ACE Inhibitor and AT₁ Antagonist Blockade of Deformation-Induced Gene Expression in the Rabbit Jugular Vein Through B₂ Receptor Activation

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Abstract—Deformation-induced endothelin-1 synthesis in endothelial cells may contribute to the intimal hyperplasia of venous bypass grafts. ACE inhibitors and angiotensin II type 1 (AT₁) receptor antagonists are capable of reducing vein graft disease. Therefore, the effects of these drugs on endothelial preproendothelin-1 (ppET-1) and smooth muscle endothelin B receptor (ET₅-R) expression were investigated in isolated perfused segments of the rabbit jugular vein. Pretreatment with ramiprilat (0.3 μmol/L) or irbesartan (0.01 to 1 μmol/L) had no effect on basal ppET-1 or ET₅-R expression but markedly attenuated the deformation-induced expression of these gene products, and these effects were reversed by the B₂ receptor antagonist icatibant (Hoe 140) and by the NO synthase inhibitor N⁵-nitro-L-arginine. Candesartan (1 μmol/L) mimicked the inhibitory effect of irbesartan. Moreover, reporter gene analysis with a rat ppET-1 promoter-luciferase construct transiently transfected into porcine aortic cultured endothelial cells revealed that the inhibitory effect of both ramiprilat and irbesartan on deformation-induced ppET-1 expression is species independent and mediated at the level of transcription. In addition, RT-PCR analysis detected only AT₁ receptor expression in the endothelium-intact rabbit jugular vein, and neither irbesartan nor ramiprilat affected endothelial NO synthase expression. Thus, ACE inhibitors and AT₁ receptor antagonists are capable of suppressing deformation-induced gene expression in the vessel wall in both an autocrine (ppET-1) and a paracrine (ET₅-R) manner via a common mechanism of action that constitutes a B₂ receptor–mediated increase in endothelial NO release. (Arterioscler Thromb Vasc Biol. 2001;21:61-66.)

Key Words: blood pressure ■ endothelin-1 ■ gene expression ■ graft failure ■ nitric oxide

Excessive mechanical strain after exposure to arterial levels of blood pressure could trigger the vasculopathy of venous bypass grafts and lead to graft failure. Moreover, pressure overload of the vessel wall seems to be involved in both hypertension-induced arterial remodeling and restenosis after PTCA. Among the various mediators implicated in these putative deformation-induced changes in smooth muscle cell (SMC) phenotype, endothelin-1 (ET-1), a potent comitogen for vascular SMCs, may play a pivotal role. Thus, rhythmic deformation has been shown to enhance the synthesis of ET-1 in cultured endothelial cells (ECs), and this effect is presumed to occur at the level of transcription of the preproendothelin-1 (ppET-1) gene. To investigate whether a pressure-induced decrease in ET-1 synthesis was accompanied by a marked increase in endothelin B receptor (ET₅-R) expression in the vascular SMCs and subsequent changes in their phenotype ranging from apoptosis in the carotid artery to proliferation in the jugular vein (see also Porter et al regarding the human saphenous vein).

Both ACE inhibitors and angiotensin II type 1 (AT₁) receptor antagonists not only are first-line antihypertensive drugs but also possess a substantial cardiac and vascular protective potential, as highlighted by several recent clinical and experimental studies. Moreover, ACE inhibitors, but not AT₁ receptor antagonists, have been shown to attenuate the release of ET-1 from cultured ECs, presumably through a kinin-dependent mechanism. We have now investigated whether an ACE inhibitor and/or an AT₁ receptor antagonist interferes with the pressure-induced increase in endothelial ET-1 synthesis and smooth muscle ET₅-R abundance in the rabbit jugular vein and, if so, which signaling mechanism may be involved therein.

Methods

In Situ Model

Segments of the external jugular vein of male New Zealand White rabbits were prepared and perfused with Tyrode’s solution as
described previously. In experiments with drug treatment, these were added to the luminal perfusate during the equilibration phase at 0 mm Hg (usually 30 minutes) before the perfusion pressure was raised to 20 mm Hg for 6 hours. At the end of the perfusion period, the segments were snap frozen in liquid N2 and stored at −80°C.

**Superfusion Bioassay**

Four venous ring segments (3 to 4 mm long) were tested simultaneously by mounting them between force transducers and a rigid support for measurement of isometric force (TSE). The rings were superfused at 1 mL/min with warmed (37°C), oxygenated (PO2 = 300 mm Hg) Krebs-Henseleit solution, pH 7.4. Passive tension was adjusted during a 30-minute equilibration period to 0.5 g. Thereafter, increasing doses of bradykinin (Bachem) or sarafotoxin 6c (Alexis) were applied as bolus injections in 30-minute intervals, and the ensuing constrictor response was monitored with the aid of a digital PC-operated analysis system (Biosys; TSE).

**Cell Culture**

ECs were isolated from porcine aortae through treatment with 1 U/mL dispase in HEPES-modified Tyrode’s solution for 7 minutes at 37°C and cultured on gelatin-coated 60-mm culture dishes (2 mg/mL gelatin in 0.1 mol/L HCl for 30 minutes at ambient temperature) in DMEM/Ham’s F-12 (1:1 v/v) containing 10 U/mL nystatin, 50 U/mL penicillin, 50 μg/mL streptomycin, 5 mmol/L HEPES, 5 mmol/L TES, and 20% FBS. They were passaged once with the use of 0.5% trypsin/0.2% EDTA (wt/vol) and seeded onto BioFlex collagen type I 6-well plates (Flexcell) that had also been coated with gelatin. They were identified according to their typical cobblestone morphology, positive immunostaining for von Willebrand’s factor, and negative immunostaining for smooth muscle α-actin (all antibodies were from Sigma-Aldrich).

**RT-PCR Analysis**

Isolation of total RNA, cDNA synthesis, and semiquantitative PCR analysis with elongation factor-1 (EF-1) as internal standard were performed as described previously. PCR conditions for the amplification of endothelial NO synthase (eNOS) cDNA were 35 cycles of 30-second denaturation at 95°C, 30-second annealing at 60°C, and 25-second primer extension at 72°C, with product size of 311 bp. The forward primer was 5′-ATCTTACCCGAGCGAGG-3′, and the reverse primer was 5′-GTCTCTGGATAGCTTCG-3′ (corresponding to nucleotide sequences 3227 to 3244 and 3537 to 3519 of the bovine eNOS gene, GenBank accession No. M93674). PCR conditions for the amplification of AT1 receptor cDNA were 40 cycles, annealing temperature of 58°C, and product size of 547 bp. The forward primer was 5′-TGAAGATCTCTGAGCCAGC-3′, and the reverse primer was 5′-GCCCTTACACAATATCGTC-3′ (corresponding to nucleotide sequences 581 to 601 and 1127 to 1108 of the human AT1 receptor gene, GenBank accession No. M87290). PCR conditions for the amplification of AT2 receptor cDNA were 40 cycles, annealing temperature of 60°C, and product size of 527 bp. The forward primer was 5′-TGATTCCCGATTTAAGTGC-3′, and the reverse primer was 5′-ACACTGAGCATATTCTCCTGG-3′ (corresponding to nucleotide sequences 226 to 247 and 771 to 749 of the human AT2 receptor gene, GenBank accession No. D16840).

**Intravascular ET-1 Concentration**

ET-1 was extracted from the weighted segments as described, and the concentration was determined by using a commercially available ELISA kit.

**Reporter Gene Analysis**

The pCMV TK luc+ expression vectors (GenBank accession No. AF027129) containing a −1329-bp rat ppET-1 promoter instead of the cytomegalovirus (CMV) promoter was provided by Prof Martin Paul (Institute of Clinical Pharmacology and Toxicology, Free University of Berlin, Germany). Cotransfections for normalization of transfection efficacy were performed with a pUC19 (GenBank accession No. M77789)–based SV40/β-galactosidase expression vector.

**Results**

**Pressure-Induced Activation of the Endothelin System**

Pressurization of the venous segments to 20 mm Hg resulted in a maximum distention with an ~3-fold increase in diameter that did not affect the mRNA level of the housekeeping reference gene EF-1. Moreover, there was no loss of ECs from the endothelium-intact segments during the 6-hour perfusion period (see Lauth et al8 and Figure 4). Raising the perfusion pressure from 0 to 20 mm Hg resulted in an average 5- to 6-fold increase in ppET-1 mRNA abundance in the rabbit jugular vein (Figures 1, 2, and 3B) that was confined to the endothelium (see Lauth et al). In good agreement with the mRNA level, the intravascular concentration of ET-1 in the endothelium-intact venous segments was elevated by a factor of 7 after a 6-hour perfusion at 20 mm Hg (Figure 2B). Moreover, there was a 4- to 5-fold increase in ETβ-R mRNA abundance (Figures 1 and 3) that, as demonstrated previously, was independent of the presence of an intact endothelium. In contrast, neither ECE-1 nor ETA-R expression appeared to be pressure sensitive (Figure 1A). In contrast to the AT1 receptor (the expression of which was pressure sensitive), no mRNA expression of the AT2 receptor could be detected by RT-PCR analysis in the endothelium-intact rabbit jugular vein, even after a 6-hour exposure to the elevated perfusion pressure (not shown).

**Effects of Ramiprilat and Irbesartan on Pressure-Induced Gene Expression**

Ramiprilat was used at a concentration (0.3 μmol/L) that was previously determined to inhibit the ACE-dependent constric-
tor response to angiotensin I in the rabbit jugular vein by >80%. Pretreatment of the venous segments with ramipril abolished both the pressure-induced increase in ppET-1 mRNA and intravascular ET-1 peptide content (n=3 in endothelium-intact segments of the rabbit jugular vein and blockade of these effects by coincubation with Hoe 140 (0.1 μmol/L, n=3 to 4). *P<0.05 vs 0 mm Hg. †P<0.05 vs control 20 mm Hg. ‡P<0.05 vs 20 mm Hg plus ramipril or irbesartan. nd indicates not determined.

Irbesartan at the maximum concentration used in the present study (1 μmol/L) completely blocked the AT1 receptor-mediated constrictor response to angiotensin II (0.1 pmol to 1.0 nmol corresponding to concentrations ranging from 0.1 nmol/L to 1.0 μmol/L) in isolated ring segments of the rabbit jugular vein (not shown). Pretreatment of the venous segments with irbesartan produced inhibitory effects on deformation-induced but not basal gene expression similar to those of ramiprilat (Figures 2 and 3), which, however, tended to be less effectively reversed with Hoe 140 (Figure 2).

The inhibitory effects of irbesartan on pressure-dependent ppET-1 and ETB-R expression were concentration dependent (Figure 3B) with a maximum between 0.1 and 1.0 μmol/L. Another AT1 receptor antagonist, candesartan, mimicked the inhibitory effect of irbesartan on both ppET-1 and ETB-R expression in response to the rise in perfusion pressure but appeared to be less potent by ~1 magnitude (Figure 3B).

As a potential mechanism of action underlying their effects on deformation-induced gene expression in the rabbit jugular vein, ramiprilat or irbesartan may upregulate NO synthase (NOS)
Effects of Ramiprilat and Irbesartan on ET\textsubscript{B}-R–Mediated Venoconstriction

Apart from RT-PCR analysis, changes in ET\textsubscript{B}-R expression in the rabbit jugular vein thus far can only be determined by assaying the biological activity of the receptor (see Lauth et al\textsuperscript{8}). Therefore, the constrictor response to sarafotoxin 6c (S6c), a selective ET\textsubscript{B}-R agonist,\textsuperscript{17} was compared in venous segments derived from the same batch but transfected with the CMV-\textsuperscript{2}x-galactosidase (mU) to account for individual differences in transfection efficacy. *P<0.05 vs static control. †P<0.05 vs stretched ECs exposed to ramiprilat or irbesartan alone. For comparison, Hoe 140 alone had no significant effect on the deformation-induced expression of the −1329-bp construct (70±18% of control, n=5).

Effects of Ramiprilat and Irbesartan on ppET-1 Gene Transcription

To verify that ramiprilat and irbesartan interfere with pressure-induced gene expression at the level of transcription, porcine aortic cultured ECs were transiently transfected with a −1329-bp rat ppET-1 promoter-luciferase reporter gene construct in porcine aortic cultured ECs under static conditions or after 6-hour exposure to cyclic strain (stretched). The double-transfected cells were preincubated for 30 minutes with vehicle (control), NO\textsubscript{2}Arg (100 μmol/L) alone, ramiprilat (0.3 μmol/L) without or with Hoe 140 (0.1 μmol/L) or NO\textsubscript{2}Arg, respectively, or irbesartan (1 μmol/L) in the absence and presence of Hoe 140 (n=3). Luciferase activity, normalized to the protein content of the sample, is expressed as relative light units measured within 30 seconds (RLU) relative to the protein-normalized activity of β-galactosidase (mU) to account for individual differences in transfection efficacy. *P<0.05 vs static control. †P<0.05 vs stretched ECs exposed to ramiprilat or irbesartan alone. For comparison, Hoe 140 alone had no significant effect on the deformation-induced expression of the −1329-bp construct (70±18% of control, n=5).

Effects of NOS and B\textsubscript{2} Receptor Blockade on ppET-1 and ET\textsubscript{B}-R mRNA Abundance

Brief exposure (30 minutes) of the venous segments to NO\textsubscript{2}Arg alone had no effect on basal ppET-1 (117±26% of control, n=3) or ET\textsubscript{B}-R (98±6% of control, n=3) mRNA expression; this was also true for segments that had been perfused at 20 mm Hg for 6 hours (not shown). However, continuous infusion of NO\textsubscript{2}Arg into the perfusate during the 6-hour period clearly upregulated basal ppET-1 mRNA abundance, albeit to a variable degree, in 3 independent experiments (cf Figure 1A). Although in 1 experiment there also appeared to be an upregulation of ET\textsubscript{B}-R mRNA under these conditions (Figure 1A), this effect of the NOS inhibitor could not be reproduced in subsequent experiments.

B\textsubscript{2} receptor antagonism alone, on the other hand, had no significant effect on either basal (107±20% and 95±6% of control, respectively, n=3) or deformation-induced ppET-1 and ET\textsubscript{B}-R expression (Figures 2A and 3A). Combined NOS and B\textsubscript{2} receptor blockade also did not affect the pressure-induced increase in ET\textsubscript{B}-R mRNA (from 274±29% to 341±74% of control, ie, 0 mm Hg, n=4), whereas ppET-1 mRNA expression was significantly enhanced (from 641±88% to 1080±154% of control, ie, 0 mm Hg, n=4, P<0.05).

Effects of Ramiprilat and Irbesartan on ppET-1 mRNA Abundance in Segments Perfused for 6 Hours at 0 mm Hg (n=4).

Figure 4. Effects of 6-hour perfusion at 20 mm Hg on eNOS mRNA expression in endothelium-intact segments of the rabbit jugular vein pretreated with vehicle (control), 1 μmol/L irbesartan, or 0.3 μmol/L ramiprilat compared with eNOS mRNA abundance in segments perfused for 6 hours at 0 mm Hg (n=4).

Figure 5. Expression of the −1329-bp rat ppET-1 promoter-luciferase reporter gene construct in porcine aortic cultured ECs under static conditions or after 6-hour exposure to cyclic strain (stretched). The double-transfected cells were preincubated for 30 minutes with vehicle (control), NO\textsubscript{2}Arg (100 μmol/L) alone, ramiprilat (0.3 μmol/L) without or with Hoe 140 (0.1 μmol/L) or NO\textsubscript{2}Arg, respectively, or irbesartan (1 μmol/L) in the absence and presence of Hoe 140 (n=3). Luciferase activity, normalized to the protein content of the sample, is expressed as relative light units measured within 30 seconds (RLU) relative to the protein-normalized activity of β-galactosidase (mU) to account for individual differences in transfection efficacy. *P<0.05 vs static control. †P<0.05 vs stretched ECs exposed to ramiprilat or irbesartan alone. For comparison, Hoe 140 alone had no significant effect on the deformation-induced expression of the −1329-bp construct (70±18% of control, n=5).
with ramiprilat (0.3 μmol/L) for 30 minutes resulted in a significant reduction in stretch-induced luciferase activity (Figure 5). This effect of the ACE inhibitor was much weaker in the presence of Hoe 140 (0.1 μmol/L) and fully reversed by 100 μmol/L NO2 Arg. Despite the presence of L-arginine in the medium (0.7 mmol/L), this concentration of NO2 Arg is sufficient to completely block NOS activity in the porcine aortic cultured ECs, as NO2 Arg is taken up by an amino acid carrier (neutral other than L-arginine (basic y’ system))20 and within minutes attains an intracellular concentration well beyond 1 mmol/L.21 Neither Hoe 140 nor NO2 Arg alone (Figure 5) significantly affected the deformation-induced expression of the ppET-1 promoter construct in the cultured ECs. Exposure of the cultured ECs to irbesartan (1 μmol/L) also significantly inhibited the deformation-induced increase in luciferase activity, and this inhibitory effect was almost fully reversed in the presence of Hoe 140 (Figure 5B). In a separate experiment, coincubation with NO2 Arg also reversed the inhibitory effect of irbesartan (not shown).

Effects of Ramiprilat and Irbesartan on the Constrictor Response to Bradykinin

The aforementioned findings clearly suggested that both ramiprilat and irbesartan inhibit the pressure (ie, deformation)-induced increase in ppET-1 and ETB-R expression in the isolated perfused rabbit jugular vein through stimulation of the endothelial B2 receptor and secondary release of NO. ACE inhibitors are known to potentiate the actions of bradykinin on ECs by interfering with the internalization of the B2 receptor and by protecting the kinin from proteolytic degradation via ACE.16,19 AT1 receptor antagonists are supposed to be devoid of such effects. To verify this contention, we compared the effects of ramiprilat and irbesartan on the B2 receptor–mediated dose-dependent constrictor response to bradykinin in the rabbit jugular vein. As shown in Figure 6, ramiprilat indeed caused an approximate 6-fold leftward shift of the dose-response curve of bradykinin, whereas irbesartan had no such effect (see also Hecker et al16).

Discussion

The findings of the present study demonstrate that the deformation-induced increase in ET-1 synthesis in native ECs, as well as the deformation-induced increase in ETB-R abundance in native SMCs, is greatly attenuated, if not abolished, by an ACE inhibitor and 2 different AT1 receptor antagonists. These compounds seem to exert their effects via a common mechanism of action that involves the stimulation of endothelial B2 receptors and the secondary release of NO from these cells acting in both an autocrine (endothelial ppET-1 expression) and a paracrine (smooth muscle ETB-R expression) manner. Other than this increase in NOS activity, there does not seem to be an additional effect (eg, on NOS expression) involved therein.

Moreover, ppET-1, but not ETB-R, expression in the isolated perfused rabbit jugular vein was upregulated after continuously blocking the endogenous synthesis of NO in the endothelium of these blood vessels. These data thus confirm and extend previous observations in cultured ECs of a suppression of ET-1 synthesis by endogenous NO22 or ACE inhibitors.13,14 In addition, they support the notion that the vasculo protective action of the ACE inhibitor class of compounds is to a significant extent due to enhancement of the B2 receptor–mediated stimulatory effect of endogenous kinins on EC autacoid production, namely that of NO.14,16,19

The inhibition by NO of endothelial ET-1 synthesis is thought to be mediated by an increase in intracellular cGMP acting on the transcription of the ppET-1 gene by an as-yet-unknown mechanism.22 Perhaps, a certain threshold concentration of NO is necessary for this effect to occur, as expression of the ppET-1 promoter-luciferase reporter gene construct was not affected by pretreatment with NO2 Arg in the cultured ECs, whereas continuous NOS blockade clearly raised the basal level of ppET-1 mRNA in the native ECs. Indeed, NOS activity in freshly isolated ECs is significantly higher compared with ECs isolated from the same vascular bed and grown under static conditions for several days.23 Moreover, NO formation in the endothelium of the perfused segments was presumably higher due to the moderate shear stress to which the native ECs are exposed to in this model. It remains to be elucidated, however, which transcription factor or factors serve as a target for the inhibitory action of NO/cGMP on ppET-1 gene expression.

In this context, it is perhaps interesting to note that brief exposure of the endothelium-intact segments of the rabbit jugular vein to NO2 Arg augmented the pressure-induced increase in ppET-1 mRNA abundance only when the B2 receptor had been additionally blocked with Hoe 140. It may be, therefore, that deformation of the native ECs not only leads to an increase in ET-1 synthesis but also triggers activation of the B2 receptor and thus the secondary formation of endothelial autacoids other than NO (eg, prostacyclin)16,19 that can also interfere with ppET-1 gene expression.

In contrast to ET-1 synthesis, an inhibition of ETB-R expression in native SMCs (no difference between endothelium-intact and denuded segments)9 by endothelial autacoids, including NO, has not yet been described. When compared with the synthesis of ET-1, however, both the endothelium-dependent inhibitory effect of ramiprilat on pressure-induced ETB-R expression and the efficacy of Hoe 140 in prevention of this effect were somewhat less pronounced. Moreover, unlike ppET-1 expression, combined NOS and B2 receptor blockade did not significantly augment the pressure-induced increase in ETB-R expression in the native SMCs. If ETB-R expression at the transcriptional level is regulated principally similar to that of ppET-1, these findings suggest either that endothelial autacoids,
including NO, are less efficacious when acting in a paracrine manner or that there are additional mechanisms that govern the deformation-induced expression of the ET_{B}-R in the native SMCs, such as the formation of angiotensin II in the endothelium.

What was truly unprecedented, however, is that both AT_{1} receptor antagonists appeared to act in the same way as the ACE inhibitor ramiprilat. Thus far, the B_{1} receptor–mediated increase in endothelial autacoid formation is thought to be a salient feature of the ACE inhibitors with respect to their protective effects in the cardiovascular system. Nonetheless, there are reports that suggest the cardioprotective effect of AT_{1} receptor antagonists in the rat and in the pig may be at least in part due to AT_{2} receptor activation and subsequent (presumably kinin-mediated) release of endothelial autacoids. This, however, may differ in the isolated rabbit jugular vein for two reasons: (1) according to RT-PCR analysis, the AT_{3}, but not the AT_{2}, receptor is abundant in endothelium-intact segments of these blood vessels; and (2) irbesartan, in contrast to ramiprilat, does not augment the smooth muscle B_{1} receptor–mediated constrictor response to bradykinin in the rabbit jugular vein. It appears, therefore, that blockade of another endothelial angiotensin receptor or perhaps an interference with the ET_{A}-R per se is responsible for the in large part NO-mediated inhibitory effect of the AT_{1} receptor antagonist on deformation-induced gene expression in the rabbit jugular vein.

The pressure-dependent increase in endothelial and, hence, intravascular ET-1 synthesis and the rise in ET_{A}-R abundance in the SMCs appear to profoundly alter the phenotype of these cells depending on the type of blood vessel studied. Recent findings from this laboratory suggest that in the rabbit jugular vein, the deformation-induced change in gene expression enhances medial SMC proliferation (see also Porter et al regarding the human saphenous vein), whereas in the rabbit carotid artery, parts of these cells undergo apoptosis, potentially leading to medial hypertrophy. The pressure-induced activation of the endothelin system in the vessel wall may thus contribute not only to the vasculopathy of venous bypass grafts but also to hypertension-induced arterial remodeling and possibly to restenosis after angioplasty. The inhibitory effects of both ACE inhibitors and AT_{1} receptor antagonists on the pressure-induced activation of the endothelin system in the vessel wall may therefore provide new opportunities for the accessory therapy of the aforementioned cardiovascular interventions or the prospective treatment of patients with borderline hypertension.

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