Endothelial Protection, AT<sub>1</sub> Blockade and Cholesterol-Dependent Oxidative Stress

The EPAS Trial

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**Background**—Statins and angiotensin type I (AT<sub>1</sub>) receptor blockers reduce cardiovascular mortality and morbidity. In the Endothelial Protection, AT<sub>1</sub> blockade and Cholesterol-Dependent Oxidative Stress (EPAS) trial, impact of independent or combined statin and AT<sub>1</sub> receptor blocker therapy on endothelial expression of anti-atherosclerotic and proatherosclerotic genes and endothelial function in arteries of patients with coronary artery disease were tested.

**Methods and Results**—Sixty patients with stable coronary artery disease undergoing elective coronary artery bypass grafting (CABG) surgery were randomized 4 weeks before surgery to: (A) control without inhibition of renin-angiotensin system or statin; (B) statin (pravastatin 40 mg/d); (C) AT<sub>1</sub> blockade (irbesartan 150 mg/d); or (D) combination of statin and AT<sub>1</sub> blocker in same dosages. Primary end point was a priori therapy-dependent regulation of an anti-atherosclerotic endothelial expression quotient Q including mRNA expression (in arbitrary units measured by real-time polymerase chain reaction) of endothelial nitric oxide synthase and C-type natriuretic peptide, divided by expression of oxidized low-density lipoprotein receptor LOX-1 and NAD(P)H oxidase subunit gp91phox in left internal mammary arteries biopsies obtained by CABG surgery; 49 patients completed the study. Statin therapy increased lnQ from 3.2±0.4 to 4.4±0.4 significantly versus control. AT<sub>1</sub> blockade showed a trend to increase lnQ to 4.2±0.5. Combination of statin and AT<sub>1</sub> blocker further increased lnQ to 5.1±0.6, but a putative interaction of both therapies in lnQ was not significant. Furthermore, preoperative therapy with statin, AT<sub>1</sub> blocker and their combination improved endothelial function in internal mammary artery rings.

**Conclusions**—Statin and AT<sub>1</sub> blocker therapy independently and in combination improve an anti-atherosclerotic endothelial expression quotient and endothelial function. (Circulation. 2006;114[suppl I]:I-296–I-301.)

**Key Words:** angiotensin ■ cardiopulmonary bypass ■ lipoproteins ■ nitric oxide synthase ■ trials

An activated renin-angiotensin system and hypercholesterolemia are well-established risk factors for coronary artery disease. Approximately 40% of patients with hypertension have hypercholesterolemia. However, hypertension is an important risk factor in patients with increased cholesterol. Pharmacological treatment of both risk factors by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) and angiotensin II type 1 (AT<sub>1</sub>) receptor blockers reduce end-organ damage and cardiovascular mortality and morbidity. However, beyond cholesterol and blood pressure lowering, additional mechanisms underlying these protective effects of both therapies including increased nitric oxide (NO) availability, reduced oxidative stress, and decreased oxidized low-density lipoprotein (ox-LDL) uptake are discussed. C-type natriuretic peptide (CNP) is expressed in the vasculature and is found in particularly high concentrations in the endothelium. Treatment with oxLDL suppressed endothelial secretion of CNP. Furthermore, abnormalities in endothelial NO synthase (eNOS) and CNP as vasodilating and growth-inhibiting factors have been demonstrated in various cardiovascular diseases including atherosclerosis, congestive heart failure, hypertension, and hypercholesterolemia.

Angiotensin II (Ang II) causes vasoconstriction by activation of the predominant AT<sub>1</sub> receptor in vascular smooth muscle cells. Furthermore, Ang II increases vascular NAD(P)H oxidase expression and superoxide anion formation. Superoxide anions can reduce vascular NO availability...
by peroxynitrite formation leading to endothelial dysfunction.\textsuperscript{11} Ang II induces LOX-1, the human endothelial receptor for oxLDL, suggesting a link between hypertension and hypercholesterolemia in the pathogenesis of atherosclerosis.\textsuperscript{12,13} Several lines of evidence support a synergistic potentiation of endothelial dysfunction and cardiovascular risk by augmented Ang II and low-density lipoprotein (LDL) levels. LDL and ox-LDL upregulate AT\textsubscript{1} receptor gene expression in vascular cells,\textsuperscript{14,15} thus promoting atherosclerosis.\textsuperscript{16} Circulating LDL can be oxidized to ox-LDL, mainly after uptake in the vessel wall, and increases AT\textsubscript{1}, gp91phox and LOX-1 expression.\textsuperscript{10,14,15,17,18} Furthermore, ox-LDL itself can induce expression of NAD(P)H oxidase subunit gp91phox and superoxide anion formation.\textsuperscript{17} Therefore, pharmacological therapy by statins and AT\textsubscript{1} receptor blockers have the potential to reduce oxidative stress and endothelial dysfunction.\textsuperscript{17,19}

AT\textsubscript{1} receptor blockers and statins are well-established therapies in the treatment of hypertension, hypercholesterolemia, and coronary artery disease. Growing evidence supports synergistic beneficial effects of both medications.\textsuperscript{20} However, whether both therapies independently or in combination affect the vascular gene expression and the endothelial function in controlled clinical trials is currently unknown. Therefore, we tested in a clinical trial in PROBE (Prospective Randomized Open Label and Blinded Evaluation) design whether statin and AT\textsubscript{1} receptor blocker therapies independently or in combination influence endothelial expression of anti-atherosclerotic and proatherosclerotic genes and endothelial function in arteries of patients with coronary artery disease. To summarize changes in vascular gene expression, an anti-atherosclerotic endothelial expression quotient Q including mRNA expression of vasoprotective genes eNOS and CNP, divided by expression of proatherosclerotic genes LOX-1 and gp91phox, was a priori approved as primary end point.

**Materials and Methods**

**Study Population and Design**

The Endothelial Protection, AT\textsubscript{1} blockade and Cholesterol-Dependent Oxidative Stress (EPAS) trial was a clinical trial in PROBE (Prospective Randomized Open Label and Blinded Evaluation) design. Sixty patients with stable coronary artery disease undergoing elective coronary artery bypass grafting (CABG) surgery were randomized 4 weeks before surgery to: (A) control without inhibition of renin-angiotensin system or statin; (B) statin (pravastatin 40 mg/d) without inhibition of renin-angiotensin system; (C) AT\textsubscript{1} blockade (irbesartan 150 mg/d) without statin; or (D) combination of statin and AT\textsubscript{1} blocker therapy in same dosages (Figure 1). Groups did not differ in clinical characteristics (Table 1). During the study period of 4 weeks, none of the patients received angiotensin-converting enzyme inhibitor therapy. All other antihypertensive pharmacological standard therapy did not differ between study groups. After 4 weeks, patients underwent elective CABG surgery and distal remnant specimens of the left internal mammary artery obtained after informed consent from 49 patients were analyzed. Primary end point was therapy-dependent regulation of an anti-atherosclerotic endothelial expression quotient Q including mRNA expression (in arbitrary units measured by real-time polymerase chain reaction [PCR]) of eNOS and CNP, divided by expression of LOX-1 and gp91phox in internal mammary arteries biopsies. In a subset of patients, endothelial function was determined in internal mammary artery rings by organ chamber experiments. The trial was approved by the local ethics committee. The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

**RNA Isolation and Reverse Transcription**

Reagents and chemicals were purchased from Sigma Chemical Co except when otherwise specified. Total RNA from biopsies of internal mammary arteries was isolated by guanidinium thiocyanate/cesium chloride centrifugation as previously described;\textsuperscript{22} 250 ng of total RNA was reverse-transcribed using SuperScript\textsuperscript{TM} II Reverse Transcriptase (Invitrogen).

**Real-Time PCR**

Primers were designed using Primer3 program according to parameters outlined in the BioRad iCycler Manual. Specificity of primers was confirmed by BLAST analysis. Gene specific PCR primers are summarized in Table 2. Eukaryotic elongation factor 2 (eEF2) was used as a reference for normalization of cDNA content of the samples. For quantification, each gene-specific PCR fragment was amplified using standard PCR techniques. The amplified PCR fragments were isolated using JETQUICK Gel Extraction Spin Kit (Genomed) and quantified by photometric measuring OD 260. The quality of the isolated gene-specific PCR fragment was analyzed by electrophoresis. The gene-specific PCR fragments were serially diluted, aliquoted, and used as external standards for real-time PCR. Real-time PCR was performed using a BioRad iQ iCycler Detection System (BioRad Laboratories, Ltd) with SYBR green fluorophore. Reactions were performed in a total volume of 20 μL including 10 μL 2 X QuantiTect Sybr Green PCR-Kit (Qiagen), 6 μmol/L of each primer and 2 μL of the previously reverse-transcribed cDNA template. Gene-specific expression was analyzed using iCycler IQ Optical System Software Version 3.0a (BioRad Laboratories, Ltd). A melt curve analysis was performed after every run to ensure a single amplified product for every reaction. All reactions were performed in at least duplicate for every patient sample. The gene-specific standard dilution series was repeated on every experimental plate in triplicate. Duplicate negative controls (no cDNA template, no
Organ Chamber Experiments

Organ chamber experiments were performed using phenylephrine-precontracted left internal mammary artery rings. The phenylephrine concentration (10^{-9} to 10^{-7} mol/L) was adjusted to obtain identical preconstriction levels. Relaxations to acetylcholine (10^{-9} to 10^{-7} mol/L) or sodium nitroprusside (SNP) (10^{-10} to 10^{-6} mol/L) were recorded. The EC_{50} was defined as concentration necessary to achieve a 50% left internal mammary artery ring relaxation. All measurements were performed by a technician blinded to patient group assignment.

Statistics

Data are shown as mean±SD, as indicated. The 4 treatment groups were compared with regard to the primary end point Q in a 2-factor (pravastatin, irbesartan) ANOVA model with interaction. Because the original Q values deviated from normality,

<table>
<thead>
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<th>TABLE 1. Clinical and Study Characteristics of Patients</th>
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<td>Sex, no. male (%)</td>
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<td>NYHA</td>
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<td>Diastolic BP after 4 weeks, mm Hg</td>
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AP indicates angina pectoris class; bpm, beat per minute; BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NYHA, New York Heart Association functional class.

Data are expressed as mean±SD. *P<0.05 vs initial value at randomization.

<table>
<thead>
<tr>
<th>TABLE 2. Characteristics of Quantitative mRNA Analyses</th>
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<td>Gene</td>
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<td>NOS3</td>
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<td>(eNOS)</td>
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<td>NPPC</td>
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<td>(CNP)</td>
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<td>OLR1</td>
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<td>(LOX1)</td>
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<td>CYBB</td>
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<td>(gp91phox)</td>
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CNP indicates C-type natriuretic peptide; EF2, eukaryotic elongation factor 2; eNOS, endothelial nitric oxide synthase; gp91phox, 91-kD glycosylated subunit of NAD(P)H oxidase; LOX-1, lectin-like oxidized LDL receptor-1.
we report results for lnQ; however, results were similar for the original scale. A value of \( P<0.05 \) was considered statistically significant. Statistical analyses were performed with SAS (Cary, NC) Version 9.1.

**Results**

**Baseline Characteristics**

When comparing the baseline values of the 4 treatment arms, no significant differences were noted in any of the measured parameters including age, height, weight, sex, smoker, New York Heart Association functional class, angina pectoris class, heart rate, systolic and diastolic blood pressure, total cholesterol, triglycerides, high-density lipoprotein cholesterol, and LDL cholesterol (Table 1). Furthermore, treatment groups did not differ in concomitant therapy with calcium antagonists, \( \beta \)-receptor antagonists, diuretics, aspirin, or nitrates.

**Effects of Therapies on Lipids and Blood Pressure**

After 4 weeks of treatment, 49 patients completed the study (11 to 14 patients per group). In the control group (group A), no significant changes in lipids and blood pressure were observed (Table 1). Pravastatin alone (group B) reduced significantly total cholesterol and LDL cholesterol during the 4 weeks of treatment (Table 1). As expected, irbesartan (group C) had no direct effect on lipid parameters. In group D with combination therapy, reduction of total cholesterol and LDL cholesterol (\( P=0.07 \)) did not reach statistical significance. However, total cholesterol was significantly lower in group D, compared with control group A and irbesartan group C after 4 weeks of treatment. Furthermore, reduction of LDL cholesterol by combination therapy (group D) reached statistical significance versus irbesartan alone (group C). No statistically significant difference of systolic and diastolic blood pressure was found among study groups after 4 weeks of \( \mathrm{AT}_1 \) receptor therapy or antihypertensive therapy without inhibition of the renin-angiotensin system.

**Effects of Therapies on Anti-Atherosclerotic Endothelial Expression Quotient**

Primary end point of the EPAS trial was the therapy-dependent regulation of an anti-atherosclerotic endothelial expression quotient \( Q \) including mRNA expression (in arbitrary units measured by real-time PCR) of eNOS and CNP, divided by expression of oxLDL receptor LOX-1 and NAD(P)H oxidase subunit gp91phox in left internal mammary arteries biopsies obtained by CABG surgery (\( P<0.05 \) vs A). Dots represent individual patients, and horizontal bars indicate the mean values with standard error. A, Control. B, Statin (pravastatin 40 mg/d). C, \( \mathrm{AT}_1 \), blocker (irbesartan 150 mg/d). D, Combination of statin and \( \mathrm{AT}_1 \), blocker therapy in same dosages.

**Effects of Therapies on Endothelial Function**

In a subset of study patients, because of availability of internal mammary artery tissue, the impact of preoperative therapy with statin, \( \mathrm{AT}_1 \), receptor blocker and their combination on endothelial function was determined (Figure 4A). In ex vivo organ chamber experiments immediately after CABG surgery, pravastatin (B), irbesartan (C), and their combination significantly improved endothelial function in rings of internal mammary arteries. Maximal dilatation in response to \( 10^{-6} \)
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Figure 4. Statin and AT1 blocker therapy improves endothelial function in patients with coronary artery disease. In a subgroup of patients, the impact of preoperative therapy with statin, AT1 blocker and their combination was analyzed on endothelial function in rings of internal mammary arteries. Dilatation (in % relaxation) was analyzed in preconstricted rings in response to increasing dosages of acetylcholine (A). Afterwards, endothelium-independent relaxation was determined in response to NO donor SNP (B). *P<0.05 vs control. **P<0.05 vs AT1 blocker + statin.

M acetylcholine was: (A) 51.5±3.2%; (B) 68.8±6.0%*; (C) 78.6±11.4%*; and (D) 86.1±7.7%* (*P<0.05 versus A).

Furthermore, combination therapy (D) significantly improved endothelial function versus statin (B), but not versus AT1 receptor blocker (C) (Figure 4A). The observed functional changes were mediated by the endothelium, because vasodilatory reaction of the left internal mammary artery in response to the endothelium-independent vasodilator sodium nitroprusside did not change after 4 weeks (Figure 4B).

Discussion

Already after 4 weeks of preoperative treatment with pravastatin, total cholesterol and LDL cholesterol were significantly reduced. In the combination therapy of pravastatin with irbesartan, these lipid parameters did not reach statistical significance, even while the absolute value were significantly lower than in the control and irbesartan group. This could be because of the slightly, but not significantly, lower baseline values of both lipid parameters in group D. Therefore, the observed improvement of the anti-atherosclerotic endothelial expression quotient Q could be partially mediated by the lipid-lowering effect in these patients. The lower LDL in group D resulting in atheroprotective effects like lower probability of vascular oxLDL formation may have contributed to the lack of effect of the combined treatment group relative to single treatment groups.

NAD(P)H oxidase-derived superoxide rapidly reacts with eNOS-derived NO, resulting in peroxynitrite formation. Because NO is known to mediate anti-atherosclerotic properties of the endothelium, eg, by inhibition of platelet aggregation, adhesion molecule expression, and vascular smooth muscle cell proliferation, the inactivation of NO by superoxide anions can contribute to the initiation of atherosclerosis. In this context, statins could mediate beneficial effects by direct lipid-lowering and pleiotropic effects. Pretreatment of human endothelial cells with statins reduced both oxLDL-induced upregulation of LOX-1 and downregulation of eNOS. The functional effects of pravastatin and irbesartan in ring studies are most probably relate to eNOS activity because eNOS expression was not changed.

Recent studies demonstrate the role of CNP as a novel endothelium-derived hyperpolarizing factor complementing other endothelial vasorelaxant mediators like NO and prostanoyclin. Therefore, oxLDL might further impair endothelial function by impaired CNP-mediated vasodilatation as well. Recently, evidence was presented that CNP reduces leukocyte recruitment by attenuation of endothelial P-selectin expression suggesting that endothelial CNP might maintain an anti-atherogenic influence on the blood vessel wall and represent a target for therapeutic intervention in inflammatory cardiovascular disorders. CNP might also have therapeutic benefits as an antagonist of graft vasospasm after coronary artery bypass surgery.

The partially improved anti-atherosclerotic endothelial expression quotient Q by AT1 receptor therapy was independent of significant changes in blood pressure. The lack of significant blood pressure decline with irbesartan is most probably because of the additional antihypertensive therapy. Several experimental findings support a protective role of AT1 receptor blockade beyond blood pressure. Potential molecular mechanisms involve the AT1 receptor-mediated increase of vascular NAD(P)H oxidase and LOX-1 by Ang II. Because of the limited amount of available vascular tissue, no additional measurement on the protein level could be performed. However, for all genes studied, in vitro and in vivo data support a strong correlation of mRNA and protein expression. Using immunohistochemistry eNOS is primarily expressed in endothelial cells of the vessel wall in untreated patients, whereas eNOS expression seems to increase during angiotensin-converting enzyme inhibitor treatment. Whether AT1 receptor blockade has a similar effect on eNOS expression in this study could not be analyzed because of limited tissue availability.

Experimental studies support an interaction of oxLDL and Ang II. OxLDL potentiates Ang II-mediated vasoconstriction. Treatment with AT1 receptor blocker attenuated aortic intimal proliferation and markedly decreased the enhanced LOX-1 expression in the aorta of hypercholesterolemic animals. In this clinical study, no significant synergy between both therapies could be shown. Furthermore, even though the internal mammary artery rarely develops atherosclerosis, it is used in several studies as a model for atherosclerosis-prone arteries. Therefore, studies including a larger number of patients and additional vessel types would be helpful to further address this question.

In conclusion, in the EPAS trial, statin and AT1 blocker therapy independently and in combination improved endothelial expression quotient of anti-atherosclerotic and proatherosclerotic genes and endothelial function, but a potentiation by interaction of both therapies was not observed. Our data support beneficial effects of both therapies in the treatment of coronary artery disease.
Sources of Funding

This study was supported by Bristol-Myers Squibb and the German Federal Ministry of Education and Research (BMBF) program NBL3 of the University of Technology Dresden (Dr Morawietz, Professorship of Vascular Endothelium and Microcirculation).

Disclosures

None.

References

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_Circulation_. 2006;114:I-296-I-301
doi: 10.1161/CIRCULATIONAHA.105.001313
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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