

Fibroblast growth factor-2 mediates ACE inhibitor-induced angiogenesis in coronary endothelium*

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Abbreviations: ACE, angiotensin converting enzyme; ACEI, angiotensin converting enzyme inhibitor(s); BK, bradykinin; CS, calf serum; CVEC, coronary venular endothelial cells; eNOS, endothelial nitric oxide synthase; FCS, fetal calf serum; FGF-2, fibroblast growth factor-2; HUA, human umbilical artery; L-NMMA, N^ω-methyl-L-arginine; PCA, porcine coronary artery.

Abstract

The beneficial effect exerted by angiotensin converting enzyme inhibitors (ACEI) on vascular endothelium has been attributed to restoration of endothelial cell survival properties, and improvement of angiogenesis. Fibroblast growth factor-2 (FGF-2) is an angiogenic factor for the microvascular endothelium, which tonically promotes endothelial cell growth and survival through an autocrine/paracrine mechanism. Here we formulate the hypothesis that FGF-2 might contribute to the prosurvival/proangiogenic effect of ACEI. We investigated zofenoprilat and in selected experiments lisinopril, as representatives of ACEI. These compounds induced formation of pseudocapillaries in vessel fragments isolated from porcine coronary and human umbilical arteries, by increasing endothelial cell growth up to 5 fold. Angiogenesis was abolished by inhibitors of nitric oxide synthase (NOS) pathway and by anti-FGF-2 antibodies. Consistently, in cultured coronary endothelial cells (CVEC) ACEI up-regulated eNOS and FGF-2, and induced MAPK ERK1/2 activation. The overexpression of eNOS/FGF-2 produced, at the functional level, enhanced cell proliferation and migration, the latter effect being dose-dependent and maximal at 0,1 μ M zofenoprilat. The importance of FGF-2 for the acquisition of the angiogenic phenotype elicited by ACEI was clearly demonstrated by the impairment of endothelial functions following transfection of CVEC with siRNA for FGF-2. Moreover, FGF-2 silencing greatly affected the nuclear translocation of the FGFR-1, highlighting the autocrine mode of action of FGF-2. At the endothelial membrane level, zofenoprilat appeared to activate the bradykinin B1 receptor, a known stimulant of FGF-2 expression. In conclusion we show that ACEI exert protective/proangiogenic effects in microvascular coronary endothelial cells by activating the endogenous FGF-2/FGFR-1 system.

INTRODUCTION

The mechanism of the protective effect of angiotensin converting enzyme inhibitors (ACEI) in cardiovascular diseases, characterized by endothelial impairment, remains poorly defined, although various hypotheses have been advanced. Among these, attention has been focused on the role of endothelial nitric oxide synthase (eNOS) in restoring endothelial cell functions, particularly angiogenesis. Here we report on the involvement of fibroblast growth factor-2 (FGF-2) which plays a central role in the prosurvival/proangiogenic effect of ACEI. The rationale for studying FGF-2 stems from the recognition that the growth factor, ubiquitously produced, is up-regulated in endothelial cells exposed to a variety of stimuli (Ziche et al., 1997, Parenti et al., 2001, Tepper et al., 2004, Cantara et al., 2004) and during stress and injury (Szebenyi and Fallon, 1999). In addition, its up-regulation mediates the vascular endothelium protective action of estrogens (Kim-Shulze et al., 1998). FGF-2, a polypeptide member of a family of heparin binding growth factors (Presta et al., 2005), has multifunctional actions, promoting cell survival in many cell types and affecting differentiation and gene expression. FGF-2 acts through an autocrine-paracrine mechanism by stimulating fibroblast growth factor receptor (FGFR-1), a membrane-associated protein, which is released from endoplasmic reticulum membranes into the cytosol and translocates to the cell nucleus by an importin-beta-mediated transport pathway along its ligand. Within the nucleus, FGFR-1 serves as a general transcription regulator, stimulating multi-gene programs for cell growth and differentiation (Maher, 1996, Reilly and Maher, 2001, Stachowiak et al., 2003).

Clinical evidence support the notion that the major therapeutic benefit of ACEI lies in their ability to repair endothelial injury, a pathological feature common to an heterogeneous group of vascular diseases (hypertension, congestive heart failure, myocardial infarction and diabetic nephropathy) (Scribner et al., 2003, Boss and Dawes, 2004, Tu et al., 2005). This concept is also grounded on a number of experimental evidences accrued through a variety of

approaches including animal disease models, in vitro endothelial cell culture, and studies on intracellular signalling pathways. Among the examples, the restoration of impaired homeostasis to vasoactive agents, the reversal of endothelial damage caused by free radicals (Mak et al., 1990, Fujita et al., 2000) or cytotoxic agents (Tokudome et al., 2000, Sacco et al., 2001).

Most importantly, ACE inhibitors have been shown to promote angiogenesis, resulting in increase of capillary density and of perfusion rate in the microvasculature, thus contributing to the restoration of vascular homeostasis. These activities have been demonstrated in cardiac hypertrophy in spontaneously hypertensive rats (Clozel et al., 1989, Olivetti et al., 1993), in rat ischemic limb (Takeshita et al., 2001, Silvestre et al. 2002), and in rabbit hindlimb ischemia, with an effect similar to that observed for the angiogenic factor vascular endothelial growth factor (Fabre et al., 1999).

Here we formulate the hypothesis that ACEI promote angiogenesis by increasing the expression and production of FGF-2 via the stimulation of eNOS. We have investigated the formation of pseudocapillaries in organ explants of human umbilical and porcine arteries continuously exposed to ACEI (zofenoprilat and lisinopril) under conditions of FGF-2 withdrawal (antibody toward FGF-2) and eNOS pharmacological blockade. At the cellular level we studied the influence of ACE inhibitors on endothelial cell growth and migration following the ablation of FGF-2 input, obtained by silencing its messenger through a mRNA interference technology and by pharmacological inhibition. We also monitored the effect of ACEI on the expression of endothelial FGF-2 and NOS mRNAs, on the activity of the MAPK ERK1/2 signal, as well as on the translocation of FGFR-1 from cell membrane to the nucleus. Finally, we examined the influence of bradykinin receptor subtypes on endothelial function activated by ACEI.

The results of this study provide evidence that the mechanism utilized by ACE inhibitors in restoring injured endothelial functions, involves activation of the endogenous FGF-2/FGFR-1 cascade, which promotes new vessel formation.

MATERIALS AND METHODS

Pseudocapillary formation from vessel rings

Pseudocapillary sprouting from vessel rings was evaluated as previously described (Morbidelli et al., 2003). Vessel rings 1-2 mm long from porcine coronary artery (PCA) and human umbilical artery (HUA) were positioned in 48-multiwell plates with the lumen oriented horizontally. They were then included in a fibrin gel obtained adding bovine fibrinogen solution (3 mg/ml) and thrombin (1.5 U/ml) into each well. The organ culture was kept at 37°C in 5% CO₂. The medium with test substances (lisinopril or zofenoprilat, in the absence/presence of pharmacological inhibitors or neutralizing antibodies) was replaced every 2 days. The extent of fibrin gel occupied by pseudocapillary structures was quantified by an inverted microscope at a magnification of 200 X, using an ocular grid. The grid was placed over the gel as many times as necessary to measure the entire gel surface covered by the pseudocapillaries. This area was then recorded and expressed by the number of grid units required to cover the pseudo-capillary surface (1 grid unit = 0.21 mm²).

Cell populations present in the pseudocapillaries were characterized and quantified at day 15. Vessel rings were removed and fibrin was lysed by adding bovine plasmin (1 U/mL, 100 µl/well); the cell suspension obtained from pseudocapillaries was centrifuged, spread on a slide, fixed in acetone/chloroform 1:1 and stained for CD31 and alfa-actin (Dako A/S, Denmark) with an amplified immunoperoxidase technique. The proportion of CD31(+) cells over total cell count was recorded.

Cell culture

Coronary venular endothelial cells (CVEC) were isolated and cultured as described (Schelling et al., 1988). Cells between passage 10 and 15 maintaining all the markers typical of

microvascular endothelium, were used in the experiments.

Silencing technique

A 21-nucleotide RNA (5'-AAACGAACTGGGCAGTATAAAA-3', gene ID NM_002006) was chemically synthesized by Qiagen (Milan, Italy). Synthetic oligonucleotides were deprotected and gel-purified as described (Elbashir et al., 2001). Control siRNA is a random siRNA (5'-AATTCTCCGAACGTGTCACGT-3') provided by Qiagen. For annealing of siRNAs, 20 μ M single strands were incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4 and 2 mM magnesium acetate) for 1 min at 90°C followed by 1 h at 37°C.

Transient transfection of siRNA in subconfluent cultures was carried out using lipofectamine (Invitrogen, USA). 104,5 μ l OPTIMEM medium (Gibco, USA) and 5,55 μ l lipofectamine per plate (6 cm diameter dish, 2 ml total stimulation volume) were preincubated for 5 minutes at room temperature. During the time for this incubation 106,7 μ l OPTIMEM medium were mixed with 1,5 μ g siRNA. The two mixtures were combined and incubated for 20 minutes at room temperature for complex formation. After incubation the entire mixture was added to the cells (taken as time 0). A second transfection was carried out after 18 h. Cells were then checked for protein expression, migration assay and immunofluorescence 36 h after beginning of transfection, and for proliferation assay 156 h after beginning of transfection.

Migration and proliferation studies

Migration and proliferation of CVEC were quantified as described (Morbidelli et al., 2003, Cantara et al., 2004). For migration studies, subconfluent adherent cells were preincubated before migration with drugs (ACE inhibitor 0,01-1 μ M, alone and in the presence of BK/B1 and B2 receptor antagonists or NOS pathway inhibitors) for the specified length of time (15 min-24 h). Silenced cells (for 36 h) were incubated with ACEI in the last 18 h of silencing. Spontaneous migration in medium containing 1% serum was evaluated after

4 h in the 48 well microchemotaxis chamber. Migrated cells were then fixed, stained and randomly counted at 40X magnification

For proliferation, 1×10^3 cells/100 μ l medium additioned with 10% calf serum (CS) were plated in 96 multiwell plates. After adherence (18 h) the supernatant was replaced with medium containing test substances. Then, zofenoprilat (1 μ M) was added in fresh medium containing 0.1% serum. Proliferation continued for further 72 h, when ACEI was added again. At day 5 after stimulation, cells were fixed, stained and randomly counted at 10X magnification.

In experiments with SU5406, after adherence cells were stimulated with FGF-2 (20 ng/ml) or ACE inhibitors (1 μ M) in the absence/presence of the pharmacological inhibitor (1 μ M). Test substances were freshly added after 72 h. Proliferation continued for further 48 h. Cells were fixed, stained and counted as reported above.

Western blotting

Western blotting was performed as described by Donnini et al. (2004). 3×10^5 cells were plated in 6 cm diameter dishes. After adhesion, cells were serum-starved overnight, then exposed to 1 μ M zofenoprilat for the specified length of time (15 min-18 h) or silenced for FGF-2 (for 36 h) as described above. In three experiments, cells were exposed to the BK/B1 antagonist (1 μ M) or the MEK inhibitor U0126 (10 μ M) for 30 min before zofenoprilat stimulation (1 μ M) for 8 h. Cells were then lysed and centrifuged. Five μ l of the supernatant from each sample was used for protein assay. 30 or 50 μ g of proteins were mixed with 4X reducing SDS-PAGE sample buffer and denatured at 100°C for 10 min. Electrophoresis was carried out in SDS/10% polyacrylamide gel. Proteins were then blotted onto activated PVDF membranes, incubated overnight with the antibodies (anti-phospho-ERK1/2 1:2000 or anti-FGF-2 1:1000) and then detected by enhanced chemiluminescence system (Amersham). Results were

normalized to those obtained by using an antibody to total ERK1/2 (for phospho-ERK1/2) and to actin (for FGF-2) as described (Cantara et al., 2004).

Immunofluorescence for FGFR-1

CVEC were plated on 1 cm round glass coverslips at the density of 2×10^4 cells in medium additioned with 10% CS. After adhesion, transfection with siRNA for FGF-2 was carried out for 36 h as described above. ACE inhibitor (1 μ M zofenoprilat) was added for 18 h. Cells were washed in PBS, fixed in 4% paraformaldehyde in PBS and permeabilized for 5 minutes with 0,5% Triton X-100. After blocking unspecific binding sites in 3% bovine serum albumin (BSA), cells were incubated overnight at 4°C with the monoclonal antibody anti-FGFR-1 diluted 1:30 in PBS containing 0.5% BSA. Cells were then washed, incubated for 2 h with a fluorescein isothiocyanate-labeled goat anti mouse IgG secondary antibody (Sigma, Chemical Co.) diluted 1:50 in PBS with 0.5% BSA and mounted in Moviol 4-88 (Calbiochem, La Jolla, CA, USA).

RT-PCR analysis

Fibroblast growth factor-2 (FGF-2) and endothelial nitric oxide synthase (eNOS) mRNA expression were measured through differential RT-PCR. Cells were stimulated for 8 h with ACE inhibitors (zofenoprilat and lisinopril, 1 and 20 μ M), the B1 receptor agonist Des-Arg9-BK (10 nM), in the absence and in the presence of NOS inhibitor. Differential RT-PCR was carried out by using the specific primers previously described (Ziche et al., 1997, Cantara et al., 2004). Primers for eNOS were 5'-GCTTGAGACCCTCAGTCAGG-3' (sense) and 5'-GGTCTCCAGTCTTGAGCTGG-3' (antisense); for FGF-2 5'-GGAGTGTGTGCTAACCGTTACCTGGCTATG-3' (sense) and 5'-TCAGCTCTTAGCAGACATTGGAAGAAAAG-3' (antisense). Results were evaluated as ratio between optical density of the target genes (eNOS, FGF-2) and GAPDH amplification products 5'-CCATGGAGAAGGCTGGGG-3' (sense) and 5'-CAAAGTTGTCATGGATGACC-3' (antisense).

Materials and reagents

Cell culture reagents, thrombin, plasmin, fibrinogen, lisinopril, Des-Arg9-BK, the selective B2 receptor antagonist (HOE140) and bradykinin were from Sigma-Aldrich (USA). Fetal calf serum (FCS) and bovine calf serum (CS) were from Hyclone, Celbio (Italy). FGF-2 was obtained from Peprotech (UK). All reagents for RT-PCR assays were from Perkin-Elmer (USA). Anti-CD31 antibody was from DakoCytomation, Glostrup, Denmark. Anti- α actin antibodies were from Oncogene Research Products, Boston MA (USA). Reagents for immunohistochemistry were from Vector (UK). Neutralizing anti-FGF-2, anti-phospho-MAPK p44/42 and total p44/42 antibodies were obtained from Cell Signaling, Celbio (Italy). The anti FGFR-1 antibody (clone 19B2) was from Upstate Biotechnology (NY, USA). The fluorescein isothiocyanate-labeled goat anti mouse IgG secondary antibody was from Sigma-Aldrich. The NOS inhibitor N^ω-methyl-L-arginine (L-NMMA), the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), the MEK inhibitor U0126 and the FGFR-1 tyrosine kinase inhibitor SU5402 were from Calbiochem (Germany). [3H]-L-arginine was from Amersham (UK). Zofenoprilat (free acid) was kindly provided by Menarini Ricerche, Florence (Italy). The selective B1 receptor antagonist (R715) was provided by Prof. D. Regoli, University of Ferrara, Italy.

Statistical analysis

Results are expressed as means \pm SEM for (n) experiments performed in triplicate. Multiple comparisons were performed by one-way analysis of variance (ANOVA) and individual differences tested by Fisher's test after the demonstration of significant inter-group differences by ANOVA.

RESULTS

ACEI promote angiogenesis in vascular organ culture

We studied the formation of pseudocapillaries from vascular explants of porcine coronary artery (PCA) and human umbilical artery (HUA) in the presence of increasing serum concentrations (1, 5 and 10% FCS) for up to two weeks in organ cultures. Pseudocapillary formation, reported in Fig. 1A as area occupied by capillaries, increased linearly as a function of serum concentration. Continuous inhibition (ten days) of ACE by zofenoprilat or lisinopril, both at 1 μ M, further accelerated ($p < 0.001$) pseudocapillary formation over the serum effect, independently of the serum concentration (Fig. 1A). Similar results were obtained in human umbilical artery (HUA) preparations (in Fig. 1B representative pictures are shown), in which ACEI increased (2,5-5 fold, $p < 0.01$) the endothelial cell population evaluated by immunohistochemical staining (Fig. 1C). As alfa-actin positive cells increased only by $9 \pm 4\%$ following drug treatment, the promotion of tube formation was accompanied by changes in endothelium:pericyte balance toward endothelium, suggesting that the effect of ACEI was directed toward the endothelium.

The large increase of endothelial cell number, noted in explants following exposure to ACEI, prompted to study the influence of FGF-2 and eNOS, signals known to be involved in the acquisition of the angiogenic phenotype, and instrumental for new capillary formation (Ziche et al., 1993, 1997). Incubation of porcine rings (0.1% FCS for 5 days) with FGF-2 produced a nearly twofold increase of pseudocapillaries (from 250 to 450 microscopic grid units; $p < 0.001$) over control rings. Zofenoprilat (1 μ M) caused capillary outgrowth, which although smaller than that observed with FGF-2, was significant ($p < 0.01$) relative to control rings (Fig. 1D). Coincubation of rings with antibody directed toward FGF-2 suppressed the capillary growth promoted by either FGF-2 or zofenoprilat, indicating that the growth promoting effect of the latter is totally dependent on the availability of FGF-2 (Fig. 1D). We also observed that

inhibition of eNOS through L-NMMA (200 μ M) abolished the pseudocapillary growth promoted by zofenoprilat, suggesting that the eNOS pathway is crucial for the expression of the angiogenic phenotype promoted by the ACE inhibitors, as previously reported by others on endothelial dysfunction (Van Belle et al., 1996). D-NMMA, as well as an irrelevant antibody (mouse IgG), were devoid of any activity (data not shown).

ACEI induce up-regulation of eNOS and FGF-2 in endothelial cells

In light of the above findings, showing the influence of the NOS and FGF-2 pathways on the pro-angiogenic activity of ACEI, we measured gene expression of eNOS and FGF-2, signals involved and sequentially linked for the acquisition of the angiogenic phenotype in cultured coronary endothelial cells (CVEC) (Ziche et al., 1997, Parenti et al., 2001). We also monitored the activation of MAPK kinase, an upstream signal relative to the mentioned genes (Cantara et al., 2004). Zofenoprilat (1 μ M) strongly induced MAPK activity, especially at long exposure times (4-18 h) (Fig. 2A).

Stimulation of quiescent endothelial cells (0.1% CS) with zofenoprilat (1-20 μ M) increased the expression of eNOS mRNA transcript to a level comparable in intensity to that exerted by the BK/B1 receptor agonist Des-Arg⁹-BK (Fig. 2B, lane n. 4), a potent inducer of eNOS pathway in CVEC (Parenti et al., 2001). Similarly, zofenoprilat and lisinopril (1-20 μ M) enhanced the expression of FGF-2 mRNA transcript (Fig. 2C, lanes 2 and 3). Since the application of the NOS inhibitor L-NMMA markedly attenuated FGF-2 upregulation induced by zofenoprilat (Fig. 2C, lane 5), we conclude that FGF-2 expression is under the control of the NOS pathway, suggesting a close linkage between these endothelium-protective signals. Since endothelial cell outgrowth and molecular responses were comparable for both compounds, we performed further experiments using only zofenoprilat.

Because ACEI have been reported to interfere with the bradykinin system by increasing the peptide availability in the endothelium (Grafe et al., 1993), as well as to interact directly with

its membrane receptors (Ignjatovic et al., 2002, 2004), we investigated whether blockade of BK/B1 receptor subtype would affect FGF-2 protein expression in CVEC. A clear upregulation of FGF-2 production was observed following endothelial cell incubation with Zofenoprilat (1 μ M). Co-incubation with B1 antagonist (R715) suppressed the ACEI enhanced expression of the growth factor (Fig. 2D).

To evaluate whether the stimulation of ERK1/2 seen in Fig. 2A could be directly due to ACEI activity, FGF-2 protein upregulation stimulated by zofenoprilat was evaluated in cells preincubated with the MEK inhibitor U0126. Treatment of the cells with U0126 completely impaired zofenoprilat induced FGF-2, clearly documenting that the MAPK pathway is upstream of ACEI induced FGF-2 upregulation (Fig. 2E).

ACEI promote endothelial cell functions via BK/B1 receptor and the eNOS pathway

Experiments on cell proliferation and migration, both hallmarks of the angiogenic phenotype in coronary endothelium, provided clear evidence that ACE inhibition directly activates endothelial cell functions.

Subconfluent and synchronized CVEC were exposed to zofenoprilat (1 μ M) either for 2 or 5 days. Significant growth enhancement was observed only following 5 days of incubation with the drug ($p < 0.01$, 31 ± 3 % increase over basal) (Fig. 3A). These results are in keeping with the observations, depicted in Fig. 1, showing the large increase of pseudocapillaries in explants exposed to zofenoprilat for 5 days and the significant amplification of endothelial component in ACEI-induced newly formed structures (Fig. 1C).

Similarly, migration of CVEC was induced by zofenoprilat in a concentration and time dependent fashion. Overnight incubation (18 h) with zofenoprilat (0.01-1 μ M) induced cell migration, maximal effect being reached at 0.1 μ M (Fig. 3B). Stimulation occurred in a time-dependent manner, as the number of migrated cells nearly doubled within 4 h (table inset of Fig. 3B). In cells preincubated for 4 h with zofenoprilat (1 μ M) and then challenged with

FGF-2 (20 ng/ml), no further increase in cell chemotaxis was found (63 ± 3 and 78 ± 1 migrated cells counted/well for 20 ng/ml FGF-2 in the absence/presence of 1 μM zofenoprilat, respectively, vs 46 ± 2 in basal condition), suggesting that maximal cell responsiveness had been attained by the preincubation.

To study the involvement of BK/B1 receptor in ACEI induced endothelial cell functions, we investigated whether blockade of BK receptor subtypes would affect endothelial cell migration promoted by these drugs. We therefore studied zofenoprilat-induced migration in the presence of fully competent concentration (1 μM) of a B1 (R715) or B2 (HOE140) receptor antagonist (Fig. 3C). Indeed, the B1 receptor antagonist significantly ($p < 0.05$) attenuated zofenoprilat induced response, while the B2 receptor antagonist was devoid of any activity.

Again, the relevance of the NOS pathway was documented in experiments in which the NOS inhibitor (L-NMMA, 200 μM), or the soluble guanylate cyclase inhibitor (ODQ, 10 μM) inhibited zofenoprilat-induced cell migration (Fig. 3C).

In conclusion these data document that BK/B1 receptor dependent NOS pathway is involved in endothelial cell migration induced by ACEI.

Endogenous FGF-2/FGFR-1 axis is required for the proangiogenic effect of ACEI

To assess the involvement of endogenous FGF-2 in mediating the proangiogenic effects of ACEI in cultured endothelium, CVEC were treated for 36 h with siRNA against FGF-2 mRNA. FGF-2 silencing was validated by western blotting, which demonstrated total inhibition of protein synthesis (Fig. 4A).

The functional response of endothelial cells, i.e. migration, was variably affected by FGF-2 silencing. We observed negligible effects on spontaneous migration, while the response to zofenoprilat was markedly impaired (60% inhibition, $p < 0.01$) (Fig. 4B).

Localization of FGFR-1, the dominant receptor in the endothelium which is internalized and imported in the nucleus upon stimulation by FGF-2, was analyzed by immunohistochemistry. In quiescent CVEC, FGFR-1 labeling was sparse and faint (Fig. 5A). Treatment with zofenoprilat (1 μ M) produced a marked enrichment of fluorescence, the bulk being localized in the nucleus (Fig. 5B). In cells silenced for FGF-2, morphological analysis documented the appearance of cytoplasmic vacuoles, signs of cell distress, and more importantly, nuclear fluorescence remained faint, regardless of drug application (Fig. 5C-D).

The relevance of FGF-2/FGFR-1 axis in ACE inhibitor induced angiogenic functions was determined in functional assay. We monitored cell growth at 5 days in endothelial cells treated with SU5402 (1 μ M), the selective inhibitor of the tyrosin kinase activity of FGFR-1, and then challenged with either FGF-2 or ACE inhibitor. Cell proliferation was induced, to a comparable extent, by all treatments relative to control cells (Fig. 5E), while SU5402 application reduced growth in FGF-2 and in zofenoprilat-treated cells (Fig. 5E).

In conclusion, these data demonstrate the requirement of the endogenous FGF-2/FGFR-1 cascade as an obligate pathway for angiogenesis induced by ACEI.

DISCUSSION

This investigation provides evidence for the intracellular signals influenced by ACEI in activating the microvascular endothelium and in inducing the angiogenic phenotype. In this study we observe that ACEI induced the over-expression of FGF-2, and promoted the internalization of its receptor, FGFR-1, a process consistent with the growth factor autocrine function in the endothelium.

ACEI are important drugs for diverse cardiovascular diseases recognizing endothelial impairment as the prevailing pathogenic mechanism. The beneficial effects exhibited by these drugs, commonly termed “protective”, have been documented to be mediated through enhanced availability of NO, resulting from stimulation of eNOS (Van Belle et al., 1996, Hatta et al., 2002). The intermediate steps lying between presentation of active drug moieties to cells, and the activation of membrane/intracellular signals and final effector molecule(s) are much less known and debated.

In this study we demonstrate that ACEI promote endothelial cell growth and migration, resulting in a marked outgrowth of new capillaries in vessel explants from human umbilical and porcine coronary arteries.

ACEI-induced activation of these endothelial functions occurs through a closely linked sequence of intracellular signals, which include over-expression of eNOS, activation of MAPK, and down-stream up-regulation of FGF-2 and its receptor (Fig. 6). That the NO-cGMP pathway is involved in the protective effects exerted by ACEI is evidenced by inhibitors of the pathway, such as L-NAME and ODQ, which abolish the beneficial action of ACEI on the endothelium. The very significant finding, reported here for the first time, is that ACEI promote over-expression of FGF-2, an angiogenic growth factor endogenously produced by endothelial cells, acting in an autocrine mode to foster their growth and survival (Gualandris et al., 1996, Tepper et al., 2004, Cantara et al., 2004). Neutralization of FGF-2, by application of neutralizing antibodies or silencing technology, obliterates the pro-

angiogenic effects of ACEI in the pseudocapillary outgrowth from organ explants and in cultured endothelial cell functions. The relevance of the FGF-2 system in the mechanism of action of ACEI is further demonstrated by the immunofluorescent localization of FGFR-1. FGFR-1 was found to translocate, under the influence of ACEI, from the membrane/cytoplasm to the nucleus, where the complex agonist/receptor serves as a general transcriptional regulator of cell growth (Stachowiak et al., 2003). In addition, blockade of FGFR-1 auto-phosphorylation by the specific receptor kinase inhibitor SU5402, appeared to curtail endothelial proliferation in response to zofenoprilat.

As evidence indicates that the NOS-cGMP pathway regulates the FGF-2 expression in endothelial cells (Ziche et al., 1997, Parenti et al., 2001), NO appears to act as a primer of FGF-2 synthesis, which assumes the role of effector signal for the acquisition of the angiogenic phenotype. The sequence of events involving the NOS-cGMP and FGF-2 explains most of the observations here reported: it accounts for the enhanced proliferation, migration and the acquisition of the angiogenic phenotype leading to new vessel formation.

An interesting finding of this study relates to the action of ACEI occurring at the endothelial cell membrane, as illustrated by the use of BK receptor subtype antagonists. In fact, the bradykinin B1 (R715), but not the B2 (HOE140) antagonist attenuated FGF-2 upregulation and endothelial cell migration elicited by ACEI, implying that the B1 subtype transduces signals to the NOS dependent FGF-2 pathway. A possible interpretation of this finding is offered by recent observations which demonstrated a direct interaction of certain ACEI with the endothelial B1 receptor, resulting in marked eNOS activation and prolonged NO release (Ignjatovic et al., 2002, 2004; Stanisavljevic et al., 2006). At variance from these authors, we found that eNOS activation and the ensuing FGF-2 overexpression require longer times of exposure (several hours) and higher (micromolar) concentration of ACEI. We think that these quantitative differences may be reconciled by considering the low constitutive expression of the B1 receptor subtype in coronary microvascular endothelium used in our study.

Interestingly, the B1 but not the B2 subtype, was previously shown to promote vigorous in vitro and in vivo angiogenic responses through the stimulation of the eNOS-FGF-2 pathway (Morbidelli et al., 1998, Parenti et al., 2001).

These results bring forward the notion that the beneficial effects of ACE inhibitors on the endothelium may not be solely associated with the converting enzyme blockade. Recently, for example, ACE inhibitors have been found to enhance the expression of COX-2 leading to production of the vasodilating prostanoids (i.e. prostacyclin) through the JNK pathway, independently from enzyme blockade (Kohlsdedt et al., 2005, Fleming et al. 2005). Conversely, ACEI, by inhibiting AngII production/AT2 activation which have been reported to negatively regulate VEGF-induced angiogenesis (Benndorf et al., 2003), may also exhibit angiogenic properties through the conventional mechanism of converting enzyme blockade. Whether the mentioned mechanisms are the expression of the inherent redundancy operating in the endothelium to repair disease injuries, remains to be investigated.

In the complex scenario of the pharmacology of angiotensin, it is worth to note that certain ACEI (e.g. captopril), at high concentrations, have been proposed as antiangiogenic tools exploiting their peptidase/protease inhibitor properties (Volpert et al., 1996, Molteni et al., 2003).

In conclusion, the results of this study clearly demonstrate that ACEI promote the formation of capillaries and activate the angiogenic programme of the microvascular endothelium. The execution of this programme occurs in an autocrine mode through the enhanced production of FGF-2 and the activation of endothelial FGFR-1.

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Footnotes:

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Legends for Figures

Figure 1: ACEI promote in vitro pseudocapillary formation by stimulating eNOS and FGF-2.

A. Porcine coronary artery rings were included in a 3D fibrin gel, covered with 1, 5 or 10% serum medium. Zofenoprilat was added at 1 μ M and refreshed every 2 days. After 10 days pseudocapillary formation was measured at the microscope, at a magnification of 200X, by an ocular grid. The area covered by pseudocapillaries was measured and expressed as mean \pm SEM of grid units. *** p <0.001 vs serum alone; n=6. **B.** Representative pictures of pseudocapillary formation from HUA rings cultured in 1% serum in the absence (a)/presence (b) of 1 μ M lisinopril. **C.** Cellular characterization of newly formed structures. Pseudocapillaries in HUA preparations were recovered at day 10 by fibrin lysis with plasmin and cells were spread on glass coverlips by cytospin. Microscopic counting of cells immunostained for CD31 and actin allowed the measurement of endothelial and smooth muscle cell components. Data, reported as % of CD31 positive cells, are the means of 5 preparations. **D.** Zofenoprilat-induced pseudocapillary formation is dependent on eNOS and FGF-2. Pseudocapillaries from PCA rings were evaluated at day 5 in 3D fibrin gels exposed to zofenoprilat (1 μ M) or FGF-2 (20 ng/ml) in the absence/presence of L-NMMA (200 μ M) or neutralizing anti-FGF-2 antibody (2 μ g/ml) in medium containing 1% FCS (n=3). * p <0.05 vs control condition, # p <0.05 vs zofenoprilat or FGF-2 alone.

Figure 2: ACEI activate gene transcription in CVEC. **A.** Cells were synchronized, treated with zofenoprilat (1 μ M, 15 min-18 h), and MAPK p44/42 phosphorylation was evaluated in cell lysates by western blotting. The effect was compared to 10% serum and normalized with total p44/42. Numbers (mean \pm SEM) represent OD ratio between phospho-ERK1/2 and total ERK1/2. **B and C.** Angiogenic factor expression induced by ACEI. RT-PCR was performed

to measure eNOS (B) and FGF-2 expression (C) on RNA extracted from serum-starved CVEC after 8 h treatment with test compounds. Target and control gene (GAPDH) amplification products were electrophoresed on a 3% agarose gel and stained with ethidium bromide. The ratio between the optical density values is reported for each sample. Results (mean±SEM) are representative of 3 independent experiments. Panel B: lane 1, control 0.1% CS; lane 2, zofenoprilat (1 µM); lane 3, zofenoprilat (20 µM); lane 4, Des-Arg9-BK (10 nM.); Panel C: lane 1, control 0.1% CS; lane 2, zofenoprilat (20 µM); lane 3, lisinopril (20 µM); lane 4, L-NMMA (1 mM); lane 5, L-NMMA (1 mM) and zofenoprilat (20 µM). **D and E.** FGF-2 protein production has been measured in CVEC lysates following zofenoprilat stimulation (1 µM, 8 h) in the absence/presence of the BK/B1 receptor antagonist or the MEK inhibitor U0126. Panel D: lane 1, control 0.1% CS; lane 2, zofenoprilat (1 µM); lane 3, R715 (1 µM); lane 4, R715 zofenoprilat. Panel E: lane 1, control 0.1% CS; lane 2, zofenoprilat (1 µM); lane 3, U1026 (10 µM); lane 4, U1026 + zofenoprilat. Numbers (mean±SEM) represent OD ratio between FGF-2 and actin (n=3).

Figure 3: ACEI promote post-capillary endothelial cell functions through BK/B1 receptor dependent eNOS pathway. **A.** Cell proliferation was evaluated at day 2 and 5 in 0.1% CS medium in the presence of 1 µM zofenoprilat. Zofenoprilat treatment: at 0 h for 2 day growth; at 0 and 72 h for 5 days growth. Data are expressed as total cell number counted/well. n=3. **p<0.05 vs basal condition. **B.** Dose-response curve for zofenoprilat induced migration. CVEC spontaneous migration was measured after 18 h incubation with zofenoprilat (0,01-1 µM). **Table inset.** Serum-starved CVEC were pretreated for different times (15 min – 24 h) with zofenoprilat (1 µM), and then seeded in the microchemotaxis chamber. Spontaneous cell migration was recorded after 4 h incubation in 1% CS medium. The effect was compared with FGF-2 (20 ng/ml) placed in the lower wells (166±6% of basal response). **p<0.01 vs 1% CS

(mean±SEM, n=4). **C.** Zofenoprilat-induced migration requires BK/B1 interaction and activation of the NOS pathway. Cells were treated with zofenoprilat (1 μM, for 18 h) in the absence/presence of B1 (R715), B2 (HOE140) receptor antagonists (1 μM each), L-NMMA (200 μM) or ODQ (10 μM), and then challenged for spontaneous migration. *p<0.05 vs control and #p<0.05 vs zofenoprilat alone.

Figure 4. Endogenous FGF-2 requirement for migration and proliferation induced by ACEI.

A. Cells were silenced for FGF-2. Western blotting was performed to validate silencing after 2 days exposure of CVEC to siRNA for FGF-2 or control siRNA. The bar graph shows the optical density of FGF-2/actin bands (means ± SEM of 3 experiments). **B.** Spontaneous migration measured in cells 18 h from silencing, and then exposed to zofenoprilat (1 μM). Data are reported as % response to zofenoprilat alone. Numbers are means±SEM of 3 experiments. ##p<0.01 vs zofenoprilat (open bar).

Figure 5. FGF-2/FGFR-1 axis is critical for ACEI induced angiogenesis. **A-D.** FGFR-1

labeling and localization in endothelial cells. CVEC were silenced for FGF-2 for 36 h and FGFR-1 localization was monitored by immunofluorescence. Panels A-B, control non transfected cells. Panels C-D, cells transfected with siRNA for FGF-2. Cells were treated with 1 μM zofenoprilat (B-D) for 18 h and labeling was compared to untreated cells (A-C). Arrows indicate positive nuclei. Original magnification 40X. **E.** Role of FGFR-1 autophosphorylation in proliferation induced by zofenoprilat. CVEC were exposed to zofenoprilat (1 μM) or FGF-2 (20 ng/ml) in the absence/presence of 1 μM SU5402. Test substances were added again at 72 h and cell proliferation was measured after a total of 5 day incubation. Data are reported as number of cells randomly counted/well and are the means±SEM of 3 experiments. **p<0.01 vs control cells and ##p<0.01 vs FGF-2 or zofenoprilat alone.

Figure 6. Schematic representation of the proposed molecular mechanisms activated by ACEI in endothelial cells and responsible for angiogenesis induction.

Fig. 1











