INTRODUCTION

Oxidized low density lipoprotein (oxLDL) has been identified as a principal mediator in the pathogenesis of atherogenesis (1, 2). Its uptake into endothelial cells is mediated by the lectin-like oxLDL receptor-1 (LOX-1, gene name OLR1) (3). LOX-1 is expressed on the cell surface of macrophages, vascular smooth muscle cell, and, importantly, in endothelial cells (4). Its functional meaning for atherogenesis has been shown in both human and animal studies (5). In a proof of concept study Mehta et al. (6) established a pro-atherosclerotic role of LOX-1 in a LOX-1 knock-out model in mice. Deletion of the OLR1 gene results in reduced formation of atherosclerotic lesions in a pro-atherogenic genetic background under a high-cholesterol diet (6). LOX-1 activation by binding of oxLDL induces profound alterations in the endothelium, which are characterized by activation of nuclear factor-kappa B (NF-κB) through reactive oxygen species (7), up-regulation of adhesion molecules (8), and endothelial apoptosis (9). Expression of LOX-1 is induced by inflammatory cytokines, oxidative stress, vasoconstrictive peptides, shear stress, and oxLDL (10, 11). Increased LOX-1 expression is associated with pro-atherogenic conditions such as hypertension, hyperlipidaemia, and diabetes (12). Moreover, an increased level of the soluble form of LOX-1, which is generated from LOX-1 by proteolytic cleavage at its membrane-proximal extracellular domain, is found in patients with acute coronary syndromes (13). As is the case with oxLDL, LOX-1 is accumulated in atherosclerotic lesions (14). Furthermore, up-regulation of LOX-1 by L-NAME-induced nitric oxide deficiency was shown in endothelial cells (15). However, although the involvement of LOX-1 in the pathogenesis of atherosclerosis is obviously proven, its role as mediator of endothelial dysfunction, as a key variable in the pathogenesis of atherosclerotic vessel disease, is still much less well evidenced. Such a role has been repeatedly postulated (11, 16) but experimental data that relate humoral factor-mediated LOX-1 expression in endothelial cells directly to endothelium-dependent vasoreactivity in humans have never been produced. As NO plays a central role in the homeostasis of the cardiovascular system (17) we addressed this issue in the present study. For this purpose, in vitro measurements of endothelium-dependent vasoreactivity (EDVR) were combined with an in vitro model in which the effects of serum from the same patients on LOX-1 and
endothelial nitric oxide synthase (eNOS) expression in human umbilical vein endothelial cells (HUVEC) was assessed.

**MATERIALS AND METHODS**

**Study design and patients**

Patients with coronary artery disease and/or risk factors for cardiovascular disease (hypertension, diabetes, hyperlipidaemia) were included in the study (n=27). Coronary artery disease was based on diagnostic cardiac catheterization and defined as stenosis of 50% or more in a major coronary artery or in a major branch. The extent of coronary artery disease was expressed as number of affected arteries: i.e. as one-, two or three-vessel disease. Exclusion criteria were myocardial infarction and impaired left ventricular function (≤60%). Hypertension was defined as a systolic blood pressure of 130 mm Hg or more, a diastolic blood pressure of 90 mm Hg or more, or self-reported use of antihypertensive drugs. Diabetes was defined as self-reported physician diagnosis of diabetes or by a serum HbA1C of >7.0%. Hyperlipidaemia was defined by total cholesterol levels of 5.2 mmol/l or higher, by triglyceride levels of 2.3 mmol/l or higher or by self-reported use of statins. All participants gave informed written consent. The study protocol complied with the Declaration of Helsinki and was approved by the local ethics committee at the University Medicine Greifswald.

**Quantification of cotinine**

As for smoking status, study participants were classified as non-smokers or current smokers based on the analysis of blood samples for the nicotine metabolite cotinine using the EIA Cotinine Serum Kit (Cozart Plc, Abington, United Kingdom) according to the manufacturer’s instructions.

**Assessment of endothelium-dependent vasoreactivity**

EDVR was assessed as FMD by measuring the increase in the diameter of the brachial artery during reactive hyperaemia after transient occlusion of the forearm. Ultrasoundography was performed by an experienced investigator who was blinded to the clinical characteristics of the patients. The brachial artery was scanned in the antecubital fossa region longitudinally. The diameter of the brachial artery above the bifurcation in the right arm was measured from B-mode ultrasound images, using a 7.5-MHz linear array transducer (HP Sonus 5500®, Hewlett Packard). Brachial artery images were obtained with positioning of the ultrasound transducer at the elbow in an area reaching from three cm above to three cm below the antecubital crease. Once an optimal transducer position had been achieved in this area, the skin surface was marked, and the arm was kept in the same position throughout the entire course of the measurements. The patients lay quietly for ten minutes before the first scan. All measurements were performed with the patients in the lying position. After baseline measurements of the brachial artery diameter, a blood pressure cuff placed around the forearm was inflated to a pressure of 250 mm Hg and released after five minutes. Next, measurements of brachial artery diameter were repeated sixty seconds after cuff deflation. Measurements were taken online from the anterior to the posterior M-line (i.e. the interface between media and adventitia) at the end of diastole, defined by the R wave on a continuously recorded electrocardiogram. Vessel diameters of four consecutive cardiac cycles were analysed for each scan, and the measurements were then arithmetically averaged. The responses of the vessel diameters to the reactive hyperaemia were expressed as absolute (FMD\textsubscript{mm}) and as a percentage of increase (FMD%) from the baseline diameter. Recurrent controls of intra-reader variations of FMD measurements are conducted semi-annually at our institution. All controls (eight patients studied twice with 24 hours between studies) revealed coefficients of variations of ≤10.0%, Spearman correlation coefficients of >0.90 and mean differences in FMD (two standard deviations) of <2.5% (±0.5%). These values are similar to those reported in other studies (18).

**Cell culture methods**

Human umbilical vein endothelial cells (HUVEC) were obtained from collagenase type II (Biochrom KG, Berlin, Germany) digested umbilical cords. Cells were cultured in endothelial cell medium (MCDB 131, Invitrogen GmbH, Karlsruhe, Germany) as described previously (19). Refreshing took place every two or three days. First- to fifth-passage cells were counted after treatment with trypsin, and were re-plated at a density of 100,000 cells per well in six well plates. The endothelial phenotype was confirmed using phase-contrast microscopy and positive staining for the endothelial-specific von Willebrand factor as analysed with flow cytometry (data not shown). All in vitro experiments were carried out within the third passage of the HUVEC after isolation. HUVEC were exposed to a mixture of equal volumes of medium and participants’ sera for four hours by the same investigator who was blinded to the identities of the participants and clinical data. Each set of experiments was done simultaneously using the same preparation of HUVEC. As control, cells were treated with media in the absence of sera. Bradykinin, prostacyclin and TNF-α were purchased from Sigma, Germany.

**Determination of eNOS and LOX-1 mRNA expression**

After a four-hours treatment with a mixture of equal volumes of medium and patients’ sera, total RNA was isolated using standard procedures including digestion with DNase according to the manufacturer’s protocol (Qiagen, Hilden, Germany). Quantification of eNOS and LOX-1 mRNA expression was performed using TaqMan® Gene Expression Assays (Applera Deutschland GmbH, Darmstadt, Germany) on the Applied Biosystems ABI Prism 7700 sequence-detection system. Additionally, GAPDH mRNA was quantified as an endogenous control to normalize for differences in the amount of total RNA in each sample. Reverse transcription was performed using 300 nanograms of RNA and 2.5 µM of random hexameres in a total volume of 100 µl according to the manufacturer’s instructions (Applera Deutschland GmbH). PCR conditions for all samples were as described previously (19, 20). Relative gene expression data were determined using the 2^−ΔΔCt method.

**Determination of eNOS and LOX-1 protein expression**

Protein analysis was performed as described previously (20). Shortly, HUVEC were washed twice with media and then homogenized in lysis buffer containing 10 mmol/l Tris (pH 7.4), 1 mmol/l sodium ortho-vanadate and 1% (w/v) sodium dodecyl sulphate. Protein concentrations in the lysates were measured using the bicinchoninic acid Protein Assay Kit (Perbio Science, Bonn, Germany). Fifty micrograms of protein were separated on 10% polyacrylamide gels according to standard procedures and transferred to a polyvinylidene difluoride membrane. A monoclonal antibody against human eNOS (Sigma-Aldrich Chemie GmbH, Taukirschen, Germany) was used as primary antibody. LOX-1 protein was detected with polyclonal antibodies against the C-type lectin-like domain (amino acid residues 143 to 273) of the human LOX-1 protein (Landsberger et al., unpublished results). Antibodies against eNOS and GAPDH were
Detection of LOX-1, eNOS, and GAPDH proteins was performed using an enhanced chemiluminescence detection system from Amersham Biosciences, Freiburg, Germany. After stripping of the membrane, equal loading of the gel was assessed by re-probing with an antibody against the human GAPDH protein (Sigma, Germany). Band intensities were normalized to GAPDH expression.

**Statistical analysis**

Sample size was calculated as following: for cell culture experiments, power was set to 80%. To detect changes in LOX-1 or eNOS RNA or protein expression by 20%, the result of the sample size calculation was five. For FMD measurements, power was set to 80% as well. Sample size was estimated to be approximately 26 for a power of 80% to detect a 2.9% difference in FMD after reactive hyperaemia assuming a common standard deviation of 5%.

**RESULTS**

**Effects of bradykinin on LOX-1 expression in human umbilical vein endothelial cells**

Regulation of EDVR is mediated among other things by vasoactive substances prostacyclin and bradykinin. As

<table>
<thead>
<tr>
<th>Patients (n=27)</th>
<th>Percentage</th>
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<tbody>
<tr>
<td><strong>Total</strong></td>
<td>100</td>
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<tr>
<td>male (n)</td>
<td>16</td>
</tr>
<tr>
<td>female (n)</td>
<td>11</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
</tr>
<tr>
<td>men years (25%-75% percentile)</td>
<td>63 (52–71)</td>
</tr>
<tr>
<td>women years (25%-75% percentile)</td>
<td>62 (60–70)</td>
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<tr>
<td><strong>Coronary artery disease (n)</strong></td>
<td>16</td>
</tr>
<tr>
<td>hypertension (n)</td>
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</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>138 ± 3</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>81 ± 2</td>
</tr>
<tr>
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<td>9</td>
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<tr>
<td>hyperlipidaemia (n)</td>
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<td><strong>Current medication</strong></td>
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<tr>
<td>beta-blocker</td>
<td>18</td>
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<tr>
<td>statin</td>
<td>16</td>
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<tr>
<td>ACE inhibitor</td>
<td>12</td>
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<tr>
<td>diuretic</td>
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<tr>
<td><strong>Serum parameters</strong></td>
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<tr>
<td>total cholesterol, mmol/l</td>
<td>5.15 ± 0.29 (n=24)</td>
</tr>
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<td>triglycerides, mmol/l</td>
<td>1.80 ±0.26 (n=23)</td>
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<tr>
<td>HDL cholesterol, mmol/l</td>
<td>1.30 ±0.08 (n=24)</td>
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<tr>
<td>LDL cholesterol, mmol/l</td>
<td>3.07 ±0.27 (n=24)</td>
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<td><strong>Smoking status</strong></td>
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<tr>
<td>non-smoker</td>
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<tr>
<td><strong>FMD%</strong></td>
<td>5.7 ± 0.7</td>
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<td><strong>LOX-1 expression, % of medium-treated HUVEC</strong></td>
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<tr>
<td>mRNA</td>
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<tr>
<td>protein</td>
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<tr>
<td><strong>eNOS expression, % of medium-treated HUVEC</strong></td>
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<tr>
<td>mRNA</td>
<td>91 ± 9</td>
</tr>
<tr>
<td>protein</td>
<td>114 ± 8</td>
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</tbody>
</table>

BP blood pressure, continuous variables are mean ± S.E.M.
inflammation is a condition that underscores many cardiovascular pathologies including endothelial dysfunction, we used TNF-α as trigger of inflammation. To test whether bradykinin or prostacyclin were able to modulate LOX-1 mRNA expression, HUVEC were treated with bradykinin or prostacyclin (each 10⁻⁷ mol/l) for four hours. As positive control, cells were treated with TNF-α (10 ng/ml). In the absence of TNF-α or bradykinin, HUVEC expressed little LOX mRNA (5±3%, P<0.01) compared to TNF-α-treated cells (100%, \( \text{Fig. 1A} \)). Treatment of HUVEC with 10⁻⁷ mol/l bradykinin had no relevant effects on baseline LOX-1 mRNA expression (7±5%, \( P<0.01 \) compared to TNF-α-treated cells). Pre-incubation of HUVEC with bradykinin 30 minutes prior to stimulation with TNF-α prevented the cytokine-induced up-regulation of LOX-1 mRNA expression (29±17%, \( P<0.01 \) vs. TNF-α-treated cells). Bradykinin-induced effects on LOX-1 mRNA expression were confirmed at the protein level (\( \text{Fig. 1B} \)). Pre-incubation of HUVEC with bradykinin 30 minutes prior to cytokine-stimulation impeded the increase in LOX-1 protein expression (63±3%, \( P<0.01 \) vs. TNF-α-treated cells) induced by TNF-α (100%). In the absence of TNF-α, basal LOX-1 protein expression (69±7%, \( P<0.01 \) vs. TNF-α-treated cells) was not significantly affected by bradykinin (58±6%, \( P<0.01 \) vs. TNF-α-treated cells). Prostacyclin-mediated effects on LOX-1 mRNA and protein expression were comparable to those mediated by bradykinin (\( \text{Fig. 1A, 1B} \)).

Clinical characteristics of the study population

Baseline clinical characteristics are shown in Table 1. Among the study population, baseline clinical characteristics were not different between women and men with the exceptions of baseline brachial artery diameters and EDVR. Baseline brachial artery diameters were significantly smaller in female patients (3.61±0.11 vs. 4.47±0.145 mm, \( P=0.003 \)). There was a tendency towards higher levels of EDVR among females (\( P=0.089 \) vs. males).

Effects of serum on LOX-1 mRNA and protein expression in vitro

To investigate whether serum of female subjects affects LOX-1 expression in human endothelial cells differently compared to serum of male patients, HUVEC were incubated with serum in an equal volume of media. After a 4-hour incubation period, total RNA was isolated and the expression of LOX-1 was quantified by reverse transcription PCR. LOX-1 protein expression was quantified by Western blotting. In general, we found that the mRNA expression of LOX-1 correlated with the protein expression of LOX-1 (\( r=0.809, P=0.001 \)). There was no significant difference in LOX-1 mRNA expression (170±64 versus 115±25%, \( P=0.554 \)) and protein expression (115±8 versus 107±4%, \( P=0.711 \)) between sera of female or male patients. For all study patients, no significant correlation was found between \( \text{in vitro} \) LOX-1 expression and flow-mediated EDV (\( \text{Fig. 3, lower panel} \), \( r=0.021, P=0.312 \)). To validate the experimental system used, we quantified eNOS mRNA and protein expression in the same samples. As observed, mRNA expression of eNOS correlated with the protein expression of eNOS (\( \text{Fig. 2, lower panel} \), \( r=0.409, P=0.034 \)). Contrary to \( \text{in vitro} \) LOX-1 expression, eNOS protein expression was associated with FMD% (\( \text{Fig. 3, lower panel} \), \( r=0.788, P=0.001 \)). In the subgroup with coronary artery disease, only reduced eNOS protein expression was associated with impaired flow-mediated EDV (\( r=0.788, P=0.001 \)) but not LOX-1 protein expression (\( r=0.409, P=0.112 \)). In the subgroup with risk factors but without coronary artery disease, reduced eNOS protein expression was also associated with impaired flow-mediated EDV (\( r=0.718, P=0.011 \)) but not LOX-1 protein expression (\( r=-0.136, P=0.673 \)).

Within the study population of 27 participants, four were classified as current smokers as substantiated by positive testing for the nicotine metabolite cotinine. Patients tested positive for cotinine had a significant reduction in flow-mediated EDV of the brachial artery (\( r=-0.386, P=0.047 \)) and eNOS protein expression (\( r=-0.389, P=0.024 \)). Further analysis revealed that flow-mediated EDV was associated with diabetes (\( r=-0.434, P=0.024 \)).

![Fig. 1](image-url)

**Fig. 1.** Effects of humoral factors on endothelial LOX-1 mRNA (A) and protein (B) expression. HUVEC were treated with bradykinin or prostacyclin (10⁻⁷ mol/l each) for 30 minutes prior to exposure to TNF-α (10 ng/ml). Total mRNA was isolated after four hours and analysed for LOX-1 and GAPDH mRNA and protein expression as outlined in Material and Methods. Data are from five independent cell preparations and are presented as the percentage of TNF-α-treated cells (mean ±S.E.M.); * \( P<0.01 \) versus TNF-α-treated cells.
DISCUSSION

In the present study, we tested the hypothesis that in vivo measurements of EDVR among patients with a known history of cardiovascular disease are inversely correlated with in vitro expression of LOX-1, which may be mediated by circulating factors in serum from the patient. Although EDVR was correlated with the in vitro expression of eNOS, an inverse association with LOX-1 expression could not be established, neither at the level of LOX-1 mRNA nor with the amount of LOX-1 protein synthesized by endothelial cells after exposure to serum from the same patients.

Previous in vitro investigations have shown that endothelial LOX-1 is up-regulated by serum proteins such as C-reactive

Fig. 2. Correlation between mRNA and protein expression in human endothelial cells. HUVEC were treated with a mixture of equal volumes of serum and medium. After four hours, total RNA and protein extracts were prepared as described in the Material and Methods section. Quantification of mRNA was performed by reverse transcription PCR, protein expression was assessed by Western blotting and subsequent densitometric analysis. Data are presented as a percentage of the expression in control cells, which were treated with medium in the absence of sera. LOX-1 and eNOS gene expression data were normalized for GAPDH expression.

Fig. 3. Correlation between flow-mediated EDV (in %) and in vitro LOX-1 protein expression (A) and eNOS protein expression (B). HUVEC were treated with a mixture of equal volumes of serum and medium. After four hours, total protein extracts were prepared as described in Material and Methods. Protein expression was assessed by Western blotting and subsequent densitometric analysis. Expression data are presented as a percentage of the expression in control cells, which were treated with medium in the absence of sera. LOX-1 and eNOS gene expression data were normalized for GAPDH expression. Flow-mediated EDV (%) was analysed as described in Materials and Methods.
protein (21) and by inflammatory mediators such as transforming growth factor-beta (TGF-β), lysophosphatidylcholine, TNF-α, and interleukin-6. Similar effects have been described for the vasopetides endothelin-1, and angiotensin II (11, 22). The same mediators, though, have likewise been implicated in the impairment of EDVR. This applies for C-reactive protein (23) as it does for inflammatory mediators (23-25) or for the impairment of EDVR. This applies for C-reactive protein (23) as mediators, though, have likewise been implicated in the discussion. As with any in vitro experiment, the model has shortcomings, which render direct extrapolation to in vivo conditions difficult. It was conceivable that LOX-1 measured in endothelial cells in vitro after treatment with patients’ serum does not adequately mirror the in vivo expression in the endothelium. However, the same experimental model has meanwhile been employed and validated in a number of studies (28, 29). Importantly, our data disclosed a significant correlation of EDVR with in vitro expression of eNOS. Not only is this in good agreement with previous studies (28, 29) but also supports the validity of our in vitro model since nitric oxide synthesis by eNOS is the main contributor to EDVR (30, 31). Impairment of NO production by eNOS is one of the causes for endothelial dysfunction, which is well recognised in cardiovascular diseases (30), but NO can also be generated by neuronal NO synthase (nNOS). However, chronic inhibition of neuronal NO synthase in spontaneously hypertensive rats, for example, does not change endothelial function of thoracic and mesenteric arteries, which were affected differently regarding contractility and structure of the vessels (32). In the same animal model, application of prazosin, an alpha-adrenergic blocker, improved the responses of coronary arteries to acetylcholine and evoked relaxation of the vessels (33). However, we have not analysed the expression of neuronal NO synthase and the effects of alpha-adrenergic blockade on LOX-1 and eNOS expression in the present study. Under the circumstance that our study investigated solely endothelial cells, the findings should be regarded as only endothelial cells-specific. Synthesis of LOX-1 in response to various stimuli is by no means confined to endothelial cells. It occurs in vascular smooth muscle cells, macrophages, and activated platelets (11). Thus, it could be assumed that the functional link between LOX-1 expression and EDVR depends on a complex array of interaction of endothelial cells with other cellular components of the blood and the vascular wall. Additionally, our model tested the effect of patients’ serum on cultivated endothelial cells. Hence, it rests on the assumption that the link of EDVR and LOX-1 expression is mediated through circulating factors such as vasopetides, cytokines and other inflammatory proteins (11, 21, 22). The impact of factors that exert a spatially limited effect within the vessel wall could have been missed, and our findings have to be interpreted accordingly. The major conclusion of our study has therefore to be that circulating factors do not mediate an association of endothelial LOX-1 expression with EDVR in human conduit vessels. There is evidence that binding to LOX-1 of oxLDL in endothelial cells induces an increased cellular production of superoxide radicals (34), which mainly act in a paracrine manner to chemically inactivate nitric oxide in endothelial cells and vascular smooth muscle cells. Furthermore, it has been demonstrated that the regulation of EDVR is a multifactorial process, which also involves factors that have, thus far, not been implicated in the regulation of LOX-1 expression. Such factors include, for example, vasoactive prostaglandins (35) and bradykinin (36). Our data lead us to infer that neither prostacyclin nor bradykinin had relevant effects on basal LOX-1 expression in healthy human endothelial cells. Under inflammatory conditions in vitro, however, pre-incubation of HUVEC with either prostacyclin or bradykinin prevented the up-regulation of LOX-1 expression induced by TNF-α suggesting that stimulation of NO production by vasoactive substances is necessary to inhibit LOX-1 up-regulation. And it may, of course, simply be that LOX-1 expression in endothelial cells is rather a feature of further-advanced, morphologically manifest atherosclerosis with its contribution to the impairment of EDVR being only of minor significance. Here, it seems important to note that even though impairment of EDVR and manifest atherosclerosis are essential components of atherogenesis, they constitute pathologically distinct processes that act independently of each other and are governed by distinct patterns of risk factors (37). To date, LOX-1 has been exclusively related to measures of manifest atherosclerotic vessel disease. The present study is, to the best of our knowledge, the first to relate LOX-1 expression in endothelial cells to a dynamic in vivo measure of vascular function as is EDVR. In this context, a specific characteristic of the regulation of LOX-1 deserves particular attention. In a study by Murase et al. laminar shear-stress has been identified as potent stimulator of in vitro LOX-1 expression in endothelial cells (38). Laminar shear-stress acts atheroprotectively by reducing inflammation in endothelial cells (39) and is also the physiologic stimulus that elicits endothelium-dependent vasodilation. This illustrates that the up-regulation of LOX-1 does not solely occur in the presence of pro-atherogenic factors and helps explain the lack of correlation between LOX-1 expression in endothelial cells and EDVR supporting the conclusion that the involvement of LOX-1 in atherogenesis is part of a more complex process in which the expression of LOX-1 in different cell types at different stages and different functional levels tends, in summary, to accelerate the development of atherosclerosis. Our results seem to be compatible with this more general conclusion. Impaired EDVR was associated significantly with smoking status confirming previous studies (40). But as only four of 27 patients were classified as current smokers, several potential limitations have to be recognized in our study. First, the number of patients enrolled was relatively small and included only patients with a known history of cardiovascular disease. Hence, the results cannot be readily extrapolated to the general population. Second, assessment of EDVR was performed by use of FMD. Other studies employed different techniques, but FMD is an established clinical tool and has been shown to be well correlated with other markers of cardiovascular disease (41). In conclusion, using an experimental approach that combined in vivo measurements with in vitro effects, our study found no evidence that humoral factors mediate a link between LOX-1 expression in endothelial cells and EDVR. Additional investigation will be required to precisely elucidate how LOX-1 is involved in the development process from impairment of EDVR to manifest atherosclerosis.

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