Oxidized-LDL through LOX-1 increases the expression of angiotensin converting enzyme in human coronary artery endothelial cells

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Abstract

\textbf{Background and objectives:} Our previous studies have shown that oxidized low-density lipoprotein (ox-LDL) and angiotensin II (Ang II) influence each other’s action in endothelial cells. This study was designed to examine the regulation by ox-LDL of the expression of angiotensin converting enzyme (ACE) gene in human coronary artery endothelial cells (HCAECs). In addition, we studied the effect of the HMG CoA reductase inhibitor simvastatin on this interaction.

\textbf{Methods and results:} Cultured HCAECs were incubated with ox-LDL (10–80 \(\mu\)g/ml) for 1–24 h. Ox-LDL increased the expression of ACE in a concentration- and time-dependent fashion. The upregulation of ACE expression in response to ox-LDL was mediated by its endothelial receptor LOX-1, since pretreatment of HCAECs with a blocking antibody to LOX-1 prevented the expression of ACE \((P<0.01)\). Native-LDL had no significant effect on ACE expression. In this process, ox-LDL-induced activation of mitogen-activated protein kinase (MAPK p42/44) played an important role, since pretreatment of HCAECs with the MAPK p42/44 inhibitor (PD98059, 10 \(\mu\)M) inhibited MAPK activation and subsequently attenuated the expression of ACE \((P<0.01 vs. \text{ox-LDL alone})\). In other experiments, we pretreated HCAECs with simvastatin (10 \(\mu\)M) and then exposed the cells to ox-LDL. Simvastatin markedly attenuated ox-LDL-induced MAPK activation, and concurrently reduced ACE expression \((P<0.01 vs. \text{ox-LDL alone})\).

\textbf{Conclusions:} Our observations provide direct evidence that ox-LDL via LOX-1 activation induces ACE gene expression in HCAECs, and MAPK activation plays a signal transduction role in this process. Simvastatin, which inhibits MAPK activation, also blocks ox-LDL-mediated upregulation of ACE.

Keywords: ACE inhibitors; Angiotensin; Endothelial function; Gene expression; Lipoproteins

1. Introduction

Renin–angiotensin system (RAS) plays an important role in atherogenesis. Angiotensin converting enzyme (ACE) is mainly expressed in endothelial cells and converts angiotensin I to angiotensin II (Ang II) \[1\]. Ang II activates at least two distinct types of cell-surface receptors, the type 1 (AT1R) and the type 2 (AT2R) \[2,3\], which exist in endothelial cells \[4,5\]. Most studies suggest that it is the AT1R activation that mediates most known biological effects of Ang II in the cardiovascular tissues \[6,7\]. In a clinical setting, both ACE and AT1R blockers improve endothelial function and reduce myocardial ischemic injury \[8,9\].

There is increasing evidence that oxidized LDL (ox-LDL) plays a critical role in endothelial injury and atherogenesis \[10,11\]. Oxidized-LDL (ox-LDL) and its receptors have been identified in the atherosclerotic vessel walls \[12\]. Ox-LDL is cytotoxic \[13\] and acts as a chemotactic factor for monocytes \[14\] leading to the accumulation of inflammatory cells and generation of oxygen-derived free radicals that can inactivate endothelium-derived nitric oxide \[15\]. Ox-LDL induces apoptosis in vascular smooth muscle cells \[16\], monocytes/
macrophages [17] and endothelial cells [18]. Based on these considerations, oxidative modification of LDL is considered a key trigger in the initiation and progression of atherosclerosis.

There is evidence from experimental and animal studies for an interaction between hypercholesterolemia and RAS in atherogenesis [19–21]. For example, AT1R expression is upregulated by ox-LDL in smooth muscle and endothelial cells [19,22]. Ang II has been shown to facilitate ox-LDL uptake by endothelial cells [5]. We have recently demonstrated upregulation of specific lectin-like receptors for ox-LDL (LOX-1) in response to Ang II [5]. These observations provide strong evidence for cross-talk between Ang II and ox-LDL. It is of note that HMG CoA reductase inhibitors, which decrease vascular events in primary and secondary prevention trials [23,24], decrease LDL was oxidized by exposure to CuSO4 (5 µmol/l free Cu2+ concentration) in phosphate-buffered saline at 37 °C for 24 h. The TBARS content of ox-LDL and native LDL was 15.2±0.53 and 0.7±0.26 mmol/l/100 µg protein, respectively (P<0.01). Ox-LDL was extensively dialyzed against tris–saline. Ox-LDL was kept in 50 µM Tris–HCl, 0.15 M NaCl and 2 mM EDTA at pH 7.4, and was used within 10 days of preparation. The level of endotoxin was measured by the E-Toxate kit (Sigma) and found to be consistently less than 0.005 EU/ml (lowest detection limit).

2. Methods

2.1. Cell culture

The methodology for culture of HCAECs has been described earlier [4,5]. The initial batch of HCAECs was purchased from Clonetics Corporation (San Diego, CA). The endothelial cells were pure based on morphology and staining for factor VIII-related antigen and acetylated LDL. These cells were 100% negative for alpha-actin smooth muscle expression. Fourth passage cells at 70% confluence were used in this study.

2.2. Study design

HCAECs were incubated with ox-LDL (10–80 µg/ml) for 1, 3, 6, or 24 h to determine the expression of ACE (mRNA and protein). The concentration and time point for maximal effect of ox-LDL were used in subsequent experiments.

To examine the receptor specificity of ox-LDL action, HCAECs were pretreated with human LOX-1 blocking antibody (JTX92, 10 µg/ml) and then exposed to ox-LDL. The details of the preparation of antibody and its specificity have been presented earlier [28]. This antibody blocks ox-LDL uptake by HCAECs. The harvested cells were used to measure the expression of ACE and MAPK activity.

To explore the molecular basis of the action of LOX-1, we studied MAPK (p42/44) signaling pathways in HCAECs. For this purpose, HCAECs were pretreated with the MAPK inhibitor (PD98059, 10 µM) for 30 min, and then the cells were exposed to ox-LDL. The harvested cells were used to measure ACE expression and MAPK activity.

Concentrations of different reagents and the duration of incubation were chosen based on previous studies [14,15,22,28].

2.3. Preparation of lipoproteins

Native LDL and ox-LDL were prepared as described earlier [4,5]. In brief, human native LDL was isolated from human blood plasma by discontinuous centrifugation. It was purified by ultracentrifugation (1.063–1.210 g / ml) to homogeneity determined on agarose gel electrophoresis. LDL was oxidized by exposure to CuSO4 (5 µmol/l free Cu2+ concentration) in phosphate-buffered saline at 37 °C for 24 h. The TBARS content of ox-LDL and native LDL was 15.2±0.53 and 0.7±0.26 mmol/l/100 µg protein, respectively (P<0.01).

2.4. Semiquantitative RT-PCR for ACE

ACE mRNA was examined by RT-PCR. Total RNA (5 µg) extracted from cultured HCAECs was reverse-transcribed with Oligo dT (Promega) and M-MLV reverse transcriptase (Promega) at 37 °C for 1 h. Two µl of the reverse-transcribed material was amplified with Taq DNA polymerase (Promega) using specific primers for human ACE. The products of PCR amplified samples were visualized on 1.2% agarose gels using ethidium bromide. Each specific mRNA band was normalized with β-actin mRNA band.

2.5. Western Analysis for ACE

HCAEC lysate from each experiment (40 µg per lane) was separated by SDS–PAGE, and transferred to nitrocellulose membranes. After incubation in blocking solution (4% non-fat milk, Sigma), membranes were incubated with 1:1000 dilution primary antibody to human ACE (Santa Cruz) for overnight at 4 °C. Membranes were washed and then incubated with 1:2000 dilution secondary antibody (Amersham) for 1 h, and the membranes were detected with the ECL system, and relative intensities of protein bands were analyzed by Scan-gel-it software [25].

2.6. Measurement of MAPK activity

HCAEC lysates were separated by 10% SDS–PAGE and...
transferred to nitrocellulose membranes. After blocking, the membranes were incubated with 1:1000 dilution phospho-specific MAPK antibodies (Calbiochem, CA) that detect \( \text{p}^{\text{42}} \text{MAPK} \) and \( \text{p}^{\text{44}} \text{MAPK} \). Thereafter, the membrane was stripped and reprobed with the MAPK antibody [18].

### 2.7. Data analysis

Data shown are from six independently performed experiments. Data are presented as mean±S.D. Statistical significance was determined in multiple comparisons among different groups of data in which ANOVA and the \( F \) test indicated the presence of significant differences. A \( P \) value\( \leq 0.05 \) was considered significant.

### 3. Results

#### 3.1. Ox-LDL and ACE expression

Incubation of HCAECs with ox-LDL (10–80 \( \mu \text{g/ml} \)) for 24 h increased the expression of ACE (mRNA and protein) in a concentration-dependent manner (Fig. 1, top panel). Incubation of HCAECs with ox-LDL (80 \( \mu \text{g/ml} \)) for 1, 3, 6 and 24 h increased the expression of ACE (mRNA and protein) in a time-dependent manner with maximal expression at 24 h (Fig. 1, bottom panel).

#### 3.2. Role of LOX-1 in the expression of ACE

Incubation of HCAECs with ox-LDL (80 \( \mu \text{g/ml} \)) markedly increased the expression of ACE. In contrast, native LDL (80 \( \mu \text{g/ml} \)) did not affect the expression of ACE. To determine the role of LOX-1 in the effects of ox-LDL, HCAECs were treated with the LOX-1 blocking antibody (10 \( \mu \text{g/ml} \)) for 30 min, and then the cells were exposed to ox-LDL for 24 h. As shown in Fig. 2, pretreatment with the LOX-1 antibody reduced the effects of ox-LDL on the expression of ACE. LOX-1 antibody alone did not affect the expression of ACE (Fig. 2).

#### 3.3. Intracellular mechanism of LOX-1-mediated ACE expression

We have earlier shown that ox-LDL activates protein

![Fig. 1. Ox-LDL and ACE expression.](https://academic.oup.com/cardiovascres/article-abstract/57/1/238/375216)

![Fig. 2. LOX-1-mediated ACE expression.](https://academic.oup.com/cardiovascres/article-abstract/57/1/238/375216)
kinase C, MAPKs and transcription factor NF-κB [4,14,18]. In this study, we confirmed that ox-LDL activated MAPKp42/44 in HCAECs (P<0.05 vs. control, n=6). This effect of ox-LDL was inhibited by pretreatment of HCAECs with simvastatin (P<0.05, n=6). High concentration of simvastatin (10 μM) gave a more potent effect than low concentration of simvastatin (1 μM) (P<0.05, n=6) (Fig. 3). To further determine the role of MAPK activation in ACE expression, we pretreated HCAECs with the MAPK inhibitor PD98059 and then exposed the cells to ox-LDL. As shown in Fig. 4, pretreatment of cells with PD98059 attenuated ox-LDL-induced ACE protein expression. MAPK inhibitor alone did not affect the baseline expression of ACE.

3.4. Simvastatin and ACE expression

As shown in Fig. 3, simvastatin inhibited ox-LDL-induced MAPKp42/44 activation. We further examined the effect of simvastatin on ACE expression. Preincubation of HCAECs with simvastatin (10 μM) significantly reduced ox-LDL-induced mRNA and protein expression of ACE in response to ox-LDL (P<0.01). Simvastatin alone did not change baseline ACE expression (Fig. 5).

4. Discussion

We demonstrate that ox-LDL upregulates the expression (mRNA and protein) of ACE in HCAECs, whereas native-LDL has no effect. This effect of ox-LDL is mediated via its receptor LOX-1, since pretreatment of HCAECs with the blocking antibody to LOX-1 prevents the expression of ACE. In this process, MAPKp42/44 activation plays an important signaling role. In addition, we show that simvas-
tatin, by inhibiting MAPK activation, attenuates ox-LDL-induced expression of ACE.

4.1. Ox-LDL, LOX-1 and ACE expression

It is quite evident that dyslipidemia and RAS activation play a crucial role in the genesis of atherosclerosis, hypertension and myocardial ischemia [19–22]. In recent studies, we have shown that ox-LDL, through NF-κB activation, upregulates AT1R, but not AT2R, expression in HCAECs [22]. On the other hand, Ang II upregulates LOX-1 gene expression in HCAECs and facilitates the uptake of 125I-ox-LDL in these cells, and this effect of Ang II can be blocked with specific blockade of AT1R [5]. Importantly, both Ang II and ox-LDL cause marked cell injury [5,22]. Ang II has also been shown to facilitate oxidization of LDL in macrophages [20]. An in vivo study from our laboratory has demonstrated upregulation of AT1R in the arteries of atherosclerotic rabbits [29]. Increased LOX-1 gene expression in hypertensive rats also supports the concept of cross-talk between ox-LDL and Ang II [30].

The present study provides direct evidence that another component of RAS, i.e. ACE, is upregulated by ox-LDL in a concentration and time-dependent manner. Importantly, we found that this effect of ox-LDL on ACE expression in HCAECs is mediated by its endothelial receptor LOX-1. Previous studies have shown that Ang II AT1R blockers inhibit LOX-1 expression in endothelial cells [5,31]. Morawietz et al. showed that the ACE inhibition decreases LOX-1 mRNA expression in internal mammary artery samples from patients undergoing coronary artery bypass surgery [31]. We believe that the interaction between ox-LDL and Ang II is a result of positive feedback. The coronary risk factors, such as stress, inflammation and smoking, result in release of free radicals, which cause oxidative modification of LDL. Ox-LDL in turn induces ACE expression, Ang II generation and AT1R upregulation that further amplifies ox-LDL generation. Activation of Ang II and ox-LDL receptors then contributes to atherosclerosis, hypertension and ischemic injury by inducing local vasoconstriction, endothelial dysfunction, and platelet aggregation. This positive feedback promotes progression of a host of pathobiological processes.

4.2. Ox-LDL, MAPK activation and ACE expression

A number of investigators have shown that the effects of ox-LDL in various tissues are mediated by its binding to receptors, such as LOX-1 and scavenger receptors [14,18,22,32]; activation of these receptors results in a cascade of intracellular signaling events. Previous studies from our laboratory [4,14,28] have shown that ox-LDL activates protein kinases and MAPKs and subsequently the transcription factor NF-κB. Inhibition of protein kinase C activation attenuates many of the effects of ox-LDL in HCAECs [28]. Others have shown that inhibition of p38MAPK in cardiac myocytes blocks the effects of ox-LDL [33]. Day et al. [34] showed that activation of MAPKp42/44 mediates ACE expression in bovine pulmonary endothelial cells. The present study confirms that ox-LDL activates MAPKp42/44, which may play an important role in ox-LDL-induced ACE gene expression in HCAECs.

4.3. Statins and ACE expression

The development of statins has been a major milestone in the primary and secondary prevention of coronary heart disease. These agents, besides lowering total and low-density lipoprotein cholesterol, have a multitude of other effects, which may have a bearing on cardioprotective effect of these agents [35]. In animals, these agents decrease the extent of atherosclerosis [36]. In clinical trials, statins improve endothelial function and reduce atherosclerosis and its complications [35]. In a number of studies [25,27,37], we have shown that simvastatin increases protein kinase B activity, increases eNOS expression and decreases adhesion molecule expression and monocyte adhesion in response to ox-LDL. In the present study, we extend the information on the pleiotropic effects of statins and demonstrate that simvastatin decreases ox-LDL-induced MAPK activation as well as ACE gene expression. It is possible that inhibition of MAPK activation is causally related to the inhibition of ACE expression, as evident from studies with the direct inhibitor of MAPK p42/44.

In summary, ox-LDL via LOX-1 activation upregulates ACE expression in human coronary endothelial cells. MAPK activation seems to play a key role in this process. This study provides another link in the concept of cross-talk between dyslipidemia and RAS.

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