Concurrent Treatment With Renin-Angiotensin System Blockers and Acetylsalicylic Acid Reduces Nuclear Factor κB Activation and C-Reactive Protein Expression in Human Carotid Artery Plaques

Katherine J.E. Sattler, MD; Julie E. Woodrum, MS; Offer Galili, MD; Monica Olson, BS; Saquib Samee, MD; Fredric B. Meyer, MD; Xiang-Yang Zhu, MD; Lilach O. Lerman, MD, PhD; Amir Lerman, MD

Background and Purpose—The local renin-angiotensin system (RAS) and cyclooxygenase-2 contribute to the activation of nuclear factor κB (NFκB) and C-reactive protein (CRP). We hypothesized that the combination of RAS blockers (RASb) and ASA reduces NFκB and CRP within atherosclerotic plaques.

Methods—Patients undergoing carotid endarterectomy were divided into groups according to treatment (RASb–acetyl-salicylic acid [ASA], ASA, RASb, and control). The expression of NFκB, CRP, and CD40L was analyzed through Western blots in the obtained plaques.

Results—Plaques from patients treated with the combination of RASb and ASA showed lower expression of NFκB (25.4 ±9.8 densitometric units [DU]) than those of the control group (57.6 ±13.2 DU, P =0.03) as well as lower expression of CRP (20.9 ±9.6 DU) than those of the other treatment groups (ASA 86.1 ±13 DU, RASb 88.4 ±31 DU, controls 67.8 ±18.6, P=0.004). A negative expression of NFκB was associated with a reduced incidence of symptoms compared with a positive expression (5/33 [15.1%] versus 14/35 [40%], P =0.031).

Conclusions—The combined treatment with RASb and ASA decreases the expression of inflammatory markers in atherosclerosis in humans. This study supports the role of the local RAS and cyclooxygenase-2 in the progression of atherosclerosis. (Stroke. 2005;36:14-20.)

Key Words: aspirin ■ atherosclerosis ■ carotid endarterectomy ■ growth factors ■ inflammation

Atherosclerosis involves the activation of inflammatory cells, cytokines, and transcription factors. An important mediator of the inflammatory processes is the transcription factor nuclear factor κB (NFκB). An increased activity of NFκB was found in atherosclerotic lesions and in patients with unstable angina. Different stimuli can lead to the activation of NFκB, including the tissue renin-angiotensin system (RAS) through the AT1 receptor. Both the p50 and p65 subunits of NFκB participate in C-reactive protein (CRP) transcription. CRP is not only an intermediate step in the inflammatory cascade, but it has itself proatherogenic properties by upregulating expression of AT1 receptor, plasminogen activator inhibitor-1, activation of vascular smooth muscle cells (VSMCs), attenuating nitric oxide production, and induction of different adhesion molecules.

An alternative pathway is the CD40/CD40L system that was found in cells involved in the development of atherosclerosis. Its activation leads to the NFκB-mediated expression of adhesion molecules and matrix metalloproteinases and induces cyclooxygenase-2 (COX-2).

Several drugs are known to modulate the processes of inflammation and possibly the atherosclerotic process. Among them are RAS–inhibiting drugs, such as angiotensin-converting enzyme inhibitors (ACE-I) and angiotensin II receptor type I blockers (ARB), and COX-2 pathway blocking agents like acetylsalicylic acid (ASA).

Although these drugs are associated with a reduction of cardiovascular events, the effect of systemic administration of these drugs on the local inflammatory pathway within the atherosclerotic plaque is not extensively studied. Therefore, we hypothesized that long-term combination of RAS blockers (RASb) and ASA lowers the plaque levels of NFκB and consecutively of CRP, thereby modifying the clinical manifestation of the disease in an advanced stage of carotid artery plaques. The expression of the independent
proatherogenic mediator CD40L was analyzed to exclude modification of marker expression due to general immunosuppressive effect of the drugs of interest.

Materials and Methods

Patients
The study was approved by the Mayo Foundation Institutional Review Board, and procedures followed institutional guidelines. Written informed consent was obtained before surgery.

Specimens were obtained from patients undergoing carotid endarterectomy for symptomatic or progressive asymptomatic carotid artery disease. Long-term intake of RASb or ASA was defined as an intake of at least 4 weeks before surgery. The patients were divided into 4 groups according to treatment with RAS–inhibiting drugs and/or ASA (patients with the combination RASb and ASA [RASb-ASA], patients with ASA alone [ASA], patients with RASb alone [RASb], patients receiving neither RASb nor ASA [control]). To rule out differences of demographic and experimental data between patients receiving ACE-I or ARB, all parameters were analyzed in patients with these drugs before combining them in the groups receiving RASb.

The clinical indication for carotid endarterectomy was met after neurological and neurosurgical examination on the basis of clinical presentation, carotid ultrasound, and MRI.

After collection, plaques were halved at the site of the plaque. One part was fixed in formalin and later embedded in paraffin, whereas the other part was frozen at −80°C until further processing. Clinical data were obtained by patient file review. Patients were considered to be symptomatic with carotid atherosclerosis when they presented with stroke, transitory ischemic attack, or amaurosis fugax ipsilateral to the plaque within 4 weeks before surgery, according to the definition of drug intake. Sample assessment and analysis were performed by observers blinded to patient categories.

Western Blots
Frozen samples were prepared as previously described.29 Monoclonal anti-CRP (Sigma, St Louis, Mo), monoclonal anti-p65 (Santa Cruz Biotechnology Inc, Santa Cruz, Calif), and polyclonal anti-CD40L (Novus Biologicals, Littleton, Colo) were used at a dilution of 1:100. Densitometry of the bands was analyzed using ImageJ (National Institutes of Health).

RNA Extraction and Reverse Transcription
Total RNA was extracted from samples of representative patients using the RNeasy Mini Kit from Qiagen Inc. Reverse transcription was done according to a previously described protocol.30 cDNA was synthesized using Invitrogen SuperScript first-strand synthesis kit.

Polymerase Chain Reaction
To analyze the expression of mRNA of CRP, real-time polymerase chain reaction (DNA engine Opticon, MJ Research) was performed using SYBR Green JumpStart Taq ReadyMix kit (Sigma) following PCR amplification. The expression of NFκB was analyzed with a dedicated kit (Agilent Technologies). Assays were performed using the manufacturer’s protocol and were performed in triplicate. The concentration of NFκB was measured in the samples and compared with the standard curve generated by the amplification of a known concentration of NFκB. The fold change in the expression of NFκB was calculated using the 2^−ΔΔCt method, which normalizes the expression of a target gene to the expression of a reference gene (β-actin) and measures the relative change in expression of the target gene in a control sample.

Immunohistochemistry
After deparaffinizing and hydrating, tissue presence of NFκB was assessed as previously reported.31 For assessing the other markers, slides were steamed with citric acid after deparaffinizing and hydrating. Primary antibody was incubated overnight at 4°C (monoclonal anti-CRP, Sigma, St Louis, Mo, 1:500; polyclonal anti-p65, Zymed, San Francisco, Calif, 1:25; and polyclonal anti-CD40L, Santa Cruz Biotechnology Inc, Santa Cruz, Calif, 1:200). In each group of slides, 1 slide served as a negative control by using mouse or rabbit IgG.

To determine the localization of the marker, serial tissue sections were stained with cell-specific antibodies (CD68 and CD3) as well as with hematoxylin/eosin.

Stained slides were viewed under a microscope (Olympus, Leeds precision Instruments) and pictures were taken with an imaging program (SPOT Advanced 3.3, Diagnostic Instruments Inc).

Statistics
Data are expressed by mean±SE for continuous variables and by frequency count and percentage for qualitative variables. Data were analyzed with Student t test for normally or with the Mann–Whitney rank sum test for nonnormally distributed data for comparison of 2 groups. Multiple groups were compared with ANOVA. Correlation was calculated with Pearson product moment correlation or with Spearman rank order correlation, depending on skewness and distribution of data. The probability of observed numerical variables was determined with Fisher exact test. Positive and negative predictive values were calculated using a contingency table. Univariate regression analysis was performed with covariables (age >70, gender, body mass index (BMI) >25, cerebrovascular event prior 4 weeks to surgery, diabetes mellitus, hypercholesterolemia, hyperlipidemia, current smoking, and hypertension), followed by a multiple linear regression analysis with identified independent covariables and the treatment modalities. Statistical significance was assumed for P<0.05.

Results
No differences were found between patients receiving ACE-I or ARB regarding clinical and demographic data or the experimental results (data not shown).

Patient Demographics
Samples were collected from 68 patients (45 males and 23 females) whose mean age was 72±1 years. Mean ASA dose was 148.8±17.5 mg per day (range 0 to 325 mg per day). MRI before surgery revealed a degree of stenosis at the site of the plaque of ≥70% in most of the patients of each group (RASb-ASA 90%, ASA 88%, RASb 100%, and control group 100% of patients).

The number of patients in each group and further characteristics and demographics of the study population are given in the Table.
Plaques from patients showing a positive NFκB Western blot were significantly more likely to be symptomatic within 1 month before surgery than with a negative NFκB expression (14/35 [40%] versus 5/33 [15.1%], respectively; P=0.031). The NFκB expression tended to be lower in asymptomatic than in symptomatic patients (29±6.3 DU versus 54±12.9 DU, respectively; P=0.075).

Although in the univariate regression analysis a significant association was found between the covariate “event” and the NFκB expression (P=0.04), the multiple linear regression analysis for “event” and the treatment modalities did not reach statistical significance.

Immunhistochemistry revealed positive NFκB expression intracellularly at plaque borders or in intra- and extraplaque microvessels (Figure 3). Mainly foam cells and endothelial cells stained positive for NFκB (Figure 3).

### C-Reactive Protein

Plaques from the patients treated with RASb-ASA showed a significant reduction in the expression of CRP compared with the other groups (15.4±8.4 DU, ASA 86.1±13 DU, RASb 88.4±31 DU, and control 67.8±18.6; ANOVA P=0.004, Figure 4). Local transcription of CRP was proven by detection of mRNA of CRP within the plaques.

A positive CRP-expression in the Western blots was observed less often in plaques of RASb-ASA than in plaques of ASA or controls (4/20 [20%] versus 19/27 [70.3%], respectively; P=0.001; versus controls: 8/14 [57.1%], P=0.036, Figure 2). Moreover, 60% (9/15) of the plaques

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**Figure 1.** NFκB expression [DU] in atherosclerotic plaques in different combinations of RASb and ASA. Top shows representative bands of Western blots. *P<0.05 vs control group.

**Figure 2.** Proportion of plaques with a positive marker expression according to medication. Gray bars for NFκB; white bars, CRP; black bars, CD40L. $\S$P<0.05 vs control group; *P<0.05 vs ASA and vs control group; #P=0.056 vs control group.

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<table>
<thead>
<tr>
<th>Demographic Factors of the 4 Study Groups</th>
<th>RASb-ASA (n=20)</th>
<th>ASA (n=27)</th>
<th>RASb (n=7)</th>
<th>Control (n=14)</th>
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<tbody>
<tr>
<td><strong>Age, y</strong></td>
<td>71±1</td>
<td>71±2</td>
<td>76±2</td>
<td>72±3</td>
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<td><strong>BMI, kg/m²</strong></td>
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<td>28±1</td>
<td>27±1</td>
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<td><strong>Systolic blood pressure, mm Hg</strong></td>
<td>137±15</td>
<td>139±4</td>
<td>144±6</td>
<td>151±7</td>
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<td><strong>Diastolic blood pressure, mm Hg</strong></td>
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<td>76±3</td>
<td>72±4</td>
<td>79±2</td>
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<td><strong>Cholesterol, mmol/L</strong></td>
<td>4.72±0.25</td>
<td>4.82±0.23</td>
<td>5.13±0.61</td>
<td>5.31±0.56</td>
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<td><strong>HDL, mmol/L</strong></td>
<td>1.18±0.07</td>
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<td>1.29±0.23</td>
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<td><strong>LDL, mmol/L</strong></td>
<td>2.65±0.25</td>
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<td>3.01±0.51</td>
<td>3.35±0.51</td>
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<td><strong>Triglycerides, mmol/L</strong></td>
<td>1.86±0.23</td>
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<td>1.78±0.64</td>
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<td><strong>Symptoms: 4 weeks before surgery, no. (%)</strong></td>
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<td>4 (14.8)*</td>
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<td>9 (64.2)</td>
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<td><strong>Transitory ischemic attack, no. (%)</strong></td>
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<td>2 (7.4)</td>
<td>2 (28.5)</td>
<td>4 (28.5)</td>
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<td><strong>Stroke, no. (%)</strong></td>
<td>2 (10)</td>
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<td><strong>Amaurosis fugax, no. (%)</strong></td>
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<td>4 (14.8)</td>
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<td><strong>Hypertension, no. (%)</strong></td>
<td>20 (100)*</td>
<td>22 (81.4)</td>
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<td><strong>Hypercholesterolemia, no. (%)</strong></td>
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<td>7 (50)</td>
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<td><strong>CAD, no. (%)</strong></td>
<td>12 (60)*</td>
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<td>3 (42.8)</td>
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<td><strong>β-blockers</strong></td>
<td>8 (40)</td>
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<td>1 (7.1)</td>
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<td><strong>Statins</strong></td>
<td>12 (60)</td>
<td>13 (48.1)</td>
<td>2 (28.5)</td>
<td>7 (50)</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CAD, coronary artery disease.

*P<0.05 vs control group.
that did not express both NFκB and CRP were from the RASb-ASA group, whereas no patient of this group showed concurrent positive expression of both CRP and NFκB (0/15; P<0.001).

Of the 15 patients with positive expression for both CRP and NFκB, none were taking RASb, 9 of these were taking ASA, and the remainder took neither RASb nor ASA.

For the treatment with RASb-ASA, the calculated positive predictive value for a negative expression of CRP was higher than in the other treatment groups (RASb-ASA 80%, ASA 29.6%, RASb 42.8%, and control 42.8%). The negative predictive values were 64.5%, 39%, 50.8% and 61.3%, respectively.

In the univariate regression analysis, no association was found between any of the covariates and the intraplaque–CRP value. Only RASb-ASA appeared to account for the ability to predict the CRP value (P=0.030).

Immunohistochemistry showed positive staining for CRP predominantly in cell-rich areas, especially in plaque shoulders, microvessels or at borders. Staining for CRP was mainly localized intracellularly in foam cells or in endothelial cells, but in rare cases positive results were also found in spindle-like cells (Figure 3). A spatial colocalization with staining for NFκB could be observed, although NFκB expression was less than the expression of CRP (Figure 3).

**CD40L**

The intraplaque expression of CD40L was chosen as a marker of ongoing inflammation, independent of RAS and COX-2. There were no significant differences among the groups in CD40L expression in the Western blots (Figure 2). Plaques of RASb-ASA showed a mean CD40L value of 62.2±7.4. Similar values were expressed in plaques of the control group (63.3±11.4), of ASA (70.5±8.2), and of RASb (85.6±11.6).

In all slides, fewer cells stained positive for CD40L than for CRP. The staining of CD40L was found intracellularly in
areas that were also positive for CRP, although there were also areas of CRP without evidence of CD40L. The expression could be demonstrated mainly in foam cells and only rarely in endothelial cells and spindle-shaped cells. CD40L–positive cells were localized in plaque shoulders, next to vessels, and less often at plaque borders (Figure 3).

Discussion

The current study demonstrates the expression of the inflammatory markers NFκB and CRP in human carotid artery plaques. Moreover, the expression of these markers was significantly decreased in patients treated with the combination of RAS–inhibiting drugs and ASA. It supports the role of the local angiotensin and COX-2 systems in atherosclerosis and may suggest a mechanism for the protective properties of the combined treatment.

The inflammatory component of atherosclerosis is drawing increasing attention, because it might be a therapeutically modifiable element of the disease. NFκB as well as CRP are both important steps in the inflammatory cascade.

NFκB is a transcription factor that mediates many processes in vascular cells, including inflammatory response and angiogenesis.3 By mediating activation of endothelial cells, the expression of cytokines and proliferation of VSMCs,3 NFκB also has proatherosclerotic effects. The significance of the expression of this transcription factor is underscored by the fact that its activated form was found in atherosclerotic lesions in different vessels.4 Furthermore, patients with unstable angina showed higher levels of NFκB than patients with stable or without angina pectoris.5,6 According, we demonstrated that negative detection of NFκB in the Western blots was associated with fewer symptoms within 4 weeks before surgery than a positive detection.

CRP is activated by the NFκB system in inflamed tissue.31,32 Recent findings of elevated CRP and mRNA expression in diseased coronary artery venous bypass grafts imply that CRP is not only a systemic but also a local marker of inflammation in atherosclerosis.33 The study supports these results, because CRP mRNA was detected within carotid artery plaques, suggesting that CRP is transcribed locally.

CRP has various proatherosclerotic properties. It attenuates the expression of endothelial nitric oxide synthase protein and mRNA13 and mediates the uptake of native low-density lipoprotein by macrophages through CD32 receptor, which subsequently stimulates formation of foam cells.14 Similar to our findings, CRP was found within atherosclerotic plaques, mainly in foam cells.33,35,36 Even in advanced atherosclerotic disease CRP contributes to disease progression, because it maintains VSMC proliferation,10 vascular inflammation37,38 and induces plaque instability.39

The induction of interleukin-6, the main regulator of CRP,40 by angiotensin II through NFκB could be demonstrated in VSMCs.41 The important role of this pathway in the processes of cardiovascular atherosclerotic disease is underscored by the observation that >90% of ACE is localized in tissue and that tissue levels of angiotensin II exceed plasma levels by far.42 Furthermore, in VSMCs the expression of COX-2 was observed after angiotensin II administration,43 which led to an increased IκBα phosphorylation–mediated proliferation and migration.44 Thus, both angiotensin II and COX-2 are involved in upregulation of NFκB and succeeding pathways and induce disease progression and plaque instability.

RAS–inhibiting drugs are successful in suppressing the effects of angiotensin II both in vitro and in vivo. AT1 receptor antagonists decrease the angiotensin II–mediated IκB phosphorylation in VSMCs.45 In a rabbit model of atherosclerosis, hypercholesterolemic animals treated with ACE-I or ARB showed decreased activity of NFκB and other inflammatory markers in the vessel wall.45,46

Similarly, ASA was found to prevent NFκB mobilization in human endothelial cells47 and to inhibit the IκB kinase–complex by binding to IκB kinase-β.48 In an animal model, ASA protected renin and angiotensinogen gene overexpressing rats from cardiac and renal damages associated with the reduced activity of NFκB.49 The relationship to the RAS could further be demonstrated in that treatment with ASA resulted in an inhibited activity of NFκB in endothelial cells stimulated with angiotensin II.50

The results of our study have further implications for treatment with both drugs in atherosclerotic diseases in vivo. We found that patients taking the combination of RASb and ASA had less expression of NFκB and/or CRP within the plaque than patients taking only 1 or none of these drugs. Furthermore, the reduction of NFκB levels in patients taking RASb and ASA or RASb only compared with the results in patients with ASA alone suggests that RASb play the major part in lowering the levels of inflammatory markers in atherosclerotic plaque. As we demonstrated in this study, ASA has the property to interfere with both NFκB activating and CRP producing pathways by blocking the above-mentioned COX-2 effect, resulting in a diminished amount of NFκB and CRP within plaque tissue when added to RASb. To our knowledge, this is the first study to show an effect of ASA on the expression of NFκB in human atherosclerotic plaque tissue.

3-hydroxy-3-methylglutaryl–coenzyme A reductase inhibitors (statins) are known to possess antiinflammatory properties.51 Although the numbers of patients taking statins were not statistically significant different among the groups, a trend for higher statin intake in the RASb-ASA group was observed. We cannot rule out a synergistic effect on the marker expression in this group.

As expected, the combination of RAS–blocking drugs and ASA did not effect the expression of CD40L within the plaque. This result supports our hypothesis that these drugs do not have a general immunosuppressive effect, but interfere at the predicted steps of the inflammatory cascades.

Summary

In this study, we demonstrated the presence of CRP, NFκB, and CD40L expression in atherosclerotic carotid artery plaques. Furthermore, we were able to demonstrate that the combination of RAS–inhibiting drugs and ASA significantly reduces the content of NFκB and CRP, therefore playing an important role in attenuating the inflammatory process within atherosclerotic plaques and inducing a stable plaque morphology. The study further supports the role of the local tissue...
RAS in the inflammatory processes within the atherosclerotic plaque. The decrease in inflammatory activity may be associated with a clinical benefit, because the long-term combination of RASb and ASA significantly reduced the occurrence of symptoms. Thus, it may be speculated that the chronic intake of this combination reduces cellular activity and induces stable plaques morphology that may lead to a beneficial and protective effect on patients with atherosclerotic carotid disease.

Acknowledgments

This work was supported by the National Institutes of Health (R01 HL 63911, K-24 HL 69840-02) and by a grant from Herz-Kreislau lnzentrum Essen, Gesellschaft für Herz-Kreislau lnorschung eV and an unrestricted grant from Merck to Katherine Sattler. Dr Amir Lerman is an Established Investigator of the American Heart Association. We thank Toni Burns, RN, and Rebecca Nelson for obtaining the tissue samples.

References

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Stroke. 2005;36:14-20; originally published online December 2, 2004;
doi: 10.1161/01.STR.0000150643.08420.78

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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