Endothelial Hypoxia-Inducible Factor-1α Promotes Atherosclerosis and Monocyte Recruitment by Upregulating MicroRNA-19a


Abstract—Chemokines mediate monocyte adhesion to dysfunctional endothelial cells (ECs) and promote arterial inflammation during atherosclerosis. Hypoxia-inducible factor (HIF)-1α is expressed in various cell types of atherosclerotic lesions and is associated with lesional inflammation. However, the impact of endothelial HIF-1α in atherosclerosis is unclear. HIF-1α was detectable in the nuclei of ECs covering murine and human atherosclerotic lesions. To study the role of endothelial HIF-1α in atherosclerosis, deletion of the Hif1α gene was induced in ECs from apolipoprotein E knockout mice (EC-Hif1α−/−) by Tamoxifen injection. The formation of atherosclerotic lesions, the lesional macrophage accumulation, and the expression of CXCL1 in ECs were reduced after partial carotid ligation in EC-Hif1α−/− compared with control mice. Moreover, the lesion area and the lesional macrophage accumulation were decreased in the aortas of EC-Hif1α−/− mice compared with control mice during diet-induced atherosclerosis. In vitro, mildly oxidized low-density lipoprotein or lysophosphatidic acid 20:4 increased endothelial CXCL1 expression and monocyte adhesion by inducing HIF-1α expression. Moreover, endothelial Hif1α deficiency resulted in downregulation of miR-19a in atherosclerotic arteries determined by microRNA profiling. In vitro, HIF-1α–induced miR-19a expression mediated the upregulation of CXCL1 in mildly oxidized low-density lipoprotein–stimulated ECs. These results indicate that hyperlipidemia upregulates HIF-1α expression in ECs by mildly oxidized low-density lipoprotein–derived unsaturated lysophosphatidic acid. Endothelial HIF-1α promotes atherosclerosis by triggering miR-19a–mediated CXCL1 expression and monocyte adhesion, indicating that inhibition of the endothelial HIF-1α/miR-19a pathway may be a therapeutic option against atherosclerosis. (Hypertension. 2015;66:00-00. DOI: 10.1161/HYPERTENSIONAHA.115.05886.)

Key Words: atherosclerosis ■ chemokines ■ endothelial cells ■ microRNAs

The adhesion of circulating monocytes to dysfunctional endothelial cells (ECs) results in the accumulation of macrophages in the subendothelial space and the formation of atherosclerotic lesions.1 Disturbed blood flow at arterial bifurcations primes the endothelium for inflammatory activation by modified lipoproteins, such as mildly oxidized low-density lipoproteins (moxLDLs).2–4 The oxidation of LDL leads to the generation of lysophosphatidic acid (LPA), which increases atherogenic monocyte adhesion by upregulating (C–X–C motif) ligand 1 (CXCL1) expression in ECs.5–7 Accordingly, CXCL1 and its receptor CXCR2 play key roles in the accumulation of macrophages in atherosclerotic lesions.8 In addition to proinflammatory nuclear factor-kB (NF-kB) signaling, the transcription factor hypoxia-inducible factor (HIF)-1α has been implicated in the regulation of CXCL1 expression.9,10

Endothelial HIF-1α activity mediates transendothelial glucose transport and hypoxia-induced angiogenesis.11,12 In addition to the upregulation of proangiogenic genes, such as vascular endothelial growth factor, the effects of endothelial HIF-1α on angiogenesis are mediated by microRNAs (miRNAs).13–15 For example, HIF-1α induces the expression of miR-107 in ECs, which promotes neoangiogenesis.16 By contrast, HIF-1α and miR-429 constitute a negative feedback loop that limits vascular endothelial growth factor expression...
in hypoxic ECs.\(^{13}\) In atherosclerosis, HIF-1α activation correlates with disease progression and lesional inflammation.\(^ {16,17}\) Vascular injury induces HIF-1α activation in smooth muscle cells and promotes neointima formation by upregulating CXCL1.\(^ {12,18}\)

Here, we investigated the role of endothelial HIF-1α in atherosclerosis. We found that HIF-1α is activated in atherosclerotic ECs and deletion of endothelial HIF-1α reduces atherosclerosis, the lesional macrophage content, and the expression of CXCL1 and miR-19a in apolipoprotein E knock-out (Apoe\(^{-/-}\)) mice. In ECs, moxLDL and LPA upregulated HIF-1α, which increased CXCL1-mediated monocyte adhesion by inducing miR-19a expression. These findings indicate that hyperlipidemia-induced HIF-1α in ECs promotes atherosclerosis by upregulating a proinflammatory miRNA.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

**HIF-1α Expression in Atherosclerosis**

HIF-1α expression was detectable in the nuclei of ECs covering human atherosclerotic lesions (Figure S1A in the online-only Data Supplement). In Apoe\(^{-/-}\) mice, nuclear HIF-1α expression was found in atherosclerotic ECs after 12 weeks of high fat diet (HFD) feeding but not in ECs from mice fed a normal diet (Figure S1B). These findings indicate that HIF-1α is activated in ECs during atherosclerosis. HFD feeding increased the Hif1a mRNA expression in the aortic arch, the thoracic and abdominal aorta, and the carotid artery of Apoe\(^{-/-}\) mice (Figure S2A). In the aortic root, however, HFD feeding did not increase the expression of Hif1a (Figure S2A) and nuclear HIF-1α accumulation was found in the majority of vascular cells in Apoe\(^{-/-}\) mice fed a normal diet (Figure S2B).

Disturbed blood flow is essential for hyperlipidemia-induced endothelial inflammation and atherosclerosis. After a decline of the Hif1a mRNA expression at 1 day and 1 week after induction of blood flow disturbances in the carotid artery of Apoe\(^{-/-}\) mice by partial ligation, the Hif1a transcript levels were increased after 2 and 4 week when compared with unligated carotid arteries (Figure S2C). Nuclear HIF-1α signals were detectable by immunostaining in ECs from partially ligated carotid arteries (Figure 1A).

**HIF-1α Promotes Endothelial Inflammation**

To investigate the role of HIF-1α on endothelial inflammation, EC-specific knockout of the Hif1a gene was induced

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**Figure 1.** Endothelial hypoxia-inducible factor (HIF)-1α induces proinflammatory gene expression. Dual HIF-1α and CD31 immunostaining in carotid arteries from Apoe\(^{-/-}\) mice (A) or from endothelial cell (EC)-Hif1a\(^{-/-}\) and EC-Hif1a\(^{+/+}\) mice (B) 6 weeks after partial ligation and feeding a high fat diet (HFD). Arrows indicate HIF-1α\(^{-/-}\) ECs. C, Gene expression in the carotid arteries 2 weeks after partial ligation and HFD feeding determined by quantitative reverse transcription polymerase chain reaction. D, Dual immunostaining for CXCL1 and CD31 in partially ligated carotid arteries. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Stars indicate the lumen. Scale bars, 50 \(\mu\)m (A and B) and 25 \(\mu\)m (D). \(^*\)P<0.05, n=5 to 8 mice per group.
in Apoe<sup>−/−</sup> (EC-Hif1a<sup>−/−</sup>) mice using the Cre-lox system. Endothelial HIF-1α immunostaining (Figure 1B) and Hif1α mRNA expression (Figure 1C) were reduced in partially ligated carotid arteries from EC-Hif1a<sup>−/−</sup> mice when compared with EC-Hif1a<sup>+/+</sup> mice. Moreover, the expression level of Cxcl1, Ccl2, and Tnfa but not those of Ccl5 and Mif were reduced in the carotid arteries of EC-Hif1α<sup>−/−</sup> mice compared with EC-Hif1α<sup>+/+</sup> mice 2 weeks after partial ligation (Figure 1C). CXCL1 expressing ECs were reduced in partially ligated carotid arteries from EC-Hif1α<sup>−/−</sup> mice, as identified by dual immunofluorescence staining of CXCL1 and CD31 (Figure 1D). A similar effect of HIF-1α on CXCL1 expression was observed in atherosclerotic lesions in the aorta after feeding an HFD (Figure 2A). Moreover, the lesional accumulation of macrophages was lower in EC-Hif1a<sup>−/−</sup> mice compared with EC-Hif1a<sup>+/+</sup> mice 2 weeks after partial ligation (Figure 2B). The lesional smooth muscle cell and collagen type I content were similar in both groups (Figure S4). To investigate whether HIF-1α in ECs contributes to vascular stenosis, the luminal volume of the partially ligated carotid arteries was quantified by computed tomographic angiography in vivo. The luminal volume was significantly reduced in the carotid arteries from EC-Hif1a<sup>−/−</sup> mice compared with EC-Hif1a<sup>+/+</sup> mice (Figure S5). Moreover, the lesion area and the lesional macrophage content were reduced in the aortas of EC-Hif1α<sup>−/−</sup> mice compared with EC-Hif1α<sup>+/+</sup> mice after feeding an HFD for 12 weeks (Figure S6). Deletion of the Hif1α gene in ECs did not affect serum cholesterol and triglyceride levels (Tables S2 and S3). Taken together, these results indicate that activation of HIF-1α in atherosclerotic ECs enhances the expression of proinflammatory cytokines.

**Endothelial HIF-1α Increases Atherosclerosis**

To study the effect of endothelial HIF-1α on atherosclerosis, lesion formation was determined in EC-Hif1α<sup>−/−</sup> mice and EC-Hif1α<sup>+/+</sup> mice. The lesion area was reduced in carotid arteries from EC-Hif1α<sup>−/−</sup> mice at 6 weeks after partial ligation and feeding an HFD (Figure 2A). Moreover, the lesional accumulation of macrophages was lower in EC-Hif1α<sup>−/−</sup> mice than in EC-Hif1α<sup>+/+</sup> mice (Figure 2B). The lesional smooth muscle cell and collagen type I content were similar in both groups (Figure S4). To investigate whether HIF-1α in ECs contributes to vascular stenosis, the luminal volume of the partially ligated carotid arteries was quantified by computed tomographic angiography in vivo. The luminal volume was significantly reduced in the carotid arteries from EC-Hif1α<sup>−/−</sup> mice compared with EC-Hif1α<sup>+/+</sup> mice (Figure S5). Moreover, the lesion area and the lesional macrophage content were reduced in the aortas of EC-Hif1α<sup>−/−</sup> mice compared with EC-Hif1α<sup>+/+</sup> mice after feeding an HFD for 12 weeks (Figure S6). Deletion of the Hif1α gene in ECs did not affect serum cholesterol and triglyceride levels (Tables S2 and S3). Taken together, these results indicate that activation of HIF-1α in ECs enhances atherosclerotic lesion formation.

**Endothelial HIF-1α Regulates CXCL1-Mediated Monocyte Adhesion**

MoxLDL releases unsaturated LPA species, such as LPA20:4, which promote atherosclerosis by upregulating endothelial CXCL1 expression. Accordingly, blocking LPA receptors using Ki16425 inhibited the moxLDL-induced release of CXCL1 from mouse aortic ECs (MAECs; Figure S7A). In contrast to LPA18:0, treatment with LPA20:4 induced the expression of Cxcl1 and its secretion from MAECs through the activation of LPA receptors (Figure S7B and S7C). Moreover, moxLDL or LPA20:4 stimulation induced Hif1α at the mRNA and protein level in MAECs (Figure 3A and 3B; Figure S8). Treatment with Ki16425 diminished both moxLDL- and LPA20:4-induced Hif1α expression in MAECs (Figure 3A and 3B). Silencing of Hif1α in MAECs using siRNA (Figure S9) decreased moxLDL- or LPA 20:4-induced Cxcl1 mRNA expression and CXCL1 protein secretion compared with nontargeting control siRNAs (Figure 3C and 3D). Knockdown of Hif1α reduced the expression of Tnfa in moxLDL-stimulated MAECs, but not in LPA20:4-stimulated MAECs (Figure 3C). The role of endothelial HIF-1α in monocyte adhesion was studied using in vitro flow chamber assays. Treatment with moxLDL or LPA20:4 increased monocyte adhesion compared with native LDL and LPA18:0 (Figure S10). The moxLDL- and LPA20:4-induced monocyte adhesion was abolished by silencing Hif1α in MAECs (Figure 3E and 3F). These results suggest that moxLDL-derived unsaturated LPAs promote CXCL1-dependent monocyte adhesion by upregulation HIF-1α.

**HIF-1α Increases Endothelial Chemokine Expression by Upregulating miR-19a**

To study the effect of endothelial HIF-1α on the expression of miRNAs during atherosclerosis, miRNA expression profiling was performed in partially ligated carotid arteries from EC-Hif1α<sup>−/−</sup> and EC-Hif1α<sup>+/+</sup> mice fed an HFD for 2 weeks using quantitative reverse transcription polymerase chain reaction arrays. Six miRNAs, including miR-19a and miR-9, were downregulated in EC-Hif1α<sup>−/−</sup> mice compared with EC-Hif1α<sup>+/+</sup> mice (Figure 4A; Table S4). The downregulation of miR-19a in EC-Hif1α<sup>−/−</sup> mice occurred mainly in ECs as determined by combined in situ polymerase chain reaction.

**Figure 2**. Endothelial Hif1α deficiency reduces disturbed flow-induced atherosclerosis. A, Lesional area was quantified in elastic van Gieson–stained sections of carotid arteries 6 weeks after partial ligation. B, Lesional macrophage accumulation was determined in partially ligated carotid arteries by Mac-2 immunostaining. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Scale bars, 100 μm. *P<0.05, n=5 to 8 mice per group.
Moreover, partial ligation increased the miR-19a expression in carotid arteries of Apoe−/− mice (Figure 4C).

In vitro, stimulation with moxLDL increased the expression of miR-19a and HIF1α in human aortic ECs (Figure 5A; Figure S11). Silencing Hif1a using siRNAs reduced the

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\begin{array}{cccccccccc}
\text{nLDL} & \text{moxLDL} & \text{Ki16425} & \text{nLDL} & \text{moxLDL} & \text{Ki16425} & \text{nLDL} & \text{moxLDL} & \text{Ki16425} & \text{nLDL} & \text{moxLDL} \\
- & + & - & - & + & + & - & + & + & - & + \\
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for miR-19a and CD31 immunostaining (Figure 4A and 4B).

Figure 4. Effect of endothelial hypoxia-inducible factor (HIF)-1α on microRNA (miRNA) expression. A, The expression of miRNAs was determined by quantitative reverse transcription polymerase chain reaction (PCR) arrays in partially ligated left carotid arteries from endothelial cell (EC)-Hif1a−/− mice and compared with that from EC-Hif1a+/+ mice fed a high fat diet for 2 weeks. The gray area indicates differentially regulated miRNAs; n=3 mice per group. B, Combined in situ PCR detection of miR-19a and immunostaining for CD31 in sections from partially ligated carotid arteries. Scale bars, 10 μm. C, MiR-19a expression in carotid arteries from Apoe−/− mice at 2 weeks after partial ligation compared with that in nonligated carotid arteries (Ctrl). **P<0.01, n=4 mice per group. RQ indicates relative quantification.
expression of miR-19a in moxLDL-stimulated human aortic ECs (Figure 5B; Figure S12). Inhibition of miR-19a using antisense oligonucleotides diminished moxLDL-induced expression of CXCL1 and CCL2 mRNAs in human aortic ECs (Figure 5C). Conversely, transfection of human aortic ECs with miR-19a mimics substantially increased the expression of CXCL1 and CCL2 (Figure 5D), triggered nuclear translocation of the NF-κB protein p65 (Figure S13), and enhanced monocyte adhesion to ECs (Figure 5E). Blocking the CXCL1 receptor CXCR2 on monocytes abrogated the effect of miR-19a on monocyte adhesion (Figure 5E). These findings suggest that reduced expression of miR-19a diminishes endothelial CXCL1 expression and NF-κB activation in EC-Hif1a−/− mice, and thereby limits atherogenic monocyte adhesion.

**Discussion**

We found that enhanced HIF-1α activation in atherosclerotic ECs promotes the expression of CXCL1 and increases atherosclerosis. MoxLDL and its derivative LPA induced CXCL1 expression and monocyte adhesion by upregulating Hif1α in ECs. This effect of HIF-1α was because of increased expression of miR-19a, which triggers NF-κB activation, CXCL1 expression, and CXCL1-dependent monocyte adhesion. Taken together, our findings indicate that hyperlipidemia-induced HIF-1α activation in ECs contributes to atherogenic monocyte recruitment by upregulating miR-19a, which increases NF-κB activation and CXCL1 expression.

Hyperlipidemia increased HIF-1α expression and activation in atherosclerotic ECs. HIF-1α accumulates in hypoxic cells mainly because of increased protein stabilization. However, ECs produce ATP mainly by glycolysis, and oxygen supply via the bloodstream to atherosclerotic ECs is not limited. Therefore, nonhypoxic stimuli of HIF-1α activation, such as tumor necrosis factor-α and angiotensin II, which increase Hif1α transcription, may be more important for the activation of HIF-1α in ECs. Unsatuated LPAs are produced during mild oxidation of LDL and induce the upregulation of Hif1α expression by activating LPA receptors in cancer cells and in smooth muscle cells. In line with these reports, our findings indicate that hyperlipidemia-induced endothelial HIF-1α activation is because of LPA receptor signaling triggered by moxLDL-derived LPAs. In addition, LPAs activate proatherogenic NF-κB signaling in ECs. Endothelial NF-κB activation may upregulate HIF-1α expression, which reciprocally amplifies NF-κB signaling and enhances angiotensin II–induced hypertensive kidney injury. In quiescent ECs, the anti-inflammatory transcription factor Krüppel-like factor 2 limits NF-κB activation and HIF-1α accumulation. However, disturbed flow downregulates endothelial Krüppel-like factor 2 expression and primes the endothelium for inflammatory activation by hyperlipidemia. Accordingly, our findings indicate that disturbed flow is a prerequisite for hyperlipidemia-induced HIF-1α expression in ECs; however, the effect of disturbed flow alone on endothelial HIF-1α activation need to be studied in more detail.

The deletion of the Hif1α gene in ECs reduced the expression of proinflammatory cytokines, such as CXCL1 and tumor necrosis factor-α. HIF-1α transcriptional activity is also required for CXCL1 expression in myeloid cells, which mediates the survival of neutrophils in mycosis. In ECs, peroxisome proliferator-activated receptor γ (PPARγ) gene in ECs reduced the expression of Hif1α. The deletion of the Hif1α gene in ECs reduced the expression of proinflammatory cytokines, such as CXCL1 and tumor necrosis factor-α. HIF-1α transcriptional activity is also required for CXCL1 expression in myeloid cells, which mediates the survival of neutrophils in mycosis. In ECs, peroxisome proliferator-activated receptor γ (PPARγ) gene in ECs reduced the expression of Hif1α.

**Figure 5.** MiR-19a promotes endothelial inflammation. A, MiR-19a expression levels in mildly oxidized low-density lipoprotein (moxLDL)- and buffer (control)-treated human aortic endothelial cells (HAECs). B, Expression levels of miR-19a in HAECs after treatment with Hif1a siRNA (siHif1a) or a nontargeting control siRNA (siNTC). CXCL1 and CCL2 mRNA expression in moxLDL-stimulated HAECs treated with inhibitors (C) or mimics (D) of miR-19a, or nontargeting oligonucleotides (control). E, Adhesion of monocytes to HAECs treated with miR-19a-mimics or control oligonucleotides. Monocytes were untreated or pretreated with CXCR2 antibodies or isotype control antibodies (control Ab). *P<0.05, **P<0.01; n=2 to 5.
expression, indicating that miR-19a plays a key role in HIF-1α-induced endothelial inflammation. MiR-19a promotes NF-κB signaling by targeting several negative regulators of NF-κB, such as NF-κB inhibitor α, and may thereby increase CXCL1 expression. Accordingly, we found that miR-19a increases NF-κB activation in ECs, indicating that HIF-1α upregulates CXCL1 expression via miR-19a-induced NF-κB activation. In line with a proatherogenic role of miR-19a, the second miR-19 family member miR-19b, which differs only in 1 nucleotide from miR-19a, increases atherosclerosis.

In conclusion, we found that endothelial HIF-1α promotes the development of atherosclerosis by mediating the effects of unsaturated LPA on endothelial inflammation and monocyte recruitment through upregulation of miR-19a.

**Perspectives**

Our finding that endothelial HIF-1α activation in ECs by hyperlipidemia promotes atherosclerosis provides further insights in the transcriptional regulation of endothelial inflammation. HIF-1α promoted NF-κB activation by upregulating miR-19a, which in turn may enhance HIF-1α activation. Therefore, targeting this mutual activation of NF-κB and HIF-1α by inhibiting miR-19a in ECs may provide a novel approach in the treatment of atherosclerosis.

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**Disclosures**

None.

**References**


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**Novelty and Significance**

**What Is New?**

- Enhanced hypoxia-inducible factor (HIF)-1α activation in atherosclerotic ECs promotes the expression of CXCL1 and increases atherosclerosis. Endothelial HIF-1α induces lysophosphatidic acid-mediated CXCL1 expression and monocyte adhesion by upregulating miR-19a.

**What Is Relevant?**

- Inhibition of the endothelial HIF-1α/miR-19a pathway may be a therapeutic option against atherosclerosis.

**Summary**

Our results indicate that hyperlipidemia upregulates endothelial HIF-1α expression by mildly oxidized low-density lipoprotein–derived unsaturated lysophosphatic acid, which induces CXCL1 expression and monocyte adhesion. The effects of HIF-1α are mediated by miR-19a, which triggers nuclear factor-κB activation, and CXCL1-dependent monocyte adhesion. Taken together, our findings indicate that hyperlipidemia-induced HIF-1α activation in endothelial cells contributes to atherogenic monocyte recruitment by upregulating miR-19a.
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ONLINE SUPPLEMENT

Endothelial hypoxia-inducible factor-1α promotes atherosclerosis and monocyte recruitment by upregulating miRNA-19a

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Supplemental Methods

Animal model

Hif1a<sup>flox/flox</sup> Apoe<sup>−/−</sup> mice were generated by crossing mice carrying a floxed Hif1a allele (The Jackson Laboratories, Bar Harbor, USA) with Apoe<sup>−/−</sup> mice. VE-Cad-Cre-ER<sup>T2</sup> mice<sup>1</sup>, which express a Tamoxifen (TMX)-inducible Cre recombinase under the endothelial specific VE-Cadherin promoter, were mated with the Hif1a<sup>flox/flox</sup> Apoe<sup>−/−</sup> mice to generate VE-Cad-Cre-ER<sup>T2</sup>/Hif1a<sup>flox/flox</sup> Apoe<sup>−/−</sup> mice. VE-Cad-Cre-ER<sup>T2</sup>/Hif1a<sup>flox/flox</sup> Apoe<sup>−/−</sup> mice (EC-Hif1a<sup>−/−</sup>) and VE-Cad-Cre-ER<sup>T2</sup>/Hif1a<sup>WT/WT</sup> Apoe<sup>−/−</sup> mice (EC-Hif1a<sup>+/+</sup>) were used throughout this study. Cre recombinase activity was induced by intraperitoneal injection of the mice with Tamoxifen (2 mg/20 g body weight; Sigma-Aldrich, Munich, Germany) dissolved in neutral oil (Migyol; Sasol, Hamburg, Germany) for 5 consecutive days. One week after the last injection, mice were either subjected to partial carotid ligation (PL) and fed a high fat diet (HFD, 0.15% cholesterol, Altromin, Lage, Germany) for 2, 4 or 6 weeks or fed the HFD for 12 weeks without partial ligation. All of the animal experiments were reviewed and approved by the local authorities in accordance with German animal protection law.

Partial ligation of carotid arteries

The left carotid arteries of 6–8-weeks old EC-Hif1a<sup>−/−</sup> and EC-Hif1a<sup>+/+</sup> mice were partially ligated and the mice were fed a HFD for the indicated time-points. Briefly, mice were anesthetized by injection with ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and the external, the internal and the occipital artery were ligated allowing blood outflow only via the superior thyroid artery. Two or six weeks after the partial ligation, the carotid arteries were harvested after perfusion fixation with 4% paraformaldehyde (PFA, Sigma-Aldrich) and embedded in paraffin. The first 200 µm of the ligated carotid artery starting from bifurcation were analyzed.

Diet-induced atherosclerosis

Female EC-Hif1a<sup>+/+</sup> and EC-Hif1a<sup>−/−</sup> mice (age 6–8-weeks) were fed a HFD for 12 weeks. Mice were anesthetized by injection with ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). The carotid arteries, aortic roots, and thoracoabdominal aorta were harvested after perfusion fixation with 4% paraformaldehyde (PFA, Sigma-Aldrich) and embedded in paraffin. The first 200 µm of the ligated carotid artery starting from bifurcation were analyzed.

Micro-computed tomography (CT) angiography

Micro-CT (TomoScope DUO, CT Imaging, Erlangen, Germany) scans was performed on partially ligated mice, as described previously<sup>2</sup>. A blood-pool contrast agent was intravenously injected and the luminal diameter of the common carotid artery was quantified within a distance of 1 mm from the bifurcation with the Imalytics Research workstation (Philips Technologie GmbH, Aachen, Germany). Three dimensional (3D) renderings of the carotid artery were created using the Definiens Developer XD software (Definiens, Munich, Germany).

Lesion quantification

Thoracoabdominal aortas were prepared en face and stained with Oil Red O stain. The Oil Red O-positive area was quantified from digital images of the aorta using image analysis software (ImageJ). The lesion area was analyzed in the carotid
arteries by elastic van Gieson (EVG) staining of serial sections (4–5 µm thick) at an interval of 100 µm.

**Immunostaining**

Consecutive sections at an interval of 120 µm were immunostained for SM22 (rabbit polyclonal antibody, Abcam, Cambridge, UK), Mac-2 (clone M3/38 antibody, Cedarlane, Ontario, Canada) and collagen type-1 (rabbit polyclonal antibody, Cedarlane). The SM22, Mac-2 and collagen type-1 positive area in the lesion was quantified using ImageJ software.

Expression of HIF-1α was determined by immunostaining using a mouse monoclonal antibody against HIF-1α (Novus Biologicals, Cambridge, UK). Double immunostaining for Hif-1α or CXCL1 (rabbit polyclonal, Abcam) and CD31 (goat polyclonal, Santa Cruz, Heidelberg, Germany) or von Willebrand factor (vWF; rabbit polyclonal Ab, Abcam, Cambridge, UK) was performed in carotid artery sections or aortic root sections by sequential incubation of primary antibodies. Non-specific IgGs served as a negative control. Primary antibodies were detected using fluorescently labelled secondary antibodies. The double immunostainings were analyzed by counting the number of CD31 and HIF-1α or CD31 and CXCL1 double positive cells in the lesions according to the presence of DAPI-(4′,6-diamidino-2-phenylindole) stained nuclei and expressed as percentage of the total number of CD31+ cells.

Human atherosclerotic lesion specimens were obtained during carotid endarterectomy and fixed in PFA. The expression of endothelial specific HIF-1α was determined by double immunostaining for vWF (rabbit polyclonal, Abcam) and Hif-1α (mouse monoclonal, Novus Biologicals). The study protocol for the collection of human plaque samples was approved by the Ethics Committee of the Medical Faculty (RWTH Aachen University). Written informed consent was obtained from all participants.

**Cell Culture**

To obtain mouse aortic ECs (MAECs), the aortic arch and the thoracic aorta were cut into small pieces, which were aseptically placed on collagen gel. The ECs that migrated onto the gel after 5–6 days were isolated by digesting the gel with 0.3% collagenase and cultured in a tissue culture flask with endothelial cell growth medium (PAA, Pasching, Austria). The purity of the ECs was determined at passage 3 by lectin staining (*Lycopersicon esculentum*; Sigma). MAECs were seeded into collagen-coated 6-well plates (Millipore, Billerica, MA, USA) and grown to appropriate density in complete growth medium.

Human aortic ECs (HAEC; PromoCell, Heidelberg, Germany) were cultured in collagen-coated dishes (Millipore) using EC growth medium (PromoCell).

Following serum starvation for 18–20 h, the culture medium was replaced with fresh growth medium. LPA20:4 (Echelon, Salt Lake City, USA, 10 µmol/L), LPA18:0 (Avanti Polar Lipids, Alabaster, USA, 10 µmol/L), mildly-oxidized LDL (moxLDL; 50 µg/ml), native LDL (nLDL; 50 µg/ml) dissolved in PBS were added to the medium and incubated at 37°C for 4–6 h. MAECs/HAECs were also treated with LPA20:4 (10 µmol/L) and the LPA receptor antagonist Ki16425 (Cayman Chemical, 100 µmol/L) or moxLDL (50 µg/ml) and Ki16425 (100 µmol/L) for 4–6 h. The medium was collected and stored at -80°C until measurement. The cells were harvested for RNA isolation.
Transfection of MAECs and HAECs

MAECs/HAECs were transfected with a small interfering RNA (siRNA) against Hif1a (Thermo Scientific, Braunschweig, Germany; Accell Mouse Hif1a (15251), Cat. # 040638-00-0002; Accell Human HIF1A (3091), Cat. # 004018-00-0005; 1µM) or a non-targeting siRNA for 72 h. MAECs were additionally incubated with or without LPA20:4 (10 µmol/L) or moxLDL (50 µg/ml) for 4 h. Cell culture medium was collected and stored at -80°C until measurement and the cells were harvested for RNA or protein isolation. Lipofectamine 2000 (Life Technologies, Darmstadt, Germany) was used to transfect HAECs with a locked nucleic acid (LNA)-miR-19a inhibitor (50 nM, miRCURY LNA™ microRNA Inhibitors; Exiqon, Vedbaek, Denmark), a miR-19a mimics (50 nM, mirVanaTM mimics; Life Technologies) or scrambled controls for 24h. Additional treatment with moxLDL (50 µg/ml) was performed for 6h, before the RNA was isolated.

For the p65 staining, HAECs were cultured in chamber slides (NuncTM Lab-TekTM; Thermo Scientific) and transfected with miR-19a-mimics or scrambled controls for 24h. HAECs were fixed with aceton/methanol (1:1) and p65 was determined using a polyclonal antibody against p65 (ab7970, Abcam).

Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from carotid arteries and from cultured ECs using the RNA easy mini kit (Qiagen, Hilden, Germany) or NucleoSpin microRNA Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Reverse transcription was performed using M-MLV reverse transcriptase (Promega, Mannheim, Germany), TaqMan microRNA reverse transcription kit or high-capacity cDNA reverse transcription kit (both from Life Technologies). Hif1a mRNA and transcripts of proinflammatory cytokines were quantified by SYBR green PCR amplification in 7900HT fast real-time PCR system (Applied Biosystems, Darmstadt, Germany) (Table S1). Gapdh and Actb were used as reference genes. MiR-19a (Cat. # 000395) expression levels were quantified using TaqMan microRNA assays and TaqMan Universal PCR Master Mix (all from Life Technologies). RNU44 (Cat. # 001094) or sno135 (Cat. # 001230) were used as reference genes. The relative expression levels were normalized to a single or multiple reference genes scaled to the sample with the lowest expression and logarithmically transformed using QbasePLUS software (Biogazelle NV, Zwijnaarde, Belgium).

MiRNA Real-time PCR Array

After reverse transcription and pre-amplification of total RNA using the Megaplex RT & Preamp Rodent Pool Set (Life Technologies), the samples were loaded onto preconfigured 384-well microfluidic TaqMan Array MicroRNA Cards for real-time polymerase chain reaction (RT-PCR) analyzing 641 mouse miRNAs, using the 7900HT Real-Time PCR System (all from Life Technologies). Data analysis was performed using QbasePLUS software (Biogazelle NV) along with multiple internal control genes. The detection limit of the individual assays was defined as a CT < 40.

Flow Chamber Assay

Monocyte adhesion to MAECs stimulated with LPA20:4 (10 µmol/L), LPA18:0 (10 µmol/L), moxLDL (50 µg/ml) or nLDL (50 µg/ml) was measured using a parallel plate
flow chamber, as described previously. MonoMac6 cells (MM6; 0.5 × 10⁶ cells/ml; Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were suspended in HBSS-medium (1× Hanks Balanced Salt Solution, 1% FCS, 0.5% BSA) and labeled with calcein AM (1 μM; Life Technologies) for 30 min. For some experiments MM6 cells were treated with an antibody against C-X-C chemokine receptor type 2 (CXCR2; 20 μg/ml, clone 48311) or an isotype control IgG (20 μg/ml, clone 20102) (both from R&D Systems, Minneapolis, MN, USA). MM6 cells were perfused over MAECs in the lower chamber using a perfusion pump (WPI-SP100I, Berlin, Germany, shear rate of 1.5 dyn/cm²). Monocyte-endothelial interactions were visualized by video-microscopy using a 10x magnification. For each experiment, recordings were made for 15 sec in one visual field and 5 random microscopic visual fields were analyzed for each experiment. After a 5 min observation period, monocytes that firmly attached to the MAECs were manually counted per visual field. Each experiment was repeated at least 3 times.

**Lipid Analysis**

The serum samples were analyzed for total cholesterol and triglycerides by dry chemistry using a Vitros 250 Analyzer (Ortho Clinical Diagnostics, Neckargemünd, Germany).

**Preparation of mildly-oxidized LDL (moxLDL)**

Human native LDL (1 mg/ml, Calbiochem, Darmstadt, Germany) was incubated with 5 μM CuSO4 at 37°C for 4 hours to prepare moxLDL. The LDL oxidation was stopped by adding 10 μM EDTA and the LDL was passed through PD-10 desalting column (GE Healthcare, Uppsala, Sweden). To prepare native LDL (nLDL) for experimental use as a negative control, all the above steps were performed except the addition of CuSO4. The protein concentration was measured using DC protein assay kit (Bio-Rad Laboratories GmbH, Munich, Germany) with BSA as a standard. The level of oxidation was evaluated by spectrophotometrically quantification the formation of thiobarbituric acid-reactive species (TBARS) at 532 nm (TBARS assay kit, Cayman Chemical, Michigan, USA). The moxLDL and nLDL were used within 14 days after preparation and stored at 4°C.

**Enzyme-linked Immunosorbent Assay (ELISA)**

The level of CXCL1 protein in the cell culture medium of MAECs was determined by a mouse CXCL1/KC ELISA kit (Ray Biotech, Inc, Norcross, USA) according to the manufacturer’s protocol. The absorbance was measured at 450 nm with a microplate reader (SPECTRA Fluor Plus, Tecan).

**Western Blot Analysis**

The level of HIF-1α protein was determined in the cell lysate of MAECs or HAECs. ECs were lysed in RIPA buffer (Sigma-Aldrich) including protease inhibitors (CompleteProtease Inhibitor Cocktail, Roche, Basel, Switzerland). The cell lysate was resolved on SDS-PAGE gels and then transferred to nitrocellulose membranes. Proteins were detected using primary antibodies against HIF-1α (clone H-206, Santa Cruz Biotechnology, CA, USA), and GAPDH (clone 6C5, Millipore), and horse radish peroxidase conjugated (HRP)-conjugated secondary antibodies (Goat Anti-Mouse IgG HRP Affinity Purified PAb, R&D Systems; Goat Anti-Rabbit IgG HRP Affinity Purified PAb, Santa Cruz). Protein bands were visualized using an enhanced chemiluminescence detection system (ECL Advance, GE Healthcare Life Sciences).
and a LAS 3000 Imager (Fuji Photo Film Co., Ltd., Tokyo, Japan) and were quantified using Multigauge software (Fuji Photo Film). Intensities of the HIF-1α bands were expressed as a percentage of those of the GAPDH bands.

**In Situ Reverse Transcriptase PCR**

Sections (5 µm thick) of carotid arteries were cooked in citrate buffer for 20 min using a microwave (600 W). One-step reverse transcriptase in situ PCR was performed using gene-specific Taq in situ primers (Sigma-Aldrich) (Table S1), SuperScript One-Step RT-PCR with PlatinumTaq (Life Technologies), and digoxigenin-11-dUTPs (Roche). After washing with SSC buffer and blocking of biotin/avidin binding sites (Blocking Kit; Vector Laboratories, Peterborough, UK), the sections were incubated with HRP anti-digoxigenin sheep F’ab fragments (Roche) for 1 h at 37°C. The probes were visualized using a tyramide-based amplification system (TSA Plus Biotin; Perkin Elmer, Waltham, MA, USA) and DyLight 549-labeled streptavidin (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA).

**Statistical Analysis**

The miRNA real-time PCR array data were analyzed using QbasePLUS software (Biogazelle NV) and are presented as mean values. All other data represent the mean ± SEM and were compared by either a 1-way or 2-way ANOVA followed by the Newman-Keuls or Bonferroni post-test, respectively, or an unpaired two-tailed Student t-test (Prism 6 software; Inc., La Jolla, CA, USA). P < 0.05 was considered to be statistically significant.
References


Supplemental Tables

Table S1: PCR primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
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<td><strong>Mouse:</strong></td>
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<td>Hif1a</td>
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<td>antisense 5'-GTGGGCAGACAGGTTAAGGCTC3-3'</td>
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<td>Cxcl1</td>
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Table S2: Serum cholesterol and triglyceride levels in EC-\(Hif1a^{+/+}\) and EC-\(Hif1a^{-/-}\) mice after partial ligation and 6 weeks HFD feeding (\(N=8\) mice per group).

<table>
<thead>
<tr>
<th>Serum levels (mmol/L)</th>
<th>EC-(Hif1a^{+/+})</th>
<th>EC-(Hif1a^{-/-})</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Cholesterol</td>
<td>18.16 ± 1.409</td>
<td>16.64 ± 1.880</td>
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<tr>
<td>Triglycerides</td>
<td>0.97 ± 0.098</td>
<td>0.84 ± 0.101</td>
<td>0.3770</td>
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Table S3: Serum cholesterol and triglyceride levels in EC-\textit{Hif1a}\textsuperscript{++/} and EC-\textit{Hif1a}\textsuperscript{−/−} mice after 12 weeks HFD feeding (N=5–7 mice per group).

<table>
<thead>
<tr>
<th>Serum levels (mmol/L)</th>
<th>EC-\textit{Hif1a}\textsuperscript{++/}</th>
<th>EC-\textit{Hif1a}\textsuperscript{−/−}</th>
<th>P-value</th>
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<tr>
<td>Cholesterol</td>
<td>18.74 ± 0.623</td>
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<td>Triglycerides</td>
<td>0.81 ± 0.143</td>
<td>0.80 ± 0.075</td>
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Table S4: Significantly downregulated miRNAs in partially ligated LCs from EC-\textit{Hif1a}^\text{-/-} compared to EC-\textit{Hif1a}^+/+ mice after 2 weeks of HFD feeding (N=3 mice per group).

<table>
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<th>log\textsubscript{10} RQ</th>
<th>neg log\textsubscript{10} P-value</th>
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<tr>
<td>miR-19a</td>
<td>-1.89</td>
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<td>miR-9</td>
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<td>2.02</td>
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<td>miR-10a</td>
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<td>1.57</td>
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<td>miR-410</td>
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<td>1.54</td>
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<td>miR-30c</td>
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<td>1.50</td>
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Table S5: Significantly upregulated miRNAs in partially ligated LCs from EC-
*Hif1a<sup>+/−</sup>* compared to EC-*Hif1a<sup>+/+</sup>* mice after 2 weeks of HFD feeding (N=3 mice
per group).

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<thead>
<tr>
<th>miRNAs</th>
<th>log&lt;sub&gt;10&lt;/sub&gt; RQ</th>
<th>neg log&lt;sub&gt;10&lt;/sub&gt; P-value</th>
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<tbody>
<tr>
<td>miR-101a</td>
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<td>miR-542-5p</td>
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<td>miR-500</td>
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<td>miR-339-3p</td>
<td>0.61</td>
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Figure S1: Endothelial HIF-1α expression in atherosclerotic lesions. (A) Dual immunostaining for HIF-1α and von Willebrand factor (vWF) in human carotid lesions. (B) Dual HIF-1α and vWF immunostaining in normal (left) and atherosclerotic aortas (right) from Apoe<sup>−/−</sup> mice. Arrows indicate HIF-1α<sup>+</sup> ECs. Nuclei were stained with DAPI. Scale bars, 25 μm (A), 10 μm (B).
Figure S2: HIF-1α expression in murine arteries. (A) Hif1a mRNA expression in different aortic regions and carotid arteries of Apoe−/− mice fed a normal diet (ND) or a high-fat diet (HFD) for 12 weeks. (B) HIF-1α immunostaining in normal aortic roots and in the lesions of the aortic roots from Apoe−/− mice fed a HFD for 12 weeks. Arrows indicate HIF-1α+ ECs. Nuclei were stained with DAPI. Scale bars, 100 µm. (C) Hif1a mRNA expression in partially ligated left carotid arteries (LC) and non-ligated right carotid arteries (RC). *P<0.05, N=4-5 mice per group.
Figure S3: Effect of endothelial Hif1a deficiency on CXCL1 expression. Immunostaining for CXCL1 in the aortas of EC-Hif1a^{+/+} and EC-Hif1a^{-/-} mice fed a HFD for 12 weeks. Nuclei were stained with DAPI. Arrows indicate cells at the luminal site. Scale bars, 25 µm.
Figure S4: Effect of endothelial *Hif1a* deficiency on disturbed flow-induced plaque composition. Representative images and quantification of Sm22 (A) and Collagen type I (B) immunostaining in EC-\(Hif1a^{+/+}\) and EC-\(Hif1a^{-/-}\) mice 6 weeks following partial carotid ligation and HFD feeding. Scale bars, 200 µm. N=5-7 mice per group.
Figure S5: Effect of endothelial Hif1a deficiency on the luminal volume in carotid arteries. The luminal volume of the partially ligated carotid arteries from EC-Hif1a+/+ and EC-Hif1a−/− mice was determined by micro-CT angiography. Representative three-dimensional reconstructions of the micro-CT angiograms are shown. LC, left carotid; RC, right carotid; Ao, Aorta. *P<0.05, N=5-8 mice per group.
Figure S6: Effect of endothelial Hif1a deficiency on diet-induced atherosclerosis. (A) Atherosclerotic lesion area was quantified by Oil red O staining in the aortas from EC-Hif1a+/+ and EC-Hif1a−/− mice fed a HFD for 12 weeks. (B) The lesional macrophage accumulation was determined by Mac-2 immunostaining and expressed as percentage of the lesion area. Scale bars, 100 μm (B). *P<0.05, N=8-10 mice per group.
Figure S7: Regulation of CXCL1 expression by moxLDL and LPA. (A) CXCL1 protein was quantified in the medium of MAECs treated with nLDL or moxLDL with or without LPA1/3 receptor antagonist Ki16425. (B) Cxcl1 mRNA expression was quantified in MAECs treated with LPA18:0 or LPA20:4 with or without Ki16425. (C) CXCL1 protein was quantified in medium of MAECs treated with LPA18:0 or LPA20:4 with or without Ki16425. *P<0.05 versus all other groups, ***P<0.0001 versus all other groups. N=3-4.
Figure S8: Effect of moxLDL and LPA 20:4 on HIF-1α protein expression. (A, B) Western blot of HIF-1α and GAPDH in MAECs stimulated with moxLDL (A) or LPA 20:4 (B). The expression levels were normalized to those of GAPDH. Unstimulated MAECs were used as a control. Samples were run on the same gel but were noncontiguous (black line). Representative blots are shown. *P<0.05, N=3-4.
Figure S9: Efficiency of HIF-1α knockdown in MAECs. (A) Expression level of Hif1a in MAECs treated with siRNA against Hif1a. An untransfected control (Control) or a non-targeting control siRNA (siNTC) was used as control. (B) Western blots of HIF-1α and GAPDH in MAECs transfected with siRNA against Hif1a or a non-targeting control siRNA (siNTC). Samples were run on the same gel but were noncontiguous (black line). Representative blots are shown. ***P<0.0001, N=3-4.
Figure S10: Effects of moxLDL or LPA20:4 on monocyte adhesion under flow conditions. Monocyte adhesion to MAECs treated with moxLDL (A) or unsaturated LPA20:4 (B) as compared to nLDL or LPA18:0. *P<0.05, **P<0.01, N=3.
Figure S11: Effects of moxLDL on HIF-1α expression in HAECs. (A) *Hif1a* expression in HAECs treated with moxLDL as compared to nLDL. *P<0.05, N=3-4.*
Figure S12: Efficiency of HIF-1α knockdown in HAECs. (A) Expression level of HIF1α mRNA in HAECs treated with siRNA against Hif1α. An untransfected control (Control) or a non-targeting control siRNA (siNTC) was used as control. (B) Western blots of HIF-1α and GAPDH in HAECs transfected with siRNA against Hif1α or a non-targeting control siRNA (siNTC). Samples were run on the same gel but were noncontiguous (black line). Representative blots are shown. **P<0.01, ***P<0.0001. N=2-5.
Figure S13: Effect of miR-19a-mimic treatment on p65 localization in HAECs. Immunostaining of p65 (green) in HAECs treated with miR-19a-mimics or control oligonucleotides (control). The nuclei were counterstained with DAPI (blue). Representative images are shown. Scale bars, 25µm.