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13. ABSTRACT (Maximum 200 Words)

Excessive consumption of dietary fat may enhance the rate of breast cancer metastasis. In addition, it is generally accepted that the upregulation of endothelial cell adhesion molecules is involved in the formation of blood-borne metastasis. Among different adhesion molecules, evidence indicates that intracellular adhesion molecule-1 (ICAM-1) may play a critical role in breast cancer metastatic formation. Our studies have demonstrated that dietary fatty acids can exert highly specific effects on NF-κB activation and expression of adhesion molecules in human endothelial cells. In addition, we indicated that linoleic acid induces ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) expression through the activation of NF-κB. More importantly, because the ability of cancer cells to interact with the endothelium appears to be a prerequisite for the potential of distant metastasis and because ICAM-1 and VCAM-1 are considered to be a crucial adhesion molecule in this process, the present study may have significant therapeutic implications in developing a novel strategy against cancer metastasis.

14. SUBJECT TERMS
Dietary fatty acids, nutrition, endothelial cells, mice, adhesion molecules, signal transduction, tumor adhesion

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Table of Contents

1. INTRODUCTION

Excessive consumption of dietary fat may enhance the rate of breast cancer metastasis. In addition, it is generally accepted that the upregulation of endothelial cell adhesion molecules is involved in the formation of blood-borne metastasis. Such a process may initiate migration of tumor cells through the endothelium into underlying tissues and thus tumor cells cannot be destroyed by the immune system. Although several adhesion molecules may be involved in this process, it appears that the overexpression of ICAM-1 (intracellular adhesion molecule-1) may play a critical role in breast cancer metastatic formation.

In our research we are the first to propose that lipid-enhanced breast cancer metastasis may be connected to the overexpression of ICAM-1. The fact that a variety of fatty acids have different effects on ICAM-1 induction may explain different effects of dietary lipids on breast cancer metastasis. In the current grant application, we propose to study mechanisms of lipid-induced ICAM expression and breast tumor cell metastatic formation on molecular, cellular and whole animal levels.

Recent evidence indicates that also other inflammatory mediators, namely vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1) can be involved in fatty acid-induced cancer metastasis. Therefore, our research has been extended to study effects of dietary lipids, primarily linoleic acid on the expression of VCAM-1 and MCP-1.

To date, our research was solely based on endothelial cell culture model system and treatments including different dietary fatty acids.

2. BODY

a. Research accomplishments associated with Task 1.

Task 1. **To identify the specific phosphorylation mechanism involved in lipid-mediated induction of ICAM-1 expression.**

The results obtained as a result of this Task indicate that exposure to linoleic acid increases protein kinase C (PKC) and mitogen-activated protein (MAP) kinase activities. In addition, inhibition of both PKC and MAP-kinase prevented linoleic acid-mediated activation of NF-κB. Endothelial cell exposure to linoleic acid also decreased cAMP levels, which indicates that c-AMP-dependent protein kinase (PKA) is an unlikely participant in fatty acid-mediated activation of NF-κB. Thus, in this research we identified two specific signal transduction mechanisms responsible for fatty acid-mediated activation of NF-κB. Which of these two pathways plays more important role in fatty acid-mediated activation of NF-κB and ICAM-1 gene expression requires further studies. Such studies may involve transfections of endothelial cells with specific NF-κB as well as IκB reporter contracts. Because endothelial cells are well known to be difficult to transfect, we developed a special technique which allows us to achieve a high-efficiency transfection of human endothelial cells. This technique was recently published by our group (Kaiser and Toborek J. Vasc. Res. 38:133-143, 2001) and it constitutes another major accomplishment resulting from this grant proposal. We were the first to report that transfection of endothelial cells can achieve as high as 32% efficiency (Figure 1). This technique also was employed in our research on NF-κB-mediated induction of ICAM-1 gene, as well as other inflammatory genes in human endothelial cells (Toborek et al., Am. J. Clin. Nutr. 75, 119-125, 2002; Lee et al., J. Nutr. Biochem. 12, 648-654, 2001; Park at al., Nutr. Cancer, 41, 126-134, 2001).

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Detailed descriptions of the obtained results are included in the appended publications.
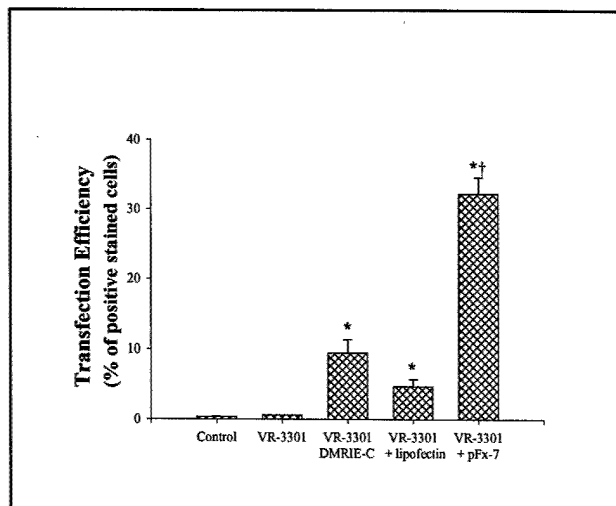

Figure 1. Efficiency of liposome-mediated transfection of human endothelial cells. Cells were transfected for 1.5 h with the VR-3301 vector (5 μg/mL) complexed with 40 μg/mL of DMRIE-C or lipofectin or with 36 μg/mL of pFx-7. *Values marked with an asterisk are significantly higher as compared to the values for control cultures or cultures transfected with naked plasmid DNA. †Values in cultures transfected in the presence of pFx-7 are significantly higher than values in other experimental groups.

b. Research accomplishments associated with Task 2.

Task 2. **To test the hypothesis that induction of ICAM-1 expression mediated by polyunsaturated but not saturated fatty acids, is the critical factor in promoting adhesion of breast tumor cells to endothelial cells and their transendothelial migration.**

Extensive studies were performed in relationship to this Task. We indicated that dietary fatty acids can exert specific effects on ICAM-1 gene expression. Exposure to both linoleic acid and linolenic acid induced a dose dependent increase in ICAM-1 mRNA levels. In addition, these two fatty acids at the concentration of 90 μmol/L stimulated induction of the ICAM-1 gene to a similar extent, i.e., by approximately 30% as measured by the density of the appropriate fluorescent bands. In contrast, exposure of endothelial cells to oleic acid decreased ICAM-1 mRNA levels to approximately 50% of control values. The results of these experiments are shown in Figure 2. The full report on dietary fatty acid-mediated expression of inflammatory genes in human endothelial cells was recently accepted for publication in the American Journal of Clinical Nutrition (Toborek et al., Am. J. Clin. Nutr. 75, 119-125, 2002) and is also appended to this Progress Report.

Figure 2C. Effects of dietary fatty acids on intercellular adhesion molecule-1 (ICAM-1) mRNA levels in human endothelial cells as measured by RT-PCR. Endothelial cells were exposed to specific fatty acids for 3 hours. β-Actin was determie to indicate that the same amount of RNA was used per sample.
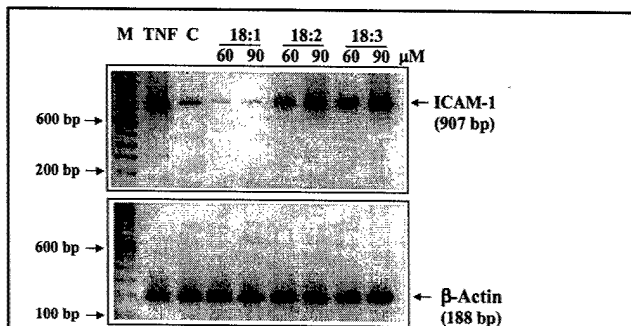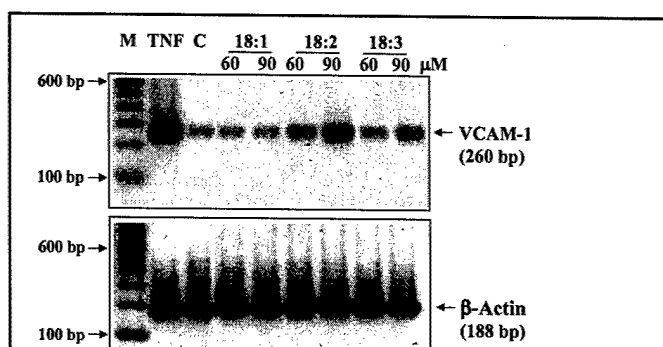

Figure 2C. Effects of dietary fatty acids on vascular cell adhesion molecule-1 (VCAM-1) mRNA levels in human endothelial cells as measured by RT-PCR. Endothelial cells were exposed to specific fatty acids for 3 hours. β-Actin was determie to indicate that the same amount of RNA was used per sample.

It appears that not only ICAM-1 but also another adhesion molecule, namely vascular cell adhesion molecule-1 (VCAM-1), may play an important role in dietary fatty acid-mediated cancer metastasis. The effects of specific unsaturated fatty acids on VCAM-1 mRNA levels in HUVEC are indicated in Figure 3. The most significant induction of the VCAM-1 gene (by 38% as measured by the density of the fluorescent bands) was observed in cells treated with 90 µmol/L of linoleic acid. In addition, exposure to 90 µmol/L of linolenic acid resulted in a slight increase in VCAM-1 mRNA levels. Treatment with oleic acid had no effect on VCAM-1 gene induction as compared to control cultures.

Our research on fatty acid-induced inflammatory responses in human endothelial cells gained significant national and international attention and recognition. Our paper on this subject published in the American Journal of Clinical Nutrition was accompanied by the Editorial Comments and has been featured on several news releases, such as Reuters Health and the Medical Post of Canada (please see the appended material).

Because of profound effects of linoleic acid on VCAM-1 gene expression, detailed studies were performed on the mechanisms of this process. We indicated that the NF-κB binding site plays the critical role in linoleic acid-induced VCAM-1 gene expression in human endothelial cells. In addition, we indicated that common anti-inflammatory drugs, such as aspirin or sodium salicylate can inhibit linoleic acid-mediated activation of NF-κB (Figure 4) as well as linoleic acid-induced VCAM-1 expression (Figure 5).
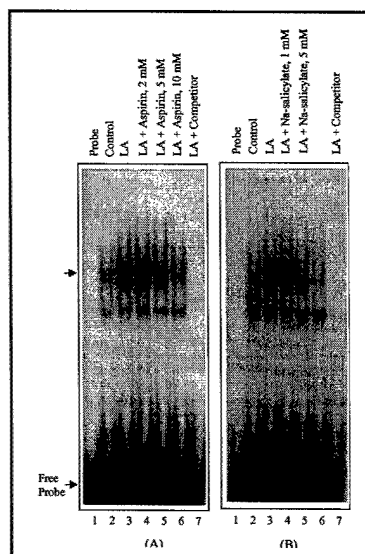

Figure 4. Pretreatment with aspirin, sodium salicylate or PDTC blocks linoleic acid (LA)-induced NF-κB DNA-binding activity in human microvascular endothelial cells (HMEC-1) as measured by EMSA. HMEC-1 were pretreated for 1 h with indicated concentrations of (A) aspirin or (B) sodium salicylate before a 2 h treatment with 50 µM of linoleic acid (lanes 4-6). Lane 1, probe alone; lane 2, treatment with 50 µM linoleic acid alone; lane 7, competition study performed by the addition of excess unlabeled oligonucleotide using nuclear extract from cells treated with 50 µM linoleic acid.

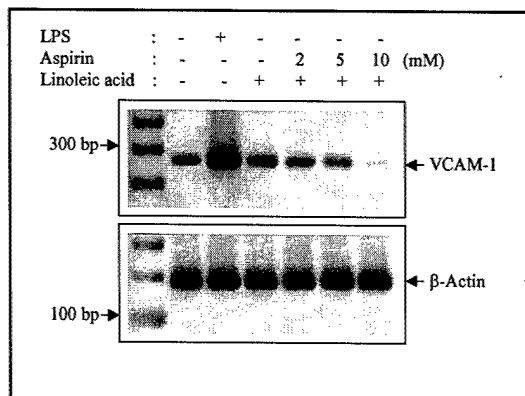Figure 5. Pretreatment with aspirin impedes the induction of VCAM-1 mRNA expression in linoleic acid-treated human microvascular endothelial cells (HMEC-1). Cells were pretreated for 1 h with indicated concentrations of aspirin for 30 min with PDTC, before a 4 h treatment with 50 µM of linoleic acid and assayed for VCAM-1 mRNA expression by RT-PCR. LPS (1 µg/mL) was used as positive control.

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b. Research accomplishments associated with Task 3.

Task 3. **To test the hypothesis that diets enriched with polyunsaturated dietary fats but not saturated fats increase metastasis formation and breast tumor development in an animal model by induction of ICAM-1 expression.**

Although our overall research related to this project progresses very well and has been very productive, our animal studies had to be postponed compared to the original research plan. A research technician and a postdoctoral scholar, who were originally assigned to perform the animal studies, left my laboratory. Thus, I had to hire new research personnel, who required additional training in animal studies. This training process is now finished, and we will be able to start the animal studies soon. Therefore, I applied for a one year, no-cost extension, which would allow us to complete this important part of our original proposal. In addition, the no-cost extension of our research project will allow us to finish other ongoing *in vitro* experiments and write final papers and research reports.

3. KEY RESEARCH ACCOMPLISHMENTS

- To identify two specific phosphorylation pathways which are induced by dietary fatty acids and participate in fatty acid-mediated activation of NF-κB.
- To establish a new transfection technique which allows the transfection of human endothelial cells with a high efficiency.
- To indicate that antioxidants and common anti-inflammatory drugs, such as aspirin, can inhibit dietary fatty acid-mediated activation of NF-κB and adhesion molecule expression in endothelial cells.
- To determine that the NF-κB binding site plays the critical role in linoleic acid-induced expression of adhesion molecules in human endothelial cells

4. REPORTABLE OUTCOMES
a. REFEREED ARTICLES

Toborek M, Lee YW, Garrido R, Kaiser S, Hennig B: Unsaturated fatty acids selectively induce an inflammatory environment in human endothelial cells. Am. J. Clin. Nutr. 75, 119-125, 2002.

Toborek M, Lee YW, Kaiser S, Hennig B: Inflammatory properties of fatty acids. Methods in Enzymology (CK Sen and L Packer; eds.) vol. 352, 198-219, 2002

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Park HJ, Lee YW, Hennig B, Toborek M: Linoleic acid-induced VCAM-1 expression in human microvascular endothelial cells is mediated by the NF-κB-dependent pathway. Nutr. Cancer 41, 126-134, 2001.

Lee YW, Park HJ, Hennig B, Toborek M: Linoleic acid induces MCP-1 gene expression in human microvascular endothelial cells through an oxidant mechanism. J. Nutr. Biochem. 12, 648-654, 2001.

Kaiser S, Toborek M: Liposome-mediated high-efficiency transfection of human endothelial cells. J. Vasc. Res. 38, 133-143, 2001.

Hennig B, Toborek M: Nutrition and endothelial cell function: implications in atherosclerosis. Nutr. Res. 21, 279-293, 2001.

Hennig B, Toborek M, McClain CJ: High-energy nutrients, fatty acids and endothelial cell function: implications in atherosclerosis. J. Am Coll. Nutr. 20, 97-105, 2001.

Hennig B, Meerarani P, Ramadass P, Watkins BA, Toborek M: Fatty acid-mediated activation of vascular endothelial cells. Metabolism 49, 1006-1013, 2000.

Hennig B, Toborek M, Boissonneault GA: Lipids, inflammatory cytokines, and endothelial cell Injury. In: Nutrition and Immunology: Principle and Practice. (ME Gershwin, B German, C Keen, editors), Humana Press, Inc., Totowa, NJ, 203-220, 2000.

b. PRESENTATIONS/ABSTRACTS

Saraswathi V, Hammock BD, Meerarani P, **Toborek M**, Hennig B: Involvement of CYP 2C9 in mediating the pro-inflammatory effects of linoleic acid in vascular endothelial cells. J. Am. Coll. Nutr. 21, 479, 2002.

Hennig B, Saraswathi V, **Toborek M**: Fatty acid-induced endothelial cell activation. Atherosclerosis 3, 123, 2002.

Saraswathi V, Hammock BD, Meerarani P, **Toborek M**, Hennig B: Mechanisms of linoleic acid-induced endothelial activation: possible involvement of epoxide metabolites in mediating the inflammatory response. FASEB J. 16, A157, 2002.

Durham CQ, Lee YW, Hennig B, **Toborek M**: Signaling mechanisms of linoleic acid-induced MCP-1 gene expression in human endothelial cells. FASEB J. 16, A211, 2002.

Reiterer G, Sali A, Meerarani P, Sarasvathi V, Kelly L, **Toborek M**, Hennig B: The plant phenolics quercetin and resveratrol protect against linoleic acid-induced endothelial cell activation. FASEB J. 16, A642, 2002.

Choi W, Lee YW, Hennig B, Robertson LW, **Toborek M**: PCB-induced inflammatory reactions in human endothelial cells: implications in cancer metastasis. FASEB J. 16, A961, 2002.

Meerarani P, **Toborek M**, Hennig B: Role of PPARγ signaling in zinc-mediated suppression of apoptosis in endothelial cells. FASEB J. 16, A975, 2002.

Kaiser S, Toborek M: High-efficiency transfection of human endothelial cells mediated by cationic lipids. Endothelium, in press.

Park H-J, Lee YW, Hennig B, Toborek M: Linoleic acid-induced VCAM-1 expression in human microvascular endothelial cells is mediated by the NF-kB-dependent pathway. FASEB J. 15, A866, 2001.

Saraswathi V, Narayan P, Hammock BD, Meerarani P, Toborek M, Hennig B. Linoleic acid-derived epoxides alter calcium and nitric oxide metabolism in endothelial cells. FASEB J. 15, A190, 2001.

Slim RM, Hammock BD, Toborek M, Robertson LW, Watkins BA, Hennig B: The role of methyl linoleic acid epoxide and diol metabolites in the synergistic toxicity of linoleic acid and PCBs to vascular endothelial cells. Toxicol. Sci., 60, 13, 2001.

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Hennig B, Meerarani P, Kaiser S, Toborek M: Inflammatory events and fatty acid-induced activation of vascular endothelial cells. FASEB J. 14, A199, 2000.

Meerarani P, Ramadass P, Toborek M, Keller J, Hennig B: The role of different fatty acids in oxidative injury and dysfunction of endothelial cells. FASEB J. 13, A1117, 1999.

Ramadass P, Meerarani P, Toborek M, Bauer HC, Bauer H, Probst G, McClain CJ, Hennig B: Protective effects of zinc against endothelial cell apoptosis induced by linoleic acid and/or TNF. FASEB J. 13, A832, 1999.

5. CONCLUSIONS

Our studies have demonstrated that dietary fatty acids can exert highly specific effects on NF-κB activation and expression of adhesion molecules in human endothelial cells. In addition, we indicated that linoleic acid induces ICAM-1 and VCAM-1 expression through the activation of NF-κB. More importantly, because the ability of cancer cells to interact with the endothelium appears to be a prerequisite for the potential of distant metastasis and because ICAM-1 and VCAM-1 are considered to be a crucial adhesion molecule in this process, the present study may have significant therapeutic implications in developing a novel strategy against cancer metastasis. These studies also provide a mechanistic insight of the role of specific dietary lipids in metastasis. Therefore, data arising from this grant proposal may allow dietary and molecular intervention to protect against breast cancer metastasis.

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Unsaturated fatty acids selectively induce an inflammatory environment in human endothelial cells[1-3]

Michal Toborek, Yong Woo Lee, Rosario Garrido, Simone Kaiser, and Bernhard Hennig

ABSTRACT

Background: Activation of the vascular endothelium by dietary fatty acids may be among the most critical early events in the development of atherosclerosis. However, the specific effects of fatty acids on inflammatory responses in endothelial cells are not fully understood.

Objective: The present study focused on the induction of inflammatory genes in human endothelial cells exposed to individual dietary fatty acids. Because of the significance of nuclear factor κB (NF-κB) and activator protein 1 (AP-1) in the regulation of inflammatory gene expression, we also determined the effects of fatty acids on NF-κB and AP-1 transcriptional activation.

Design: Human umbilical vein endothelial cells were exposed to dietary mono- and polyunsaturated 18-carbon fatty acids. Transcriptional activation of NF-κB and AP-1 was determined in human umbilical vein endothelial cells transfected with reporter constructs regulated by these transcription factors. Induction of the inflammatory genes was studied by use of reverse transcriptase–polymerase chain reaction.

Results: Of the fatty acids studied, linoleic acid stimulated NF-κB and AP-1 transcriptional activation the most. In addition, treatment with this fatty acid markedly enhanced messenger RNA levels of tumor necrosis factor α, monocyte chemoattractant protein 1, vascular cell adhesion molecule 1, and intercellular adhesion molecule 1. Treatment with linolenic acid stimulated only a moderate induction of the genes encoding for these inflammatory mediators, and exposure to oleic acid either had no effect or resulted in decreased inflammatory gene messenger RNA. In addition, exposure to both linoleic and linolenic acids strongly stimulated induction of the phospholipid hydroperoxide glutathione peroxidase gene.

Conclusion: Specific unsaturated dietary fatty acids, particularly linoleic acid, can selectively stimulate the development of a proinflammatory environment within the vascular endothelium. *Am J Clin Nutr* 2002;75:119–25.

KEY WORDS Fatty acids, inflammatory genes, transcription factors, human endothelial cells, atherosclerosis, nuclear factor κB, activator protein 1

INTRODUCTION

Activation or dysfunction of the vascular endothelium is one of the first events in the development of atherosclerosis (1, 2), and

See corresponding editorial on page 4.

selected dietary fatty acids may be among the most critical factors that induce these processes. For example, lipids, including selective fatty acids, may cause injury to the endothelium (reviewed in reference 3). It has been proposed that hydrolysis of triacylglycerol-rich lipoproteins mediated by lipoprotein lipase, a key enzyme in lipoprotein metabolism that is associated with the luminal site of endothelial cells, may be an important source of high concentrations of fatty acid anions near the endothelium (4, 5). In support of this notion, it was shown that lipoprotein lipase activity is increased in atherosclerotic lesions (5–7). Lipoprotein lipase–derived remnants of lipoproteins isolated from hypertriglyceridemic subjects as well as selective unsaturated fatty acids can disrupt endothelial integrity (8, 9). Because the lipid composition of plasma and tissues is closely related to dietary fat intake (10), exposure of endothelial cells to individual fatty acids can be directly influenced by the types of fatty acids consumed in the diet (10, 11).

Strong evidence indicates that exposure to selected dietary unsaturated 18-carbon fatty acids can directly affect endothelial cell metabolism. Significant amounts of data have been accumulated to show that linoleic acid (18:2n−6) can induce marked injury to endothelial cells. For example, it was reported that this fatty acid can disrupt endothelial cell integrity, alter functions of gap-junctional proteins (12), increase concentrations of intracellular calcium, and induce cellular oxidative stress (13). In addition, the treatment of endothelial cells with linoleic acid and tumor necrosis factor α (TNF-α) can activate caspase 3 activity and induce apoptotic cell death (14, 15). The role of other dietary unsaturated 18-carbon fatty acids in endothelial cell metabolism is less well understood. However, evidence indicates that dietary oleic acid can protect endothelial cells against hydrogen peroxide–induced oxidative stress (16) and reduce the susceptibility of LDLs to oxidative modifications (17).

Atherosclerosis is an inflammatory disease of the vascular wall (18). Inflammatory reactions in endothelial cells are regulated primarily through the production of chemokines [eg, monocyte chemoattractant protein 1 (MCP-1)], inflammatory cytokines (eg, TNF-α), and adhesion molecules [eg, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1)]. Expression of these inflammatory mediators and their effects are closely interrelated. In addition, overexpression of MCP-1 (19), TNF-α (20), and ICAM-1 and VCAM-1 (21) is a common feature of atherosclerotic processes.

Inflammatory genes, such as those encoding for MCP-1, TNF-α, ICAM-1, and VCAM-1, are regulated by a variety of transcription factors (2, 22). It appears that nuclear factor κB (NF-κB) and activator protein 1 (AP-1) play critical roles in these regulatory processes. The binding sites for these transcription factors were identified in the promoter regions of various inflammatory genes (22–25), and increased amounts of NF-κB were found in atherosclerotic vessels (26, 27). In addition, selected fatty acids, such as linoleic acid, can activate NF-κB in endothelial cells (13). Moreover, effects mediated by NF-κB and AP-1 appear to be interrelated. For example, it was shown that TNF-α–mediated induction of VCAM-1 expression requires both activated NF-κB and AP-1 (23).

Phospholipid hydroperoxide glutathione peroxidase (PHGPx) is an antioxidant enzyme involved in detoxification of lipid hydroperoxides in cellular membranes and lipoproteins (28). Thus, this enzyme may play a critical role in antioxidant protection against oxidative stress induced by unsaturated fatty acids.

Although it is known that selected fatty acids can induce oxidative stress and activate transcription factors responsive to oxidative stress (13), the specific effects of unsaturated fatty acids on inflammatory responses in endothelial cells are not fully understood. Therefore, the focus of the present study was to examine the induction of the inflammatory genes in human endothelial cells exposed to specific 18-carbon, mono- and polyunsaturated fatty acids. In addition, because of the significance of NF-κB and AP-1 in the regulation of the inflammatory genes, the effects of unsaturated fatty acids on the activity of these transcription factors were also determined.

MATERIALS AND METHODS

Human umbilical vein endothelial cell cultures and fatty acid treatments

Human umbilical vein endothelial cells (HUVECs) were isolated as described previously (29) and cultured in enriched M199 medium, which included 25 mmol HEPES/L, 54.3×10^3 U heparin/L, 2 mmol L-glutamine/L, 1 μmol sodium pyruvate/L, 200×10^3 U penicillin/L, 200 mg streptomycin/L, 0.25 mg amphotericin B/L (GibcoBRL, Grand Island, NY), 0.04 g endothelial cell growth supplement/L (Becton Dickinson, Bedford, MA), and 20% fetal bovine serum (HyClone, Logan, UT). Cells were determined to be endothelial in origin by their cobblestone morphology and uptake of fluorescently labeled acetylated LDL (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Molecular Probes, Eugene, OR). All experiments were performed on cells from passage 2. Confluent cell cultures were treated with ≤180 μmol/L of oleic acid (18:1n−9), linoleic acid, or linolenic acid (18:3n−3) (Nu-Chek Prep, Elysian, MN). Fatty acid–enriched experimental media were prepared as described earlier (9).

To study the dose-dependent effects of specific unsaturated fatty acids on messenger RNA (mRNA) levels of genes critical in the endothelial cell inflammatory response, HUVECs were exposed to 60, 90, and 180 μmol fatty acids/L. Preliminary experiments showed that fatty acids consistently exerted a maximum effect on inflammatory gene induction at the concentration of 90 μmol/L. Therefore, experiments with 180 μmol fatty acids/L were discontinued and data are presented only from studies in which HUVECs were exposed to 60 and 90 μmol unsaturated fatty acids/L.

Transfections and reporter gene assay

Transfections were performed as described earlier (30). Briefly, HUVECs were seeded in 12-well plates and grown to 50–60% confluency in normal growth medium. Then, aliquots of normal M199 medium were mixed with 36 mg/L of a liposome pFx-7 (Invitrogen, Carlsbad, CA) and with 10 mg/L of NF-κB– or AP-1–responsive plasmids (pNFκB-Luc or pAP1-Luc, respectively) containing a luciferase reporter gene (Stratagene, La Jolla, CA). The transfection mixtures were incubated at 37°C for 30 min to allow DNA-lipid complexes to form. Endothelial cell cultures were washed with M199 medium to remove serum, and 1 mL transfection solution was added for 1.5 h to each well of the 12-well plate. After incubation, transfection solutions were aspirated and replaced with growth medium for 24 h. Then, transfected cultures were treated with specific unsaturated fatty acids for 24 h. Control groups consisted of transfected HUVEC cultures that were not exposed to fatty acids.

Luciferase activity was measured by use of the Luciferase Assay System (Promega, Madison, WI). Briefly, culture media were removed and HUVECs were washed with phosphate-buffered saline and incubated with cell culture lysis reagent. Cell lysates were centrifuged ($12000 \times g$, 2 min, 4°C) to remove membrane debris, and 10 μL of the cell extracts was mixed with 100 μL luciferase assay reagent containing luciferin and ATP in a luminometer with automatic injection. Values are expressed in relative light units (RLU)/μg protein.

Transfection efficiency was monitored as described earlier (30) by transfection of endothelial cells with the VR-3301 vector, which contains human placental alkaline phosphatase as the reporter gene. Under the described conditions, transfection efficiency was determined to be 32% (30). All transfection studies were repeated 3 times by using 6 wells in 12-well plates per experimental group.

Reverse transcriptase–polymerase chain reaction analyses

Reverse transcriptase–polymerase chain reaction (RT-PCR) was performed as described earlier (29, 31). Briefly, treated HUVECs were lysed and the total RNA was extracted with use of RNA STAT-60 (Tel-TEST, Inc, Friendswood, TX) according to the procedure supplied by the manufacturer. Isolated RNA was quantitated by measuring absorbance at 260 nm. A standard reverse transcription reaction was performed at 42°C for 60 min in 20 μL of 5 mmol $MgCl_2$/L; 10 mmol tris-Cl/L, pH 9.0; 50 mmol KCl/L; 0.1% Triton X-100; 1 mmol dNTP/L; 1×10^6 U recombinant RNasin ribonuclease inhibitor/L; 15×10^6 U AMV reverse transcriptase/L; and 0.5 μg oligo(dT)$_{15}$ primer (Promega). The sequences of the primer pairs used for PCR amplification of the studied genes are shown in **Table 1**. For quantitation, levels of mRNA of the studied inflammatory genes

TABLE 1

Sequences of the primer pairs used in the reverse transcriptase–polymerase chain reactions[1]

Studied mediator	Sequence of the primer pairs (5'–3')
MCP-1[2]	
Forward	CAG CCA GAT GCA ATC AAT GC
Reverse	GTG GTC CAT GGA ATC CTG AA
TNF-α[2]	
Forward	GTG ACA AGC CTG TAG CCC A
Reverse	ACT CGG CAA AGT CGA GAT AG
ICAM-1	
Forward	GGT GAC GCT GAA TGG GGT TCC
Reverse	GTC CTC ATG GTG GGG CTA TGT CTC
VCAM-1[2]	
Forward	ATG ACA TGC TTG AGC CAG G
Reverse	GTG TCT CCT TCT TTG ACA CT
PHGPx	
Forward	TGT GCG CGC TCC ATG CAC GAG T
Reverse	AAA TAG TGG GGC AGG TCC TTC TCT
β-Actin	
Forward	AGC ACA ATG AAG ATC AAG AT
Reverse	TGT AAC GCA ACT AAG TCA TA

[1]ICAM-1, intercellular adhesion molecule 1; MCP-1, monocyte chemoattractant protein 1; PHGPx, phospholipid hydroperoxide glutathione peroxidase; TNF-α, tumor necrosis factor α; VCAM-1, vascular cell adhesion molecule 1.

[2]Primer pairs purchased from R&D Systems, Minneapolis.

and the gene encoding for PHGPx were related to β-actin mRNA. The PCR mixture consisted of 2 μL of a product of the reverse transcription reaction, a Taq PCR Master Mix Kit (Qiagen, Valencia, CA), and 20 pmol of primer pairs in a total volume of 50 μL. For each individual gene, a linear range of PCR amplification was established and the induction of the target gene was studied within the range.

The following thermocycling conditions were used to determine the induction of the genes encoding for the studied inflammatory mediators:

MCP-1: 94 °C for 4 min; followed by 94 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s (repeated 25 times); followed by an extension at 72 °C for 10 min;

TNF-α: 94 °C for 4 min; followed by 94 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s (repeated 28 times); followed by an extension at 72 °C for 10 min;

ICAM-1: 94 °C for 4 min; followed by 94 °C for 45 s, 60 °C for 45 s, 72 °C for 60 s (repeated 28 times); followed by an extension at 72 °C for 7 min;

VCAM-1: 94 °C for 60 s, 55 °C for 60 s, 72 °C for 60 s (repeated 25 times); and

PHGPx: 94 °C for 4 min; followed by 94 °C for 40 s, 66 °C for 60 s, 72 °C for 2 min (repeated 20 times); followed by an extension at 72 °C for 7 min.

Induction of the β-actin gene was determined by using the same number of cycles and thermocycling conditions as for the target genes. Under these RT-PCR conditions, the β-actin transcript increased linearly in the range of 15–40 PCR cycles.

PCR products were separated by 2%-agarose gel electrophoresis, stained with SYBR Green I (Molecular Probes), and visualized by using phosphorimaging technology (FLA-2000; Fuji, Stamford, CT). The relative intensity of fluorescence (ratio of the intensity of the band corresponding to the target gene to

that corresponding to the β-actin gene) was quantified with IMAGE GAUGE 3.0 software (Fuji) and expressed as average pixel intensity. Experiments were repeated 4 times on different days, and the values of relative fluorescence from the 4 experiments were statistically analyzed.

Statistical analysis

Statistical analysis was performed by using SYSTAT 8.0 (SPPS Inc, Chicago). One-way analysis of variance was used to compare mean values among the treatments. When the overall F values were significant, analysis of variance was followed by post hoc Bonferroni tests to compare means from different treatments. A P value <0.05 was considered significant.

RESULTS

Unsaturated fatty acids selectively induce NF-κB and AP-1 transcriptional activation

The effects of specific unsaturated fatty acids on NF-κB transcriptional activation are shown in **Figure 1A**. Treatment of
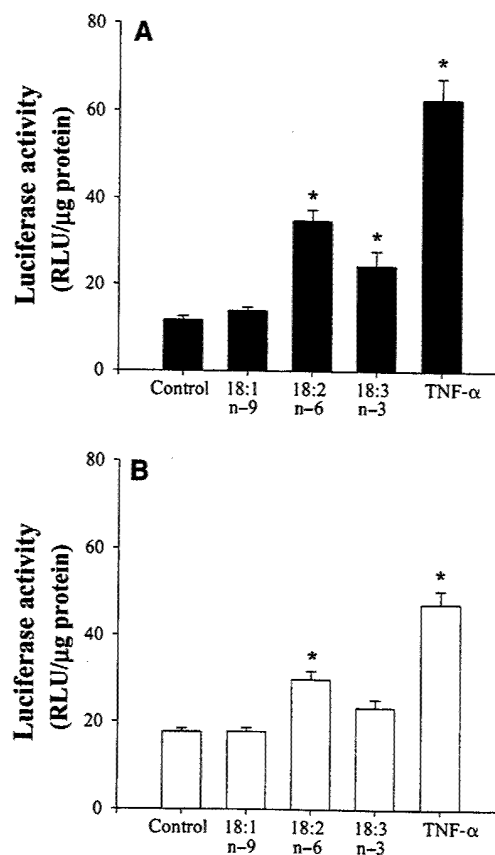

FIGURE 1. Mean (±SEM) fatty acid–induced nuclear factor κB (NF-κB)–related (A) and activator protein 1 (AP-1)–related (B) transcription in human endothelial cells. Transcriptional activation was measured by luciferase activity in human umbilical vein endothelial cells transfected with an NF-κB–responsive or AP-1–responsive luciferase reporter construct and exposed to specific unsaturated fatty acids (90 μmol/L) for 24 h. Tumor necrosis factor α (TNF-α) treatment was used as a positive control. RLU, relative light units. *Significantly different from control cultures, $P < 0.05$.
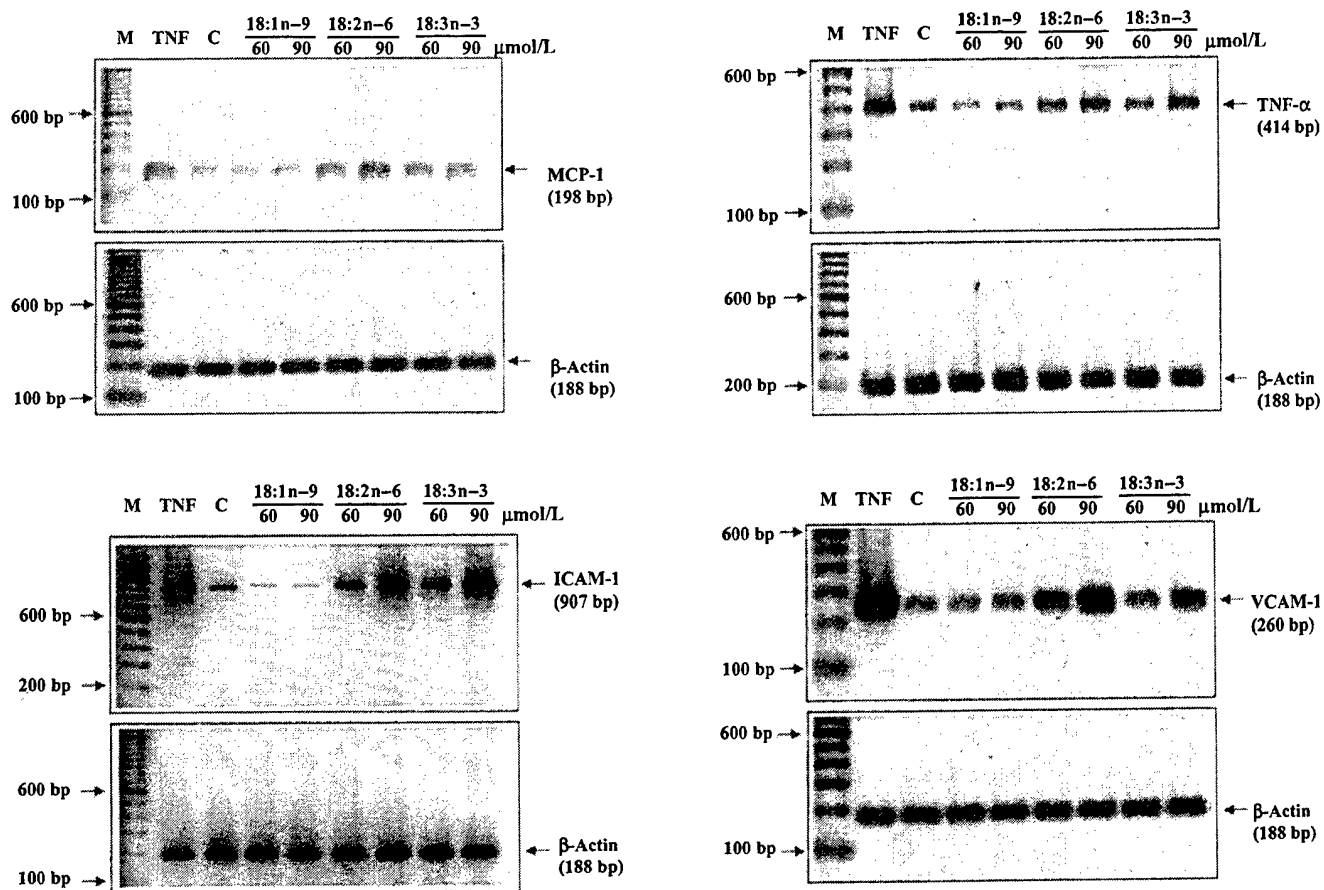
FIGURE 2. Effects of dietary fatty acids on monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor α (TNF-α), intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) messenger RNA levels in human endothelial cells as measured by reverse transcriptase–polymerase chain reaction (RT-PCR). Human umbilical vein endothelial cells were exposed to specific unsaturated fatty acids for 3 h. β-Actin was used to indicate that the same amount of RNA was used per sample. The amplified PCR products were electrophoresed on a 2%–tris-borate EDTA agarose gel, stained with SYBR Green I (Molecular Probes, Eugene, OR) and visualized by using phosphorimaging technology (FLA-2000: Fuji, Stamford, CT). bp, base pair: M, marker: C, control.

endothelial cells with oleic acid did not significantly affect luciferase activity in cells transfected with pNFκB-Luc. Compared with control cultures, linolenic acid exerted only a moderate effect on NF-κB transcriptional activation; however, treatment of transfected endothelial cells with linoleic acid resulted in a pronounced increase in luciferase activity, indicating a marked increase in transcriptional activation of NF-κB.

Similar results were observed in endothelial cells transfected with pAP1-Luc (Figure 1B). Among the fatty acids tested, linoleic acid stimulated AP-1 transcriptional activation most markedly compared with control cultures. In contrast, linolenic acid exerted more moderate effects, and oleic acid did not significantly affect luciferase expression.

Unsaturated fatty acids selectively induce the genes encoding for MCP-1 and TNF-α

The effects of treatment with selected unsaturated fatty acids on MCP-1 mRNA levels are shown in **Figure 2**. Among the tested fatty acids, linoleic acid at the concentration of 90 μmol/L stimulated the most pronounced induction of the MCP-1 gene (51 ± 1.97% above the control values as measured by the density of the fluorescent bands). Indeed, MCP-1 mRNA levels in endothelial cells treated with 90 μmol linoleic acid/L for 3 h

were in the range observed in cells exposed to 20 μg TNF-α/L, which was used as a positive control. MCP-1 mRNA levels also increased in endothelial cells treated with 60 and 90 μmol linolenic acid/L (by 24 ± 2.46% and 30 ± 5.25%, respectively). In contrast, induction of the MCP-1 gene in endothelial cells exposed to oleic acid was approximately at the range observed in unstimulated endothelial cells.

The effects of treatment with selected unsaturated fatty acids on TNF-α mRNA levels are also shown in Figure 2. Similarly to the results for MCP-1 gene induction, treatment of HUVECs with linoleic acid markedly induced TNF-α mRNA levels (21 ± 3.22% above control values). In addition, linolenic acid at the dose of 90 μmol/L stimulated similar induction of the TNF-α gene. Independent of the dose used, treatment with oleic acid did not significantly affect TNF-α mRNA levels in cultured HUVECs.

Unsaturated fatty acids selectively induce the genes encoding for adhesion molecules

The effects of treatment with selected unsaturated fatty acids on ICAM-1 mRNA levels is shown in Figure 2. Exposure to both linoleic acid and linolenic acid induced similar dose-dependent increases in ICAM-1 mRNA levels. Specifically, linoleic and linolenic acids at the concentration of 90 μmol/L stimulated
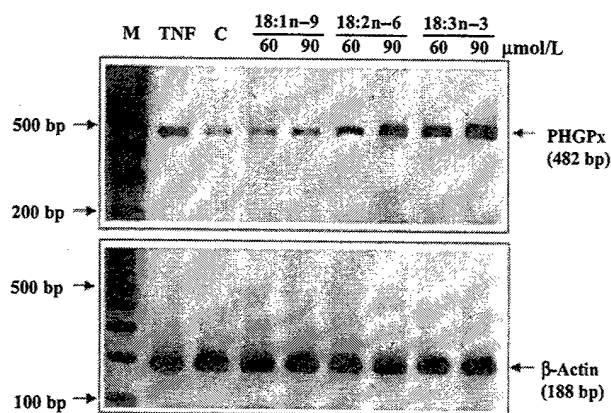
FIGURE 3. Effects of dietary fatty acids on phospholipid hydroperoxide glutathione peroxidase (PHGPx) mRNA levels in human endothelial cells as measured by reverse transcriptase–polymerase chain reaction (RT-PCR). Human umbilical vein endothelial cells were exposed to specific unsaturated fatty acids for 24 h. RT-PCR was performed as described in the legend to Figure 2. bp, base pair; M, marker; TNF, tumor necrosis factor α; C, control.

induction of the ICAM-1 gene by 32 ± 2.54% and 30 ± 3.34%, respectively. In contrast, exposure of HUVECs to oleic acid decreased ICAM-1 mRNA levels to ≈50% of control values.

The effects of specific unsaturated fatty acids on VCAM-1 mRNA levels in HUVEC are also shown in Figure 2. The most significant induction of the VCAM-1 gene (by 38 ± 2.20%) was observed in cells treated with 90 μmol linoleic acid/L. In addition, exposure to 90 μmol linolenic acid/L resulted in a slight increase in VCAM-1 mRNA levels (14 ± 1.88%). Treatment with oleic acid had no significant effect on VCAM-1 gene induction compared with control cultures.

Unsaturated fatty acids induce the gene encoding for PHGPx

The effects of selected fatty acids on PHGPx mRNA levels in HUVECs are shown in **Figure 3**. Compared with the control, treatment with oleic acid increased PHGPx mRNA levels by ≈30%. However, both linoleic and linolenic acids strongly, and dose dependently, stimulated induction of PHGPx gene in HUVECs. In fact, treatment with 60 and 90 μmol linoleic acid/L enhanced PHGPx mRNA levels by 60 ± 7.58% and 104 ± 5.04%, respectively. Furthermore, exposure to 60 and 90 μmol linolenic acid/L increased the induction of the PHGPx gene by 108 ± 6.48% and 121 ± 4.36%, respectively.

DISCUSSION

Mono- and polyunsaturated 18-carbon fatty acids provide a unique model for studying the cellular effects of fatty acids that differ in unsaturation independent of carbon length (9). In addition, the unsaturated fatty acids used in the present study are major dietary fatty acids. Endothelial cells were exposed to fatty acids at concentrations of 60 or 90 μmol/L, with an albumin concentration in the experimental media of 60 μmol/L. Normal plasma fatty acid concentrations can range from ≈90 to 1200 μmol/L; however, most fatty acids are bound to plasma components, mostly albumin (32, 33). In fact, the main factor in the availability of fatty acids for cellular uptake is determined by the ratio of fatty acids to albumin. Normally, this ratio can range from 0.15 to 4 under various conditions, with an average of ≈1 (32, 33). Thus, the experimental conditions used in the present study, which resulted in a ratio of fatty acids to albumin of 1 or 1.5, were within the physiologic range.

One of the most important functions of the vascular endothelium is to regulate inflammatory reactions (1). The development of inflammatory reactions is a normal defense mechanism in response to injury or activation of the vessel wall. The physiologic significance of such reactions is to maintain and repair the normal structure and function of the vessel wall. However, excessive inflammatory reactions with the development of a positive feedback inflammatory cycle can lead to severe tissue damage and are associated with vascular pathology, including the development of atherosclerotic plaques (34).

Induction of genes encoding for mediators of the inflammatory response, ie, inflammatory cytokines, chemokines, and adhesion molecules, can initiate leukocyte infiltration of the vessel wall. These mediators of the inflammatory response interact closely with each other in vivo. For example, ICAM-1 and VCAM-1 facilitate leukocyte adhesion to the vascular endothelium and both MCP-1 (35) and, to a lesser extent, TNF-α (36, 37) are potent chemoattractant factors that play a significant role in recruiting lymphocytes and monocytes into the vessel wall. In addition, TNF-α is a strong inducer of inflammatory reactions and can stimulate overexpression of MCP-1, inflammatory cytokines, and the adhesion molecules ICAM-1 and VCAM-1 (38). In fact, these strong proinflammatory properties of TNF-α were the reason that this cytokine was used as the positive control in our present study. In addition, the inflammatory genes examined in the present study, ie, those encoding for VCAM-1, ICAM-1, TNF-α, and MCP-1, are regulated by similar transcription factors, with dominant roles of NF-κB and AP-1 (22–25).

The importance of NF-κB and AP-1 in the induction of inflammatory reactions prompted us to study the effects of specific fatty acids on the transcriptional activity of these transcription factors in human endothelial cells. Among the unsaturated fatty acids studied, linoleic acid induced both NF-κB and AP-1 transcriptional activation most markedly. These data agree with our previous results in which our use of an electrophoretic mobility shift assay showed a marked activation of NF-κB (13) and AP-1 (39) in endothelial cells exposed to linoleic acid. It is possible that fatty acid–induced endothelial cell oxidative stress and disturbances in the glutathione redox status are responsible for the activation of these oxidative stress–responsive transcription factors. Intercellular glutathione is the major nonprotein thiol compound that regulates the cellular redox status. Depletion of glutathione concentrations and alterations in the equilibrium between the reduced and oxidized derivatives of glutathione can stimulate activation of NF-κB (40). To support this notion, we showed that exposure of endothelial cells to unsaturated fatty acids can result in a marked decrease in cellular glutathione concentrations and activation of NF-κB (9, 13). In addition, the glutathione precursor N-acetylcysteine prevented fatty acid–induced activation of NF-κB (41).

Glutathione peroxidases are a family of antioxidant enzymes that utilize glutathione in the reduction of hydrogen peroxide and alkyl hydroperoxides. Among the various glutathione peroxidases, PHGPx plays a unique role. In addition to reducing hydrogen peroxide and soluble hydroperoxides, PHGPx is the only antioxidant enzyme that can reduce hydroperoxy fatty acids that are integrated in cellular membranes (42) or lipoproteins (43).

PHGPx was also shown to be involved in silencing activities of cyclooxygenase or 5- and 15-lipoxygenases (44, 45), enzymes involved in the metabolism of unsaturated fatty acids. Results of the present study showed that exposure of endothelial cells to specific unsaturated fatty acids can markedly stimulate induction of PHGPx mRNA. In addition, the fatty acid–stimulated increases in PHGPx mRNA levels appeared to be correlated with the amount of unsaturated bonds in fatty acid molecules. For example, linolenic acid, followed by linoleic acid, enhanced induction of the PHGPx gene most markedly.

The present study provides compelling evidence that linoleic acid can induce profound inflammatory responses in cultured human endothelial cells. In fact, among all the unsaturated fatty acids studied, linoleic acid stimulated induction of inflammatory gene mRNA most markedly. Because expression of the inflammatory genes is regulated primarily by NF-κB and AP-1, a strong induction of NF-κB and AP-1 transcriptional activation by linoleic acid may explain the marked induction of the studied genes. In addition, not only linoleic acid but also specific oxidative products of this fatty acid can exert proinflammatory effects (46, 47). However, we observed that the lipoxygenase metabolites of linoleic acid, such as 13-hydroperoxyoctadecadienoic acid (13-HPODE) or 13-hydroxyoctadecadienoic acid (13-HODE), induce a different pattern of inflammatory responses in endothelial cells than does free linoleic acid. Specifically, exposure of HUVECs to 13-HPODE or 13-HODE does not induce the expression of VCAM-1 or E-selectin (48). In addition, polyunsaturated fatty acids, such as linoleic acid, can be nonenzymatically converted to 4-hydroxynonenal. However, exposure of HUVECs to 4-hydroxynonenal markedly stimulates apoptosis of vascular endothelial cells but does not result in activation of NF-κB or induction of adhesion molecules (49). Thus, even though linoleic acid can be converted to oxidized metabolites, it appears unlikely that 13-HPODE, 13-HODE, or 4-hydroxynonenal can contribute significantly to inflammatory reactions induced by this fatty acid. On the other hand, the effects of other metabolites of polyunsaturated fatty acids, eg, derivatives of the cytochrome P450 pathway, on inflammatory reactions in human endothelial cells remain to be determined. Our recent data suggest that epoxide metabolites of linoleic acid may have proinflammatory properties (50).

Although our data clearly indicate that specific unsaturated fatty acids can induce proinflammatory effects in endothelial cells, opposite results were reported when cells were exposed to selected n−3 or n−6 fatty acids for ≤72 h and coexposed to inflammatory cytokines, such as interleukin 1β (IL-1β) or TNF-α, for an additional 12 h. When such experimental approaches were used, preexposure to fatty acids inhibited cytokine-induced expression of inflammatory mediators, such as VCAM-1, on the surface of endothelial cells (51). Similar inhibition of ICAM-1 expression was also observed in cells pretreated with 13-HPODE before stimulation with IL-1β. However, simultaneous administration of 13-HPODE with IL-1β or TNF-α resulted in additive effects on ICAM-1 production (48). We showed that preexposure of endothelial cells to linoleic acid can cross-amplify TNF-α–mediated induction of cellular oxidative stress and endothelial cell dysfunction (13) but does not potentiate or even inhibit NF-κB–dependent transcription (13, 41). To explain this phenomenon, it was proposed that fatty acid–induced activation of NF-κB could lead to increased numbers of NF-κB inhibitory subunits, which, in turn, could prevent further activation of this transcription factor in cells exposed to cytokines at later time points (13).

In contrast with linoleic and linolenic acids, which exerted strong or moderate proinflammatory responses, respectively, oleic acid diminished inflammatory gene mRNA levels in endothelial cells. These data agree with previous reports on antioxidant effects mediated by oleic acid. For example, a diet enriched in oleic acid markedly decreases LDL susceptibility to oxidation and LDL-protein modification in mildly hypercholesterolemic patients (10). Similar results were obtained in experimental animals fed a diet enriched in oleic acid (52). Extensive evidence also indicates the protective and antioxidant effects of oleic acid on endothelial cell activation. Cellular treatment with this fatty acid protects endothelial cells against cytokine-induced VCAM-1, ICAM-1, or E-selectin overexpression (53). In addition, supplementation with oleic acid protects endothelial cells against hydrogen peroxide–induced cytotoxicity (16) and against dysfunction of the endothelial barrier as mediated by oxidized LDL (54).

In conclusion, the present study showed that specific unsaturated dietary fatty acids can induce highly individual effects on endothelial cell activation and contribute differently to induction of the inflammatory genes in vascular endothelial cells. Among the fatty acids studied, linoleic acid stimulated inflammatory gene mRNA most markedly. In contrast, oleic acid appeared to silence the induction of various proinflammatory genes in endothelial cells. These results showed that specific unsaturated dietary fatty acids, such as linoleic acid and to a lesser extent linolenic acid, can stimulate the development of proinflammatory environments within the vascular endothelium. ☙

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Links between food and vascular disease[1,2]

John C Rutledge

The article by Toborek et al (1) in this issue of the Journal is this group's next logical step in a theme targeting the actions of ingested lipids on the vascular system (2, 3). Over the past decade, these investigators have contributed a substantial body of literature investigating the dietary links to vascular physiology and pathophysiology. The current article reports that specific unsaturated dietary fatty acids, particularly linoleic acid, can stimulate a proinflammatory environment within the vascular endothelium. For many years this concept has remained elusive in the context of how a diet-vascular interaction could occur in this complex system. The authors have pursued this concept in a systematic and logically progressive fashion. Their work and this article specifically show that specific lipids can, without any modification, perturb vascular endothelial cells and promote a proinflammatory environment (4, 5). This finding separates this study from much previous work indicating that lipids must be modified to stimulate and activate endothelial cells.

These studies provide part of the foundation for future studies of diet-vascular interactions in more complex organ systems and whole organisms, including humans. These future studies are essential for determining the pathophysiologic relevance of the present experiments performed in cell culture. Additionally, basic mechanistic studies are needed to determine the exact sequence in which certain ingested lipids activate endothelial cells (6) and the modalities and therapies by which the process of endothelial cell activation can be prevented or attenuated (7). Additional interesting experiments may determine the mechanisms by which endothelial cell activation is amplified by specific lipids, such as linoleic acid.

It is clear that multiple mechanisms exist by which the vascular endothelium can be activated and the vascular wall injured. The studies by Hennig et al (2) show that specific lipids, ie, fatty acids, can injure vascular endothelium without modification. Thus, it is clear that our reliance on measuring classic blood lipid indexes, such as total cholesterol, triacylglycerol, HDL cholesterol, and LDL cholesterol, remains rudimentary. Future analysis may include more comprehensive dietary phenotyping and plasma lipid composition, including specific phospholipids and fatty acids. Although the authors have identified a specific culprit in the activation of the vascular endothelium, many more of these "bad actors" may exist in the diet. In the coming years, our major challenge will be to identify additional pathogenic lipids and other blood components that activate endothelial cells, investigate their mechanisms, and develop treatment regimens to prevent the development of vascular disease, the most costly disease in our country in terms of both lives lost and health care dollars.

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Some unsaturated fats promote inflammation: study

By Suzanne Rostler

NEW YORK, Jan 03 (Reuters Health) - A type of unsaturated fatty acid found in corn and safflower oil appears to activate genes that promote inflammation inside blood vessels, a risk factor for heart disease, new research suggests.

This fatty acid, known as linoleic or omega-6 fatty acid, also appeared to reduce cells' ability to protect themselves from oxidative damage. Oxidative damage is caused by free radicals, byproducts of the body's normal processes that can damage body tissues.

While unsaturated fatty acids in general are thought to be healthier than saturated fat, the findings support previous research showing that omega-6 fatty acid can injure cells lining the insides of blood vessels, leading to inflammation.

"If people consume high amounts of antioxidants, unsaturated fats could be healthier than saturated fats," Dr. Michal Toborek of the University of Kentucky in Lexington, a study author, told Reuters Health. He stressed that other types of unsaturated fatty acids have been shown to protect against heart disease.

"The real problem is that people eat high-fat diets, which are rich in omega-6 fatty acids, without balancing such diets with antioxidants," compounds that neutralize free radicals, Toborek added.

To investigate how certain fatty acids lead to inflammation, the researchers exposed human cells that line the insides of blood vessels, making up the endothelium, to several different unsaturated fatty acids in a test tube. Omega-6 fatty acid substantially increased the activity of two proteins that triggered an inflammatory gene, while linolenic (omega-3) acid, found in fish oil, had only a moderate effect on these proteins.

Both omega-3 and omega-6 stimulated the formation of a protein blocking an enzyme that normally protects against free radicals and allows cells to defend themselves against oxidative stress.

Oleic acid, the predominant fatty acid in olive oil, either had no effect or helped block processes leading to inflammation, the researchers report in the January issue of the American Journal of Clinical Nutrition.
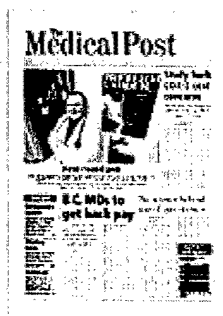
"Specific unsaturated dietary fatty acids can induce highly individual effects on endothelial cell activation and contribute differently to induction of the inflammatory genes in vascular endothelial cells," the study concludes.

In an interview, Toborek said that more research into the health effects of different types of fat is sorely needed.

"This is an important issue because more and more Americans consume fast and convenient foods, which contain high amounts of omega-6 fatty acids," he said.

SOURCE: American Journal of Clinical Nutrition 2002;75:119-125.

Medical Post

NUTRITION

Linoleic acids may inflame endothelial cells

By Nancy Deutsch

LEXINGTON, KY. – Contrary to popular belief, not all unsaturated fatty acids are healthier than their saturated counterparts.

Unsaturated fatty acids have been touted as healthier for their ability to protect against heart disease by decreasing plasma cholesterol, but select types may do more harm than good, a new study suggests.

Lead researcher Dr. Michal Toborek of the University of Kentucky, found one specific dietary unsaturated fatty acid known as linoleic acid can initiate oxidative stress and inflammation in human endothelial cells.

"The real problem is people eat high-fat diets, which are rich in linoleic acid, without balancing such diets with antioxidants," he said.

Researchers exposed human umbilical vein endothelial cells to dietary mono- and polyunsaturated 18-carbon fatty acids.

Linoleic acid, most commonly found in corn oil, was found to be the most likely to stimulate the development of a pro-inflammatory environment within blood vessels.

The study was undertaken because the specific effects of fatty acids on inflammatory responses in endothelial cells is not well understood, and some previous research had indicated linoleic acid could disrupt the integrity of vascular endothelial cells, according to Dr. Toborek.

The authors plan further studies.

"This is an important issue, because more and more Americans consume fast and convenient foods which contain high amounts of linoleic acid," Dr. Toborek sais.

This study appeared in the *American Journal of Clinical Nutrition.*

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Latest News

Some Unsaturated Fats Promote Inflammation
Excerpt By Suzanne Rostler, Reuters Health

NEW YORK (Reuters Health) - A type of unsaturated fatty acid found in corn and safflower oil appears to activate genes that promote inflammation inside blood vessels, a risk factor for heart disease, new research suggests.

This fatty acid, known as linoleic or omega-6 fatty acid, also appeared to reduce cells' ability to protect themselves from oxidative damage. Oxidative damage is caused by free radicals, byproducts of the body's normal processes that can damage body tissues.

While unsaturated fatty acids in general are thought to be healthier than saturated fat, the findings support previous research showing that omega-6 fatty acid can injure cells lining the insides of blood vessels, leading to inflammation.

``If people consume high amounts of antioxidants, unsaturated fats could be healthier than saturated fats," Dr. Michal Toborek of the University of Kentucky in Lexington, a study author, told Reuters Health. He stressed that other types of unsaturated fatty acids have been shown to protect against heart disease.

``The real problem is that people eat high-fat diets, which are rich in omega-6 fatty acids, without balancing such diets with antioxidants," compounds that neutralize free radicals, Toborek added.

To investigate how certain fatty acids lead to inflammation, the researchers exposed human cells that line the insides of blood vessels, making up the endothelium, to several different unsaturated fatty acids in a test tube. Omega-6 fatty acid substantially increased the activity of two proteins that triggered an inflammatory gene, while linolenic (omega-3) acid, found in fish oil, had only a moderate effect on these proteins.

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different types of fat is sorely needed.

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SOURCE: American Journal of Clinical Nutrition 2002;75:119-125.

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Omega 6 sería uno de los responsables

Algunas grasas insaturadas aumentarían riesgo de infarto

El ácido linoleico también parece reducir la capacidad de las células de protegerse a sí mismas del daño oxidativo.

El ácido linoleico, también conocido como Omega 6 y que se encuentra en el aceite de maíz y de maravilla, podría activar genes que promueven la inflamación de vasos sanguíneos, aumentando el riesgo de enfermedades cardíacas.

La investigación, realizada por investigadores de la Universidad de Kentucky en Lexington (EE.UU.), indica que dicho ácido graso insaturado también parece reducir la capacidad de las células de protegerse a sí mismas del daño oxidativo, proceso responsable del deterioro de los tejidos del cuerpo.

El doctor Michal Toborek, autor del estudio, indicó que las personas que consumen dietas ricas en Omega 6 pueden balancear sus efectos con alimentos que contengan antioxidantes, como cítricos y verduras.
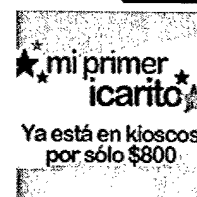
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Linoleic Acid-Induced VCAM-1 Expression in Human Microvascular Endothelial Cells Is Mediated by the NF-κB-Dependent Pathway

Hyen Joo Park, Yong Woo Lee, Bernhard Hennig, and Michal Toborek

Abstract: Vascular cell adhesion molecule-1 (VCAM-1) has been reported to play an important role in cancer metastasis via the adhesive interaction between tumor cells and endothelial cells. In this study, we examined the effects of linoleic acid on VCAM-1 expression and its transcriptional regulatory mechanism in human microvascular endothelial cells (HMEC-1). Time- and dose-dependent increases of VCAM-1 mRNA levels were observed in linoleic acid-treated HMEC-1 as detected by reverse transcriptase-polymerase chain reaction. Flow cytometry analysis showed a significant and dose-dependent upregulation of VCAM-1 expression in HMEC-1 stimulated with linoleic acid compared with controls. To clarify the transcriptional regulatory pathway, we investigated the role of nuclear factor-κB (NF-κB) in the expression of VCAM-1 by linoleic acid in HMEC-1. Nuclear extracts from HMEC-1 stimulated with linoleic acid showed a dose-dependent increase in binding activity to the NF-κB consensus sequences. These effects were preventable by cotreatment with inhibitors of NF-κB activity, such as sodium salicylate, aspirin, or pyrrolidine dithiocarbamate. In addition, pretreatment with NF-κB inhibitors markedly suppressed the ability of linoleic acid to induce VCAM-1 gene expression. The role of NF-κB in linoleic acid-induced VCAM-1 expression was confirmed by functional promoter studies in HMEC-1 transfected with reporter constructs of the VCAM-1 promoter with or without mutated NF-κB binding site. These results indicate that linoleic acid upregulates VCAM-1 expression in HMEC-1 through the NF-κB-dependent pathway.

Introduction

Dietary fat is considered to be one of the main risk factors of carcinogenesis. For example, a positive correlation was reported between dietary fat intake and increased risks for the development of breast, colon, and prostate cancers (1–3). However, the role of dietary fat in the development of human breast cancer has recently been questioned. Although data obtained from animal studies (reviewed in Ref. 4), international correlation analyses (5,6), and meta-analysis of dietary fat intervention studies (7) strongly indicate the association between fat consumption and the development of breast cancer, data from prospective cohort studies on dietary fat and breast cancer (8–10) suggested that dietary fat might not be a risk factor for human breast cancer. Among different dietary fatty acids, it appears that linoleic acid (C18:2, n-6) can promote carcinogenesis (11–14). 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 (15).

The formation of blood-borne metastasis is a complex process that requires several steps. However, a growing body of evidence indicates that the direct adhesive interaction between tumor cells and endothelial cells is the critical event in metastasis formation (16,17). This process requires the binding of tumor cells to specific adhesion molecules on the surface of endothelial cells followed by migration of tumor cells through the endothelium into underlying tissues (16). Evidence indicates that, among several adhesion molecules that can be involved in this process, vascular cell adhesion molecule-1 (VCAM-1) may play one of the most important roles. For example, it was demonstrated that VCAM-1 facilitated adhesion of metastatic breast tumor cells to endothelial cells stimulated by shear stress (18). In patients with breast cancer or gastric cancers, serum levels of soluble VCAM-1 were closely correlated with disease progression (19,20). Upregulation of VCAM-1 was also shown to be involved in adhesion of RAW117 lymphoma cells (21) or melanoma cells to hepatic sinusoidal endothelial cells (22). In addition, evidence indicated the role of VCAM-1 in adhesion of B9/BM1 cells to bone marrow-derived endothelial cells (23) and adhesion of DU145 cells (the cell line derived from cerebral metastasis of prostate carcinoma) to human brain microvascular endothelial cells (24).

VCAM-1 is a 110-kDa member of the immunoglobulin gene superfamily first described as a cytokine-inducible endothelial adhesion protein (25). It facilitates tumor cell adhesion through binding of an integrin counterreceptor, very

late antigen-4 (26). Functional studies on the activity of the VCAM-1 gene promoter have shown that regulation of VCAM-1 gene expression in endothelial cells appears to be complex and related to the type of stimuli. For example, VCAM-1 induction by inflammatory cytokines, such as interleukin (IL)-1β or tumor necrosis factor-α (TNF-α), as well as by lipopolysaccharide (LPS), strongly relies on activation of nuclear transcription factor-κB (NF-κB) (27,28). In contrast, recent evidence indicated that IL-4-induced VCAM-1 expression is independent of NF-κB activation (29,30). Thus the specific role of NF-κB in linoleic acid-induced overexpression of the VCAM-1 gene is uncertain and was chosen as the subject of the present study.

Because of the significance of dietary linoleic acid and VCAM-1 expression in cancer metastasis, the aim of the present study was to investigate the molecular signaling pathways involved in linoleic acid-induced VCAM-1 upregulation in human microvascular endothelial cells. We have determined that linoleic acid can induce VCAM-1 expression at the mRNA and protein levels. Furthermore, we provide evidence that linoleic acid-stimulated expression of the VCAM-1 gene is mediated by activation of NF-κB.

Materials and Methods

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 medium (Sigma, St. Louis, MO) enriched with 10% fetal bovine serum, 1% penicillin-streptomycin, 1 μg/ml hydrocortisone, and 0.01 μg/ml epidermal growth factor in a 5% CO_2 atmosphere at 37°C. Linoleic acid (>99% pure; Nu-Chek Prep, Elysian, MN) was added to the medium as described previously (31). In the present study, linoleic acid was used at ≤50 μM, i.e., levels that do not induce cytotoxic effects in vascular endothelial cells (32).

In selected experiments, HMEC-1 were pretreated for 1 h with salicylates (aspirin or sodium salicylate) or for 30 min with pyrrolidine dithiocarbamate (PDTC). Salicylates were used at ≤10 mM and PDTC at ≤25 μM.

Electrophoretic Mobility Shift Assay

Nuclear extracts from HMEC-1 were prepared according to the method of Beg et al. (33). Binding reactions were performed in a 20-μl volume containing 4–10 μg of nuclear protein extracts, 10 mM Tris·HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, 2 μg of poly(dI-dC) (nonspecific competitor), and 40,000 cpm of ^{32}P-labeled specific oligonucleotides that contained the consensus sequence for NF-κB site (5′-AGTTGAGGGGACTTTCCCAGG-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 stud-

ies 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.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted by the use of TRIreagent (Sigma) and reverse-transcribed at 42°C for 60 min in 20 μl of 5 mM $MgCl_2$, 10 mM Tris·HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1 mM dNTP, 1 U/μl of recombinant RNasin ribonuclease inhibitor, 15 U/μg of avian myeloblastosis virus reverse transcriptase (RT), and 0.5 μg of oligo(dT)$_{15}$ primer. For amplification of VCAM-1 and of β-actin (a housekeeping gene), the following primer combinations were used: 5′-ATGACATGCTTGAGCCAGG-3′ and 5′-GTGTCTCCTTCTTTGACACT-3′ (VCAM-1; expecting 260-bp fragment) (34) and 5′-AGCACAATGAAGATCAAGAT-3′ and 5′-TGTAACGCAACTAAGTCATA-3′ (β-actin; expecting 188-bp fragment) (35). The polymerase chain reaction (PCR) mixture consisted of a *Taq* PCR Master Mix Kit (Qiagen, Valencia, CA), 2 μl of the RT reaction, and 20 pmol of primer pairs in a total volume of 50 μl. 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 15–30 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).

Flow Cytometry for VCAM-1 Determination

VCAM-1 protein expression was determined by flow cytometry. Briefly, HMEC-1 were grown to confluence on six-well culture plates and treated with linoleic acid for 12 h. HMEC-1 were then washed with Hanks' buffer and gently harvested by trypsin-EDTA. Cells were washed twice with phosphate-buffered saline (PBS) and incubated for 1 h on ice with saturating amounts of monoclonal mouse anti-human VCAM-1 antibody labeled with fluorescein isothiocyanate (R & D Systems, Minneapolis, MN). Fluorescein isothiocyanate-labeled mouse anti-human IgG1 was used as the isotype control (R & D Systems). After incubation with antibodies, samples were washed twice with ice-cold PBS and analyzed with 10,000 cells/sample in a fluorescence-activated cell sorter (Becton Dickinson, San Jose, CA). After correction for unspecific binding (isotype control), specific mean fluorescence intensity was expressed as the indicator of VCAM-1 protein expression.

Transient Transfection and Reporter Gene Assays

Transient transfections of HMEC-1 were performed using pFx-7 (Invitrogen, Carlsbad, CA) as described earlier (36). Cells were transfected with 10 μg of the VCAM-1 pro-

moter constructs with or without mutated NF-κB site (pF3-mNF-κB-CAT3 and pF3-CAT3, respectively) and cotransfected with 4 μg of the pGL3-Luc control vector (Promega, Madison, WI) to normalize transfection rates. The reporter constructs, pF3-mNF-κB-CAT3 and pF3-CAT3, were kind gifts from Dr. Andrew S. Neish (Emory University School of Medicine). Generation of these constructs was described and characterized earlier (27,37). After transfection, cultures were maintained in normal growth medium for 24 h and then exposed to 50 μM linoleic acid for an additional 24 h in MCDB 131 medium enriched with 5% fetal bovine serum. After treatment exposure, cells were washed twice with PBS and lysed in 100 μl of reporter lysis buffer (Promega). Chloramphenicol acetyltransferase (CAT) activity was determined using the method of Gorman et al. (38). The cell lysates, normalized for protein levels, were incubated for 4 h at 37°C with a reaction mixture composed of 125 mM Tris·HCl (pH 7.8), 0.83 mM acetyl coenzyme A, and 3 μl of [^{14}C]chloramphenicol (25 μCi/ml; Amersham Pharmacia Biotech, Piscataway, NJ). Then acetylated and nonacetylated forms of chloramphenicol were extracted with ethyl acetate and separated by thin-layer chromatography using the solvent system with chloroform-methanol (95:5, vol/vol). After autoradiography, the zones corresponding to acetylated or nonacetylated chloramphenicol were cut from the plates, and radioactivity was counted in a liquid scintillation counter for quantitation of CAT activity. The CAT activity was normalized according to luciferase activity, which was determined using the Luciferase Assay System (Promega) according to the manufacturer's protocol.

Statistical Analysis

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

Results

Linoleic Acid Activates NF-κB in HMEC-1

To determine whether linoleic acid can activate NF-κB in HMEC-1, cells were exposed to this fatty acid for 2 h, and NF-κB binding was analyzed by electrophoretic mobility shift assay (EMSA) using nuclear extracts of the treated cells. Results of these experiments are shown in Fig. 1, A and B. Figure 1A depicts the effects of linoleic acid on the binding activity of NF-κB in HMEC-1. A slight endogenous activity of NF-κB was observed in control cultures (Fig. 1A, Lane 2). However, when the cells were stimulated with 50 μM linoleic acid, a marked increase in NF-κB binding activity was detected. This binding was specifically inhibited by an unlabeled competitor DNA containing the consensus NF-
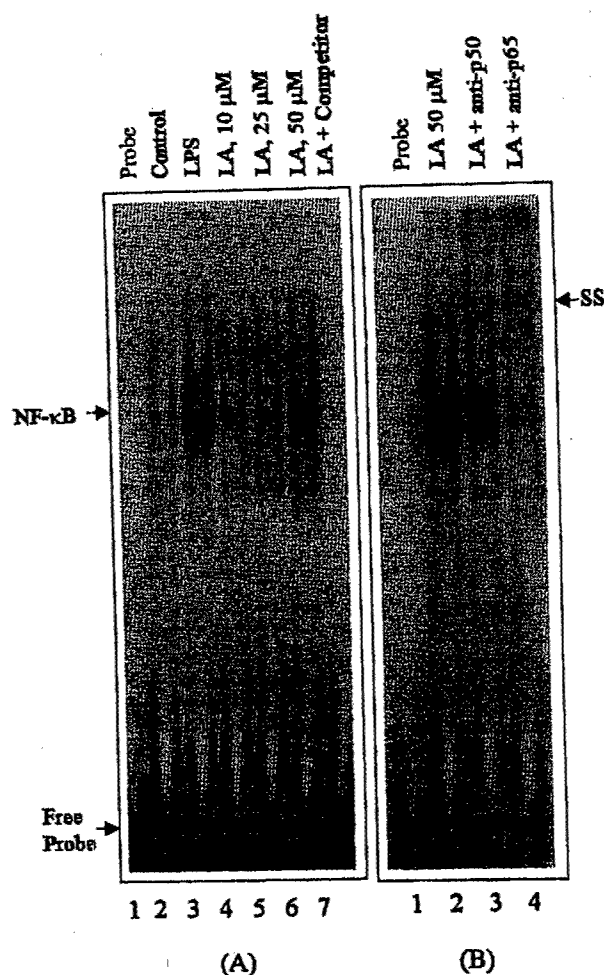

Figure 1. A: treatment with 50 μM linoleic acid (LA) enhances nuclear factor-κB (NF-κB) binding in human microvascular endothelial cells (HMEC-1) as analyzed by electrophoretic mobility shift assay. HMEC-1 were untreated (Lane 2) or treated for 2 h with increasing doses of linoleic acid (Lanes 4–6). Competition study was performed by addition of excess unlabeled oligonucleotide (Lane 7) using nuclear extracts from cells treated with 50 μM linoleic acid. Lane 1, probe alone; Lane 3, lipopolysaccharide (LPS, 1 μg/ml, positive control). B: supershift analysis of LA-induced NF-κB binding activity in HMEC-1. Nuclear extracts were prepared from cells treated with 50 μM linoleic acid for 2 h (Lanes 2–4) and incubated with anti-p50 antibody (Lane 3) or anti-p65 antibody (Lane 4) for 25 min before addition of ^{33}P-labeled probe. Lane 1, probe alone. SS, bands shifted by specific antibodies.

κB sequence (Fig. 1A, Lane 7). In addition, the identity of NF-κB binding was confirmed by supershift experiments with antibodies against specific NF-κB subunits, p50 and p65 (Fig. 1B, Lanes 3 and 4).

To further study linoleic acid-induced activation of NF-κB, HMEC-1 were pretreated for 1 h with aspirin or sodium salicylate or for 30 min with PDTC before a coexposure to linoleic acid for 2 h. Both salicylates and PDTC are widely used as inhibitors of NF-κB activation. Pretreatment with aspirin (Fig. 2A), sodium salicylate (Fig. 2B), or PDTC (Fig. 2C) resulted in dose-dependent inhibitions of NF-κB activation in HMEC-1 exposed to linoleic acid.
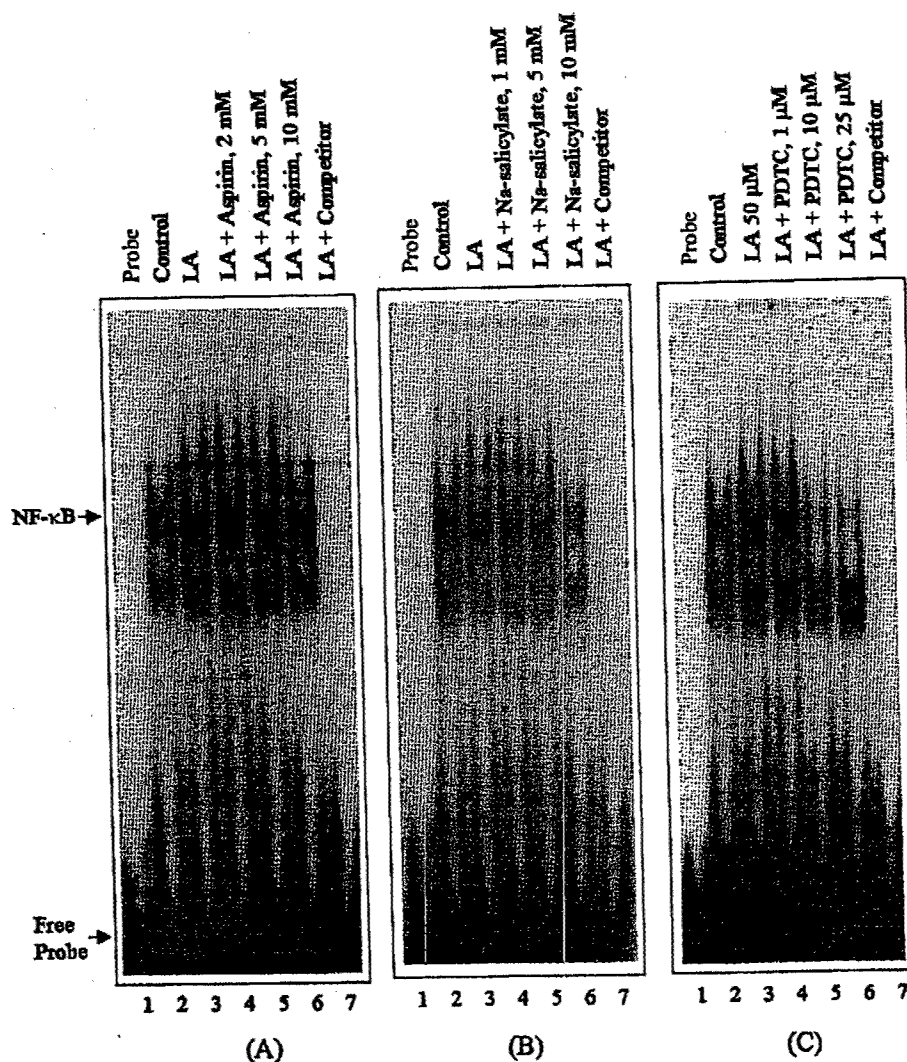
Figure 2. Pretreatment with aspirin, sodium salicylate, or pyrrolidine dithiocarbamate (PDTC) blocks linoleic acid-induced NF-κB DNA-binding activity in HMEC-1 as measured by electrophoretic mobility shift assay. HMEC-1 were pretreated for 1 h with indicated concentrations of aspirin (A) or sodium salicylate (B) or for 30 min with PDTC (C) before 2 h of treatment with 50 μM linoleic acid (Lanes 4–6). Lane 1, probe alone; Lane 3, 50 μM linoleic acid alone; Lane 7, competition study performed by addition of excess unlabeled oligonucleotide using nuclear extract from cells treated with 50 μM linoleic acid.

Linoleic Acid Induces VCAM-1 Expression in HMEC-1

To investigate whether exposure to linoleic acid can induce expression of VCAM-1 in microvascular endothelial cells, HMEC-1 were treated with 50 μM linoleic acid for increasing time periods, and the VCAM-1 mRNA level was determined by semiquantitative RT-PCR. As shown in Fig. 3, low levels of constitutively expressed VCAM-1 mRNA were detected in control cells (no linoleic acid supplementation). On the other hand, mRNA transcripts for VCAM-1 were clearly increased in linoleic acid-treated cells. Upregulation of VCAM-1 expression was observed after 1 h of exposure to linoleic acid, reaching maximal levels at 4 h (Fig. 3A).

Figure 3B shows that 4 h of exposure of HMEC-1 to linoleic acid resulted in a dose-dependent increase in the VCAM-1 mRNA. The most marked VCAM-1 expression was observed in HMEC-1 cultures treated with 50 μM linoleic acid. Additional increase in linoleic acid concentration did not further potentiate VCAM-1 expression (data not shown).

Figure 4 indicates the effects of increasing concentrations of linoleic acid treatment on VCAM-1 protein expression as measured by flow cytometry. In agreement with RT-PCR data, VCAM-1 protein was constitutively expressed in untreated HMEC-1. However, in cells treated with linoleic acid for 12 h, expression of this adhesion molecule was markedly upregulated in a dose-dependent manner.

Linoleic Acid-Induced VCAM-1 Expression in HMEC-1 Is Mediated by NF-κB

To determine whether linoleic acid-mediated activation of NF-κB is involved in upregulation of VCAM-1, expression of the VCAM-1 gene was studied in HMEC-1 pre-
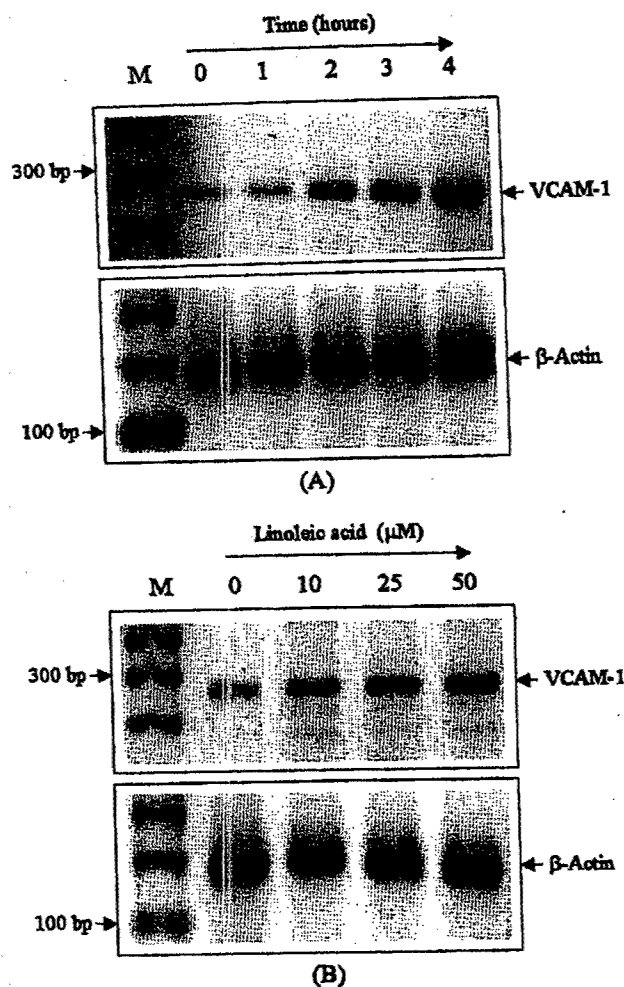
Figure 3. Time- and dose-dependent upregulation of vascular cell adhesion molecule-1 (VCAM-1) mRNA expression in HMEC-1 induced by linoleic acid as measured by reverse transcriptase-polymerase chain reaction (RT-PCR). HMEC-1 were exposed to 50 µM linoleic acid for indicated period of time (A) or treated with increasing concentrations of linoleic acid for 4 h (B). PCR products were analyzed by 2% agarose gel electrophoresis and visualized using phosphoimaging. Predicted sizes of RT-PCR products for VCAM-1 and β-actin are 260 and 188 bp, respectively. M, molecular weight markers (100-bp DNA ladder).

Figure 4. Linoleic acid increases VCAM-1 protein expression in HMEC-1 as measured by flow cytometry. HMEC-1 were exposed to increasing concentrations of linoleic acid for 12 h. Values are means ± SD expressed as relative fluorescence intensity and corrected for unspecific binding. *, Significantly different from untreated control ($P < 0.05$).

treated with different doses of NF-κB inhibitors and exposed to 50 µM linoleic acid for 4 h. Similar to our studies presented in Fig. 2, aspirin, sodium salicylate, and PDTC were employed to inhibit NF-κB. Effects of these NF-κB inhibitors on linoleic acid-induced overexpression of the VCAM-1 gene are reflected in Fig. 5. As indicated, 1 h of pretreatment with increasing doses of aspirin (Fig. 5A) or sodium salicylate (Fig. 5B), as well as 30 min of treatment with PDTC (Fig. 5C), markedly and in a dose-dependent manner decreased linoleic acid-mediated stimulation of the VCAM-1 gene.

To further determine that the NF-κB binding site plays the critical role in linoleic acid-induced VCAM-1 gene expression in HMEC-1, cells were transfected with the construct of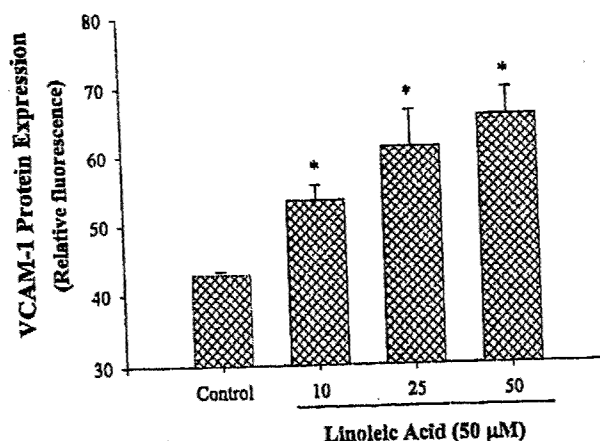 normal VCAM-1 promoter (pF3-CAT3) or with a similar construct that had a mutated NF-κB binding site (pF3-mNF-κB-CAT3). As indicated in Fig. 6, exposure to linoleic acid induced CAT activity only in cells transfected with the pF3-CAT3 construct. In contrast, mutation of the NF-κB site completely inhibited linoleic acid-induced stimulation of CAT activity in HMEC-1 transfected with the pF3-mNF-κB-CAT3 construct.

Discussion

Adhesive interactions between vascular endothelial cells and tumor cells play a critical role in the process of metastatic tumor dissemination. This process is mediated by adhesion molecules, which are expressed on the surface of endothelial cells, and specific integrin receptors, which are present on tumor cells. After adhesion, tumor cells can migrate across the vascular endothelium and establish new metastatic colonies. In addition, this process protects tumor cells against destruction by cells of the immune system (17). Thus, although adhesion molecules do not directly influence carcinogenesis, they can markedly stimulate blood-borne tumor metastasis. It appears that, among different adhesion molecules involved in endothelial cell-tumor cell interactions, VCAM-1 may play one of the most important roles (19–24). Furthermore, determination of VCAM-1 expression can serve as an important marker in cancer diagnosis. It is well known that angiogenesis, i.e., the formation of new capillaries from preexisting blood vessels, is essential for tumor growth and metastasis (39). Because adhesion molecules, including VCAM-1, are expressed on the surface of newly formed vascular endothelium, their elevated levels can indicate an active angiogenesis (19). Thus overexpression of VCAM-1 can have two distinctive features in cancer biology and diagnosis: 1) it can stimulate metastasis through facilitation of tumor cell adherence to the vascular endothe-
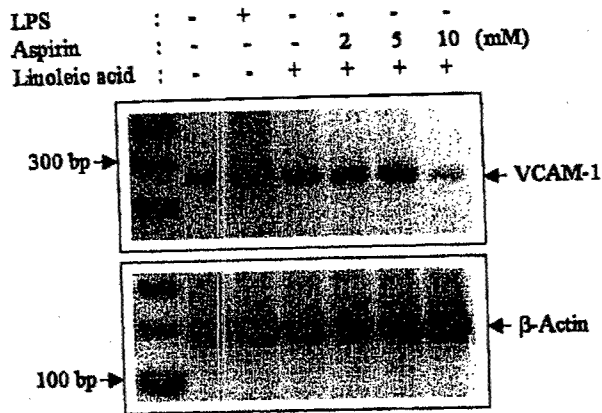
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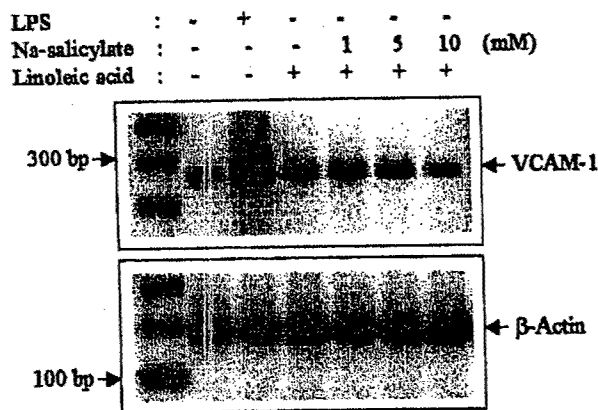
(B)
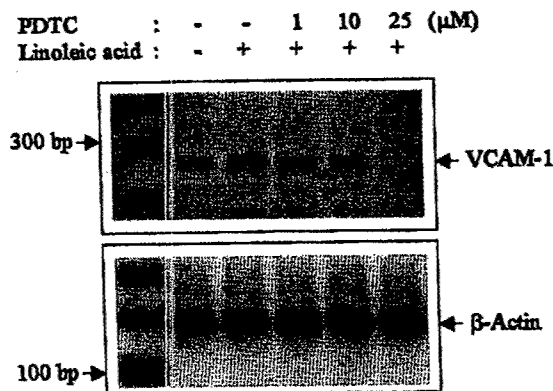
(C)

Figure 5. Pretreatment with aspirin, sodium salicylate, or PDTC impedes induction of VCAM-1 mRNA expression in linoleic acid-treated HMEC-1. Cells were pretreated for 1 h with indicated concentrations of aspirin (A) or sodium salicylate (B) or for 30 min with PDTC (C) before 4 h of treatment with 50 µM linoleic acid and assayed for VCAM-1 mRNA expression by RT-PCR. LPS (1 µg/ml) was used as positive control.

lium, and 2) it can serve as a marker of angiogenesis, which is associated with tumor growth.

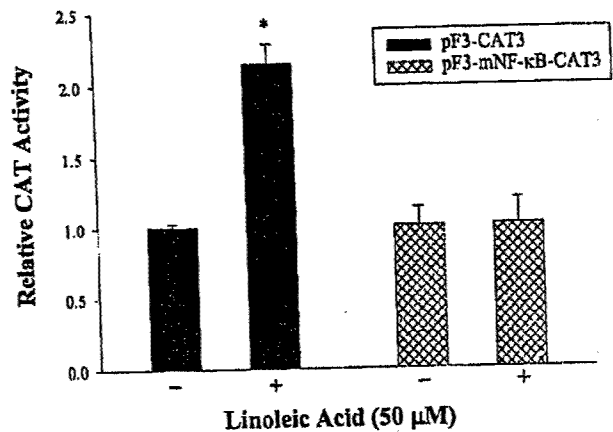**Figure 6.** Functional analysis of NF-κB binding site of the human VCAM-1 promoter in linoleic acid-treated HMEC-1. Cells were transfected with pF3-CAT3 or pF3-mNF-κB-CAT3 construct and untreated or treated with 50 µM linoleic acid for 24 h. Mutation of NF-κB site in VCAM-1 promoter construct completely inhibited linoleic acid-induced chloramphenicol transferase (CAT) activity. *, Significantly different from untreated control ($P < 0.05$).

A number of clinical and animal studies have implicated the intake and composition of dietary fats in expression of endothelial cell adhesion molecules, including upregulation of VCAM-1 (40–43). In the average American diet, fat accounts for 35–40% of energy (44), and such overconsumption of foods rich in fat may be a major risk for cancer development and metastasis. Although the role of dietary fat in human breast cancer has been recently questioned (8–10), dietary factors, including excessive intake of fat, are considered to contribute to 35% of all cancers (1). Part of the carcinogenic and prometastatic effects of dietary fat can be related to modulation of the functions of the vascular endothelium. It appears that, among different dietary fatty acids, linoleic acid can alter endothelial cell metabolism most significantly (45) and, thus, induce the development of cancer metastasis (12,13,15). Because it is an unsaturated fatty acid, linoleic acid can undergo peroxidative pathways initiated by hydrogen abstraction followed by oxygen attack on the generated lipid alkyl radical (46). Several reports suggest that linoleic acid can act as a potent prooxidant in endothelial cells in culture. For example, linoleic acid can enhance radical adduct formation in endothelial cells exposed to iron-induced oxidative stress (47), decrease glutathione levels (31), and increase peroxisomal β-peroxidation (48), a pathway that leads to the production of hydrogen peroxide. Degradation of linoleic acid via peroxidative pathways also can lead to formation of highly cytotoxic products, such as linoleic acid hydroperoxides or 4-hydroxy-2-(E)-nonenal (49). Metabolism of polyunsaturated fatty acids through lipoxygenase-mediated processes also may play an important role in cancer biology. For example, it was shown that 12(S)-hydroxyeicosatetraenoic acid, a metabolite of arachidonic acid generated in the reaction catalyzed by 12-lipoxygenase, can influence angiogenesis and formation of cancer metastasis (50). However, endothelial cell effects of linoleic acid have been primarily studied in

cells isolated from major vascular vessels, such as pulmonary artery (31,32,51) or umbilical veins (52). It is well known that the structure and functions of endothelial cells that originated from different tissues and vessels can differ markedly (53). Therefore, the present study focused on mechanistic effects of linoleic acid on induction of VCAM-1 in human microvascular endothelial cells, i.e., the cell type that is most relevant to cancer metastasis.

In the present study we report that treatment of HMEC-1 with linoleic acid results in an increase of the steady-state concentration of the VCAM-1 mRNA in a time- and dose-dependent manner (Fig. 3). In addition, flow cytometry analysis showed that linoleic acid-induced upregulation of the VCAM-1 gene is correlated with a significant and dose-dependent overexpression of VCAM-1 protein in HMEC-1 (Fig. 4). These results are in agreement with earlier reports that indicated upregulation of another adhesion molecule, such as intercellular adhesion molecule-1, in endothelial cells treated with linoleic acid (52). Recent evidence also indicated that an oxidized derivative of linoleic acid, 13-hydroperoxyoctadecadienoic acid, can induce VCAM-1 gene expression in endothelial cells (54). On the other hand, a 72-h preexposure of endothelial cells to selected n-3 or n-6 fatty acids, followed by a cotreatment with IL-1β or TNF-α for an additional 12 h, resulted in an inhibition of cytokine-induced VCAM-1 expression compared with cells that were not pretreated with fatty acids (55). However, a very different experimental setting used in that study was, most likely, responsible for this discrepancy with our present results.

The current study also reveals that treatment of HMEC-1 with linoleic acid can activate NF-κB. These results are in agreement with earlier reports on NF-κB activation by linoleic acid in porcine pulmonary artery endothelial cells (51, 56). It is possible that linoleic acid-mediated induction of oxidative stress (51), a decrease in cellular glutathione (31), and alterations of cellular redox status (31,51) are responsible for activation of NF-κB. To support the role of oxidative stress in linoleic acid-induced activation of NF-κB, this effect was attenuated by salicylates and PDTC (Fig. 2). Aspirin and sodium salicylate have been shown to specifically inhibit the activation of NF-κB by preventing the degradation of IκB, an NF-κB inhibitory subunit, and blocking the translocation of NF-κB into the nuclear compartment (57, 58). PDTC, the radical-scavenging thiol compound, is also widely used as an inhibitor of NF-κB activation (59,60).

NF-κB binding sites are located in the promoter regions of the genes encoding for adhesion molecules, including VCAM-1 (61). Two adjacent κB sites located at positions −77 and −63 relative to the transcription start site were identified in the VCAM-1 promoter (27,28). The role of these κB binding sites in the induction of the VCAM-1 gene is not fully understood and may depend on the type of stimulus. For example, NF-κB binding appears to be critical in TNF-α or lipopolysaccharide-induced VCAM-1 expression (27,28,61). In contrast, IL-4-mediated induction of the VCAM-1 gene is independent of NF-κB activation. This phenomenon was reported in endothelial cells (29) and in other types of vascular

cells (30). These conflicting reports on the role of NF-κB activation in VCAM-1 gene expression prompted us to investigate the role of this transcription factor in linoleic acid-mediated stimulation of VCAM-1 in HMEC-1. In the present study, two different lines of experiments proved that linoleic acid-induced activation of NF-κB and induction of the VCAM-1 gene are interrelated. First, pretreatment of the HMEC-1 with inhibitors of NF-κB activation, such as salicylates or PDTC, completely inhibited linoleic acid-induced VCAM-1 expression (Fig. 5). Second, reporter gene assays were performed using normal VCAM-1 promoter reporter construct as well as similar construct but with mutated NF-κB binding site. As indicated in Fig. 6, mutation of the NF-κB binding site in the VCAM-1 promoter region completely abolished linoleic acid-induced expression of the reporter gene. These results specifically indicate the importance of NF-κB activation in linoleic acid-induced expression of the VCAM-1 gene. On the other hand, low doses of linoleic acid, such as 10 or 25 μM, stimulated VCAM-1 expression (Figs. 3 and 4) but were not sufficient to activate NF-κB. To explain this discrepancy, it should be pointed out that the promoter region of the VCAM-1 gene contains binding sites not only for NF-κB, but also for several other transcription factors, such as AP-1, SP-1, GATA-1, or Ets (27,28). It is possible that, in low concentrations of linoleic acid, these other transcription factors may participate in induction of the VCAM-1 gene. In contrast, in higher concentrations of linoleic acid, such as 50 μM, it appears that activation of NF-κB is the critical factor in induction of VCAM-1 expression.

In conclusion, our studies have demonstrated that linoleic acid induces VCAM-1 expression in HMEC-1 through the activation of NF-κB. More importantly, because the ability of cancer cells to interact with the endothelium appears to be a prerequisite for the potential of distant metastasis and because VCAM-1 is considered to be a crucial adhesion molecule in this process, the present study may have significant therapeutic implications in developing a novel strategy against cancer metastasis. Finally, these studies provide a mechanistic insight into the role of specific dietary lipids in metastasis.

Acknowledgments and Notes

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ELSEVIER

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. © 2001 Elsevier Science Inc. All rights reserved.

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 μg/ml hydro-

cortisone and 0.01 μg/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 μM or with N-acetyl-cysteine (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 μl volume containing 6 μg of nuclear protein extracts, 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, 2 μg of poly[dI-dC] (nonspecific competitor) and 40,000 cpm of ^{32}P-labeled specific oligonucleotides that contained the NF-κB sequence specific for the NF-κB 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 $\times$ 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 μl 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/μl of recombinant RNasin ribonuclease inhibitor, 15 units/μg of AMV reverse transcriptase, and 0.5 μg of oligo(dT)$_{15}$ primer [22]. For amplification of MCP-1 and of β-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' (β-actin; expecting 188-bp fragment) [23]. The PCR mixture consisted of a Taq PCR Master Mix Kit (Qiagen, Valencia, CA), 2 μl of the reverse transcriptase reaction, and 20 pmol of primer pairs in a total volume of 50 μl. 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
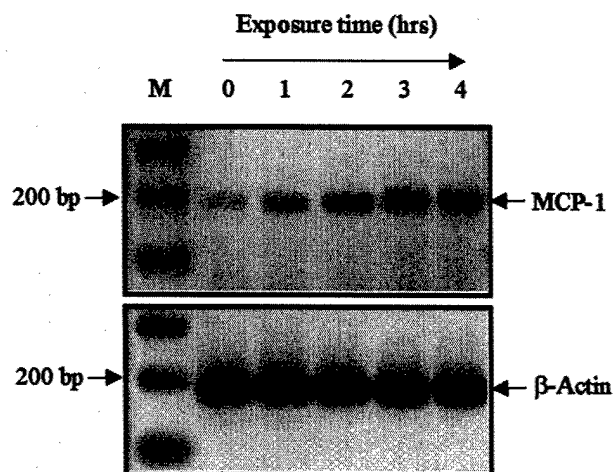
Fig. 2A. Time-dependent upregulation of MCP-1 mRNA expression by linoleic acid in human microvascular endothelial cells (HMEC-1). Cells were exposed to 50 μM linoleic acid for up to 4 h. The levels of MCP-1 mRNA were determined by RT-PCR. PCR products were analyzed by 2% agarose gel electrophoresis and visualized using phosphoimaging. The predicted sizes of RT-PCR products for MCP-1 and β-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 $\pm$ 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 μM.

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 μM of linoleic acid.

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 μM 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).
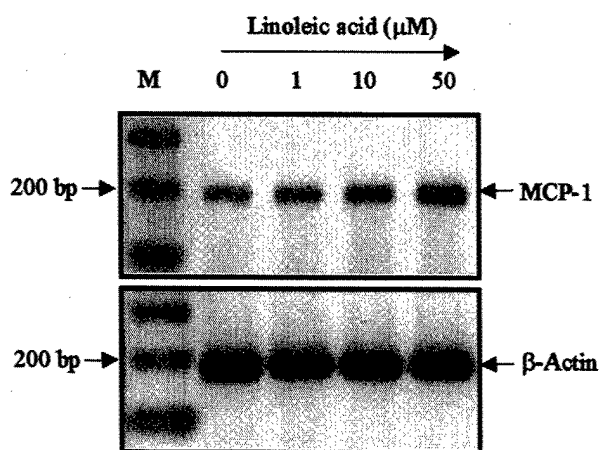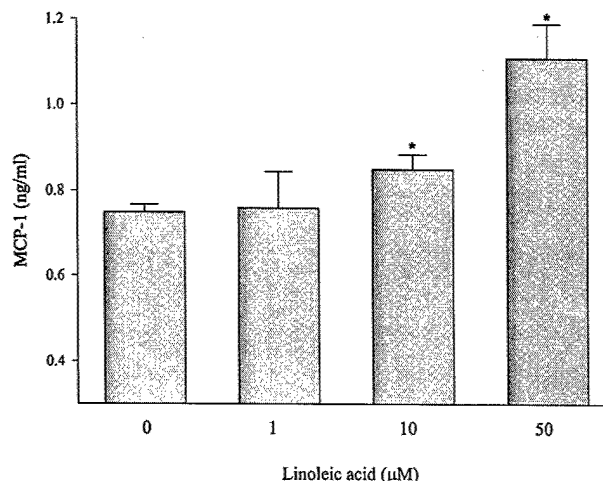

Figure 2B. Dose-dependent upregulation of MCP-1 mRNA expression by linoleic acid in human microvascular endothelial cells (HMEC-1). Cells were exposed to increasing concentrations of linoleic acid for 4 h. The levels of MCP-1 mRNA were determined as described in the legend to Figure 2A.

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

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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Liposome-Mediated High-Efficiency Transfection of Human Endothelial Cells

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Abstract

Liposome-mediated transfection of endothelial cells provides a valuable experimental technique to study cellular gene expression and may also be adapted for gene therapy studies. However, the widely recognized disadvantage of liposome-mediated transfection is low efficiency. Therefore, studies were performed to optimize transfection techniques in human endothelial cells. The majority of the experiments were performed with primary cultures of human umbilical vein endothelial cells (HUVEC). In addition, selected experiments were performed using human brain microvascular endothelial cells and human dermal microvascular endothelial cells. To study transfection rates, HUVEC were transfected with the pGL3 vector, containing the luciferase reporter gene, complexed with several currently available liposomes, such as different Perfect Lipid (pFx) mixtures, DMRIE-C, or lipofectin. The optimal transfection rate was achieved in HUVEC transfected for 1.5 h with 5 µg/ml of DNA plasmid in the presence of 36 µg/ml of pFx-7. In addition, transfection with the VR-3301 vector encoding for human placental alkaline phosphatase revealed that, under the described conditions, transfection efficiency in HUVEC was approximately 32%. Transfections mediated by other liposomes were less efficient. The usefulness of the optimized transfection technique was confirmed in HUVEC transfected with NF-κB or AP-1-responsive constructs and stimulated with TNF or LPS. We conclude that among several currently available liposomes, pFx-7 appears to be the most suitable for transfections of cultured human endothelial cells.

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Introduction

Cellular transfections (physical-chemical methods of introducing genes into cells) provide powerful experimental tools to study gene regulation in vivo and in vitro [1]. In addition, transfection techniques are used to deliver foreign DNA in gene therapy strategies [2, 3]. Stable transfections refer to the production of a population of cells in which the gene of interest is stably expressed in the cell. Thus, the gene is not only introduced into the cell but also is integrated into the host DNA and reproduced during cell cycles or cell division. The second general type of transfection is transient transfection, during which plasmid DNA is introduced into a cell population but no stable cell lines are isolated. Instead, gene expression is studied shortly after the transfection procedure, usually within 24–72 h [4]. The advantage of the second approach is the simplicity of the technique and the fact that the same

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preparation of DNA can be introduced into various cell types. Because cellular membranes create barriers for large and highly charged DNA molecules to enter cellular compartments, several techniques have been developed to facilitate cellular transfections. Transfection methods include calcium-phosphate precipitation, electroporation, detergent-DNA complexes, DNA-DEAE complexes, microinjection, virus-mediated transfection, introduction of DNA via particle bombardment and lipid-mediated transfection [2, 5]. In transfections performed in vitro in cultured cells, cationic lipids have become standard carriers of plasmid DNA [6].

Endothelial cells are a promising target in somatic gene therapy in cardiovascular disorders, ischemic disease [7] and cancer [8, 9], since the endothelium is involved in these pathological stages and endothelial cells are accessible for gene transfer via circulation [10]. Several experimental and clinical studies have demonstrated the therapeutic potential of somatic gene therapy in vascular diseases. For example, in the treatment of restenosis, positive results were obtained when animals were transfected with the genes encoding for vascular endothelial growth factor, nitric oxide synthase, thymidine kinase, retinoblastoma, growth arrest or antisense oligonucleotides against transcription factors [10, 11]. In atherosclerosis, gene therapy strategies have been used in the treatment of vascular proliferation, endothelial dysfunction, thrombosis, and ischemia as well as in modification of the blood/biomaterial interface [12]. It has also been reported that transfer of genes encoding for cyclooxygenase and endothelial nitric oxide synthase can protect against intimal hyperplasia in angioplasty-injured carotid arteries [13]. Clinical trials indicated that substantial therapeutic benefits could be obtained by intramuscular injections of naked DNA plasmid encoding for human vascular endothelial growth factor in patients with severe peripheral arterial disease [14].

The most efficient transgene expression can be achieved by using adenoviruses [15]. In fact, with adenovirus vector, recombinant genes can be delivered to approximately 100% of endothelial cells of normal human vessels in organ cultures [16]. However, adenoviral vectors can induce injury to the vessel wall. For example, in arteries transduced with replication-defective adenoviral vector AdRSVn-LacZ, a marked accumulation of macrophages and increased intimal cellularity were reported. In addition, in hypercholesterolemic cynomolgus monkeys, this vector caused an increase in vessel wall inflammation and progression of early atherosclerotic lesions [17]. Viral transduction can also induce changes in endothelial cell phenotype [18]. Therefore, nonviral transfections, including cationic liposomes, remain attractive carriers to facilitate the entry of foreign DNA into endothelial cells.

The aim of the present study was to optimize a transfection technique using different, currently available cationic liposomes in cultured human endothelial cells. Transfection rate was established using liposomes complexed with the pGL3 vector, driven by the simian virus 40 (SV40) promoter and containing the luciferase reporter gene. In addition, the efficiency of transfection was studied by employing the VR-3301 vector driven by the cytomegalovirus (CMV) promoter ligated to the human placental alkaline phosphatase reporter gene (hpAP). We found that cultured human endothelial cells can be efficiently transfected.

Materials and Methods

Endothelial Cell Cultures

Human umbilical vein endothelial cells (HUVEC) were isolated as described previously [19]. They were maintained in growth medium containing M199, 25 mM HEPES, 54.3 U/ml heparin, 2 mM L-glutamine, 1 µM sodium pyruvate, 200 U/ml penicillin, 200 µg/ml streptomycin, 0.25 µg/ml amphotericin B (all reagents from Gibco BRL, Grand Island, N.Y., USA), 40 µg/ml endothelial cell growth supplement (ECGS, Becton Dickinson, Bedford, Mass., USA), and 20% FBS (HyClone Laboratories, Inc., Logan, Utah, USA).

Cells were determined to be endothelial by their cobblestone morphology and uptake of fluorescent labeled acetylated LDL (1,1'-dioctadecyl-3,3,3'3'-tetramethyl-indocarbocyanine perchlorate; Molecular Probes Inc., Eugene, Oreg., USA). All experiments were conducted with cells from passage two.

Selected experiments also were performed using human aortic endothelial cells (HAEC), immortalized human brain microvascular endothelial cells (HBMEC) and immortalized human dermal microvascular endothelial cells (HMEC-1). HAEC were purchased from Clonetics Corp., (Walkersville, Md., USA) and cultured in medium supplied by the manufacturer. HBMEC (a generous gift from Dr. M. Fiala, UCLA School of Medicine) were isolated from a brain biopsy of an adult female with epilepsy and immortalized by transfection with SV40 large-T antigen. They were cultured in RPMI-1640 medium (Gibco BRL), supplemented with 10% FBS (HyClone Laboratories), 10% NuSerum IV (Becton Dickinson), 1% nonessential amino acids, 1% vitamins, 5 U/ml heparin, 1 mM sodium pyruvate, 2 mM L-glutamine (all reagents from Gibco BRL), and 30 µg/ml ECGS (Becton Dickinson) [20, 21].

HMEC-1 (a generous gift from Dr. E. Smart, University of Kentucky) were isolated from dermal microvessels and immortalized by transfection with SV40 large-T antigen. They were cultured in MCDB-131 medium (Gibco BRL) supplemented with 10% FBS (HyClone Laboratories), 200 U/ml penicillin, 200 µg/ml streptomycin (Gibco BRL), 10 ng/ml endothelial growth factor (Calbiochem, San Diego, Calif., USA) and 1 mg/ml hydrocortisone (Sigma).

Each experiment was performed at least in triplicate on at least four (and up to 12) independent cultures.

Liposome Carriers for Transient Transfection and Transfection Procedure

The PerFect Lipid Transfection kit (Invitrogen, Carlsbad, Calif., USA), DMRIE-C, and lipofectin (Gibco BRL, Grand Island, N.Y., USA) were used for transfections of endothelial cells. The PerFect Lipid Transfection kit provides eight different compositions of lipids (pFx 1–8), and each of these lipids was employed in the present study. Molecular weights of different pFx mixtures vary from 847 (pFx-4) to 2,617 (pFx-8). The molecular weight of pFx-7, the liposome used in the majority of our experiments, is 1,011. DMRIE-C (molecular weight 646) was used because it resembles a lipid carrier which was previously used successfully for endothelial cell transfections [5]. Lipofectin (molecular weight 669.5), which was employed in our earlier study [22], is widely used in transfection of endothelial cells. Selected experiments also were performed using cytofectin GCV (Glen Research, Sterling, Va., USA), DAC-30 (Eurogentec, Sersing, Belgium), and SuperFect (Qiagen, Valencia, Calif., USA). These additional liposomes were selected based on a recent report which demonstrated that cytofectin GCV or SuperFect can mediate uptake of antisense oligonucleotides in cultured human iliac artery endothelial cells with high efficiency [23].

For transfection studies, endothelial cells were seeded in 12-well plates and grown to 50–60% confluency in normal growth medium. To perform transfections, aliquots of normal M199 were mixed with different concentrations of specific lipid carriers in polystyrene tubes, mixed with plasmid DNA and incubated at 37°C for 30 min to allow the formation of DNA-lipid complexes. Endothelial cell cultures were washed three times with M199 to remove serum, and 1 ml of transfection solution was added to each well of the 12 well plates. Controls consisted of endothelial cells incubated with plasmid DNA alone or liposomes complexed with a carrier plasmid. After incubation, transfection solutions were aspirated and replaced with growth medium. Cells were maintained in these conditions for 48 h before assays for reporter genes were performed.

pGL3 Vector and Luciferase Reporter Gene Assay

To monitor the transfection rate, endothelial cells were transfected with individual liposomes complexed with the pGL3 Luciferase Reporter Vector (Promega, Madison, Wisc., USA). This vector contains the SV40 promoter and enhancer sequence and firefly luciferase as a reporter gene. Following the transfection process, luciferase activity was measured by Luciferase Assay System (Promega) according to the instructions supplied by the manufacturer. Briefly, culture media were removed and cells were washed three times with PBS and incubated for 10 min with 60 µl of Cell Culture Lysis reagent. Attached cells were then scraped, centrifuged to remove membrane debris, transferred to new tubes, and stored at –80°C until analysis. For luciferase assay, 10 µl of the cell extracts were mixed with 100 µl of Luciferase Assay Reagent containing luciferin and ATP in a luminometer with automatic injection. Light emission was measured every 0.5 s, for 10 s. Values are expressed in RLU/µg protein. Cellular proteins were measured using Bradford reagent (Bio-Rad, Hercules, Calif., USA).

VR-3301 Vector and Alkaline Phosphatase Reporter Gene Assay

To establish transfection efficiency, endothelial cells were transfected with VR-3301 vector (Vical Inc., San Diego Calif., USA) mixed with pFx-7, DMRIE-C or lipofectin. The VR-3301 vector contains CMV promoter/enhancer which regulates expression of the hpAP gene. Transfected endothelial cells were fixed in 4% paraformaldehyde for 1 h and then washed 3 times with PBS. Following heat inactivation of endogenous alkaline phosphate isoenzymes of non-placental origin (30 min at 65°C), cells were stained for hpAP using an azo dye coupling technique [24]. Briefly, 0.2 ml of naphtol AS-MX phosphate (0.25% alkaline solution, Sigma) were mixed with 4.8 ml of 0.1 M Tris-HCl buffer (pH 10.0) and 10 mg of fast red TR salt (Sigma). The stain mixture was filtered immediately before use, and cells were stained for the presence of hpAP for 15 min at room temperature. In independent sets of experiments, fluorescence of transfected cells was determined either by flow cytometry (in cell suspension) or fluorescent microscopy (in cells cultured on glass-bottom dishes) using rhodamine filter sets. Data are expressed as a percentage of cells in which activity of hpAP was detected.

Employment of the Optimized Transfection Conditions to Study Activation of Transcription Factors in Endothelial Cells

To determine whether the optimized transient transfection technique is useful in studies on transcription factor activation in endothelial cells, HUVEC were transfected for 1.5 h with 5 µg of NF-κB or AP-1 reporter plasmids (Stratagene, La Jolla, Calif., USA) mixed with 36 µg/ml of pFx-7. NF-κB responsive plasmid contained five repeats of NF-κB enhancer elements, and AP-1-responsive plasmid contained seven repeats of AP-1 enhancer elements, linked to basic TATA element and the firefly luciferase reporter gene. Following transfection, cells were incubated in normal growth medium for 24 h. Then, HUVEC were treated with either TNF (10 ng/ml) or LPS (1 µg/ml) in a medium containing 10% FBS for 24 h. At the end of the incubation time, cells were washed with PBS, lysed, and measured for luciferase activity using Luciferase Assay Reagent (Promega).

Cell Proliferation (5-Bromo-2'-Deoxyuridine Incorporation Assay)

Endothelial cell proliferation was determined by the 5-bromo-2'-deoxyuridine (BrdU) incorporation assay according to the procedure supplied by the manufacturer (Roche Diagnostics, Mannheim, Germany). This assay takes advantage of the incorporation of BrdU, instead of thymidine, into the DNA of proliferating cells. Briefly, immediately following transfections, endothelial cells were incubated for 12 h with 10 µM BrdU diluted in normal growth medium. Then, cultures were fixed and incubated with monoclonal anti-BrdU antibody labeled with peroxidase. Following a 30-min incubation, tetramethylbenzidine was added as a substrate for peroxidase and, after a 10-min interval time required for color development, absorbance was read at 370 nm. The results were expressed as percentage of control.

Statistical Analysis

Statistical analysis was performed using SYSTAT 8.0 (SPPS Inc., Chicago, Ill., USA). One-way or two-way ANOVA was used to compare the mean values among the treatments. Two-way ANOVA was employed in statistical analysis of all experiments which included at least two variables, such as time and different treatment factors. When the overall F values were significant, ANOVA was followed by a posthoc Bonferroni test to compare means from different treatments. Statistical probability of $p < 0.05$ was considered significant.
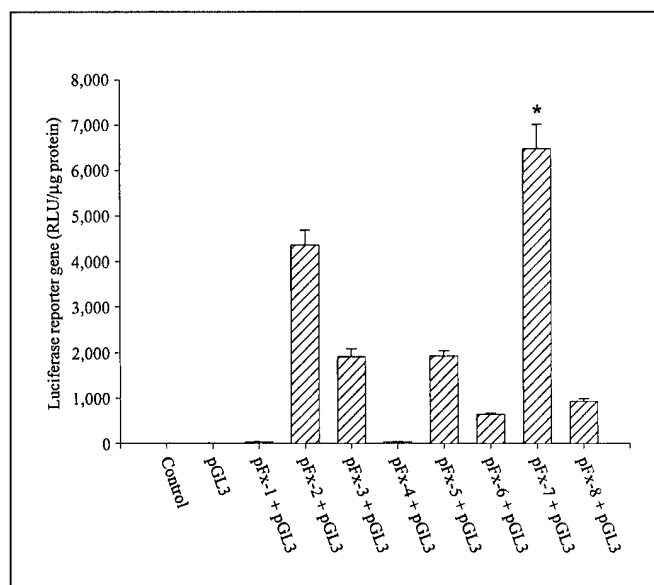
Fig. 1. Comparison of transfection rates mediated by different pFx mixtures. Cells were transfected for 3 h with 5 μg/ml of the pGL3 vector complexed with 36 μg/ml of individual pFx lipids. Transfections were followed by a 48-hour recovery period in normal growth medium, after which the reporter gene assay was performed. Values are mean ± SEM. *Values in cultures transfected by pFx-7 are significantly higher than values from groups transfected with other pFx lipids.

Fig. 2. The effect of liposome concentrations on transfection rates in HUVEC. Cells were transfected for 1.5 h with 5 μg/ml of the pGL3 vector complexed with different concentrations of pFx-7, DMRIE-C or lipofectin. Values are mean ± SEM. *Values are statistically significant as compared to the values in the group transfected with the preceding concentration of a given liposome. †Values in cultures transfected in the presence of 36 μg/ml pFx-7 are significantly higher than transfection rates in other experimental groups.

Results

Transient Transfection Rates Mediated by Different pFx Liposomes

To determine the most effective pFx liposome as a mediator of transient transfection of endothelial cells, HUVEC were transfected with 5 μg/ml of the pGL3 vector complexed with 36 μg/ml of each liposome provided in the PerFect Lipid Transfection kit. Figure 1 indicates transfection rates, as determined by luciferase activity, mediated by individual pFx liposomes. Transfections were performed for 3 h, followed by a 48-hour recovery process. Only minimal transfection rates (range of 3–7 RLU/μg protein) were determined in HUVEC exposed to the pGL3 vector alone. Except for pFx-1 and pFx-4, all remaining pFx liposomes successfully mediated transfection of HUVEC. However, the most marked transfection rate was observed in cells transfected with pFx-7. Therefore, optimization of transfection conditions was performed with this liposome.
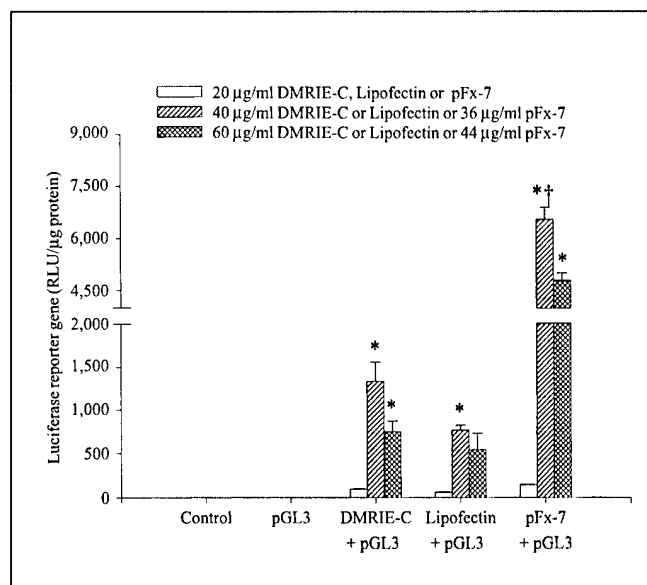
Comparison of Transfection Rates Mediated by DMRIE-C, Lipofectin, or pFx-7 and Optimization of Liposome Concentrations

DMRIE-C reagent and lipofectin are commercially available liposomes, widely used to initiate transient or stable transfections. To establish the most suitable liposome carrier and the optimal liposome concentration for transient transfection of endothelial cells, the pGL3 vector (5 μg/ml) was complexed with different concentrations of DMRIE-C, lipofectin or pFx-7. Transfections were performed for 1.5 h, followed by a 48-hour recovery period. Results of these experiments are reflected in figure 2. Liposomes at the concentrations of 20 μg/ml (or lower – data not shown) appeared to be ineffective in HUVEC transfection. However, an increase in liposome concentrations from 20 to 40 μg/ml for DMRIE-C or lipofectin and to 36 μg/ml for pFx-7 resulted in an increase of transfection rates, as measured by luciferase activity. In particular, a dramatic increase (approximately 250 times) in transfection rate was detected in HUVEC transfected with pGL3 complexed with pFx-7 at the concentration of 36 μg/ml. The rate of transfection mediated by this con-

centration of pFx-7 was approximately 8.5 times higher compared to transfection induced by 40 µg/ml lipofectin. In addition, the transfection rate achieved by pFx-7 exceeded that mediated by 40 µg/ml DMRIE-C by almost 5 times. Further increase in concentrations of DMRIE-C, lipofectin or pFx-7 decreased transfection rates. It appears that a marked cytotoxicity observed in endothelial cell cultures exposed to high doses of liposomes was responsible for this phenomenon.

In separate experiments, transfection rates mediated by pFx-7 at the dose of 36 µg/ml were compared to those mediated by cytofectin GCV (used at the concentration range of 1–40 µg/ml), DAC-30 (concentration range of 5–30 µg/ml), and SuperFect (concentration range of 20–80 µg/ml). Among these liposomes, transfection of HUVEC mediated by pFx-7 also resulted in the highest transfection rates (data not shown).

Liposome-Mediated Toxicity in Cultured Endothelial Cells

When introduced into cell cultures, liposomes can induce cytotoxic effects which depend on lipid concentration and transfection time. Therefore, their toxic effects were also measured in cultured endothelial cells. BrdU incorporation assay, which reflects cell proliferation, was used in these studies. As indicated in table 1, treatments with lipofectin appeared to be most toxic in cultured endothelial cells. Diminished incorporation of BrdU was observed in endothelial cells incubated with 40 or 60 µg/ml of lipofectin for as short as 1 h. In addition, when cells were treated with lipofectin for 3 h, even lower doses of this liposome decreased proliferation of endothelial cells.

Transfection mediated by pFx-7 resulted in a moderate toxicity. Endothelial cell proliferation was not statistically decreased when this liposome was used at the doses of up to 36 µg/ml for 1 or 1.5 h. However, a higher dose (i.e., 44 µg/ml) of pFx-7 as well as a 3 h incubation time markedly diminished incorporation of BrdU in transfected HUVEC (table 1). In general, the most marked cytotoxicity was observed when endothelial cells were exposed to high doses of liposomes for 3 h. Liposome-mediated toxic effects similar to those detected in HUVEC were observed in cultures of HAEC (data not shown).

Although 1.5-hour treatments with liposomes at concentrations which mediated the optimal transfection rates as reported in figure 2 did not affect BrdU incorporation, they resulted in morphological changes of cultured endothelial cells. Because the character of these changes was similar for all studied liposomes, they are documented

Table 1. Toxic effects of different transfection carriers as measured by the incorporation of BrdU assay

Transfection carrier	Exposure time		
	1 h	1.5 h	3 h
Lipofectin, µg/ml			
10	98.7 ± 3.94	94.1 ± 1.97	69.9 ± 0.82[a,b]
20	97.1 ± 4.70	91.7 ± 2.57	59.4 ± 4.75[a,b]
40	79.4 ± 8.49[a]	74.1 ± 4.96[a,c]	47.3 ± 4.69[a,b]
60	60.9 ± 4.06[a]	54.3 ± 0.33[a,c]	47.4 ± 5.47[a]
DMRIE-C, µg/ml			
10	98.5 ± 6.37	90.5 ± 1.31	96.6 ± 2.10
20	105.2 ± 4.45	87.3 ± 7.32	76.2 ± 1.47[a,c]
40	101.7 ± 2.69	86.1 ± 1.38[a]	74.4 ± 3.17[a,b]
60	102.4 ± 1.22	81.2 ± 2.01[a,b]	64.7 ± 2.55[a,b]
pFx-7, µg/ml			
12	98.4 ± 3.30	92.2 ± 3.07	93.5 ± 4.28
24	95.1 ± 2.63	86.9 ± 2.30	85.6 ± 3.22
36	88.7 ± 5.82	84.2 ± 4.79	66.7 ± 0.98[a,c]
44	65.0 ± 3.11[a,c]	61.5 ± 4.94[a–c]	52.6 ± 2.69[a,c]

Values are mean ± SEM and are expressed as percentage of control.
[a] Statistically different as compared to control, i.e., non-transfected cells.
[b] Statistically different as compared to the values in the group transfected with the same concentration of a given liposome for the preceding exposure time.
[c] Statistically different as compared to the values in the group transfected for the same exposure time with the preceding concentration of a given liposome.

only for pFx-7, the liposome which produced the highest transfection rates in HUVEC. Figure 3A reflects morphological alterations of HUVEC, as observed under a phase-contrast microscope, after a 1.5-hour incubation with 36 µg/ml pFx-7 complexed with 5 µg/ml of the pGL3 vector. Cytotoxic effects of this complex included cellular shrinkage and detachment. However, a 48-hour recovery period following transfection, during which cells were maintained in normal growth medium, allowed HUVEC to regain normal morphological features. This phenomenon is shown in figure 3B, a photograph of the same culture as depicted in figure 3A, but taken after the recovery period.
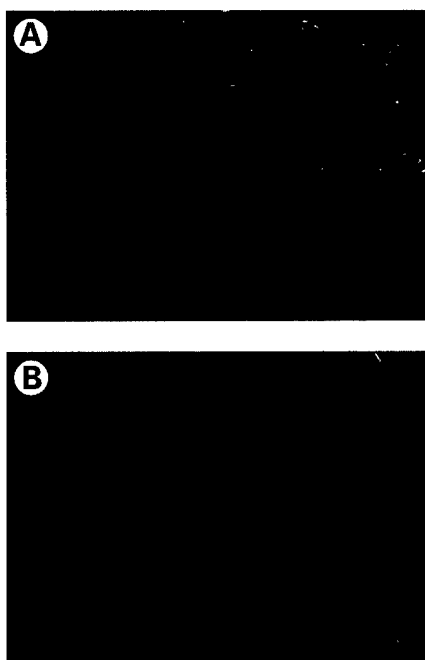
Fig. 3. The effect of pFx-7-mediated transfection on HUVEC morphology as observed under a phase-contrast microscope. Cells were transfected for 1.5 h with the pGL3 vector complexed with 36 µg/ml of pFx-7. **A** Cell morphology at the end of the 1.5-hour transfection period. **B** Cell morphology at the end of the 48 hour recovery period in which cells were maintained in normal medium.

Optimization of Plasmid DNA Concentration and Transfection Time for Transient Transfection of Endothelial Cells

Both the amount of plasmid DNA used for transfection and transfection time are important factors which can determine the transfection rate. Figure 4 shows transfection rates in HUVEC transfected with different amounts of the pGL3 vector complexed with pFx-7 at the concentration of 36 µg/ml as well as with DMRIE-C or lipofectin at the concentration of 40 µg/ml. Maximum transfection rate was observed in cells transfected with 5 µg/ml of plasmid DNA. In cells transfected with either 2 or 10 µg pGL3/ml, transfection rates were minimal as compared to 5 µg/ml of the pGL3 vector.

To determine the optimal transfection time, HUVEC were transfected for 1, 1.5 or 3 h, followed by a 48-hour recovery period. Time-dependent effects on liposome-mediated transfection are also shown in figure 4. As reflected in this figure, most successful HUVEC transfections resulted from 1.5-hour transfection time. Transfection rates in cells exposed to liposomes for that period of time were constantly higher than those in HUVEC transfected for 1 h. In addition, extension of transfection time to 3 h did not result in higher transfection rates. It appears that cytotoxicity of liposomes, as reported in table 1, could affect transfection rates in HUVEC transfected for 3 h.

Fig. 4. Comparison of transfection rates mediated by DMRIE-C, lipofectin, or pFx-7 under different concentrations of plasmid DNA and transfection times. HUVEC were transfected for 1, 1.5 or 3 h with different concentrations of the pGL3 vector complexed with DMRIE-C or lipofectin at the concentration of 40 µg/ml or with pFx-7 at the concentration of 36 µg/ml. Values are mean ± SEM. Luciferase activities in control (nontransfected) cells and in cells transfected with naked pGL3 were negligible and were not plotted. *Values in cultures transfected with 5 µg pGL3/ml are significantly higher than values from groups transfected with other amounts of plasmid DNA. †Values in cultures transfected for 1.5 or 3 h in the presence of 36 µg pFx-7/ml complexed with 5 µg pGL3/ml are significantly higher than transfection rates in other experimental groups.
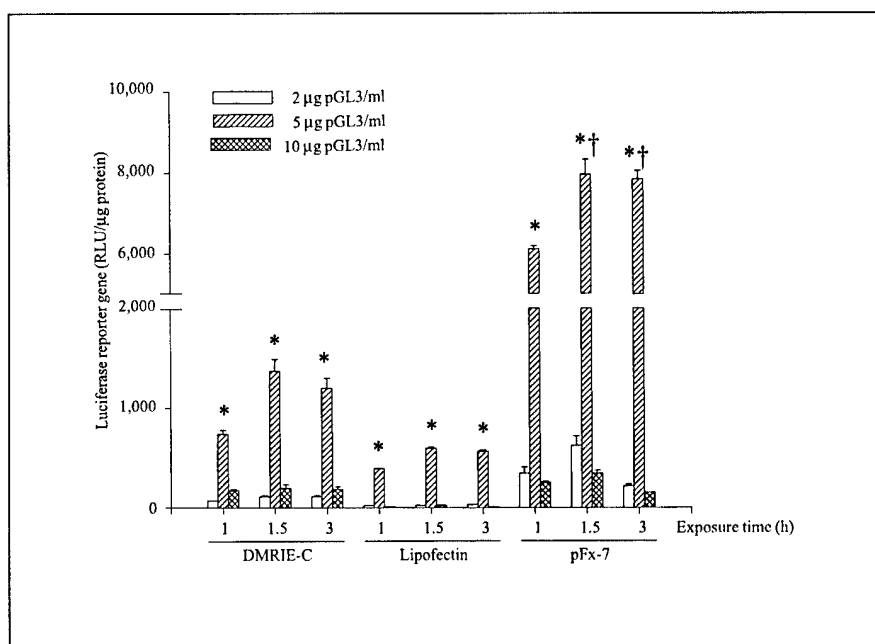
Efficiency of Transient Transfection in Endothelial Cells

Previously described experiments allowed us to determine the optimal transfection conditions for HUVEC using individual liposomes , i.e., pFx-7 at the concentration of 36 µg/ml, DMRIE-C or lipofectin at the concentration of 40 µg/ml, a transfection time of 1.5 h, and plasmid DNA concentration of 5 µg/ml. Using these experimental settings, transfection efficiency was measured by determination of activity of human placental alkaline phosphatase (hpAP) in HUVEC transfected with the VR-3301 vector, encoding for hpAP, and complexed with pFx-7, DMRIE-C or lipofectin. A fluorescent marker of hpAP activity, the fast red TR salt, was employed in these studies, and fluorescence was measured by either flow cytometry (in cell suspension) or fluorescent microscopy. Figure 5A shows the results of the quantitative analysis of transfection efficiency performed by flow cytometry. Under the described conditions, transfection efficiency in HUVEC mediated by pFx-7 was determined to be 34.4%. In contrast, transfection efficiency in endothelial cells transfected with DMRIE-C or lipofectin was much lower, i.e. approximately 9.5 or 4.7%, respectively. In control cultures and in cultures exposed to the naked plasmid DNA, positive staining for hpAP was negligible. Figure 5B depicts HUVEC positively stained for the presence of alkaline phosphatase (arrows) as observed under the fluorescent microscope.

Effectiveness of the Optimized Transfection Technique to Study Activation of Transcription Factors in HUVEC and for Transient Transfection of Different Endothelial Cell Types

One of the major applications of transient transfections is to study activation of transcription factors and mechanisms of gene regulation. Therefore, our optimized transfection technique (i.e., pFx-7, 36 µg/ml; plasmid DNA concentration, 5 µg/ml; transfection time, 1.5 h followed by a 48-hour recovery period) was employed in such an experimental setting. HUVEC were transfected with NF-κB- or AP-1-responsive plasmids containing the firefly luciferase reporter gene, and luciferase activity was determined in cells stimulated with TNF (10 ng/ml) or LPS (1 µg/ml). The results of these experiments are shown in figure 6. Both TNF and LPS significantly increased luciferase activity in HUVEC transfected with NF-κB or AP-1-responsive plasmids. These data are consistent with TNF or LPS-induced activation of NF-κB or AP-1 in HUVEC, as determined by electrophoretic mobility shift assay (data not shown).
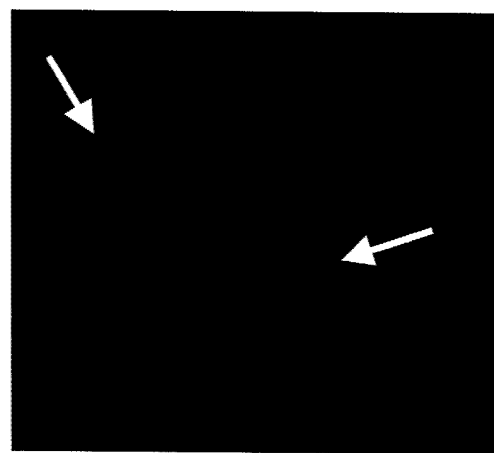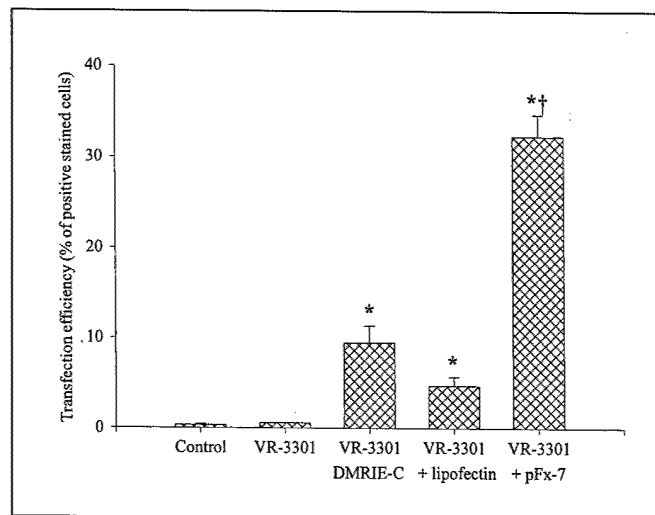

Fig. 5. A Efficiency of liposome-mediated transfection in HUVEC as measured by flow cytometry. Cells were transfected for 1.5 h with the VR-3301 vector (5 µg/ml) complexed with 40 µg/ml of DMRIE-C or lipofectin or with 36 µg/ml of pFx-7. *Values marked with an asterisk are significantly higher as compared to the values for control cultures or cultures transfected with naked plasmid DNA. †Values in cultures transfected in the presence of pFx-7 are significantly higher than values in other experimental groups. **B** An example of HUVEC positively stained for hpAP as observed under a fluorescent microscope (rhodamine filter). Transfection was mediated by pFx-7 under conditions as described in the legend to **A**.

Structure and functions of endothelial cells originated from different tissues differ markedly [25]. Therefore, experiments were performed in which the optimized transfection technique was employed to compare transfection rates in different types of endothelial cells, namely in HUVEC, HAEC, HBMEC, and HMEC-1. The optimized transfection conditions (i.e., pFx-7, 36 µg/ml; pGL3,
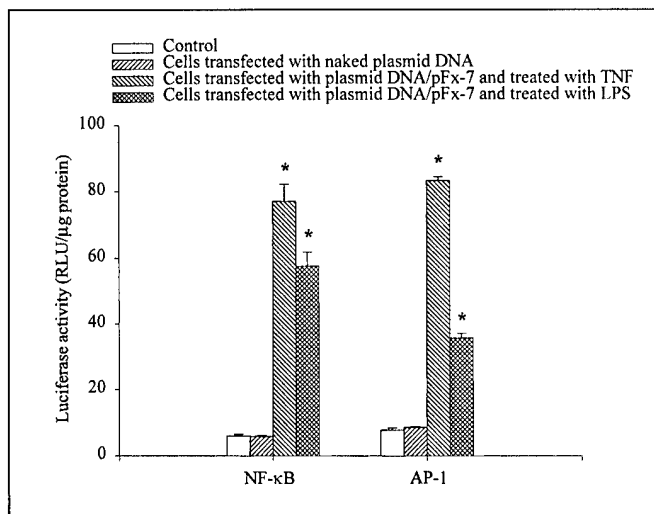
Fig. 6. The effectiveness of the optimized transfection conditions to study activation of transcription factors in HUVEC. Cells were transfected for 1.5 h with 5 μg/ml of the NF-κB or AP-1-responsive constructs complexed with 36 μg/ml of pFx-7. Transfections were followed by a 24-hour recovery period in normal growth medium, after which cells were treated either with TNF-α (10 ng/ml) or LPS (1 μg/ml) for 24. Values are mean ± SEM. *Values marked with an asterisk are significantly higher as compared to those of control cultures or cultures transfected with naked plasmid DNA.
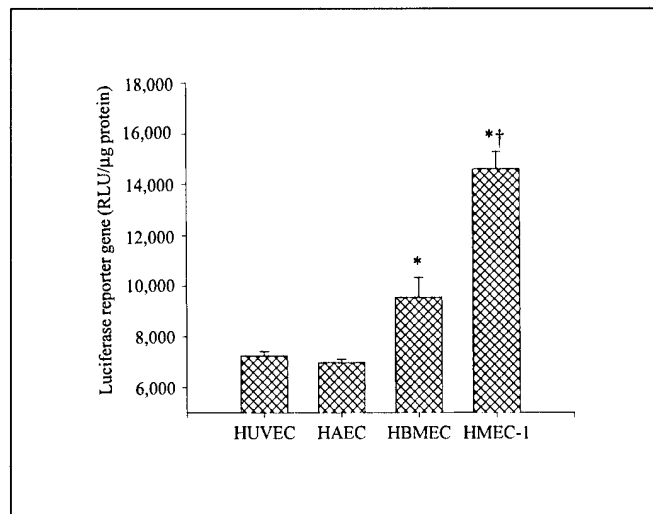
Fig. 7. A comparison of transfection rates in different types of human endothelial cells. HUVEC, HAEC, HBMEC, and HMEC-1 were transfected for 1.5 h with 5 μg/ml of the pGL3 vector complexed with 36 μg/ml of pFx-7. Luciferase activities in control (nontransfected) cells and in cells transfected with naked pGL3 were negligible and were not plotted. Values are mean ± SEM. *Transfection rates in HBMEC and HMEC-1 are significantly higher than those in HUVEC. †Transfection rates in HMEC-1 are significantly higher than those in other experimental groups.

5 μg/ml; transfection time, 1.5 h followed by a 48-hour recovery period) were employed in these experiments. As depicted in figure 7, among studied endothelial cell types, pFx-7 mediated the highest transfection rates in the immortalized endothelial cell lines, in particular in HMEC-1. There were no differences in transfection rates between HUVEC and HAEC.

Discussion

Cationic liposomes are positively charged lipids which can be mixed with negatively charged DNA to form lipid-DNA complexes. The most important advantages of mediating transfection with liposomes are that they are easy to prepare, they can transfer genes of various sizes and they are not infectious [6]. The most recognized disadvantage of liposome-mediated transfection is low efficiency of transfection. However, it is possible that the development of new generations of cationic lipids and transfection methods may overcome this limitation. In addition, better understanding of the mechanisms of liposome-mediated transfection may also contribute to the development of experimental methods which would allow for higher transfection efficiency [26].

Several factors can affect liposome-mediated transfection, including cell type, culture conditions, lipid composition of the liposomes, promoter type, reporter gene type, and amount of transfected plasmid DNA and DNA/lipid ratio. The dependency of transfection on the type of endothelial cells was observed in the present study. In the present study, we observed that transfection rates in HUVEC were approximately at the same rate as in HAEC but significantly lower as compared to immortalized endothelial cell lines (fig. 7). This is in agreement with a widely accepted phenomenon that cell lines are easier to transfect than primary cell cultures, such as HUVEC. However, it should be noted that endothelial cells, in general, are difficult to transfect. This may relate to the fact that endothelial cells represent a physiologic barrier against invasion of the vessels and underlying tissues by exogenous substances. During liposome-mediated transfection, lipids can fuse with cell membranes and thus deliver DNA into the cytoplasm. Liposome-mediated transfections are usually more efficient in dividing cells, because the nuclear membrane, which prevents DNA

from entering the nucleus, is not present during replication [6]. For this reason, transfections performed in the present study were initiated at approximately 55–65% confluency, i.e., in a state when cultured endothelial cells divide rapidly. However, it should be pointed out that liposomes can also transfect non-replicating cells [2].

Although liposome-delivered foreign DNA can enter the nucleus, it is not incorporated into the host genome. Therefore, liposome-mediated transfections are not mutagenic. The transfected plasmids remain as episomal nonreplicating minichromosomes and are gradually degraded [4]. In the present study, the reporter gene assays were performed 48 h following transfection, the standard interval for measuring reporter gene expression in cell cultures [4].

Because of the heterogeneity of cellular membranes, for optimal transfection, different types of cells require liposomes characterized by specific lipid profiles. In fact, lipid composition is the most critical factor determining the efficiency of liposome-mediated transfection. In the present study it was determined that among several commercially available liposomes pFx-7 is the most suitable lipid carrier for transfection of HUVEC. The optimal transfection rate was achieved when cells were incubated for 1.5 h with 36 μg pFx-7/ml complexed with 5 μg of plasmid DNA (fig. 2, 4). Although relatively high concentrations of pFx-7 induced cytotoxic effects in HUVEC, maintaining cells in normal growth medium for 48 h following transfection allowed for full recovery of morphological features (fig. 3). Among studied liposomes, incubation of endothelial cells with lipofectin resulted in most marked inhibition of endothelial cell proliferation (table 1). This is in agreement with an earlier report in which high toxicity of this liposome also was observed in cultured human endothelial cells [23].

In addition to comparing transfection efficiency in HUVEC mediated by different liposomes, transfections with the pGL3 vector alone were also included in the present study. It has been reported that injection with naked DNA plasmid encoding for VEGF into skeletal muscle was beneficial in patients with critical limb ischemia [14]. In addition, exposure of neurons to naked decoy κB DNA inhibited amyloid β-peptide-induced NF-κB activation [27]. However, in the present study transfection of HUVEC with naked DNA produced only a minimal effect. This is in agreement with the earlier report in which transfection efficiency with naked DNA plasmid was reported as low as approximately 0.08% [5].

Rates of liposome-mediated transfection are dependent on amounts of plasmid DNA and thus on the ratio of DNA/cationic lipids. Our studies revealed that the transfection rate of HUVEC can be enhanced with an increase in the amount of transfected DNA up to 5 μg DNA/ml (fig. 4). Further increases in the amount of plasmid DNA, and thus alteration of the DNA/liposome ratio, decreased efficiency of transfection. Similar results were obtained in the earlier studies [5]. Therefore, 5 μg DNA/ml was the standard amount of plasmid DNA used in the majority of the reported experiments.

The type of promoter which regulates the transgene expression can greatly influence transfection efficiency [28]. For example, using a plasmid regulated by the human β-actin promoter, it was reported that efficiency of transfection of HUVEC by electroporation was approximately 0.68%, by lipofectin approximately 0.45%, and by other transfection methods, including calcium phosphate and DEAE-dextran-mediated transfection, also below 1% [29]. In contrast, lipofectin-mediated transfection of HUVEC with a plasmid regulated by a strong respiratory syncytial virus (RSV) viral promoter resulted in transfection efficiency as high as 10–20% [30]. Highly efficient transfection of approximately 20% was also achieved in HUVEC transfected with a plasmid regulated by the CMV promoter, using γAP-DLRIE/DOPE liposomes [5]. Constructs employed in the present study also contained strong promoters. The pGL3 vector is regulated by the SV40 promoter and the VR-3301 vector contains the CMV promoter. Because these strong promoters use transcription factors which are present in host cells, they can be constitutively active in transfected cells. For example, the CMV promoter contains binding elements for common transcription factors, such as cyclic adenosine monophosphate and NF-κB [2]. These transcription factors remain active at the baseline level even in non-stimulated cells. In addition, one may suggest that cellular stress connected with transfection may further stimulate activation of these transcription factors. It should be noted that the CMV promoter can provide better transfection rates in HUVEC compared to the RSV promoter. This was demonstrated in experiments in which HUVEC were transfected with plasmids encoding for the same reporter gene (hpAP) but driven either by the CMV or the RSV promoter [5].

In the present study, transfection conditions were optimized using the pGL3 vector regulated by the SV40 promoter and encoding for firefly luciferase. Firefly luciferase has been recognized to be the reporter gene of choice for transfection studies in cells resistant to uptake of foreign DNA [31]. The transgene is simple to measure and has no background levels in animal tissues. In contrast,

our preliminary experiments with β-galactosidase revealed background activity of this enzyme in cultured HUVEC (data not shown). Determination of luciferase activity also has the advantage of being several orders of magnitude more sensitive than other common reporter gene assays, such as activities of chloramphenicol acetyltransferase, β-galactosidase or alkaline phosphatase [4, 31]. However, to determine the efficiency of transfection, the vector encoding for hpAP was used. This experimental approach allowed us to stain and count the transfected cells. Transfection efficiency of 32% achieved in HUVEC in the present study is higher than in earlier studies which reported efficiencies of approximately 20% [5, 30]. However, it should be noted that much higher transfection efficiency can be achieved for liposome-mediated transfection of endothelial cells with antisense oligonucleotides. For example, it was reported that cytofectin GCV or SuperFect can mediate the uptake of antisense oligonucleotides to more than 95% of cultured human iliac artery endothelial cells [23]. In contrast, these liposomes appeared to be less effective in facilitation of transfection of plasmids, such as the pGL3 vector, into HUVEC (data not shown).

In the present study, a strong correlation between transfection rates and transfection efficiency was observed. The high transfection rates mediated by pFx-7 were associated with high transfection efficiency in endothelial cells transfected in the presence of this liposome. In contrast, transfections mediated by either DMRIE-C or lipofectin resulted in moderate transfection rates and efficiency.

In summary, efficient transfection conditions have been established for a transient transfection of human endothelial cells. The optimal transfection conditions, resulting in the transfection efficiency of approximately 32%, were achieved with cationic liposome pFx-7 used at the concentration of 36 µg/ml for 1.5 h. Although these transfection conditions were connected with some cytotoxicity, a 48-hour period of maintaining endothelial cells in normal growth medium allowed the cells to recover fully. We conclude that pFx-7 can be used as an efficient transfection agent to deliver foreign DNA into human endothelial cells.

Acknowledgements

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