is a “rapid-acting” transcription factor that can be activated through signal-induced phosphorylation and subsequent degradation of its inhibitor protein IκB (14). It has been shown that NF-κB activation in humans and rats enhances NF-κB binding to the promoter region of COX-2 and induces the expression of COX-2 gene (3, 29, 37). Several lines of evidence obtained from the present study supported our hypothesis that the induction of COX-2 expression in the liver was mediated via NF-κB activation. First, there was a
significant increase in COX-2 mRNA and protein levels in the liver of hyperhomocysteinemic rats. The enzyme activity of COX-2 was increased in the same liver tissue. Second, NF-κB binding to DNA was markedly elevated in the liver of hyperhomocysteinemic rats. Further analysis revealed that activation of NF-κB in the liver at the initial phase of hyperhomocysteinemia was due to increased phosphorylation of its inhibitor protein IκBα, leading to a reduction in the total IκBα protein. Concomitantly, serum ALT level was significantly elevated and inflammatory foci appeared in the liver tissue in hyperhomocysteinemic rats, indicating that liver injury had occurred. Third, administration of NF-κB inhibitor (PDTC) to hyperhomocysteinemic rats not only prevented NF-κB activation but also abolished COX-2 expression in the liver. Inhibition of NF-κB mediated COX-2 expression also ameliorated liver injury in hyperhomocysteinemic rats. These results suggested that NF-κB activation played an important role in the induction of COX-2 expression in the liver during the initial phase of hyperhomocysteinemia, which, in turn, contributed to liver injury (Fig. 9).

Hyperhomocysteinemia-induced liver injury may be mediated through different signaling pathways and mechanisms at various stages of hyperhomocysteinemia. Our previous study has showed that AP-1 is the major transcription factor involved in hepatic inflammatory response by induction of MCP-1 expression in 4-week hyperhomocysteinemic rats (48). Hamelet and colleagues have also reported that hepatic NF-κB activation is mediated through calcium-dependent proteases but not phosphorylation-dependent mechanism in CBS deficiency-resulted hyperhomocysteinemic mice (16). Results from the present study demonstrated that NF-κB mediated hepatic COX-2 expression occurred as early as the first week after the development of hyperhomocysteinemia in rats.

Oxidative stress due to increased generation of free radicals has been indicated as one of the
potential triggers for the development of hepatic steatosis, steatohepatitis and fibrosis (13, 35).
Oxidative stress is closely linked to the activation of transcription factors and inflammatory response. Increased free radical generation was found to be responsible for alcohol-induced hepatic NF-κB activation, steatosis and necrosis in mice (22). In the present study, the level of superoxide anions was markedly increased in the liver of hyperhomocysteinemic rats due to increased production and decreased metabolism. Administration of TEMPO, a known superoxide anion scavenger, to hyperhomocysteinemic rats reduced the level of superoxide anions in the liver. Such a treatment effectively abolished NF-κB mediated COX-2 expression in the liver. Moreover, inhibition of oxidative stress led to the recovery of liver function which was impaired during hyperhomocysteinemia. Taken together, it is plausible that oxidative stress may serve as one of the potential mechanisms responsible for inflammatory response and liver injury during the initial phase of hyperhomocysteinemia (Fig. 9).

PERSPECTIVES AND SIGNIFICANCE
The present study demonstrates, for the first time, that the induction of COX-2 expression in the liver of hyperhomocysteinemic rats is mediated via NF-κB activation. Our results provide novel evidence that oxidative stress and inflammatory response contribute to liver injury during the initial phase of hyperhomocysteinemia. Identification of molecular mechanisms that are responsible for early liver injury may lead to a better therapeutic approach to prevent the progression of liver damage in patients with chronic diseases.
GRANTS

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Figure legends

Fig. 1. Measurement of serum and liver homocysteine
Hcy levels in the serum (A) and liver (B) were measured in rats fed a regular diet (control) or a high-methionine diet (hyperhomocysteinemia, HHcy) for 1 week or 4 weeks. Results are expressed as mean ± SE (n=8). *P<0.05 when compared with control values.

Fig. 2. COX-2 mRNA and protein expression in livers
Rats were fed a regular diet (control) or a high-methionine diet (HHcy) for 1 week or 4 weeks. (A) COX-2 mRNA was measured by a real-time PCR analysis. (B) COX-2 protein was determined by Western immunoblotting analysis. Results are expressed as mean ± SE (n=8). *P<0.05 when compared with control values (expressed as 100%).

Fig. 3. Effect of NF-κB inhibitor on COX-2 expression in livers
Rats were fed a regular diet (control) or a high-methionine diet (HHcy) for 1 week. In one set of experiments, rats fed a regular diet (PDTC) or a high-methionine diet (HHcy + PDTC) were given PDTC (100 mg/kg, i.p.) 16 h before euthanasia. (A) COX-2 mRNA was measured by a real-time PCR analysis. (B) COX-2 protein was determined by Western immunoblotting analysis. (C) COX-2 activity was determined. Results are expressed as mean ± SE (n=8). *P<0.05 when compared with values obtained from control group (expressed as 100%). #P<0.05 when compared with values obtained from HHcy group.
Fig. 4. Activation of NF-κB in the liver

Liver nuclear proteins were isolated from rats fed a regular diet (control) or a high-methionine diet (HHcy) for 1 week. In one set of experiments, rats fed a regular diet (PDTC) or a high-methionine diet (HHcy + PDTC) were given PDTC (100 mg/kg, i.p.) 16 h before euthanasia. (A) NF-κB/DNA binding activity was determined by EMSA. Lane 1: unlabeled probe, Lane 2: Control, Lane 3: HHcy, Lane 4: HHcy+PDTC, Lane 5: PDTC. (B) Serum ALT was measured. Results are expressed as mean ± SE (n=8). *P<0.05 when compared with values obtained from the control group. #P<0.05 when compared with values obtained from the HHcy group.

Fig. 5. Liver morphologic examination

Liver tissue samples from rats fed a regular (Control) or a high-methionine diet (HHcy) for 1 week were examined with hematoxylin and eosin (H&E) staining to detect morphological changes. One group of rats fed a high-methionine diet was given PDTC (100 mg/kg, i.p.) 16 h before euthanasia (HHcy + PDTC). **Arrows** indicate inflammatory foci. (A) Magnification, ×100; (B) magnification, ×200; and (C) magnification, ×400.

Fig. 6. Determination of total IκBα and phospho-IκBα proteins in the liver

Rats were fed a regular diet (control) or a high-methionine diet (HHcy) for 1 week. Total IκBα proteins (A) and phospho-IκBα proteins (B) were determined by Western immunoblotting analysis. β-actin was used as an internal control to confirm equal amount of protein loading to the gel. Results are expressed as mean ± SE (n=8). *P<0.05 when compared with values obtained from the control group (expressed as 100%). #P<0.05 when compared with values obtained from the HHcy group.
Fig. 7. Effect of superoxide anion scavenger on COX-2 expression and NF-κB activation in the liver

Rats were fed a regular diet (control) or a high-methionine diet (HHcy) for 1 week. In one set of experiments, rats fed with a regular diet (TEMPO) or a high-methionine diet (HHcy + TEMPO) was injected with TEMPO (1.5 mmol/kg, i.p.) 6 h before euthanasia. (A) Superoxide anion levels in the liver were measured by lucigenin chemiluminescence assay. (B) NF-κB/DNA binding activity was determined by EMSA. Lane 1: unlabeled probe, Lane 2: Control, Lane 3: HHcy, Lane 4: HHcy + TEMPO, Lane 5: TEMPO. (C) COX-2 protein was measured by Western immunoblotting analysis. (D) Serum ALT was measured. Results are expressed as mean ± SE (n=8). *P<0.05 when compared with values obtained from the control group. #P<0.05 when compared with values obtained from HHcy group.

Fig. 8. Hepatic activities of NADPH oxidase, superoxide dismutase and catalase.

Rats were fed a regular diet (control) or a high-methionine diet (HHcy) for 1 week. (A) NADPH oxidase, (B) Superoxide dismutase (SOD) and (C) catalase activities were determined in the liver tissue and expressed as percentage of control. Results are expressed as mean ± SE (n=8). *P<0.05 when compared with values obtained from the control group.

Fig. 9. Proposed mechanism of hyperhomocysteinemia-induced liver injury.

Hyperhomocysteinemia causes liver injury via inflammatory response characterized by the induction of a proinflammatory factor, COX-2, expression. Oxidative stress caused by hyperhomocysteinemia results in the activation of NF-κB and subsequently elevation of COX-2
expression. Inhibition of oxidative stress by 4-hydroxy-tetramethyl-piperidine-1-oxyl (TEMPO) or prevention of NF-κB activation by pyrrolidine dithiocarbamate (PDTC) is able to abolish hepatic COX-2 expression and improve liver function in hyperhomocysteinemic rats.
Fig. 1

A
Serum Hcy Concentration (μmol/L)

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B
Liver Hcy Concentration (nmol/g)

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Fig. 2

A

COX-2 mRNA expression (% of Control)

Control | HHcy | Control | HHcy
---|---|---|---
1 week | 100 | 400 |*
4 weeks | 100 | 300 |*

B

COX-2/β-actin

Control | HHcy | Control | HHcy
---|---|---|---
1 week | 100 | 150 |*
4 weeks | 100 | 200 |*
Fig. 3

A

COX-2 mRNA expression (% of Control)

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<th>PDTC</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-2</td>
<td></td>
<td>*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

COX-2 protein expression (% of Control)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HHcy</th>
<th>HHcy+PDTC</th>
<th>PDTC</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-2</td>
<td></td>
<td>*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

β-actin
Fig. 5

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HHcy</th>
<th>HHcy+PDTC</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>B</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>C</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
</tbody>
</table>

A (×100)  
B (×200)  
C (×400)
Fig. 6

A

\[ \text{i\&B}_\alpha \]

\[ \beta\text{-actin} \]

Control | HHcy

\[ \begin{array}{c}
0 \\
20 \\
40 \\
60 \\
80 \\
100 \\
120 \\
0 \\
20 \\
40 \\
60 \\
80 \\
100 \\
120 \\
\end{array} \]

B

\[ \text{p}\text{i\&B}_\alpha \]

\[ \beta\text{-actin} \]

Control | HHcy

\[ \begin{array}{c}
0 \\
20 \\
40 \\
60 \\
80 \\
100 \\
120 \\
140 \\
0 \\
20 \\
40 \\
60 \\
80 \\
100 \\
120 \\
140 \\
\end{array} \]
Fig. 7

A) Superoxide anion level (% of Control)

Control   HHcy   HHcy+TEMPO   TEMPO

B) NF-κB/DNA binding activity (% of Control)

Control   HHcy   HHcy+TEMPO   TEMPO
C

**COX-2**

Control HHcy HHcy+TEMPO TEMPO

COX-2 protein expression (% of Control)

D

**ALT (U/L)**

Control HHcy HHcy+TEMPO TEMPO

* *
Fig. 8

A

Catalase activity (U/mg protein)

0 200 400 600 800 1000

Control HHcy

B

SOD activity (U/mg protein)

0 10 20 30 40

Control HHcy

C

NADPH oxidase activity (U/mg protein)

0 10 20 30 40

Control HHcy

*
Fig. 9

Hyperhomocysteinemia → Oxidative Stress (superoxide anion) → Oxidative stress inhibitor (TEMPO) → NF-κB activation → Pro-inflammatory factors (COX-2) → Inflammatory response → Liver injury

NF-κB inhibitor (PDTC)