Upregulation of Endothelin Receptor B in Human Endothelial Cells by Low-Density Lipoproteins

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Low-density lipoproteins (LDLs) represent the most important treatable risk factors for coronary artery disease. Although it has been previously shown that hypercholesterolemia stimulates the endothelin system, the effects of increased levels of LDL on endothelial endothelin receptors have not been previously studied. In particular, the influence of native and oxidatively modified LDLs (nLDLs and oxLDLs) and the regulatory mechanisms in endothelial cells are currently unknown. Human endothelial cells almost exclusively express the endothelin receptor type B (ET_B). Therefore, the effect of nLDL and oxLDL on the expression of ET_B was studied in primary cultures of human umbilical vein endothelial cells (HUVEC). HUVEC were stimulated by nLDL and oxLDL in a time-dependent (1–12 hrs) and dose-dependent (25–100 μg/ml) manner. To analyze signal transduction pathways involved in the regulation of ET_B, protein kinase C (PKC) was inhibited using 100 n*M* Ro-31-8220. The mRNA expression of ET_B was determined by quantitative reverse transcription–polymerase chain reaction and ET_B protein expression by Western blot. Native LDL induced ET_B mRNA after 1 hr (100 μg/ml, 199 ± 35%, $n = 15$, $P < 0.05$ vs. control). Stimulation of HUVEC with oxLDL increased ET_B mRNA expression (1 hr, 100 μg/ml oxLDL: 308 ± 48%, $n = 15$, $P < 0.05$ vs. control) as well. Induction of ET_B was also found on the protein level. nLDL was even more potent than oxLDL in inducing ET_B protein expression. Induction of ET_B expression by oxLDL is mediated by PKC. These data demonstrate that low-density lipoproteins even independent of oxidative modification are potent inducers of ET_B receptors at the mRNA and protein level in HUVEC. Given the nitric oxide-releasing capacity of endothelial ET_B receptors, this effect may represent a possible vasoprotective mechanism. Exp Biol Med 231:766–771, 2006

Key words: endothelin-1; endothelin receptor B; endothelium; low-density lipoprotein

Introduction

Low-density lipoproteins (LDLs) represent the most important treatable risk factors for coronary artery disease (1). Increased levels of native low-density lipoprotein (nLDL) can promote the influx of lipoproteins into the intima, leading to fatty streak formation (2, 3). The modification of nLDL by reactive oxygen species to oxidized low-density lipoprotein (oxLDL) can further promote the local progression of atherosclerosis (4). However, how different lipoproteins modulate the regulation of components of the endothelin system is still not well understood (5).

Endothelin is expressed in the vascular wall in endothelial cells and vascular smooth muscle cells as well as in macrophages accumulating in atherosclerotic plaques (6). Endothelin-1 (ET-1) binds to G-protein–coupled endothelin receptors A or B (ET_A, ET_B) and activates the phospholipase C phosphatidylinositol 1,4,5-trisphosphate pathway and Ca^{2+}, promoting vasoconstriction (7). Human umbilical vein endothelial cells (HUVEC) almost exclusively express ET_B (8, 9), causing in intact endothelial cells mainly nitric oxide (NO) release.

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In hypercholesterolemic patients, endothelin-1 release is increased (10, 11). In experimental studies, hypercholesterolemic stimuli activate the endothelin system in endothelial cells (12, 13) and vascular smooth muscle cells (14). However, the effects of increased levels of LDL on endothelial ET_B receptor have not been previously studied. In particular, the influence of nLDL, oxLDL, and the regulatory mechanisms in endothelial cells are currently unknown. Moreover, the potential role of protein kinase C for the regulation has not been studied. Therefore, the effect of nLDL and oxLDL on the expression of ET_B and the role of protein kinase C (PKC) in this process was analyzed in primary cultures of HUVEC.

Materials and Methods

Cell Culture. All cell culture reagents and chemicals were purchased from Sigma-Aldrich (Munich, Germany) unless otherwise specified. Primary cultures of HUVEC were isolated using collagenase IV. To minimize variations of primary cultures, different batches of primary isolates were pooled and cultured on gelatine-coated plates in medium M199 (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum.

Stimulation of HUVEC with nLDL and oxLDL. nLDLs and oxLDLs were isolated as previously described (15). Confluent primary cultures of HUVEC were stimulated with nLDL or oxLDL in a time-dependent (1–12 hrs) and concentration-dependent (25–100 μg/ml) manner. Each experiment was accompanied by a control from the same HUVEC preparation incubated for the same period of time without lipoproteins.

Quantitation of mRNA Expression by Reverse Transcription–Polymerase Chain Reaction (RT-PCR). Total RNA from endothelial cells was isolated by guanidinium isothiocyanate/cesium chloride centrifugation. RNA concentration was determined spectrophotometrically. Human ET_B mRNA expression was quantified by RT-PCR and normalized to 18S rRNA (16). In brief, 500 ng RNA was reverse transcribed using random hexamer primers and SuperScript II RNase H^- reverse transcriptase (Invitrogen) at 42°C for 1 hr. PCR was performed using 400 n*M* of specific sense and antisense primers, 1× Taq reaction buffer, and 20 n*M* each of dNTP and 1 U rTaqDNA polymerase (Amersham Biosciences Europe, Freiburg, Germany). After denaturation at 95°C for 2 mins, PCR was performed using the following protocol: 30 secs at 95°C, 30 secs at specific annealing temperature, and 30 secs at 72°C. After final extension at 72°C for 3 min, PCR products were separated by standard agarose gel electrophoresis with ethidium bromide. Optical density of amplified PCR fragments was quantified using AIDA software (Raytest; Isotopenmessgeraete GmbH, Berlin, Germany) and normalized to the density of 18S rRNA PCR fragments. To confirm the specificity of the ET_B PCR primers, amplified fragments were cloned and sequenced (data not shown).

Protein Isolation and Western Blot. After stimulation with nLDL or oxLDL, cells were washed twice with phosphate-buffered saline, and the membrane protein fraction was isolated as previously described (17). The protein concentration was determined with BCA Protein Assay Reagent (Perbio Science, Bonn, Germany). Equal amounts of membrane protein (20 μg/lane) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (Roth, Karlsruhe, Germany). Membranes were incubated with a polyclonal ET_B antibody (18) and secondary horseradish peroxidase-linked rabbit Ig (Amersham Biosciences Europe). Protein expression was detected with ECL Western blotting detection reagent (Amersham Biosciences Europe) and quantified by densitometry.

Inhibitor Studies. Protein kinase C was inhibited using 100 n*M* Ro-31-8220 (Calbiochem, San Diego, CA) as described (16). After preincubation with or without inhibitor for 1 hr, lipoproteins were applied on HUVEC for the time indicated. Each experiment was accompanied by two controls from the same HUVEC preparation incubated for the same period of time without lipoproteins: (i) without inhibitor, and (ii) with inhibitor at identical concentrations. From these experiments, ET_B mRNA expression was determined by RT-PCR.

Statistics. Data are given as mean ± SEM; *n* indicates the number of independent experiments. Statistical analysis was performed by Student's *t* test, Mann-Whitney Rank Sum test, or ANOVA followed by Bonferroni's method (multiple comparisons) when appropriate (Sigma Stat software; SPSS Inc., San Rafael, CA, USA). $P < 0.05$ was considered statistically significant.

Results

Induction of ET_B by Native and Oxidized Low-Density Lipoprotein in Human Endothelial Cells. HUVEC were stimulated by nLDL and oxLDL in a time-dependent (1–12 hrs) and dose-dependent (25–100 μg/ml) manner. The mRNA expression of ET_B was determined by quantitative RT-PCR and ET_B protein expression by Western blot. Native LDL induced ET_B mRNA after 1 hr (100 μg/ml, 199 ± 35%, $n = 15$, $P < 0.05$ vs. control; Fig. 1A). Stimulation of HUVEC with oxLDL increased ET_B mRNA expression (1 hr, 100 μg/ml oxLDL: 308 ± 48%, $n = 15$, $P < 0.05$ vs. control) as well (Fig. 1B). Induction of ET_B mRNA by nLDL and oxLDL returned to baseline after 12 hrs. The induction of ET_B by lipoproteins was also found on the protein level after 2 hrs of stimulation (Fig. 2). Native LDL was even more potent than oxLDL in inducing ET_B protein expression.

Effect of Protein Kinase C Inhibition on Lipid-Induced ET_B Expression. To determine potential signaling mechanisms involved in the lipoprotein-dependent upregulation of ET_B, inhibitor studies were performed.

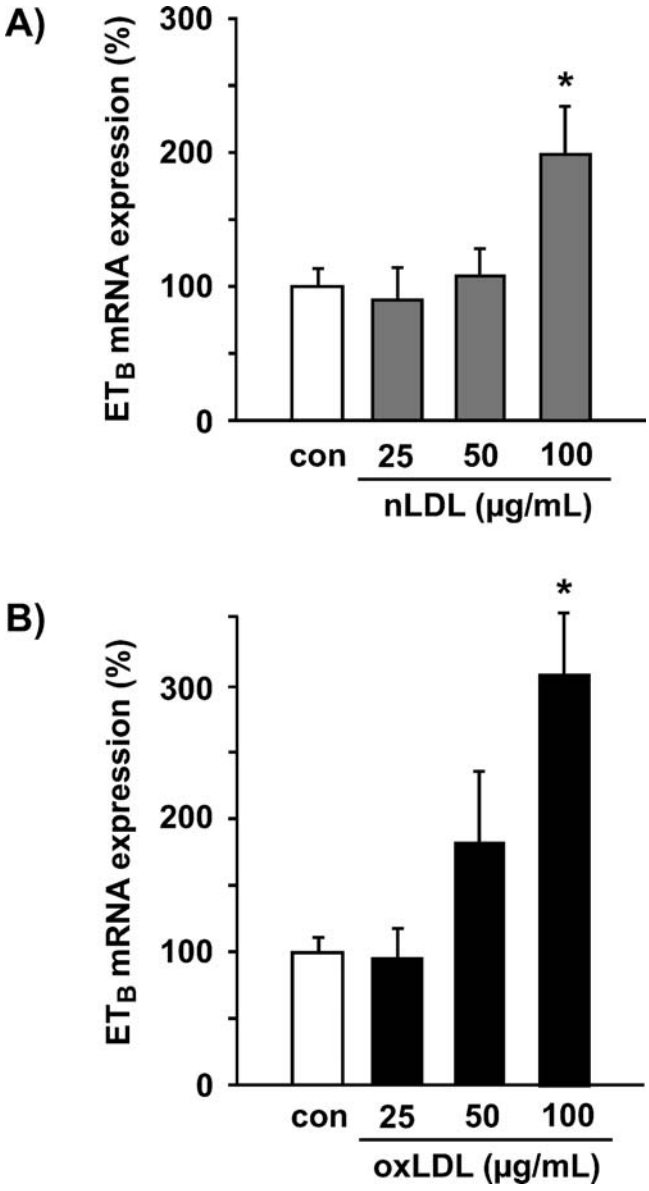

Figure 1. Induction of ET_B receptor mRNA expression by nLDL and oxLDL in human endothelial cells. Human umbilical vein endothelial cells (HUVEC) were exposed to increasing concentrations (25–100 µg/ml) of nLDL (A) and oxLDL (B) for 1 hr. ET_B mRNA was determined in equal amounts of RNA by RT-PCR, and normalized to 18S rRNA expression. All samples were normalized to internal controls (con) incubated in parallel for the same period of time. Values are given as mean ± SEM in relative units; $n \geq 5$; $^*P < 0.05$ vs. control.

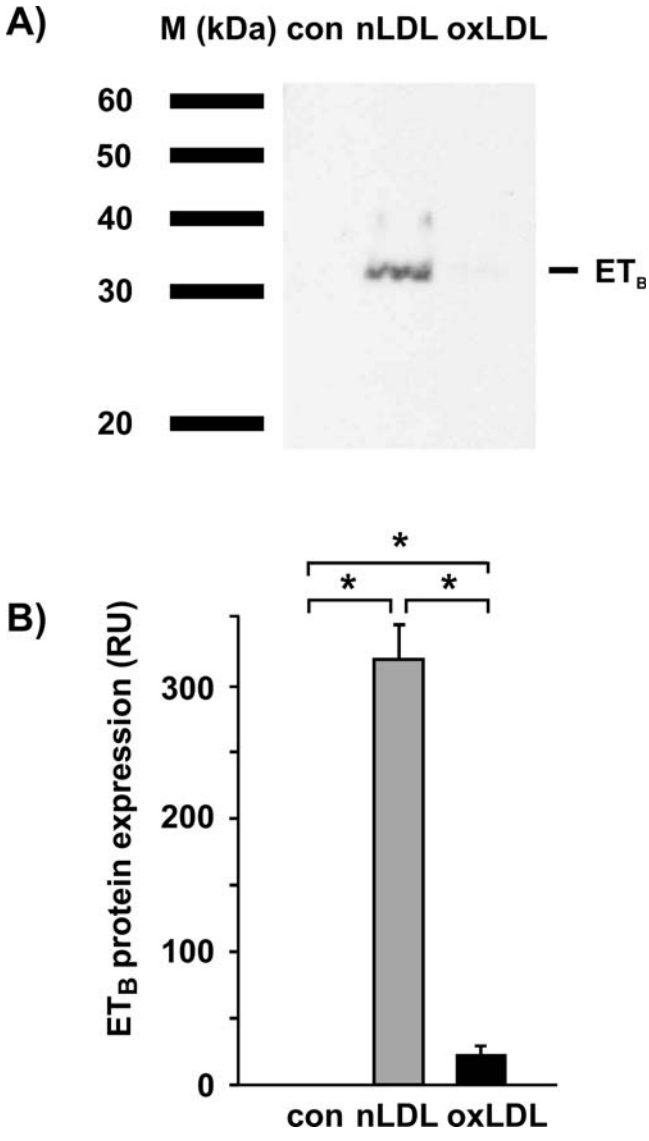

Figure 2. Effects of low-density lipoproteins on ET_B protein expression in human umbilical vein endothelial cells. Cells were exposed to 100 µg/ml nLDL and oxLDL for 2 hrs. Samples were normalized to internal control (con) incubated in parallel for the same period of time without lipoprotein. The protein expression of ET_B was determined by Western blot (Lane M, marker; Lane con, control; Lane nLDL, HUVEC stimulated with nLDL; Lane oxLDL, HUVEC stimulated with oxLDL (A). Values are given as mean ± SEM in relative units; $n = 6$; $^*P < 0.05$ (B).

HUVEC were exposed to 100 µg/ml nLDL or oxLDL for 1 hr in the presence or absence of the nonisoform selective PKC inhibitor Ro-31-8220 (100 n*M*). The corresponding data are shown in Figure 3. The increased ET_B expression in response to oxLDL was reduced with PKC inhibition ($P <$ 0.05 vs. lipoprotein-induced expression). In contrast, PKC inhibitor Ro-31-8220 had no significant effect on nLDL-induced ET_B expression (not shown).

Discussion

This is the first study showing that lipoproteins induce transcription and protein expression of the ET_B receptor in human endothelial cells. We tested in detailed dose-response experiments the impact of native and oxidized LDL on ET_B expression in endothelial cells. Our data suggest that a certain threshold of both lipoproteins is necessary before the mRNA expression of ET_B is significantly induced. This could correspond to an initially limited number of LDL or oxLDL receptors on native

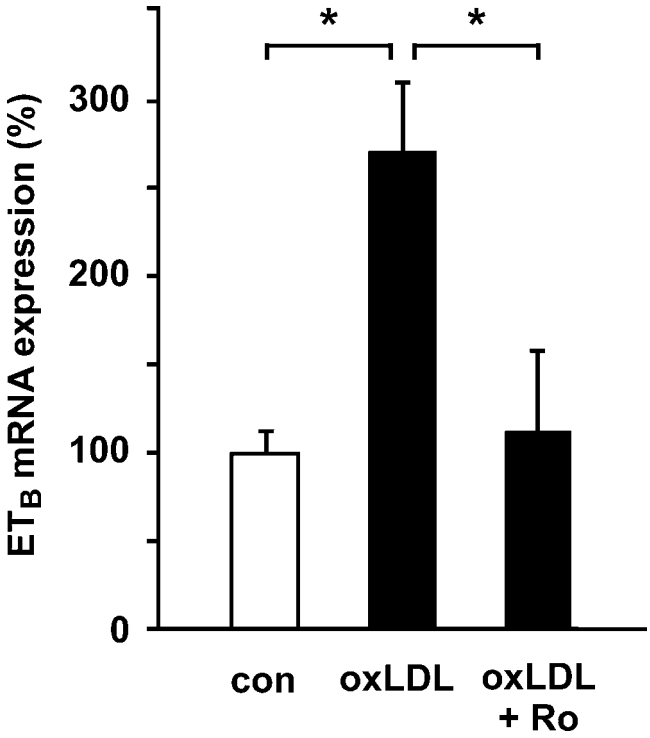

Figure 3. Induction of the ET_B receptor by oxidized low-density lipoprotein involves protein kinase C. HUVEC were exposed to 100 μg/ml oxLDL for 1 hr. PKC was inhibited by 100 n*M* Ro-31-8220 (Ro), and ET_B mRNA expression was determined in equal amounts of RNA by RT-PCR and normalized to 18S rRNA expression. All samples exposed to lipoproteins were normalized to internal controls (con) incubated in parallel for the same period of time without or with the corresponding inhibitor concentration. Values are given as mean ± SEM in relative units; $n \geq 3$; $^*P < 0.05$.

endothelial cells, which have to be activated by their agonists, or a threshold of intracellular signaling to activate transcription factors to stimulate ET_B mRNA expression. We recently analyzed the effect of native and oxidized LDL on endothelin-converting enzyme-1, preproendothelin-1 and endothelin-1 peptide release in endothelial cells. Comparing the effects of both lipoproteins on other components of the endothelin system, oxLDL had a stronger effect on induction of ppET-1 mRNA. However, even as oxLDL showed a nonsignificant trend to stimulate the release of ET-1 stronger than nLDL, both lipoproteins were finally equally potent in the induction of the endothelin system in human endothelial cells (19). nLDL and oxLDL showed similar potencies to induce ET_B mRNA expression. However, nLDL induced ET_B on the protein level much more strongly than did oxLDL. This augmented ET_B protein level in response to nLDL might involve additional mechanisms on the posttranscriptional level, e.g., by increasing protein stability or inhibiting degradation. To our knowledge, no experimental data addressing this specific topic have been published so far. Differential effects of nLDL and oxLDL have been described in earlier studies. nLDL has been shown to induce genes like the angiotensin II type 1 receptor in vascular smooth muscle cells (20), whereas oxLDL had no such effect (21). In macrophages, nLDL and oxLDL differentially induce macrophage scavenger receptors and reduce macrophage lipoprotein lipase secretion and mRNA expression by mechanisms involving mRNA stability (22, 23). Recently, a regulation of the LDL receptor on the level of mRNA stability has been demonstrated involving the extracellular signal-regulated protein kinase (ERK) signaling pathway through interactions of cis-regulatory sequences of 3′ UTR and mRNA binding proteins (24). Which of these mechanisms is involved in the observed differential regulation of the ET_B receptor in our study will be the focus of future studies.

Only a few studies have described an upregulation of endothelial ET_B so far. Our group has previously reported that laminar shear stress upregulates ET_B mRNA by a PKC-dependent mechanism (16). The induction of ET_B by oxLDL in this study seems to involve a PKC-dependent pathway as well. Furthermore, PKC was critically involved in the expression of ET_B in organ culture of rat basilar arteries (25). PKC plays also a role in the endothelial release of ET-1 and the enhanced arterial myogenic tone in response to oxLDL, but not nLDL (26). Therefore, our findings further support a role of PKC in the activation of endothelin system in response to hypercholesterolemia (27).

In vascular smooth muscle cells, ET_B was upregulated by nLDL and oxLDL as well (14). The clinical relevance of this finding is supported by enhanced vasoconstrictor response to ET_B stimulation in patients with atherosclerosis suggesting an upregulation of vascular smooth muscle ET_B receptors in atherosclerosis (28). Binding studies *in vitro* suggest that ET_B receptors are upregulated in the tunica media of atherosclerotic human coronary arteries (29, 30). In patients with other risk factors for coronary heart disease such as hypertension (31) and in patients with atherosclerosis (32) dual ET_A/ET_B receptor blockade improved endothelium-dependent vasodilatation, suggesting increased bioavailability of nitric oxide when blocking both receptors.

The endothelial ET_B receptor has been reported to mediate NO release (33) and ET-1–mediated survival of endothelial cells (34). Furthermore, ET_B could serve as a clearance receptor of augmented circulating ET-1 serum concentration and locally increased vascular ET-1 immunoreactivity in coronary endothelial dysfunction and atherosclerosis (35–37). Therefore, the induction of ET_B by lipoproteins, especially by nLDL, might be considered as a compensatory atheroprotective mechanism. This concept is further supported by the finding that chronic ET_A antagonism even while leading to increased ET-1 plasma levels preserves coronary endothelial function and increases NO most probably by an ET_B-mediated mechanism in hypercholesterolemia (38).

In conclusion, our data demonstrate that low-density lipoproteins even independent of oxidative modification are potent inducers of ET_B receptors at the mRNA and protein

level in HUVEC. Given the NO-releasing capacity of endothelial ET_B receptors, this effect may represent a possible vasoprotective mechanism.

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