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Homocysteine Augments Cytokine-Induced Chemokine Expression in Human Vascular Smooth Muscle Cells: Implications for Atherogenesis

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Abstract—Hyperhomocysteinemia is an independent risk factor for atherosclerosis and atherothrombosis. While in vitro studies have revealed a number of homocysteine-mediated alterations in the thromboregulatory properties of endothelial cells, comparatively little is known about homocysteine-modulated smooth muscle cell function. We observed that exposure of human aortic smooth muscle cells to pathophysiologically relevant concentrations of homocysteine results in concentration-dependent increases in cytokine-induced MCP-1 and IL-8 secretion. RNase protection assays revealed that both MCP-1 and IL-8 mRNA concentrations are increased in homocysteine-treated smooth muscle cells when compared to cells activated with cytokines alone. Homocysteine treatment also increased cytosolic-to-nuclear translocation of the p65 and p50 subunits of the Rel/NF-κB family of transcription factors but had no effect on AP-1 activation. Cumulatively, these data suggest that homocysteine may increase monocyte recruitment into developing atherosclerotic lesions by upregulating MCP-1 and IL-8 expression in vascular smooth muscle cells.

KEY WORDS: homocysteine; hyperhomosteine; atherosclerosis; atherothrombosis; cytokines.

INTRODUCTION

Homocysteine, a sulfhydryl-containing amino acid produced as an intermediate in the methionine conservation cycle, is a participant in both the single-carbon folate and transulfuration pathways. Homozygous cystathionine-β-synthase deficiency is very rare but is the most common cause of marked hyperhomocysteinemia (up to 500 μM). Patients who suffer with this disease which is marked by developmental delay, osteoporosis, and ectopic lenses, are prone to severe premature atherosclerotic vascular disease and vascular occlusion (1). Acquired and heterozygous forms of hyperhomocysteinemia are relatively common. These hyperhomocysteinemia states are accompanied by an elevation in plasma methionine and a reduction in plasma cysteine concentrations. Even relatively modest hyperhomocysteinemia (15 μM–50 μM) is now recognized to be an independent risk factor for premature atherosclerotic coronary, cerebral, and peripheral vascular disease (2). As many as 40% of patients who suffer from complications of cerebrovascular or coronary artery atherosclerosis are hyperhomocysteinemic.

During the past decade in vitro studies have provided insight into potential mechanisms through which homocysteine contributes to the development of atherosclerosis and thrombosis. Most of these studies have focused on homocysteine-mediated proatherothrombotic endothelial dysfunction. In contrast to the extensive body of data which bear on mechanisms of homocysteine-mediated endothelial dysfunction, comparatively little is known about potential mechanisms of homocysteine-mediated vascular smooth muscle dysfunction. Homocysteine exposure has been reported to increase collagen synthesis by rabbit aortic smooth muscle cells and to stimulate vascular smooth muscle cell proliferation (3). In turn, atherosclerotic lesions exhibit

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increased collagen content and proliferation of smooth muscle cells (4). Recent studies indicate that homocysteine triggers the expression of inducible nitric oxide synthase (iNOS) and concentration-dependent increases in NO production by vascular smooth muscle cells (5). It has been hypothesized that locally produced NO may promote atherosclerosis by increasing local oxidative stress.

The present study indicates that exposure of vascular smooth muscle cells to pathophysiologically relevant concentrations of homocysteine results in augmentation of cytokine-induced MCP-1 and IL-8 secretion and corresponding increases in MCP-1 and IL-8 mRNA concentrations. Electrophoretic mobility shift assay data also indicate that homocysteine induces nuclear factor-kappa B (NF-κB) (p50/p65) cytosolic to nuclear translocation but has no effect on activator protein-1 (AP-1).

METHODS

D,L-homocysteine and lipopolysaccharide (KPS) from *E. coli* (serotype 0127:B8) were purchased from Sigma (St. Louis, Missouri). Recombinant human tumor necrosis factor-α(TNF-α), interferon-γ (IFN-γ) and interleukin-1β (IL-1β) were obtained from R&D Systems (Minneapolis, Minnesota). Extraction of nuclear protein, electrophoretic mobility shift assays and RNAse protection assays were performed as previously described (6)–(8). Human aorta vascular smooth muscle cells, media, and media supplements were purchased from Clonetics (Walkersville, Maryland). The cells were grown and maintained as previously described (7).

Treatment of Smooth Muscle Cells for Enzyme Immunoassay (EIA) Experiments. Smooth muscle cells were exposed to the specified concentrations of homocysteine (between 31μmol and 1 mmol) for 4 h. Subsequently, either TNF-α (1 ng/ml or 10 ng/ml, as indiated or a mixture consisting of LPS (30 μg/ml), IL-1β (50 ng/ml) and IFN-γ (50 ng/ml) were added to the culture media and the cells were incubated for an additional 8 h. Conditioned media were collected at the end of this 8 h incubation period and subjected to either MCP-1 or IL-8 EIAs.

MCP-1 and IL-8 EIAs. Smooth muscle cells were grown to confluence on 96-well flat bottom polystyrene plates (Costar). Capture plates of MCP-1 or IL-8 were prepared by coating sterile 96-well flat bottom ELISA plates (Corning, Corning, New York) with anti-human MCP-1 or anti-human IL-8 antibody, respectively (R&D Systems, 50 μl/well, 0.8 μg/ml in PBS). The plates were incubated at 4°C overnight, washed with Wash Buffer (0.05% Tween-20 in PBS, pH 7.4) and blocked overnight at 4°C with blocking buffer (0.05% Tween-20, 1% BSA in PBS, pH 7.4) to reduce nonspecific binding. Capture plates were stored at 4°C until use. One hundred μl aliquots of the conditioned cell media and human recombinant MCP-1 or IL-8 standards (R&D systems) were loaded into capture plates and incubated at 4°C overnight. Biotinylated anti-human MCP-1 or anti-human IL-8 detection antibody (R&D Systems) was added to each well (0.1 μg/ml, 100 μl per well) and plates were incubated at room temperature for 2 h. After 3 washes, streptavidin horseradish peroxidase was added (Neutralite avidin, Southern Biotechnology Associates, Birmingham, Alabama; 100 μl/well, 1:4000 dilution) and incubated for 30 min at room temperature. Wells were washed to remove unbound conjugated peroxides and then incubated with 100 μl/well of peroxidase substrate (ABTS®, Boehringer Mannheim Biochemica, Germany). After 0.5–4 h absorbance at 405 nm was measured using an automated microplate reader (EL_x808, Bio-Tek Instruments, Winooski, Vermont). Concentrations of MCP-1 or IL-8 were calculated by 4 parameter curve fitting regression analysis of rhMCP-1 or rhIL-8 standard curves using KC3 software (Bio-Tek Inc.).

Statistical Analysis. All values are expressed as the means ± SE. Data were analyzed using Friedman two way analysis of variance (ANOVA) with Bonferroni comparison of means. Probability (P) values of < 0.05 were considered significant.

RESULTS

Homocysteine Augments Cytokine-Induced MCP-1 Expression. Exposure of smooth muscle cells to homocysteine resulted in concentration-dependent increases in MCP-1 secretion when the cells were subsequently stimulated with either LPS, IFN-γ and IL-1β (Figure 1) or TNF-α (Figure 2). Smooth muscle cells exposed to medium alone secreted a small amount of MCP-1 over the 12 h period of the experiment. Secretion of MCP-1 was induced in smooth muscle cells pretreated with medium for 4 h and subsequently exposed to LPS, IFN-γ and IL-1β. When smooth muscle cells were treated with varying concentrations of homocysteine (62.5 μM to 1 mM) prior to LPS, IFN-γ and IL-1β exposure, significant increases in MCP-1 concentrations were detected. Maximum chemokine concentra-

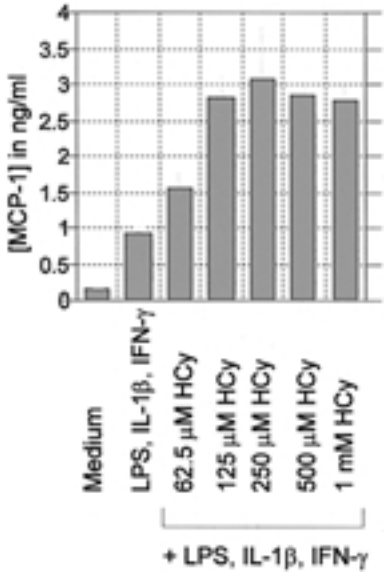

Fig. 1. Augmentation of cytokine-induced MCP-1 expression by homocysteine. Smooth muscle cells were treated for 4 h with the indicated concentrations of homocysteine, and subsequently a mixture consisting of LPS, IFN-γ and IL-1β was added. Conditioned media were collected at 8 h following addition of stimuli and analyzed by MCP-1 EIA as described in "METHODS." Asterisk* indicates significant difference from positive controls (MCP-1 concentrations in conditioned media from cells that had been incubated in presence of LPS, IFN-γ, and IL-1) as determined by Bonferroni comparison of means (27).

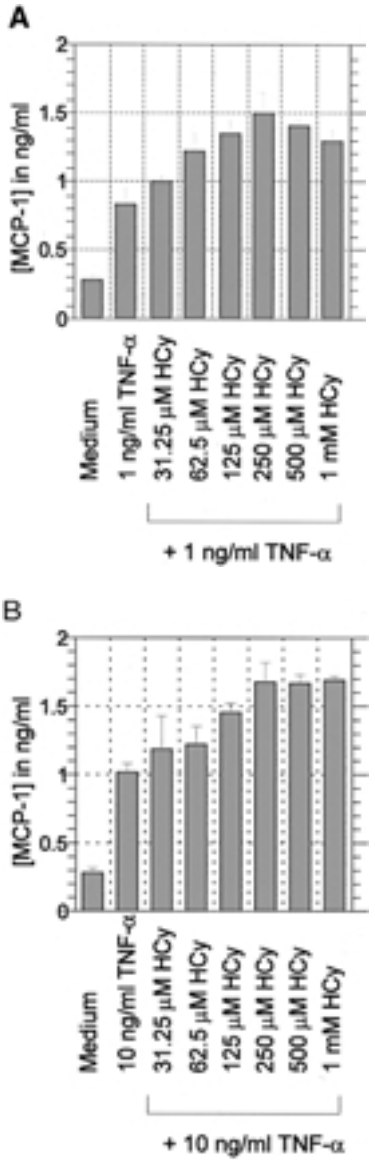

Fig. 2. Augmentation of TNF-α-induced MCP-1 expression by homocysteine. Smooth muscle cells were treated for 4 h with varying doses of homocysteine, and subsequently TNF-α at either 1 ng/ml (Figure 2A) or 10 ng/ml (Figure 2B) was added. Conditioned media were collected at 8 h following addition of stimuli and analyzed by MCP-1 EIA as described in "METHODS." Asterisk* denotes significant difference from positive controls (MCP-1 concentrations in conditioned media from cells that had been incubated in presence of TNF-α) as determined by Bonferroni comparison of means (27).

tions were measured at a concentration of 150 μM homocysteine, when MCP-1 concentrations were increased more than 2-fold over controls. Figures 2a and 2b are representative of a series of similar EIA experiments performed with TNF-α-treated smooth muscle cells (1 ng/ml and 10 ng/ml TNF-α respectively). Pretreatment with homocysteine at concentrations ranging from 62.5 μM to 1 mM resulted in significantly elevated MCP-1 concentrations (compared to smooth muscle cells pretreated only with medium prior to TNF-α activation) when cells were subsequently activated with 1 ng/ml TNF-α. Pretreatment with homocysteine at concentrations ranging from 125 μM to 1 mM resulted in significantly elevated MCP-1 concentrations (compared to smooth muscle cells pretreated only with medium prior to TNF-α activation) when cells were subsequently activated with 10 ng/ml TNF-α. For both concentrations of TNF-α tested, MCP-1 expression was maximally induced with 250 μM homocysteine.

Homocysteine Augments Cytokine-Induced IL-8 Expression. Analogous results for IL-8 were observed in aortic smooth muscle cells exposed to homocysteine followed by LPS, IFN-γ and IL-1β (Figure 3). Smooth muscle cells treated with LPS, IL-1β and IFN-γ secreted approximately twice the amount of IL-8 compared to

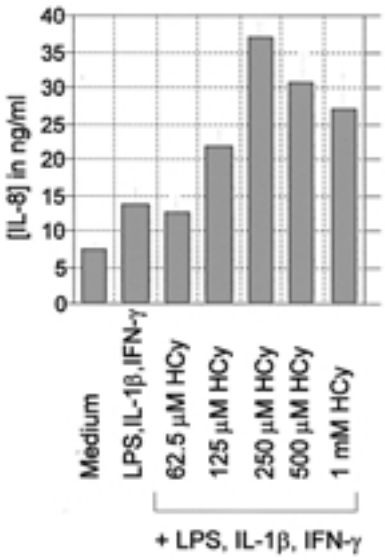

Fig. 3. Augmentation of cytokine-induced IL-8 expression by homocysteine. Smooth muscle cells that had been pretreated for 4 h with the indicated concentrations of homocysteine (31.25 μM to 1 mM) were incubated in the presence of a mixture of LPS, IL-1β and IFN-γ for 8 h. The conditioned media were collected and analyzed by IL-8 EIA as described in "METHODS." Asterisk* denotes significant difference from positive controls (IL-8 concentrations in conditioned media from cells that had been incubated in presence of LPS, IFN-γ, and IL-1) as determined by Bonferroni comparison of meams (27).

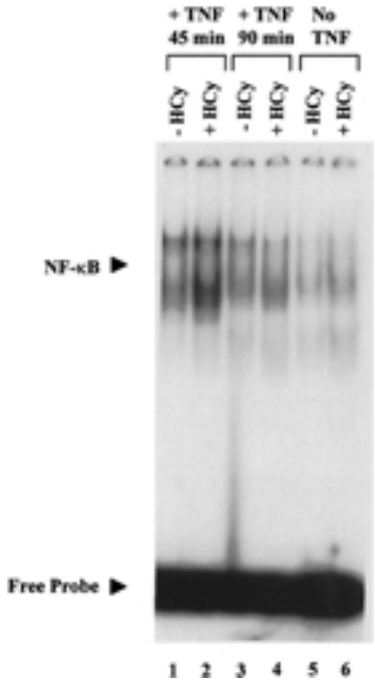

Fig. 4. Electrophoretic mobility shift analysis of nuclear extracts derived from smooth muscle cells. Cells were exposed to 250 μM homocysteine or medium followed by 1. TNF-α for 45 min (lanes 1 and 2) 2. TNF-α for 90 min (lanes 3 and 4) 3. medium for 90 min (lanes 5 and 6). Nuclear extracts were incubated in presence of a radiolabeled double-stranded NF-κB consensus oligonucleotide probe for 30 min at room temperature and samples were run on a nondenaturing 4% polyacrylamide gel.

media-treated controls. Pretreatment with 250 μM of homocysteine resulted in maximum IL-8 concentrations that were increased by 170% when compared to cells pretreated only with prior to activation by LPS, IL-1β and IFN-γ.

Homocysteine Increases Cytokine-Induced NK-κB Translocation. Martin et al. (9) recently reported that MCP-1 gene expression in endothelial cells in response to cytokine stimulation depends on the cooperative interaction of the redox-sensitive transcription factors NF-κB and AP-1. NF-κB is a ubiquitous factor present in the cytosol of most quiescent cells in a complex with an inhibitor, IκB-α (10, 11). When the cell is appropriately stimulated, IκB-α is degraded, thus releasing NF-κB and allowing it to translocate to the nucleus where it can bind to cis-acting κB-sites in the promoters and enhancers of genes, thereby modulating their transcription. Nuclear extracts prepared from smooth muscle cells exposed to 250 μM homocysteine or medium alone for 4 h followed by TNF-α exposure for 45 min and 90 min, respectively, were subjected to electrophoretic mobility shift assays with a κB consensus oligonucleotide. As seen in Figure 4, there were significant increases in binding to the NF-κB recognition sequence in smooth muscle cells preexposed to homocysteine (lanes 2 and 4) as compared to smooth muscle cells exposed to medium alone (lanes 1 and 3) prior to cytokine activation. Competition with a 50-fold excess of unlabeled (cold) NF-κB abrogated the gel shift whereas addition of an unlabeled irrelevant oligonucleotide probe (Oct1) did not affect NF-κB detection (data not shown).

Characterization of NF-κB/Rel Family Members Activated by Homocysteine and Cytokines. In order to delineate which members of the NF-κB/Rel family of transcription factors are activated in smooth muscle cells upon exposure to homocysteine and cytokines, supershift assays utilizing antibodies for the p65, p50, p52, c-Rel and Rel B subunits were performed. Representative results from these EMSA analyses can be seen in Figure 5. Lanes 1–6 depict supershift assays performed with nuclear extracts from smooth muscle cells incubated for 4 h in presence of medium followed by a 45 min exposure to TNF-α. In lane 7, nuclear extract

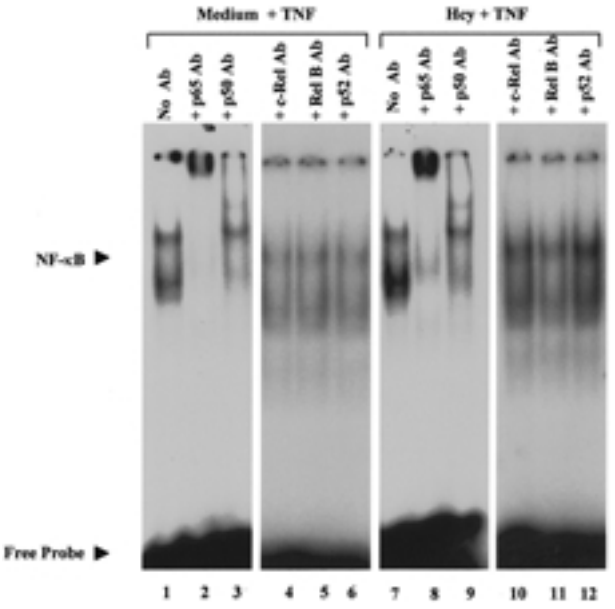

Fig. 5. Supershift analyses of nuclear extracts derived from cytokine-activated smooth muscle cells. Cells were subjected to a 4 h exposure to either medium (lanes 1–6) or homocysteine (250 μM) followed by a 45 min incubation in presence of TNF-α. Nuclear extracts were prepared as described in "METHODS." Extracts were preincubated with antibodies to either p65 (lanes 2 and 8), p50 (lanes 3 and 9), c-Rel (lanes 4 and 10), Rel B (lanes 5 and 11) or p52 (lanes 6 and 12) for 30 min prior to addition of the NF-κB oligonucleotide probe. Samples were run on a nondenaturing 4% polyacrylamide gel. Lanes 1 and 7 depict binding activities of nuclear extracts without preincubation with supershift antibodies.

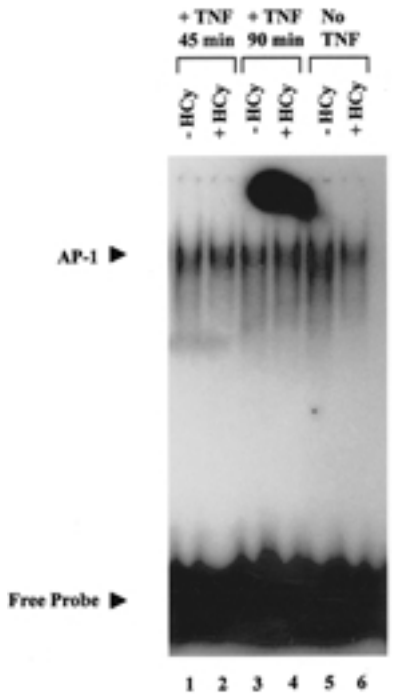

Fig. 6. AP-1 gel shift analysis of nuclear extracts derived from TNF-α-activated smooth muscle cells. Cells were exposed to 250 μM homocysteine or medium followed by 1. TNF-α for 45 min (lanes 1 and 2) 2. TNF-α for 90 min (lanes 3 and 4) 3. medium for 90 min (lanes 5 and 6). Nuclear extracts were incubated in presence of a radiolabeled double-stranded AP-1 consensus oligonucleotide probe for 30 min at room temperature and samples were run on a nondenaturing 4% polyacrylamide gel.

from smooth muscle cells that had been subjected to a 4 h incubation in presence of homocysteine followed by 45 min of TNF-α was subjected to electrophoresis without prior incubation with supershift antibodies. Supershifts occurred when nuclear extract-oligonucleotide probe complexes were incubated with either anti-p65 (lane 8) or anti-p50 (lane 9) antibodies. Incubation with antibodies to either c-Rel (lane 10), Rel B (lane 11), or p52 (lane 12), failed to induce supershifts of the DNA-protein complexes.

Homocysteine does not Affect AP-1 Activation. AP-1 is a redox-sensitive transcription factor composed of homo- or heterodimers of members of Fos and Jun families which bind to the TPA (12-O-tetradecanoylphorbol-13-acetate) responsive element (TRE) motif to mediate gene transcription (12). It has been shown to be indispensable for maximum cytokine induction of MCP-1 in endothelial cells (9). Figure 6 depicts a representative EMSA experiment with an AP-1 consensus oligonucleotide. No significant differences in AP-1 activation were observed in nuclear extracts from homocysteine-treated smooth muscle cells (Figure 6, lanes 2 and 4) when compared to cells that were only incubated in presence of medium prior to cytokine stimulation (Figure 6, lanes 1 and 3).

Homocysteine Treatment Augmnts MCP-1 and IL-8 mRNA Concentrations. In order to determine whether increased levels of IL-8 and MCP-1 mRNAs were present in homocysteine pretreated smooth muscle cells we utilized a multi-probe RNase protection assay. Figures 7 and 8 depict time courses for the appearances of MCP-1 and IL-8 mRNAs in smooth muscle cells incubated in the presence of homocysteine (250 μM) or medium followed by a mixture of LPS, IL-1β and IFN-γ (Figure 7) or TNF-α (Figure 8), respectively. Low concentrations of IL-8 and MCP-1 mRNAs were present in unstimulated smooth muslce cells (Figure 7, lane 7; Figure 8, lane 7). At 2, 4, and 6 h following exposure to LPS, IL-1β and IFN-γ, both IL-8 and MCP-

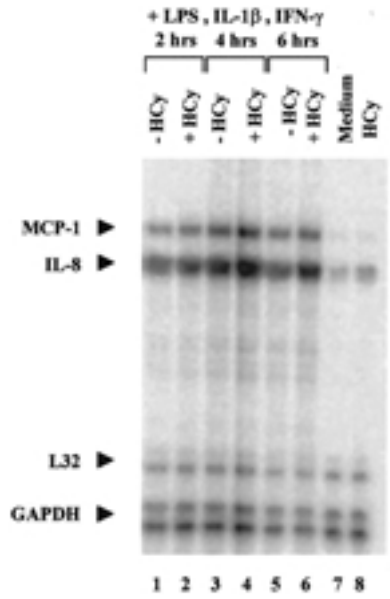

Fig. 7. MCP-1 and IL-8 mRNAs in cytokine-activated smooth muscle cells. RNase protection assay was utilized in order to detect specific transcripts for both chemokines and for the "housekeeping genes, L32 and GAPDH, as controls. Cells were exposed to 250 mM homocysteine or medium for 4 h followed by a mixture of LPS, IL-1β, and IFN-γ for 2 h (lanes 1 and 2), 4 h (lanes 3 and 4), or 6 h (lanes 5 and 6). Lanes 7 and 8 represent mRNAs from smooth muscle cells that had been incubated in presence of medium alone or 250 μM homocysteine for 10 h, respectively.

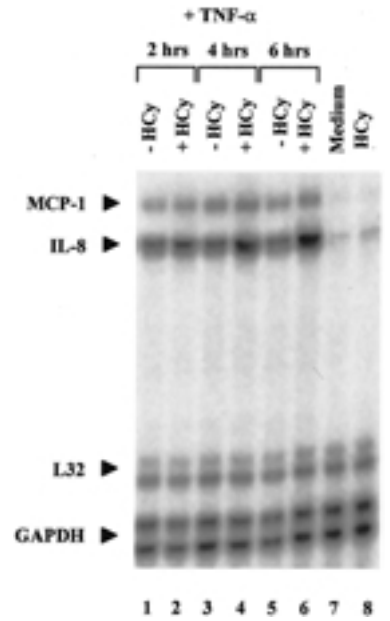

Fig. 8. MCP-1 and IL-8 mRNAs in TNF-α-activated smooth muscle cells. RNase protection assay was utilized in order to detect specific transcripts for both chemokines and for the "housekeeping" genes, L32 and GAPDH, as controls. Cells were exposed to 250 μM homocysteine or medium for 4 h followed by TNF-α for 2 h (lanes 1 and 2), 4 h (lanes 3 and 4), or 6 h (lanes 5 and 6). Lanes 7 and 8 represent mRNAs from smooth muscle cells that had been incubated in presence of medium alone or 250 μM homocysteine for 10 h, respectively.

1 transcripts were significantly upregulated in homocysteine pretreated cells (Figure 7, lanes 2, 4, 6) as compared to controls (smooth muscle cells exposed to medium prior to LPS, IL-1β and IFN-γ stimulation; Figure 7, lanes 1, 3, 5). Maximum concentrations of both IL-8 and MCP-1 mRNAs were detected following a 4-h exposure of smooth muscle cells to homocysteine followed by a 4-h incubation in presence of LPS, IL-1β and IFN-γ (Figure 7, lane 4). As can be seen in Figure 8, homocysteine pretreatment for 4 h followed by TNF-α exposure for 2, 4, and 6 h respectively (lanes 2, 4 and 6), resulted in significantly elevated IL-8 and MCP-1 mRNA concentrations when compared to media-pretreated cells (lanes 1, 3, and 5).

DISCUSSION

The recruitment, proliferation, and activation of multiple cell types (i.e. monocytes, T-lymphocytes, smooth muscle cells, endothelial cells) within lesions are critical features of the chronic inflammatory and fibroproliferative responses central to atherosclerosis (13), (14). MCP-1, a member of the C-C or beta subfamily of chemokines is abundant in macrophage-rich areas of atherosclerotic lesions and is believed to be atherogenic, in part by promoting the firm adhesion of leukocytes to endothelium that facilitates chemotactic transmigration (14). Aiello et al. (15) recently reported that MCP-1 expression by leukocytes, predominantly macrophages, hastens the progression of atherosclerosis in apolipoprotein E-deficient mice by increasing both macrophage numbers and oxidized lipid accumulation.

The C-X-C chemokine IL-8 has also recently been recognized to promote atherogenesis by virtue of its ability to trigger the firm adhesion of monocytes to vascular endothelium (16). Interleukin-8 is secreted by macrophages, endothelial and smooth muscle cells and, like homocysteine, is a potent mitogenic stimulus for smooth muscle cells (17). Interleukin-8 has angiogenic properties and is chemotactic for T-lymphocytes which are present in atherosclerotic lesions (18). Rus et al. (19) measured protein levels and gene expression of IL-8 in the human arterial atherosclerotic wall and observed

that IL-8 protein concentrations are increased significantly in fibrous plaques compared to normal arterial media and intima. In addition, IL-8 transcripts are also expressed in the walls of human arteriosclerotic vessels. Wang et al. (20) reported that THP-1 macrophages incubated with acetylated LDL exhibit increased IL-8 mRNA concentrations. Interleukin-8 induction has also been observed in fresh human monocyte-derived macrophages cells exposed to acetylated LDL. Immunohistochemical and in situ hybridization studies using human coronary atheromas have revealed IL-8 mRNA in macrophage-rich areas. Macrophages from atherosclerotic plaques exhibit an enhaced capacity to produce IL-8 compared to normal blood monocytes (21). Moreover, intimal macrophages express the growth-related oncogene (GRO) and IL-8 receptor mlL-8RH/CXR2 in advanced murine and human atherosclerotic lesions (22).

Elevated plasma homocysteine concentrations were first linked to atherosclerosis three decades ago (23) but the mechanisms by which homocysteine exerts its atherothrombotic action remain controversial. Accumulating evidence suggests that hyperhomocysteinemia, through the oxidation of homocysteine, leads to endothelial injury and dysfunction mediated by reactive oxygen intermediates (ROIs) such as superoxide anion, hydrogen peroxide (H_2O_2), and hydroxyl radical (24). These ROIs in turn have been identified as potent activators of the redox-sensitive transcription factor NF-κB in a variety of cell lines (10). NF-κB is critical for the expression of many genes involved in atherogenesis including those encoding pathophysiologically relevant cytokines (IL-1, TNF-α), chemokines (IL-8, MCP-1, GRO α), growth factors (granulocyte macrophage colony stimulating factor (GM-CSF), and adhesion molecules (vascular cell adhesion molecule 1 (VCAM-1) (25), (26).

Most published studies regarding the effect of hyperhomocysteinemia on smooth muscle cells have foucsed on the mitogenic properties of homocysteine. We observed that pathophysiologically relevant concentrations of homocysteine increase cytokine-mediated MCP-1 and IL-8 expression by smooth muscle cells at both the protein and mRNA level. To our knowledge this is the first report addressing the effects of homocysteine on chemokine expression by smooth muscle cells. Welch et al. (5) recently reported that exposure of vascular smooth musle cells to homocysteine prior to cytokine stimulation leads to an increase in NF-κB-mediated *Nos*2 transcription. Catalase and superoxide dismutase reduced cytokine activation of NF-κB after homocysteine exposure by 35% and 40%, respectively, suggesting that ROIs generated during oxidation of homocysteine facilitate cytokine-dependent activation of NF-κB (5). In addition, a significant increase in hydrogen peroxide (H_2O_2) formation was detected in conditioned media from homocysteine-treated smooth muscle cells. Cumulatively, the studies reported by Welch et al. (5) and our data suggest that exposure of smooth muscle cells to pathophysiologically relevant concentrations of homocysteine results in increased cytosolic-to-nuclear translocation of NF-κB which in turn results in the increased expression of several relevant inflammatory mediators.

Upregulation of MCP-1 expression by homocysteine may accelerate atherogenesis by facilitating increased recruitment of monocytes into sites of atheroma development. Activated lesional monocyte-macrophages promote the progression of atherosclerosis by scavenging and oxidizing LDL, thus producing proinflammatory oxidized lipids and by expressing a variety of growth factors, cytokines, complement proteins and proteases. MCP-1 modulates not only monocyte (and T lymphocyte) recruitment but also monocyte activation, including the expression of some complement proteins, IL-1, and the procoagulant tissue factor, thereby potentially contributing to thrombotic events associated with atherosclerosis (14). The mitogenic properties of both IL-8 and homocysteine may facilitate an autocrine pathway in smooth cells: IL-8 elaboration by activated smooth muscle cells may result in increased numbers of smooth muscle cells which may in turn secrete even greater concentrations of IL-8. Since IL-8 can induce the firm adhesion of rolling monocytes onto monolayers expressing E-selectin (16) and is chemotactic for T-cells, upregulation of IL-8 expression by homocysteine would likely also result in increased recruitment of monocytes and T-hymphocytes to sites of atherosclerotic lesions.

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