Suppression of Rage as a Basis of Simvastatin-Dependent Plaque Stabilization in Type 2 Diabetes

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Objective—Receptor for advanced glycation end products (AGEs) (RAGE) plays a central role in the process of plaque rupture in diabetic patients. Recently, it has been reported that RAGE may be downregulated by improving glycemic control. In contrast, despite being well known that RAGE may be induced in human vessels in a glucose-independent fashion, also by myeloperoxidase (MPO)-dependent AGE generation, no data exist regarding the possibility of a pharmacological modulation of glucose-independent RAGE generation. Thus, the aim of this study was to characterize the effect of simvastatin on the expression of RAGE and RAGE-dependent plaque-destabilizing genes in human atherosclerotic plaques.

Methods and Results—Seventy type 2 diabetic patients with asymptomatic carotid artery stenosis (>70%) were randomized to American Heart Association (AHA) step 1 diet plus simvastatin (40 mg/d) or AHA step 1 diet alone for 4 months before endarterectomy. Plaque expression of MPO, AGEs, RAGE, NF-κB, COX-2, mPGES-1, matrix metalloproteinase (MMP)-2 and MMP-9, lipid and oxidized LDL (oxLDL) content, procollagen 1, and interstitial collagen was analyzed by immunohistochemistry and Western blot;zymography was used to detect MMP activity. Plaques from the simvastatin group had less (P<0.0001) immunoreactivity for MPO, AGEs, RAGE, p65, COX-2, mPGES-1, MMP-2, and MMP-9, lipids and oxLDL; reduced (P<0.0001) gelatinolytic activity; increased (P<0.0001) procollagen 1 and collagen content; and fewer (P<0.0001) macrophages, T-lymphocytes, and HLA-DR+ cells. Of interest, RAGE inhibition by simvastatin, observed not only in plaque sections but also in plaque-derived macrophages, was reverted by addition of AGEs in vitro.

Conclusions—This study supports the hypothesis that simvastatin inhibits plaque RAGE expression by decreasing MPO-dependent AGE generation. This effect in turn might contribute to plaque stabilization by inhibiting the biosynthesis of PGE2-dependent MMPs, responsible for plaque rupture. (Arterioscler Thromb Vasc Biol. 2006;26:2716-2723.)

Key Words: diabetes mellitus • metalloproteinases • myeloperoxidase • RAGE • statins

Diabetes is associated with severe atherosclerosis in humans and represents a leading cause of morbidity and mortality.1 Chronic perturbation of diabetic vasculature leads to increased number, size, and complexity of atherosclerotic plaques. Furthermore, lesion instability is enhanced in diabet es and mediates increased incidence and severity of clinical events.2

In the recent years, there is increasing evidence that in diabetic patients, RAGE (receptor for advanced glycation end products [AGEs]) plays a central role in the cascade of events that result in accelerated atherosclerotic plaque formation, as well as plaque erosion and fissuring. In fact, it has been recently demonstrated that administration of the extracellular ligand-binding domain of the receptor, soluble (s) RAGE, to apolipoprotein (apo) E-null mice with the diagnosis of hyperglycemia suppressed both accelerated development of atherosclerotic plaques3 and progression of established lesions toward complexity.4,5 Furthermore, we recently extended these observations to humans by demonstrating that RAGE may promote the evolution of atherosclerotic plaques toward instability by inducing PGE2-dependent metalloproteinase (MMP)-2 and MMP-9 production.6

Worth noting is the observation that the pro-atherogenic role of RAGE may be modulated in vivo. In fact, we have demonstrated that RAGE may be downregulated by improving glycemic control.6 Furthermore, Marx et al7 have recently
showed that thiazolidinediones (TZD), insulin-sensitizer drugs, can inhibit the RAGE pathway in humans. However, no data still exist regarding the possibility that drugs affecting myeloperoxidase (MPO) may reduce the RAGE-dependent plaque instability despite it being well known that enhanced deposition of AGEs (the RAGE ligands) occurs in diabetic blood vessels driven by MPO activity in macrophages, and the fact that it may upregulate RAGE expression after ligation, the latter realizing a positive loop of auto-amplification of this inflammatory reaction. Thus, it is time to hypothesize that pharmacological inhibition of MPO in the plaque environment may turn off RAGE expression and related pro-inflammatory mechanisms, thereby slowing down atherosclerotic plaque instability.

In this light, recent clinical trials have shown that statins strongly reduce cardiovascular events and mortality in diabetics. Clinical benefits of statins are greater than those expected on the basis of only modest change in arterial stenosis severity. These clinical data suggest that statins may somehow stabilize plaques against disruption, and in fact we and others have recently demonstrated that statins may decrease MMP activity and increase collagen content in human plaques. However, the specific molecular mechanism(s) by which statins may influence MMP generation in diabetic plaques has not yet been completely elucidated.

The possibility that suppression of RAGE by statins might represent a mechanism of glucose-independent plaque stabilization in diabetics led us to investigate whether it would modulate MMP production and extracellular matrix metabolism in atherosclerotic plaques. Here, we reported reduced RAGE expression in carotid plaques of diabetic patients randomized to simvastatin, possibly caused by reduction in AGE generation resulting from the suppression of MPO activity.

Methods

For detailed methods, please see the online Materials and Methods, available at http://atvb.ahajournals.org

Patients

We studied 70 of 122 consecutive, not previously examined or included in clinical trials, type 2 diabetic surgical inpatients (37 mol/L, 33F: 69±4 years), enrolled to undergo carotid endarterectomy for extracranial high-grade (>70%) internal carotid artery stenosis. All patients were “asymptomatic” according to North American Symptomatic Carotid Endarterectomy Trial (NASCET) classification, and had low-density lipoprotein (LDL) cholesterol ranging between 70 mg/dL and 100 mg/dL. All the patients had fair glycemic control, as reflected by glycohemoglobin (HbA1c) <8%. Study patients were representative of those commonly admitted to our Vascular Surgery Unit for extracranial internal carotid artery stenosis. Patients taking chronic statin therapy in the last two months were excluded from the study. Eligible patients were randomized to 4-month treatment with American Heart Association (AHA) step 1 diet plus simvastatin (Sinvacor, Merck Sharp & Dohme) 40 mg/d or AHA step 1 diet alone. Finally, after the treatment period all patients underwent endarterectomy. Lipid profile was measured at baseline and immediately before endarterectomy. Percentage of carotid diameter reduction, procedural methods, risk factors, and concomitant therapy did not differ between the two groups (Table). In particular, by the time of surgery, all patients were taking 100 mg daily of aspirin. The study was approved by local ethics review committees.

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<th>Characteristics of Study Patients</th>
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ECA indicates carotid endarterectomy; IHD, ischemic heart disease; NSAID, nonsteroidal antiinflammatory drug. *P<0.0001.

Written informed consent was obtained from all patients before each examination.

Immunohistochemistry

After the surgical procedure, samples were immediately frozen in isopentane and cooled in liquid nitrogen (LN3). Serial sections were prepared and immunohistochemistry to detect CD68 macrophages, CD3 lymphocytes, HLA-DR, myeloperoxidase, AGEs, RAGE, p65, COX-2, mPGES-1, type 1 procollagen, oxLDL, MMP-2, and MMP-9 was performed as previously described.

Western Blot

Tissue extracts were subjected to western blot analysis, as previously described. The antibodies for MPO, AGEs, RAGE, p65, COX-2, mPGES-1, MMP-2, and MMP-9 were used.

Zymography

Zymographic analyses on 7.5% acrylamide gel containing 0.1% gelatin on plaque homogenates or plaque-derived macrophages was performed as previously described.

Oil Red O Staining for Lipid Content

Two parallel sections from each plaque specimen were incubated in isopropanol, then in Oil Red O (Carlo Erba, Milan, Italy) solution and rinsed in water. One of the sections was counterstained with hematoxylin.

Sirius Red Staining for Collagen Content

Tissue sections were rinsed with distilled water, incubated with Sirius red (Sigma) in saturated picric acid and collagen content was detected as previously described.

EIA Determinations

Plaque content of PGE2 were measured by using a commercially available enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, Mich), according to the manufacturer’s instructions.

NF-κB Activity

Nuclear extracts from plaque specimens from diet- or statin-treated patients were obtained as described by Ohlsson et al. Subsequently,
to study NF-κB DNA binding activity in the 2 groups of plaques, chemiluminescent EMSA was performed on a biotin-labeled oligonucleotide containing an NF-κB DNA-binding consensus sequence using the LightShift EMSA kit (Pierce).\textsuperscript{19} according to the manufacturer’s protocols. In addition, we also analyzed the expression of activated p50 subunit by specific Trans-AM NF-κB p50 transcription factor assay kit (Active Motif, Rixensart, Belgium).\textsuperscript{5,20}

**Assessment of Myeloperoxidase Activity**
Myeloperoxidase activity was measured as described by Sehirli et al.\textsuperscript{21}

**Extraction of Macrophages From Atherosclerotic Plaques**
Macrophages were selectively extracted from plaques as described by de Vries et al.\textsuperscript{22} Then, immunocytochemistry, Western blot, and zymography were performed as illustrated. Results are representative of 3 different experiments.

**Isolation and Culture of Blood Monocytes**
Peripheral blood monocytes were purified and cultured from 5 healthy blood donors as previously described.\textsuperscript{17} Control or stimulated (lipopolysaccharide, 1 μg/mL; IL-1β, 10 ng/mL) monocytes (5×10⁶ cells/4 mL RPMI 1640) were incubated for 48 hours at 37°C in the presence or absence of 10 μmol/L simvastatin (Merck Sharp & Dohme). AGEs (800 μg/mL) were also added to some of the cultures. AGEs were prepared as described by Basta et al.\textsuperscript{19} MMP-2 and MMP-9 activity and expression were analyzed in collected media by zymography and in scraped monocytes by Western blot respectively. The results are representative of 3 experiments using cells from different donors.

**Statistical Analysis**
For clinical data and histological examination, variables were compared by application of χ² test. The significance of difference in biochemical marker expression and inflammatory cell infiltration between diet-treated and simvastatin-treated patients was analyzed by Student t test. The strength of the association between plasma HbA1c and plaque RAGE expression after simvastatin treatment was assessed by linear regression analysis. Data are expressed as percentage or mean±SD. All calculations were performed using the SPSS 12.0 computer program.

**Results**

**Clinical Results**
Percentages of carotid diameter reduction after treatment did not differ between the 2 groups (−1.3±0.8% versus −1±0.9%). Baseline lipid levels were similar in the 2 groups (Table). At the end of the study, total cholesterol and LDL cholesterol were significantly reduced in patients treated with simvastatin (−28% and −21%, respectively), whereas they did not change in patients randomized to diet alone. High-density lipoprotein cholesterol did not change significantly in the 2 groups of patients after treatment period (+4% in the simvastatin group). Baseline HbA1c was similar in the 2 groups (7.8±0.2% versus 7.7±0.2%), and unchanged after 4 months of treatment with simvastatin (7.7±0.2% versus 7.7±0.3%). No patient in either group developed any clinical event during the study.

**Inflammatory Infiltration**
Immunocytochemistry revealed inflammatory infiltration in all specimens examined, more evident in the shoulder of diet-treated plaques. Plaque area occupied by macrophages and T cells was significantly greater (P<0.0001) in diet-treated rather than in simvastatin-treated plaques (Table; supplemental Figure I, available online at http://atvb.ahajournals.org). Inflammatory cells in plaques from diet group were always characterized by strong expression of HLA-DR, a selective marker of cell activation, which contrasted markedly with the low expression of HLA-DR in plaques from simvastatin group.

**Myeloperoxidase Expression and Activity Is Reduced by Simvastatin in Diabetic Plaques**
Expression of MPO and its bioproducts AGEs was markedly lower in the diabetic plaques treated with simvastatin as compared with plaques treated with diet alone (23±3% versus 7±1%, and 29±4% versus 12±3%, respectively, n=35, mean±SD; P<0.0001) (Figure 1). Worth noting is the fact that the reduction in MPO expression was always associated with a comparable reduction in myeloperoxidase activity in atherosclerotic plaques from patients treated with simvastatin (8.75±0.9 versus 3.1±0.5 U/g tissue). MPO staining was observed mainly in inflammatory cells infiltrating the shoulder of the plaques.

**RAGE Expression Is Reduced by Simvastatin in Diabetic Plaques**
After ligation with RAGE, AGE may upregulate RAGE expression, thus realizing a positive loop of auto-amplification of this inflammatory reaction. So, we investigated the association between AGE suppression by simvastatin and RAGE expression in diabetic plaques. Immunohistochemistry revealed strong RAGE immunoreactivity in diabetic plaques (Figure 2A), significantly more abundant in lesions treated with diet alone than in those treated with simvastatin (30±4% versus 9±3%, n=35, mean±SD; P<0.0001). RAGE areas characterized as macrophage-rich were localized prominently in the shoulder region, in the periphery of the lipid core and in the proximity of vasa vasorum. In fact, RAGE staining at high magnification indicated its localization in activated macrophages.
RAGE Suppression by Simvastatin Is a Glucose-Independent Phenomenon

Western blot analysis confirmed RAGE expression in plaques (Figure 2B), markedly higher in diabetic plaques treated with diet alone (8433 ± 11006 132 versus 1437 1H1006 126 densitometric units (DU), mean ± SD, n = 35; P < 0.0001). Notably, RAGE reduction in plaques treated with simvastatin was completely unrelated to glycemic control, as reflected by the absence of any correlation (R² = 0.08, P = NS) between plasma HbA1c and plaque RAGE content (quantified by densitometric analysis).

The Effect of Simvastatin on Diabetic Plaques Is Mediated by NF-κB Inhibition

After ligation with its ligands, RAGE may activate target genes via stimulation of NF-κB, consequently we investigated the association between RAGE inhibition by simvastatin and NF-κB activation in plaques. NF-κB activation as reflected by the analysis of the activated form of both p50 subunit was significantly higher in diet- than in simvastatin-treated plaques (22 ± 2 versus 9 ± 2 pg/μg of nuclear extracts, n = 35; P < 0.0001; supplemental Figure IIA), and showed a strong concordance with RAGE expression. EMSA shift assays also showed that the greatest DNA binding activity was with nuclear extracts from patients treated with diet alone, whereas strongly reduced binding was detected in those treated with statin (4208 ± 224 versus 1256 ± 147 DU, mean ± SD, P < 0.001) (supplemental Figure IIB). Staining for p65α was significantly higher in plaques treated with diet alone (25 ± 3% versus 6 ± 2%, n = 35; P < 0.0001) (supplemental Figure IIC), and accumulated mainly in macrophages infiltrating the plaque shoulder.

The Expression of RAGE Target Genes Is Reduced by Simvastatin

COX-2 and mPGES-1 staining was more abundant in lesions from diet-treated patients (24 ± 3% versus 5 ± 1%, and 20 ± 2% versus 4 ± 1%, respectively, mean ± SD, n = 35, P < 0.0001) (Figure 3A); furthermore, COX-2/mPGES-1 accumulated in activated macrophages and smooth muscle cells at the shoulder regions. Western blot confirmed the higher expression of COX-2/mPGES-1 in plaques from diet group as compared with plaques from simvastatin group (7544 ± 134 versus 1876 ± 111, and 6987 ± 124 versus 1567 ± 117 DU, respectively, mean ± SD, n = 35, P < 0.0001; Figure 3B).

The Activity of RAGE Target Genes Is Reduced by Simvastatin

Because a direct and accurate comparison of the levels of COX-2/mPGES-1 might be limited by variable antibody affinities, we also confirmed results concerning COX-2/mPGES-1 expression by EIA measurements of bioproduct PGE2 plaque content. In fact, PGE2 was present in higher amounts in diet-treated plaques with respect to simvastatin-treated plaques (186 ± 17 versus 42 ± 4 ng/mg tissue, mean ± SD, n = 35; P < 0.0001).
Diabetic Plaques

than in simvastatin-treated lesions (24 ± 3% versus 8 ± 3%, n = 35, mean ± SD; P < 0.0001) (Figure 4C). Procollagen levels were always inversely associated with myeloperoxidase and RAGE levels (R² = -0.41, P < 0.0001). Similarly, Sirius Red polarization showed considerably higher content of interstitial collagen in tissue sections from simvastatin group compared with sections from diet group (21 ± 3% versus 9 ± 2%, n = 35, mean ± SD; P < 0.0001).

Finally, total lipids and oxidized LDL (oxLDL) plaque content was significantly lower in plaques from simvastatin-treated patients (5 ± 2% versus 23 ± 6%, and 4 ± 1% versus 24 ± 3%, respectively, n = 35, mean ± SD; P < 0.0001).

Colocalization of Myeloperoxidase with RAGE, COX-2/mPGES-1, and MMPs in Plaque Macrophages

In the first experiment, serial sections of diet-treated plaques were incubated with primary antibodies for CD68, MPO, RAGE, COX-2, mPGES-1, MMP-2, and MMP-9. Within the lesion, all proteins accumulated in the shoulder as well as in the periphery of the lipid core. In 2 other experiments, immunofluorescence double labeling associated the expression of MPO with RAGE, COX-2, mPGES-1, and MMPs in CD68+ macrophages both in plaque sections and in plaque-derived macrophages (supplemental Figure III). Thus, these analyses confirmed the concomitant presence of MPO, RAGE, and downstream effectors in macrophages at the vulnerable region of diabetic plaques.

Macrophages Isolated From Diet-Treated and Simvastatin-Treated Plaques Recapitulate Differences in Enzyme Expression Observed In Vivo

The lower RAGE levels observed in simvastatin-treated versus diet-treated plaques could be because of a lower relative abundance of macrophages in the former tissues (Table). Alternatively, reduced MPO expression by macrophages exposed to simvastatin could also account for the observed differences. To address this point, we assessed the expression of MPO, RAGE, and downstream enzymes by immunocytochemical and Western blot analyses in macrophages isolated from 5 diet-treated and 5 simvastatin-treated plaques. A lower positivity for MPO, RAGE, COX-2, mPGES-1, and MMPs was observed by immunocytochemistry in macrophages from simvastatin-treated plaques. In contrast, a 5-fold higher signal (P < 0.0001) was shown by Western blot in macrophages from diet-treated diabetic plaques (6234 ± 145 versus 877 ± 55 DU for MPO; 5345 ± 123 versus 768 ± 66 DU for RAGE; 6324 ± 106 versus 1034 ± 65 DU for COX-2; 5487 ± 102 versus 733 ± 43 DU for mPGES-1; 6756 ± 121 versus 787 ± 65 DU for MMP-2; 5956 ± 154 versus 834 ± 63 DU for MMP-9; supplemental Figure IV). We conclude that the phenotype of macrophages from plaques from simvastatin group differs from that of cells from diet-treated plaques and that the differences are maintained after plating, suggesting the occurrence of sustained inhibition signals in macrophages exposed to simvastatin in vivo.

Impact of Simvastatin on the Expression and Activity of PGE₂-Dependent MMPs in Diabetic Plaques

Immunohistochemistry revealed that positivity for MMP-2 and MMP-9 was significantly more abundant in diet-treated than in simvastatin-treated lesions (24 ± 3% versus 6 ± 1%, and 22 ± 2% versus 7 ± 2%, n = 35; P < 0.0001) (Figure 4A). These data were also confirmed by Western blot (6598 ± 121 versus 1104 ± 89, and 6676 ± 78 versus 1165 ± 67 DU, n = 35, mean ± SD; P < 0.0001). In addition, zymography (Figure 4B) demonstrated that extracts from diet-treated plaques contained higher amounts of the activated form of MMP-2 and MMP-9 (2559 ± 67 versus 543 ± 45, and 2734 ± 83 versus 565 ± 67 DU, n = 35, mean ± SD; P < 0.0001).

Effect of Simvastatin on Plaque Extracellular Components

Immunohistochemistry analysis showed markedly higher content of procollagen type 1 in simvastatin-treated than in diet-treated plaques (22 ± 3% versus 8 ± 3%, n = 35, mean ± SD; P < 0.0001) (Figure 4C). Procollagen levels were always inversely associated with myeloperoxidase and RAGE levels (R² = -0.41, P < 0.0001). Similarly, Sirius Red polarization showed considerably higher content of interstitial collagen in tissue sections from simvastatin group compared with sections from diet group (21 ± 3% versus 9 ± 2%, n = 35, mean ± SD; P < 0.0001).

Finally, total lipids and oxidized LDL (oxLDL) plaque content was significantly lower in plaques from simvastatin-treated patients (5 ± 2% versus 23 ± 6%, and 4 ± 1% versus 24 ± 3%, respectively, n = 35, mean ± SD; P < 0.0001).
Simvastatin Regulates RAGE Expression in Inflammatory Cells Through Inhibition of AGEs

To determine if simvastatin may suppress RAGE and related pro-inflammatory genes through MPO inhibition, we examined the effect of simvastatin on RAGE expression in blood monocytes in vitro (supplemental Figure V). Lipopolysaccharide caused an enhancement ($P<0.0001$) in MPO (6975 ± 144 versus 1432 ± 132 DU), RAGE (6567 ± 122 versus 1011 ± 87 DU), COX-2 (6232 ± 156 versus 1111 ± 135 DU), mPGES-1 (6123 ± 142 versus 1231 ± 78 DU), MMP-2 (5437 ± 159 versus 1222 ± 89 DU), and MMP-9 (6854 ± 165 versus 1211 ± 121 DU) levels over those detected in control monocytes. Induction of these proteins by lipopolysaccharide was significantly ($P<0.0001$) inhibited by simvastatin (1145 ± 65, 1267 ± 107, 1549 ± 88, 1476 ± 45, 1322 ± 57, and 1332 ± 96 DU, respectively); however, the inhibition of RAGE and related genes was completely reversed by the addition of AGEs. Similar results were also observed when IL-1β was used as a stimulus. Inhibition of RAGE by simvastatin thus appears secondary to the reduction of AGE biosynthesis.

Discussion

We previously reported that RAGE contribute to the clinical instability of atherosclerotic plaques in diabetics by promoting plaque rupture induced by PGE$_2$-dependent MMPs, key enzymes in the final step of this process. Now, in the present report, we provide evidence for the critical involvement of RAGE in the process of plaque stabilization realized by simvastatin. In particular, the present findings are the first to our knowledge to: (1) demonstrate a direct inhibitory effect of simvastatin on RAGE expression in human atherosclerotic lesions; (2) show in humans the possibility of RAGE regulation in a glucose-independent fashion; and (3) associate the inhibition of RAGE with the reduction of MMP activity observed after statin therapy.

Lower expression of RAGE was found in specimens obtained from carotid lesions of diabetic patients randomized to simvastatin compared with specimens obtained from patients randomized to diet alone. Notably, the hypothesis that RAGE suppression by simvastatin may have a protective impact on plaque phenotype is also supported in this study by its correlation with the parallel reduction in MMP expression, and with the strong increment in plaque collagen content after simvastatin therapy.

In previous studies we$^{14}$ and others$^{15,23}$ reported the ability of statins to reduce atherosclerotic plaque inflammatory burden and to slow lesion evolution toward rupture. In particular, a recent work by Martín Ventura et al$^{21}$ showed that a 1-month intensive treatment with atorvastatin reduced both local and systemic inflammatory mediators in patients undergoing carotid endarterectomy, as evaluated by plaque content of macrophages, MCP-1, COX-2, and circulating levels of MCP-1 and PGE$_2$. However, these studies did not provide any evidence regarding involvement of RAGE in pathophysiology of statin-dependent plaque stabilization. RAGE is a transmembrane receptor that, after ligation with AGEs, may induce NFκB activation, ultimately promoting COX-2/mPGES-1 induction and PGE$_2$-dependent MMP biosynthesis. Thus, concomitant AGE generation and RAGE expression may be necessary for the biosynthesis of PGE$_2$-dependent MMPs in the setting of atherosclerotic diabetic plaques.

Interestingly, macrophages of the shoulder region contain most of the RAGE within the lesion. This finding may have functional importance, because RAGE can regulate the expression of different genes in different cell types. In particular macrophages, not present in normal arterial tissue, produce an array of metalloproteinases, including the PGE$_2$-dependent MMP-2 and MMP-9,$^{19}$ considered 2 of the most important in the process of atherosclerotic plaque rupture. Thus, we have facts that allow us to confidently hypothesize that increased COX-2/mPGES-1 induction and PGE$_2$ generation in plaque macrophage as consequence of RAGE overexpression may enhance the synthesis of MMPs in the same cell, possibly representing a crucial step in the pathophysiology of diabetic plaque instability. In this light, our description of an association between the strong reduction of these metalloproteinases in plaque treated with simvastatin and the inhibition of RAGE in the same cells support the hypothesis that such “RAGE-related” mechanism of MMP inhibition by simvastatin may operate in vivo in diabetic patients.

Usually, the expression of RAGE in human diabetic plaques has been strictly associated with glycemic control. In fact, it has been previously demonstrated that RAGE expression is higher in diabetic patients with poor glycemic control$^6$ and that RAGE may be downregulated both by amelioration of glycemic control$^6$ and by insulin sensitizers.$^7$ Now, in this study, we provide the first demonstration, to our knowledge, in humans that RAGE expression in plaque may also be modulated in a glucose-independent manner by inhibition of macrophage myeloperoxidase. This observation appears to be of interest because it could even be potentially extended to other clinical settings related to the pro-atherogenic role of RAGE, suggesting that direct generation of AGE in plaques by macrophage myeloperoxidase may be operative and potentially relevant in the setting of all those clinical conditions, such as hypercholesterolemia$^{14}$ and hypertension$^{24}$ characterized by high infiltration of activated macrophages in atherosclerotic plaques. Furthermore, because AGE-RAGE interaction results in upregulation of RAGE, and NF-κB activated by RAGE may further amplify RAGE expression in inflammatory cells by a direct, positive control on gene promoter,$^{25}$ RAGE downregulation in plaque macrophages treated with simvastatin may establish a negative auto-inhibitory autocrine and paracrine feedback loop, thus suppressing this inflammatory response leading to progressive plaque destabilization.

In this study, the hypothesis that RAGE suppression by simvastatin is largely dependent on the reduction of AGE generation by myeloperoxidase is also supported by additional in vitro experiments, and by the observation that RAGE reduction in plaques is associated with comparable reduction in AGE content. In addition, the experiments on plaque-derived macrophages clearly demonstrated that the reduction in RAGE expression after simvastatin therapy was related to the enzymatic inhibition of these cells, and not merely the consequence of the reduction in the number of macrophages infiltrating the plaques.
Usually, myeloperoxidase has been strongly and independently associated with cardiovascular disease. In fact, expression of human myeloperoxidase in macrophages promoted atherosclerosis in hypercholesterolemic mice. Furthermore, individuals with total or subtotal myeloperoxidase deficiency appear less likely to develop cardiovascular disease. Again, individuals harboring a promoter polymorphism associated with a 2-fold reduction in myeloperoxidase expression appear cardioprotected, with markedly reduced angiographic evidence of coronary artery disease, myocardial infarction, and cardiac death. Finally, plasma and serum levels of myeloperoxidase have been shown to strongly predict risks of subsequent myocardial infarction and death.

Now, our data may provide a new molecular mechanistic hypothesis for explaining, at least in part, the higher risk of atherothrombotic events associated with myeloperoxidase in these clinical studies.

Finally, we also believe to be of interest the fact that our results were obtained in diabetic patients with normal levels of LDL cholesterol at baseline, an observation that fully supports the results of recent clinical trials and that provides further support against the existence of a LDL cholesterol threshold, below which lowering it would not reduce risk in diabetics.

In conclusion, this study proposes a solid hypothesis for addressing the missing link between statin therapy and plaque stabilization in diabetics by demonstrating the inhibition of the functional myeloperoxidase/AGE/RAGE axis in human atherosclerotic lesions after simvastatin therapy, and by providing evidence that it is associated with plaque stabilization possibly by suppression of the MMP-induced matrix degradation promoting plaque rupture. These findings are also potentially important from a practical standpoint, because they raise the interesting possibility that modification of the RAGE signaling by statins might provide a novel form of therapy for plaque stabilization of diabetic patients with atherosclerotic disease and prevention of acute ischemic syndromes.

Sources of Funding

This study was supported in part by grants from the Italian Ministry of Research (PRIN 2004), and by EC FP6 funding (LSHM-CT-2004-005033). This publication reflects only the authors’ views. The Commission is not liable for any use that may be made of information herein.

Disclosures

None.

References


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Arterioscler Thromb Vasc Biol. 2006;26:2716-2723; originally published online October 12, 2006;
doi: 10.1161/01.ATV.0000249630.02085.12
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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METHODS

Patients. We studied 70 of 122 consecutive, no previously examined or included in clinical trials, type 2 diabetic surgical inpatients (37M, 33F; 69±4yr), enlisted to undergo carotid endarterectomy for extracranial high-grade (>70%) internal carotid artery stenosis. All patients were “asymptomatic” according to North American Symptomatic Carotid Endarterectomy Trial (NASCET) classification,\textsuperscript{16} and had LDL-cholesterol (LDL-C) ranging between 70 mg/dL and 100 mg/dL. All the patients had fair glycemic control, as reflected by glycated hemoglobin (HbA\textsubscript{1c}) <8%. Patients taking chronic statin therapy in the last two months were excluded from the study. Eligible patients were randomized to 4-month treatment with AHA step 1 diet plus simvastatin (Sinvacor, Merck Sharp & Dohme) 40 mg/d or AHA step 1 diet alone. Finally, after the treatment period all patients underwent endarterectomy. Fasting plasma total cholesterol (TC), HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), triglycerides (TG), and HbA\textsubscript{1c} levels were measured at baseline and immediately before endarterectomy. Asymptomatic carotid stenosis was detected on the basis of systematic clinical examination of patients with coronary or peripheral disease. These asymptomatic patients never had an ischemic episode in the territory of the carotid stenosis, but carotid endarterectomy has been shown to be beneficial in asymptomatic patients,\textsuperscript{34} particularly in those with carotid diameter reduction about 70% or more as shown by the Asymptomatic Carotid Surgery Trial (ACST).\textsuperscript{35} Percentage of carotid diameter reduction, procedural methods, risk factors and concomitant therapy did not differ between the two groups (Table 1). In particular, by the time of surgery, all patients were taking 100 mg daily of aspirin. Surgeons and the study coordinator knew the allocated treatments, while the experimental tests on plaques were performed by operators blinded to treatment groups. The study was approved by local ethics review committees. Written informed consent was obtained from all patients before each examination.
**Immunohistochemistry.** After the surgical procedure, samples were immediately frozen in isopentane and cooled in liquid nitrogen. Serial sections were prepared as previously described.\(^1\) Briefly, frozen tissues were sectioned using a cryostat and serial fresh sections were fixed in cold acetone (-20°C) for 10 min. Endogenous peroxidase activity was reduced by preincubation with 0.3% hydrogen peroxide in PBS. Subsequently, the sections were washed three times in PBS-Tween 20 (PBS-T) and then incubated with PBS containing 1% BSA (Sigma Chemical Co, St. Louis, Missouri) to minimize nonspecific binding. Consecutive sections then were incubated with the following antibodies: anti-myeloperoxidase, anti-AGEs, anti-RAGE, anti-p65, anti-COX-2, anti-mPGES-1, anti-type 1 procollagen, anti-oxLDL, anti-MMP-2 and anti-MMP-9 for 60 min. Biotinylated goat polyclonalized IgG (Dako) was used as a secondary antibody (1:100) for 30 min at room temperature. After three washes with PBS-T, we treated individual sections with horseradish peroxidase-labeled streptavidin (Dako) for 30 min, washed the sections three times with PBS-T and determined peroxidase activity with 3,3-diaminobenzidine tetrahydrochloride (Dako). Serial sections, adjacent to those used for reaction with the above described antibodies, were incubated with the primary antibodies anti-CD68, anti-CD3, anti-HLA-DR, to detect inflammatory infiltrate. Omission of primary antibodies and staining with isotype-matched control immunoglobulins served as negative controls. Individual sections were also counterstained with Gill's Hematoxylin (Sigma Chemical Co). In addition, four sections from each plaque were examined for the presence of plaque ulceration (defined as intimal defect larger than 1000 \(\mu\)m in width, exposing the necrotic core of the atheromatous plaque) and intraplaque hemorrhage (defined as bleeding within the plaque of greater than 1500 \(\mu\)m in diameter). Two expert pathologists blinded to the patients' treatment analyzed the specimens. The intra- and inter-observer variability was 5%. 
**Quantitative Histological Analyses.** CD3-positive T cells were counted individually and expressed as the number of cells per mm$^2$ section area as determined by computer-aided planimetry (see below). This approach was not feasible in the case of macrophages because these cells were often present in dense, nearly confluent infiltrates, thus making it difficult to delineate individual cells. Instead, we determined the area occupied by CD68-positive cells planimetrically and calculated the percentage of macrophage-rich areas. The value for ±2 SD of the mean of the differences between 2 blinded observers was 3% for macrophage planimetry and 6 cells per mm$^2$ section area for T cell counts. Analysis of immunohistochemistry was performed with a personal computer-based quantitative 24-bit (16.2 million unique combinations) color image analysis system. Briefly, electronic images were digitized with a Leica CCD DC100 color video camera (Solms, Germany) into a 1 kilopixel x 1 kilopixel image buffer of the AlphaEase 5.02 image analysis system (Alpha Innotech Corp., San Leandro, California, USA). A color threshold mask for immunostaining was defined to detect the red color by sampling, and the same threshold was applied to all specimens. The percentage of the total area with positive color for each section was recorded. The same operator performed all morphometric measurements.

**Western Blot.** Tissue extracts (50 µg protein) were subjected to electrophoresis on 10% SDS-polyacrylamide gels, as described.\textsuperscript{17} Resolved proteins were transferred to 0.45 µm nitrocellulose membranes (Amersham Biosciences) in blotting buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% methanol). The membranes were then blocked with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.3% Tween 20 (TBS-T) containing 10% nonfat dried milk for at least 2 h. The blots then were washed three times with TBS-T and incubated overnight with the primary antibodies in TBS-T containing 10% nonfat dried milk. The following antibodies were used: anti-myeloperoxidase, anti-AGEs, anti-RAGE, anti-p65, anti-COX-2, anti-mPGES-1, anti-MMP-2 and anti-MMP-9. The blots were washed (3 x 20 min), and
then incubated at room temperature for 1 h with a HRP-linked anti-rabbit antibody (Amersham Biosciences) at 1:1000 dilution in TBS-T containing 10% nonfat dried milk. The washes were repeated using TBS-T and the immunoreactive bands were detected using ECL (Amersham Biosciences) according to the manufacturer’s instructions. Co-immunodetection of β-actin was performed to confirm that equal amounts of protein were loaded in individual lanes. We exposed the blots to X-ray film for 1 to 15 min at room temperature and then quantified the immunoreactive bands using computer-assisted densitometry (Alpha Ease 5.02). The results are expressed as arbitrary densitometric units (DU).

**Zymography.** Zymographic analyses on 7.5% acrylamide gel containing 0.1% gelatin on plaque homogenates or plaque-derived macrophages was performed as previously described. This method detects both activated and zymogen forms of MMPs. In fact, in the presence of SDS otherwise inactive forms can lyse the substrate contained in the gel due to detergent-induced conformational change. Briefly, samples for SDS-PAGE were not boiled before the electrophoresis under nonreducing conditions. After electrophoresis, the substrate gels were soaked twice for 30 minutes with Triton-X100 solution (2.5%) at room temperature to remove SDS. Gels were then incubated in 50 mmol/L Tris-HCl, pH 7.4, 0.15 mol/L NaCl, 5 mmol/L CaCl₂, 0.02% NaN₃ for 24 hours at 37°C. Conditioned medium of human fibrosarcoma cell line HT1080 was used as positive control with known gelatinolytic activity. The lysis of the substrates in the gels was visualized by staining with 2.5% Coomasie brilliant blue (Sigma).

**Oil Red O Staining for lipid content.** Two parallel sections from each plaque specimen were incubated in 60% isopropanol for 2 minutes and then in Oil Red O (Carlo Erba, Milan, Italy) solution for 20 minutes and rinsed in water. One of the sections was counterstained with hematoxylin.
**Sirius Red Staining for collagen content.** Frozen tissue sections were rinsed with distilled water and incubated with 0.1% Sirius red (Sigma) in saturated picric acid for 90 minutes, as previously described.\(^{14}\) Sections were rinsed two times with 0.01 N HCl for 1 minute and then immersed in distilled water. After dehydration with 70% ethanol for 30 seconds, the sections were observed under polarized light after coverslipping. The sections were photographed with identical exposure settings for each section.

**EIA determinations.** Plaque content of PGE\(_2\) were measured by using a commercially available EIA kit (Cayman Chemical, Ann Arbor, MI), according to the manufacturer’s instructions.

**Electrophoretic mobility shift assay.** Nuclear extracts from plaque specimens from diet- or statin-treated patients were obtained as described by Ohlsson et al.\(^{18}\) Briefly, plaque samples were homogenized in on ice-cold hypo-buffer (1 M Hepes, 1 M MgCl\(_2\), 1 M KCl, 1 M DTT, 1:1000 protease inhibitors) supplemented with DTT and detergent with a Ultra Turrax homogenizer. The samples were incubated on ice for 30 min and centrifuged at 850g for 15 min. The supernatant (cytosolic fraction) was immediately transferred to a prechilled tube. The pellet was gently resuspended in Hypo-buffer, then incubated on ice for 15 min. 1% NP40 was added to the pellet, that was immediately vortexed for 10 s and then centrifuged for two min. After removal of supernatant, pellet was resuspended in ice cold buffer containing 1 M Hepes, 1 M MgCl\(_2\), 25% glycerol, 1 M NaCl, 0.5 M EDTA, 1 M DTT, 1:1000 protease inhibitors, then vortexed and kept in cold room for 30 min on a roking platfrom. The extract was centrifuged at 14,000g for 15 min at 4 °C, and the supernatant was assayed for protein concentration by using a Bradford assay kit (Bio-Rad Laboratories, Hercules, CA).

Next, in order to study NF-\(\kappa\)B DNA binding activity, chemiluminescent EMSA was performed on a biotin-labeled oligonucleotide containing an NF-\(\kappa\)B DNA-binding consensus sequence, 5’-AGT TGA GGG GAC TTT CCC AGG C-3’ (Panomics, Inc.), and a unlabeled
oligonucleotide, 5'-AGT TGA GGC GAC TTT CCC AGG C-3' (Panomics, Redwood City, CA) using the Lightshift EMSA kit (Pierce), according to the manufacturer's protocols. In addition, we also analyzed the expression of activated p50 subunit by specific Trans-AM™ NF-κB p50 transcription factor assay kit (Active Motif, Rixensart, Belgium).

Assessment of myeloperoxidase activity. Myeloperoxidase activity was measured as described by Sehirli et al. Briefly, atherosclerotic plaques were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0), and centrifuged at 41,400 g (10 min); pellets were suspended in 50 mM PB containing 0.5 % hexadecyl-trimethylammonium bromide (HETAB). After three freeze and thaw cycles with sonication between cycles, the samples were centrifuged at 41,400 g for 10 min. Aliquots (0.3 mL) were added to 2.3 mL of reaction mixture containing 50 mM PB, o-dianisidine, and 20 mM H$_2$O$_2$ solution. One unit of enzyme activity was defined as the amount of the myeloperoxidase that caused a change in absorbance measured at 460 nm for 3 min. Results are expressed as U/g tissue.

Extraction of macrophages from atherosclerotic plaques. Macrophages were selectively extracted from plaques as described by de Vries et al. Briefly, fresh lesions were incubated with an enzyme mixture containing collagenase, elastase, and trypsin inhibitor in Hanks’ buffered HEPES buffer (pH 7.4) containing 0.3% BSA. Lesions were incubated for 60 min at 37°C, and the resulting suspension was filtered by using a nylon sieve (180 µm) to isolate macrophages. The filtrate was centrifuged at 700 g for 10 min at 10°C. the remaining tissue was incubated with fresh enzyme mixture for 1 hour at 37°C. The pellet containing foam cells was loaded onto a discontinuous Nycodenz gradient. Gradients were centrifuged at 1200 g at 10°C. Pure cell populations of macrophages were obtained from the 5% and 10% layer of the density gradient. Cells were allowed to adhere overnight in DMEM containing penicillin/streptomycin, L-glutamine, supplemented with 5% BCS. Flow cytometry analysis of purified cell preparations using a Phycoerythrin-
conjugated anti-CD68 monoclonal antibody (Clone Y1/82A, BD Biosciences Pharmingen) showed that >98% of the selected cells were positive for CD68. Then, immunocytochemistry, Western blot and zymography were carried out as illustrated above. Results are representative of three different experiments.

**Isolation and culture of blood monocytes.** Peripheral blood monocytes were purified and cultured from 5 healthy blood donors as previously described. Control or stimulated (LPS, 1 µg/mL; IL-1β, 10 ng/mL) monocytes (5 x 10^6 cells/4 mL RPMI 1640) were incubated for 48 hours at 37°C in the presence or absence of 10 µM simvastatin (Merck Sharp & Dohme). AGEs (800 µg/mL) were also added to some of the cultures. AGEs were prepared as described by Basta et al. At the end of the incubation, media were collected and the activity of released MMP-2 and MMP-9 was analyzed by zymography; monocytes were scraped, collected, lysed, and enzyme expression evaluated by Western blot. The results are representative of three or more experiments using cells from different donors.

**Statistical Analysis.** For clinical data and histological examination, variables were compared by use of the χ^2 test. The significance of difference in biochemical marker expression and inflammatory cell infiltration between diet-treated and simvastatin-treated patients was analyzed by Student’s t test. The strength of the association between plasma HbA1c and plaque RAGE expression after simvastatin treatment was assessed by linear regression analysis. Data are expressed as percentage or mean±SD. All calculations were performed using the SPSS 12.0 computer program.
Supplemental references


Supplemental figure legends.

**Figure I.** Immunohistochemical analysis (5x) on carotid plaque sections from patients treated for 4 months with diet or simvastatin, performed by using antibodies for CD68 to visualize macrophages, and CD3 to detect T-lymphocytes. Similar regions of the plaque are showed. These results are typical of 35 plaques from diet-treated patients and 35 simvastatin-treated patients.

**Figure II.** Expression of activated NF-κB by specific Trans-AM™ p50 subunit assay kit in nuclear extracts from plaques of statin-or diet-treated patients (A). DNA binding activity of nuclear proteins to an NF-κB specific oligonucleotide evaluated by chemiluminescent EMSA. *Lane 1,* Biotin-EBNA control DNA (negative control); *lane 2,* Biotin-EBNA control DNA + EBNA extract (positive control); *lane 3,* Biotin-EBNA control DNA + EBNA extract + excess unlabeled EBNA DNA (negative control); *lane 4,* Biotin-NF-κB probe alone; *lane 5,* Biotin-NF-κB probe + nuclear extract from patient treated with diet; *lane 6,* Biotin-NF-κB probe + nuclear extract from patient treated with statin (B). Immunohistochemistry (x10) for activated p65 in diet-treated or simvastatin-treated diabetic plaques. Similar regions of the
plaque are showed (C). These results are typical of 35 diet-treated and 35 simvastatin-treated plaques.

**Figure III.** Confocal laser scanning microscopy showing double immunofluorescence on plaque-derived macrophages of myeloperoxidase (*green staining*) and CD68, RAGE, mPGES-1 and MMP-9 (*red staining*). Yellow staining indicates colocalization areas. These results are typical of 35 diet-treated and 35 simvastatin-treated plaques.

**Figure IV.** Representative Western Blot for MMP-2 and MMP-9 on plaque-isolated macrophages in patients treated with diet alone or simvastatin. Western blot for β-actin is also shown as a protein loading control (n=5) (A). Densitometric analysis showing the mean of densitometric units for protein expression of MMP-2 and MMP-9 in each group. Results are normalized to β-actin. *P<0.0001 (B).

**Figure V.** Representative western blot and densitometric analyses of the protein expression of myeloperoxidase, RAGE, COX-2, mPGES-1, MMP-9 in monocytes isolated from peripheral blood of healthy donors (n=5). Cells were left untreated, stimulated with 1 µg/mL LPS for 48 hours, or pre-incubated with 10 µM simvastatin for 2 hours and thereafter stimulated with 1 µg/mL LPS. AGEs (800 µg/mL) were added to some cultures. Monocytes were harvested at 48 hours for Western blot analysis. Results are expressed in densitometric arbitrary units as mean ± SD, and normalized to β-actin. *P<0.0001 LPS vs RPMI 1640 (control monocytes); #P<0.0001 LPS vs LPS plus simvastatin.
Simvastatin Diet

**A**

![Western Blot Analysis](image)

- MMP-2: 72 KDa
- MMP-9: 92 KDa
- β-actin: 42 KDa

**B**

![Bar Graphs](image)

- MMP-2 and MMP-9 densitometric units

*Fig. IV*
**A**

Graph showing the p50 levels (pg/μg nuclear extracts) for Diet and Simvastatin treatments.

**B**

Western blot analysis with lanes labeled 1 to 6. NF-κB bands are visible for each lane.

**C**

Immunohistochemical staining showing DIET and SIMVASTATIN treatments with p65 staining.
Fig. 7