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# Linoleic acid induces MCP-1 gene expression in human microvascular endothelial cells through an oxidative mechanism

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**Abstract**

Linoleic acid is a dietary fatty acid that appears to play an important role in activation of the vascular endothelium under a variety of pathological conditions, including development of atherosclerosis or cancer metastasis. Evidence indicates that inflammatory responses may be an underlying cause of endothelial cell pathology induced by linoleic acid. However, the profile of inflammatory mediators and the potential mechanisms involved in inflammatory reactions stimulated by the exposure to linoleic acid are not fully understood. The present study focused on the mechanisms of linoleic acid-induced expression of monocyte chemoattractant protein-1 (MCP-1) gene in human microvascular endothelial cells (HMEC-1). Treatment of HMEC-1 with increasing doses of linoleic acid markedly activated an oxidative stress-responsive transcription factor, nuclear factor-κB (NF-κB). In addition, exposure to linoleic acid induced a time- and concentration-dependent overexpression of the MCP-1 gene. Increased MCP-1 mRNA levels were observed in HMEC-1 treated with linoleic acid at doses as low as 10 μM. Linoleic acid-induced overexpression of the MCP-1 gene was associated with a significant elevation of MCP-1 protein levels. Most importantly, preexposure of HMEC-1 to antioxidants, such as pyrrolidine dithiocarbamate (PDTC) or N-acetylcysteine (NAC), attenuated linoleic acid-induced MCP-1 mRNA expression. The obtained results indicate that linoleic acid triggers MCP-1 gene expression in human microvascular endothelial cells through oxidative stress/redox-related mechanisms. 

*Keywords:* dietary fatty acids; vascular endothelium; cancer metastasis; atherosclerosis; oxidative stress

## 1. Introduction

Induction of inflammatory genes plays an important role in the physiological and pathological functions of the vascular endothelium. For example, the overexpression of adhesion molecules on the surface of endothelial cells may stimulate adhesion and migration of both tumor cells or monocytes/macrophages across the vascular endothelium [1]. In addition, increased expression of chemokines, such as monocyte chemoattractant protein-1 (MCP-1) may play a critical role in the biology of vascular dysfunction. A member of the CC chemokine family, human MCP-1 stimulates chemotaxis and transmigration of monocytes, lymphocytes, and granulocytes [2]. Increased production of MCP-1 may be involved in a variety of processes, including early phases of atherosclerosis [3,4] and cancer metastasis [5–7].

There are at least two distinct mechanisms by which MCP-1 may participate in cancer metastasis: MCP-1 may induce the unidirectorial migration of inflammatory cells [2]. MCP-1 may be chemotactic to tumor cells [5]. This latter effect was demonstrated using MCF-7 cells, a cell line obtained from human breast carcinoma [5]. The chemotactic influence of MCP-1 on tumor cells was shown to be mediated by a receptor-stimulated signaling pathway [8]. Thus, it appears that MCP-1 can directly attract tumor cells and induce tumor cell migration across the vascular endothelium with the subsequent generation of tumor metastasis. In addition to such direct effects, chemotactic properties of MCP-1 towards leukocytes may also indirectly affect tumor metastasis. Leukocytes attracted and activated by MCP-1 in the proximity of the endothelium can migrate across the endothelium and degrade extracellular matrix proteins, which separate the endothelium from the underlying layers of the vascular wall [9,10]. Such a process can markedly facilitate invasion of tumor cells, a process associated with the development of metastasis. To support the role of

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MCP-1 in tumor metastasis, it was demonstrated that levels of this chemokine were elevated in serum of ovarian cancer patients [6] and in urine of patients with bladder cancer [7]. In fact, the urinary MCP-1 levels were strongly correlated with tumor stage, grade, and distant metastasis [7].

Selected dietary fatty acids can modulate inflammatory responses in numerous tissues, including the vascular endothelium [11]. However, it appears that the effects mediated by individual fatty acids are very specific, and are influenced by diet and types of dietary fat. Among different dietary fatty acids, linoleic acid may play one of the most critical roles in induction of alterations of endothelial cell metabolism [11,12]. It was reported that this fatty acid can disrupt endothelial cell integrity, alter functions of gap-junctional proteins [13], increase levels of intracellular calcium, and induce cellular oxidative stress [14]. In clinical studies, a positive correlation was found between linoleic acid levels in the phospholipid fractions of human coronary arteries and ischemic heart disease [15] as well as between concentrations of linoleic acid in adipose tissue and the degree of coronary artery disease [16]. Evidence also indicates that dietary linoleic acid also can promote carcinogenesis. In fact, it was demonstrated that when the dietary content of linoleic acid exceeded 4–5% of total calories, any additional fat linearly increased chemically-induced tumor incidence [17,18]. In addition to its role in carcinogenesis, dietary linoleic acid can also enhance the metastatic formation of mammary tumors. For example, a linoleic acid-enriched diet increased the rate of metastasis of mammary cancer to the lung in rats [19]. However, detailed mechanisms of linoleic acid-stimulated cancer metastasis are not fully understood, and we hypothesize that induction of vascular endothelial cell inflammatory genes, such as genes encoding for adhesion molecules or chemokines, including MCP-1, may markedly contribute to carcinogenesis and cancer metastasis induced by this fatty acid.

Because of the importance of MCP-1 induction in vascular biology, and because of the involvement of linoleic acid in the pathology of the vascular endothelium, the present study was designed to examine the regulatory mechanisms of linoleic acid-induced MCP-1 gene expression in microvascular endothelial cells. We demonstrate that linoleic acid can trigger overexpression of the MCP-1 gene, leading to increased MCP-1 production, through an oxidative stress-related mechanism.

## 2. Methods

### *2.1 Endothelial cell culture*

Human microvascular endothelial cells (HMEC-1) were a generous gift from Dr. Eric Smart (University of Kentucky Medical Center). HMEC-1 were cultured in MCDB 131 media (Sigma, St. Louis, MO) enriched with 10% fetal bovine serum, 1% penicillin/streptomycin, 1 $\mu$g/ml hydrocortisone and 0.01 $\mu$g/ml epidermal growth factor in a 5% $CO_2$ atmosphere at 37°C. Linoleic acid (>99% pure) was obtained from Nu-Chek Prep (Elysian, MN). The medium was enriched with linoleic acid as described previously [20].

In selected experiments, HMEC-1 were pretreated for 30 min with pyrrolidine dithiocarbamate (PDTC, Sigma, St. Louis, MO) at the levels of up to 25 $\mu$M or with N-acetylcysteine (NAC, Sigma, St. Louis, MO) at the levels of up to 50 mM.

### *2.2. Electrophoretic mobility shift assay (EMSA)*

Nuclear extracts from HMEC-1 were prepared according to the method of Beg *et al* [21] as described earlier [22]. Binding reactions were performed in a 20 $\mu$l volume containing 6 $\mu$g of nuclear protein extracts, 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, 2 $\mu$g of poly[dI-dC] (nonspecific competitor) and 40,000 cpm of $^{32}$P-labeled specific oligonucleotides that contained the NF- $\kappa$B sequence specific for the NF- $\kappa$B site binding site in the MCP-1 promoter (5′-AGA GTG GGA ATT TCC ACT CA-3′). The resultant protein-DNA complexes were resolved on native 5% polyacrylamide gels using 0.25 × TBE buffer (50 mM Tris-Cl, 45 mM boric acid, 0.5 mM EDTA, pH 8.4). Competition studies were performed by the addition of a molar excess of unlabeled oligonucleotide to the binding reaction. Rabbit polyclonal anti-p50 and anti-p65 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were employed in supershift experiments.

### *2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)*

Total RNA was extracted by the use of TRI reagent (Sigma, St. Louis, MO) and reverse-transcribed at 42°C for 60 min in 20 $\mu$l of 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1 mM dNTP, 1 unit/$\mu$l of recombinant RNasin ribonuclease inhibitor, 15 units/$\mu$g of AMV reverse transcriptase, and 0.5 $\mu$g of oligo(dT)$_{15}$ primer [22]. For amplification of MCP-1 and of $\beta$-actin (a housekeeping gene), the following primer combinations were used: 5′-CAG CCA GAT GCA ATC AAT GC-3′ and 5′-GTG GTC CAT GGA ATC CTG AA-3′ (MCP-1; expecting 198-bp fragment; R&D Systems, Minneapolis, MN) and 5′-AGC ACA ATG AAG ATC AAG AT-3′ and 5′-TGT AAC GCA ACT AAG TCA TA-3′ ($\beta$-actin; expecting 188-bp fragment) [23]. The PCR mixture consisted of a Taq PCR Master Mix Kit (Qiagen, Valencia, CA), 2 $\mu$l of the reverse transcriptase reaction, and 20 pmol of primer pairs in a total volume of 50 $\mu$l. Thermocycling was performed according to the following profile: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, repeated 20 times. Amplification was linear within the range of 15–25 cycles. PCR products were separated by 2% agarose gel electrophoresis, stained with SYBR® Green I (Molecular Probes, Eugene, OR) and

visualized using phosphoimaging technology (FLA-2000, Fuji, Stamford, CN).

### 2.4. Measurement of MCP-1 production

MCP-1 concentrations in cell culture supernatants were determined using a Quantikine® Human MCP-1 Immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's recommendations. This assay employs the quantitative sandwich enzyme immunoassay technique using a murine monoclonal antibody against human MCP-1 and a polyclonal secondary antibody conjugated with horseradish peroxidase. The minimum detectable concentration of MCP-1 was less than 5.0 pg/ml.

### 2.5. Statistical analysis

Routine statistical analysis of data was completed using SYSTAT 7.0 (SPSS Inc., Chicago, IL). One-way ANOVA was used to compare responses among the treatments. The treatment means were compared using Bonferroni least significant difference procedure. Statistical probability of $p < 0.05$ was considered significant.

## 3. Results

### 3.1. Linoleic acid activates NF-κB binding in microvascular endothelial cells

NF-κB is an oxidative stress-responsive transcription factor, which is involved in transcriptional regulation of a variety of inflammatory genes [24]. In addition, activation of NF-κB can serve as a sensitive marker of oxidative stress and alterations in cellular redox status. To determine if linoleic acid can activate NF-κB in HMEC-1, cells were exposed up to 50 μM of this fatty acid for 2 h and NF-κB binding was analyzed by EMSA, using nuclear extracts from the treated cells. As shown in Figure 1, a slight endogenous activity of NF-κB was observed in control cultures (lane 2). However, when the HMEC-1 were stimulated with linoleic acid, a marked increase in NF-κB binding activity was detected (lanes 3–5). This binding was completely inhibited by an unlabeled competitor DNA containing the consensus NF-κB sequence (lane 6). In addition, the identity of NF-κB binding was confirmed by experiments in which nuclear extracts isolated from linoleic acid-treated cultures were incubated with antibodies against specific NF-κB subunits prior to adding the radioactive NF- κB oligonucleotide probe. As indicated, incubation with both anti-NF-κB p50 or anti-NF-κB p65 antibody resulted in a marked decrease in intensity of the NF-κB band (lanes 7 and 8).

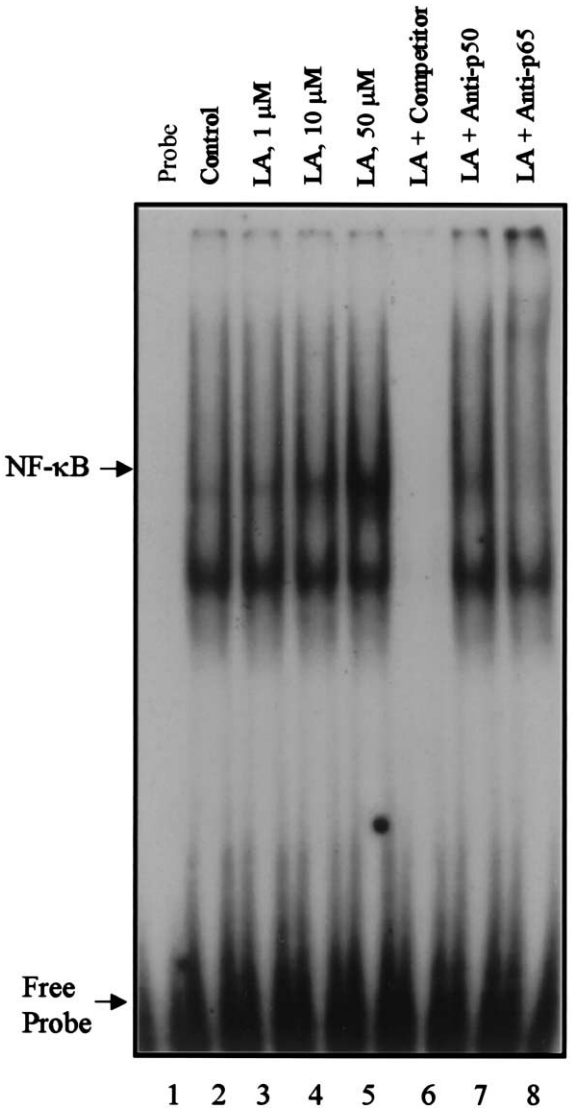


Fig. 1. Linoleic acid enhances NF-κB binding in human microvascular endothelial cells (HMEC-1). Cells were either untreated (lane 2) or treated with increasing doses of linoleic acid (lanes 3–5) for 2 h. Nuclear extracts were analyzed by EMSA. Competition study and supershift analysis were performed by the addition of excess unlabeled oligonucleotide (lane 6) and anti-NF-κB antibody (anti-p50 and anti-p65, lanes 7 and 8, respectively), using nuclear extracts from HMEC-1 stimulated by 50 μM of linoleic acid for 2 h.

### 3.2. Linoleic acid stimulates MCP-1 gene expression and protein production in microvascular endothelial cells

Figure 2 indicates the effects of linoleic acid on MCP-1 mRNA expression in HMEC-1 using a semi-quantitative RT-PCR technique. As indicated, low levels of MCP-1 mRNA were observed in control cell cultures. In addition, treatment of HMEC-1 with 50 μM of linoleic acid markedly and in a time-dependent way increased accumulation of MCP-1 mRNA (Figure 2A). Upregulation of the MCP-1 mRNA expression was already detected 1 h after linoleic acid treatment and reached the maximum levels at 3 and 4 h. Figure 2B indicates that

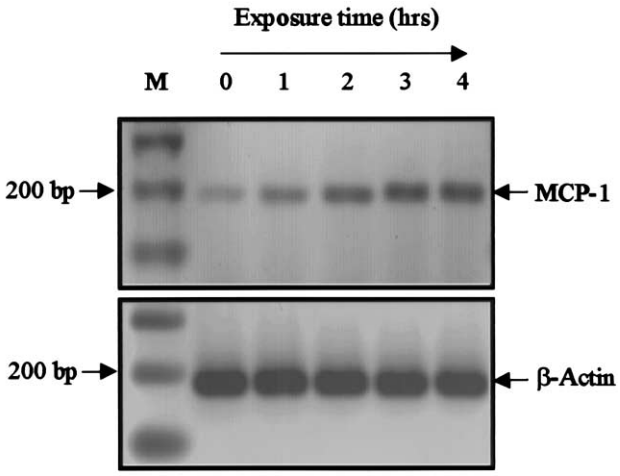


Fig. 2A. Time-dependent upregulation of MCP-1 mRNA expression by linoleic acid in human microvascular endothelial cells (HMEC-1). Cells were exposed to 50 $\mu$M linoleic acid for up to 4 h. The levels of MCP-1 mRNA were determined by RT-PCR. PCR products were analyzed by 2% agarose gel electrophoresis and visualized using phosphoimaging. The predicted sizes of RT-PCR products for MCP-1 and $\beta$-actin (represented by arrows) are 198 bp and 188 bp, respectively. M, molecular weight markers (100-bp DNA ladder).

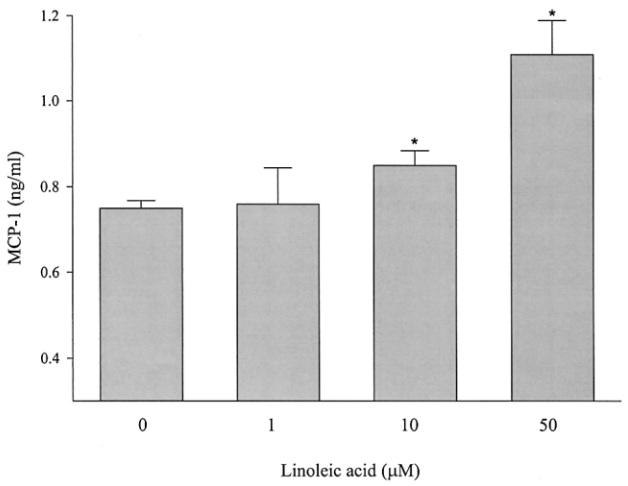


Fig. 3. Linoleic acid increases production of MCP-1 protein in human microvascular endothelial cells (HMEC-1). Cells were treated with increasing concentrations of linoleic acid for 16 h. Concentration of MCP-1 was measured by ELISA in the aliquots of culture media. Values represent mean ± SD. *Statistically significant compared to the control group ($P < 0.05$).

linoleic acid-induced stimulation of the MCP-1 mRNA is dose dependent. Maximal induction of the MCP-1 gene was detected in HMEC-1 exposed to linoleic acid at the dose of 50 $\mu$M.

The quantitative sandwich enzyme immunoassay technique was employed to determine whether linoleic acid-mediated induction of the MCP-1 gene is paralleled by a concomitant production of MCP-1 protein. Concentration of MCP-1 protein was determined in culture supernatants from HMEC-1 treated with different doses of linoleic acid for 16 h (Figure 3). Consistent with the data on MCP-1 gene expression, treatment with linoleic acid resulted in a dose-dependent upregulation of MCP-1 protein levels. Significant elevations of MCP-1 levels were observed in cultures exposed to 10 and 50 $\mu$M of linoleic acid.

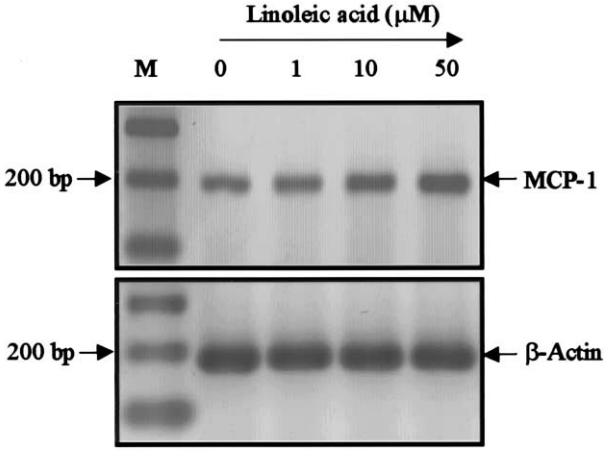


Figure 2B. Dose-dependent upregulation of MCP-1 mRNA expression by linoleic acid in human microvascular endothelial cells (HMEC-1). Cells were exposed to increasing concentrations of linoleic acid for 4 h. The levels of MCP-1 mRNA were determined as described in the legend to Figure 2A.

### *3.3. Antioxidants attenuate linoleic acid-induced MCP-1 gene expression*

To determine whether linoleic acid-mediated MCP-1 gene expression is mediated by an oxidative stress-related mechanism, HMEC-1 were pretreated for 30 min either with pyrrolidine dithiocarbamate (PDTC) or with N-acetylcysteine (NAC), followed by a 4 h treatment with 50 $\mu$M of linoleic acid. Both PDTC and NAC are widely used as antioxidant compounds to study redox regulation of intracellular signaling pathways and of cell function [25,26]. As shown in Figure 4A, PDTC attenuated linoleic acid-induced MCP-1 mRNA levels. Similar effects were observed when HMEC-1 were pretreated with NAC prior to exposure to linoleic acid (Figure 4B).

## 4. Discussion

Linoleic acid is the major dietary fatty acid present in high concentrations in corn, soy, sunflower, or safflower oils. It is estimated that it provides approximately 7–8% of the average dietary energy intake [27]. Such a high consumption of linoleic acid may markedly affect endothelial cell metabolism. It is widely recognized that the lipid composition of plasma lipoproteins is closely related to dietary fat intake [28]. In addition, it has been proposed that hydrolysis of triglyceride-rich lipoproteins mediated by lipoprotein lipase, a key enzyme in lipoprotein metabolism

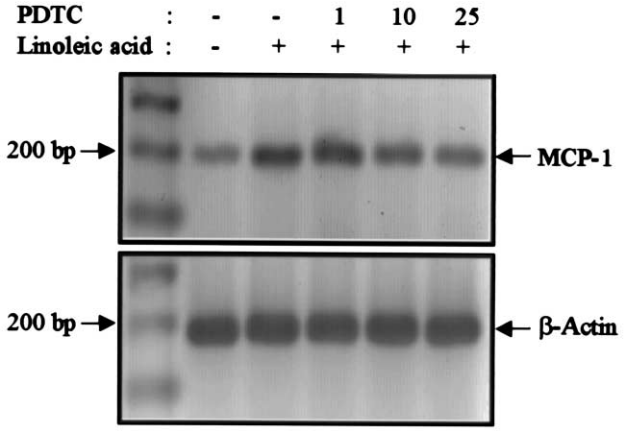


Fig. 4A. Pyrrolidine dithiocarbamate (PDTC) inhibits linoleic acid-induced induction of MCP-1 mRNA in human microvascular endothelial cells (HMEC-1). Cells were pretreated with indicated amounts of PDTC for 30 min before a 4 h treatment with 50 $\mu$M linoleic acid and analyzed for MCP-1 mRNA by RT-PCR as described in the legend to Figure 2A.

that is associated with the luminal site of endothelial cells, may be an important source of high concentrations of fatty acid anions in the proximity to the endothelium [29,30]. Therefore, the fatty acids which are exposed to endothelial cells are correlated with the type of fat that is being consumed [28,31].

Linoleic acid may be one of the most important dietary factors which can activate the vascular endothelium, a process which is involved in a variety of pathological conditions, such as early atherosclerotic changes or induction of cancer metastasis. Research from our laboratories demonstrated that exposure of endothelial cells to this fatty acid can induce profound inflammatory responses, demonstrated by increased production of adhesion molecules and inflammatory cytokines [32–34]. In line with these earlier reports, the results of the present study indicate that exposure of

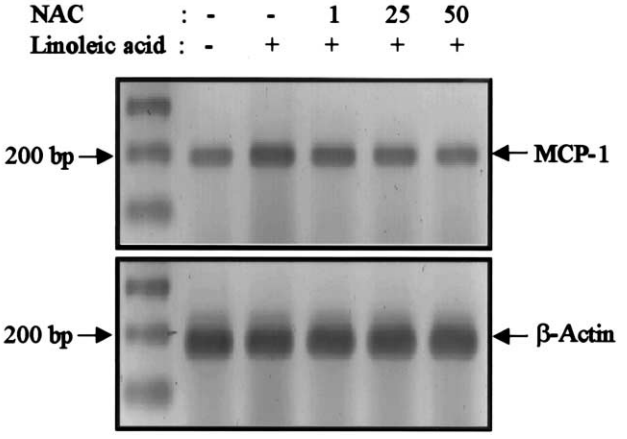


Figure 4B. N-Acetylcysteine (NAC) inhibits linoleic acid-induced induction of MCP-1 mRNA in human microvascular endothelial cells (HMEC-1). Cells were pretreated with indicated amounts of NAC for 30 min before a 4 h treatment with 50 $\mu$M linoleic acid and analyzed for MCP-1 mRNA by RT-PCR as described in the legend to Figure 2A.

HMEC-1 to linoleic acid can induce MCP-1 gene expression through an oxidative stress-related mechanism. We demonstrated that pretreatments of endothelial cells either with PDTC or with NAC attenuated linoleic acid-induced elevation of the MCP-1 mRNA levels (Figure 4). Several lines of evidence can explain this phenomenon. For example, polyunsaturated fatty acids, and in particular linoleic acid, are potent prooxidants. In fact, linoleic acid is considered to be the predominant substrate for lipid peroxidation processes both in lipoproteins, such as low-density lipoproteins (LDL), as well as in tissues [35]. Linoleic acid was demonstrated to a) enhance radical adduct formation in endothelial cells exposed to iron-induced oxidative stress [36], b) decrease glutathione levels [20], and c) increase peroxisomal $\beta$-peroxidation [37], a pathway that leads to the production of hydrogen peroxide. Degradation of linoleic acid *via* the cytochrome-P450 pathway also can lead to formation of highly prooxidative and proinflammatory derivatives, such as epoxides and diol metabolites [38]. In support of the hypothesis that expression of human MCP-1 might be regulated by oxidative stress-related mechanisms it was demonstrated that red wine with high antioxidant capacity can inhibit MCP-1 expression and reduce neointimal thickening after balloon injury of the aorta in cholesterol-fed rabbits [39].

Evidence indicates that not only linoleic acid but also a variety of its oxidative derivatives can induce profound proinflammatory responses [38,40]. However, the present study indicated that already a 2 h exposure to linoleic acid was sufficient to markedly elevate the MCP-1 mRNA levels in HMEC-1. Such a very short exposure time suggests that induction of the MCP-1 gene may be caused by a direct effect of linoleic acid rather than by its oxidative metabolites.

Transcriptional mechanisms of linoleic acid-induced MCP-1 gene expression are not fully understood; however, they may involve activation of transcription factors whose binding sites are present in the promoter region of the MCP-1 gene. Evidence indicates that putative binding sites for NF-$\kappa$B, AP-1, SP-1 and GAS exist in the 5′-flanking region of the human MCP-1 gene [41]. Although activation of these transcription factors appears to be redox-responsive, they are regulated by different and specific mechanisms. It is generally accepted that activation of NF-$\kappa$B is regulated by increased cellular oxidative stress and/or alterations of glutathione metabolism [42,43]. Evidence indicates that exposure of endothelial cells to linoleic acid can markedly affect glutathione levels. In fact, we observed a significant decrease in cellular glutathione content and increased ratio between oxidized and reduced glutathione in peripheral endothelial cells exposed to this fatty acid [20]. In addition to linoleic acid-induced activation of NF-$\kappa$B, we have evidence that treatment with this fatty acid can markedly stimulate NF-$\kappa$B-dependent transcription [14,44]. However, our earlier reports on vascular effects of linoleic acid have been based on cells isolated from major vascular vessels, such as pulmonary artery [14,20,33,38,44] or um-

bilical veins [22,32,34]. It is well known that endothelial cells from different tissues and vessels can differ markedly in their structure and functions [45]. In the present study, we report that linoleic acid can activate NF-κB in microvascular endothelial cells, i.e., the type of endothelial cells that provide a most relevant experimental model to study vascular mechanisms of cancer metastasis. In addition, it should be noted that in the present study linoleic acid-mediated NF-κB activation was detected using the NF- κB oligonucleotide probe specific for the NF-κB binding site of the human MCP-1 promoter region.

AP-1 is another transcription factor that is activated by alterations of cellular redox status. However, the specific mechanisms of such activation appear to be complex. AP-1 is composed of the Jun and Fos gene products, which can form heterodimers (Jun/Fos) or homodimers (Jun/Jun). It has been demonstrated that under specific experimental conditions, both oxidants and antioxidants can lead to activation of this transcription factor [46,47]. For example, oxidation of cysteine residues of c-Fos and c-Jun (Fos Cys-154 and Jun Cys-272, respectively) can convert the AP-1 subunits into inactive forms and inhibit binding activity of this transcription factor [48]. However, oxidative stress also can induce the mitogen-activated protein kinase (MAPK) cascade which can lead to AP-1 activation [49]. Linoleic acid and its oxidative derivatives can stimulate both c-Fos and c-Jun mRNA expression, as well as activate MAPK in rat aortic smooth muscle cells [50]. In addition, in support of the possible involvement of NF-κB and AP-1 activation in linoleic acid-induced MCP-1 gene in HMEC-1, the critical role of these transcription factors in MCP-1 gene expression was demonstrated in cells stimulated with tumor necrosis factor-α (TNF-α) [51,52].

The promoter region of the MCP-1 gene also contains GAS and SP-1 binding sites [41,53]. However, their possible involvement in linoleic acid-induced overexpression of the MCP-1 gene is not fully understood. It is known that the transcription factor STAT1α specifically interacts with GAS binding sites. Our unpublished observations indicate that activation of STAT1α can be regulated by cellular oxidative status. However, there is no existing evidence whether this transcription can be activated by linoleic acid treatment in cultured microvascular endothelial cells. In addition, evidence indicates that activation of the SP-1 transcription factor can be regulated by the cellular redox status and that it plays a critical role in interleukin-4-mediated induction of the vascular adhesion molecule-1 (VCAM-1) gene expression [54]. However, at the present time it is unknown if a similar mechanism also is involved in linoleic acid-mediated overexpression of the MCP-1 gene in HMEC-1.

In conclusion, the present study provides compelling evidence that linoleic acid can induce MCP-1 expression in human microvascular endothelial cells, a cell model used for studying mechanisms of cancer metastasis. These data may contribute to a better understanding how dietary lipids can induce production of the inflammatory mediators in the microvasculature and contribute to a variety of pathological alterations, such as cancer metastasis.

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