Homocysteine induces monocyte chemoattractant protein-1 expression by activating NF-κB in THP-1 macrophages

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Received 25 October 2000; accepted in final form 29 January 2001

Wang, Guoping, Yaw L. Siow, and Karmin O. Homocysteine induces monocyte chemoattractant protein-1 expression by activating NF-κB in THP-1 macrophages. Am J Physiol Heart Circ Physiol 280: H2840–H2847, 2001.—Homocysteinemia is an independent risk factor for cardiovascular disorders. The recruitment of monocytes is an important event in atherosclerosis. Monocyte chemoattractant protein-1 (MCP-1) is a potent chemokine that stimulates monocyte migration into the intima of arterial walls. The objective of the present study was to investigate the effect of homocysteine on MCP-1 expression in macrophages and the underlying mechanism of such effect. Human monocyte cell (THP-1)-derived macrophages were incubated with homocysteine. By nuclelease protection assay and ELISA, homocysteine (0.05–0.2 mM) was shown to significantly enhance the expression of MCP-1 mRNA (up to 2.6-fold) and protein (up to 4.8-fold) in these cells. Homocysteine-induced MCP-1 expression resulted in increased monocyte chemotaxis. The increase in MCP-1 expression was associated with activation of nuclear factor (NF)-κB due to increased phosphorylation of the inhibitory protein (IkB-α) as well as reduced expression of IkB-α mRNA in homocysteine-treated cells. In conclusion, our results demonstrate that homocysteine, at pathological concentration, stimulates MCP-1 expression in THP-1 macrophages via NF-κB activation.

chemotaxis; inhibitory protein; IkB-α; atherosclerosis; nuclear factor-κB

HOMOCYSTEINEMIA is one of the important risk factors for atherosclerosis (5, 7, 44). Atherosclerosis is the principal contributor to the pathogenesis of myocardial and cerebral infarction, which are the leading causes of death in developed countries. Abnormal elevations of homocysteine levels up to 0.1–0.25 mM in blood have been reported in patients with homocysteinemia (13). Elevated homocysteine levels have been observed in a significant proportion of patients with coronary artery disease (up to 30–40%) (25). Several plausible mechanisms for homocysteine-induced atherosclerosis have been suggested, including endothelial dysfunction, increased proliferation of smooth muscle cells, promotion of lipoprotein oxidation and platelet activation, and enhanced coagulability as well as increased cholesterol synthesis in hepatocytes (26).

One of the important features of atherosclerosis is monocyte infiltration into the injured arterial wall followed by differentiation into macrophages (30). These macrophages then take up large amounts of lipids to become foam cells (13, 14). Monocyte chemoattractant protein-1 (MCP-1) is a potent chemokine that stimulates the migration of monocytes into the intima of arterial walls (24). MCP-1 mRNA and protein are detectable in atherosclerotic lesions in both human and experimental animals (9, 46). Although many factors have been identified to induce MCP-1 expression, the effect of homocysteine on the expression of MCP-1 in atherosclerotic lesions is not entirely clear. The biochemical events leading to the infiltration of monocytes into the arterial wall in homocysteinemic patients remain to be elucidated.

Recent studies (3, 21, 36) suggest that the transcription factor nuclear factor (NF)-κB plays an important role in upregulating the expression of MCP-1 and other inflammatory factors in atherosclerotic lesions. The promoter region of the MCP-1 gene consists of several putative binding sites for transcription-activator factors including NF-κB (40). NF-κB is normally present in the cytoplasm in an inactive form associated with an inhibitory protein named IkB (21, 33, 36). Although several inhibitor proteins have been identified (IkB-α, IkB-β, IkB-γ, and p105), IkB-α is the best-characterized form of IkB (36). After stimulation, there is a rapid phosphorylation of IkB-α and subsequent degradation of IkB-α by the proteosome, leading to the release of NF-κB followed by its translocation into the nucleus. Once inside the nucleus, NF-κB dimers bind to the κB-binding motifs in the promoters or enhancers of the genes encoding cytokines (21, 36). The activation of p50/p65, (p65)2, or c-Rel/p65 protein complexes have been detected in various types of cells (36, 40). Protein kinases responsible for phosphorylation of IkB-α are still under extensive investigation. Recently, one of the possible protein kinases, called IkB kinase, has been identified (14).

The finding that macrophages accumulate in atherosclerotic lesions suggests that the recruitment of monocytes is enhanced during the development of athero-
sclerosis (13, 14). The accumulated macrophages in atherosclerotic lesions are capable of producing various cytokines including MCP-1. Although MCP-1 expression in endothelial cells may play an important role in the initiation of monocyte infiltration into the arterial wall, it is plausible that the MCP-1 produced by macrophages may facilitate the further recruitment of additional monocytes into atherosclerotic lesions. In the present study, we investigated the effect of homocysteine on MCP-1 expression as well as elucidated the mechanism(s) by which homocysteine regulates MCP-1 expression, leading to enhanced monocyte chemotaxis. Our results clearly demonstrate that homocysteine, at pathophysiological concentrations, stimulates MCP-1 expression via the activation of NF-κB.

MATERIALS AND METHODS

Cell culture. THP-1 cells, a human monocytic cell line, were purchased from the American Type Culture Collection. Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum. For experiments, THP-1 cells were cultured in six-well dishes in the presence of phorbol 12-myristate 13-acetate (PMA) for 16 h to induce differentiation of these cells into macrophage-like cells (defined as THP-1 macrophages) (16, 42).

Nuclease protection assay and ELISA. After THP-1 macrophages were incubated with DL-homocysteine (Sigma) for various time periods, total RNA was isolated from cultured cells with TriZOL Reagent (Life Technologies). Assays were performed with a nuclease protection assay kit (Ambion). In brief, the isolated RNA (10 μg) was hybridized with 32P end-labeled MCP-1 or IκB-α oligonucleotide probes overnight at 30°C followed by nuclease digestion (23). A 28S rRNA oligonucleotide probe (Ambion) was used as an internal control. After digestion, the protected fragments were resolved on a denaturing 12% polyacrylamide gel containing 8 M urea followed by transfer to a nitrocellulose membrane. The membrane was then probed with rabbit anti-IκB-α antibody or anti-phosphorylated IκB-α antibodies (New England Biolabs). Bands corresponding to IκB-α or phosphorylated IκB-α proteins were visualized using enhanced chemiluminescence reagents (Amersham Pharmacia; Boston, MA) and analyzed with a gel documentation system (Bio-Rad Gel Doc1000 and Multi-Analyst version 1.1). Values are expressed as relative expression of MCP-1 mRNA or IκB-α mRNA normalized to 30S rRNA levels. The amount of MCP-1 protein secreted by cultured cells into the medium was determined by ELISA (R&D Systems).

For MCP-1 mRNA stability experiments, cells were incubated in the absence or presence of homocysteine for 4 h, and actinomycin D was added at a final concentration of 5 μg/ml (22). Cells were further cultured for various time periods, and mRNA was analyzed as described above.

Monocyte chemotaxis. Monocyte chemotaxis was measured using a 48-well Micro Chemotaxis Chamber (Neuro Probe; Gaithersburg, MD) (6, 18, 32, 43). First, THP-1 macrophages were cultured for 6 h in the absence (control) or presence of homocysteine. After incubation, the medium (defined as conditioned medium) was transferred to the lower chamber of the Micro Chemotaxis Chamber. The lower and upper chambers were separated by a polycarbonate membrane with a pore size of 5 μm (Neuro Probe). An aliquot of THP-1 monocyte cell suspension (2 × 10⁶ cells/ml) was added to the upper chamber, and the cells were allowed to transmigrate for 2 h. After transmigration, the surface of the membrane facing the THP-1 cell suspension was scraped and washed three times according to the manufacturer’s instructions. The migrated cells, on the side of the membrane facing the conditioned medium, were fixed and then stained with hematoxylin. The number of migrated monocytes was determined with the use of light microscopy. THP-1 macrophages possess human monocyte-like characteristics (38). Many investigators (8, 11, 39) have used those cells to study the expression of MCP-1 and chemotactic activity. For example, oxidized low-density lipoprotein (LDL)/lipopolysaccharide was shown to stimulate THP-1 cell chemotaxis via p38 mitogen-activated protein kinase activation (15), and Han et al. (10) recently reported that LDL enhances MCP-1-mediated chemotaxis in THP-1 monocytes.

Electrophoretic mobility shift assay and supershift assay. Nuclear proteins were isolated by a method described previously (31). An electrophoretic mobility shift assay (EMSA) was performed to determine NF-κB/DNA-binding activity (20). In brief, nuclear proteins (10 μg) were incubated with the reaction buffer for 15 min at room temperature followed by incubation with a 32P end-labeled oligonucleotide containing a sequence for a NF-κB/DNA-binding site (5’-AGAGTGG-GAATTCCACTCA-3’) (40). The reaction mixture was separated in a nondenaturing 6% polyacrylamide gel, which was later exposed to the X-ray film at −70°C. The binding of labeled oligonucleotide to nuclear proteins was blocked by adding unlabeled oligonucleotide to the reaction mixture. This was to confirm that the binding of 32P end-labeled oligonucleotide to NF-κB was sequence specific. A supershift assay was performed with antibodies (2 μg) against p50, p65, c-Rel, or normal rabbit IgG. Another transcription factor, activator protein-1 (AP-1), was also analyzed by EMSA (17) with a consensus sequence for the AP-1/DNA-binding site according to the manufacturer’s instruction (Promega).

Western immunoblotting analysis of IκB-α. The cellular levels of IκB-α proteins were determined by Western immunoblotting analysis (4). Briefly, cellular proteins were separated by SDS-12.5% polyacrylamide gel electrophoresis followed by electrophoretic transfer of proteins from the gel onto a nitrocellulose membrane. The membrane was then probed with rabbit anti-IκB-α or anti-phosphorylated IκB-α antibodies (New England Biolabs). Bands corresponding to IκB-α or phosphorylated IκB-α proteins were visualized using enhanced chemiluminescence reagents (Amersham Pharmacia; Boston, MA) and analyzed with a gel documentation system (Bio-Rad Gel Doc1000 and Multi-Analyist version 1.1).

Statistical analysis. The results were analyzed using a two-tailed independent Student’s t-test. The level of statistical significance was set at P < 0.05.

RESULTS

Effect of homocysteine on MCP-1 expression. THP-1 macrophages were incubated with homocysteine for various time periods, and the MCP-1 mRNA expression was determined by nuclease protection assay. As shown in Fig. 1A, the expression of MCP-1 mRNA was significantly enhanced after homocysteine treatment. The increase in the levels of MCP-1 mRNA reached a maximum at 4 h of incubation. The amount of MCP-1 protein produced by cultured cells was then determined by ELISA. As shown in Fig. 1B, the amount of MCP-1 protein in the medium collected from homocysteine-treated cells was significantly elevated compared with the control and reached the highest amount at 6 h of incubation. The levels of MCP-1 protein in the cultured medium gradually returned to basal levels after 24 h of incubation. Although the exact mechanism by which MCP-1 protein levels returned to the basal levels at 24 h of incubation is unknown, the degradation of
MCP-1 and the metabolism of homocysteine during the course of incubation may contribute to the decrease in the MCP-1 levels in the culture medium. It has also been reported by other investigators (42) that the expression of MCP-1 was increased in THP-1 macrophages in response to homocysteine.

Fig. 1. Effect of homocysteine on monocyte chemoattractant protein (MCP)-1 mRNA and protein expression in THP-1 macrophages. 
A: THP-1 macrophages were incubated in the absence (control) or presence of homocysteine (0.1 mM) for 2 to 24 h. After incubation, nuclease protection assays were performed. Top, typical autoradiograph of MCP-1 mRNA and 28S rRNA as determined by nuclease protection assay. Bottom, the graph of quantitative amount of MCP-1 mRNA (normalized to 28S rRNA) produced over time. 
B: THP-1 macrophages were incubated in the absence (control, ■ and dashed lines) or presence of homocysteine (0.1 mM, ▲ and solid lines) for various time periods. At the end of incubation, the amount of MCP-1 protein secreted by THP-1 macrophages to culture media was determined by ELISA. The results are depicted as means ± SD (error bars) of 5 separate experiments. *P < 0.05 compared with control values.

Fig. 2. Effect of homocysteine at various concentrations on MCP-1 mRNA expression. 
A: after THP-1 macrophages were incubated with homocysteine at various concentrations for 4 h, nuclease protection assays were performed. Values are expressed as the relative expression of MCP-1 mRNA normalized to 28S rRNA levels. Cells without homocysteine treatment were used as the control. The results are depicted as means ± SD (error bars) of 5 separate experiments. *P < 0.05 compared with control values. 
B: THP-1 macrophages were incubated in the absence (control, ■ and dashed lines) or presence of homocysteine (0.1 mM, ▲ and solid lines) for various time periods. Actinomycin D (at a final concentration of 5 µg/ml) was then added, and cells were further incubated for 2–8 h. The MCP-1 mRNA levels were assessed by a nuclease protection assay. Results are expressed as a percentage of control (at the point before addition of actinomycin D) and depicted as means ± SD (error bars) of 5 separate experiments.
phages during early incubation with stimuli (LDL and an inhibitor of acyl-CoA, cholesterol acyltransferase) and returned to basal level 20 h after incubation (42).

THP-1 macrophages were then incubated with various concentrations of homocysteine for 4 h. As shown in Fig. 2A, the ratio of MCP-1 mRNA to 28S rRNA was significantly increased in THP-1 macrophages treated with homocysteine in a concentration-dependent manner, reflecting a significant increase (1.5- to 2.6-fold) in levels of MCP-1 mRNA compared with the control. To differentiate whether homocysteine prolonged the stability of MCP-1 mRNA, THP-1 macrophages were incubated with actinomycin D, a transcriptional inhibitor, in the absence or presence of homocysteine. As shown in Fig. 2B, the relative rate of MCP-1 mRNA decay remained the same in THP-1 macrophages treated with homocysteine compared with control cells. Thus it appears that the homocysteine could induce MCP-1 expression at a transcriptional level.

Effect of homocysteine-induced MCP-1 production on monocyte chemotaxis. To investigate whether homocysteine-induced MCP-1 expression resulted in increased monocyte chemotaxis, culture media were collected from THP-1 macrophages pretreated with homocysteine (defined as macrophage-conditioned medium). As shown in Fig. 3, homocysteine-induced MCP-1 expression resulted in a significant increase in THP-1 monocyte chemotaxis. Furthermore, anti-MCP-1 antibody treatment (0.5 μg/ml) completely abolished the stimulatory effect of conditioned media on monocyte chemotaxis (Fig. 3), indicating that MCP-1 was the major chemoattractant protein in THP-1 macrophages treated by homocysteine.

Effect of homocysteine on NF-κB/DNA-binding activity. To investigate the involvement of NF-κB activation in homocysteine-induced MCP-1 expression in THP-1 macrophages, EMSA was performed. As shown in Fig. 4A, NF-κB/DNA-binding activity was significantly elevated in cells treated with homocysteine for 30 min and reached a peak at 90 min of incubation with homocysteine. To determine which components of NF-κB family were activated, a supershift assay using specific antibodies against individual subunits was performed. As shown in Fig. 4B, addition of anti-p50, -p65, c-Rel, or nonspecific rabbit IgG. Antibodies against p50, p65, or c-Rel supershifted the NF-κB/DNA complex. Absence of antibodies. C: EMSA performed to determine activator protein (AP)-1/DNA-binding activity.
NF-κB/DNA band. To investigate whether homocysteine also activated another transcription factor such AP-1, AP-1/DNA-binding activity was determined. As shown in Fig. 4C, AP-1-binding activity was not significantly changed after homocysteine stimulation.

Effect of NF-κB inhibitors on homocysteine-mediated MCP-1 expression and monocyte chemotaxis. To determine whether NF-κB activation was necessary for homocysteine-mediated MCP-1 expression, two NF-κB inhibitors, N α-p-tosyl-l-lysine chloromethyl ketone (TLCK) or N-acetyl-l-cysteine (NAC), were used (20). As shown in Fig. 5A, the expression of MCP-1 mRNA was significantly elevated in cells preincubated with homocysteine (0.1 mM). Both inhibitors completely abolished the homocysteine-mediated MCP-1 mRNA expression. Second, the effect of the inhibitors on homocysteine-mediated NF-κB activation was examined. These two inhibitors also abolished the effect of conditioned media on monocyte chemotaxis (Fig. 5B). Furthermore, these inhibitors also abolished the stimulatory effect of conditioned media on monocyte chemotaxis (Fig. 5C).

Effect of homocysteine on IκB-α expression. The activation of NF-κB might be caused by enhanced phosphorylation and degradation of the inhibitor protein IκB-α. As shown in Fig. 6A, the level of phospho-IκB-α...
was significantly elevated in cells incubated with homocysteine for 15 min and returned to the basal level at 30 min of incubation. We also investigated whether the activation of NF-κB might be caused by a reduction in the protein levels of IκB-α in homocysteine-treated cells. As shown in Fig. 6B, homocysteine treatment caused a significant reduction in the levels of IκB-α protein. The expression of IκB-α mRNA in cells treated with homocysteine was then analyzed. As shown in Fig. 7, homocysteine treatment caused a significant reduction in the levels of IκB-α mRNA. This inhibitory effect was observed after THP-1 macrophages were incubated with homocysteine for 30 min. The maximum inhibitory effect was obtained from cells treated with homocysteine for 60–90 min.

DISCUSSION

Many prospective epidemiological studies have indicated that homocysteinemia is involved in atherogenesis. The recruitment of monocytes into the arterial wall is regarded as an early event during the development of atherosclerosis. MCP-1 plays an important role in monocyte chemotaxis. At present, the mechanism by which an elevated homocysteine level promotes monocyte infiltration and macrophage accumulation during atherogenesis is not fully understood. The results obtained from the present study have clearly demonstrated that 1) homocysteine stimulates MCP-1 expression in human monocyte-derived macrophages, which results in increased monocyte chemotaxis; 2) such stimulatory effect is mediated via the activation of NF-κB; and 3) homocysteine increases the phosphorylation of IκB-α protein as well as reduces the expression of IκB-α mRNA and protein, leading to the activation of NF-κB. Taken together, these results provide a notion that NF-κB activation plays an important role in homocysteine-induced MCP-1 expression, leading to enhanced monocyte chemotaxis.

The finding that macrophages and foam cells accumulate in atherosclerotic lesions suggests that the recruitment of monocytes is enhanced during the process of atherosclerosis. The recruitment of monocyte/macrophages appears to be mediated by MCP-1, which exerts its action mostly through interaction with the CCR2 receptor (2, 24). The importance of MCP-1 and its receptor CCR2 in the development of atherosclerosis was further revealed in CCR2-deficient mice (1). Boring et al. (1) recently reported a dramatic reduction in atherosclerotic lesion formation in apolipoprotein E-null mice (genetically modified to develop atherosclerosis) that also lacked CCR2. During the development of atherosclerosis, the site of origin of the inflammatory signals, including MCP-1, is thought to be the vessel wall itself (28). Many cells have the potential to secrete MCP-1 in vitro as well as in vivo (24). The expression of MCP-1 mRNA and protein has been detected in macrophages, endothelial cells, and smooth muscle cells of human and animal atherosclerotic lesions (46). We (34) recently reported that homocysteine stimulates MCP-1 expression in cultured endothelial cells, leading to enhanced monocyte adhesion to endothelial cell. Endothelial expression of MCP-1 is thought to initiate the subendothelial migration of monocytes in early atherosclerotic lesions (24). Upon stimulation, macrophages are able to produce significant amount of MCP-1 in atherosclerotic lesions (24, 46). The present study demonstrates that homocysteine also stimulates MCP-1 expression and secretion in macrophages. As a consequence, homocysteine-induced MCP-1 production, monocyte chemotactic activity was significantly increased.

Recent evidence (36, 40) suggests that the activation of NF-κB is involved in the induction of MCP-1 gene expression. However, the role of this transcription factor in homocysteine-induced MCP-1 expression is largely unknown. In the present study, several lines of evidence clearly indicate that NF-κB is activated in homocysteine-treated macrophages. First, the results from EMSA demonstrated that activation of NF-κB by homocysteine treatment (maximal at 1.5 h) preceded the induction of MCP-1 mRNA expression (maximal at 4 h). Second, the findings that NF-κB inhibitors were able to completely abolish homocysteine-induced MCP-1 mRNA expression as well as subsequent monocyte chemotaxis suggested that the activation of NF-κB might be a prerequisite for homocysteine-induced MCP-1 expression in THP-1 macrophages. Although the predominant NF-κB isofrom is thought to be a p50/p65 heterodimer, multiple NF-κB isoforms have also been detected in various cells (6, 27, 40). For example, the assembly and activation of the tumor...


necrosis factor-responsive element of murine MCP-1 gene expression is mediated through NF-κB p65 (43), whereas Chlamydia pneumonia infection of smooth muscle and endothelial cells was shown to activate p50/p65 heterodimers (6). Ueda et al. (40) reported that the binding of (p65)2 and c-Rel/p65 to NF-κB-binding sites of the MCP-1 gene elevated the transcription of the human MCP-1 gene. Results from the present study also revealed an activation of p50, p65, and c-Rel proteins in THP-1 macrophages after homocysteine stimulation. We speculate that p50/p65, (p65)2, and c-Rel/p65 might be candidates likely to mediate homocysteine-induced MCP-1 expression in THP-1 macrophages.

The pathways of NF-κB activation have been intensely elucidated by many investigators. Most studies (33, 36) have demonstrated that upon stimulation, IκB-α is rapidly phosphorylated, leading to the ubiquitination and subsequent degradation of IκB-α. Released NF-κB can then translocate to the nucleus and regulate the expression of target genes. In the present study, the level of phospho-IκB-α was significantly elevated in THP-1 macrophages treated with homocysteine for 15 min but returned to the basal level at 30 min of incubation (Fig. 6A). We also observed that the levels of IκB-α mRNA decreased significantly after 30 min of incubation in cells with homocysteine (Fig. 7) followed by a further reduction in IκB-α protein levels (Fig. 6B). Therefore, rapid phosphorylation of IκB-α protein might serve as a signal for degradation of this inhibitory protein during an early stage of incubation with homocysteine. The initial increase in the level of phospho-IκB-α and a subsequent decrease in IκB-α mRNA and protein expression may be responsible for the activation of NF-κB, eventually leading to enhanced MCP-1 expression. Although another ubiquitous transcription factor (AP-1) has been indicated to participate in various immune and acute phase responses (17, 43), the results obtained from the present study suggests that AP-1 activation may not be involved in homocysteine-induced MCP-1 expression in THP-1 macrophages.

The advantages for using PMA-activated THP-1 cells as a macrophage model in our study were severalfold. First, THP-1 cells are homogeneous, and, upon PMA activation, they are highly differentiated and demonstrate macrophage-like characteristics (37, 41). Second, upon differentiation, THP-1 macrophages express scavenger receptors (35, 42). Such a property allowed many investigators (35, 42) to study lipid (cholesterol) loading and foam cell formation in these cells, which are important features during the development of arteriosclerosis. Furthermore, macrophages are one of the major sources for producing MCP-1 in the injured vascular wall. Many investigators (10, 15, 45) have also studied the expression of MCP-1 and the activation of NF-κB as well as the regulation of other cytokines and transcriptional factors in PMA-activated THP-1 macrophages. In addition, the homogeneity of the THP-1 cell line allows comparison of findings obtained from different experiments.

Homocysteinemia refers to a plasma homocysteine level above the normal concentration (5–15.9 μmol/l) (7, 19, 29). Abnormal elevations of homocysteine levels up to 0.1–0.25 mM in blood have been reported in patients with homocysteinemia (12, 13). The concentrations of homocysteine used in the present study to display its stimulatory effect on NF-κB activation and MCP-1 expression were similar to those found in the plasma of patients with moderate homocysteinemia. Moderately elevated levels of plasma homocysteine are regarded as an independent risk factor for atherosclerosis (12).

In summary, the present study has clearly demonstrated that homocysteine, at pathophysiological concentrations similar to those found in the plasma of patients, induces the phosphorylation of IκB-α protein and reduces the expression of IκB-α mRNA and protein, which in turn activates NF-κB in macrophages. The activation of NF-κB is necessary for homocysteine-induced MCP-1 gene expression, leading to enhanced MCP-1 protein production and subsequent monocyte chemotaxis. These findings may provide us with one of the important mechanisms by which homocysteine causes atherosclerosis.

This study was supported by Research Grant Council of Hong Kong Grants HKU 7288/98M and HKU/7346/00M (to K. O).

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