Heme Oxygenase 1 Determines Atherosclerotic Lesion Progression Into a Vulnerable Plaque

Caroline Cheng, PhD*; Annemarie M. Noordeloos, MSc*; Viktoria Jeney, MSc; Miguel P. Soares, PhD; Frans Moll, PhD, MD; Gerard Pasterkamp, PhD, MD; Patrick W. Serruys, PhD, MD; Henricus J. Duckers, PhD, MD

Background—The molecular regulation for the transition from stable to vulnerable plaque remains to be elucidated. Heme oxygenase 1 (HO-1) and its metabolites have been implicated in the cytoprotective defense against oxidative injury in arterogenesis. In this study, we sought to assess the role of HO-1 in the progression toward plaque instability in carotid artery disease in patients and in a murine model of vulnerable plaque development.

Methods and Results—Atherectomy biopsy from 112 patients with clinical carotid artery disease was collected and stratified according to characteristics of plaque vulnerability. HO-1 expression correlated closely with features of vulnerable human atheromatous plaque (P<0.005), including macrophage and lipid accumulation, and was inversely correlated with intraplaque vascular smooth muscle cells and collagen deposition. HO-1 expression levels correlated with the plaque destabilizing factors matrix metalloproteinase-9, interleukin-8, and interleukin-6. Likewise, in a vulnerable plaque model using apolipoprotein E−/− mice, HO-1 expression was upregulated in vulnerable versus stable lesions. HO-1 induction by cobalt protoporphyrin impeded lesion progression into vulnerable plaques, indicated by a reduction in necrotic core size and intraplaque lipid accumulation, whereas cap thickness and vascular smooth muscle cells were increased. In contrast, inhibition of HO-1 by zinc protoporphyrin augmented plaque vulnerability. Plaque stabilizing was prominent after adenoviral transduction of HO-1 compared with sham virus–treated animals, providing proof that the observed effects on plaque vulnerability were HO-1 specific.

Conclusions—Here we demonstrate in a well-defined patient group and a murine vulnerable plaque model that HO-1 induction reverses plaque progression from a vulnerable plaque to a more stable phenotype as part of a compensatory atheroprotective response. (Circulation. 2009;119:3017-3027.)

Key Words: atherosclerosis ■ coronary disease ■ inflammation ■ genes ■ vasculature

Atherosclerosis is the leading cause of mortality and comorbidity in Western countries. Progressive atherosclerotic lesion destabilization with subsequent rupture, acute thrombus formation, and coronary artery occlusion is the main mechanism in the pathogenesis of myocardial infarction and sudden coronary death.1,2 Autopsy studies suggest that these atherosclerotic lesions, which are prone to rupture, typically consist of a necrotic/lipid core, covered by a thin fibrous cap with severe infiltration of macrophages in the shoulder regions.3 Rupture of these vulnerable atherosclerotic plaques is initiated by biomechanically induced tears in the thin fibrous cap or shoulder regions.4,5 Subsequent exposure of the thrombogenic necrotic core results in an acute vascular occlusion. Unfortunately, most of the vulnerable lesions show only a 30% luminal occlusion by coronary angiography, and detection by conventional imaging modalities of these rupture prone plaques has proved to be difficult. The identification of new molecular biomarkers and regulators for this specific plaque phenotype may help in understanding of the progression of atherosclerosis to the vulnerable lesions and may provide new tools for diagnosis and intervention.6

Clinical Perspective on p 3027

Heme oxygenase 1 (HO-1), the inducible isoform of the family of heme oxygenases, degrades heme into the metabolites carbon monoxide, biliverdin, and ferrous iron. Endogenous HO-1 expression can be detected in advanced...
human atherosclerotic lesions localized in endothelial cells, macrophages, and foam cells.7,8 The expression of HO-1 is induced by a number of proatherogenic stimuli including increased blood pressure,9,10 smoking,11,12 and oxidized lipids,13 which also are regarded as risk factors for atherosclerosis. In animal models, induction of HO-1 (by heme administration or adenovirus-mediated transgene overexpression) impedes the development of atherosclerotic lesions, whereas inhibition of HO-1 (by zinc protoporphyrin IX [ZnPPIX]) stimulates atherogenesis.8,14 Likewise, apolipoprotein E (ApoE)/HO-1 double knockout mice demonstrate accelerated atherosclerosis compared with ApoE knockout mice when subjected to a high-cholesterol diet.15 Taken together, these antiatherogenic properties of HO-1 suggest a prominent role for HO-1 in the genetic regulation in the development of atherosclerosis. It is hypothesized that the atheroprotective properties of HO-1 are based partly on its immune-modulating properties. Overexpression of HO-1 suppresses serum levels of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and monocyte chemotactic protein-1, and inhibits endothelial expression of adhesion molecules E-selectin and vascular cell adhesion molecule-1, whereas, the anti-inflammatory cytokine IL-10 is stimulated.16,17 The inflammatory process is considered a crucial factor for initiating atherosclerotic development and eventual destabilization of the lesion into a vulnerable plaque phenotype. Therefore, we postulated that HO-1 may be an important regulator of advanced atherosclerotic lesion progression and eventual plaque destabilization. In the present study, we correlate HO-1 expression with phenotypes of atherosclerotic lesions in carotid endarterectomy (CEA) material obtained from patients with documented cardiovascular disease.18,19 HO-1 protein expression was specifically increased in atherosclerotic lesions and correlated closely with the instable plaque phenotype, as well as with the expression levels of intimal proinflammatory markers. This was confirmed in a validated murine vulnerable plaque model in which HO-1 induction prevented plaque progression into vulnerable lesions by increasing fibrous cap thickness and intimal vascular smooth muscle cell (VSMC) accumulation, whereas the necrotic core area and intraplaque lipid deposition were reduced.

Methods

Analysis of Human Atherosclerotic Plaques in Human CEA Specimens

CEA specimens were obtained from a biobank that collects endarterectomy-derived specimens of patients with symptomatic carotid artery disease (Athero Express Biobank, Utrecht, Netherlands).6 The study was approved by an institutional review committee, and subjects gave informed consent. Collected specimens are routinely processed for immunohistological analysis as well as for protein/RNA extraction and are subsequently quantified by 2 blinded observers for the presence of characteristics indicative of vulnerable plaque morphology, as reported earlier.18 For an extended version of Methods, see the online-only Data Supplement. For a detailed description of the animal experiments, see Figure I in the online-only Data Supplement.

Statistical Analysis

SPSS (version 16.0; SPSS Inc) was used for all analyses. For the human study, the Kruskal-Wallis test was used for data sets with nongaussian distribution and ordinal data. For dichotomous variables, the χ² test was used. For the murine study, the 1-way ANOVA test was conducted when >2 unpaired samples were compared. When only 2 unpaired samples were tested, the unpaired t test was performed. In all cases, P values <0.05 were considered significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

HO-1 Is Specifically Upregulated in Human Vulnerable Atherosclerotic Lesions

Average HO-1 expression levels in the CEA material was 0.98±0.12 pg/mL. CEA patients were divided into quartiles of HO-1 expression: The first quartile contained 28 patients (HO-1 expression: median, 0.30 pg/mL; range, 0.15 pg/mL), the second quartile contained 27 patients (HO-1 expression: median, 0.44 pg/mL; range, 0.20 pg/mL), the third quartile contained 29 patients (HO-1 expression: median, 0.73 pg/mL; range, 0.44 pg/mL), and the fourth quartile contained 28 patients (HO-1 expression: median, 1.61 pg/mL; range, 8.05 pg/mL) (Figure IIA in the online-only Data Supplement). The baseline characteristics of these patients are shown in the Table. The distribution of carotid artery disease risk factors did not differ among the HO-1 quartiles.

Increasing protein levels of HO-1 were associated with a characteristic vulnerable plaque phenotype (P=0.004; Figure 1A). More specifically, increasing percentages of lipids and macrophages in the carotid lesions correlated with HO-1 protein expression (P=0.006 and P=0.005; Figure 1B and 1C), whereas increasing percentages of collagen and VSMCs in the lesions correlated with decreasing levels of HO-1 (P=0.04 and P<0.0005; Figure 1D and 1E). Double labeling in immunohistological analysis suggested that HO-1 expression was localized mainly in the base of the intimal lesion (Figure 2A) and colococalized with residing macrophages, whereas HO-1 expression in VSMCs was hardly detectable (Figure 2B and 2C).

HO-1 Expression Correlates With Distinct Molecular Markers of Plaque Vulnerability in Human CEA Material

Next, we assessed the relation between HO-1 levels and protein expression of local matrix metalloproteinase (MMP) or various inflammatory cytokines, which previously were shown to promote plaque vulnerability. HO-1 expression levels in the carotid lesions correlated with MMP-9 expression levels (P=0.02) but had only limited effects on MMP-2 protein levels (P=0.06; Figure 3A and 3B). Likewise, HO-1 levels were associated with IL-6 and IL-8 protein levels (P<0.01; Figure 3C and 3D). In contrast, no clear relation was detected with MMP-8 and IL-10 (P=0.13 and P=0.52, respectively; data not shown).

HO-1 Expression Levels Are Associated With Plaque Thrombogenicity in Human CEA Material

Luminal or intraplaque thrombus formation is an established characteristic sign of plaque vulnerability.20–22 We
therefore evaluated the relation between thrombus formation and the expression levels of HO-1. High intraplaque protein expression of HO-1 was correlated with the presence of thrombus in the assessed human carotid lesions ($P = 0.04$; Figure 4).

**Table. Baseline Characteristics of Patients**

<table>
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<tr>
<th>HO-1 Quartile</th>
<th>HO-1 Quartile II</th>
<th>HO-1 Quartile III</th>
<th>HO-1 Quartile IV</th>
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<td>0.73 and 0.44</td>
<td>1.61 and 8.05</td>
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<td>n (N=112 patients)</td>
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<td>27</td>
<td>29</td>
<td>28</td>
</tr>
<tr>
<td>Male, n/N (n=81, 72.3%)</td>
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<td>20/27</td>
<td>19/29</td>
<td>22/28</td>
</tr>
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<td>65 and 9</td>
<td>68 and 8</td>
<td>68 and 8</td>
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<td>27/27</td>
<td>29/29</td>
<td>24/28</td>
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<td>20/27</td>
<td>16/29</td>
<td>16/28</td>
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<td>Hypercholesterolemia, n/N (n=17, 15.2%)</td>
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<td>6/27</td>
<td>1/29</td>
<td>6/28</td>
</tr>
<tr>
<td>Smoker, nonsmoker vs current vs past, median and range</td>
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<td>2.0 and 2.0</td>
<td>2.0 and 2.0</td>
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<td>4.6 and 5.0</td>
<td>4.4 and 3.8</td>
<td>5.2 and 4.1</td>
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</table>

**Pharmaceutical Induction of HO-1 Inhibits Vulnerable Plaque Development in ApoE$^{-/-}$ Mice Without Modulating Lesion Size**

The observations in the human carotidectomy material suggest that HO-1 expression is upregulated in advanced athero-

![Figure 1](http://circ.ahajournals.org/)

**Figure 1.** Intraplaque HO-1 levels correlated strongly with the characteristic parameters of vulnerable plaque. A, An association between HO-1 expression and plaque vulnerability was observed in the human CEA samples ($P = 0.004$). HO-1 was associated with increased intimal lipid deposition (B) and macrophages (C) ($P = 0.006$, and $P = 0.005$, respectively) but was inversely correlated with intimal collagen (D) and VSMCs (E) ($P = 0.04$ and $P < 0.0005$, respectively). Plaque variables were divided into 4 quartiles ranging from 0.5 (representing the group of patients with the lowest percentage) to 2 (representing the group of patients with the highest percentage) on the $y$ axes. On the $x$ axes, HO-1 protein expression levels are shown, divided into 4 quartiles of expression ranging from 1 (representing the group of patients with the lowest percentage) to 4 (representing the group of patients with the highest percentage).
sclerotic plaques with a vulnerable phenotype. Induction of HO-1 expression could aid in the stabilization of the atheromatous plaque. To study this, HO-1 expression was induced by cobalt protoporphyrin IX (CoPPIX) or HO-1 activity was inhibited by ZnPPIX in a mouse model for vulnerable atherosclerotic plaque formation. ApoE knockout mice were fed a high-cholesterol diet and implanted with a carotid cast as previously described. This flow-modifying device induces atherosclerotic lesions with a vulnerable phenotype in the proximal segment, whereas downstream from the device stable atherosclerotic lesions are formed. The proximal vulnerable lesions typically comprise a low content of plaque-stabilizing components, including collagen and VSMCs, and high percentages of plaque-destabilizing components, including lipids and macrophages, which taken together compose a lesion phenotype typically seen in human vulnerable plaques described by Virmani et al.

Endogenous HO-1 mRNA expression was increased in murine atherosclerotic vessel segments compared with contralateral naive carotid arteries and was highest in the proximal lesions with the vulnerable phenotype compared with the distal lesions with a stable phenotype (Figure 5A) at 9 weeks after implantation. Immunohistological assessment confirmed these findings as the relative intraplaque HO-1 surface area was extended 5-fold in the vulnerable versus the stable murine lesions (Figure 5B). HO-1 was expressed only at low levels in naive carotid arteries.

CoPPIX treatment to induce HO-1 expression or ZnPPIX treatment for HO-1 inhibition was initiated at 8 weeks of Western diet (6 weeks after cast implantation), when the early lesions in the carotids do not yet show the histomorphological characteristics of vulnerable plaque development. Histomorphological analysis was performed after 3 weeks of CoPPIX or ZnPPIX treatment and 9 weeks of cast placement. HO-1 protein levels were increased in vascular segments of CoPPIX-treated mice compared with the saline-treated group by 20-fold as measured by Western blot analysis of aorta segments. HO-1 expression levels were elevated as early as 2 days after initiation of CoPPIX injections (Figure 5C). In contrast, ZnPPIX injection reduced HO-1 protein levels in the vessel segments (Figure IIB in the online-only Data Supplement). Induction of HO-1 protein was associated with an increase in serum bilirubin levels (by 51%; $P<0.05$; Figure 5D), indicative of HO activity, whereas ZnPPIX injections reduced the serum levels of bilirubin by 38% ($P<0.05$; Figure 5D). In addition, HO activity measurements in pooled aorta samples of the different groups showed similar effects. CoPPIX increased HO activity by 5.8-fold compared with saline-injected control, whereas ZnPPIX reduced HO activity by 27% (Figure IIC in the online-only Data Supplement).

HO-1 induction by CoPPIX or inhibition by ZnPPIX did not affect the neointima/media ratio of advanced vulnerable lesions (Figure 6A). However, induction of HO-1 increased the relative fibrous cap thickness (by 237%; $P<0.05$; Figure 6B) and caused a significant decrease in necrotic core/intima ratio (by 42%; $P<0.05$; Figure 6C). HO-1 induction was further associated with the induction of plaques reminiscent of stable lesions, indicated by a diminished lipid deposition (by 35%; $P<0.05$; Figure 6D), and an increase in VSMCs residing in the intima (by 66%; $P<0.05$; Figure 6E). In contrast, HO-1 inhibition by ZnPPIX decreased relative cap thickness (by 51%; $P<0.05$; Figure 6B), whereas the necrotic core/intima ratio was increased (by 40%; $P<0.05$; Figure 6C). In addition, HO-1 inhibition increased the lipid content of the vulnerable plaque (by 65%; $P<0.05$; Figure 6D), whereas the intimal VSMC surface area was reduced (by 57%; $P<0.05$; Figure 6E).

Although intimal CD68$^+$ macrophage infiltration was reduced by 37% (Figure 6F) in response to CoPPIX injections, no significant difference was observed. Likewise, ZnPPIX inhibition of HO-1 did not affect the relative intraplaque CD68$^+$ area (Figure 6F). In addition, HO-1 induction or
inhibition did not affect intimal collagen formation in the atherosclerotic lesions (Figure 6G). HO-1 induction by CoPPIX or inhibition by ZnPPIX had no effect on lesion size or plaque phenotype of stable lesions (data not shown).

Intravascular Adenoviral Transfection of an HO-1 Expression Vector Inhibits Vulnerable Plaque Development in ApoE<sup>−/−</sup> Mice Without Modulating Lesion Size

To determine the role of HO-1 overexpression during vulnerable plaque development, HO-1 expression was induced by adenoviral vector–mediated transfection of HO-1 at 6 weeks after cast placement. HO-1 overexpression was validated by quantitative polymerase chain reaction analysis of carotid vessel segments treated with sham or HO-1 adenovirus. A 4-fold increase in HO-1 protein expression was induced in the HO-1-adenovirus–treated vessels compared with uninfected or ΔE1A sham adenovirus–infected carotid arteries 1 week after intra-arterial injection (Figure IID in the online-only Data Supplement). In concordance with our previous findings, the intima/media ratio of the vulnerable lesions did not differ between the HO-1 adenovirus and ΔE1 sham adenovirus transfected groups (Figure 7A).

HO-1 adenovirus–treated animals showed a significant increase in relative fibrous cap thickness (of 70%; P<0.05; Figure 7B), whereas the necrotic core/intima ratio was diminished (by 62.5%; P<0.05; Figure 7C). Transgenic overexpression of HO-1 also decreased lipid accumulation in the vulnerable plaque (by 40.4%; P<0.05; Figure 7D), whereas relative intraplaque VSMCs were increased (by 139.4%; P<0.05; Figure 7E) compared with ΔE1 sham adenovirus transfected controls. Similar to the findings in the CoPPIX–treated group, HO-1 adenovirus transgenic overexpression did not affect CD68<sup>+</sup> macrophage accumulation in vulnerable plaque (Figure 7F), nor did it alter intimal collagen formation (Figure 7G).

Discussion

The present data on human CEA atherosclerotic plaques and on murine vulnerable lesions suggest that HO-1 expression is
HO-1 was designated as a protein involved in heme protein degradation but more recently was suggested to play a more versatile role because the heme catabolic products were shown to be cytoprotective. HO-1 has been associated with early atherogenesis, as shown in previous clinical studies. Kaneda and coworkers showed in a study with 554 patients that short (GT)n repeats in the HO-1 gene promoter with elevated HO-1 expression was predictive of a beneficial outcome in coronary atherosclerotic disease progression. Likewise, the ability of blood-derived mononuclear cells to express HO-1 correlated with short (GT)n promoter repeats suggested that high HO-1 expression levels protect against the initiation of atherosclerosis. Previously, increased HO-1 expression was also detected in advanced atherosclerotic lesions. However, data on the genetic regulation of advanced atherosclerotic lesions and the progression into vulnerable plaques are lacking.

In the present study, we sought to define the role of HO-1 in the genetic regulation of vulnerable plaque formation. Intraplaque HO-1 expression in carotid artery disease patients correlated with plaque vulnerability, as assessed by the intimal distribution of plaque components. These observations were corroborated by correlations with proatherogenic cytokines IL-6 and IL-8 and with MMP-9, previously shown to be involved in collagen breakdown that weakens the atherosclerotic cap and adds to the vulnerability of the advanced atherosclerotic lesion. Increased HO-1 levels in lesions with an atheromatous/vulnerable plaque phenotype suggested that HO-1 could be upregulated to modulate plaque morphology and stability. In agreement with this finding, HO-1 was expressed in endothelial cells overlying advanced atherosclerotic lesions, whereas endothelial cells derived from early lesions did not express HO-1 in a small group of patients. Immunohistological analysis of these human atherosclerotic lesions by others and in the present study showed that HO-1 was expressed mainly by the macrophages/foam cells residing or infiltrating in the neointima.

In addition, atherosclerotic vulnerable lesions are characterized by an increase in frequency and extent of intraplaque hemorrhages because of intimal neovascularization, increased permeability of the vasa vasorum, or extravasation of hemoglobin due to small ruptures. Clearance and degradation of hemoglobin by infiltrated CD163 macrophages again induce HO-1 expression. Schae and coworkers described colocalization of HO-1 and macrophages that express the hemoglobin scavenger receptor CD163 in human atherosclerotic lesions. In vitro, HO-1 expression was induced by CD163 internalization after hemoglobin binding in macrophages. In the present analysis of human carotid vulnerable plaque, HO-1 was specifically upregulated in vulnerable lesions with the highest thrombogenicity, suggesting that intimal hemorrhages could stimulate HO-1 expression in this type of lesions, presumably as a compensatory mechanism.

The induction of HO-1 that occurs in the vulnerable plaque strongly suggests a role for this enzyme in the regulation of plaque destabilization and stabilization. Previously, it was shown that adenoviral gene transfer of HO-1 inhibits initiation of atherogenesis, as suggested by a reduction in intimal size of lesions located in the aortic root and aortic arch in ApoE−/− mice. Induction of HO-1 by hemin injections also decreased the lesion size in low-density lipoprotein receptor−/− mice, whereas HO-1 inhibition by Sn-protoporphyrin IX promoted lesion development compared with the saline-treated control animals. In HO-1/ApoE double knockout mice, accelerated atherosclerotic lesion formation was observed, whereas the ApoE knockout control mice developed lesions with the characteristics of a fatty streak after 8 weeks of a Western diet. However, the role of HO-1 in the molecular regulation of plaque progression into an advanced complex lesion with plaque vulnerability remains poorly understood.

The function of HO-1 in the progression of early atherosclerotic lesions into advanced vulnerable plaques was therefore investigated in an established vulnerable plaque model.
developed in the mouse with the use of a shear stress-modifying device placed around the carotid arteries of ApoE knockout mice. Cast placement has been shown to induce low shear stress proximal to and oscillatory shear stress distal to the device, which will induce atherosclerotic lesions that are histologically reminiscent of human vulnerable plaques in the proximal region and stable plaques distal to the cast. In this murine model, endogenous HO-1 expression was indeed increased in the vulnerable plaque region compared with the downstream stable plaque region. HO-1 induction by CoPPIX injections was initiated at 6 weeks after cast placement. Previous studies have indicated that at this time point, lesions in the proximal segments are already developed with an advanced phenotype with VSMC infiltration and cap formation, although a necrotic core is still absent. Although late HO-1 induction had no effect on lesion size, further progression of the vulnerable plaque phenotype was prevented in the HO-1 CoPPIX and HO-1 adenovirus groups, indicated by a decrease in the necrotic content, and reduced lipid deposition in the vulnerable lesions compared with the saline and D/EIA sham adenovirus groups. In agreement with this finding, HO-1 deficiency was associated with increased oxidized low-density lipoprotein uptake by macrophages and lipid accumulation in foam cells in vitro. Accumulation and apoptosis of foam cells in the atherosclerotic lesion led to extracellular lipid deposition and the formation of the necrotic core characteristic of a vulnerable plaque. The observed decrease in intimal lipids in response to HO-1 upregulation could have aided in plaque stabilization by limiting the necrotic core area. In agreement with these findings, HO-1 inhibition by ZnPPIX indeed augmented lipid accumulation and increased necrotic core size.

HO-1 upregulation by CoPPIX or adenovirus-mediated gene transfer was also associated with a significant increase in the percentage of intimal VSMCs and the relative cap thickness of a more stabilized atherosclerotic lesion. HO-1 might prevent cap thickening by inhibition of VSMC proliferation. HO-1 expression inhibited progression of wire- or balloon injury–induced restenosis by VSMC cell-cycle arrest via its catabolic end products. However, Yet and coworkers showed in an autologous vein graft transplantation model that neointimal VSMCs in HO-1/-vein grafts were severely reduced by increased VSMC death as detected by a terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling assay. In addition, HO-1 deletion significantly decreased viability of VSMCs after oxidative stress stimulation in vitro and in vivo associated with hemodynamic pressure in the vein graft model. Taken together, these data support the idea that HO-1 expression may protect VSMCs from oxidative stress–induced cell death. Because of the distinct morphology of the vulnerable plaque, the VSMC-rich fibrous cap is indeed exposed to high vascular strain and
apoptosis during the cardiac cycle. This renders the lesion prone to rupture by sheer mechanical force but also weakens the cap by inducing apoptosis in the residing VSMCs. In addition, high secretion levels of proinflammatory cytokines including TNF-α by macrophages residing at the boundaries of the necrotic core could provide an additional source of oxidative stress for VSMCs. However, TNF-α alone is unable to trigger apoptosis in VSMCs because it also activates the nuclear factor-κB–mediated cell survival pathway. VSMC programmed cell death induced by additional factors, however, can be facilitated by the presence of TNF-α. Recent studies have indicated that the sensitivity of VSMCs to free fatty acid– and oxysterol-induced apoptosis could be amplified by TNF-α stimulation. In our present study, intimal
Figure 7. Adenovirus-mediated HO-1 transduction protects advanced atherosclerotic lesions against plaque destabilization. Morphometric and immunohistochemical analyses of vulnerable plaques in sham adenovirus (ΔE1A-Ad) or HO-1 adenovirus (HO-1-Ad) transfected ApoE−/− mice are shown. In the right column, representative cross sections are shown. In the left column, bar graphs show the effects of HO-1 transduction on intima/media (I/M) ratio (A), relative cap thickness (ie, mean cap thickness at plaque shoulders and midregion/maximal intimal thickness) (B), necrotic core/intima ratio (C), percent intimal lipids (D), percent intimal VSMCs (E), percent intimal collagen (F), and percent intimal CD68+ macrophages (G) in vulnerable plaque lesions. *P<0.05 vs ΔE1-Ad. n=10 for each group. In the photographs, dotted lines and asterisk represent the lumen area.
VSMC accumulation was significantly preserved in the arteries by HO-1 induction, whereas HO-1 inhibition had an opposite effect. This finding suggested that HO-1 promotes VSMC survival in the fibrous cap and neointima by protecting the cells against oxidative stress damage, surpassing the cytostatic effect of HO-1. Currently, we are studying the role of HO-1 in porcine VSMCs in vitro, and preliminary data suggest that HO-1 induction can protect against TNF-α-presensitized cell death. Further studies are being conducted to better understand the role of HO-1 in VSMC survival.

In conclusion, the present study provides evidence that HO-1 expression defines the progression of an advanced atherosclerotic lesion into a vulnerable plaque, both in human carotid atherosclerotic lesions (in 112 patients) and in a hyperlipidemic vulnerable plaque mouse model. HO-1 expression in vulnerable plaques is enhanced as a compensatory atheroprotective response, in which HO-1 prevents plaque instability by impeding lipid deposition and necrotic core growth and by prolonging VSMC survival in the fibrous cap. Genetic or pharmaceutical enhancement of HO-1 levels could protect this type of lesion from rupture, thereby reducing the risks on subsequent acute coronary events.

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Disclosures
None.

References

**CLINICAL PERSPECTIVE**

Sudden rupture of vulnerable atherosclerotic lesions at critical sites in the vasculature accounts for the high incidence of myocardial infarction and stroke in patients suffering from carotid artery disease. In this article, intraplaque heme oxygenase 1 levels were closely associated with increased plaque vulnerability in human carotid endarterectomy material. In addition, pharmacologically and genetically introduced alterations in heme oxygenase 1 levels in a murine model of vulnerable plaque determined lesion outcome by affecting plaque stability. These present findings suggest that novel therapies based on increasing heme oxygenase 1 levels in patients with carotid artery disease could benefit patient outcome by stabilizing the plaque phenotype.
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Material and Methods:

Analysis of the human atherosclerotic plaques in human CEA specimens

CEA specimens are harvested, embedded in paraffin, and immunohistochemically examined for the presence of macrophages, VSMCs, collagen, and lipids. In addition, plaque thrombogenicity is assessed by the presence of intraluminal thrombus or intraplaque erythrocyte deposition, in stained hematoxylin/eosin sections. In addition, adjacent sections of the collected CEA atherosclerotic plaques are freshly frozen for protein analysis. Protein samples of 112 patients were randomly selected from the Athero Express biobank and analyzed. Plaques were subdivided into fibrous, fibro-atheromatous, and atheromatous lesions, based on plaque morphology as assessed on hematoxylin/eosin stained sections. For analysis of plaque thrombogenicity, the CEA material was divided into lesions with no, minor, moderate and heavy thrombogenicity by scoring for the presence of erythrocytes in the intimal area.

ELISA analysis of protein expression

Proteins were extracted from atherosclerotic segments adjacent to the segments used for immunohistochemical analysis by Tri-Pure Isolation Reagent (Roche, the Netherlands). Total protein concentration was determined by a colorimetric protein assay, followed by optical spectroscopy (BioRad, the Netherlands). HO-1, IL6, and IL8 protein expression levels in lysates was assessed with a commercial quantitative sandwich ELISA assay (for HO-1; EKS-800 kit, Stressgen, USA, for IL6, IL8, MMP2 and MMP9; Sanquin, the
Netherlands). The association between HO-1 protein expression and patient CEA characteristics was analyzed using a Kruskal-Wallis one-way analysis of variance for ranks. Data are presented as mean ± SEM unless stated differently. Value of $P<0.05$ was considered to indicate significance.

**Immunohistological analysis by confocal microscopy**

For the human CEA samples, paraffin embedded sections were stained with anti-human CD68 (Hycult, The Netherlands), CD163 (clone 106D, Sanbio, The Netherlands), α-Actin Cy3 (Dako, Belgium), and HO-1 (OSA-110, Stressgen, The Netherlands), and with secondary antibodies labeled with Alexa Fluor (568nm, 488nm, Molecular Probes, Invitrogen Inc., The Netherlands). Processed sections were visualized by tile analysis on the confocal microscope and quantified using LSM software (LSM510 NLO/FCS, Zeiss, The Netherlands).

**Vulnerable plaque model in ApoE–/- mice**

All experiments were performed in compliance with institutional (Erasmus University Medical Center, Rotterdam, The Netherlands) and national guidelines. ApoE–/- mice on a C57BL/6J background (age 12-15 weeks) were obtained from Jackson Laboratory (Bar Harbor, USA). The design of this animal study is described in Supplemental figure 1. (A) Two weeks before surgery, all animals were placed on a Western-type diet containing 15% (w/v) cocoa butter and 0.25% (w/v) cholesterol (diet W, Hope Farms, The Netherlands). (B) Two weeks later, animals were anaesthetized by isoflurane inhalation, and the right common carotid artery was dissected from
circumferential connective tissues. A cast was placed around the right common carotid artery, after which the wounds were closed with vicryl sutures, and the animals were allowed to recover. (C) Six weeks after cast placement, animals were randomized to either treatment with phosphate-buffered saline (Cambrex, UK, N=15), cobalt protoporphyrin in order to induce endogenous HO-1 expression (CoPPIX, Frontier Scientific Inc., Canada, ip injection, 5mg/kg q2d, in 0.2M NaOH, pH 7.4, N=10), or zinc protoporphyrin to inhibit endogenous HO-1 (ZnPPIX, Frontier Scientific Inc., Canada, ip injection, 5mg/kg q2d, in 0.2M NaOH, pH 7.4, N=10). After 21 days of treatment, animals were sacrificed. The carotid arteries were harvested and processed for histological analysis. For measuring HO-1 activity, the aortas were isolated and immediately snapfrozen in liquid nitrogen. The tissue samples were pooled per group, homogenized in 3 mL of homogenizing buffer (200 mM KH2PO4, 135 mM KCl, 0.1 mM EDTA, pH 7.4), and sonicated at 4°C. The supernatant was transferred to ultracentrifuge tubes and 5 mL of homogenizing buffer was added, followed by an ultracentrifugation (100,000 x g, 4 °C, 60 minutes, Beckman L7-35 Ultracentrifuge, 70 Ti rotor). The microsomal pellet was resuspended in 320 ml of HO activity buffer (100 mM KH2PO4, 2 mM MgCl2, pH 7.4). After a second sonication step the samples were centrifuged, and the supernatant was used to determine HO activity. The reaction mixture for HO activity measurement contained rat liver cytosol (as a source of biliverdin reductase, 2 mg/assay), hemin (20 mmol/L), glucose 6-phosphate (2 mmol/L), glucose 6-phosphate dehydrogenase (0.2 U/reaction) and NADPH (0.8 mmol/L) in a total volume of 400 ml. After the reaction at 37 °C for 1 hour, the formed bilirubin was extracted with 1 ml of chloroform by vigorous vortexing 3 times for 10 seconds. After centrifugation optical
densities at 464 nm and 530 nm of the organic phase were determined and HO activity was calculated as pmol bilirubin formed/mg of tissue/60 min.

Blood samples were drawn by orbital puncture and total serum levels of bilirubin were determined according to the protocol of Bartels and Bohmer\(^1\). The effects of HO-1 overexpression induced by adenoviral mediated transfection on vulnerable plaque development was assessed (D). At six weeks after cast placement, 20 µl of 1x10\(^{10}\) pfu/ml adenoviral suspension (either HO-1-Ad or ΔE1-Ad, N=10 per group) was injected in the common carotid artery via the external carotid artery after ligation of the proximal common carotid artery and the internal carotid artery in order to induce a stagnant fluid condition. After an incubation time of 15 minutes, the temporal ligations were removed, and a permanent ligation was placed at the injection site at the external carotid artery before closure of the wound. 21 days later, these animals were sacrificed and the carotid arteries were harvested for analysis. For validation of HO-1 overexpression in HO-1-Ad transduced arteries, a separate group of ApoE \(\sim/-\) animals were transfected with Ad- ΔE1 or HO-1-Ad. 1 week after transfection, these animals were sacrificed and the carotid arteries were processed for qPCR analysis.

**Gene expression analysis**

Freshly isolated vessel segments were divided into the stable and vulnerable plaque regions. TotalRNA was extracted using the RNeasy kit (Qiagen, The Netherlands), and reversed transcribed into cDNA. QPCR analysis was performed using a real-time fluorescence determination in the iCycler iQ Detection System (Biorad, The Netherlands). Primers for HO-1 were designed using online 3primer software. Target
gene expression levels were expressed relative to the housekeeping gene hypoxanthine guanine phosphoribosyl transferase (HPRT) as an endogenous control. HO-1 forward primer sequence: ACGCATATACCCGCTACCTG, HO-1 reverse primer sequence: CCAGAGTGTTCATTGAGCA.

Tissue preparation and histological analysis

Carotid arteries were embedded in OCT (Sakura Finetek, The Netherlands) and snap-frozen in liquid nitrogen. The abdominal aorta from each animal was excised, pulverized using a small glass pestle before sonication in NP40 lysis buffer on ice (Ultrasonic bath VWR, the Netherlands, 30 minutes at maximum power). Quantification of histological data was performed on 7 µm cryosections, at 140 µm intervals. In addition to standard H/E staining, immunohistochemical analysis was performed, including assessment of macrophages (anti-CD68 antibody, Santa Cruz Biotechnology Inc., The Netherlands), smooth muscle cells (anti-α-actin antibody, Sigma, Zwijndrecht, The Netherlands), and HO-1 (anti-HO-1, OSA-110, Stressgen, The Netherlands). Lipid and collagen deposition were visualized by Oil-red-O and picrosirius red stainings, respectively. Data analysis was performed using a commercial image analysis system (Impak C, Clemex Technologies, Canada). Intima/media ratio and necrotic core/intima ratios were analyzed in sequential H/E-stained carotid sections. Necrotic area was defined as neointimal areas devoid of cellular tissue. Relative fibrous cap thickness was defined as the ratio of the average cap thickness at the shoulder and mid-plaque region divided by maximal intima cross-sectional thickness. The percentage of the different plaque components including HO-1, was calculated as the surface area that were positive for each specific indicative
expressed as a percentage of the total intimal surface area. Statistical analysis was performed using Student’s t-test. Data are presented as mean ± SEM. P values less than 0.05 were considered to be significant.

**Western blot analysis**

Total protein concentration was determined using a colorimetric protein assay followed by optical spectroscopy (BioRad, the Netherlands). HO-1 protein expression was analyzed by standard Western Blot analysis (Biorad, The Netherlands) and was subsequently visualized using a fluorescent detection system (Li-cor Biosciences, The Netherlands).

**Supplemental Figures:**

Figure 1: Schematic overview of the experimental animal model. (A) C57bl/6 ApoE -/- mice were fed a cholesterol rich, high fat diet starting from 2 weeks before cast implantation (T=-2 weeks). (B) The cast device was implanted into the right carotid artery (T= 0 weeks). (C) Saline, CoPPIX or ZnPPIX was injected i.p. every two days starting from week 6 (T= 6 weeks). The animals were sacrificed 9 weeks post cast-implantation, and the carotid artery segments were harvested for immunohistological analysis. The control group was injected with saline from T= 6 weeks. The HO-1 induced group was injected with CoPPIX from T = 6 weeks. The HO-1 inhibition group was injected with ZnPPIX from T = 6 weeks. (D) For the ΔE1-Ad or HO-1-Ad groups, adenoviral transduction of an empty expression vector (ΔE1-Ad) or HO-1 expression cDNA vector (HO-1-Ad) was mediated by intra-arterial injection of 1X10^{10} pfu/ml
adenoviral suspension (either HO-1 or sham virus) into the common carotid artery at T= 6 weeks.

**Figure 2:** (A) HO-1 level distribution in CEA specimens. Boxplots show data distribution of HO-1 in human atherosclerotic lesions per quartile as assessed by ELISA analysis. The median is indicated by a thick black line. (B) Western blot analysis show HO-1 protein levels in pooled aortas of saline, CoPPIX or ZnPPIX treated ApoE-/- mice after 3 weeks of ip injections. (C) HO activity measurement of HO activity in pooled aorta samples of saline, CoPPIX or ZnPPIX treated ApoE-/- mice after 3 weeks of ip injections (N=5 per group). Data are shown as pmol bilirubin formed/mg of tissue/hour. (D) qPCR assessment of HO-1 expression in HO-1-Ad transduced carotid vessel segments of ApoE-/- mice compared to A-sham treated animals and untreated contralateral carotid arteries after 1 week of transfection. *P<0,05 versus control. †P<0,05 versus ΔE1-Ad.
References: