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Comparative Effects of AT1-Antagonism and Angiotensin-Converting Enzyme Inhibition on Markers of Inflammation and Platelet Aggregation in Patients With Coronary Artery Disease

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OBJECTIVES We evaluated whether renin-angiotensin system (RAS) blockade attenuates cardiovascular events.

BACKGROUND Because inflammation and enhanced thrombogenesis are hallmarks of atherosclerosis, we assessed whether RAS inhibition elicits anti-inflammatory and anti-aggregatory effects.

METHODS Interleukin 6 (IL-6), high-sensitivity C-reactive protein (hsCRP), metalloprotease 9 (MMP-9), and interleukin 10 (IL-10) were determined in patients with coronary artery disease (CAD) and arterial hypertension six to eight weeks after coronary angioplasty (low-density lipoprotein serum levels <150 mg/dl). Patients were randomized double-blind to either 20 mg enalapril (ENAL, n = 27) or 300 mg irbesartan (IRB, n = 21) for 3 months. Blood samples were drawn at baseline and at three months. Thromboxane A2-induced platelet aggregation was determined turbidimetrically; urine bicyclo-prostaglandin E2 (PGE_2) and inflammatory markers were measured by enzyme-linked immunosorbent assay technique.

RESULTS Both treatment regimens enhanced serum IL-10 levels (IRB $p < 0.001$, ENAL $p < 0.03$) and reduced serum MMP-9 protein (IRB $p < 0.001$, ENAL $p < 0.05$) and MMP-9 activity (IRB $p < 0.005$, ENAL $p < 0.05$). Only IRB reduced serum IL-6 and hsCRP levels significantly compared with baseline ($p < 0.01$), whereas ENAL did not (hsCRP $p < 0.02$ IRB vs. ENAL, $p < 0.01$ IRB vs. ENAL). Platelet aggregation was only reduced by IRB ($p < 0.001$, ENAL $p < 0.06$, IRB vs. ENAL $p < 0.001$) while urine PGE_2 levels remained unchanged.

CONCLUSIONS Angiotensin-converting enzyme (ACE) inhibition and angiotensin II type 1 receptor (AT1) blockade reduced serum MMP-9 protein/activity to a similar extent, and only AT1 blockade reduced hsCRP, IL-6, and platelet aggregation in patients with CAD. Thus, AT1-blockade appears to exert stronger systemic anti-inflammatory and anti-aggregatory effects compared with ACE inhibition. (J Am Coll Cardiol 2004;44:362–8) © 2004 by the American College of Cardiology Foundation

Inflammation and enhanced thrombogenesis are hallmarks of atherosclerosis (1) and markers of inflammation; for example, interleukin 6 (IL-6) and C-reactive protein (CRP) (2) are elevated in patients with coronary artery disease (CAD). In contrast, cytokines such as interleukin (IL)-10 elicit anti-atherosclerotic effects in vitro and in vivo (3–6). Interleukin-10 has deactivating effects on macrophages, T-cells, and cyclooxygenase-2 (COX-2) and metalloproteases (MMPs) (7–10). Interleukin-10 further decreases serum cholesterol levels (11) and may thereby contribute to atherosclerotic plaque's stabilization by increasing its collagen content and decreasing MMP activity (9–12). Therefore, the balance of pro- (IL-6, CRP) and anti-inflammatory (IL-10) mediators may be important for both the stability of atherosclerotic lesions and the perpetuation of atherosclerotic plaques (3,12,13).

Long-term blockade of the renin-angiotensin system (RAS), as shown in the Heart Outcome and Prevention Evaluation (HOPE) or in the EUROPA study (EURopean trial On reduction of cardiac events with Perindopril in stable coronary Artery disease), significantly reduced cardiovascular events such as unstable angina, myocardial infarction, or stroke in patients with established atherosclerosis (14,15). In this regard, recent observations from our laboratory raised the possibility that the beneficial effects of angiotensin-converting enzyme (ACE) inhibitors may be in part related to an interaction of RAS components with inflammatory cytokines, such as IL-6 (16). Importantly, IL-6 serum levels were shown to be elevated in patients with unstable angina (17) and have been implicated in the prognosis of patients with acute coronary events (2).

Furthermore, the HOPE investigators reported that patients with elevated urine 11-dehydro-thromboxane B2

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Abbreviations and Acronyms

ACE = angiotensin-converting enzyme
ANG II = angiotensin II
AT1 = angiotensin II type 1 receptor
BP = blood pressure
CAD = coronary artery disease
COX = cyclooxygenase
ENAL = enalapril
EUROPA = European Trial on Reduction of Cardiac Events With Perindopril in Stable Coronary Artery Disease
hsCRP = high-sensitivity C-reactive protein
HOPE = Heart Outcome and Prevention Evaluation
IL = interleukin
IRB = irbesartan
MMP = metalloprotease
PG = prostaglandin
RAS = renin-angiotensin system
TXA2 = thromboxane A2

(TXB2) had a higher risk of acute coronary events (18). Because prostaglandins (PGs) may be generated by COX (19,20) and thereby combine inflammation with thrombosis (1), it was suggested that TXA2 (and its urine derivate 11-dehydro-TXB2) may also contribute to the development of acute coronary syndromes in patients with established CAD.

Besides ACE inhibitors, angiotensin II type 1 (AT1) receptor antagonists elicit beneficial effects in various experimental models of atherosclerosis in primates (21) by suppressing plaque formation and surrogate markers of atherogenesis. Moreover, results from our laboratory demonstrated that AT1-blockade by losartan may reduce platelet aggregation in vitro and in vivo via its metabolite EXP3179 (22). Therefore, potential systemic anti-inflammatory, anti-aggregatory, and anti-collagenolytic effects of long-term ACE inhibition with AT1-recceptor blockade were compared in a double-blind randomized study in patients with CAD. Interleukin-10 serum levels were determined as potential anti-atherosclerotic surrogate markers. In order to evaluate the involvement of COX-dependent pathways, urine bicyclo prostaglandin E2 (PGE_2) was determined.

MATERIALS AND METHODS

Study subjects. Sixty patients with established CAD and arterial hypertension were enrolled. Patients were screened while scheduled for elective percutaneous transluminal coronary angioplasty in our department and enrolled six to eight weeks after coronary angioplasty. All patients had angiographically documented CAD without flow-limiting coronary stenosis (<50%). Patients agreed to participate in this double-blind randomized study and were assigned to enalapril or irbesartan according to a randomization list. Medication was blinded by the Department of Pharmacology. None of the patients reported symptoms of angina or heart failure at the time of inclusion into the study. Patients received echocardiography and exercise stress tests before randomization as required safety parameters by the local ethical committee. Patients receiving ACE-inhibitors, AT1-receptor blockers, HMG-CoA reductase inhibition, or nonsteroidal anti-inflammatory drugs (NSAID) were excluded, with the exception of 100 mg of aspirin. Patients with chronic renal failure, low-density lipoprotein serum levels >150 mg/dl, or hypotension (systolic blood pressure [BP] <90 mm Hg) were excluded. Patients were randomized to either 2 × 10 mg enalapril (group A, ENAL) or 2 × 150 mg irbesartan (group B, IRB) per day; the dosage was established based on their BP-lowering potencies. One patient in the enalapril group did not complete the study because of symptomatic hypotension. Eleven patients were excluded from the analysis because of study protocol violations. Of those, eight patients started with NSAID and three patients started with HMG CoA reductase inhibitors during the study. Four patients in the irbesartan group and six patients in the enalapril group had diabetes (Table 1); of those, one patient in each group received oral medication.

Table 1. Characteristics of Study Subjects

	Irbesartan	Enalapril
Subjects	21	27
Male/female	16/5	20/7
Age	56 ± 8	58 ± 13
Body mass index (kg/m^2)	27.5 ± 4	28.5 ± 6
Diabetics	4	6
Baseline blood pressure (mm Hg)		
Systolic	147 ± 35	143 ± 23
Diastolic	88 ± 16	84 ± 16
3-month blood pressure (mm Hg)		
Systolic	133 ± 19†	133 ± 22†
Diastolic	83 ± 9*	80 ± 12*

*$p < 0.05$ vs. baseline diastolic pressure, †$p < 0.01$ vs. baseline systolic blood pressure. Systolic and diastolic blood pressure was not significant between groups.

Blood sampling and laboratory analysis. At baseline and after three months serum and urine samples were collected. Blood samples were drawn in seated position from the antecubital vein and serum/urine samples were stored at −80°C before use. Interleukin-6 (pg/ml), hsCRP, MMP-9 (ng/ml), and MMP-9 activity (ng/ml) were used as markers of inflammation, IL-10 (pg/ml) as a potential antagonist: IL-6, IL-10, and total MMP-9 were measured using an enzyme-linked immunabsorbent assay technique (Quantikine, R and D Systems, Minneapolis, Minnesota). Intra-assay reproducibility from triplicate analysis was below 5% in all samples.

Metalloprotease activity. Metalloprotease-9 activity was analyzed using a fluorometric assay (Fluorokine, R and D Systems) designed to quantitatively measure enzyme activity. Therefore, a monoclonal antibody specific for MMP-9 was coated onto a microplate, standards and samples were added into the wells, and any MMP-9 was bound by the immobilized antibody. After any unbound substances were washed away, an activation reagent (p-aminophenylmercuric acetate) was added to all samples.

After a wash, a fluorogenic substrate linked to a quencher molecule was added and any active enzyme present would cleave the peptide linker between the fluorophore and the quencher molecule. This cleavage eliminates the distance-dependent resonance energy transfer between the fluorophore and the quencher molecule, allowing a fluorescent signal that is proportional to the amount of enzyme activity in the sample. The kit is designed to measure the levels of both endogenous active MMP-9 in the samples and the MMP-9 in those samples that can be activated by APMA during the assay procedure. Each sample was measured in triplicets; data are given as mean ± SEM.

Platelet aggregation. Whole blood was collected in 10% acid-citrate-dextrose at baseline and at 3 months, and platelet-rich plasma was generated as previously described (22). Platelets were stimulated with U46619 (10^{-7} M), a TXA2 analog. Aggregation was determined turbimetrically (aggregometer APACT Inc., Achensburg, Germany). Thrombin (10 U/ml) was used as TXA2-independent stimulus of platelet aggregation. Each experiment was performed in duplicate; results were given as mean ± SEM.

Bicyclo PGE_2 analysis. Bicyclo PGE_2 was assayed by a commercially available enzyme-linked immunosorbent assay kit (Cayman Chemicals, Ann Arbor, Michigan) (23,24). Data were analyzed in triplicets and are given as mean ± SEM.

High-sensitivity CRP. Plasma samples obtained at baseline and after three months were thawed and assayed for CRP using a high-sensitivity assay with a coefficient of variation below 5% (Dade Behring using a BN II nephelometer analyzer, FDA-approved). The limits of detection (0.02 mg/l) and quantification (0.15 mg/l) were reported recently (25).

Statistical analysis. Sample size was calculated for an independent *t* test for comparison of means assuming 50% difference in platelet aggregation, two-tailed of 0.05 for platelet aggregation and for analysis of variance for the remaining parameters. Sixty patients were required to obtain a power of 80% to detect a 30% difference in platelet aggregation as primary end point. The *t* test was performed two-sided; p values <0.05 were considered statistically significant.

RESULTS

Clinical parameters of study subjects are summarized in Tables 1 and 2. Groups did not differ with regard to body mass index, gender, cardiovascular risk factors, or medication. None of the patients reported symptoms of angina or heart failure at the time of inclusion into the study, and therefore the treatment regimens could not test the impact on clinical symptoms. At baseline and after three months of therapy, no differences with regard to serum electrolytes, renal function, or blood cell counts were recorded between the groups. Systolic BP was significantly lower at three months compared with baseline conditions; treatment effects were not different between the groups (Tables 1 and 2). Diastolic BP was significantly reduced to a similar extent in both groups. There were no significant changes in left ventricular function and exercise stress test for both groups before and after three months of therapy. In both groups, serum levels of IL-10 were significantly elevated after three months of therapy as compared with baseline levels (Fig. 1) (IRB vs. ENAL n.s.). In contrast, both treatment regimens resulted in significantly reduced circulating levels of MMP-9 (IRB vs. ENAL n.s.) and MMP-9 activity (Fig. 2) (IRB vs. ENAL n.s.). However, only IRB reduced IL-6 and hsCRP levels significantly compared with baseline ($p < 0.01$) and to ENAL (hsCRP: IRB vs. ENAL $p < 0.02$; IL-6: IRB vs. ENAL $p < 0.01$) (Fig. 3). Angiotensin I type 1-receptor blockade reduces thromboxane A2 (TXA2)-induced platelet aggregation in vitro and in vivo (22). To address whether this latter involves a cyclo-oxygenase dependent mechanism, TXA2-induced platelet aggregation as well as urine bicyclo (PGE_2 was determined. Figure 4 shows that only IRB reduced TXA2-induced platelet aggregation significantly compared with baseline ($p < 0.001$). Moreover, the IRB effect on platelet aggregation was significantly different compared to ENAL ($p < 0.001$). Urine bicyclo PGE_2 was determined because circulating PGE_2 is rapidly degraded to 13,14-dihydro-15-keto PGE_2 (PGE_2-M, $t_{1/2}$ = 3 min), being converted to prostaglandin A products

Table 2. Baseline Laboratory Parameters of All Study Subjects

	Irbesartan	Enalpril
Total cholesterol (mg/dl)	201 ± 15	187 ± 22
LDL (mg/dl)	115 ± 17	124 ± 22
Creatinine (μmol/l)	89 ± 27	83 ± 22
K^+ (mmol/l)	4.4 ± 0.4	4.6 ± 0.3
Na^+ (mmol/l)	142 ± 3	142 ± 4
Hb (g/dl)	14.6 ± 1.5	14.6 ± 1.8
Leucocytes ($10^3/\mu l$)	6.4 ± 2.6	7.4 ± 1.9

n.s. for all parameters depicted.
LDL = low density lipoprotein.

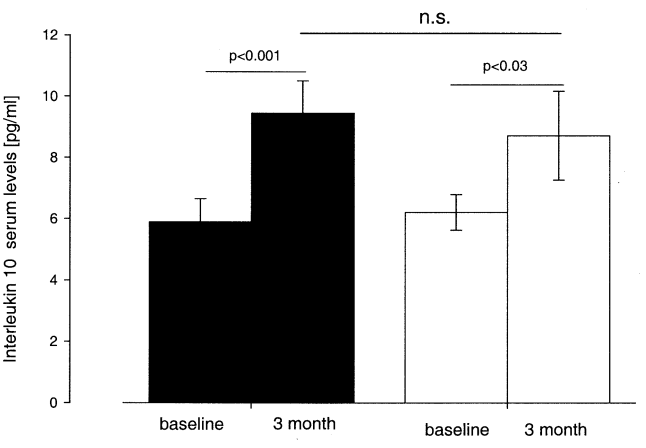

Figure 1. Impact of renin-angiotensin system inhibition on interleukin-10 (IL-10). Patients were treated for 3 months with either 20 mg enalapril (ENAL, **white bars**) or 300 mg of irbesartan (IRB, **black bars**); IL-10 serum concentrations were determined by enzyme-linked immunosorbent assay technique at baseline and at 3 months. Data represent mean ± SEM.

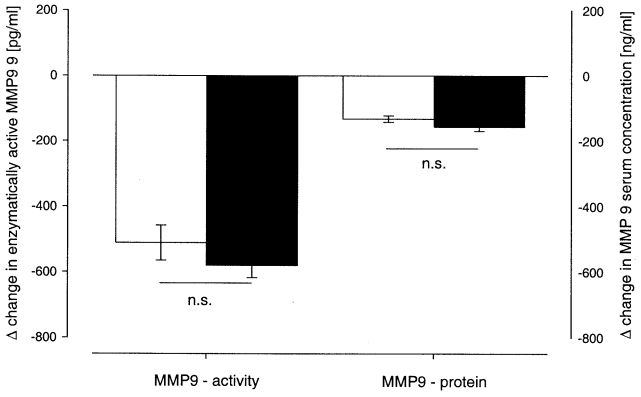

Figure 2. Renin-angiotensin system inhibition and metalloprotease 9 (MMP9). Concentration of serum MMP9 protein was determined by enzyme-linked immunosorbent assay and its serum activity was fluorometrically analyzed by enzyme immunosorbent assay zymography. Patients were treated for 3 months with either 20 mg enalapril **(white bars)** or 300 mg of irbesartan **(black bars)** and blood samples were drawn at baseline and at 3 months. Bar graphs summarize individual Δ-changes in serum MMP9 and MMP9 activity over a three-month observation period. Data are given as mean ± SEM.

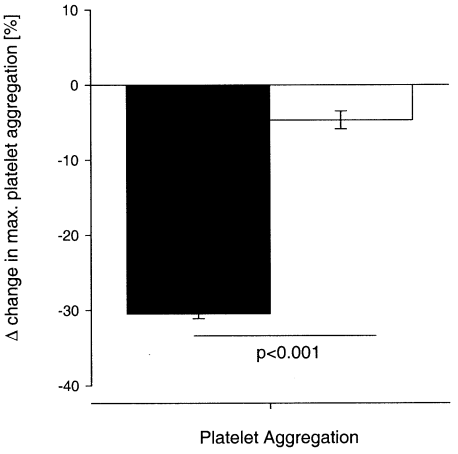

Figure 4. Renin-angiotensin system inhibition and platelet aggregation. Platelet-rich plasma from patients treated with either 20 mg enalapril **(white bars)** or 300 mg irbesartan **(black bars)** was isolated (all received 100 mg acetyl salicylic acid). Platelet aggregation was determined turbimetrically after U46619 stimulation (a synthetic thromboxane A2-analog). Bar graph summarizes individual Δ-changes of platelet aggregation in patients with coronary artery disease and hypertension. Data represent mean ± SEM.

in considerable extent (25,26). Urinary PGE_2 excretion remained unchanged between the groups and as compared to baseline conditions suggesting a cyclo-oxygenase-independent mechanism (Fig. 5).

DISCUSSION

The present study shows that RAS blockade, ACE inhibition, and AT1-receptor blockade enhance serum levels of IL-10 and reduce MMP-9 protein/activity in patients with angiographically documented CAD. However, circulating levels of IL-6, hsCRP and TXA2-induced platelet aggregation were only reduced by AT1 receptor blockade. These findings suggest that the continual activation of an acute-phase reaction in patients with CAD (as determined by elevated hsCRP and IL-6 levels) may be suppressed more effectively by long-term AT1 blockade than by ACE inhibition.

Serum markers of inflammation such as hsCRP and IL-6 are elevated in patients with unstable angina (2,17) and are predictors of recurrent cardiovascular events (2,17,27). In acute and chronic inflammatory processes, IL-6 promotes B-cell differentiation, acute-phase protein synthesis, smooth muscle cell proliferation (28), and the release of metalloproteases involved in extracellular matrix degradation. Interleukin-6 regulates the expression of adhesion molecules and other cytokines such as IL-1 beta and tumor necrosis

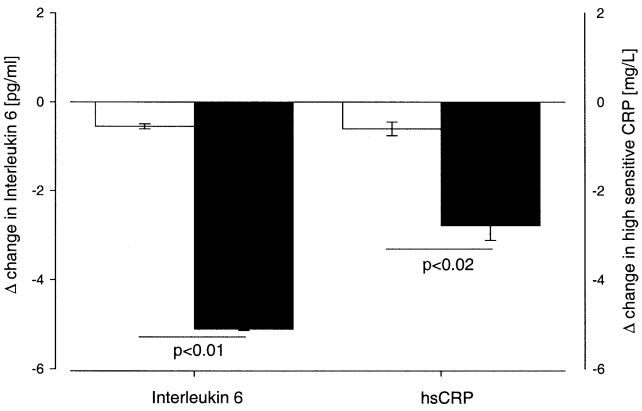

Figure 3. Impact of renin-angiotensin system inhibition on surrogate markers of inflammation. Bar graphs summarize individual Δ-changes in serum high-sensitivity C-reactive protein (hsCRP) and interleukin-6 levels over a three-month observation period in patients with coronary artery disease and hypertension. Patients were treated with either 20 mg of enalapril **(white bars)** or 300 mg Irbesartan **(black bars)** and interleukin-6 serum levels were determined by enzyme-linked immunosorbent assay. Data are given as mean ± SEM.

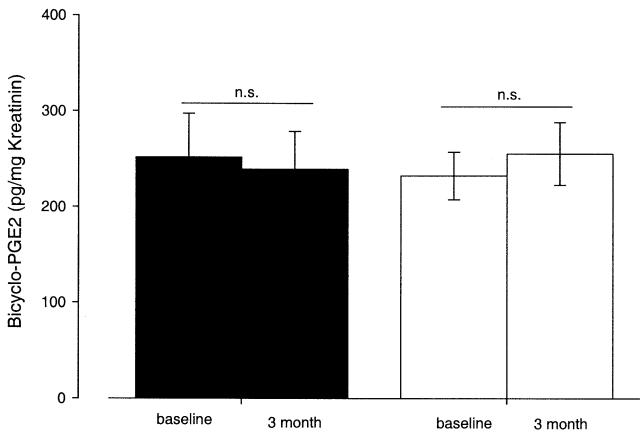

Figure 5. Renin-angiotensin system inhibition and urine bicyclo-prostaglandin E2 (PGE2). High-sensitivity C-reactive protein was determined in urine samples from patients treated with either 20 mg enalapril or 300 mg irbesartan and normalized to serum creatinine levels. All patients received 100 mg acetyl salicylic acid. Prostaglandin E2 was determined by enzyme-linked immunosorbent assay. Both treatment regimens had no significant impact on urine bicyclo-PGE2, which is predominantly secreted by platelets, indicating a cyclo-oxygenase-independent mechanism. **White bars** = enalapril; **black bars** = irbesartan.

factor alpha (28). These effects may amplify the inflammatory reaction at the atherosclerotic lesion and subsequently contribute to the plaques' fibrous cap decomposition (13,29).

Although most of these observations were obtained in vitro, it is possible that circulating serum IL-6 may activate circulating inflammatory cells (e.g., monocytes) to enter the vessel wall, inducing cell differentiation and the presentation of adhesion molecules at the luminal site of the vessel wall. Moreover, circulating IL-6 may easily diffuse into the vessel wall and act locally by inducing monocyte recruitment, activating MMPs, and enhancing the expression of adhesion molecules (28). Thus, reducing circulating levels of IL-6 with long-term AT1-receptor blockade, as reported in the present study, may be a pathophysiologic mechanism involved in atherosclerotic plaque progression and instability and may therefore be of therapeutic interest.

In addition, an activated RAS is associated with a higher incidence of acute coronary syndromes (30). Moreover, ACE is expressed at the shoulder region of atherosclerotic lesions (31) and ACE activity is enhanced in coronary samples of patients with unstable angina (32). Both IL-6 and angiotensin II (ANG II) are co-localized in stable and unstable plaques (16), and ANG II enhances synthesis and release of IL-6 (16,33). In this regard, our present study shows that AT1-receptor blockade reduces plasma levels of IL-6 in patients with CAD. Whether or not this effect reflects the local pathophysiology at the atherosclerotic lesions or represents a more systemic effect needs further investigation.

Although ACE inhibition does not reduce IL-6 and CRP, the effect of ACE inhibition and AT1-blockade on MMP-9 protein and activity was comparable. Thus, while the effects of AT1-blockade may be related to blockade of ANG II and its receptor, the ACE-inhibitor effect may be related to bradykinin, because long-term ACE inhibition enhances circulating bradykinin levels and consequently enhances the release of nitric oxide. There is experimental evidence that nitric oxide abolishes MMP-9 activity and increases tissue inhibitors of metalloproteases (34). Thus, enalapril and irbesartan may reduce MMP-9 activity (despite dissimilar effects on hsCRP or IL-6) by different mechanisms. In the case of AT1-blockade, the more complete blockade of ANG II-AT1-receptor-mediated effects may be the predominant mechanism, whereas ACE inhibition may elicit this effect via bradykinin and nitric oxide. Whether or not the effect of AT1-blockade also involves the stimulation of the ANG II type 2 receptor remains to be determined.

In addition, long-term AT1-receptor blockade diminishes TXA2-induced platelet aggregation in the present study. In patients with CAD, TXA2 may play a critical role based on its vasoconstrictor and mitogenic potencies (35). A major urinary metabolite of TXA2 is elevated in patients with unstable angina (35,36), and the major portion of this metabolite is believed to come from activated platelets. Eikelboom et al. (18) reported that those patients enrolled in the HOPE trial whose urinary 11-dehydro TXB2 levels were in the highest quartile had a higher risk of cardiovascular events compared with those subjects in the lowest quartile. Similarly, we report that AT1-receptor blockade reduces TXA2-induced platelet aggregation but may be cyclo-oxygenase independent because urinary PGE_2 concentrations were unaffected. Although the underlying mechanism needs further investigation, this effect may possibly be mediated either by a direct inhibition of irbesartan at the TXA2-receptor (22) or by active metabolites of irbesartan (37). Similarly, beneficial effects of ACE inhibitors on platelet aggregation have been determined ex vivo by whole-blood aggregometry (not in platelet-rich plasma with specific stimuli, as shown in the present study), but the underlying mechanisms remained unclear (38). Moreover, we have demonstrated that AT1-receptor blockade specifically reduced TXA2-dependent effects in platelets, an effect that has not been reported for ACE inhibitors and that may be beyond the blockade at the AT1-receptor. Nevertheless, the present observations were made in a relatively small population of patients and therefore confirmation in large patient populations is warranted.

Mean values for hsCRP levels were slightly elevated in both groups, an observation that has been reported previously (39). Moreover, the main focus of our study was to compare long-term ACE inhibition with long-term AT1-receptor blockade on markers of inflammation because previous observations in patients with mild to moderate CAD reported not only that pro-inflammatory markers such as VCAM-1 or tumor necrosis factor alpha are increased, but also that irbesartan may reduce these markers of inflammation significantly (40,41). Despite a very similar reduction of BP (Table 1), the decrease of hsCRP and IL-6 was significantly different between the treatment groups.

Moreover, one could speculate that the suppressive effect of AT1-receptor blockade and ACE inhibition may be dose related and may be dependent on the tissue affinity of the ACE inhibitor used (i.e., enalapril vs. ramipril or perindopril). These effects, however, do not involve systemic BP effects, because both drugs (300 mg of irbesartan and 20 mg of enalapril) elicited similar BP-lowering effects in our study. Nevertheless, 300 mg irbesartan may produce additional tissue effects that are ANG II-AT1-receptor dependent and AT1-receptor independent. In this regard, it is of particular interest that atheroclerostic lesions contain ANG II-forming enzymes (with ACE activity), such as chymase and cathepsin G (42), which represent an alternative pathway of ANG II-formation unaffected by ACE inhibitors with various tissue affinity. Therefore, we suggested that inhibitors of the AT1-receptor may elicit a more complete blockade of ANG II-proinflammatory and procoagulatory effects at the AT1-receptor level as compared to ACE inhibitors by blocking ANG II-AT1 receptor-dependent effects and by developing anti-inflammatory pleiotropic effects via anti-inflammatory metabolites.

Finally, it should be noted that it is unclear whether serum composition of pro- or anti-inflammatory markers and MMPs reflect the local vascular inflammatory state at the atherosclerotic lesion. Circulating cytokines such as IL-10 or IL-6, however, may well diffuse easily into the atherosclerotic wall and thereby modulate activation and/or differentiation of circulating or adherent inflammatory cells (e.g., monocytes to active macrophages) or cause T-lymphocytes to enter the atherosclerotic wall. Thus, elevated serum levels of both pro- or anti-inflammatory cytokines may have the potential to modulate local inflammatory processes that subsequently would contribute to plaque destabilization or passivation.

Nevertheless, although convincing experimental and small-scale clinical studies with AT1-antagonists exist, large-scale clinical trials with AT1-antagonists in patients with CAD are still missing and we cannot exclude a late and individual decline in some of the parameters in our patients. Therefore, actual clinical recommendation for patients with CAD should primarily focus on ACE inhibitors for RAS inhibition, based on the results from the HOPE and EUROPA trials.

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