Homocysteine Induces Heme Oxygenase-1 Expression via Transcription Factor Nrf2 Activation in HepG2 Cells

Monireh Mani[1], Taghi Golmohammadi[*1], Shahnaz Khaghani[1], Zahra Zamani[2], Kayhan Azadmanesh[3], Reza Meshkani[1] and Parvin Pasalar[1]

[1]Dept. of Biochemistry, School of Medicine, Tehran University of Medical Science, Tehran; [2]Dept. of Biochemistry, Pasteur Institute of Iran, Tehran; [3]Dept. of Virology, Pasteur Institute of Iran, Tehran, Iran

ABSTRACT

Background: Elevated level of plasma homocysteine has been related to various diseases. Patients with hyperhomocysteinemia can develop hepatic steatosis and fibrosis. We hypothesized that oxidative stress induced by homocysteine might play an important role in pathogenesis of liver injury. Also, the cellular response designed to combat oxidative stress is primarily controlled by the transcription factor Nrf2, a principal inducer of anti-oxidant and phase II-related genes. **Methods:** HepG2 cells were treated with homocysteine in different time periods. Glutathione content was measured by flowcytometry. Using electrophoretic mobility shift assay (EMSA) and Western-blotting, anti-oxidant response element (ARE)-binding activity of Nrf2 for heme ocygenase-1 (HO-1) was demonstrated. Real time RT-PCR and Western-blotting were performed to evaluate whether homocysteine was able to induce mRNA and protein expression of HO-1. **Results:** The role of Nrf2 in cellular response to homocysteine is substantiated by the following observations in HepG2 cells exposed to homocysteine (i) Western-blotting revealed that Nrf2 is strongly stabilized and became detectable in nuclear extracts. (ii) EMSA demonstrated increased binding of Nrf2 to oligomers containing HO-1 promoter-specific ARE-binding site. (iii) Real time RT-PCR and Western-blotting revealed increased mRNA and protein expression of inducible gene HO-1 after treatment with homocysteine. **Conclusion:** Data presented in the current study provide direct evidence that the immediate cellular response to oxidative stress provoked by homocysteine is orchestrated mainly by the Nrf2-ARE pathway. Therefore, induction of Nrf2-ARE-dependent expression of HO-1 could be a therapeutic option for hepatic cells damage induced by homocysteine. *Iran. Biomed. J. 17 (2): 93-100, 2013*

Keywords: Heme oxygenase-1, HepG2 cells, Oxidative stress

INTRODUCTION

Homocysteine is a thiol-containing amino acid, which produces at the cross road of methionine metabolism. Liver is the tissue where methionine is metabolized. Elevated plasma homocysteine or hyperhomocysteinemia has been implicated as a risk factor for atherosclerosis, cerebrovascular, peripheral vascular diseases and hepatic dysfunction [1, 2].

The enzymes 5, 10 methylenetetrahydrofolate reductase and cystathionine β-synthase play critical roles in regulating plasma homocysteine. Patients with hyperhomocysteinemia due to deficiency in these enzymes can develop hepatic steatosis and fibrosis [3]. Consistent with these findings, homozygous cystathionine β-synthase -deficient mice having hyperhomocysteinemia also develop hepatic steatosis

[4, 5]. It is also shown that in chronic alcoholism, hyperhomocysteinemia due to insult in methionine metabolism can contribute to the development of alcoholic liver disease, that is characterized by fatty liver, steatohepatitis, fibrosis, cirrhosis and potentially hepatocellular carcinoma [6, 7].

Little is known about the cellular and biochemical mechanisms of homocysteine-induced hepato-toxicity, but it has been confirmed that oxidative stress plays an important role in different types of liver injury [8, 9]. To counteract damage induced by oxidative stress, cells have developed an adoptive defense mechanism that leads to rapid and efficient induction of detoxifying and anti-oxidative stress enzymes. Among them, induction of a family of anti-oxidant detoxification enzymes is a key element in maintaining cellular redox homeostasis and reducing oxidative damage. One of these enzymes is heme

*Corresponding Author; Tel.: (+98-21) 8895 3004; Fax: (+98-21) 6405 3384; E-mail: golmoham@sina.tums.ac.ir

oxygenase 1 (HO-1). Increased homocysteine levels may affect the expression of intracellular defensive genes such as HO-1, which is induced in response to stressful conditions [10]. The HO-1 expression contributes to protection against oxidative liver damage and is a potential therapeutic target for hepato-protection [11]. Data indicate that anti-oxidant gene responsible for encoding this enzyme is coordinately regulated through consensus cis-acting element called anti-oxidant response element (ARE) in its 5'-flanking promoter region [12, 13].

Transcription factor Nrf2, a member of the cap'n collar family of transcription factors, is present in the cytoplasm attaching to an actin-binding protein named Klech-like ECH-associated protein [14]. After activation, it dissociates from Klech-like ECH-associated protein 1 and translocates into the nucleus, where it activates transcription of several detoxification and anti-oxidant genes [15]. It has been demonstrated that the Nrf2-dependent adaptive response provides a pivotal defense mechanism against oxidative stress [16]. However, a direct link between homocysteine-mediated HO-1 induction and activation of Nrf2 expression via stimulation of ARE-binding activity remains to be examined. The current study was designed to investigate whether homocysteine induces the expression of HO-1 in HepG2 cells, and whether this induction is mediated by ARE present within its promoter.

MATERIALS AND METHODS

Cell culture, treatment and cell viability assay. The HepG2 cells (National Cell Bank, Pasteur Institute of Iran) were grown in DMEM medium containing 10% heat inactivated fetal calf serum and 100 U/ml penicillin G sodium, 100 µg/ml streptomycin and L-glutamine in humidified atmosphere containing 5% CO_2 at 37ºC. For RNA extraction and glutathione detection, the cells were seeded at a density of 5×10^5 per well in 6-well culture plates. For isolation of whole cell and nuclear protein extract, cells were treated in 25-cm^2 flasks. D,L-homocysteine (Sigma Chemical Co, USA) was used at a final concentration of 50 µM corresponding to 25 µM L-homocysteine. The culture medium was replaced with fresh media and the different treatments were initiated. The untreated cells were used as control. The cells were treated with homocysteine for 15 min, 30 min, 45 min, 3 h, 6 h and 9 h. For cell viability assay, HepG2 cells (1×10^6 cells/ml) were incubated in control or test medium supplemented with 5% fetal calf serum at 37ºC for 18 h. Cell viability was assessed by counting cells on a hemocytometer after suspension in PBS (pH 7) containing 0.5% Trypan blue stain. The data shows the

means ± S.D. from three independent experiments.

Flow cytometry. Cellular glutathione (GSH) levels were measured using the thioltracker Violet GSH detection reagent (Invitrogen/Molecular Probes, Eugene, OR, USA) according to the manufacturer's protocol. Cells were seeded in a 6-well plate, incubated overnight and treated the following day. After designated treatment times, the cells were washed with PBS and incubated in Dulbecco's PBS containing 10 µM (final concentration) thioltracker Violet at 37ºC for 30 min. Afterwards, cells were analyzed on a flow cytometer (Partec, Germany). Analysis of cellular GSH content was restricted to PI negative (intact) cells.

RNA extraction and real time quantitative PCR. Total RNA was extracted from HepG2 cells using RNeasy cell mini kit (Qiagen GmbH, Hiden, Germany) according to the manufacturer's instruction. RNA samples with A260/A280 ratios above 1.8 were stored at -70°C for further analysis. Total RNA (3 µg) was subjected to reverse transcription using QuantiTect Revese Transcription Kit (Fermentase, Vilnius, Lithuania). Real time quantitative PCR was done using SYBR Premix Taq (Takara, Japan) and primer for HO-1 (QT00092645, QIAGEN GmbH, Hiden, Germany). For data normalization, the transcript for the constitutive gene product β-actin (QT01680476, QIAGEN GmbH, Hiden, Germany) was used. Each cycle consisted of denaturation at 95ºC for 5s and combined annealing and extension at 60ºC for 30 s. Each sample type was run in duplicate and each experiment was performed four times. The expression levels of the gene were quantified using REST 2009 Software using the real time PCR data.

Western-blot analysis. For nuclear extraction preparation, HepG2 cells were washed twice with PBS (pH 7.4). Cells were then harvested in 1 ml of PBS and centrifuged at 2,800 ×g at 4°C for 10 min. The cell pellet was resuspended in 250 µl of cold buffer A consisting of 10 mM HEPES (pH 7.9),1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol and protease inhibitor cocktail (Roche, Mannheim, Germany). The pellet was then incubated on ice for 15 min to allow cells to swell. Then, 10% Nonidet P-40 was added and the tube was vortex-mixed for 10 s. The homogenate was then centrifuged at ,800 ×g at 4°C for 10 min. The resulting nuclear pellet was resuspended in 30 µl of cold buffer B consisting of 5 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol , 26% glycerol and protease inhibitor. The pellet was then incubated on ice for 15 min and vortex-mixed for 10-15 s every two min. The nuclear extract was finally centrifuged at 14800 ×g at 4°C for 5 min. The protein concentration was measured by Bradford's method. For

preparation of the whole cell extract, HepG2 cells not treated (control) or treated with homocysteine (as indicated previously) were washed in PBS, resuspended in 200 µl of radioimmuno precipitation assay buffer (150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8) and protease inhibitor cocktail. Afterward, it was incubated on ice for 10 min. The cells were then centrifuged at 9300 ×g for 10 min and the supernatant was taken as the whole cell extract. The amount of protein was measured with Bradford's method. Sample proteins from whole cell lysates (30 µg) and nuclear extracts (40 µg) were loaded onto 12% sodium dodecyl sulfate polyacrylamide gel for HO-1 and 10% polyacrylamide gel for Nrf2. They separated by electrophoresis, transferred to a nitrocellulose membrane and blocked. Immunoblot analysis was carried out using anti-HO-1 (1/1000) polyclonal antibody (Abcam Co., UK), anti-Nrf2 (1/500) polyclonal antibody (Abcam Co., UK). Anti-β-actin (1/3000) polyclonal antibody and anti-lamin B (1/2000) polyclonal antibody (Abcam Co., UK) were used to confirm the equal loading of proteins prepared from HepG2 cells after different treatments. The second antibody was goat polyclonal secondary antibody to rabbit IgG and was horseradish peroxidase conjugate. Visualization was accomplished by the enhanced chemiluminescence (ECL plus Western-blotting detection system, GE Healthcare). The intensity of bands was calculated with image J software (version 1.46a).

Electrophoretic mobility shift assay (EMSA). Synthetic complementary oligonucleotides (Sigma, USA) were 3'-biotinylated using the biotin 3'-end DNA labeling Kit (Pierce, USA) according to the manufacturer's instructions. After labeling, complementary strands were mixed together in an equimolar ratio and allowed to anneal at 37ºC for 2 h to form double-stranded probe. The sequences of the oligonucleotides, 5'-GATCTTTTATGCTGTGTCATGGTTT-3' and its complementary strand 5'-AAACCATGACACAGC ATAAAAGATC-3' were used as the probe. Binding reactions were carried out at room temperature for 20 min in the presence of 50 ng/µl poly (dI-dc), 0.05% Nonidet P-40, 5 mM MgCl$_2$, 10m M EDTA and 2.5% glycerol in 1× binding buffer (Lightshift chemilu-minescent EMSA Kit, Pierce) using 20 fmol biotin end-labeled target DNA and 10 µg of nuclear extract (as described previously). In antibody super shift assays, 1 µl of 1µg/µl Nrf2 antibody (Santa Cruz, sc-722X) was added to the reaction mixture prior to the addition of the probe. A 200-fold excess of unlabeled oligonucleotide (competitor) was added where

necessary. Assays were loaded onto native 5% polyacrylamide gels pre-electrophoresed in 0.5× Tris borate/EDTA for 60 min and electrophoresed at 100 V before being transferred onto a positively charged nylon membrane in 0.5× Tris borate/EDTA at 100 V for 30 min. The membrane was UV cross-linked and the biotin end-labeled probe was detected with streptavidin-horseradish peroxidase using a luminol enhancer solution (Lightshift chemiluminescent EMSA Kit, Pierce, USA) according to the manufacturer's instruction.

Statistical analysis. Data analysis was done using SPSS version 16. Data are presented as the means ± S.E. (standard error). Significance of differences between control and treated groups was determined by one-way analysis of variance (ANOVA). Least significant difference post-hoc analysis was performed for comparing the groups. For all analyses, *P* values less than 0.05 were considered significant.

RESULTS

Effect of homocysteine on cell viability. Exposure of HepG2 cells (1 × 10^6 cells/ml) to 50 µM homocysteine for up to 18 h had no effect on cell viability (control [live cells: 93 ± 0.7] vs. treatment group with 50 µM homocysteine [live cells: 93 ± 1.0]).

Homocysteine reduced GSH level. To determine if homocysteine cause oxidative stress in HepG2 cells, we evaluated the intercellular levels of the anti-oxidant thiol glutathione using flow cytometry. Incubations with 50 µM D,L-homocysteine resulted in a reduction in thioltracker violet dye fluorescence compared to that seen in untreated cells (Fig. 1). GSH content from 46% in untreated cells significantly decreased to about 19% an 35% within 15 min and 45 min of homocysteine exposure respectively (*P*<0.001). We did not observe any toxicity using this concentration of homocysteine.

Homocysteine induced Nrf2 protein expression. Since Nrf2 nuclear translocation is a key event in the activation of Nrf2; we examined Nrf2 nuclear accumulation in HepG2 cells treated with homocysteine. The HepG2 cells were incubated with homocysteine (50 µM) and the nuclear cell extracts were electrophoresed. Proteins were transferred and the transferred proteins were visualized and analyzed by Image J software. Western-blot analysis showed that homocysteine led to Nrf2 accumulation in nuclear extract by 1.31, 1.92 and 2.21 folds in HepG2 cells treated with homocysteine after 15, 30 and 45 min in compared with control (Fig. 2A). The statistical
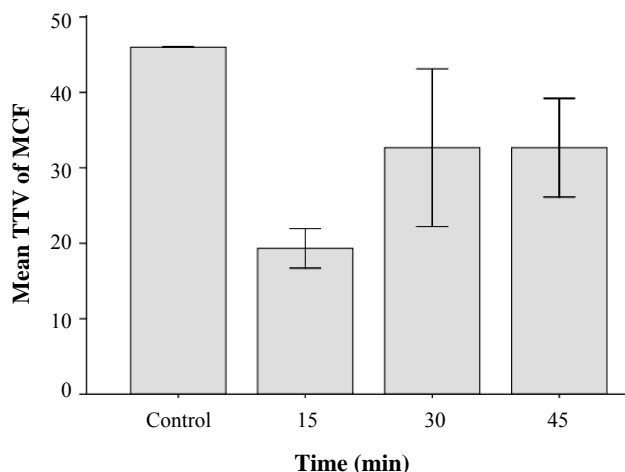
Fig. 1. Intercellular levels of cellular glutathione using flow cytometry in HepG2 cells exposed to homocysteine. HepG2 cells were treated with D/L homocysteine (50 μM) for the indicated times and harvested for flow cytometric analysis of intracellular glutathione content using mean channel fluorescence (MCF) of thioltracker Violet dye (TTV). Analysis of cellular GSH content was restricted to PI negative (intact) cells. Values are means ± S.E and statistically different from control.

analysis also showed that there was a significant difference between Nrf2 nuclear accumulation level in different treatment times ($P<0.001$).

Homocysteine up-regulates HO-1 expression via activation of Nrf2. Several investigators have defined Nrf2 as a major transcription factor regulating ARE-driven phase 2 gene expression [12, 16]. Therefore, we attempted to determine whether homocysteine could activate Nrf2 in association with HO-1 up-regulation in HepG2 cells. To elucidate the role of Nrf2 in transcriptional activation of ARE, EMSA was performed using oligomers containing HO-1 promoter-specific ARE-binding site. HepG2 cells were exposed to homocysteine (50 μM) for the indicated time periods. Incubation of the nuclear extract from the cells with biotin-labeled HO-1 ARE oligonucleotide resulted in the enhanced HO-1 ARE-binding activity of Nrf2. The nuclear DNA-binding of Nrf2 was increased by homocysteine treatment. The ARE-binding complex appeared at 60 min of homocysteine treatment and was maintained through 120 min. Densitometric evaluation showed that level of nuclear DNA-binding of Nrf2 was increased by 1.8 and 3.3 folds after 60 and 120 min of the treatment. There was a significant difference between binding nuclear protein level at 1 and 2 h with control ($P<0.01$). Addition of an anti-Nrf2 antibody indicates involvement of the Nrf2 transcription factor at this site (Fig. 2B).

Effect of homocysteine on HO-1 mRNA expression and protein expression. To determine the effect of Nrf2 activation on gene expression, levels of HO-1 gene (one of the Nrf2 target genes) were analyzed. To evaluate HO-1 mRNA expression, cells were treated with homocysteine and quantitative real time RT-PCR analysis was performed. As shown in Figure 3, mRNA expression of HO-1 in HepG2 cells exposed to homocysteine was increased in 3 and 6 h compared with the control and declined to basal level after 9 h. Levels of HO-1 were increased 3 folds ±1.5 and 5.7 folds ±1.82 in 3 and 6 h, respectively. The statistical analysis showed that there was a significant difference between expression level at 6 h and control ($P = 0.001$). The statistical analysis also showed that there was a significant difference between expression levels in different treatment time ($P<0.05$). To confirm the result obtained from real time RT-PCR experiment, Western-blot analysis was performed. The HepG2 cells were treated with homocysteine (50 μM) for 3, 6 and 9 h. The cell extracts were electrophoresed, and then the separated proteins were incubated with primary and secondary antibodies. Finally, the transferred proteins were visualized. The results showed that the levels of HO-1 protein started to decline 3 h after exposure to homocysteine and then increased by 1.32 and 2.1 folds in HepG2 cells treated with homocysteine for 6 and 9 h, respectively (Fig. 4). The statistical analysis showed that there was a significant difference between HO-1 protein level at 6 and 9 h with control ($P<0.001$ and $P = 0.004$, respectively). The statistical analysis also showed that there was a significant difference between HO-1 protein level in different treatment time ($P<0.001$).

DISCUSSION

Elevated levels of homocysteine play an important role in the pathogenesis of various diseases including hepatic dysfunction. The underlying mechanisms of how hyperhomocysteinemia contributes to patho-genesis of liver injury are still poorly understood and may be multifactorial.

We used HepG2 cells as a cellular model to consider the role of the self-protecting systems in the cells which arises upon exposure to homocysteine. In the present study we used 50 μM D,L-homocysteine for the treatment of the cells. This concentration of homocysteine that can be found in hyper-homocysteinemic individual [17] did not affect overall cell number or viability as we showed by Trypan blue assay. Studies on the other cultured cells also indicate that exogenous homocysteine in concentration up to 5 mM do not impair cell viability and only increase intracellular levels of homocysteine [18, 19].
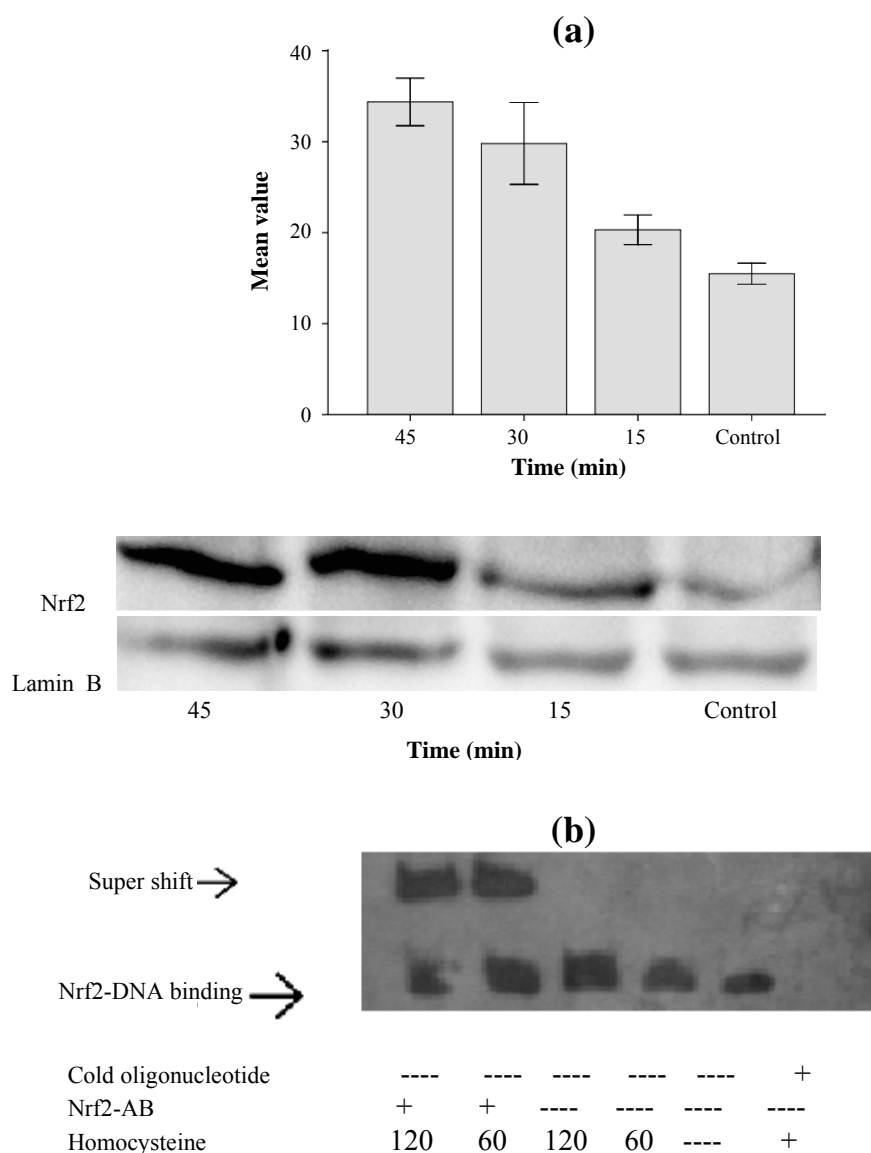
(a)

(b)

Cold oligonucleotide	----	----	----	----	----	+
Nrf2-AB	+	+	----	----	----	----
Homocysteine	120	60	120	60	----	+

Fig. 2. Nrf2 protein expression in HepG2 cells exposed to homocysteine. **(a)** Western-lot of samples from HepG2. Values represent the means ± S.E. of three separate experiments. The bar graph shows the quantization of Nrf2 protein. The nuclear cell extracts were electrophoresed, protein transferred, and blotted with the polyclonal antibody Nrf2, a major band appearing at approximately 68 kDa. Lamin B was used as loading control. Exposure to homocysteine (50 μM) for the indicated time periods causes Nrf2 nuclear accumulation. Quantification of band intensity was performed by Image J (version 1.46a). **(b)** Gel shift assay using oligomers containing the HO-1 promoter-specific ARE-biding site and nuclear fractions of HepG2 cells that were exposed to homocysteine (50 μM) for the indicated time periods (min). Competitor (200 folds excess) and antibodies were applied as indicated.

Using quantitative real time PCR analysis and Western-blotting, an increase in mRNA and protein levels for HO-1 was demonstrated. We identified that exposure to homocysteine at micro molar level is inducer of HO-1 in HepG2 cells. We found that HO-1 induction occurs via an ARE within the HO-1 promoter. Furthermore, we found that this induction is dependent on Nrf2-ARE pathway. The promoter region of the HO-1 gene contains multiple copies of ARE [13]. To elucidate the role of Nrf2 in transcriptional activation of ARE in response to homocysteine,

electromobility shift assay (EMSA) was performed. Using EMSA, we were able to demonstrate that homocysteine induces increased binding of Nrf2 nuclear proteins in HepG2 cells to ARE containing oligonucleotide specific for the HO-1 promoter. It is well established that Nrf2 nuclear accumulation is indicator of Nrf2 activation [20, 21]. Consistent with the results of EMSA, Western-blot analysis and increasing Nrf2 nuclear proteins after stimulation with homocysteine revealed that transcriptional factor Nrf2 plays a critical role in cellular response against
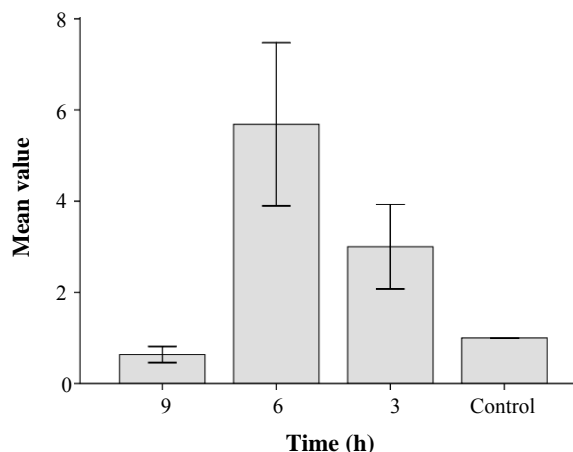
Fig. 3. Quantitative real-time RT-PCR analysis of samples from HepG2. Values represent the means ± S.E. of four separate experiments. The bar graph shows the quantization of HO-1 gene expression.

homocysteine-induced oxidative stress. Our data also indicated that Nrf2 is rapidly stabilized and mobilized to the nucleus in response to homocysteine treatment. The translocation of Nrf2 protein into nucleus following homocysteine treatment was associated with a marked increase in expression of HO-1.

Increased expression of HO-1 as an anti-oxidant has been reported in many studies [10, 11]. Induction of HO-1 expression under regulation of Nrf2-ARE is consistent with observation of Huang *et al.* [22], who reported that up-regulation of HO-1 expression inhibited oxidative stress in association with increased Nrf2-ARE-binding activity in hepatocytes. This study showed that some protective compounds inhibit oxidative stress in hepatocytes by up-regulation of HO-1. On the other hand, up-regulation of HO-1 in chronic liver diseases has been suggested in different studies [23-25]. In this context, the results obtained by Wei *et al.* [23] in patients with liver cirrhosis showed that HO-1 mRNA and protein expression were increased in hepatocytes of cirrhotic rat liver. Bessa *et al.* [24] showed that HO-1 mRNA expression in patients with chronic liver disease was significantly increased. There was a significant relationship between the increased HO-1 expression and oxidative stress biomarkers in patients with chronic liver diseases. Regarding clinical cases with non-alcoholic steatohepatitis, Malaguarnora *et al.* [25] showed that HO-1 expression was significantly increased in these patients, and the increase reflected the severity of the disease. Moreover non-alcoholic steatohepatitis patients with lower glutathione level exhibited higher expression of HO-1.
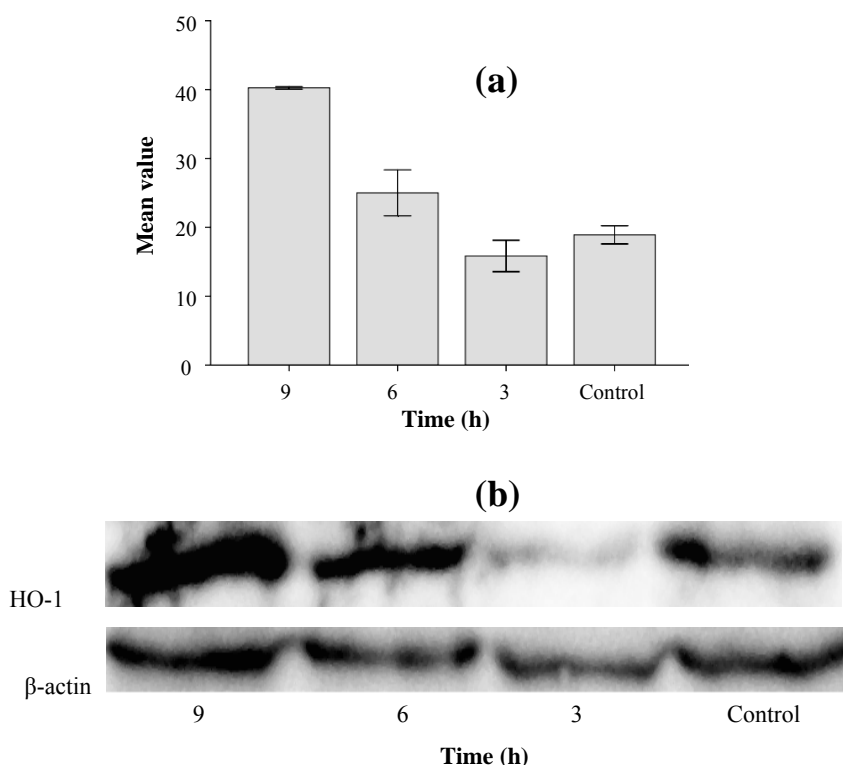

(a)

(b)

Fig. 4. Expression of HO-1 protein in HepG2 cells exposed to homocysteine. **(a)** Western blot of samples from HepG2. Values represent the means ± S.E. of three separate experiments. The bar graph shows the quantization of HO-1 protein. **(b)** Western blot analysis of HO-1 in HepG2 cells. The cells were incubated with 50 μM homocysteine for 3, 6 and 9 h, and the cell extracts were electrophoresed, protein transferred, and blotted with the polyclonal antibody to HO-1. HO-1 is a major band appearing at approximately 32 kDa. β -actin was used as loading control.

Regarding alcoholic steatohepatitis, Yao *et al.* [26] have shown that the induction of HO-1 by *Ginkgo biloba* is associated with a decrease in liver damage caused by ethanol feeding in rats. This is probably due to the enhanced anti-oxidative capacity against ethanol-induced oxidative stress and maintenance of cellular redox balance [27, 28]. Similar results were also obtained by Nepal *et al.* [29] in ethanol-induced apoptosis in HepG2 cells. They showed that HO-1 induction protects HepG2 cells from apoptosis. This protective effect is mediated via Nrf2 pathway.

Our results showed that Nrf2-dependent HO-1 induction is an adoptive response against oxidative stress induced by homocysteine. This was supported by a recent study by Abiko *et al.* [30] who reported that exposure of HepG2 cells to arsenic caused persistent induction of HO-1 accompanied by prolonged Nrf2 activation. Taken together, the results obtained from our study and the others [22, 27-30] showed that HO-1 induction not only is a general response in hepatocytes against oxidative stress but also is evoked by different protective compounds which have the potential to protect hepatic cells against oxidative stress-mediated chronic diseases.

Our study has suggested that the pathophysiological effects of homocysteine on hepatic cells are mediated via oxidative stress. The evidence from the present study suggests that the effects of homocysteine on hepatic cells are mediated via oxidative stress. First there was a significant decrease in glutathione (GSH) content in HepG2 cells treated with homocysteine. GSH is the most prevalent non-protein thiol and plays a fundamental role in defending against cellular injuries induced by oxidative stress in liver [31]. In this study, GSH levels significantly decreased after 15 min of treatment. This initial decline shows that to combat against oxidative stress induced by homocysteine, glutathione might be the first line of anti-oxidant defense. Perhaps this lowered GSH level which was caused the stimulation of Nrf2/ARE system and induced HO-1 expression. Second, homocysteine induces Nrf2/ARE pathway. This pathway is important in the cellular anti-oxidant defense system and is evoked when oxidative stress happens [20].Third, HO-1 mRNA and protein expression are up-regulated following oxidative stress and cellular injury [32].

Exposure to homocysteine causes GSH depletion, and changes in the redox status of the cell, which triggers nuclear translocation of Nrf2 and activation of the Nrf2/ARE pathway, in turn leading to up-regulation of HO-1. This suggests that an auto-regulation anti-oxidant mechanism exists against homocysteine and it is largely dependent on the activation of Nrf2 pathway. From this study, we conclude that HO-1 mRNA and protein expression are significantly increased in HepG2 cells exposed to homocysteine. Our results also show that this increase is under regulation of Nrf2/ARE pathway. As Nrf2/ARE pathway has been shown to provide protection against several oxidative impacts in different organs [33-35], induction of HO-1 under regulation of Nrf2/ARE might be a therapeutic option for liver dysfunctions in cases with severe hyperhomocys-teinemia. However, clinical studies are needed to clarify the exact role of HO-1 system in defending against homocysteine-induced oxidative stress and the potential clinical application of inducing the HO-1 system.

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