Homocysteine stimulates nuclear factor κB activity and monocyte chemoattractant protein-1 expression in vascular smooth-muscle cells: a possible role for protein kinase C

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Monocyte chemoattractant protein-1 (MCP-1) is a potent chemokine that stimulates the migration of monocytes into the intima of arterial walls. Although many factors that induce MCP-1 expression have been identified, the effect of homocysteine on the expression of MCP-1 in atherogenesis and the underlying mechanisms are not entirely clear. The objective of the present study was to investigate the role of homocysteine in MCP-1 expression in human aorta vascular smooth-muscle cells (VSMCs). After VSMCs were incubated with homocysteine for various time periods, a nuclease protection assay and ELISA were performed. Homocysteine (0.05–0.2 mM) significantly increased the expression of MCP-1 mRNA (up to 2.7-fold) and protein (up to 3.3-fold) in these cells. The increase in MCP-1 expression was associated with the activation of protein kinase C (PKC) as well as nuclear factor κB (NF-κB). Further investigation demonstrated that the activation of NF-κB was the result of a PKC-mediated reduction in the expression of inhibitory protein (IκBα) mRNA and protein in homocysteine-treated cells. Oxidative stress might also be involved in the activation of NF-κB by homocysteine in VSMCs. In conclusion, the present study has clearly demonstrated that the activation of PKC as well as superoxide production followed by activation of NF-κB is responsible for homocysteine-induced MCP-1 expression in VSMCs. These results suggest that homocysteine-stimulated MCP-1 expression via NF-κB activation may play an important role in atherogenesis.

Key words: atherosclerosis, calcium, IκBα, oxidative stress, phosphorylation.

INTRODUCTION

Homocysteinaemia is an important risk factor for atherosclerosis [1–5]. Abnormal elevations of homocysteine levels up to 0.1–0.25 mM in blood have been reported in patients with homocysteinaemia [6–7]. Plausible mechanisms of homocysteine-induced atherosclerosis include 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 [4,8,9].

One of the important features of atherosclerosis is monocyte infiltration into the injured arterial wall followed by monocyte differentiation into macrophages. These macrophages then take up large amount of lipids to become foam cells [10–12]. The migration of monocytes into the intima of arterial walls can be stimulated by a chemokine monocyte chemoattractant protein-1 (MCP-1) [13–19]. The expression of MCP-1 and other inflammatory factors in atherosclerotic lesions can be up-regulated by the transcription factor nuclear factor κB (NF-κB) [20–23]. Among the several inhibitor proteins identified (IκBα, IκBβ, IκBγ and p105), IκBα is the best-characterized form of IκB [23,24]. Upon stimulation with various NF-κB stimuli, IκBα is rapidly phosphorylated, leading to the ubiquitination and subsequent degradation of IκBα, as well as translocation of NF-κB to the nucleus (activated NF-κB) [23–26]. Activated NF-κB has been detected in macrophages, endothelial cells and smooth-muscle cells of human atherosclerotic lesions [20]. In addition, studies from other laboratories as well as ours have demonstrated that the expression of MCP-1 can be regulated by the protein kinase C (PKC) signalling pathway [27,28].

Functional changes in vascular smooth-muscle cells (VSMCs) have been implicated in the development of atherosclerosis [29,30]. The proliferation of VSMCs can lead to excessive production of extracellular matrix, generation of cytokines and growth factors, including MCP-1 [29,30]. 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 [18,19]. At present, little is known about the role of homocysteine in MCP-1 expression. In the present study we investigated the effect of homocysteine, at concentrations similar to those found in the plasma of patients, on MCP-1 expression in human aorta VSMCs. We also performed experiments to elucidate the underlying mechanism for such effect.

EXPERIMENTAL

Culture of VSMCs

A human aorta VSMC cell line was purchased from the American Type Culture Collection (A.T.C.C.). The cell line (HA-VSMC, catalogue no. CRL-1999) used in the present study was a smooth-

Abbreviations used: MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor κB; PKC, protein kinase C; IκB, inhibitory protein κB; VSMCs, vascular smooth-muscle cells; EMSA, electrophoretic-mobility-shift assay; Tos-Lys-CH_2Cl, N^{α}-*p*-tosyl-L-lysylchloromethane ('TLCK'); NAC, *N*-acetyl-L-cysteine; SOD, superoxide dismutase; RT-PCR, reverse-transcription PCR; TNFα, tissue necrosis factor-α; Ro-32-0432, bisindolylmaleimide XI hydrochloride.

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muscle cell line initiated from the normal aorta of an 11-month-old Caucasian female. Progeny from the distribution stock of HA-VSMC (P16) have been shown to undergo approx. 15–20 population doublings (P31-36) from the frozen state before the onset of senescence in longevity studies conducted during characterization. Cells were cultured in F-12K Nutrient medium (Gibco BRL) containing 10% foetal bovine serum, and results were obtained from cells between passages 18 and 22.

Nuclease protection assay

Total RNA was isolated from cultured cells with TRIzol Reagent (Life Technologies). Assays were performed with a nuclease protection assay kit (Ambion, Austin, TX, U.S.A.). In brief, the isolated RNA (10 μg) was hybridized with ^{32}P-end-labeled MCP-1 oligonucleotide probe (ClonTech Laboratories, Palo Alto, CA, U.S.A.) overnight at 30 °C, followed by nuclease digestion according to the manufacturer's instruction [31]. A positive control, 28 S rRNA oligonucleotide probe (Ambion), was used as an internal control. After digestion, the protected fragments were resolved on a denaturing polyacrylamide gel (12%) containing 8 M urea followed by transfer on to filter paper and exposure to X-ray film. The bands corresponding to MCP-1 mRNA or 28 S rRNA were analysed using a gel documentation system (Bio-Rad Gel Doc1000 and Multi-Analyst® version 1.1). Values are expressed as the relative expression of MCP-1 mRNA normalized to 28 S rRNA levels.

Determination of MCP-1 protein by ELISA

The amount of MCP-1 protein secreted into the culture medium by cultured cells was determined by ELISA (R & D systems, Minneapolis, MN, U.S.A.). The polystyrene microplate (96 wells) was coated with a murine monoclonal anti-MCP-1 antibody, and recombinant human MCP-1 was used as the standard.

Electrophoretic-mobility-shift assay (EMSA) and supershift assay

Nuclear proteins were isolated by a method described previously [32]. EMSA was performed to determine the NF-κB–DNA binding activity [33,34]. In brief, nuclear proteins (10 μg) were incubated with the reaction buffer for 15 min at room temperature, followed by incubation with ^{32}P-end-labelled oligonucleotide containing the consensus sequence for the NF-κB–DNA binding site (5′-GGGGACTTTCC-3′) (Life Technologies). The reaction mixture was separated in a non-denaturing polyacrylamide gel (6%) that was later exposed to the X-ray film at −70 °C. The binding of labelled oligonucleotide to nuclear proteins was blocked by adding unlabelled specific competitor oligonucleotide to the reaction mixture (Life Technologies). This was to confirm that the binding of ^{32}P-end-labelled oligonucleotide to NF-κB was sequence-specific. A supershift assay was also performed to characterize the specific NF-κB subunits in the complex [30]. Specific antibodies (2 μg) against p50 and/or p65 or normal rabbit IgG (Santa Cruz Biotechnology) were added to the nuclear proteins prior to the EMSA.

Assay for PKC activity

Cell lysate was prepared as described previously [28]. PKC activity was determined using a synthetic peptide as a substrate (Ac-FKKSFKL-NH_2, where Ac is acetyl and NH_2 is an amide group) [28]. The reaction mixture contained 50 μM peptide substrate in 20 mM Hepes (pH 7.5)/1 mM dithiothreitol/100 μM [γ-^{32}P]ATP ($\approx$ 1500 c.p.m./pmol)/5 mM $MgCl_2$/0.5 mM $CaCl_2$ and sonicated dispersions of brain phosphatidylserine (140 μM) and diacylglycerol (3.8 μM). After 10 min incubation at 30 °C, the reaction was terminated by spotting an aliquot of the reaction mixture on to Whatman P81 ion-exchange-chromatography paper, followed by four washes with 0.4% (v/v) phosphoric acid and a 95% (v/v) ethanol rinse. The radioactivity (^{32}P) incorporated into the peptide substrate was determined by liquid-scintillation counting.

Analysis of IκB-α mRNA expression

The expression of IκB-α mRNA in cultured cells was examined by semi-quantitative reverse-transcription (RT-) PCR analysis [30]. The primers used in the PCR reactions [(1) 5′-GCTCGG-AGCCCTGGAAGC-3′; (2) 5′-GCCCTGGTAGGTAACTCT-3′] were synthesized by ClonTech. RT-PCR was performed. Total RNA (2 μg) was used in the RT reaction. The reaction mixture for PCR contained 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.4 μM 5′ primer, 0.4 μM 3′ primer, 2 units of *Taq* DNA polymerase and 1 μg of cDNA product from the RT reaction. After 35 cycles of PCR amplification (94 °C for 30 s, 55 °C for 45 s and 72 °C for 90 s), an additional 7 min extension was carried out at 72 °C. The products of PCR were separated by 1.8%-agarose gel (containing ethidium bromide) electrophoresis and visualized under UV light using a gel documentation system (Bio-Rad Gel Doc1000). Human β-actin was used as an internal standard to verify equal PCR product loading for each experiment. The primers for β-actin used in the PCR-reaction [(1) 5′-GTGGGGCGCCCCAGGCACCA-3′; (2) 5′-CTCCTTAAT-GTCACGCACGATTT-3′] were synthesized by ClonTech. After the RT-PCR reaction, the IκBα signal was normalized by comparison with the β-actin signal from the same sample [35,36]. Values are expressed as a ratio of IκBα to β-actin.

Western immunoblotting analysis of IκBα protein

The cellular levels of IκBα protein were determined by Western immunoblotting analysis [37]. Briefly, cell proteins were separated by SDS/12.5% (w/v)-PAGE, followed by electrophoretic transfer of proteins from the gel to a nitrocellulose membrane. The membrane was probed with rabbit anti-IκBα or anti-phosphorylated IκBα antibodies (New England Biolabs, Beverly, MA, U.S.A.). Bands corresponding to IκBα or phosphorylated IκBα proteins were visualized using enhanced chemiluminescence (ECL®; Amersham) reagents and analysed with a gel documentation system (Bio-Rad Gel Doc1000 and Multi-Analyst® version 1.1).

Statistical analysis

The results were analysed by using a two-tailed independent Student *t*-test. The level of statistical significance was set at $P < 0.05$.

RESULTS

Homocysteine stimulates MCP-1 expression

Incubation of cells with homocysteine (0.1 mM) for various time periods resulted in a significant increase in MCP-1 mRNA expression at 4 h which reached a maximum at 12 h (Figure 1A). This stimulatory effect of homocysteine on MCP-1 mRNA expression occurred in a concentration-dependent manner (0.05–0.2 mM) (Figure 1B). In accordance with these results, homocysteine treatment significantly increased the amount of MCP-1 secreted by cultured cells, and the highest amount of MCP-1 secretion (3.3-fold increase) was observed after 24 h

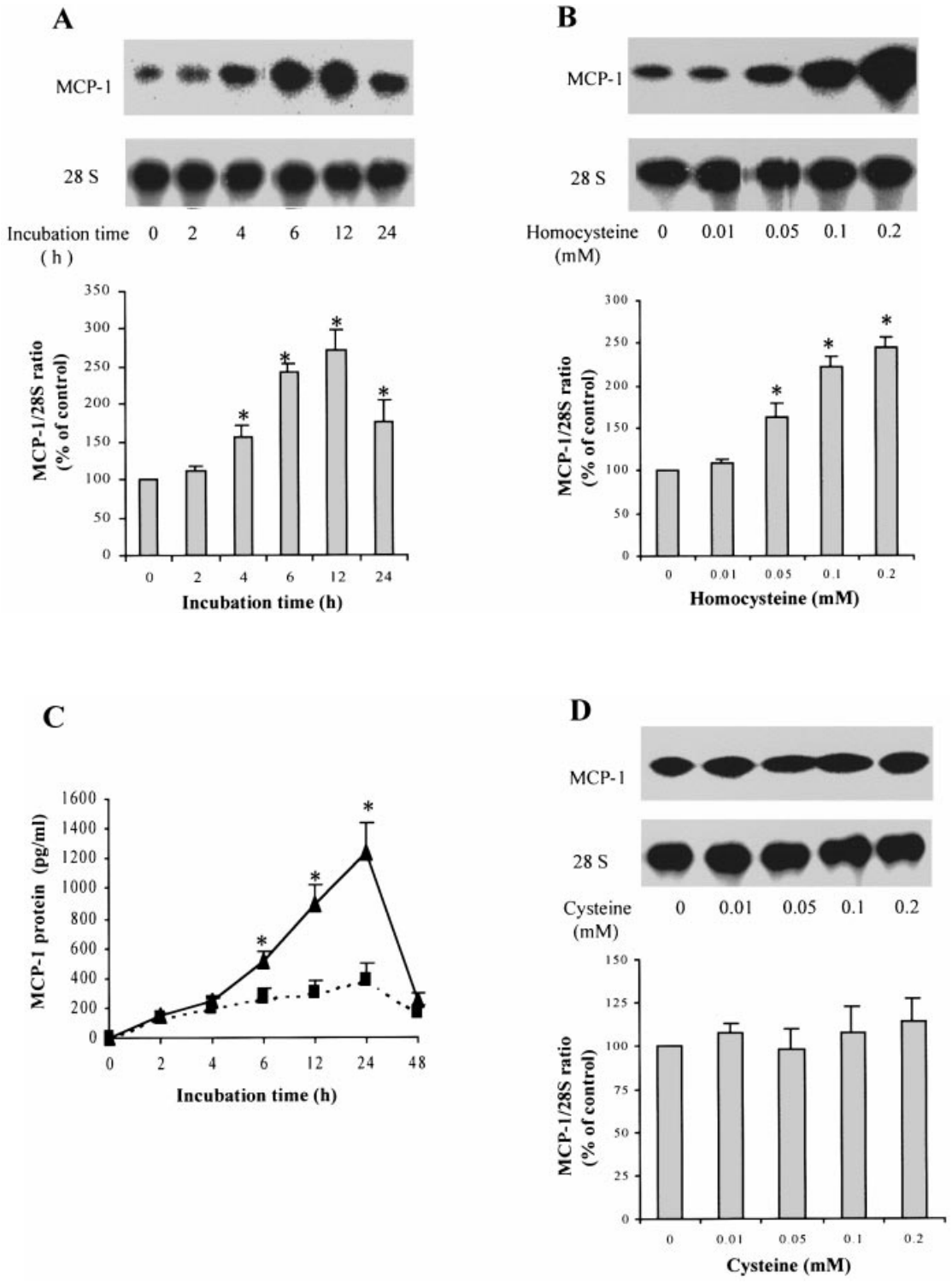


Figure 1 Effect of homocysteine on the expression of MCP-1 mRNA and protein

(**A**) Cells were incubated in the absence (control) or presence of homocysteine (0.1 mM) for 2–24 h. After incubation, nuclease-protection assays were performed to determine MCP-1 mRNA expression. Cells without homocysteine treatment were used as the control. (**B**) After cells were incubated with 0.01–0.2 mM homocysteine at various concentrations for 6 h, a nuclease-protection assay was performed. Values are expressed as the relative expression of MCP-1 mRNA normalized to 28 S rRNA levels. Cells without homocysteine treatment were used as the control. (**C**) Cells were incubated in the absence (■) or presence (▲) of homocysteine (0.1 mM) for various time periods. At the end of incubation, culture media were collected and the amount of MCP-1 protein secreted by these cells into culture media was measured by ELISA. (**D**) After cells were incubated with cysteine (0.01–0.2 mM) for 6 h, a nuclease-protection assay was performed. Values are expressed as the relative expression of MCP-1 mRNA normalized to 28 S rRNA levels. Cells without cysteine treatment were used as the control. Results are depicted as the means ± S.D. (error bar) for five separate experiments. $^*P < 0.05$ when compared with control values.

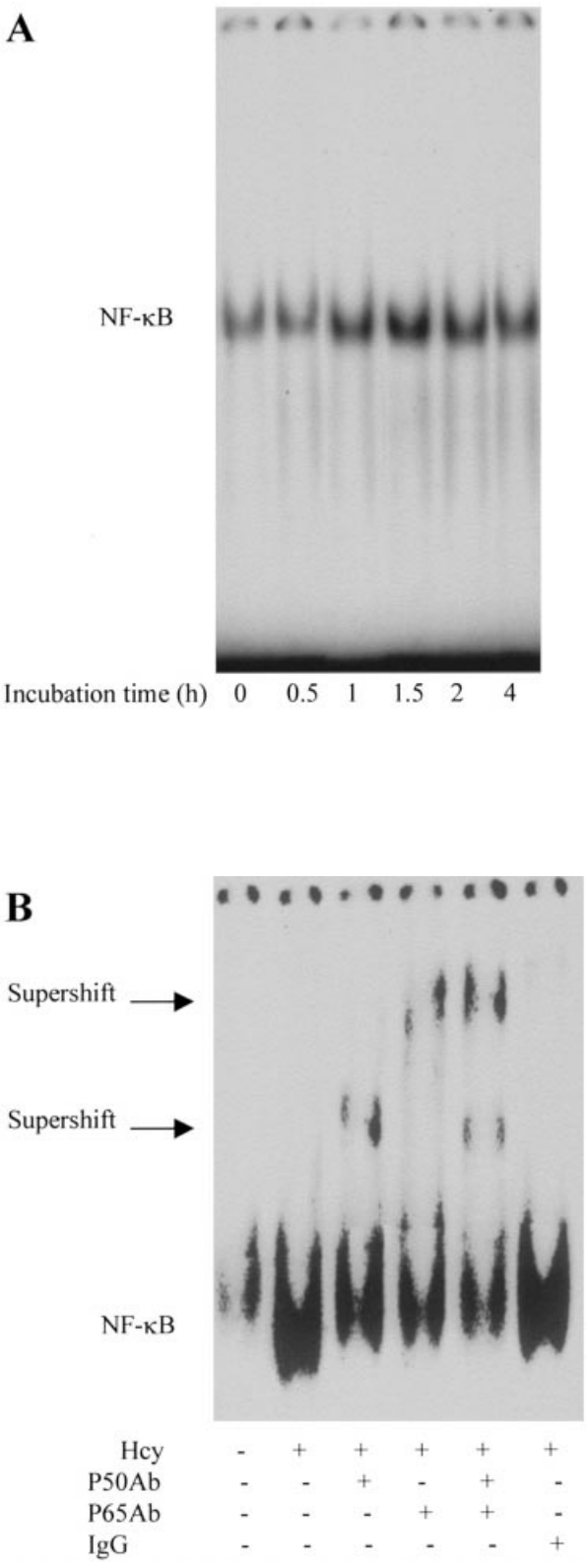


Figure 2 Effect of homocysteine on NF-κB/DNA binding activity

(**A**) After cells were incubated in the absence (control) or presence of homocysteine (Hcy, 0.1 mM) for various time periods, nuclear proteins were isolated and EMSA was performed to determine the NF-κB–DNA binding activity. The autoradiograph is a representative EMSA from three separate experiments. (**B**) Supershift assay was performed in the presence of antibodies against p50 and/or p65 or non-specific rabbit IgG. Antibodies against p50 or p65 caused a shift of the NF-κB–DNA band.

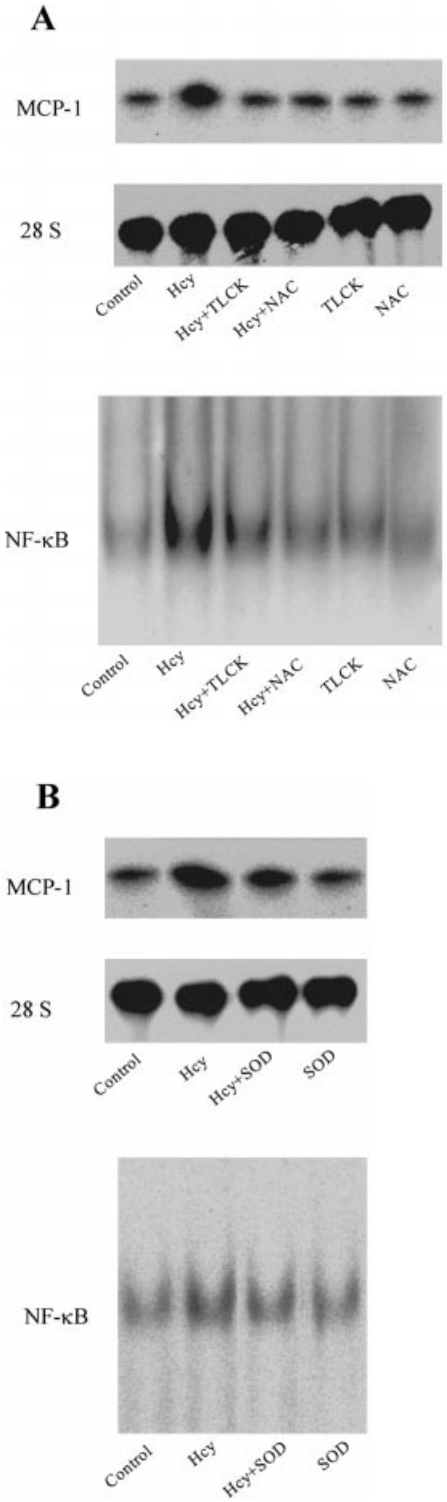


Figure 3 Effect of inhibitors of NF-κB activation or SOD on homocysteine-mediated MCP-1 mRNA expression and NF-κB–DNA binding activity

(**A**) Cells were preincubated with inhibitors (10 μM Tos-Lys-CH_2Cl ('TLCK') or 10 mM NAC) for 15 min, followed by incubation with homocysteine (Hcy, 0.1 mM) for another 6 h. After incubation, total RNA was isolated and a nuclease-protection assay was performed. In another set of experiment, cells were preincubated with inhibitors (10 μM Tos-Lys-CH_2Cl or 10 mM NAC) for 15 min, followed by incubation with homocysteine (0.1 mM) for another 1 h. Nuclear proteins were isolated and the EMSA was performed to determine NF-κB activity. Cells incubated in the absence of homocysteine were used as controls. (**B**) Similar experiments were performed on cells preincubated with SOD (300 units/ml). Autoradiographs shown are representative of three separate experiments.

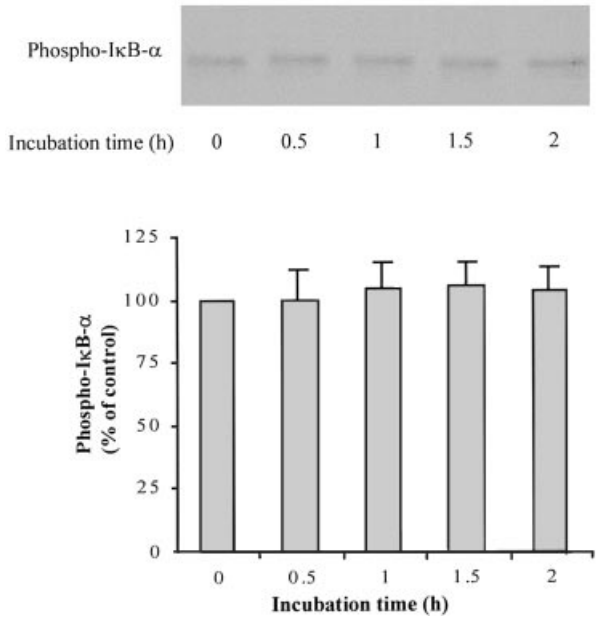


Figure 4 Effect of homocysteine on the expression of phospho-IκBα protein

Cells were incubated in the absence (control) or presence of homocysteine (0.1 mM) for various time periods. Intracellular levels of phospho-IκBα protein were measured by Western immunoblotting analysis with anti-(phosphorylated IκBα) antibodies. Results are expressed as means ± S.D. (error bar) for three separate experiments.

incubation (Figure 1C). To determine whether the stimulation of MCP-1 expression by homocysteine was a generalized effect of thiol-containing amino acids, cells were treated with cysteine (0.01–0.2 mM). As shown in Figure 1(D), cysteine treatment did not affect MCP-1 mRNA levels in cultured cells, indicating that the stimulatory effect observed was related specifically to homocysteine.

Homocysteine activates NF-κB–DNA binding activity

To investigate whether homocysteine stimulated NF-κB–DNA binding activity, cells were incubated with homocysteine (0.1 mM) for various time periods and nuclear proteins were prepared for EMSA. As shown in Figure 2(A), incubation of cells with homocysteine significantly stimulated the NF-κB–DNA binding activity at 1 h and was maintained up to 4 h. An increase in the NF-κB–DNA binding activity was observed in homocysteine-treated cells in a concentration-dependent manner (0.05–0.2 mM) (results not shown). To determine which subunit of NF-κB was activated in homocysteine-treated cells, a supershift assay using specific antibodies against individual NF-κB subunits was performed. As shown in Figure 2(B), addition of p65 and/or p50 antibodies resulted in supershifts, indicating that both p65 and p50 subunits were activated in homocysteine-treated cells. The non-specific IgG did not result in any shift in the NF-κB/DNA band (Figure 2B).

To determine whether NF-κB activation was necessary for homocysteine-mediated MCP-1 expression, two inhibitors of NF-κB activation, N^{α}-*p*-tosyl-L-lysylchloromethane (Tos-Lys-CH_2Cl; 'TLCK') and NAC (*N*-acetyl-L-cysteine), were used (Sigma) [34]. As shown in Figure 3(A), both inhibitors could abolish the homocysteine-induced MCP-1 mRNA expression as

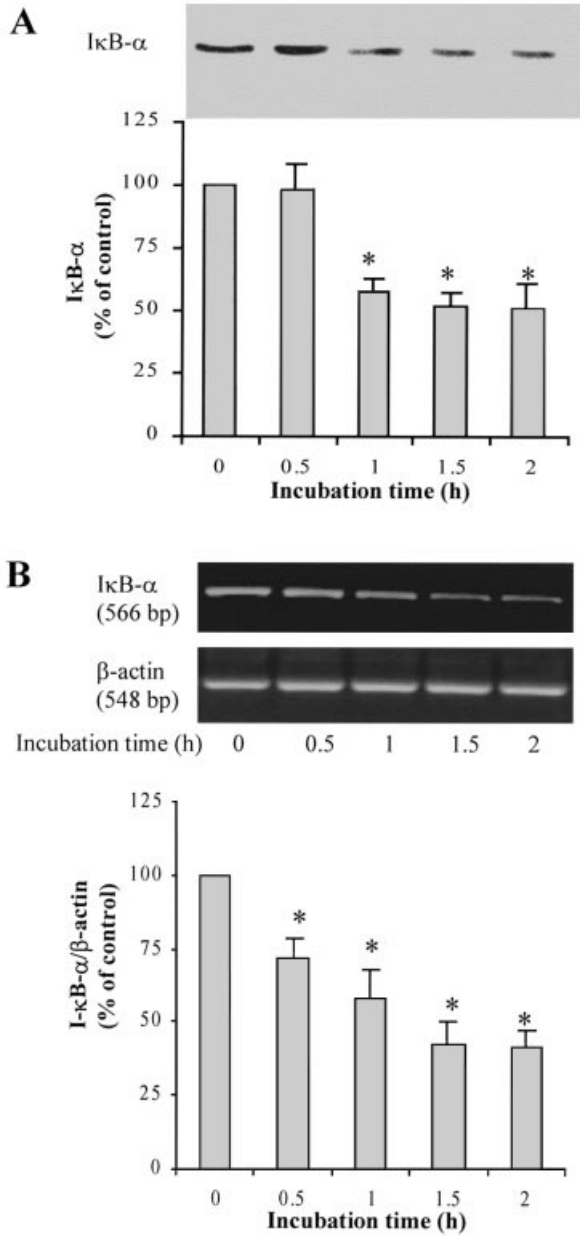


Figure 5 Effect of homocysteine on the expression of IκBα protein and mRNA

Cells were incubated in the absence (control) or presence of homocysteine (0.1 mM) for various time periods. (**A**) Intracellular levels of IκBα protein were determined by Western immunoblotting analysis with anti-IκBα antibodies. (**B**) IκBα mRNA expression was determined by semiquantitative RT-PCR analysis. Results are expressed as means ± S.D. (error bar) for three separate experiments. $^*P < 0.05$ when compared with control values.

well as block the homocysteine-stimulated NF-κB activation. These results suggested that homocysteine stimulated MCP-1 expression via the activation of NF-κB. To investigate whether the activation of NF-κB and subsequent MCP-1 expression by homocysteine involved oxidative stress, cells were incubated with homocysteine in the absence or presence of superoxide dismutase (SOD), an antioxidant enzyme. As shown in Figure 3(B), addition of SOD partially reversed the stimulatory effect of homocysteine on NF-κB activation.

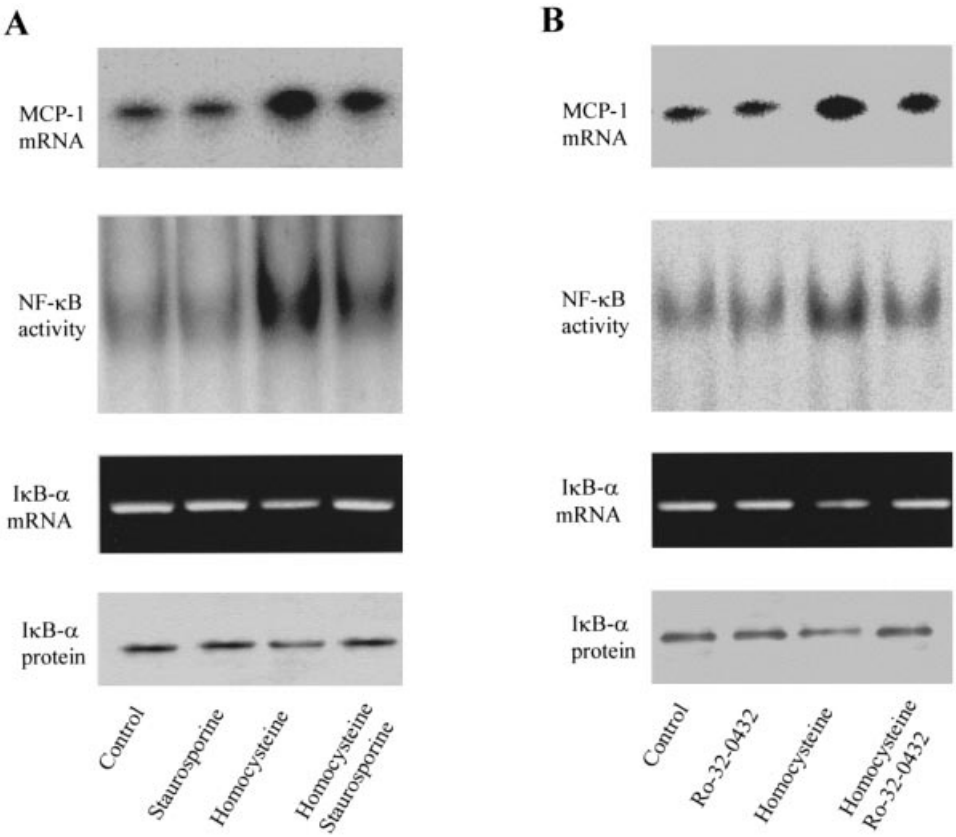


Figure 6 Effect of PKC inhibitors on the expression of MCP-1, IκBα and the activity of NF-κB

(**A**) Cells were incubated with homocysteine (0.1 mM) for 6 h in the absence or presence of staurosporine (1 nM). After incubation, nuclease protection assays were performed to determine the expression of MCP-1 mRNA. In a separate set of experiments, cells were incubated with homocysteine (0.1 mM) for 1 h in the absence or presence of staurosporine (1 nM). After incubation, the EMSA was performed to determine the NF-κB activity. Intracellular levels of IκB-α mRNA were determined by a semi-quantitative RT-PCR analysis. Intracellular levels of IκBα protein were determined by a Western immunoblotting analysis with anti-IκB-α antibodies. (**B**) Similar experiments were performed in cells treated with Ro-32-0432 (100 nM). Autoradiographs shown are representative of three separate experiments. Cells incubated in the absence of homocysteine or staurosporine were used as a control.

Homocysteine inhibits IκBα expression

The activation of NF-κB might be caused by enhanced phosphorylation of the inhibitor protein IκBα. Therefore the effect of homocysteine on the phosphorylation status of IκBα was investigated. As shown in Figure 4, homocysteine treatment did not affect the levels of phosphorylated IκBα. As a control, similar experiments were performed with tissue necrosis factor-α (TNFα), an agonist for NF-κB activation via enhanced phosphorylation of the IκBα. The levels of phosphorylated IκBα protein were significantly elevated in TNFα-treated cells (results not shown). To investigate whether the activation of NF-κB was caused by a reduction in the protein level of IκB-α (non-phosphorylated) in homocysteine-treated cells, cellular IκBα protein was determined. Homocysteine treatment caused a significant decrease in the level of IκBα protein in VSMCs at 1–2 h (Figure 5A). The expression of IκBα mRNA in cells treated with homocysteine was also analysed. As shown in Figure 5(B), homocysteine treatment caused a significant reduction in the level of IκBα mRNA in VSMCs at 0.5–2 h.

Homocysteine activates calcium-dependent PKC

The role of PKC in homocysteine-induced MCP-1 expression was investigated by using staurosporine, a PKC inhibitor [28,38]. Staurosporine (1 nM) abolished the homocysteine-induced MCP-1 mRNA expression as well as NF-κB activation (Figure 6A). This was also accompanied by blocking of the inhibitory effect of homocysteine on the expression of IκBα mRNA and IκBα protein (Figure 6A). Another more selective cell-permeable PKC inhibitor [39], Ro-32-0432 (bisindolylmaleimide XI hydrochloride; Alexis Corporation, Grünberg, Germany), was also used. This inhibitor selectively inhibits PKC α, β and γ isoforms. The presence of Ro-32-0432 (100 nM) in the culture medium abolished the effect of homocysteine on MCP-1 mRNA expression, NF-κB activation as well as the expression of IκBα mRNA and IκBα protein (Figure 6B). In cells treated with homocysteine, the calcium-dependent PKC activity was significantly elevated (Figure 7A). To investigate the contribution of calcium to homocysteine-induced PKC activation, cells were treated with verapamil (10 nM), a calcium-channel blocker [28,40,41]. In the presence of verapamil, the homocysteine-stimulated PKC activity was reduced by 40 % (results not shown). Verapamil also reduced the stimulatory effect of homocysteine on MCP-1 secretion in these cells (46 % reduction as compared with homocysteine-treated cells) (Figure 7B). Similar results were obtained after cells were treated with another calcium-channel blocker, diltiazem (an L-type calcium channel blocker) [28,42] (Figure 7B). These results suggested that the calcium-dependent PKC signalling pathway could be involved in the inhibitory effect of homo-

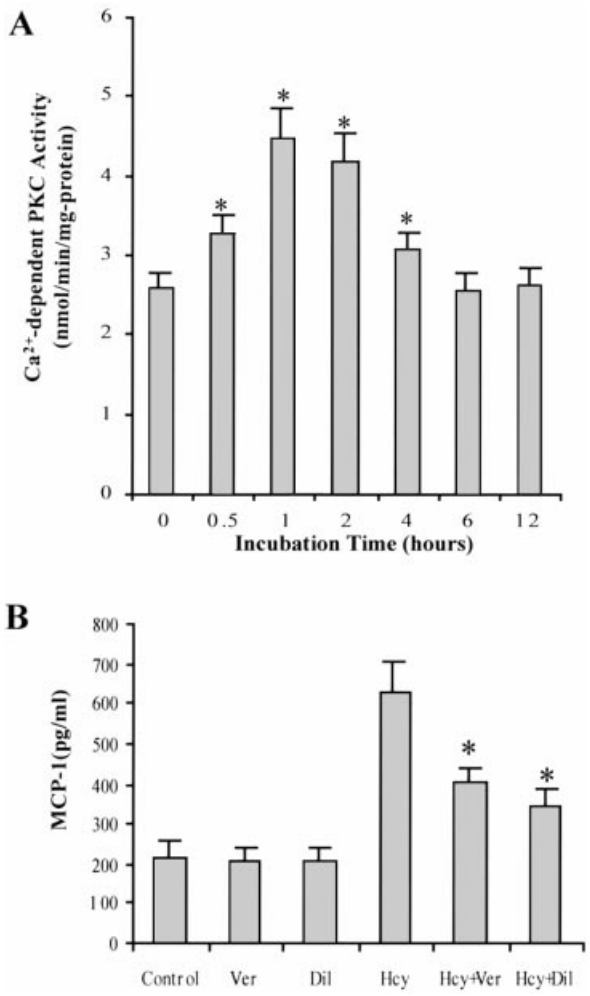


Figure 7 Effect of homocysteine on PKC activity

(**A**) Cells were incubated with homocysteine (0.1 mM) for various time periods. After incubation, cellular PKC activity was measured. The results are depicted as means ± S.D. (error bar) for five separate experiments. $^*P < 0.05$ when compared with control values. (**B**) Cells were incubated with verapamil (Ver) or diltiazem (Dil) for 15 min, followed by incubation with homocysteine (0.1 mM) for 6 h. After incubation, the amount of MCP-1 protein secreted into culture media was determined by ELISA. Cells incubated in the absence of homocysteine (Hcy) and verapamil (Ver) were used as controls. The results are depicted as means ± S.D. (error bar) for five separate experiments. $^*P < 0.05$ when compared with homocysteine-treated cells.

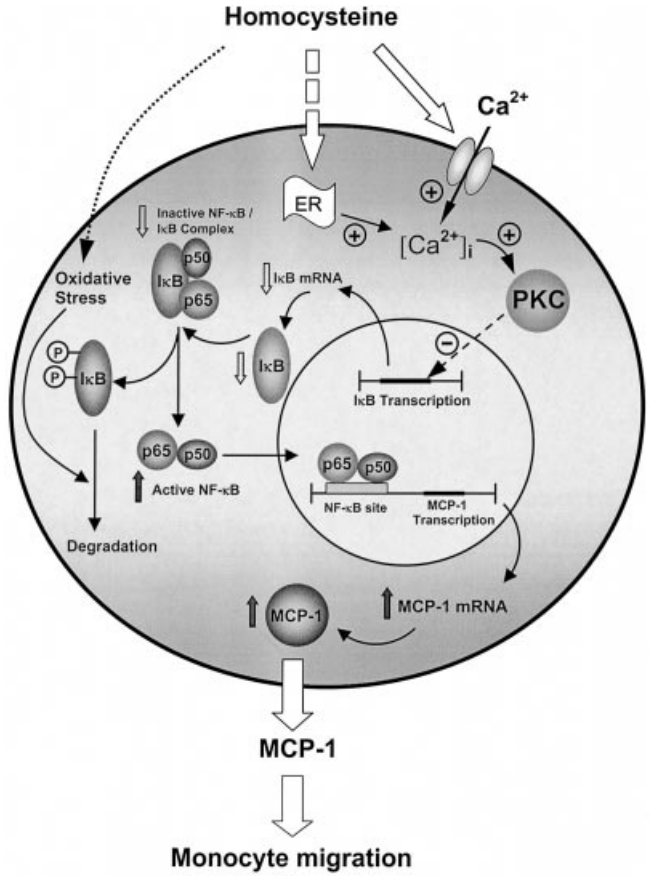
cysteine on IκB-α expression, which, in turn, could stimulate NF-κB activity and MCP-1 expression.

DISCUSSION

The recruitment of monocytes into the arterial wall is regarded as an early event during the development of atherosclerosis. At present, the mechanism by which elevated homocysteine levels promote monocyte infiltration and macrophage accumulation in atherosclerosis is not yet fully understood. The present study clearly demonstrates that: (1) homocysteine stimulates MCP-1 expression in human VSMCs; (2) such stimulatory effect is due to the activation of NF-κB via oxidative stress and a reduction in IκBα expression; and (3) homocysteine-stimulated calcium influx in VSMCs contributes partially to the activation of PKC, which, in turn, causes a reduction in the expression of IκBα. Taken together, these results indicate that NF-κB activation may play an important role in homocysteine-induced MCP-1 expression leading to monocyte/macrophage accumulation in atherosclerotic lesions.

Recent evidence suggests that the activation of NF-κB is involved in the induction of MCP-1 gene expression [23–25]. However, the role of this transcription factor in homocysteine-induced MCP-1 expression is largely unknown. In the present study we investigated the role of NF-κB activation in the homocysteine-induced MCP-1 production in VSMCs. Several lines of evidence indicate that NF-κB is involved in the homocysteine-induced MCP-1 expression. First, the results from EMSA clearly demonstrated that the activation of NF-κB by homocysteine preceded the induction of MCP-1 mRNA expression (Figures 1A and 2A). Secondly, the finding that inhibitors of NF-κB activation significantly reversed homocysteine-induced MCP-1 mRNA expression suggested that the activation of NF-κB was necessary for homocysteine-induced MCP-1 expression (Figure 3). In the present study, the possible mechanisms of homocysteine-stimulated activation of NF-κB were also explored. Although phosphorylation of IκB-α has been shown to play an important role in the activation of NF-κB, this process did not seem to be affected in VSMCs by homocysteine treatment. On the other hand, levels of IκBα mRNA decreased significantly after 0.5 h incubation of VSMCs with homocysteine, which was followed by a corresponding reduction in IκBα protein levels (after 1 h incubation). Such decrease in IκBα expression might contribute, in part, to an activation of NF-κB in homocysteine-treated cells. Recently, radical oxygen species such as superoxide have been implicated to stimulate IκBα degradation and NF-κB activation in endothelial cells [43] as well as in smooth muscle cells [44]. Homocysteine-induced superoxide production had been indicated as one of the mechanisms causing cell damage in the vascular wall [45,46]. SOD can antagonize the effect of free radicals [47]. Wilcken et al. [47] recently reported a positive relationship between circulating extracellular SOD and homocysteine in homocysteinuric patients. They proposed that an elevation of extracellular SOD could represent a protective antioxidant response to homocysteine-induced oxidative changes [47]. As shown in the present study, SOD was able to partially reverse the stimulatory effect of homocysteine on NF-κB activation, as well as on MCP-1 expression. These results indicate that homocysteine might increase superoxide production in VSMCs, leading to increased degradation of IκBα and the activation of NF-κB.

The present study also demonstrated that the decrease in IκB-α mRNA expression in VSMCs was mediated by a PKC signalling mechanism. When PKC inhibitors were included in experiments, the stimulatory effect of homocysteine on MCP-1 expression and NF-κB activation was abolished. Furthermore, the PKC activity was significantly elevated in homocysteine-treated cells. The lack of a stimulatory effect of homocysteine on MCP-1 expression in the presence of Ro-32-0432, which is selective for the calcium-dependent PKC isoforms, points towards a possible calcium-dependent mechanism for the action of homocysteine. It was recently reported that homocysteine could initiate the release of intracellular calcium, leading to increased collagen production in VSMCs [48]. It was suggested that homocysteine-induced calcium-signalling cascades might be due to an increase in calcium influx and/or release from endoplasmic-reticulum stores [48]. In accordance with those results [48], we observed that blockage of calcium influx in VSMCs with a calcium-channel blocker could partially abolish the stimulatory effect of homocysteine on MCP-1 expression. We speculate that homocysteine might stimulate calcium influx and/or release from intracellular stores, leading to an activation of PKC in VSMCs, since the calcium channel



Scheme 1 Proposed mechanisms of homocysteine-induced MCP-1 expression in VSMCs

blocker used could only inhibit calcium influx. However, results obtained from the present study could not confirm whether homocysteine also induced calcium release from the endoplasmic recticulum. Furthermore, the mechanism by which homocysteine-stimulated PKC activation leads to a reduction in the expression of IκBα in these cells remains to be determined.

On the basis of the results obtained from the present study, as well as those of others, we propose in Scheme 1 (a) mechanism(s) by which homocysteine stimulates MCP-1 expression in VSMCs. Homocysteine elicits calcium influx and/or release from endoplasmic reticulum ('ER') leading to a rise in intracellular calcium and the activation of PKC. Once activated, PKC exerts an inhibitory effect on IκBα mRNA expression, which, in turn, activates NF-κB. On the other hand, homocysteine treatment may also enhance superoxide production in VSMCs, which, in turn, stimulates IκBα degradation and hence NF-κB activation. The activation of NF-κB results in an increased expression of MCP-1 in VSMCs.

The concentrations of homocysteine used in the present study to display a stimulatory effect on NF-κB activation and MCP-1 expression were similar to that found in the plasma of patients with moderate homocysteinaemia [6,7]. Moderately elevated levels of plasma homocysteine are regarded as an independent risk factor for atherosclerosis [5,47,51]. Plasma homocysteine is found primarily in three molecular forms: homocysteine, disulphide homocysteine and the mixed disulphide homocysteine-cysteine [3]. These three molecular species exist in free and protein-bound forms. Reduced or free homocysteine (non-protein-bound) normally constitutes only 1 % of the total plasma level [3]. In the present study, homocysteine added to the culture medium might form disulphide bonds among homocysteine molecules themselves or form disulphide bonds with cysteine. Furthermore, homocysteine molecules might interact with other components present in the culture medium or interact with proteins/molecules secreted by cells, as well as components of cell membranes. Although many studies on the *in vitro* effect of homocysteine have been reported, it is not clear whether free homocysteine, mixed or protein-bound homocysteine or its metabolites, or in various combinations, exert the observed diverse effects on vascular cells.

During the development of atherosclerosis, the origin of inflammatory signals, including MCP-1, is thought to be the vessel wall itself [49]. We observed previously that homocysteine stimulated the expression of MCP-1 in cultured endothelial cells [50]. It is generally believed that endothelial expression of MCP-1 initiates the migration of monocytes in the arterial wall [13–17]. The evidence obtained from the present study indicates that homocysteine can also stimulate MCP-1 expression in VSMCs. We speculate that homocysteine-induced endothelial MCP-1 expression may be associated with early atherosclerosis by stimulating monocyte transmigration to the subendothelial space and differentiation into macrophages. On the other hand, MCP-1 produced in VSMCs may facilitate the recruitment of additional monocytes to atheroslcerotic lesions in patients with homocysteinaemia.

In conclusion, the present study has clearly demonstrated that homocysteine-induced MCP-1 expression is mediated via the activation of NF-κB in VSMCs. These findings may provide us with one of the important mechanisms by which homocysteine causes atherosclerosis.

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