Signaling via the Angiotensin-Converting Enzyme Enhances the Expression of Cyclooxygenase-2 in Endothelial Cells

Karin Kohlstedt, Rudi Busse, Ingrid Fleming

Abstract—Angiotensin-converting enzyme (ACE) inhibitors elicit outside-in signaling via ACE in endothelial cells. This involves the CK2-mediated phosphorylation of ACE on Ser1270 and the activation of the c-Jun N-terminal kinase (JNK)/c-Jun pathway, resulting in an enhanced endothelial ACE expression. Because cyclooxygenase-2 (COX-2) expression is reported to be increased in subjects treated with ACE inhibitors, we determined the role of ACE signaling in this phenomenon and the transcription factors involved. In lungs from mice treated with the ACE inhibitor ramiprilat for 5 days, COX-2 expression was increased. A similar (1.5- to 2-fold) increase in COX-2 protein was detected in primary cultures of human endothelial cells treated with ramiprilat. In an endothelial cell line stably expressing human somatic ACE, ramiprilat increased COX-2 promoter activity, an effect not observed in ACE-deficient cells or cells expressing a nonphosphorylatable ACE mutant (S1270A). The ramiprilat-induced, ACE-dependent increase in COX-2 expression and promoter activity (both 1.5- to 2-fold greater than control) was prevented by the inhibition of JNK. Ramiprilat significantly enhanced the DNA binding activity of activator protein-1 in cells expressing ACE but not S1270A ACE. Activator protein-1 decoy oligonucleotides prevented the ACE inhibitor-induced increase in COX-2 promoter activity and protein expression. As a consequence of the ramiprilat-induced increase in COX-2 expression, prostacyclin and prostaglandin E2, but not thromboxane A2, production was increased and was inhibited by the COX-2 inhibitor celecoxib. These results indicate that ACE signaling may underlie the increase in COX-2 and prostacyclin levels in patients treated with ACE inhibitors. (Hypertension. 2005;45:126-132.)

Key Words: angiotensin-converting enzyme ■ cyclooxygenase ■ endothelium ■ prostacyclin ■ ramipril ■ signal transduction

Inhibitors of the angiotensin-converting enzyme (ACE), such as ramipril, exert beneficial effects on endothelial function and vascular remodeling and protect against the progression of atherosclerosis and the occurrence of cardiovascular events in humans. Although the latter effects are generally attributed to a decrease in the ACE-mediated generation of angiotensin II and the accumulation of bradykinin, a number of the effects of ACE inhibitors cannot be accounted for by inhibition of the enzyme per se.

One consequence of prolonged ACE inhibitor therapy is an increase in the expression of the enzyme itself. Increased ACE levels have been demonstrated in lung tissue and plasma from ACE inhibitor-treated rats and in the serum from patients who distinctly benefit from ACE inhibitor therapy. The finding that ACE inhibitors enhance the phosphorylation of ACE on Ser1270 within the short cytoplasmic tail of the enzyme and thus induce the phosphorylation and nuclear translocation of c-Jun, together with the report that the increase in ACE expression detected in phorbol ester-stimulated endothelial cells can be attributed to the activity of an activator protein-1 (AP-1) complex containing a c-Jun homodimer, suggests that a novel ACE-dependent signaling cascade can affect gene expression.

The expression of cyclooxygenase-2 (COX-2) is regulated by c-Jun, and because prostacyclin (PGI2) production is increased in subjects treated with ACE inhibitors, we hypothesized that an ACE signaling pathway may affect COX-2 expression and thus vasodilator prostanoi production. To assess the role of ACE signaling in the regulation of COX-2 expression, we determined the effects of ramipril/ra-miprilat in vivo (mouse lung), in primary cultures of human endothelial cells that express ACE, as well as in 3 porcine aortic endothelial cell lines. The latter endothelial cells were either ACE-deficient or overexpressed either human somatic ACE or a nonphosphorylatable ACE mutant in which Ser1270 was mutated to alanine (S1270A).

Materials and Methods

Celecoxib was a generous gift from Professor G. Geisslinger (Pharmazentrum, Frankfurt), and the JNK inhibitor SP600125 was from Tocris (Bristol, UK). All other substances were obtained from Sigma.

Cell Culture

Human umbilical vein endothelial cells were isolated and cultured as described. Because ACE expression decreases with time in culture,
all of the experiments in human endothelial cells were performed using primary cultures. Porcine aortic endothelial cells stably transfected with ACE or the S1270A ACE mutant were generated and cultured as described.\(^\text{17}\) Although the porcine endothelial cells no longer endogenously expressed ACE or functional angiotensin II and bradykinin receptors, they expressed a number of characteristic endothelial cell proteins (von Willebrand factor, CD31, the endothelial nitric oxide synthase and vascular endothelial cadherin).

**Animals**

To analyze the effect of ACE inhibition on COX-2 expression in vivo in male mice (C57 black 6; 6 weeks; Charles River, Wilmington, Mass), ramipril (5 mg/kg per day) was included in the drinking water over 5 days. Thereafter, the mice were anesthetized (isofuran 1.5%) and euthanized by a transverse cut through the large abdominal vessel. The lungs were perfused rapidly with ice-cold phosphate-buffered saline and snap-frozen homogenized as described.\(^\text{11}\)

**Immunoblotting**

Cells or lung homogenates were either boiled in SDS-sample buffer or lysed in nonidet lysis buffer, left on ice for 10 minutes, and centrifuged at 10 000g for 10 minutes. Supernatants were heated in SDS-PAGE sample buffer and separated by SDS-PAGE.\(^\text{17}\) Proteins were detected using their respective antibodies and were visualized by enhanced chemiluminescence using a commercially available kit (Amersham). The values obtained were normalized to tubulin or CD31. The bar graphs summarize data obtained in 6 to 8 independent experiments. \(* P<0.05, \text{**} P<0.001\) vs control (CTL).

**Luciferase Reporter Gene Assay**

Cells were transiently transfected with a COX-2 promoter construct (kindly provided by Margarete Goppelt-Struebe, Erlangen, Germany) together with a LacZ construct (Invitrogen, Karlsruhe, Germany) using calcium phosphate precipitation as described.\(^\text{17}\) After stimulation, the cells were lysed and luciferase activity was assayed using a commercially available kit (Promega, Mannheim, Germany). The values obtained were normalized to \(\beta\)-galactosidase activity (Tropix, Bedford, Mass) or to protein content. In some experiments, cells were cotransfected with flag-tagged dominant-negative JNK (pcDNA3.1Flag-JNK1-APF), wild-type JNK (pcDNA3.1Flag-JNK1), or empty vector (pcDNA3.1)\(^\text{18}\) 24 hours before transfection with the promoter construct.

**Prostaglandin Assays**

6-Keto-PGF\(_{1\alpha}\) (6-Keto-PGF\(_{1\alpha}\)) and prostaglandin E\(_2\) (PGE\(_2\)) levels were determined in the supernatants of primary cultures of human endothelial cells using commercially available kits (PGI\(_2\): Amersham Bioscience; PGE\(_2\): Assay Designs Inc.).

**Electrophoretic Mobility Shift Assay**

Double-stranded oligonucleotides containing the sequence of the binding site for AP-1 (5’-CGCTGTAGTGTCAGCCGAAATTTTTCGGCTGACTCATCAAGCG-3’), the AP-1 scrambled oligonucleotides (5’-CGCTTGATCTTAACGGGAATTTTTCCGGCTAGTAATCATCAAGCG-3’), and the AP-1 oligonucleotides were synthesized by BioSpring (Frankfurt, Germany). Preliminary experiments assessing the effects of the oligonucleotides on the DNA-binding activity of AP-1 and NF-\(\kappa\)B were performed to determine the specificity of the oligonucleotides at the concentration used.

**Statistical Analysis**

Data are expressed as mean\(\pm\)SEM and statistical evaluation was performed using Student\’s \(t\) test for unpaired data or 1-way ANOVA followed by a Bonferroni \(t\) test when appropriate. Values of \(P<0.05\) were considered statistically significant.

**Results**

**Effect of an ACE Inhibitor on the Expression of COX-2 in Primary Cultures of Human Endothelial Cells and in the Mouse Lung**

To assess the effect of ramipril on the expression of COX-2 in endothelial cells, primary cultures of human umbilical vein
endothelial cells were stimulated with ramiprilat (100 nmol/L, 4 to 72 hours) and harvested in SDS sample buffer. Ramiprilat induced a significant increase in the expression of COX-2, which was first evident 4 hours after the addition of the ACE inhibitor and remained elevated over the rest of the experimental period (72 hours; Figure 1A). Although COX-1 was expressed in human endothelial cells, ramiprilat failed to increase COX-1 expression (data not shown). A significant increase in the expression of COX-2 was also detected in the lungs of mice treated with ramipril over 5 days (Figure 1B).

**Effect of Ramiprilat on COX-2 Promoter Activity in Porcine Aortic Endothelial Cells**

To determine whether the effect of ramiprilat on COX-2 protein expression in vivo and in vitro was mediated by the ACE signaling pathway, we compared the effect of ramiprilat on COX-2 promoter activity in porcine aortic endothelial cells deficient in ACE and in cells stably expressing either human somatic ACE or the nonphosphorylatable ACE mutant (S1270A). Promoter activity was assessed as the cell lines did not express COX-2 under basal conditions or after cell stimulation (data not shown).

Ramiprilat (100 nmol/L, 24 hours) significantly enhanced COX-2 promoter activity in cells expressing human somatic ACE (Figure 2A). However, the ACE inhibitor failed to affect COX-2 promoter activity in ACE-deficient cells or in cells expressing the S1270A ACE mutant. The effect of ramiprilat on COX-2 promoter activity was comparable with that of interleukin (IL)-1β, but weaker than that of phorbol 12-myristate 13-acetate (Figure 2B).

**Effect of JNK Inhibition on COX-2 Upregulation by Ramiprilat in Endothelial Cells**

Pretreatment of primary cultures of human endothelial cells with the JNK inhibitor, SP600125 (5 μmol/L), prevented the ramiprilat-induced (100 nmol/L, 24 hours) increase in COX-2 expression (Figure 3A).

To determine whether the ramiprilat-induced increase in JNK activity can enhance the activity of the COX-2 promoter, experiments were performed in the ACE-expressing endothelial cell line. Transfection of these cells with a control vector or wild-type JNK failed to affect the ramiprilat-induced increase in promoter activity, whereas transfection with a dominant-negative JNK completely prevented the effect of the ACE inhibitor (Figure 3B).

**Role of AP-1 in the Ramiprilat-Induced Upregulation of COX-2**

Because COX-2 expression in endothelial cells is reportedly influenced by the transcription factors AP-1 and the cAMP-response element binding protein, we determined the effect of ramiprilat on the activity of these transcription factors in the ACE-overexpressing endothelial cell line. Ramiprilat did not affect the DNA binding activity of cAMP-response element binding protein (data not shown), but significantly enhanced the DNA binding activity of AP-1 (Figure 4A). In contrast, the ACE inhibitor did not increase AP-1 activity in ACE-deficient cells or in endothelial cells expressing the S1270A ACE mutant.

To directly demonstrate the involvement of AP-1 in the ramiprilat-induced upregulation of COX-2, we assessed the effects of AP-1 decoy oligonucleotides. ACE-overexpressing endothelial cells were incubated with either control oligonucleotides (AP-1 scrambled) or AP-1 decoy oligonucleotides immediately after transfection with the COX-2 promoter and before the addition of the ACE inhibitor. The ramiprilat-induced increase in COX-2 promoter activity was unaffected.
by scrambled oligonucleotides but was prevented by the AP-1 decoy oligonucleotides (Figure 4B).

AP-1 decoy oligonucleotides also prevented the ramiprilat-induced increase in endogenous COX-2 expression in primary cultures of human endothelial cells (Figure 5A). The ramiprilat-induced increase in COX-2 expression was accompanied by an increase in the accumulation of the stable PGI2 metabolite, 6-keto PGF1α, in the cell supernatant. The specific COX-2 inhibitor celecoxib (1 μmol/L) attenuated the basal production of 6-keto PGF1α in solvent-treated cells by 75±3% (n=5, P<0.01) and completely prevented the ACE inhibitor-induced increase in the autacoid so that in the presence of celecoxib 6-keto PGF1α levels did not differ between the control and ramiprilat-treated groups (Figure 5B).

Although ramipril also enhanced the celecoxib-sensitive generation of PGE2 (by 20±5%, n=8, P<0.01), the absolute levels measured were much lower than those of PGI2, which is in agreement with several previous studies.21,22 PGI2 production by ramipril-stimulated cells ranged from 6 to 12 ng/mL, whereas PGE2 levels were between 100 and 220 pg/mL. We were unable to detect any effect of ramiprilat on the production of thromboxane A2 (data not shown).

Discussion

The results of the present investigation demonstrate that the binding of the ACE inhibitor, ramipril, to ACE directly elicits a signaling cascade that results in the activation of the transcription factor AP-1 and an increase in the expression and activity of COX-2 in endothelial cells. This signaling...
The positive effects of ACE inhibitor therapy are generally attributed to the inhibition of ACE activity and the subsequent decrease in the production of angiotensin II and the accumulation of bradykinin. However, numerous experimental studies clearly indicate that some of the effects of ACE inhibitors cannot be directly attributed to changes in the concentration of either angiotensin II or bradykinin. We have recently reported that ACE is an ectoenzyme and also possesses properties that implicate its involvement in cellular signaling. For example, the cytoplasmic tail of ACE, more specifically Ser1270, is phosphorylated, and the binding of an ACE inhibitor to ACE increases its phosphorylation and elicits the activation of the ACE-associated kinases CK2 and JNK. As a consequence of the activation of JNK in ACE inhibitor-treated cells, c-Jun is translocated to the nucleus and the expression of ACE is increased. The latter effect is not limited to an artificial cell culture system but can be reproduced in mice given an ACE inhibitor in the drinking water, and has been documented in individuals treated with ACE inhibitors. Although this evidence certainly indicates an ACE-dependent signaling process. By interfering with the activity of JNK and AP-1, it was possible to prevent the ramiprilat-induced increase in COX-2 promoter activity and protein expression, but again only in cells expressing wild-type ACE.

At first glance, it is not evident that an increase in the expression of COX-2 can be associated with vasoprotective effects because COX-2 is still considered as the “bad” COX isoform, which is generally induced in response to an inflammatory reaction. On the basis of findings obtained in other cell systems, it has been generally assumed that COX-2 plays a detrimental role in cardiovascular homeostasis. Although it is clear that COX-2–derived thromboxanes would be expected to induce vasoconstriction and potentiate an inflammatory state, the enzyme generates mainly PGI2 when it is expressed in endothelial cells. We detected only low levels of PGE2 in primary cultures of ramiprilat-treated human endothelial cells and were unable to detect thromboxane production. Further evidence that COX-2–derived PGI2 is beneficial comes from studies reporting that selective COX-2 inhibition attenuates acetylcholine-induced vasodilatation in the forearm circulation of patients with essential hypertension and diminishes the positive effects of ACE inhibitors on blood pressure.

Reports of a link between ACE inhibitor treatment and enhanced endothelium-dependent vasodilatation have long been linked with the increased production of PGI2. In fact, there are several mechanisms by which ACE inhibitors have been proposed to affect the synthesis of PGI2. For example, because a short-term effect of ramiprilat (peaking within 10 to 60 minutes) on PGI2 production could be abolished by the B2 kinin receptor antagonist, icatibant, part of the response has been attributed to the activation of the B2 kinin receptor signaling pathway. However, such a rapid effect is more likely to be related to the activation of phospholipase A2 enzymes in response to an ACE inhibitor-induced elevation in intracellular calcium than to an alteration in COX expression. Although the evidence linking some of the acute effects of ACE inhibitors with the transactivation of the B2 kinin receptor or alterations...
in B₂ kinin receptor sequestration to caveolae is convincing, the long-term effects of ACE inhibitors on COX-2 expression reported here is unlikely to be related to the accumulation of bradykinin. The ACE-overexpressing cell line used to assess the effects of ramiprilat on COX-2 promoter activity does not express bradykinin receptors. A bradykinin-independent, long-term effect of ACE inhibitors on PGI₂ production has previously been reported in rats made hypertensive with a nitric oxide synthase inhibitor and treated with quinapril. In the latter study, the ACE inhibitor-induced improvement in flow-induced dilatation in small mesenteric arteries was sensitive to COX-2 inhibitor-induced improvement in flow-induced dilatation, but not to the B₂ kinin receptor antagonist. Because we have shown that ramipril increases COX-2 expression in mice in vivo, it is tempting to suggest that ACE signaling underlies the previously reported ACE inhibitor-induced increase in PGI₂ production.

Taken together, the results of the present investigation indicate that the enhanced production of PGI₂, which is a well-documented consequence of ACE inhibitory therapy, can be attributed to an ACE signaling cascade involving the phosphorylation of ACE and the activation of JNK, AP-1, and COX-2. Although it remains to be determined which additional intracellular mediators participate in the ACE signaling pathway, COX-2–derived PGI₂ may mediate effects other than/in addition to vasodilator function. There is at least circumstantial evidence supporting a role for COX-2 in other ACE-dependent responses inasmuch as the ACE inhibitor captopril was found to increase renal renin production in wild-type and COX-1⁻/- mice but not in COX-2⁻/- mice.

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