Sulfhydryl angiotensin converting enzyme inhibitor promotes endothelial cell survival through nitric oxide synthase, fibroblast growth factor-2 and telomerase cross-talk*

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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; FGF-2, fibroblast growth factor-2; L-NMMA, Nω-methyl-L-arginine; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PI-3K, phosphoinositide-3 kinase; ROS, reactive oxygen species; TERT, telomerase reverse transcriptase; VEGF, vascular endothelial growth factor.

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Abstract

The protective effect exerted by angiotensin converting enzyme inhibitors (ACEI) in cardiovascular diseases caused by endothelial injury/aging, has been attributed to the restoration of endothelial cell functions. Recently, we demonstrated a central role of fibroblast growth factor (FGF-2)/FGF receptor-1 system in mediating the acquisition of an angiogenic phenotype in coronary microvascular endothelium exposed to ACEI. Here we report on the rescuing effect of ACEI on impaired endothelium and the intracellular signaling mechanisms which lead endothelial cells to enter apoptosis and to senesce. Conditions mimicking pathological cell damage (serum deprivation) lead to endothelial apoptosis as evidenced by increased caspase-3 activity. ACEI enhanced cell survival through activation of prosurvival and antiaging signals involving Akt phosphorylation, endothelial nitric oxide synthase (eNOS) expression and activation, FGF-2 and telomerase catalytic subunit (TERT) up-regulation and delayed senescence. In microvascular endothelial cells exposed to ACEI, Akt/eNOS pathway dependent FGF-2 was necessary for gene transcription of TERT. These protective effects were particularly evident for SH containing ACEI reported to exhibit potent antioxidant effects (namely zofenoprilat). In conclusion, ACEI with antioxidant properties upregulate eNOS, FGF-2 and TERT mRNA, which favor endothelial cell survival, prolonging their lifespan, thus restoring endothelial cell functions following vascular damage. These effects could explain the beneficial effects of these drugs in different cardiovascular diseases associated with endothelial injury and aging.
Introduction

Since their introduction in clinical use, the mechanism of inhibitors of angiotensin converting enzyme (ACEI) has evolved from the widely held tenet that their therapeutic benefit is exclusively associated with the decrease of vascular tone consequent to the inhibition of angiotensin II formation. Discrepancies, disputing the initial hypothesis, arise from clinical evidence indicating that ACEI provide significant benefits (e.g., decreased mortality) in a large number of patients suffering from cardiac heart failure, acute myocardial infarction and diabetes complications, in face of marginal decreases of blood vessels tone (Yusuf et al., 2000, Boss and Dawes, 2004, Kjeldsen and Julius, 2004, Brugts et al., 2009). The common thread linking these diverse diseases is dysfunction of the endothelium, widely recognized as one of the factors implicated in these pathologies. Reports in the literature illustrate the improvement of damaged endothelium afforded by ACEI, as well as their properties to promote vascular remodeling and to diminish the burden of cardiovascular risk factors (Galderisi and de Devitiis, 2008). The underlying mechanism of the protection exhibited by ACEI, has been attributed to their ability to influence the enzyme endothelial nitric oxide synthase (eNOS), favoring the proper assembly of the enzyme complex, and the engagement of its downstream effectors (Dimmeler et al., 1997b). ACEI have been documented to restore the functioning of eNOS from the molecular injuries inflicted by oxidative stress, a feature typical of the diseased endothelium, utilizing a complex set of intracellular signals (Münzel and Keaney, 2001, Ignarro et al., 2002, Kohlstedt et al., 2004, 2005).

Recently, we have contributed in delineating the pattern of molecules lying downstream eNOS, describing the up-regulation of fibroblast growth factor-2 (FGF-2) by ACEI in coronary endothelium. FGF-2 over-expression appears to act in an autocrine mode, triggering the FGF-
2/FGFR-1 system, hence promoting endothelial cell survival and the development of the angiogenic phenotype (Donnini et al., 2006).

The marked effect exerted by ACEI on the survival properties of coronary endothelium led us to investigate other signals which might contribute to the overall protection. We focused on Akt and telomerase reverse transcriptase (TERT) activities, both signals being involved in the survival processes operant in a wide variety of cells (Breitschopf et al., 2001, Jakob and Haendeler, 2007). TERT is especially interesting in the context of the diseased endothelium repair, because this reverse transcriptase enzyme opposes the apoptotic drive of oxidative stress and delays vascular senescence (Murasawa et al., 2002, Brandes et al., 2005). We therefore performed a study on cultured post-capillary coronary endothelial cells of bovine origin (CVEC), cultured in extremely low serum (0.1% CS), analogous to the deprived nutrient condition likely to occur in ischemic cardiac pathologies. We investigated both functional endothelial parameters (survival and neo-capillaries formation) and signals relevant to cell survival and death (Akt, NO/cGMP, FGF-2, TERT and caspase-3) and the influence of a panel of ACEI either bearing an SH group in their moiety (captopril, zofenoprilat) or devoid of it (enalaprilat, lisinopril), on these processes.
Methods

Pseudocapillary formation from vessel rings

Pseudocapillary sprouting from vessel rings of human umbilical artery (HUA) and characterization of the cells present in the pseudocapillaries were evaluated as described (Donnini et al., 2006).

Cell cultures

Coronary venular endothelial cells (CVEC) were isolated and cultured as described (Schelling et al., 1988). FGF-2-/—endothelial cells were kindly provided by Prof. P. Mignatti (New York University School of Medicine, USA), and cultured as described (Yu et al., 2008).

Survival studies

One thousand cells/well (of a 96 multiplate) were resuspended in 10% calf serum (CS), and after adherence (3-4 h) the supernatant was replaced with medium containing ACEI (0.01-10 μM) and 0.1% CS. Cells were kept in culture for 5 consecutive days and media with test substances was added freshly at 36 and 72 h. Cells were then fixed, stained and randomly counted at 10X magnification (Donnini et al., 2006). Cell survival in response to ACEI was also assessed by the MTT test (Pezzatini et al., 2007). Data of absorbance units at 540 nm were reported as % of basal response.

Detection of apoptosis

Apopain/caspase 3 activity was measured in cell lysates (Cantara et al., 2004). Cells (density of 1x10⁵ cells/6 cm diameter dish) were serum-starved overnight. Stimulation was carried out for 6 h in medium containing 0.1% serum additioned with 1 and 10 μM zofenoprilat in the absence/presence of 3 mM L-NMMA. Then adherent and in suspension cells were pooled and lysed. Apopain/caspase-3 activity was monitored in vitro using the FluorAceTM Apopain assay (BIO-RAD, Milan, Italy) following manufacturer instructions.
Senescence-associated β-galactosidase (SA-β–gal) activity assay

CVEC were seeded in 6-well multiplates. After adherence and serum starved, cells were exposed to the stimuli for 3 days. SA-β–gal activity was measured by the use of Cellular Senescence Assay KAA002 (Chemicon-Millipore, Milan, Italy) following the manufacturer instructions. A total of 400 cells were counted manually, and the percentage of senescent cells over the total cell number was determined.

Western blotting for phospho-Akt, phospho-eNOS and cleaved caspase-3

Western blotting was performed as described by Donnini et al., (2006). 3x10^5 cells were plated in 6 cm diameter dishes. After adhesion, cells were serum-starved overnight, then exposed to ACEI or FGF-2 (10 min-6 h). Electrophoresis (50 μg of protein/sample) was carried out in SDS/10 or 15% polyacrylamide gels. Proteins were then blotted onto nitrocellulose membranes, incubated overnight with anti-phospho-Akt antibody, anti-phospho-eNOS (Ser1177) and caspase-3 (each at 1:1000) and then detected by enhanced chemiluminescence system (Amersham, GE Healthcare, Milan, Italy). Results were normalized to those obtained by using an antibody against total Akt.

Nitric oxide synthase activity and cGMP production

Sparse and synchronized CVEC were stimulated with different ACEI (1 μM, 18 h). cGMP accumulation was evaluated in cell lysates by EIA kits (Cayman, CABRU, Milan, Italy) as described (Cappelli et al., 2004).

eNOS, FGF-2 and TERT expression of by quantitative RT-PCR

Subconfluent endothelial cells were let to adhere in 6 cm diameter plates. After 24 h of serum starving, CVEC were stimulated with ACEI (1-10 μM) for 6 h. Total RNA was extracted using RNeasy Kit (Qiagen, Milan, Italy). Total RNA (1 μg) was reverse transcribed using iScript™
cDNA Synthesis kit (BIO-RAD, Milan, Italy), with the following thermal profile: 25°C for 5 min, 42°C for 30 min and 85°C for 5 min. For all of the further analyses, the cDNA was diluted in water to obtain a final concentration of 30 ng/μl.

For each screened gene, iQ™ SYBER® Green Supermix (BIO-RAD) and unlabelled primers designed with Beacon Design programme (BIO-RAD) and purchased from Applied Biosystems were used: eNOS forward 5’-GCT TGA GAC CCT CAG TCA GG-3’, reverse 5’-GGT CTC CAG TCT TGA GCT GG-3’; FGF-2 forward 5’-TCA GCT CTT AGC AGA CAT TGG AAG AAA AAG-3’, reverse 5’-GGA GTG TGT GCT AAC CGT TAC CTG GCT ATG-3’; and TERT forward 5’-ATG GGG ACA TGG AGA ACA AG-3’, reverse 5’-GTG AAC CTG CGG AAG ACG GT-3’. β-actin (forward 5’-TGA ATG GAC AGC CAT CAT GGA C-3’, reverse 5’-TCT CAA GTC AGT GTA CAG GAA AGC-3’) was used as reference gene. 300 nM of each primer was used together with 20 μl of iQ™ SYBER® Green Supermix (BIO-RAD) and water in a total volume of 25 μl. After enzyme activation for 10 min at 95° C, 40 two-step cycles were performed (15 sec, 95° C; 1 min, 60° C) in the iCycler detector (BIO-RAD). Differentially expressed genes were quantified by using the threshold cycle (Ct) and the comparative method (2^ -ΔΔCt) as described (Livak and Schmittgen, 2001). Data are reported as fold increase of basal condition (0.1% CS).

Silencing technique

A 21-nucleotide RNA [sense r(ACG AAC UGG GCA GUA UAA A)dTdT, antisense r(UUU AUA CUG CCC AGU UCG U)dTdT, FGF-2 gene ID NM_002006] and control (non silencing) siRNA (catalog n. 1022076) were chemically synthesized by Qiagen (Milan, Italy). Transient transfection of siRNA in subconfluent endothelial cultures and silencing efficacy were performed as previously described (Donnini et al., 2006).
Materials and reagents

Cell culture reagents and captopril were from Sigma Life Science (Milan, Italy). Fetal calf serum (FCS) and calf serum (CS) were from Hyclone (Celbio, Milan, Italy). VEGF and FGF-2 were from Peprotech (London, UK). NOC12, the NOS inhibitor Nω-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 PI3K inhibitor LY294002 were from Calbiochem (Merck, Nottingham, UK). Anti phospho-Akt (Ser473), anti Akt, anti phospho-eNOS (Ser1177), anti caspase-3 and neutralizing anti-FGF-2 antibodies were obtained from Cell Signalling (Celbio, Milan, Italy). Zofenoprilat, enalaprilat and lisinopril were kindly provided by Menarini Ricerche, Florence (Italy).

Statistical analysis

Results are expressed as means ± S.E.M. 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

Prosurvival effect of ACEI on microvascular endothelium

Given the favorable effect exerted by ACEI on endothelial dysfunction (Scribner et al., 2003), we investigated whether these positive activities could be observed in the survival of endothelial cells cultured in oxidative stress condition as serum deprivation (Martin et al., 2005). We studied a panel of ACEI, either bearing the SH group in their moiety (captopril and zofenoprilat) or those devoid of this functional group (e.g. enalaprilat and lisinopril).

Exposure of endothelial cells for 5 consecutive days to increasing concentrations of the ACEI (0.01-10 μM) highlighted different prosurvival properties of the ACEI (Fig. 1A). MTT data demonstrate that zofenoprilat was the most effective producing maximal effect at 1 and 10 μM. Captopril was less potent than zofenoprilat, inducing maximal survival at 10 μM. The ACEI lacking the SH group were less effective, producing a bell shaped dose-response curve with maximal effect at 0.1 μM (Fig. 1A).

We then evaluated the effect of these compounds on the formation of pseudocapillaries from vascular explants of human umbilical artery (HUA) in the presence of low serum concentration (0.1% FCS) for up to two weeks in organ cultures. All compounds, tested at 1 μM, induced pseudocapillary sprouting, expressed as area occupied by capillaries at 10 days after continuous addition of drugs (Fig. 1B). Significant differences among the compounds were noted, the SH containing compounds (captopril and zofenoprilat) producing the richest vascularization (2 to 3 fold increase relative to 0.1% FCS), while enalaprilat and lisinopril were much less efficacious.

Evaluation of the endothelial marker CD31 in pseudocapillaries showed a time-related decline in those grown in low serum (from 15 ± 3% of CD31 positive cells at day 10 vs 10 ± 2% at day 15, n=5), which was reversed by zofenoprilat (from 20 ± 2% CD31 positive cells at day 10 to 30 ± 4% at day 15, n=5). Thus the drug promotes the development of endothelial cell rich pseudocapillaries.
In light of the above results, we selected zofenoprilat for further studies on cultured endothelial cells.

Apopain/caspase-3 activity, an early marker of cells entering apoptosis, determined in low serum (0.1% CS), was greatly increased relative to cells grown in higher serum concentrations (Fig. 2A). Zofenoprilat (1-10 µM for 6 h) reduced caspase-3 activity, reaching significance \((p < 0.01)\) at 10 µM (Fig. 2A). The protective effect of zofenoprilat on the apoptosis signal was linked to a functioning NOS pathway, since enzyme inhibition by L-NMMA reported caspase-3 levels to the basal activity \((p < 0.05, \text{Fig. } 2B)\).

Since in conditions of growth factor deprivation, cell apoptosis and senescence are closely linked (Brandes et al., 2005, Wang et al., 2007), we determined the effect of zofenoprilat (1-10 µM, for 3 days) on senescence associated β-galactosidase (SA-β-gal) activity. The presence of SA-β-gal positive endothelial cells, enhanced by serum deprivation, was markedly reduced by the drug (>50%), which exerted a protective effect comparable to that of the angiogenic/prosurvival growth factor FGF-2 (20 ng/ml) (Fig. 2C).

As molecular target of endothelial survival/longevity, we examined TERT by measuring its mRNA expression. In preliminary experiments in CVEC exposed to zofenoprilat (10 µM), we determined that peak expression of the messenger occurred at 6 h \((1.8 \pm 0.4, 6 \pm 0.9 \text{ and } 4.8 \pm 0.3 \text{ fold increase, at } 1, 6 \text{ and } 18 \text{ h, respectively, } n=3)\). Upon incubation with ACEI (all at 10 µM) we observed varying degrees of TERT mRNA expression (Fig. 2D). Zofenoprilat increased TERT mRNA (6 fold), approaching the level reached by FGF-2 (20 ng/ml), while enalaprilat appeared to be the weakest among the compounds tested. VEGF was devoid of any activity, as previously reported (Kurz et al., 2003) (Fig. 2D). Moreover, the effects exerted by zofenoprilat and enalaprilat were also observed in cultured HUVEC (above described conditions), in which TERT mRNA expression increased 5.4 ± 0.17 and 1.4 ± 0.34 fold over basal condition, respectively.
Activation of PI-3K/Akt and eNOS signalling in endothelium exposed to ACEI

We extended the analysis to prosurvival signals related to the FGF-2, since its upregulation appears to be crucial for the protection exerted by ACEI on the endothelium (Donnini et al., 2006). We examined PI-3K/Akt phosphorylation, eNOS expression and phosphorylation state/activity, and the expression of TERT mRNA in serum deprived cultured coronary endothelial cells.

Zofenoprilat (10 $\mu$M) enhanced the phosphorylation of Akt (5-6 fold over control), activation being slow but persistent (up to 6 h), whereas that produced by FGF-2 was rapid, declining with time (Fig. 3A and B).

Assessment of eNOS activation downstream of Akt, measured by phosphorylation of eNOS in Ser1177 (Dimmeler et al., 1999), evidenced that zofenoprilat increased eNOS activation (Fig. 3C). Further, treatment of the cells with the PI-3K inhibitor LY294002, obliterated the phosphorylation of Akt and eNOS produced by zofenoprilat, indicating that PI-3K/Akt is the target of the ACEI.

Next, we evaluated both the expression of eNOS (through RT-PCR), and its enzyme activity by measuring cGMP accumulation in response to the compounds under study in serum deprived endothelial cells. Among the compounds, only zofenoprilat induced a strong increase in eNOS expression (16 fold), whereas both lisinopril and enalaprilat failed to upregulate it (Table 1).

Similarly, zofenoprilat (1 $\mu$M) nearly doubled the amount of cGMP produced by cells, while neither lisinopril nor enalaprilat were effective on cGMP accumulation, providing evidence that the enhanced enzyme expression levels translated in increased NO production. Indeed, the effect of zofenoprilat on eNOS enzymatic activity (measured by conversion of L-arginine in L-citrulline after 15 min of incubation) was sensitive to the inhibitory action of L-NMMA (30 ± 5 and 5 ± 2 % increase over basal NOS activity by 1 $\mu$M zofenoprilat in the absence and presence of 2 mM L-NMMA, respectively).

Consistent with the role of PI-3K/Akt and eNOS pathways on the senescence and survival signals activated by the ACEI, PI-3K inhibition abolished FGF-2 and TERT upregulation (Fig. 3D and E) as well caspase-3 activity (Fig. 3F). Further, simultaneous treatment of the cells with zofenoprilat
with the MEK inhibitor U0126 (10 μM each, 6 h) resulted in obliteration of TERT upregulation (1.8 ± 0.6 fold increase, n=3), indicating a role for ERK1-2 activated by ACEI in TERT expression.

The eNOS/cGMP axis exerted a tight control on zofenoprilat-induced telomerase overexpression, as its blockade with L-NMMA and ODQ abolished the activation of the messenger formation (Fig. 3G). Conversely, incubation of cells with the NO donor drug NOC12 alone, markedly promoted TERT transcription (p<0.01) (Fig. 3G).

These data document that zofenoprilat significantly activates PI-3K/Akt and eNOS signaling, which subsequently promotes MAPK activation and FGF-2 and TERT upregulation and prosurvival outcome.

Central role of endogenous FGF-2 in ACEI effects on survival and senescence

As ACEI upregulate FGF-2 formation (Fig. 3D and see also Donnini et al., 2006) necessary for ACEI-mediated cell survival (Fig. 4A), we wondered whether the overexpression of the growth factor might induce the observed activation of TERT mRNA and long term survival. When the zofenoprilat-induced FGF-2 rise was prevented through FGF-2 silencing, we detected no increase of TERT mRNA in response to the drug, suggesting a causal link between these events (Fig. 4B).

Since FGF-2 stored in the extracellular matrix can be rapidly released by different stimuli (Finetti et al., 2008, Murakami et al., 2008), we assessed its specific role in ACEI effect in FGF-2 null endothelial cells (FGF-2−/) and by a neutralizing anti-FGF-2 antibody.

In FGF-2−/ cells exposed to zofenoprilat, the phosphorylation of Akt as well as that of eNOS in Ser1177 was impaired, and the long term prosurvival effect elicited by zofenoprilat lost even at the high doses (Fig. 5A and B). Moreover, in the absence of FGF-2 we could not find the antiapoptotic effect of the ACEI (the percent of caspase-3 cleavage over total caspase-3 was 20 ± 4 and 17 ± 5 %, in 0.1% CS and after 1 μM zofenoprilat treatment, respectively, n=3). Consistently, neutralization of FGF-2 from extracellular stores also resulted in ablation of zofenoprilat induced TERT mRNA expression (Fig. 5C), documenting a crucial role for stored FGF-2 in the effects elicited by ACEI.
Collectively, these data strongly support the central role of endogenous FGF-2, both released from pre-stored pools and de novo synthesized, in the control of Akt/eNOS signalling leading to increased survival and reduced senescence.
Discussion

This study delineates a pattern of closely interrelated signals which when stimulated by ACEI activate the microvascular endothelium to form capillaries, induce cell survival and delay endothelial replicative senescence. Because the experimental conditions used in this work, i.e. coronary venous endothelial cells cultured in extremely low serum (0.1% CS), induce a large increase of reactive oxygen species (ROS) (Martin et al., 2005), we focused on the PI-3K/Akt and eNOS pathways, both signals known to regulate ROS production (Chen et al., 2008). The endothelial protective actions exerted by ACEI on a wide range of human pathologies (Yusuf et al., 2000, Kjeldsen and Julius, 2004, Napoli et al., 2008) lead us to investigate signals involved in cell survival (FGF-2) and on the senescence process (TERT). We show that, among the ACEI examined here, those bearing an SH group in their moiety (captopril, zofenoprilat), exhibit a distinct pro-survival effect, whereas those devoid of it (enalaprilat, lisinopril) are much less active.

The greatest differences were observed in the ability to promote the eNOS messenger RNA expression and to stimulate NO production. In fact, whereas zofenoprilat induced a large increase of eNOS expression (sixteen fold), that elicited by enalaprilat or lisinopril was barely over control (two-fold). Correspondingly, we observed varying degrees of enhanced NO production in cells exposed to ACEI, which in the case of zofenoprilat was higher (3 fold) than in control. The magnitude of these signaling changes has an impact on cell functions. Examples are the promotion of endothelial survival and the acquisition of the angiogenic phenotype, demonstrated by the rich capillary sprouting, following zofenoprilat sub-chronic exposure. Thus a correlation might be drawn between the upregulation of the eNOS pathway and endothelial protective functions by SH-containing drugs. Interestingly, the reversal of endothelial dysfunction by ACEI, a concept proposed two decades ago (Rajagopalan and Harrison, 1996), continues to be a matter of study as shown by a recent clinical trial in moderately hypertensive patients, reporting significant improvements, by zofenoprilat, of parameters related to oxidative stress (Napoli et al., 2008).
The rapid and steady activation of PI-3K/Akt pathway, signal related to eNOS activation, observed mainly for zofenoprilat, is of interest as it may promote the alignment of the eNOS molecule in a conformation conducive to efficient NO production, thus contributing to overcoming the stress-induced enzyme uncoupling. The ACEI-induced enhancement of Akt phosphorylation and of NO production have been reported to suppress the prevailing apoptotic program present in serum deprived endothelial cells, as in this study (Dimmeler et al., 1997a, Mital et al., 2002).

The other salient finding of this work concerns the ability of ACEI, particularly zofenoprilat, to reverse early signs of cell replicative senescence produced by the hostile nutrient deprived environment. Indeed, application of ACEI to endothelial cells promoted TERT overexpression (3 to 6 fold), concomitant with a reduction of the SA-β-gal activity accumulation. The mechanism whereby ACEI promote TERT, in the present conditions, appears to be related to the activation of the FGF-2 pathway, since its silencing or neutralization abrogates TERT expression. While the evidence indicating downregulation of telomerase by oxidative stress is overwhelming (Haendeler et al., 2004, Voghel et al., 2008, Erusalimsky and Skene, 2009), interventions which favour its up-regulation are limited to NO donor drugs (Vasa et al., 2000), statins (Haendeler et al., 2004), low dose aspirin (Hu et al., 2008), estrogen (Grasselli et al., 2009), and FGF-2 (Kurz et al., 2003). The direct stimulation of hTERT expression by NO, either endogenously produced or delivered by NO donor agents as shown here, has been recently disputed (Erusalimsky and Skene, 2009). These discrepancies, albeit important, have little influence on our interpretation since we propose that the endothelial telomere system is regulated by the steady FGF-2 output, the effects of which are antagonized by inhibitors of Akt, NO and MAPK pathways.

The mechanism outlined in the present and previous work (Donnini et al., 2006) illustrates the central action of FGF-2 in counteracting the arrest of endothelial replicative capacity through the up-regulation of the telomere system. This study further reinforces the necessity of the endogenous FGF-2, both released for the pre-stored pools and synthesized de novo, for endothelial survival and functioning. These ACEI-induced “protective effects”, not accounted by the inhibition of
angiotensin converting enzyme, are class related, although SH bearing drugs are far more efficacious protective agents. Other signalling pathways have been recently described (Münzel and Keaney, 2001, Ryan and Sigmund, 2004, Kohlstedt et al., 2004, 2005). These reports have demonstrated the presence in the vascular endothelium of a specific ACE pathway, with numerous ramifications, which include the enhanced synthesis of the cardio-protective prostacyclin (Kohlstedt et al., 2005) and may concur to the overall beneficial effects of ACEI.

In light of these novel findings which provide a rationale for the ACEI-promoted benefits, the question arising is whether the current ACEI therapeutic regimens are adequate to sustain the full expression of the above mechanisms in terms of dosage and kinetic profile.
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References


derivative through depressing integrin beta4 in vascular endothelial cells. *Endothelium* **14**: 325-332.

Footnotes:

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Legends for figures

**Figure 1**: Prosurvival effect of ACEI on endothelial cells. **A.** Effect of different ACEI on the survival of microvascular endothelium. CVEC were incubated with increasing concentrations (0.01-10 μM) of drugs and survival was measured by MTT test at day 5. Data are reported as % of basal response. n=3. *, p < 0.05 vs 0.1% CS condition. **B.** Human umbilical artery rings were included in a 3D fibrin gel, covered with 0.1 or 5% serum medium. ACEI were added at 1 μM (in low serum condition) and medium with test substances was 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 grid units. n=5. *, p < 0.05 and ***, p < 0.001 vs 0.1% FCS alone.

**Figure 2.** ACEI impair serum deprivation-induced apoptosis of endothelial cells and prevent their ageing. **A.** The effect of zofenoprilat on caspase-3 activity was evaluated in sparse, serum-starved CVEC after 6 h of incubation with 1 and 10 μM of the drug in the presence of 0.1% CS. Data are expressed as increase in fluorescence of caspase activity/sample after 4 h incubation. n=3. *, p < 0.05 and **, p < 0.01 vs 0.1% CS. **B.** NOS inhibition reverts the caspase-3 activity reduced by zofenoprilat. Cells were pre-treated with 3 mM L-NMMA before zofenoprilat addition (10 μM for 6 h). n=3. *, p < 0.05 vs 0.1% CS and #, p < 0.05 vs zofenoprilat alone. **C.** Zofenoprilat inhibits endothelial senescence. CVEC were incubated with different concentrations of zofenoprilat (1-10 μM) and FGF-2 (20 ng/ml) for 3 days. Cell senescence was evaluated by SA-β-gal staining. Representative pictures of SA-β-gal positive cells (blue) are reported. After 3 days in culture, the number of SA-β-gal positive cells was counted on a total of 400 cells. n=3. *, p < 0.01 vs control. **D.** ACEI and growth factors differently induce TERT transcription. ACEI (10 μM), FGF-2 and VEGF (20 ng/ml each) were tested on TERT mRNA expression measured by real time RT-PCR.
Data are reported as fold increase in telomerase expression (corrected for actin transcription) over control condition (0.1% CS). n=4. *, p < 0.05 and **, p < 0.01 vs 0.1% CS.

Figure 3. Molecular mechanisms activated in microvascular endothelium by ACEI: role of PI-3K/Akt and eNOS pathway. A-B. Akt phosphorylation by FGF-2 and ACEI. A representative blot is reported. The bar graphs represent the optical densities related to the ratio phospho-Akt/Akt. n=3. *, p < 0.05 vs time 0. C. Role of PI-3K activity on Akt and eNOS phosphorylation. Cells were incubated with 1 μM LY294002 and then stimulated with 10 μM zofenoprilat for 10 min. FGF-2 (20 ng/ml) was used as positive control. Phospho-Akt and phospho-eNOS (Ser1177) were then detected by western blotting. The blot is representative of 3 with overlapping results. D. PI-3K activity controls FGF-2 mRNA expression. Cells were treated with 1 μM LY294002 and then stimulated with 10 μM zofenoprilat for 6 h. FGF-2 mRNA transcription was monitored by RT-PCR. n=3. *, p < 0.05 vs 0.1% CS alone, #, p < 0.01 vs zofenoprilat alone. E. ACEI induced TERT transcription is under the control of Akt activation. CVEC were exposed for 6 h to zofenoprilat (10 μM) in the absence/presence of LY294002 (1 μM). n=3. *, p < 0.05 vs 0.1% CS alone, #, p < 0.01 vs zofenoprilat alone. F. Zofenoprilat reduces caspase-3 cleavage in an Akt dependent manner. CVEC were stimulated with 10 μM zofenoprilat in the absence/presence of 1 μM LY294002 for 6 h. Caspase-3 cleavage was evaluated by western blotting using an antibody recognizing the total and cleaved caspase-3. The blot is representative of 3 with overlapping results. G. Role of NOS/cGMP pathway in the control of telomerase expression. CVEC were treated with 200 μM L-NMMA or 10 μM ODQ for 30 min before zofenoprilat or enalaprilat (10 μM) addition. Some cells were also treated with 10 μM NOC12 alone. Telomerase expression was evaluated after 6 h by RT-PCR. n=3. *, p < 0.05 and **, p < 0.01 vs 0.1% CS alone; #, p < 0.01 vs zofenoprilat alone.
Figure 4. FGF-2 upregulation is essential for cell survival and telomerase transcription. A. CVEC silenced for FGF-2 for 36 h (FGF-2 mRNA levels were 10 fold less than in not treated cells or cells treated with siControl, while FGF-2 protein expression was reduced by 90 ± 4% respect to siControl treatment) were seeded for survival assay. Zofenoprilat (10 μM) was freshly re-added at 36 and 72 h and cell number was counted after 5 days. Data are reported as number of cells counted/well (n=6 in triplicate). The number of cells counted was: 140 ± 2 and 197 ± 8 in 0.1% CS and zofenoprilat alone, respectively. *, p < 0.05 vs siControl alone, and #, p < 0.05 vs zofenoprilat in siControl cells. B. mRNA for TERT was measured in cells silenced for FGF-2 and stimulated for 6 h with zofenoprilat (10 μM). n=6. Data are expressed as fold increase relative to 0.1% CS condition without any treatment. *, p < 0.05 vs siControl alone and #, p < 0.05 vs zofenoprilat in siControl cells.

Figure 5. Endogenous FGF-2 is necessary for the ACEI endothelial protective functions. A. FGF-2−/− cells (Yu et al., 2008) were stimulated with 10 μM zofenoprilat or FGF-2 (20 ng/ml) for 10 min. Phospho-Akt and phospho-eNOS (Ser1177) were then detected by western blotting. The blot is representative of 3 with overlapping results. B. Zofenoprilat was unable to promote survival (evaluated as MTT after 5 days of incubation) of FGF-2−/− cells, while the effect of exogenously added FGF-2 is statistically significant (**, p < 0.05 vs 0.1% CS). Data are reported as % of basal response. n=3. C. TERT transcription was measured in CVEC treated with a neutralizing anti-FGF-2 antibody and stimulated for 6 h with zofenoprilat (10 μM). n=3. *, p < 0.05 vs 0.1% CS and #, p < 0.05 vs zofenoprilat alone.
Table 1: eNOS expression and cGMP levels in endothelial cells exposed to ACEI

<table>
<thead>
<tr>
<th>Preincubation (18 h)</th>
<th>eNOS expression (fold increase)</th>
<th>Stimuli for cGMP (15 min)</th>
<th>cGMP levels (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>1.00</td>
<td>none</td>
<td>0.2 ± 0.05</td>
</tr>
<tr>
<td>none</td>
<td>--</td>
<td>NOC12</td>
<td>0.83 ± 0.08 **</td>
</tr>
<tr>
<td>Enalaprilat</td>
<td>1.50</td>
<td>none</td>
<td>0.33 ± 0.02</td>
</tr>
<tr>
<td>Lisinopril</td>
<td>2.08</td>
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<td>0.40 ± 0.08</td>
</tr>
<tr>
<td>Zofenoprilat</td>
<td>16.79</td>
<td>none</td>
<td>0.59 ± 0.01 *</td>
</tr>
<tr>
<td>Zofenoprilat</td>
<td>--</td>
<td>ODQ</td>
<td>0.13 ± 0.10 #</td>
</tr>
</tbody>
</table>

Cells have been treated with different ACEI (1 μM) for 18 h. eNOS mRNA expression was evaluated by real-time RT-PCR. Data are reported as fold increase over basal condition. In parallel dishes, following ACEI incubation and washing, basal cGMP levels have been measured in the supernatant after further 15 min of incubation. The NO donor drug NOC12 (10 μM) has been used as positive control. The soluble guanylate cyclase inhibitor ODQ (10 μM) has been used to check assay specificity. n= 3. *, p < 0.05 and **, p < 0.01 vs 0.1% CS medium; #, p < 0.05 vs zofenoprilat alone.
Fig. 1

A

![Graph showing survival vs. concentration for different drugs]

B

![Graph showing pseudocapillary formation for different conditions]

- Zofenoprilat
- Lisinopril
- Enalaprilat
- Captopril

Survival (% of basal response) vs. Concentration (µM)

Pseudocapillary formation (microscopic grid units)

- 0.1% FCS
- 5% FCS
- Zofenoprilat
- Lisinopril
- Enalaprilat
- Captopril

* Significant difference
Fig. 2

A

Caspase-3 activity (fluorescence increase)

<table>
<thead>
<tr>
<th></th>
<th>1% CS</th>
<th>0.1% CS</th>
<th>1 uM</th>
<th>10 uM</th>
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</thead>
<tbody>
<tr>
<td>Zofenoprilat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

Caspase-3 activity (fluorescence increase)

<table>
<thead>
<tr>
<th></th>
<th>None</th>
<th>L-NMMA 3 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% CS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 uM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Zofenoprilat
Fig. 2

C

0.1% CS  

Zofenoprilat (10 μM)

% senescent cells

0  5  10  15  20

0.1% CS  FGF-2  1 uM  10 uM

Zofenoprilat

D

TERT expression (fold increase)

0  2  4  6  8

0.1% CS  Zofenoprilat  Enalaprilat  FGF-2  VEGF

*  **
Fig. 3

A

FGF-2 (20 ng/ml)

P-Akt

Akt

B

Zofenoprilat (10 μM)

P-Akt

Akt

C

Zofenoprilat

FGF-2

LY294002

P-Akt

P-eNOS (Ser1177)

Akt

- + - - + + -

- - + - - + +

- - - + + + +
Fig. 4

A

Survival (cells counted/well)

- siFGF-2
- siControl

0.1% CS
Zofenoprilat

B

TERT expression (fold increase)

- siFGF-2
- siControl

0.1% CS
Zofenoprilat