HDAC9 regulates ox-LDL-induced endothelial cell apoptosis by participating in inflammatory reactions

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1. ABSTRACT

Atherosclerosis is the most common cause of cardiovascular diseases worldwide. The endothelial cell apoptosis elicited by oxidized low-density lipoprotein (ox-LDL), which contributes to endothelial damage and inflammation, is a particularly important event in the early stage of atherosclerosis. However, the mechanism underlying ox-LDL-induced endothelial cell apoptosis remains unclear. Here we found that HDAC9 expression was increased at both the mRNA and protein levels accompanied by dose-dependent ox-LDL-induced endothelial cell apoptosis. Depletion of HDAC9 by its specific shRNA significantly antagonized ox-LDL-induced cell apoptosis and suppressed the expression of ox-LDL-induced inflammatory factors, such as TNF-α and MCP1. These data suggest that HDAC9 is an important epigenetic factor regulating ox-LDL-induced endothelial cell apoptosis and inflammatory factor expression. These results suggest that HDAC9 may participate in ox-LDL-induced endothelial damage and inflammation during atherosclerosis development.

2. INTRODUCTION

The important role of the vascular endothelial wall is maintenance of blood vessel stability, and endothelial cell apoptosis contributes to endothelial dysfunction and destabilization of atherosclerotic plaques and thrombosis under pathological conditions, which lead to the development of atherosclerosis (1). It has been reported that endothelial cell apoptosis is stimulated by oxidized low-density lipoprotein (ox-LDL), hypoxia, oxidative stress, and other factors (2). Currently, a leading interest in atherosclerosis is to clarify the mechanism underlying stimuli-induced endothelial cell apoptosis, and identify an effective way of preventing endothelial cell damage and promoting endothelial cell recovery (3, 4).

Among several pro-apoptotic factors present in atherosclerosis, an elevated level of ox-LDL is one of the major risk factors for atherosclerosis. Ox-LDL participates in the formation and progression of lesions by eliciting lipid accumulation, local inflammation, and toxic events, and causing vascular wall injury and death, plaque erosion, and subsequent atherosclerosis (5, 6). Ox-LDL also enhances endothelial expression of adhesion molecules,
which recruit inflammatory cells to migrate through the endothelial barrier, followed by endothelial dysfunction and anti-apoptotic protein loss (6-8). Therefore, ox-LDL appears to be particularly important in the pathophysiology of atherosclerosis, and ox-LDL-induced endothelial cell apoptosis has been widely used to investigate the mechanisms underlying this process (9, 10). Different signaling pathways, such as JNK and p38 MAPK, trigger subsequent apoptotic programs such as activation of the caspase protease family, regulate pro-apoptotic stimuli-induced endothelial cell apoptosis (11, 12). Furthermore, some downstream factors, such as VPO1, FRP, and Cathepsin L, have been shown to regulate ox-LDL-induced endothelial cell apoptosis by mediating the Bcl-2 and Caspase-3 pathways (4, 9, 10). However, the molecular mechanism underlying ox-LDL-induced endothelial cell apoptosis still remains unclear. Accordingly, identifying mechanisms that can prevent or reverse endothelial dysfunction has become a long-term goal.

At the molecular level, ox-LDL was also shown to promote the expression of adhesion molecules, heat shock proteins, and coagulation proteins, to suppress the production of endothelium-derived factors and to induce pro-inflammatory cytokines and growth factors (13). A further role of ox-LDL in atherosclerosis is to initiate and affect the expression of inflammatory mediators such as C-reactive protein (CRP), interleukin (IL)-6, and tumor necrosis factor (TNF-α) in human umbilical vein endothelial cells (14-17). However, the correlation between inflammation response and ox-LDL-induced endothelial cell apoptosis has not been elucidated. It has been widely reported that HDAC inhibition by its inhibitors can reduce the inflammatory response in various injured normal tissues and inflammation-related tumors (16, 18-20). This strongly suggests that specific HDAC proteins should be targeted by HDAC inhibitors to attenuate ox-LDL-induced inflammatory responses. HDAC9, which has been shown to be associated with T regulatory cell function and inflammation (21, 22), is the target candidate in the present study. Recently, it was demonstrated that HDAC9 represses cholesterol efflux and activates macrophages in atherosclerosis development (23), suggesting that HDAC9 may play an important role in the ox-LDL-induced endothelial response.

Here we investigated the role of HDAC9 in ox-LDL-induced endothelial cell apoptosis and the potential mechanisms involved. We found that HDAC9 expression was increased in ox-LDL-induced cell apoptosis. As expected, HDAC9 knockdown inhibited ox-LDL-induced endothelial cell apoptosis and inflammatory factor activation.

3. MATERIALS AND METHODS

3.1. Cell culture and reagents

Human umbilical vein endothelial cells (HUVECs) were originally purchased from the ATCC. HUVECs were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified incubator at 37˚C in a 5% CO₂ atmosphere. Ox-LDL was purchased from Biomedical Technologies (Stoughton, MA, USA). To investigate the effects of ox-LDL on endothelial cell apoptosis and HDAC9 expression, the cells were treated with the indicated concentrations of ox-LDL for 24 h.

3.2. RNA extraction and real-time PCR analysis

Total RNA was extracted using TRIzol reagent following the manufacturer’s instructions. 2 μg total RNA was reverse transcribed to cDNA using the FastQuant RT Kit (Tiangen, China) according to the manufacturer’s protocol. Real-time PCR was performed with SYBR Green real-time PCR master mix (Toyobo, Japan) to determine the relative gene expression. The results were normalized to GAPDH. The primers are shown in Table 1.

3.3. Western blot analysis

Cultured cells were exposed to ox-LDL treatments. At the end of stimulation, the treated cells were collected for western blot analysis. Protein concentrations were measured using the BSA method as a standard. Equal amounts of total protein were separated by SDS-PAGE, and the blots were probed with antibodies against HDAC9 (1:200, Abcam, UK) and GAPDH (1:2000, Abcam, UK). Blots were visualized using a chemiluminescence kit (Thermo Scientific, USA).

3.4. Annexin V/PI double-staining assay

HUVECs (2×10⁵/well) were cultured in six-well plates to 70–80% confluence. Cells were then treated with the indicated concentrations of ox-LDL. Cells were collected for western blot analysis. Protein concentrations were measured using the BSA method as a standard. Equal amounts of total protein were separated by SDS-PAGE, and the blots were probed with antibodies against HDAC9 (1:200, Abcam, UK) and GAPDH (1:2000, Abcam, UK). Blots were visualized using a chemiluminescence kit (Thermo Scientific, USA).

3.5. HDAC9 knockdown in HUVECs

The lentiviral-vector mediated shRNA targeting HDAC9 (LV-shHDAC9) and lentiviral-vector non-specific control (LV-NC) were purchased from Shanghai Hanbio (Shanghai, China). The shRNA target sequences were: HDAC9, 5’-GAAAGACACUCCAACUAU-3’; NC, 5’-CGUACGCGGAUUACUCGA-3’. The lentivirus was packaged in HEK293T cells and shRNA-containing lentivirus was delivered to transfect HUVECs. The knockdown efficiency of HDAC9 shRNA was determined by western blot analysis. The stably-transfected cells were then treated with ox-LDL (100 μg/mL) for 24 h and
3.6. Cytotoxicity and cell viability assays

The cell cytotoxicity assay was performed with a colorimetric tetrazolium salt-based assay. To determine the cytotoxicity of ox-LDL, HUVECs were cultured in a 96-well plate for 24 h and then incubated with different concentrations of ox-LDL for 24 h, rinsed with PBS, and incubated for another 72 h in complete medium. To detect photothermal cytotoxicity, HUVECs were irradiated with a 980-nm laser at a fluence of 60-120 J cm$^{-2}$. Cell cytotoxicity was assessed 12 h after laser irradiation with CCK8. The absorbance value at 450 nm (OD450), was read with a 96-well plate reader (Infinite M200, Switzerland) to determine the viability of the cells.

3.7. Statistical analysis

All experiments were independently repeated at least 3 times. Values are shown as the mean ± SE. Student’s unpaired t-test was used to establish significance between the groups. P values were used to evaluate the statistical significance: *, P<0.05; **, P<0.01; ***, P<0.001.

4. RESULTS

4.1. Ox-LDL inhibits endothelial cell viability and induces endothelial cell apoptosis in a dose-dependent manner

Ox-LDL activates endothelial cell apoptosis through multiple signaling pathways, such as PKC, PTK, BCL-2, and FAS (24, 25), while the mechanisms underlying the pathophysiological consequences of ox-LDL remain unclear. To explore this, HUVECs were used as an in vitro model to determine the role of ox-LDL. HUVECs were incubated with increasing concentrations of ox-LDL (0, 10, 30, 50, 100, 300 μg/mL) for 24 h, and the cells were then subjected to cell viability and cytotoxicity assays. As shown in Figure 1A, ox-LDL inhibited endothelial cell viability in a dose-dependent manner (≥50 μg/mL), demonstrating that endothelial cell growth was significantly suppressed by ox-LDL.

Furthermore, ox-LDL exhibited marked dose-dependent cytotoxicity in HUVECs (Figure 1B). The ox-LDL-treated HUVECs displayed characteristics of apoptosis including cell shrinkage and nuclear condensation. Therefore, cell apoptosis, which is always accompanied with cytotoxicity, was determined following ox-LDL treatment. Annexin V/PI staining analysis was conducted to quantify the percentages of early (PI$^{-}$/annexin V$^{+}$) and late (PI$^{+}$/annexin V$^{+}$) apoptosis (Figure 2A) in ox-LDL-treated HUVECs. As expected, ox-LDL treatment resulted in increased percentages of late apoptotic cells, especially when the ox-LDL concentration was more than 100 μg/mL. Similarly, the percentage of early apoptotic cells was strongly upregulated in 100 or 300 μg/mL Ox-LDL, while no significant effects were observed in ≤50 μg/mL ox-LDL-treated HUVECs (Figure 2B). Collectively, ox-LDL inhibited endothelial cell viability, induced cell cytotoxicity and apoptosis in a dose-dependent manner.

4.2. The mRNA levels of inflammatory factors were upregulated by ox-LDL exposure

It was previously shown that ox-LDL affected the expression of inflammatory mediators such as C-reactive protein (CRP), interleukin (IL)-6, and tumor necrosis factor (TNF-α) in HUVECs (14-17). In view of the tight correlation between ox-LDL and the inflammatory response in atherosclerosis, the expression of multiple inflammatory factors, including TNF-α, IL1-β, TGF-β1, IL8, IFN-β, MCP1, MIP1-α, MIP1-β, and PDGF-BB were determined following ox-LDL stimulation. Notably,

Table 1. Primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense 5’-3’</th>
<th>Anti-sense 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDAC9</td>
<td>AGTAGAGGGCATCGAGAAGA</td>
<td>GGAGTGTCTTCTGGTGTGAT</td>
</tr>
<tr>
<td>TNFα</td>
<td>TGCACTTTGGAGATGCGG</td>
<td>ACTCGGGGCTTCGAAAGATG</td>
</tr>
<tr>
<td>IL1β</td>
<td>CCCTAAACAGATAGTGCTCTTCTT</td>
<td>GGTGTCGAGATTTGAGT</td>
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<td>TGFβ</td>
<td>CTGACAGTGACGATCAAAG</td>
<td>AAGTTGGCGATTTGAGT</td>
</tr>
<tr>
<td>IL8</td>
<td>CTCTCTTGGCGATCTCTTCTT</td>
<td>GGTGGAAGTTTGGAGT</td>
</tr>
<tr>
<td>IFNβ</td>
<td>CTAGCACTTTGGGAAGTGAAG</td>
<td>TTGCCCTCAGGTAATGCAGA</td>
</tr>
<tr>
<td>MCP1</td>
<td>TCTGTCCGCTGCTGCTAG</td>
<td>GGGACTGAGTGGACGCTGC</td>
</tr>
<tr>
<td>MIP1α</td>
<td>TGGCAACCAGTTCTGCTAC</td>
<td>TTTCTGACCCACGCTCAAC</td>
</tr>
<tr>
<td>MIP1β</td>
<td>AAGCTGCTGCTGCTGCTTCTT</td>
<td>GCCTGCTGCTTGGTGT</td>
</tr>
<tr>
<td>PDGFB</td>
<td>CTCTGCTGCTACCTGCGTCTTT</td>
<td>TGCTACGTTGAGGCACTGCGC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ATGACCCCAGCTGCACCATT</td>
<td>GGTCCTACGTTGAGGGCCATG</td>
</tr>
</tbody>
</table>

the number of apoptotic cells was measured by apoptosis assays.
HDAC9 on ox-LDL induced endothelial cell apoptosis

The expression levels of TNF-α, IL1-β, IL8, and MCP1 were markedly upregulated by higher concentrations of ox-LDL (100 and or 300 μg/mL) (Figure 3), corresponding to the apoptotic phenotype elicited by ox-LDL treatment (Figure 2). This demonstrated that the inflammatory response may be associated with ox-LDL-induced apoptosis.

4.3. HDAC9 knockdown antagonizes the ox-LDL-induced cell viability suppression and cell apoptosis promotion

Given the inflammatory response of HUVECs in ox-LDL-induced apoptosis, we next identified the downstream target of ox-LDL during this process. Considering the possible function of HDAC9 in inflammation, cholesterol efflux and macrophages in atherosclerosis (21-23), the role of HDAC9 in ox-LDL-induced apoptosis and the inflammatory response was preferentially investigated in the present study.

The expression pattern of HDAC9 responding to ox-LDL treatment was examined by real-time PCR and western blot analysis. We found that ox-LDL enhanced the expression of HDAC9 in a dose-dependent manner at the mRNA (Figure 4A) and protein levels (Figure 4B), suggesting that HDAC9 expression change was correlated with ox-LDL-induced apoptosis.

We hypothesized that HDAC9 may mediate ox-LDL-elicited effects, including cell viability suppression and cell apoptosis promotion. We used lentivirus-mediated HDAC9 shRNA to efficiently knockdown the expression of HDAC9 in HUVECs, which was confirmed by western blot results (Figure 5A). Control shRNA or HDAC9 shRNA-expressing HUVECs were treated with 100 μg mL ox-LDL for 24 h and then cell viability and cytotoxicity were determined by corresponding assays. Although cell viability was inhibited and cytotoxicity was increased in both groups of transfected HUVECs following ox-LDL treatment, the ox-LDL-elicited suppression of cell viability in HDAC9-depleted cells was much weaker than that in control HUVECs (Figure 5B), and ox-LDL-induced cytotoxicity was also impaired in HDAC9 shRNA-expressing cells (Figure 5C). This suggests that ox-LDL-induced cell viability suppression and cell apoptosis promotion were antagonized by HDAC9 knockdown. However, the ox-LDL-induced effects were only impaired and not fully blocked by HDAC9 depletion (Figure 5B and 5C), demonstrating that HDAC9 partially mediates ox-LDL-induced cell viability suppression and cell apoptosis promotion in HUVECs.

The possibility of HDAC9 mediating ox-LDL-induced apoptosis was then investigated. Control shRNA and HDAC9 shRNA-expressing HUVECs treated with 100 μg/mL ox-LDL for 24 h were subjected to Annexin V/PI staining assays. Interestingly, ox-LDL-induced early apoptosis was significantly decreased in HDAC9-depleted cells, while ox-LDL-elicited late apoptosis was fully blocked in HDAC9-knockdown HUVECs when compared with the control group (Figure 6). It is possible that HDAC9 may play an important role in ox-LDL-induced late apoptosis.

4.4. HDAC9 depletion impaired the ox-LDL-induced inflammatory factor expression

To further explore the relationship between HDAC9 and the ox-LDL-induced inflammatory response, the expression of inflammatory factors was examined in control and HDAC9-depleted HUVECs with or without ox-LDL treatment. It was found that ox-LDL-induced upregulation of TNF-α and MCP1 was hindered by HDAC9 knockdown (Figure 7), demonstrating that HDAC9 mediates the ox-LDL-induced inflammatory response. Taken together, these results suggest that HDAC9-mediated inflammatory reactions were tightly associated with the ox-LDL-induced effects in HUVECs, highlighting the essential role of HDAC9 in ox-LDL-deregulated endothelial cells.

5. DISCUSSION

This study revealed that ox-LDL induced endothelial cell apoptosis and the expression of HDAC9...
in endothelial cells in a concentration-dependent manner concomitant with increased cytotoxicity and inflammatory reactions. These effects were inhibited by HDAC9 depletion in HUVECs. These findings suggest, for the first time, that HDAC9 plays a critical role in ox-LDL-induced endothelial cell apoptosis and mediates the ox-LDL-elicited inflammatory response in endothelial cells.

**Figure 2.** Ox-LDL induces endothelial cell apoptosis in a dose-dependent manner. The apoptosis of HUVECs in increasing concentrations of ox-LDL treatment was determined by Annexin V/PI double-staining assays. A set of representative results are shown in (A) and the number of apoptotic cells is displayed in (B).

**Figure 3.** The mRNA levels of inflammatory factors were upregulated following ox-LDL exposure. Real-time PCR analysis was conducted to determine the relative expression levels of TNF-α, IL1-β, TGF-β, IL8, IFN-β, MCP1, MIP1-α, MIP1-β, and PDGF-BB.
Vascular endothelial cells are normally resistant to Fas-mediated apoptosis (26), and endothelial cell apoptosis is observed in the early stages of atherosclerosis. Ox-LDL and its components have been detected in human atherosclerotic plaques (27), and ox-LDL induced endothelial cell apoptosis in our study (Figure 2) and other studies (6, 14). We also classified ox-LDL-induced apoptosis into early and late apoptosis and found that ox-LDL treatment led to enhanced early apoptosis as well as late apoptosis (Figure 2). In addition, cytotoxicity and cell viability were also determined and ox-LDL was found to inhibit cell growth and increase cytotoxicity (Figure 1). These findings demonstrate that these effects are critical in the pathophysiology of ox-LDL toxicity in endothelial cells and atherosclerosis.

There are several mechanisms that may account for the increased apoptosis of endothelial cells by ox-LDL. Ox-LDL may alter the expression levels of BCL-2 family members, which positively or negatively regulate...
FAS-mediated apoptosis (28-30). Several factors have been reported to modulate Fas-mediated cell death including sentrin, VPO1, GD3 ganglioside, and nitric oxide, which may be modulated by ox-LDL (9, 31-33). Although there have been many suggestions concerning the role of inflammation in atherosclerosis (25, 34, 35), the relationship between ox-LDL and inflammatory activation has rarely been studied. In the present study, we found that multiple inflammatory factors, such as TNF-α, IL1-β, IL8, and MCP1, were increased by ox-LDL (Figure 3), accompanied by ox-LDL-induced apoptosis. It has been consistently reported that MCP1 was induced by ox-LDL in endothelial cells (36). This result links ox-LDL-induced apoptosis and the inflammatory response in endothelial cells. We attempted to identify the downstream effector of ox-LDL in the ox-LDL-induced effects in relation to inflammation. Coincidently, HDAC inhibition reduces the inflammatory response in inflammation-related tumors (16, 18-20), and HDAC9 plays important roles in inflammation and macrophages in atherosclerosis development (21, 23). These findings inspired us to investigate the role of HDAC9 in the ox-LDL-induced endothelial response. As expected, HDAC9 expression was significantly induced by ox-LDL treatment (Figure 4), and HDAC9 depletion antagonized ox-LDL-induced cell viability suppression and cell apoptosis promotion (Figure 5 and 6). Importantly, ox-LDL-induced upregulation of TNF-α and MCP1 was impaired by HDAC9 knockdown. These data demonstrate that HDAC9 mediates the ox-LDL-induced
effects in endothelial cells. However, ox-LDL functions in cell viability, cell apoptosis, and the inflammatory response were not fully mediated by HDAC9 (Figure 4-6). Possible reasons for this are as follows: HDAC9 was partially knocked down by HDAC9 shRNA (Figure 5A); other unknown factors (or other HDAC members) also participate in the ox-LDL-induced effects. An interesting observation was that HDAC9 depletion fully blocked ox-LDL-induced late apoptosis. It is possible that HDAC9 shRNA first suppressed ox-LDL-induced early apoptosis,

Figure 6. HDAC9 depletion antagonizes ox-LDL-induced cell apoptosis. The control shRNA (LV-NC) and HDAC9 shRNA (LV-shHDAC9)-expressing cells were cultured with or without ox-LDL supplementation for 24 h and cell apoptosis was determined. A set of representative apoptotic assay results is shown in (A) and the calculated apoptotic cell number is shown in (B).
leading to the slow down of the whole apoptotic process, or HDAC9 shRNA blocked the transition from early apoptosis to late apoptosis. This hypothesis requires further study in the future.

It is currently difficult to assess the overall impact of ox-LDL-induced effects on HDAC9 in atherosclerosis. In this context, HDAC9 may have a protective role in response to ox-LDL-induced apoptosis. In addition, oxidized lipids can increase the sensitivity of endothelial cells to death signals. HDAC9 may mediate ox-LDL-affected endothelial cell sensitivity. As ox-LDL triggers inflammatory processes, HDAC9 may contribute to atherosclerosis resulting in exaggerated hyperlipidemia. These results show that ox-LDL inhibited cell viability and promoted cell apoptosis in endothelial cells, and HDAC9 mediated ox-LDL-induced apoptosis and inflammatory reactions. Our study may provide a mechanistic rationale for the pathophysiology of atherosclerosis and inflammatory cell accumulation that is characteristic of fibroproliferative disorders of the vessel wall. HDAC9 might be a potential target for protecting against ox-LDL-induced apoptosis in endothelial cells, and the occurrence of atherosclerosis.

6. ACKNOWLEDGEMENTS

Xu Han and Xiang Han are co-first authors.

7. REFERENCES


10. Liu S, Shen H, Xu M, Liu O. FRP inhibits...
ox-LDL-induced endothelial cell apoptosis through an Akt-NF-(kappa)B-Bcl-2 pathway and inhibits endothelial cell apoptosis in an apoE-knockout mouse model. Am J Physiol Endocrinol Metab 299(3), E351-63 (2010) DOI: 10.1152/ajpendo.00005.2010


13. Steinberg D. Low density lipoprotein oxidation and its pathobiological significance. J Biol Chem 272(34), 20963-6 (1997) DOI: 10.1074/jbc.272.34.20963


Doi not found.

DOI: 10.1074/jbc.272.41.25783

Doi not found

DOI: 10.1126/science.277.5332.1652

DOI: 10.1074/jbc.272.39.24125

DOI: 10.1161/01.CIR.0000041433.94868.12

DOI: 10.1016/j.rdc.2005.01.006

DOI: 10.1016/j.bbrc.2004.04.056

**Abbreviations:** Ox-LDL: oxidized low-density lipoprotein; CRP: C-reactive protein; TNF-α: tumor necrosis factor; HUVECs: human umbilical vein endothelial cells

**Key Words:** Ox-LDL, TNF-α, HUVECs