Statins Control Oxidized LDL-Mediated Histone Modifications and Gene Expression in Cultured Human Endothelial Cells

Philippe Dje N’Guessan, Fabian Riediger, Kremena Vardarova, Stefanie Scharf, Julia Eitel, Bastian Opitz, Hortense Slevogt, Wilko Weichert, Andreas C. Hocke, Bernd Schmeck, Norbert Suttorp, Stefan Hippenstiel

Objective—Activation of the endothelium by oxidized low-density lipoprotein (oxLDL) has been implicated in the development of atherosclerosis. Histone modifications impact on the transcriptional activity state of genes. We tested the hypothesis that oxLDL-induced inflammatory gene expression is regulated by histone modifications and experienced the effect of statins on these alterations.

Methods and Results—OxLDL-related interleukin-8 (IL-8) and monocyte-chemoattractant protein-1 (MCP-1) secretion in endothelial cells was reduced by statins but enhanced by histone deacetylase inhibitors. OxLDL induced lectin-like oxidized LDL receptor-1 (LOX-1) and extracellular regulated kinases (ERK1/2)-dependent acetylation of histone H3 and H4 as well as phosphorylation of histone H3, both globally and on the promoters of il8 and mcp1. Pretreatment of oxLDL-exposed cells with statins reduced the above mentioned histone modification, as well as recruitment of CREB binding protein (CBP) 300, NF-κB, and of RNA polymerase II but prevented loss of binding of histone deacetylase (HDAC)-1 and -2 at the il8 and mcp1 gene promoters. OxLDL reduced HDAC1 and 2 expression, and statins partly restored global HDAC-activity. Statin-related effects were reverted with mevalonate. In situ experiments indicated decreased expression of HDAC2 in endothelial cells in atherosclerotic plaques of human coronary arteries.

Conclusions—Histone modifications seem to play an important role in atherosclerosis. (Arterioscler Thromb Vasc Biol. 2009;29:380-386.)

Key Words: endothelium ■ cytokines ■ statins ■ histone ■ HDAC

Atherosclerosis is a chronic inflammatory disease of the arterial wall. Increased oxLDL serum levels are considered as an important risk factor for atherosclerosis, and oxLDL accumulation in the vessel wall has been suggested to trigger endothelial inflammation. Expression of cytokines and adhesion molecules by activated endothelium promotes monocyte/lymphocyte infiltration into the subendothelium thus stimulating inflammation of the vessel wall. In particular, strong chemoattractants and proinflammatory cytokines like IL-8 and MCP-1 liberated by inflamed endothelium contribute to leukocyte attraction in atherosclerosis. LOX-1 is increasingly linked to atherosclerotic plaque formation. LOX-1 activation by oxLDL stimulates endothelial proinflammatory gene expression and production of superoxide radicals.

Increasing evidence indicates that histone modifications are important for the transcriptional activity state of genes in many cellular processes. In chromatin, 146 base pairs of DNA are wrapped 1.65 turns around a histone octamer (H2A, H2B, H3, H4). Transcription repression or gene activation is regulated by specific covalent modifications of accessible N-terminal histone tails including acetylation (mostly lysine) and phosphorylation (serine/threonine). For example, histone acetylases (HATs) increase histone acetylation thereby reducing DNA-histone binding and facilitating gene transcription whereas histone deacetylases (HDACs) act in the opposing way. Phosphorylation at Ser-10 on histone acetylation at Lys-14 of H4 seem to have a special impact on gene regulation. These modifications were, eg, implicated in lipopolysaccharide-stimulated activation of dendritic cells as well as in Listeria monocytogenes- or Chlamyphila pneumoniae-induced activation of human endothelial cells.

In this study we tested the hypothesis that oxLDL regulated inflammatory gene expression in endothelial cells by histone modifications. Results presented indicate that histone modifications impact on oxLDL-LOX-1/ERK1/2-related expression of proinflammatory genes in endothelial cells. Moreover,
Statins prevented oxLDL-related modifications of histones and restored HDAC activity in oxLDL-exposed cells. Understanding of histone-related control of gene expression may help to develop new therapeutic interventions in inflammatory diseases of the vessel wall.

**Materials and Methods**

For a detailed description please see the supplemental materials (available online at http://atvb.ahajournals.org).

**Material and Reagents**

All material, chemicals, and reagents used were of analytic grade.

**Culture of Human Aortic Endothelial Cells and Human Umbilical Vein Endothelial Cells**

Human aortic endothelial cells (HAECs) and human umbilical vein endothelial cells (HUVECs) were cultured as described previously.14,15

**Cytokine Measurement**

IL-8 and MCP-1 were quantified using a sandwich-ELISA Kit.

**RNA Interference in HUVECs**

HUVECs were transfected with control nonsilencing siRNA or siRNA targeting LOX-1.

**Chromatin Immunoprecipitation**

HUVECs were stimulated and chromatin immunoprecipitation (ChIP) for *il8* and *mcp1* promoter was performed as described previously12,16 with antibodies against Ac-H4, P-Ser10/Ac-Lys14-H3, p65, RNA polymerase II, HDAC1, and HDAC2. Equal input DNA control was assessed.

**Western Blotting**

To determine siRNA-mediated knock-down of LOX-1, HDAC-1, and -2 expression or diverse histone modifications (Acetyl-H4 and P-Ser10/Ac-Lys14-H3), Western blots were performed. Proteins were visualized by incubation with secondary IRDye800- or Cy5.5-labeled antibodies, respectively (Odyssey infrared imaging system, LI-COR Inc.).12,15–17

**Patients, Tissue, and Immunohistochemistry**

Tissue of coronary arteries was used in this retrospective study. Location and grade of arteriosclerotic vessel alterations was established on standard H&E stained sections. An immunohistochemical detection of HDAC1 and -2 in the tissue samples was performed. Quantitative analysis of immunostaining by digital image analysis was performed as described previously.18

**HDAC Activity Assay**

Global HDAC-activity was measured by fluorometric detection with an HDAC activity assay (BIOMOL) as described previously.17

**Statistics**

Data are shown as the mean±SEM of at least 3 independent experiments. For data analysis, appropriate tests were applied. *P<0.05* was considered to be significant as indicated by an asterisk (*) or an H Key (#). If not indicated otherwise test was performed versus control (*) or stimulated probe versus inhibitor treated probe (♯).

**Results**

OxLDL Induced IL-8 and MCP-1 Expression in HUVECs and HAECs

OxLDL-related release of proinflammatory cytokines by endothelial cells may promote inflammation of the vessel wall contributing to initiation and progression of atherosclerosis.1–3 We analyzed the regulation of gene expression of two proinflammatory model cytokines, IL-8 and MCP-1, which are dose-dependently induced in oxLDL-exposed endothelial cells (supplemental Figure I).

**Histone Modifications Contribute to oxLDL Related Cytokine Expression in Endothelial Cells**

Increasing evidence indicates that the transcription of inflammatory genes is regulated by specific histone modifications.11,12 Stimulation of HUVECs with oxLDL time-dependently induced global acetylation of H4 and phosphorylation/acetylation of H3 at Ser-10/Lys-14 (Figure 1A). To test whether oxLDL-related histone modifications are of functional importance for the observed cytokine induction, cells were incubated with HDAC inhibitors TSA (0.01 ng/mL) and SAHA (1 nmol/L), concentrations which do not induce significant cytokine expression in endothelial cells per se12,13 (Figure 1) followed by oxLDL (50 μg/mL) stimulation. As shown in Figure 1, TSA as well as SAHA significantly enhanced oxLDL-induced expression of IL-8 (Figure 1C) and MCP-1 (Figure 1D) in HUVECs and HAECs. In accordance, TSA and SAHA increased global acetylation of H4, and exposure of cells to oxLDL and TSA increased H4-acetylation in HUVECs (Figure 1B). Thus,
Histone acetylation seems to contribute to oxLDL-related cytokine release in human endothelial cells.

**LOX-1 and ERK1/2 Pathway Are Important for oxLDL-Related Histone Modifications in Endothelial Cells**

Endothelial cells mediate the uptake of oxLDL by LOX-1 which accumulates in atherosclerotic lesions. Therefore, we analyzed the role of LOX-1 for oxLDL induced histone modifications (Figure 2A and 2B). LOX-1–specific siRNA inhibited LOX-1 protein expression in HUVECs (Figure 2A). In HUVECs with silenced LOX-1, we found reduced global acetylation of H4 and phosphorylation/acetylation of H3 at Ser-10/Lys-14 (Figure 2B). In contrast, nonsilencing siRNA (control siRNA) neither reduced LOX-1 expression nor inhibited the above-mentioned histone modifications (Figure 2A and 2B). These observations suggest that LOX-1 is essential for oxLDL-induced global acetylation of H4 and phosphorylation/acetylation of H3 at Ser-10/Lys-14 in HUVECs.

ERK1/2-related signaling has been shown to be important for oxLDL–LOX-1–mediated inflammation. Thus, we next analyzed the impact of the ERK1/2 pathway on oxLDL-related histone modifications. Inhibition of the ERK1/2 pathway blocked oxLDL-induced phosphorylation/acetylation of histone H3 (Ser10/Lys14) and acetylation of histone H4 (Lys8) (Figure 2 C).

**Statins Control the oxLDL-Induced Cytokine Release Through Blockade of Histone Modifications**

The beneficial effects of clinically used statins, which blocked 3-hydroxy-3-methylglutaryl coenzyme A (HMG Co-A), depended at least in part on the inhibition of the release of proinflammatory cytokines. Pretreatment of endothelial cells with simvastatin (5 μmol/L) or fluvastatin (1 μmol/L) for 24 hours reduced oxLDL-related release of IL-8 (Figure 3A) and MCP-1 (Figure 3B). Preincubation of cells with both statins blocked oxLDL-related modification of histone H3 (Ser-10/Lys-14) as well as of H4 as shown by Western blot (Figure 3A). Furthermore, statin effects on oxLDL-induced histone modifications are reverted by mevalonate (500 μmol/L). Thus, statins may control inflammation by impacting on histone modifications. Within the time and dose frames used in this study, simvastatin, fluvastatin, mevalonate, and oxLDL did not significantly reduce endothelial cell numbers or induced morphological signs of cytotoxicity (data not shown).

**OxLDL-Related Histone Modifications of the IL8 and MCP1 Gene Promoter and Effect of Statins on These Processes**

To gain more insight into the impact of histone modifications on oxLDL-related cytokine regulation, we analyzed *il8* and
Ac-H4, Ser-10/Lys-14 H3, CBP/P300, is known to be essential for IL-8 and MCP-1 expression23,24. As it could be seen in Figure 4B, preincubation with 5 \( \mu \)g/mL simvastatin decreased protein expression (Figure 4), we assessed its effect on oxLDL-stimulated endothelial cells as well as binding of Pol II at the promoters indicating the start of gene transcription (Figure 4A). We observed recruitment of p65/RelA to the promoters in oxLDL-stimulated endothelial cells as well as the recruitment of the basic gene transcription machinery of the cell, including RNA polymerase II (Pol II), and NF-κB/RelA were detected by ChIP at gene promoters (A). Analysis of Simva preincubation (B) and effect of TSA, SAHA, (C) and Simva (D).

**Figure 4. oxLDL-related histone modifications at the il8 and mcp1 gene promoter.** Ac-H4, Ser-10/Lys-14 H3, CBP/P300, HDAC1, HDAC2, RNA polymerase II (Pol II), and NF-κB/RelA were detected by ChIP at gene promoters (A). Analysis of Simva preincubation (B) and effect of TSA, SAHA, (C) and Simva (D).

Because statins inhibit oxLDL-related IL-8 and MCP-1 expression, we studied the binding pattern of the HAT-CBP/p300 as well as HDAC1 and HDAC2 on the promoters of il8 and mcp1 (Figure 4A). In oxLDL exposed cells we noticed recruitment of CBP/p300 and reduced binding of HDAC1 and HDAC2 at both promoters (Figure 4A).

Activation of transcription factor NF-κB p65/RelA subunit is known to be essential for IL-8 and MCP-1 expression23,24, as well as the recruitment of the basic gene transcription machinery of the cell, including RNA polymerase II (Pol II).24 We observed recruitment of p65/RelA to the il8 and mcp1 promoters in oxLDL-stimulated endothelial cells as well as binding of Pol II at the promoters indicating the start of gene transcription (Figure 4A).

Because statins inhibited oxLDL-related IL-8 and MCP-1 protein expression (Figure 4), we assessed its effect on histone modifications at the two promoters. As it could be seen in Figure 4B, preincubation with 5 \( \mu \)mol/L simvastatin reduced phosphorylation/acetylation of H3 at Ser-10/Lys-14 at the il8 and mcp1 promoters (Figure 4A). In parallel, simvastatin reverted the composition of histone modifying enzymes back to a gene silencing state in oxLDL-exposed cells by reducing HAT-CBP/p300 recruitment (Figure 4B) and promoting binding of acetylation-suppressing HDAC1 and HDAC2 (Figure 4B). This is accompanied by loss of p65/RelA and Pol II recruitment in simvastatin preincubated cells (Figure 4B). As exemplary shown in Figure 4C and 4D, TSA and SAHA increased the recruitment of Pol II at il8 promoter, whereas as expected simvastatin had no effect on that process (Figure 4D).

**Effect of oxLDL on HDAC1, HDAC2 Expression, and Global HDAC Activity in Endothelial Cells**

Our data indicate that increased histone acetylation contribute to oxLDL-induced proinflammatory gene expression in endothelial cells. Reduced expression and activity of HDACs may contribute to increased transcription of inflammatory genes in oxLDL exposed endothelium in atherosclerosis as suggested for a chronic inflammatory lung disease.25 In line with this hypothesis, we noticed that prolonged incubation of endothelial cells with 50 \( \mu \)g/mL oxLDL decreased protein expression of HDAC1 and HDAC2 (Figure 5A). Furthermore, we assessed HDAC1 and HDAC2 expression in human coronary artery endothelium. At sites free of atherosclerosis, constitutive expression of both HDACs in the nuclei of endothelial cells was observed (Figure 5B, III and V). Interestingly, in endothelial cells covering atherosclerotic lesions of coronary arteries a slight reduction of HDAC2 expression was noted (Figure 5B, VI and 5C). Moreover, global HDAC activity was decreased in oxLDL-exposed human endothelial cells (Figure 6A), and preincubation of cells with statins reversed, at least in part, the oxLDL-related reduction of HDAC activity in endothelial cells in vitro (Figure 6B). Addition of mevalonate reverted statin-related effects on oxLDL-induced HDAC activity (Figure 6B).

**Discussion**

This study evidenced that statins modulate oxLDL-induced inflammatory activation of human endothelial cells by interfering with LOX-1-ERK1/2 signaling pathway leading to histone modifications. OxLDL-induced expression of proatherosclerotic cytokines IL-8 and MCP-1 was reduced by statins, whereas HDAC inhibitors enhanced cytokine liberation. Pretreatment with statins reduced global oxLDL-induced histone modifications as well as specific modifications at the il8 and mcp1 gene promoters accompanied by loss of NF-κB p65/RelA and RNA polymerase II promoter recruitment. Furthermore, the statin-related effects were reverted by incubation of cells with mevalonate.

Impaired endothelial function is observed in a variety of pathological conditions of the vessel wall such as hypertension and atherosclerosis,26 and the endothelium is a primary target in several inflammatory diseases.27 In particular, oxLDL accumulation in vascular vessel wall has been suggested to trigger endothelial inflammation and promoting atherosclerosis.1,2 In our model, incubation of endothelial cells with oxLDL induced the release of the chemoattractants and proinflammatory cytokines IL-8 and MCP-1. These cytokines are thought to also play important roles in atherosclerosis: In aortic arches of atherosclerotic mice, for example, expression of MCP-1 was plaque progression-dependently increased.28 MCP-1 serum levels were elevated in patients with coronary artery disease, myocardial infarction, and unstable angina.
Enhanced IL-8 production was observed in human abdominal aortic aneurysms, and IL-8 plasma levels were increased in coronary heart disease patients. In addition, IL-8 was significantly increased in infarct-related coronary artery thrombi and atherosclerotic plaque specimens obtained with a transluminal extraction catheter from cases of acute myocardial infarction. Because release of IL-8 and MCP-1 was also observed in oxLDL incubated macrophages, these cytokines may play an important role in the host response to inflammation within the vessel wall and we therefore analyzed them as model cytokines.

Recent studies imply that histone modifications control eukaryotic gene transcription by affecting transcription factor binding, recruitment of the basal transcription machinery, and promoter transactivation. Tight wrapping of DNA around histone octamers appears to obstruct binding of the transcription machinery. Acetylation and phosphorylation of histones change the polarity of histone tails. Particularly, histone acetylation facilitates uncoiling of DNA and binding of transcription factors and the basal transcription machinery thereby promoting gene transcription. Here we show that oxLDL induced the acetylation of histone H4, phosphorylation (P-Ser-10), and acetylation (Ac-Lys-14) histone H3. Furthermore, the release of IL-8 and MCP-1 was enhanced by inhibition of histone deacetylation in human venous and aortic endothelial cells. Stimulus-induced phosphorylation of H3 at Ser-10 has been reported to be associated with the activation of promoters of mammalian immediate-early genes.

**Figure 5.** In vitro and in situ HDAC expression. A, HUVECs were stimulated with oxLDL. HDAC1 and HDAC2 expression were analyzed. B, Coronary arteries were examined for atherosclerotic lesions as shown by H&E staining (I, II). Endothelial HDAC1 (III, IV) and HDAC2 (V, VI) expression was analyzed (B; C).

**Figure 6.** Effect of statins on HDAC activity in oxLDL stimulated endothelial cells. Quantification of global HDAC activity in oxLDL-exposed HUVECs (A). HUVECs were preincubated with Simva or Fluva and stimulated with oxLDL in presence or absence of HMG-CoA reductase inhibitor mevalonate (Mev) and global HDAC activity was assessed (B).
genes. Some evidence has been provided that H3 phosphorylation at Ser-10 may have a role in the regulation of transcription by acting as a signal for subsequent acetylation of lysines and, in particular, histone H3 Lys-14. Moreover, Agalioti et al demonstrated that both phosphorylation/acetylation (Ser-10/Lys-14) of histone H3 and acetylation of Lys-8 at histone H4 were necessary for the recruitment of general transcription factors and hence for gene transcription. In this line, we provided evidence that histone acetylation is necessary for L. monocytogenes as well as C. pneumoniae-related release of IL-8, but not IFN-γ by human endothelial cells. Furthermore a study done by Choi and coworkers has shown in trichostatin A an histone acetyltransferase inhibitor exacerbates atherosclerosis in LOX-1-deficient mice. Overall, histone modifications seem to impact on gene expression in inflamed endothelium, including oxLDL-induced release of IL-8 and MCP-1 and are therefore important in the pathogenesis of atherosclerosis.

OxLDL activates endothelial cells via LOX-1–related ERK1/2 activation. Indeed, we found that oxLDL-induced histone modifications depended on LOX-1 and ERK1/2. Because some reports have demonstrated that the ERK1/2 pathways was involved in phosphorylation of histone H3 and activity of histone acetyltransferases, it might be reasonable to suggest that oxLDL controlled the above-mentioned histone modifications through LOX-1–induced ERK1/2 activation.

Histone acetylation seems to be regulated by a balanced action of HATs like CBP/p300 facilitating acetylation and HDACs acting in the opposite way. In oxLDL-exposed cells, we noted the recruitment of HAT CBP/p300 but a loss of binding of HDAC1 and 2 at the mcp1 and il8 gene promoters. In parallel, NF-κB p65/RelA (which is known to be critical for IL-8 expression) as well as Pol II were both recruited to the promoters of both genes. In line with these observations, previous studies indicated a central role of histone phosphorylation and acetylation for IL-8 expression. As Agalioti et al have shown, phosphorylation of H3 at Ser-10, acetylation of H3-Lys-14 and H4-Lys-8 were followed by recruitment of bromodomain-containing factors TFIID and SWI/SNF and subsequent gene transcription. Additionally, decreased of HDAC1 binding have demonstrated to be important for Legionella pneumophila induced IL-8 release in lung epithelium. However, as seen in other studies and this report, the time-course of the overall histone modifications observed for H3 and H4 did not directly reflect the time-course of specific modifications observed at specific gene promoters of, eg, il8 and mcp1. Overall, these studies implicate a sequence of oxLDL-related histone phosphorylation, histone acetylation, chromatin remodeling, and successful gene transcription.

We demonstrated that simvastatin reduced oxLDL-related phosphorylation/acetylation of H3 as well as acetylation of H4, but, however, showed only minor effects on acetylation of H4 at the il8 and mcp1 gene promoters. In addition, exposure to simvastatin reverts the binding pattern of histone modifying enzymes back to that observed in resting cells, eg, reduced detection of CBP/p300 but enhanced the binding of HDAC1 and -2. Moreover, this was accompanied by loss of NF-κB p65/RelA and Pol II binding at both analyzed gene promoters. In line with these observations, we recently reported that C. pneumoniae-related histone modifications and subsequent proinflammatory cytokine release is inhibited by simvastatin.

It has been suggested that the beneficial effects of HMG-CoA reductase inhibiting statins on endothelial and vascular function depended on both cholesterol reduction as well as cholesterol independent mechanism. Because the results obtained by using statins in our study could be reverted through addition mevalonate, observed effects may be mediated, at least in part, through inhibition of small GTP-binding Rho proteins as evidenced for C. pneumoniae-infected endothelium.

Current evidence suggested that chronic inflammatory processes (in the lung) might impact on the expression on histone modifying enzymes. We now noted in oxLDL-exposed endothelial cells reduced expression of HDAC1 and HDAC2 as well as reduced global HDAC activity. Moreover, investigation of HDAC1 and HDAC2 expression in situ in atherosclerotic lesions of human coronary arteries indicated reduced expression of HDAC2. The expression of HDAC2 is reduced in the peripheral lung and in alveolar macrophages of patients with COPD, and we recently demonstrated reduced expression of HDAC2 and reduced HDAC activity in lung epithelial cells infected with Moraxella catharrhalis. Thus, during (chronic) inflammation, reduced HDAC expression may pave the way to hyperacetylation of proinflammatory genes with subsequently enhanced expression of those genes resulting in chronic tissue inflammation as observed in atherosclerosis and COPD. Remarkably, statins, at least in part, reverted HDAC activity in vitro in a cholesterol-dependent manner, and may thereby contribute to suppression of inflammatory gene expression. However, a more detailed analysis of more samples and specimen from diverse vascular lesions has to be performed to quantify HDAC expression in endothelial cells in atherosclerosis in humans.

In conclusion, we provide evidence that histone modifications play an important role in oxLDL-induced cytokine production by human endothelial cells. The above-described pathway may contribute significantly to the pathogenesis of chronic vascular lesions and coronary heart disease. This study also suggests that some beneficial effects of statins in cardiovascular diseases may be based on the control of histone modifications of inflammatory genes.

Acknowledgments

The excellent technical assistance of Sylvia Schapke, Franke Schreiber, and Jacqueline Hellwig is greatly appreciated. Parts of this work will be included in the MD thesis of F. Riediger.

Sources of Funding

This work was supported by the Deutsche Forschungsgemeinschaft to S.H. (Hi-789/6-1) and by the Bundesministerium für Bildung und Forschung (BMBF) to B.S. (BMBF-FORSYS Junior Group), N.S., and S.H. (BMBF-network PROGRESS).

Disclosures

None.
References


Statins Control Oxidized LDL-Mediated Histone Modifications and Gene Expression in Cultured Human Endothelial Cells

Philippe Dje N’Guessan, Fabian Riediger, Kremena Vardarova, Stefanie Scharf, Julia Eitel, Bastian Opitz, Hortense Slevogt, Wilko Weichert, Andreas C. Hocke, Bernd Schmeck, Norbert Suttrop and Stefan Hippenstiel

Arterioscler Thromb Vasc Biol. 2009;29:380-386; originally published online January 2, 2009;
doi: 10.1161/ATVBAHA.108.178319

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/29/3/380

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2009/01/27/ATVBAHA.108.178319.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Fig I SUPPLEMENTAL MATERIAL

A

- HUVEC

- HAEC

B

- IL-8 [ng/ml]

- MCP-1 [ng/ml]

- oxLDL [µg/ml]

- TNFα
MATERIALS AND METHODS

Material and Reagents

ECBM (Endothelial Cell Basal Medium) was provided by PAA, Austria. FCS, trypsin-EDTA-solution and antibiotics were obtained from Life Technologies (Karlsruhe, Germany). Simvastatin, fluvastatin, mevalonate, SAHA, and TSA were obtained from Merck Biosciences GmbH (Bad Soden, Germany) and TNFα from R & D System (Wiesbaden, Germany). OxLDL was purchased from Biotrend (Cologne, Germany). The characteristic of the used OxLDL is mentioned below:

Unesterified Cholesterol ~8%
Cholesterol ester~ 37%
Triglyceride ~11%
Phospholipid~22%
Protein~22%
MWT...~2.3 million

Each vial of 1ml contains 5 milligrams of protein and ~23 milligrams of whole LDL Particle.

All other chemicals used were of analytical grade and obtained from commercial sources.

Culture of Human aortic endothelial cells (HAEC) and human umbilical vein endothelial cells (HUVEC)

HAEC were obtained from Clonetics (San Diego, CA, USA), and HUVEC were isolated from human umbilical cord veins. Both cell types were cultured as described previously1,2. HUVEC or HAEC were incubated with oxLDL as indicated in MCDB131 or EGM-2, respectively.
Cytokine measurement

IL-8 and MCP-1 concentrations in the supernatants of stimulated HUVEC or HAEC were quantified using a commercially available sandwich-ELISA Kit (R&D Systems, Wiesbaden, Germany) according to the manufacturer’s instructions.

RNA interference in HUVEC

Control non-silencing siRNA (sense UUCUCCGAACGUGUCACGUtt, antisense ACGUGACACGUUCGGAGAAtt), siRNA targeting LOX-1 (sense CCUAAACUCACCA-GAAAAAGtt, antisense CUUUUUCUGGUGAGUUAGGtt) were purchased from Ambion (Austin, TX, USA). HUVECs were transfected by using Amaxa Nucleofector™ (Amaxa, Köln, Germany) according to the manufacturer's protocol (HUVEC Nucleofector Kit) with 2 µg siRNA per 10⁶ cells.

Chromatin Immunoprecipitation (ChIP)

Briefly, HUVECs were stimulated and ChIP was performed as described previously³;⁴ with antibodies from Cell Signaling Technology, MA, USA (Ac-H4 and P-Ser10/Ac-Lys14-H3), and Santa Cruz Biotechnology, CA, USA (p65, RNA polymerase II, HDAC1, and HDAC2). Equal amounts of input DNA was controlled by gel electrophoresis. The following promoter-specific primers were used: IL-8 sense 5’–AAGAAAAACTTTTGTCATACTCCG–3’, antisense 5’–TGGCTTTTTATATCATCACCCTAC–3’. MCP-1 sense 5’–GCCTTTGCATATATCAGACAG–3’, antisense 5’–CAGGCTTGTGCC GAGATGTTC–3’.
Western blotting

To determine siRNA-mediated knock-down of LOX-1, Western blots were performed. Briefly, HUVEC were transfected or stimulated as indicated. Cells were lysed in buffer containing Triton X-100, subjected to SDS-PAGE and blotted on Hybond-ECL membrane (Amersham Biosciences, Freiburg, Germany). Immunodetection of target proteins was carried out with specific antibodies against LOX-1 (Santa Cruz Biotechnology, CA, USA). For the quantification of HDAC-1 and –2 expressions, HUVECs were treated with oxLDL as mentioned above. For the detection, HDAC-1 and –2 antibodies were used (Santa Cruz Biotechnology, CA, USA).

For histone analysis, cells were lysed, and H₂SO₄ was added to a final concentration of 0.2 M as described³. Protein was precipitated with trichloroacetic acid, subjected to SDS-PAGE and blotted on Hybond-ECL membrane (Amersham Biosciences, Freiburg, Germany). Immunodetection was carried out with antibodies specifically detecting Acetyl-H4 and P-Ser-10/Ac-Lys-14-H3 (Cell Signaling Technology, MA, USA). In all experiments, H3, H4 (Cell Signaling Technology, MA, USA) or actin (Santa Cruz Biotechnologies, Santa Cruz, CA) was detected simultaneously to confirm equal protein loading. Proteins were visualized by incubation with secondary IRDye 800- or Cy5.5-labeled antibodies, respectively (Odyssey infrared imaging system, LI-COR Inc., Bad Homburg, Germany)²⁻⁵.

Patients, tissue and immunohistochemistry

Archival tissue of coronary arteries taken for diagnostic purpose from explanted hearts of 5 patients who received heart transplantation was used in this retrospective study. Use of the tissue for scientific purposes was approved by the ethics committee of the Charité - Universi-
sitätsmedizin Berlin, Germany. Each tissue block consisted of several cross sections taken from the anterior descending branch of the left coronary artery. Tissue was fixed in formalin, embedded in paraffin and freshly cut. Identification of location and grade of arteriosclerotic vessel alterations was established on standard H&E stained sections. For immunohistochemical detection of HDAC isoforms on tissue samples, prediluted HDAC1 Ab (1:11, Abcam, Cambridge, UK) or HDAC2 Ab (1:5000, Abcam, Cambridge, UK) were used on 5 μm paraffin sections, respectively. For antigen retrieval, deparaffinized slides were placed in 0.01 M sodium citrate buffer, pH 6.0 and boiled for 5 min in a pressure cooker. After several rinses in TBS and pre-treatment with blocking reagent (DAKO, Glostrup, Denmark) for 5 min, slides were incubated with primary antibody in antibody diluting solution (Zymed, San Francisco, CA, USA) for 20 min at room temperature and subsequently at 4°C overnight. After washing, bound antibody was detected by applying a streptavidin-biotin/alkaline phosphatase system (BioGenex, San Ramon, CA, USA) due to a standard protocol with standard antibody dilutions as supplied by the manufacturers. For visualization, a fast red system (Sigma, Deisenhofen, Germany) was used. The slides were cover slipped using Aquatex (Merck, Gernsheim, Germany).

Quantitative analysis of immunostaining by digital image analysis

Image analysis was performed as described previously⁶. Briefly, measurement was performed with a custom-designed filter for red alkaline phosphatase system in optical quality (central wavelength 525 nm; half band width 10 nm ± 2 nm; Chroma Technology Corp., Brattleboro, USA). 8-bit (range: 0-255) gray-scale images (objective: 40x/numerical aperture 1.3 oil; Zeiss Plan-Neofluar) were acquired using a cooled CCD-camera (AxioCam MRm, Zeiss, Jena, Ger-
many) mounted on an Axioskop 2 mot (Zeiss), and processed with ImageProPlus® 4.5 (Media Cybernetics, Silver Springs, USA). Background measurement was performed to evaluate the influence of nonspecific antibody binding, and a shade of gray of 60 was set as threshold for positive staining. For the measurement of HDAC isoform expression endothelial cells covering morphologically normal vessel areas (control) were compared to endothelial cells covering overt atherosclerotic lesions on the same slide. For each, control and atherosclerosis, at least 15 cells (n = 5) were analyzed. Signal intensities of control cells were set 100% and changes of atherosclerotic lesions were expressed as percentage of control. For direct visualization of immunostaining intensity a pseudocolor scale was applied. Images for H&E staining were taken with a cooled high-range-color AxioCam HRc (Zeiss); (objective: 10x/numerical aperture 0.3; Zeiss Plan-Neofluar).

**HDAC activity assay**

Global HDAC-activity was measured by fluorometric detection with an HDAC activity assay (BIOMOL, Hamburg, Germany) as described previously.

**Statistics**

Data are shown as the mean ± SEM of at least three independent experiments. A one-way ANOVA was used for data analysis of Fig. 1, 2 (C; D), 4 (C; D), 6C, 7. Main effects were then compared by a Newman-Keuls' post-test. Mann-Whitney U test for non-parametric data was used for Fig. 6B/C. A value of p < 0.05 was considered to be significant as indicated by an asterisk (*) or an H Key (#). If not indicated otherwise test was performed vs. control (*) or stimulated probe vs. inhibitor treated probe (#).
RESULTS

OxLDL induced IL-8 and MCP-1 expression in HUVEC and HAEC

OxLDL-related release of pro-inflammatory cytokines by endothelial cells may promote inflammation of the vessel wall contributing to initiation and progression of atherosclerosis\(^7\)\(^{-9}\). We analyzed the regulation of gene expression of two pro-inflammatory model cytokines, IL-8 and MCP-1, which are dose-dependently induced in oxLDL-exposed endothelial cells (Fig 1 Supplement).
**Figure Legends**

**Figure 1:** Histone modifications contribute to oxLDL related cytokine expression in endothelial cells. HUVEC were incubated with oxLDL (50µg/ml) for the indicated time periods. Histone modifications were detected by Western blot using antibodies specifically detecting Ac-H4, phosphorylated/acetylated (Ser-10/Lys-14) H3 (A; B). TSA and SAHA were used as positive control and a synergistic effect of oxLDL and TSA was also addressed by stimulation of HUVEC with both substances (B). Moreover HUVEC and HAEC were pre-incubated with the HDAC inhibitor TSA (0.01ng/ml) or SAHA (1nM) and then stimulated with oxLDL (50µg/ml) for 16 h. Release of cytokines (IL-8 and MCP-1) was measured in the supernatant by ELISA (C; D). For A and B representatives out of three independent experiments were shown. Data are shown in ELISA (C; D) as means ± SEM of at least three independent experiments. *, p < 0.05 for oxLDL stimulated vs. unstimulated control. #, p < 0.05 for oxLDL stimulated vs. TSA/SAHA-exposed cells.

**Figure 2:** LOX-1 and ERK1/2 pathway are important for oxLDL related Histone modifications in endothelial cells

To assess the role of LOX-1 for oxLDL control activation of Histone modifications, HUVECs were transfected with control siRNA or specific siRNAs targeting LOX-1 and gene silencing abilities were assessed by Western blot (A). Additionally LOX-1 targeted HUVECs were incubated with oxLDL (50µg/ml) and histone modifications were detected by Western blot using antibodies specifically detecting Ac-H4, phosphorylated/acetylated (Ser-10/Lys-14) H3 (B). To investigate the contribution of ERK1/2 to oxLDL-induced histone modifications, HUVECs were pre-incubated for 1 h with the chemical ERK1/2 inhibitor U0126 10µM before
incubation with oxLDL (50µg/ml) for 30 min. Histone modifications were detected by Western blot using antibodies specifically detecting Ac-H4, phosphorylated/acetylated (Ser-10/Lys-14) H3 (C).

Representatives of three independent blots with similar results are shown (A, B, C).

**Figure 3:** Statins control oxLDL induced cytokine release through blockade of histone modifications. To assess the effect of statins on oxLDL-induced histone modifications, HUVECs were pre-incubated for 24 h with simvastatin or fluvastatin before incubation with oxLDL for 30 min. Histone modifications were detected by Western blot using antibodies specifically detecting Ac-H4, phosphorylated/acetylated (Ser-10/Lys-14) H3 (A; B). Furthermore HUVEC and HAEC were pre-incubated for 24 h with simvastatin (Simva) (5 µmol/l) or fluvastatin (fluva) (1 µmol/l) and stimulated with oxLDL (50 µg/ml) for 16 h. Cytokine release was measured in the supernatant by ELISA (C; D). To test whether the observed effects of the statins were due to the inhibition of the HMG-CoA reductase, mevalonate (Mev) (500µmol/l) was supplemented to the abovementioned stimulation protocol (B; C; D). Data are shown as means ± SEM of at least three independent experiments for the ELISA. *, p < 0.05 from oxLDL stimulated vs. unstimulated control. #, p < 0.05 for oxLDL stimulated vs. simvastatin/fluvastatin-exposed cells. Representatives of three independent blots with similar results are shown (A; B).

**Figure 4:** oxLDL related histone modifications of the *il8* and *mcp1* gene promoter and effect of statins on these processes. HUVEC were incubated with oxLDL (50µg/ml) for the indicated time periods. Binding of modified histone (acetylated H4, phosphorylated/acetylated
Ser-10/Lys-14 on H3), as well as CBP/P300, HDAC1, HDAC2, RNA polymerase II (Pol II) and NF-κB/RelA were detected at the \textit{il8} and \textit{mcp1} gene promoter by ChIP (A). In addition HUVEC were pre-incubated for 24 h with simvastatin (Simva) (5µmol/l) and stimulated with 50µg/ml oxLDL for 30 min [excepted CBP/P300 and p65/RelA (60 min)]. The binding pattern of different targets to \textit{il8} and \textit{mcp1} promoters was analysed by ChIP (B). Inhibition of recruitment of phosphorylated/acetylated (Ser-10/Lys-14) H3, CBP/p300, p65/RelA, and Pol II but not of acetylated (H4) to \textit{il8} and \textit{mcp1} promoters were observed in the simvastatin pre-treated cells. As exemplary shown in (C) and (D) the effect of TSA, SAHA as well as simvastatin alone on the recruitment of pol II at \textit{il8} promoter in HUVEC was addressed. Representative gels out of three independent experiments are shown.

Figure 5: \textit{In vitro} and \textit{in situ} HDAC expression in endothelial cells. (A) HUVECs were stimulated with oxLDL (50µg/ml) for the indicated time periods. HDAC1 and HDAC2 expression were analysed by Western blot (representative blot out of 3 independent experiments). (B) Coronary arteries of five patients were examined for atherosclerotic lesions as shown by H&E staining (I, II). Quantitative endothelial HDAC1 (III, IV) and HDAC2 (V, VI) expression was digitally analysed and pseudocolor depiction of nuclear expression of both isoforms was demonstrated as indicated by arrowheads. (C) HDAC expression is depicted as percent of gray scale values normalized to baseline controls (set as 100%). HDAC2 expression was slightly, but significantly reduced in atherosclerotic lesions (B VI and C). Mean ± SEM of 5 independent experiments are given. *p<0.05 vs. control. In 5 B representatives specimens from a control area compared to an atherosclerosis area are shown.
**Figure 6:** Effect of statin on HDAC activity in oxLDL stimulated endothelial cells. (A) HUVECs were stimulated with oxLDL (50µg/ml) for the indicated time periods and global HDAC activity was quantified in an HDAC activity assay. Moreover HUVECs were pre-incubated for 24 h with simvastatin (Simva) (5µmol/l) or fluvastatin (Fluva) (1µmol/l) and stimulated with oxLDL (50µg/ml) for 16 h (B). HDAC activity was quantified in an HDAC activity assay. To test whether the observed effects of the statins were due to the inhibition of the HMG-CoA reductase, mevalonate (Mev) (500µmol/l) was supplemented to the above-mentioned stimulation protocol (B). *, p < 0.05 from oxLDL stimulated vs. unstimulated control. #, p < 0.05 for oxLDL stimulated vs. simvastatin/ fluvastatin-exposed cells. Representatives out of three independent experiments are shown.

**Figure I Supplement:** oxLDL-induced concentration dependent release of IL-8 and MCP-1 in HUVEC and HAEC. HUVEC and HAEC were incubated for 16 h with oxLDL (10, 20, 50µg/ml). IL-8 and MCP-1 release were measured in the supernatant by ELISA. Cells stimulated with TNFα (50 ng/ml) were used as positive control. Data are shown as means ± SEM of at least three independent experiments. *, p < 0.05 compared cells with and without oxLDL incubation.