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Rapid Letter

Effect of Oxidized Low-Density Lipoprotein on Differential Gene Expression in Primary Human Endothelial Cells

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ABSTRACT

Oxidative modification of low-density lipoprotein (LDL) plays an important role in the initiation and progression of atherosclerosis. It has been proposed that the biological action of oxidized LDL (ox-LDL) may be partially attributed to its effect on a shift of the pattern of gene expression in endothelial cells. To examine the transcriptional response to ox-LDL, we applied cDNA array technology to cultured primary human endothelial cells challenged with oxidized human LDL. A twofold or greater difference in the expression of a particular gene was considered a significant difference in transcript abundance. Seventy-eight of the 588 genes analyzed were differentially expressed in response to the treatment. Ox-LDL significantly affected the expression of genes encoding for transcription factors, cell receptors, growth factors, adhesion molecules, extracellular matrix proteins, and enzymes involved in cholesterol metabolism. The alteration of the expression pattern of several genes was substantiated *post hoc* using RT-PCR. The experimental strategy identified several novel ox-LDL-sensitive genes associated with a "response to injury" providing a conceptual background to be utilized for future studies addressing the molecular basis of the early stages of atherogenesis. *Antioxid. Redox Signal.* 5, xxx–xxx.

INTRODUCTION

LOW-DENSITY LIPOPROTEIN (LDL) is a well established factor involved in the risk of vascular disease (1, 41). Oxidation of LDL is considered a key event associated with endothelial injury and dysfunction involved in "early-stage" atherogenesis (28). Although native LDL has been implicated in proatherogenic endothelial changes, oxidized LDL (ox-LDL) in the vessel wall is thought to be a primary determinant of the increased adhesiveness of endothelial cells to leukocytes and exaggerated vessel wall permeability. These events are associated with increased monocyte/macrophage migration into the subendothelial space (1, 11). In the intima, the modified apolipoprotein B-100 component of the LDL particle serves as a ligand for the scavenger receptors of macrophages (25, 38), which accumulate ox-LDL in a nonsaturable manner. This process leads to foam cell formation and the beginning of a fatty streak. However, the initial pathological impact of ox-LDL at the endothelial level is thought to be mediated through a proinflammatory shift of the pattern of gene expression. Thus, the analysis of differential gene expression in the endothelium is critical to our understanding of the sequence of events leading to the formation of atherosclerotic lesions. This approach has been recently utilized to study the molecular basis of endothelial cell dysfunction induced by different kinds of stimuli, related either to the development of cardiovascular disease, such as shear stress (4, 20), tumor necrosis factor-α (23), and nicotine (20), or to infections, such as lipopolysaccharide (49) and *Chlamydia pneumoniae* infection (6).

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In order to gain a more comprehensive overview of the response of endothelial cells to ox-LDL, we determined the expression profile of selected families of genes known to play a role in molecular models of atherosclerosis. The findings obtained by the cDNA arrays were confirmed by using reverse transcription–polymerase chain reaction (RT-PCR) as an independent technique. The current study corroborates the hypothesis that ox-LDL induces a "response to injury" type of change in differential gene expression and suggests several novel candidate genes for further studies addressing the molecular basis of the early phases of endothelial dysfunction.

MATERIALS AND METHODS

Cell culture

Primary human umbilical vein endothelial cells (HUVEC) were obtained from the umbilical cord vein as described previously (17). Umbilical cords were kindly provided by the nursery of "Fatebenefratelli" hospital of Rome. HUVEC were utilized for experiments at 90–100% apparent confluence within passages 3–6. Passages were performed according to standardized protocols (31, 32) and by diluting the cell population 1:3. The identity of endothelial cells was confirmed by routine analysis for the expression of typical endothelial genes as indicated in the original article (17).

LDL preparation and oxidation

Blood was collected in EDTA (1 mg/ml) containing vacutainer tubes from the antecubital vein of fasting healthy volunteers. The LDL fraction, corresponding to a density of 1.019–1.063 g/ml, was isolated from plasma by sequential ultracentrifugation in salt solutions, according to Havel *et al.* (14), using a Beckman T-100 bench-top ultracentrifuge (T-100.3 rotor). The LDL fraction was stored under nitrogen at 4°C and used within 1 week of isolation. Lipoprotein concentration was expressed in terms of protein content. Protein was measured by the Lowry method (19) using bovine serum albumin as the standard.

Before the experiments, LDL was dialyzed in the dark for 24 h at 4°C against three changes of buffer (1 L each) containing 0.01 *M* phosphate-buffered saline, 0.0027 *M* KCl, and 0.138 *M* NaCl, pH 7.4. Dialyzed LDL (200 μg of protein/ml) was oxidized with 5 μ*M* $CuCl_2$ in phosphate-buffered saline at 37°C. Oxidation was followed by monitoring the increased formation of conjugated dienes at 234 nm using a Beckman DU 70 spectrophotometer at 37°C. In order to standardize the level of oxidation, LDL was administered to HUVEC when its oxidation reached the midpoint of the propagation phase, at a final concentration of 25 μg/ml. To assess further the extent of oxidation of LDL in terms of the modified surface charge on the apolipoprotein B-100, lipoprotein electrophoresis was performed using a Beckman Paragon electrophoresis system (Fig. 1).

HUVEC cells were incubated with ox-LDL for 6 h. Control cells were maintained in culture for the same length of time without ox-LDL. In the study related to RT-PCR assessment of mRNAs expression (see below), HUVEC were incubated with both native LDL and ox-LDL at the same final concentration.

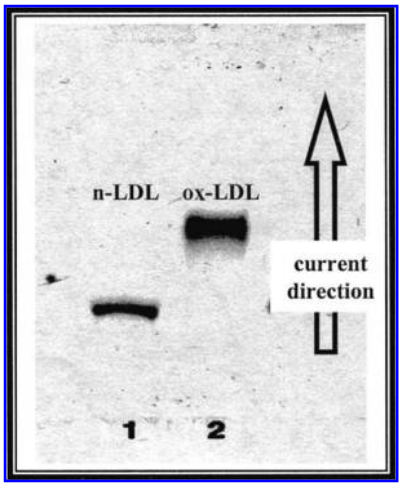

FIG. 1. Agarose gel electrophoresis of native (n-LDL) and Cu^{2+}-oxidized human LDL (ox-LDL). Treatment with 5 μ*M* $CuCl_2$ results in an increased mobility confirming the occurrence of oxidative changes in the surface charge of the apoG100 protein. Lane 1: n-LDL; lane 2: ox-LDL.

Complex probe preparation and hybridization to Atlas cDNA array

Total RNA was isolated from HUVEC using RNeasy Mini Kit (Qiagen). Complex cDNA probes were prepared by radioactive reverse transcription in the presence of [α-^{32}P]dATP (NEN) using 3′ primers corresponding to genes represented in the Atlas human cardiovascular array (Clontech). After hybridization at 68°C overnight, membranes were washed and autoradiographed to reveal the expression profiles as described previously (10). Results were evaluated using the Atlas Image Software. The intensity of the hybridization signal was obtained by subtracting the average intensity of the background. The relative expression of a gene was based on the normalization of its hybridization signal to the signals obtained from a set of housekeeping genes. Studies on the reproducibility and variability of array results indicate that a twofold or greater difference in the expression of a particular gene could be considered a real difference in transcript abundance (9, 15). A difference in gene expression between the ox-LDL-treated and controls was therefore considered significant, at a ratio of twofold or more and if both readings had a signal intensity above 1,000 units Previous studies on the application of cDNA array indicated that the coefficient of variation for differential gene expression in cultured cells is 10–15% (47).

RT-PCR

RNA from control and ox-LDL-treated HUVEC was reverse-transcribed by using Moloney Murine Leukemia Virus Reverse Transcriptase (GIBCO, Life Technologies) according to standardized protocols and then amplified using Taq DNA polymerase (Promega). Details regarding the amplification protocol for each gene are given in Table 1, together with the sequences of primers utilized. An aliquot from each PCR reaction was electrophoresed in a 1.7% agarose gel containing

TABLE 1. EXPERIMENTAL CONDITIONS FOR RT-PCR ANALYSIS OF INDIVIDUAL TRANSCRIPTS

Gene	*Amplicon length*	*Primer sequence*	*PCR conditions*
G3PDH	900	5′ CCACAGTCCATGCCATCAC 3′ 5′ TCCACCACCCTGTTGCTGTA 3′	3 min at 95°C (1st cycle); 30 s at 94°C; 30 s at 50°C; 1 min at 72°C (25 cycles); 10 min at 72°C as final extension
CTGF	289	5′ GTACCGGCCCGGTTAGTATCATCAGATCG 3′ 5′ GGCTTGTTACAGGCAAATTCACTTGCCACAAG 3′	3 min at 95°C (1 cycle); 30 s at 94°C; 30 s at 57°C; 1 min at 72°C (35 cycles); 10 min at 72°C as final extension
PDGFAa	433	5′ GCATCCGGGACCTCCAGCGACTCCT 3′ 3′ AGGCTTGTGGTCGCGCAGGCGCACT 3′	3 min at 95°C (1st cycle); 30 s at 94°C; 30 s at 57°C; 1 min at 72°C (35 cycles); 10 min at 72°C as final extension
PDGFR	465	5′ TCTCCCGTCTTCTGCCTCCCACTCCCATA 3′ 5′ GTCTAATTGGTCCTACAGAACCTAGTCAGGTT 3′	3 min at 95° C (1st cycle); 30 s at 94°C; 30 s at 55°C; 1 min at 72°C (35 cycles); 10 min at 72°C as final extension
FTase β	313	5′ CACAGCTTGGAACTGCTAGATGAACC3′ 5′ AGGTTGCTTCAGGGAGTACAAATACTG 3′	3 min at 95°C (1st cycle); 30 s at 94°C; 30 s at 50°C; 1 min at 72°C (35 cycles); 10 min at 72°C as final extension
MMP9	296	5′ CCCATTTCGACGATGACGAGTTGTG 3′ 5′ GGAGTAGGATTGGCCTTGGAAGATG 3′	3 min at 95°C (1st cycle); 30 s at 94°C; 30 s at 52°C; 1 min at 72°C (35 cycles); 10 min at 72°C as final extension
Fibronectin	196	5′ GAACCATCAAGCCAGATGTCAGAAGC 3′ 5′ TGCCATGATACCAGCAAGGAATTGGG 3′	3 min at 95°C (1st cycle); 30 s at 94°C; 30 s at 52°C; 1 min at 72°C (33 cycles); 10 min at 72°C as final extension
GATA-2	237	5′ GCGCAGCAAGGCTCGTTCCTGTTCAGAA 3′ 5′ CGCCATAAGGTGGTGGTTGTCGTCTGACAA3′	3 min at 95°C (1st cycle); 30 s at 94°C; 30 s at 58°C; 1 min at 72°C (30 cycles); 10 min at 72°C as final extension

0.2 μg/ml ethidium bromide. The gel was photographed under UV light and PCR bands quantified using a laser densitometer linked to a computer analysis system. Gene expression data were normalized against glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and expressed as the absolute ratio between signals. The number of polymerization cycles was chosen within the range providing linear amplification of the cDNA. Moreover, for each gene, increasing amounts of cDNA were amplified in order to check the linearity of the resulting signal. The details of the PCR procedures are given in Table 1.

All RT-PCR experiments were conducted in triplicate. The intraassay variability of PCR (expressed as a ratio of gene expression relative to the expression of the housekeeping gene) was <10%.

RESULTS AND DISCUSSION

The major goal of the present study was to provide a comprehensive database of differential expression of genes related to atherosclerosis in human endothelial cells after treatment with ox-LDL, a well established factor associated with the risk of vascular disease (1, 41). The oxidation of LDL by Cu^{2+} represents the most widely used standardized technique for the preparation of oxidatively modified lipoprotein, which enables comparison with previous studies (5, 7, 21, 39). Data in the literature indicate that the uptake of ox-LDL by different cell types (13, 30) occurs within 1 h. An exposure time to ox-LDL of 6 h was considered appropriate to study changes in mRNA expression of the majority of genes in the HUVEC.

Significant hybridization signals from ^{32}P-labeled cDNA probes from HUVEC were obtained for >50% of the genes spotted onto the membrane. Plasmid and bacteriophage DNAs, which served as negative controls, showed no hybridization signals. Of the 588 genes evaluated, 78 genes displayed a greater than twofold change in expression levels, 57 genes were up-regulated, and 21 genes were down-regulated in response to ox-LDL.

Figure 2 summarizes changes in the differential gene expression induced by ox-LDL, whereas a detailed breakdown of genes differentially expressed in response to ox-LDL and divided into functional classes is given in Table 2.

Transcription factors

Treatment of HUVEC with ox-LDL resulted in a twofold increase in GATA-2 mRNA levels as compared with control.

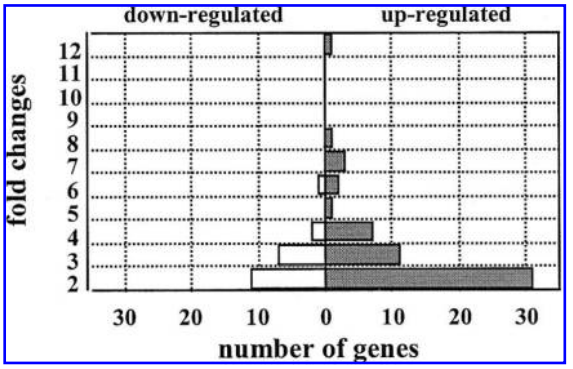

FIG. 2. Summary of the changes in the pattern of mRNA expression induced by 6 h of treatment with Cu^{2+}-oxidized human LDL (ox-LDL) in HUVEC. Only changes greater than ±2-fold were considered significant and reported. See text for experimental details.

GATA-2 is a transcription factor known to play a major role in cell adhesion expression.

P-selectin, which is synthesized by endothelial cells and acts as an adhesion receptor for leukocytes, is partially regulated by GATA-2 (26). In agreement with this observation, the expression of the mRNA encoding for P-selectin and its ligand was also up-regulated by ox-LDL in the current study. The mRNA levels of Fos-related antigen 2, a member of the family of immediate-early genes, were up-regulated in ox-LDL-treated HUVEC. All members of the *fos* family act by binding to activator protein-1 (AP-1) sites as heterodimeric complexes (35). It is interesting to note that one of the AP-1-like sites in the promoter of the cell adhesion protein intercellular adhesion molecule-1 (ICAM-1) is highly responsive to cholesterol (46).

Exposure of HUVEC cells to ox-LDL also induced the expression of the gene encoding for the structurally related sterol regulatory element binding protein-1 (SREBP1) and SREBP2. In addition to regulating rate-limiting enzymes in cholesterol synthesis, it has been recently shown that SREBPs activate every step of the cholesterol synthesis pathway, which contributes to efficient cholesterol synthesis in mammalian cells (33).

Nuclear factor-κB (NF-κB) and AP-1 transcription factors have been reported to be involved in endothelial dysfunction. These transcription factors are regulated at the posttranscriptional level, and their activity can therefore not be detected using cDNA array technology. However, an independent set of experiments (data not shown) confirmed that the treatment of HUVEC with ox-LDL resulted in an increase in the nuclear translocation of NF-κB and AP-1, an effect that was not detectable following treatment with native LDL.

Adhesion molecules, metalloproteinases (MMPs) and extracellular matrix proteins

Under the conditions investigated, the expression of a range of genes encoding for adhesion molecules were affected by ox-LDL. The combination of an up- and down-regulation of genes encoding for adhesion molecules indicates a complex pattern of response to ox-LDL in endothelial cells. The expression of the gene encoding for leukocyte adhesion glycoprotein p150 was down-regulated, whereas mRNA levels of vinculin, cell-surface glycoprotein MUC 18, and both the precursor and the ligand of P-selectin were higher when compared with untreated controls. An increase of the expression of P-selectin in response to ox-LDL in endothelial cells has already been reported by Zhao and Xu (50).

MMPs are a family of proteases capable of degrading virtually all the components of the extracellular matrix. Several lines of evidence support the potential role of MMPs in human atherosclerosis and plaque disruption. In agreement with our results in endothelial cells, ox-LDL up-regulated MMP2 and MMP9 in macrophages (45), thereby possibly contributing to a matrix breakdown in the atherosclerotic plaques that predisposes the vascular wall to plaque disruption and/or vascular remodeling.

A further characteristic of atherosclerosis is the production of extracellular matrix proteins. In the current study, ox-LDL exposure resulted in an increased expression of a number of these proteins, such as fibronectin. The increase of extracellular matrix proteins induced by ox-LDL might in turn facilitate platelet and lymphocyte adhesion, and the migration and proliferation of smooth muscle cells in the atherosclerotic lesion as described previously (22).

Cell receptors and growth factors

The expression of a number of genes encoding for receptors of inflammatory chemokines (chemokine receptor like-2, chemokine receptor type-2, chemokine receptor type-6) were up-regulated in response to the ox-LDL treatment. In endothelial cells, the observed increase in chemokine receptor expression may lead to an increased recruitment of monocytes into the subendothelial space.

A fivefold increase in the thromboxane A_2 (TXA2) receptor (TXA2R) mRNA was also induced in response to ox-LDL treatment. TXA2 is known to be a potent vasoconstrictor and platelet activator, and its cellular action is thought to be mediated via the TXA2R, which affects MCP-1 production by inducing NF-κB and AP-1 activation (16).

Growth factors are biologically active mediators that bind to specific receptors on target cells and regulate genes involved in cell growth, wound healing, and regeneration. We found a significant increase of the expression of platelet-derived growth factor α (PDGFA), PDGFA subunit precursor, and connective tissue growth factor (CTGF) expression in endothelial cells following ox-LDL exposure. An increasing body of evidence suggests that PDGF and CTGF play a key role in development of atherosclerotic lesions (8), and their chronic overexpression may lead to vascular dysfunction (29, 34).

A twofold increase in the expression of genes encoding for advanced glycosylation end product (AGE) specific receptor (RAGE) precursors was also evident. These compounds are thought to represent the link between hyperglycemia and the development of microvascular and neuropathy complications (35, 43). We observed a significant reduction (−3.3 with respect to the baseline level) of phospholipase A_2 (PLA2) expression. PLA2 is an integral component of the stress response system, maintaining cell membrane phospholipid composition and

TABLE 2. GENES DIFFERENTIALLY EXPRESSED BY HUVEC

Fold increase	*Protein/Gene/Swiss protein code*	*Function*
	Cell Receptors and Growth Factors	
−2.4	Adrenocorticotropic hormone receptor/***ACTHR***; Q01718; synonym: melanocortin 2 receptor/***MC2R***; adrenocorticotropin receptor	ACTH receptor associated with G proteins
2.0	Neuropilin-1/***NRP1***; O14786; synonym: vascular endothelial cell growth factor 165 receptor/***VEGF156R***	Growth factor membrane receptor involved in cardiovascular organogenesis
2.1	Chemokine receptor-like 2/***CMKRL2***; Q99527; synonym: IL8-related receptor DRY12/***DRY12***; flow-induced endothelial G protein-coupled receptor (FEG1); G protein-coupled receptor/***GPR30***	Chemokine receptor
2.1	B1 bradykinin receptor/***BDKRB1***; P46663; synonym: BK-1 receptor/***BradyB1***	Bradykinin receptor
2.1	Interleukin-13 receptor α-1 subunit precursor/***IL13RA1***; P78552	Interleukin receptor
2.2	Advanced glycosylation end product-specific receptor precursor/***RAGE***; Q15109	Receptor for glycosylated proteins
2.4	Small inducible cytokine/***SCYA20***; O00585; synonym: β-chemokine Exodus 2, macrophage inflammatory protein 3α (MIP-3-α)/***MIP3A***	Lymphocyte growth factor
2.4	Protein farnesyl transferase α-subunit (Ftase-α)/***FNTA***; P49354	Prenyl transferase enzyme involved in RAS prenylation.
2.5	Vasoactive intestinal polypeptide receptor 2 precursor/***VIPR2***; P41587, Q15870, Q13053; synonym: pituitary adenylated cyclase activating polypeptide receptor 3 (PACAPR3); helodermin-preferring VIP receptor	VIP receptor
2.5	Vascular endothelial growth factor B precursor/***VEGFB***; P49765; synonym: VEGF-related factor/***VRF***	EC growth factor
2.6	Thyroid hormone receptor α-2/***THRA***; ***ERBA2;*** P10827	Nuclear receptor
2.7	Thrombopoietin precursor/***THPO;*** Q92954; synonym: megakaryocyte colony stimulating factor (MSF)	Megakaryocyte growth factor
2.8	Vascular endothelial growth factor receptor 2 precursor (VEGFR2)/***FLK1***; P35968, Q14178; synonym: kinase insert domain receptor/***KDR***	Growth factor, cytokine, and chemokine receptor
−2.8	Endothelin-converting enzyme 1/***ECE1***; P42892	Membrane-associated enzyme
2.9	Platelet basic protein precursor (PBP)/***PPBP***; synonym: connective tissue activating peptide III (CTAP III)/***CTAP3***; low-affinity platelet factor IV (LA PF4); β-thromboglobulin (β-TG)/***TGB1***; P02775	Growth factor induces mitosis, glycolysis, and prostaglandin E_2 secretion
3.3	CC chemokine receptor type 9 (C-C CKR-9)/***CMKBR9***; ***CCR9***; O00537	Chemokine receptor
3.6	Connective tissue growth factor precursor/***CTGF***; P29279	Growth factor secreted by EC, induces chondrocyte proliferation and differentiation
3.9	Atrial natriuretic peptide receptor B precursor (ANPB)/***ANPRB***; P20594; synonym: guanylate cyclase B (GCB)	Hormone receptor

(Continued)

TABLE 2. GENES DIFFERENTIALLY EXPRESSED BY HUVEC *(CONTINUED)*

Fold increase	*Protein/Gene/Swiss protein code*	*Function*
4.6	Vasopressin V1a receptor/***AVPR1A***; P37288; synonym: vascular/hepatic-type arginine vasopressin receptor; antidiuretic hormone receptor1A (ADHR1A)	Hormone vasopressin receptor
4.6	Vasoactive intestinal polypeptide receptor 1 precursor/***VIPR1***; P32241; synonym: pituitary adenylate cyclase activating polypeptide type II receptor (PACAPR2)	Hormone VIP receptor, activates adenylate cyclase enzyme
4.7	Thromboxane A_2 receptor/***TBXA2R***; P21731; synonym: prostanoid TP receptor	Thromboxane A_2 receptor, strongly stimulates platelet adhesion
4.8	C-X-C chemokine receptor type 6/***CXCR6***; P51684	Chemokine receptor
7.8	Platelet-derived growth factor receptor α-subunit/***PDGFRA***; P16234; synonym: CD140A antigen	PDGFA receptor, with tyrosine kinase activity
8.8	Platelet-derived growth factor A subunit precursor/***PDGFA***; P04085	Mitogenic factor for mesenchymal cells
12.2	Protein farnesyl transferase β-subunit (FTase-β) ***FNTB***; P49356	Prenyl transferase enzyme involved in RAS prenylation
	Adhesion Molecules	
−2.6	Integrin αX/***ITGAX***; P20702; synonym: leukocyte adhesion glycoprotein p150, 95 α subunit precursor; leukocyte adhesion receptor p150, 95; CD11C antigen; leu-M5	Fibrinogen receptor, mediates cellular interactions during inflammation
2.3	Vinculin/***VCL***; P20702	Cellular adhesion protein, binds actin microfilaments to the plasmatic membrane
2.8	P-selectin precursor/***SELP***; P16109; synonym: granule membrane protein 140 (GMP140); PADGEM; CD62P antigen; leukocyte-endothelial cell adhesion molecule 3 (LECAM3)	Ca^{2+}-dependent receptor, mediates the interaction of activated EC or platelets with leukocytes
2.9	Macrosialin precursor/***CD68***; P34810	Mediates intercellular interactions by binding to selectins
3.2	Cell-surface glycoprotein/***MUC 18***; P43121; synonym: melanoma-associated antigen A32; CD146 antigen; melanoma adhesion molecole/***MCAM***	Adhesion molecule
3.6	Cadherin-16/***CDH16***; P43121; synonym: KSP-cadherin	Ca^{2+}-dependent adhesion protein
4.6	P-selectin glycoprotein ligand 1/***SELPLG***; Q14242	Binds P selectin and mediates rolling of neutrophils and lymphocytes on EC
	Matrix Proteins and Proteases	
−6.3	Carboxypeptidase N catalytic chain/***CPN1***; P15169	Amino- and carboxypeptidases
−4.2	Collagenase 3 precursor/***MMP13***; P45452; synonym: matrix metalloproteinase 13 (MMP13)	Metalloproteinase, cleaves collagen type I
−4.0	Collagen α-1 (XVIII) subunit/***COL18A1***; P39060	Gene product containing endostatin and inhibiting EC proliferation and angiogenesis
−3.9	Plasma kallikrein/***KLK3***; P01043; synonym: kininogen	Activates factor XII, cleaves bradykinin from HMW kyninogen, cleaves pro-renin.
−3.4	Matrilysin/***MMP7***; P09237; synonym: matrix metalloproteinase 7 (MMP7)	Metalloproteinase, cleaves casein, fibronectin and gelatin types I, II, V
−2.6	Collagen 19 α-1 subunit precursor/***COL19A1***; Q13676	Extracellular matrix protein

TABLE 2. GENES DIFFERENTIALLY EXPRESSED BY HUVEC *(CONTINUED)*

Fold increase	*Protein/Gene/Swiss protein code*	*Function*
2.1	Matrix metalloproteinase 17 (MMP17)/***MMP17***; Q14850; synonym: membrane-type matrix metalloproteinase 4 (MT-MMP4)	Metalloproteinase, degrades extracellular matrix
2.3	α-2 type V collagen subunit/***COL5A2***; P05997	Extracellular matrix protein
2.4	Matrix metalloproteinase-19/***MMP19***; Q99580; synonym: MMP RASI; MMP-18	Metalloproteinase, degrades extracellular matrix
3.0	72-kDa type IV collagenase/***CLG4A***; P08253; synonym: matrix metalloproteinase 2/***MMP2***	Metalloproteinase, cleaves gelatin type I and collagen types IV, V, VII, X
4.5	92-kDa type IV collagenase/***CLG4B***; P14780; synonym: matrix metalloproteinase 9/***MMP9***	Metalloproteinase, involved in osteoblast reabsorption
5.9	Fibronectin precursor (FN)/***FN***; P02751	Extracellular matrix protein, binds collagen, actin, and heparin
6.3	Collagen α 4 subunit precursor/***COL4A4***; P53420	Extracellular matrix protein, major component of the basal membrane
	Lipid Metabolism	
−3.8	Farnesyl-diphosphate farnesyl transferase/***FDFT1***; P37268; synonym: squalene synthetase	Catalyzes squalene synthesis
−2.1	Cytochrome P450 IB1/***CYP1B1***; Q16678	Monooxygenase, oxidizes steroids, fatty acids, and xenobiotics
−2.1	Cytochrome P450 IIB6/***CYP2B6***; P20813	Monooxygenase, oxidizes steroids, fatty acids, and xenobiotics
−2.1	Estradiol 17 β-dehydrogenase 2/***EDH17B2***; P37059	Involved in hormone metabolism
−2.0	Hydroxyacyl-CoA dehydrogenase; 3-ketoacyl-CoA thiolase; enoyl-CoA hydratase β-subunit/***FADB***; P55084	Involved in lipid metabolism
2.0	Diphosphomevalonate decarboxylase/***MVD***; P53602; synonym: mevalonate pyrophosphate decarboxylase (MPD)	Involved in isoprenoid synthesis
2.0	Alcohol dehydrogenase 5 ? polypeptide/***ADH***; P11766	Catalyzes the formation of aldeydes, ketones, and NADH
2.1	Prostaglandin G/H synthase 1/***PTGS1***; P35354; synonym: COX-1	Catabolizes arachidonic acid
2.1	Catalytic AMP-activated protein kinase alpha 2/***PRKAA2***; ***AMPK2***; P54646	Regulates fatty acid synthesis by phosphorylating acetyl-CoA carboxylase
2.6	Nonspecific lipid-transfer protein precursor (NSLTP)/***SCP2***; P22307; synonym: sterol carrier protein-2; sterol carrier protein X (SCPX)	Carrier protein for phospholipids, cholesterol, and other lipids
2.6	NADH-cytochrome B5 reductase/***DIA1***; P00387	Desaturates and extends fatty acids, involved in xenobiotic metabolism
3.4	Rab geranylgeranyl transferase α subunit/***RABGGTA***; Q92696	Catalyzes the transfer of geranylgeranyl groups on RAB proteins
3.9	Oxysterol-binding protein/***OSBP***; P22059	Involved in steroid metabolism
6.4	Catalytic AMP-activated protein kinase α-1/***PRKAA1***; ***AMPK1***; O00286	Regulates fatty acid synthesis by phosphorylating acetyl-CoA carboxylase
	Transcription Factors	
−3.2	COUP transcription factor 1/***TFCOUP1***; P10589; synonym: v-erbA related protein (EAR3)/***ERBAL3, EAR3***	Binds to ovalbumin promoter and parlindrome sequences 5′ AGGTCA 3′

(Continued)

TABLE 2. GENES DIFFERENTIALLY EXPRESSED BY HUVEC *(CONTINUED)*

Fold increase	*Protein/Gene/Swiss protein code*	*Function*
−2.0	Endothelial pas domain protein 1/***EPAS1***; Q99814; synonym: member of pas protein 2 (MOP2)	Transcription factor involved in the regulation of oxygen-regulated genes
2.1	Endothelial transcription factor GATA2/***GATA2***; P23769	Transcription factor involved in the regulation of endothelin gene, binds to 5′ AGATAG 3′ sequence
2.3	Orphan nuclear receptor EAR-2/***EAR2***; P10588; synonym: v-erbA related protein (EAR2)	Transcription factor
2.8	Transcriptional enhancer factor (TEF1)/***TEAD1, TEF1***; P28347; synonym: protein GT-IIC; transcription factor 13 (TCF13)	Transcription factor, its activity is mediated by TIF factors
3.2	Nuclear receptor ROR–α/***RORA***; P35397; synonym: transcription factor RZR-α (RZRA); RAR-related orphan receptor α-1 (ROR-α1; RORA1) + RORA4	Transcription factor, binds to HRE sequences
3.4	Retinoic acid receptor RXR-β/***RXRB***; P28702	Transcription factor, involved in the response to retinoic acid
4.6	Sterol regulatory element-binding transcription factor 1/***SREBF1***; P36956	Transcription factor, binds SRE-1 sequences, regulates genes involved in cholesterol homeostasis
7.1	Fos-related antigen 2 (FRA2)/***FRA2, FOSL2***; P15408	Member of the FOS family proteins
7.4	Sterol regulatory element-binding transcription factor 2/***SREBF2***; Q12772	Transcription factor, binds SRE-1 sequences, regulates genes involved in cholesterol homeostasis
	Nonclassified	
−3.7	Proliferating cell nuclear antigen/***PCNA***; P12004	DNA polymerase and topoisomerase
−3.6	Annexin VIII/***ANX8***; P13928	Anticoagulant
−3.2	Sodium-independent organic anion transporter/***SLC21A3***; P46721; synonym: organic anion transporting polypeptide/***OATP***	Mediates sodium-independent transport of organic anions
−2.5	Serum albumin precursor/***ALB***; P02768	Plasmatic protein, essential for osmotic pressure regulation
−2.0	BCL-2-related protein A1/***BCL2A1***; Q16548; synonym: BFL1 protein/***BFL1***; hemopoietic-specific early response protein; GRS protein/***GRS***	Member of the bcl-2 family proteins, delays apoptosis
2.2	Insulin-responsive glucose transporter type 4/***GLUT4, SLC2A4***; P14672	Insulin-regulated glucose carrier
2.8	Cardiac and skeletal muscle sodium channel protein α-subunit/***SCN6A***; Q01118	Voltage-dependent sodium ionic channel
3.3	Thiazide-sensitive sodium-chloride cotransporter/***TSC***; ***SLC12A3***; P55017; synonym: Na-Cl symporter	Sodium chloride carrier

Listed are the genes differentially expressed by HUVEC after 6 h of incubation with Cu^{2+}-oxidized human LDL. Genes have been divided into functional classes.

providing the substrate for the synthesis of eicosanoids. PLA2 therefore acts as a mediator of different processes, such as inflammation, immune responses and NADPH oxidase activation. An increased PLA2 activity is often evident in membranes of ischemic tissues (see 36) and endothelial cells challenged by reactive oxygen species (44).

RAS is a family of small GTP-binding proteins playing an important regulatory role that mediates intracellular signaling pathway correlated to PDGF receptor (PDGFR) activation. In the cell, functional RAS is associated with the plasma membrane. This is achieved by posttranslation farnesylation catalyzed by the enzyme farnesyl transferase (FTase) (48). In the present study, we found that treatment with ox-LDL was associated with a significant up-regulation of mRNA levels encoding for both α and β subunits of FTase.

Protein FTase is a member of the protein prenyl transferase family of lipid-modifying enzymes (2). This family of enzymes catalyzes the formation of thioether bonds between the C1 atom of farnesyl isoprenoid lipid and a cysteine residue at or near the C terminus of the acceptor protein. The overexpression of the β subunit has been correlated to the hyperstimulation of cell proliferation and cancerogenic transformations induced by growth factors (24), and has been revealed in tumors together with the mutation of k-ras (3). FTase has in fact been proposed as a possible target for the development of antitumor and vascular therapies in malignant potential tumors and in carcinoma cases in association with the k-ras mutation (40). These findings suggest that there may be a direct relationship between the k-ras dysfunction and expression of FTase β-subunit gene (40).

The current study provides experimental evidence that ox-LDL could induce alterations in the signaling pathway mediated by PDGFR and FTase, which could in turn lead to metabolic dysfunctions of the endothelium. As proposed by Goalstone *et al.* for insulin (12), ox-LDL could alter endothelium homeostasis by enhancing the influence of other growth factors and adrenergic agonists, cytokines, and advanced glycosylation end products, in the RAS signal transduction cascade. Further studies are warranted to investigate whether the up-regulation of FTase also increases the activity of RAS associated with the plasma membrane in HUVEC challenged with ox-LDL.

Expression analysis of individual transcripts

Methods based on subtractive enrichment procedures can sometimes yield false positive results, and thus the expression pattern of individual clones should be further verified by an external methodology (20, 49). Therefore, we have used *post hoc* analysis in order to determine the expression changes of eight genes present on the cardiovascular array in response to ox-LDL using RT-PCR in HUVEC. Most importantly, the alteration of the expression pattern of all genes monitored was substantiated by RT-PCR, confirming in all cases that the occurrence of the changes was in the same direction as that indicated by the cDNA array (see Table 3). Thus, the current RT-PCR data clearly support the reliability of our microarray analysis.

In conclusion, our study enabled the identification of candidate genes possibly involved in atherogenesis in human endothelial cells challenged with ox-LDL. Treatment of HUVEC with ox-LDL altered the expression of genes encoding for transcription factors, cell receptors, adhesion molecules, extracellular matrix proteins, and enzymes involved in cholesterol metabolism that compose a "response to injury" type of effect. This is consistent with a proinflammatory environment associated with alterations in endothelial cell metabolism described in the early stages of atheroma formation (18, 42). Furthermore, important interactions between these gene products have been described. Future studies will address the effect of *in vivo* oxidatively modified LDL (LDL−), which is considered to be the atherogenic form of LDL in the blood stream (27, 37), on differential gene expression in human endothelial cells.

TABLE 3. EXPRESSION OF INDIVIDUAL TRANSCRIPTS FROM HUVEC TREATED WITH OX-LDL

Gene	*cDNA array*	*RT-PCR*
CTGF	++	1.87
PDGF	+++	2.65
PDGFR	+++	2.65
FTase β	+++	2.40
P-selectin	+	1.75
Fibronectin	+++	2.86
MMP9	+++	2.52
GATA-2	+	2.04

The expression of individual transcripts from HUVEC treated with Cu^{2+}-oxidized LDL (ox-LDL) by means of RT-PCR is presented. Results have been normalized to the G3PDH signal and are expressed as the value relative to untreated cells. See text for experimental details. Data obtained from cDNA array are indicated as follows: +, two- to threefold increase; ++, three- to fourfold increase; +++, greater than fourfold increase.

ABBREVIATIONS

AGE, advanced glycosylation end products; AP-1, activator protein-1; CTGF, connective tissue growth factor; EC, endothelial cells; FTase, farnesyl transferase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HUVEC, primary human endothelial cells; ICAM-1, intercellular adhesion molecule-1; LDL, low-density lipoprotein; MMP, metalloproteinase; NF-κB, nuclear factor-κB; ox-LDL, oxidized LDL; PDGFA, platelet-derived growth factor-α; PDGFR, platelet-derived growth factor receptor; PLA2, phospholipase A_2; RAGE, AGE-specific receptor; RT-PCR, reverse transcription–polymerase chain reaction; SREBP, sterol regulatory element binding protein; TXA2, thromboxane A_2; TXA2R, thromboxane A_2 receptor.

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