ACE inhibition with perindopril and endothelial function. Results of a substudy of the EUROPA study: PERTINENT

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on behalf of the EUROPA Investigators on behalf of the PERTINENT Investigators and the Statistical Committee

Abstract

Objectives: EUropean trial on Reduction Of cardiac events with Perindopril in stable coronary Artery disease [EUROPA] demonstrates reduction in cardiovascular mortality and myocardial infarction for a possible vascular and antiatherosclerotic effect of angiotensin-converting enzyme (ACE) inhibition with perindopril. Our objective was to study the effect of perindopril on endothelial function and to verify its link to risk and occurrence of cardiovascular events.

Methods: Blood from 1200 EUROPA patients was withdrawn at baseline and after 1 year of treatment with either perindopril or placebo to measure von Willebrand factor and from 45 healthy subjects and 87 EUROPA patients to study endothelial function at the cellular level by cultivating in vitro human umbilical vein endothelial cells. In this setting, we determined protein expression/activity of endothelial nitric oxide synthase and the rate of apoptosis. Plasma levels of angiotensin II, bradykinin, tumor necrosis factor α, and nitrite/nitrate were also measured.

Results: The occurrence of cardiovascular events was related to von Willebrand factor at baseline (P=0.013) that also significantly decreased after 1 year’s treatment (P<0.001). Perindopril upregulated 19% and 27% protein expression/activity of endothelial nitric oxide synthase (P<0.05) as well as reduced the rate of apoptosis by 31% (P<0.05). There was also a significant reduction in levels of angiotensin II, increase in bradykinin, reduction in tumor necrosis factor α, and increase in nitrite/nitrate (P<0.05 for all).

Conclusions: Abnormal endothelial function occurs in patients with coronary artery disease, and this can be counteracted by angiotensin-converting enzyme inhibition with perindopril. These effects could contribute, at least in part, to explaining the results of the main EUROPA Study.

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Keywords: Inhibition; Perindopril; Endothelial function; von Willebrand factor

1. Introduction

The EUropean trial on Reduction Of cardiac events with Perindopril in stable coronary Artery disease (EUROPA)
demonstrated a reduction in cardiovascular mortality and myocardial infarction with angiotensin-converting enzyme (ACE) inhibition by perindopril (8 mg/day) in patients with coronary artery disease (CAD) [1]. The background hypothesis for the EUROPA study was a possible vascular and antiatherosclerotic effect of long-acting ACE inhibition as shown in different experimental studies [2,3].

In order to verify the mechanism of action of perindopril in secondary prevention, several substudies were conducted as part of the EUROPA program [4–6]. Among them, PERTINENT (PERindopril—Thrombosis, Inflammation, Endothelial dysfunction and Neurohormonal activation Trial) [7] examined the effects of perindopril on endothelial function, thrombosis, and inflammation. We report here the results of PERTINENT relevant to endothelial function which was studied: (i) by evaluating the predictive role of von Willebrand factor (vWF), a procoagulant product of the endothelium which is also a marker of endothelial function [8], in EUROPA patients treated for 1 year with perindopril 8 mg/day or placebo; and (ii) more directly, at cellular level, in EUROPA patients treated for 1 year with perindopril.

**2. Materials and methods**

Measurements were performed both before the screening visit (baseline) (n=1157 patients) and after 1 year of treatment with either perindopril (8 mg/day) or placebo (n=996 patients) from four countries (Italy, Greece, Portugal, and Spain) as well as in 45 healthy subjects and 87 CAD patients included in the EUROPA study. The biological end points were: changes in protein expression and activity of endothelial nitric oxide synthase (eNOS), rate of apoptosis, and protein expression of Bcl-2 associated protein X (Bax) and B cell lymphoma/leukemia 2 (Bcl-2). Plasma levels of angiotensin II (Ang II), bradykinin (BK), tumor necrosis factor α (TNF-α) and nitrite/nitrate (NOx) were also determined as they are known to affect endothelial function and to be modified by ACE inhibition.

**2.1. Blood processing**

Blood was collected in the morning from fasting patients (before administration of the daily dose of the treatment) after at least 30 min supine rest. Blood was drawn after venipuncture with a 21 gauge indwelling plastic/PTFE cannula and it was added with sodium citrate (3.8%), centrifuged to separate plasma and stored in aliquots at −20 °C until analysis. vWF was measured by a commercial Enzyme-Linked Immuno Sorbent Assay (ELISA) (American Diagnostic, Connecticut, USA) and standardized by reference from the National Institute for Biological Standards and Controls, Potters Bar, UK and is reported as %/Unit. Antigenic TNF-α was determined by a sandwich ELISA with commercially available kits (Biosource EASIA and R&D Systems, respectively). NOx was measured in serum as previously described [9].

Two aliquots of blood were separately collected from 45 healthy subjects and 87 CAD patients from Italian centers:

a) one was stored for 1 h, and then centrifuged at 1700 g for 15 min at room temperature. The obtained serum was frozen at −80 °C with cell culture and TNF-α measurement;

b) the other aliquot was placed in prechilled plastic tubes containing (final concentrations): 3.3 mM Na2EDTA,2H2O and, as protease inhibitors, 0.3 mM aprotinin, 4.2 mM leupeptin, 0.5 mg/mL perindoprilat, 1 mM Ro 42–5292. These inhibitors are needed to block the degradation of BK and the delivery of Ang II by endogenous enzymatic systems after sampling. The blood solution was gently mixed and immediately centrifuged at 4 °C for 15 min at 1700 g. The separated plasma was frozen at −80 °C until subsequent analyses.

The determination of Ang II and BK peptides consisted in a 3-step procedure.

1. Solid-phase extraction: Ang II and BK were extracted from plasma according to the method of Hermann et al. [10] modified using Oasis/Waters cartridges as solid phase.

2. HPLC separation: the dry residue was dissolved in 0.3 M acetate buffer, filtered (0.45 μm) and 200 μL were used for high-performance liquid chromatography (HPLC) separation following the procedure of Campbell et al. [11] modified as previously described [12].

3. Radioimmunoassay: evaporated specimens were used for peptide quantitation by standard RIA, using two kits from Peninsula Laboratories. For Ang II, the coefficient of variation (7.1%) for within-assay precision was established by running in the same RIA eight aliquots of a single plasma sample containing 20 pg/mL Ang-(1–8) octapeptide. The coefficient of variation (12.5%) for between-assay precision was determined by measuring the Ang-(1–8) octapeptide content of the same plasma on 15 different assays. For BK, the coefficient of variation (9.0%) for within-assay precision was established by running in the same RIA eight aliquots of a single plasma sample containing 20 pg/mL BK-(1–9). The coefficient of variation (14.1%) for between-assay precision was determined by measuring the BK-(1–9) content of the same plasma on 15 different assays. Normal Ang II and BK values in our laboratory are in the range of 4–17.5 pg/mL and 11–25 pg/mL, respectively.
Two separate series of experiments were conducted to further investigate the specific role of Ang II, BK and TNF-α on eNOS expression and rate of apoptosis:

a. pooled serum from control subjects (n=144) was added with peptides at three concentrations (Fig. 5) reflecting those (low, median and high) detected in CAD patients (further details in Results section);
b. serum from perindopril treated patients was added with 1 μM BK B2 receptor blocker (to establish the role of bradykinin).

2.2. Cell cultures

HUVECs were isolated from human umbilical cords according to Jaffe et al. [13] and cultivated as previously described [14].

eNOS: After protein extraction of HUVECs incubated for 48 h with 20% serum from either the control group or the patients from the EUROPA study, eNOS Western blotting and radiometric measurement of eNOS activity were performed as previously described [14,15].

Assessment of apoptosis: HUVECs were incubated for 72 h with 20% serum of either control group or patients from the EUROPA study. As a positive control, 48-h serum-starved HUVECs were used.

Quantitative assessment of apoptosis by flow cytometry: Apoptotic cells were detected by flow cytometry as previously described [14]. Apoptotic cells were detected on a PI histogram of cells as a hypodiploid peak.

Qualitative assessment of apoptosis by fluorescence microscopy: After treatment, cells were washed and spun on slides. For viewing 4,6-diamino-2-phenylindole (DAPI)-positive apoptotic cells showing typical chromatin condensation and apoptotic body formation, a fluorescence microscope was used (Nikon Optiphot-2 equipped with mercury lamp excitation and a specific DAPI filter).
Bax and Bcl-2: Western blotting was performed by using mouse monoclonal anti-human Bax and Bcl-2 antibodies (R&D and Serotec, both 1:1000) as primary antibodies. In order to compare baseline with follow-up protein expression, results are expressed as a ratio to the internal control obtained by serum subtraction.

2.3. Statistical analysis

Descriptive statistics were expressed as mean±SD. Comparisons between groups were performed with the Student’s t test or with the nonparametric Mann–Whitney test in the case of continuous variables while with $\chi^2$ test or Fisher’s exact test for categorical variables. Correlations between continuous variables were evaluated by means of Pearson’s or Spearman correlation coefficients. A logistic regression was performed with predictors variables as the most important risk factors to evaluate if the patients who dropped-out from the study are different from the others.

The relationship between the difference baseline minus 1-year values of the various variables in relation to treatment and baseline value was analyzed with analysis of covariance (ANCOVA). An ANCOVA including the relevant known risks factors or confounding factors (i.e., age, sex, diabetes, systolic and diastolic blood pressure, statin therapy, allocation to perindopril or placebo) was also performed. The prognostic role of vWF on survival was performed applying a log-rank test. The effect of treatment and its interaction with vWF was evaluated by a Cox proportional hazard model. This analysis included not only the prognostic factors defined in the protocol but also the interaction between treatment and diabetes already investigated in another substudy [6]. The selection of the most important prognostic factors was performed in the different models using the Akaike Information Criterion (AIC). For each test, the significance level was established at $P<0.05$.

2.4. Sample size calculation

A total of about 1200 patients were determined a priori based on the case and control patients as those with and without events as occurred in the main EUROPA study [1]. For the study at cellular level, a total of 87 patients (and 45 healthy subjects) were selected based on “logistic” facilities related to the blood sampling and processing for this explorative part of the substudy.

3. Results

The baseline characteristics of patients enrolled are reported in Table 1. There were no significant differences between active and placebo in either groups or from the overall EUROPA study population ($P=NS$). The analysis of possible differences between patients who remained in the

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**Table 1: Baseline Characteristics of Patients**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWF</td>
<td>1.37 (1.07-1.75)</td>
<td>0.013</td>
</tr>
<tr>
<td>Age</td>
<td>1.51 (1.09-2.10)</td>
<td>0.014</td>
</tr>
<tr>
<td>SBP</td>
<td>1.14 (0.86-1.51)</td>
<td>n.s.</td>
</tr>
<tr>
<td>DBP</td>
<td>1.20 (0.82-1.58)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Sex</td>
<td>0.54 (0.23-1.22)</td>
<td>n.s.</td>
</tr>
<tr>
<td>DIA</td>
<td>2.01 (1.25-3.27)</td>
<td>0.004</td>
</tr>
<tr>
<td>STA</td>
<td>1.29 (0.85-1.97)</td>
<td>n.s.</td>
</tr>
<tr>
<td>TREAT</td>
<td>0.95 (0.63-1.47)</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

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**Fig. 2. Prognostic role of von Willebrand factor (vWF) for cardiovascular events. Abbreviations: EUROPA, EUropean trial on Reduction of cardiac events with Perindopril in stable coronary Artery disease.**

**Fig. 3. Univariate and multivariate analyses of von Willebrand factor (vWF) adjusted by confounding factors: hazard ratio (5–95% confidence intervals); $P$ values. Reference category for sex=M. The effect of diabetes is shown as main effect plus treatment interaction. Abbreviations: SBP, systolic arterial pressure; DBP, diastolic arterial pressure; DIA, diabetes; STA, statin treatment; TREAT, treatment.**
study and those who dropped-out showed that there were no differences. Moreover, in the subset of patients in whom vWF was measured, the cardiovascular event rate was lower (7% and 8% in perindopril and placebo, respectively) than that of the total EUROPA population (8% and 9.9% in perindopril and placebo, respectively) due to the smaller sample size (1/10). However, the relative risk reduction showed a similar trend to that in the overall study.

The 45 healthy subjects—the control group for the cellular level study—had similar age, gender, and blood pressure as the patients (61 ± 8 years; eight female (18%); 131 ± 10/80 ± 6 mm Hg, respectively). None had clinical signs of acute or chronic illness or was receiving any treatment.

Fig. 1 shows that, at baseline, vWF levels were at the upper limit of the normal range (44−158%/Unit) with the mean values being 145.0 ± 37.0 and 142.0 ± 33.9%/Unit in the placebo and perindopril group, respectively (P = NS). In diabetic patients (15%), vWF levels were significantly higher than in non-diabetic patients (151 ± 35 versus 142.0 ± 35%/Unit, respectively; P = 0.002). The univariate survival analysis showed that the occurrence of cardiovascular events was related to vWF at baseline (hazard ratio [HR]: 1.37 (1.07−1.75); P = 0.013) (Figs. 2 and 3). The same analysis also shows the predictive role for age and diabetes (Fig. 3).

The multivariate survival analysis on vWF, age, SBP, DBP, sex, diabetes, use of statins and treatment allocation confirmed the predictive role for cardiovascular events of vWF and age. Diabetes—which was a prognostic predictor in the univariate analysis—lost its prognostic role in the multivariate analysis due to the higher impact of the treatment effect in diabetic patients (the coefficient of the Cox model for diabetes is 1.17 (0.33) while −1.06 for the interaction between treatment and diabetes (Fig. 3).

vWF levels significantly decreased after 1 year of treatment with perindopril 8 mg/day versus placebo (P < 0.001) (Fig. 1). An ANCOVA model, adjusted for the previously defined confounding factors including statins, confirmed that the changes in vWF levels were related to treatment (coefficient −5.97; SE 2.27; P = 0.012), diabetes (coefficient −5.74; SE 2.27; P = 0.012), and age (coefficient −0.42; SE 0.09, P < 0.001).

Incubation of HUVECs with serum taken at baseline from patients with CAD showed a significant down-regulation of eNOS protein expression and activity (−26% and −30%, respectively; both P < 0.01 compared with incubation with serum from the control group). At 1 year, the down-regulation of eNOS protein expression and activity was modulated by the treatment with perindopril: up-regulation by 19% and 27% for eNOS protein expression (P = NS) and activity (P < 0.05), respectively, was observed (Table 2).

This modulation was at least in part mediated by the activation of BK B2 receptors since the use of icatibant—a

Table 2
Parameters of endothelial function evaluated in vitro and neurohumoral activity in plasma

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Controls (n = 45)</th>
<th>CAD patients (n = 87)</th>
<th>P-values*</th>
<th>P-values*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline (n = 87)</td>
<td>1 year (n = 87)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cGMP expression (α units/mg protein)</td>
<td>7.4 ± 6.0</td>
<td>7.4 ± 6.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cGMP activity (μmol/min/mg protein)</td>
<td>7.4 ± 6.0</td>
<td>7.4 ± 6.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoptosis (%)</td>
<td>7.4 ± 6.0</td>
<td>7.4 ± 6.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bax (μmol/L)</td>
<td>7.4 ± 6.0</td>
<td>7.4 ± 6.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl-2 (μmol/L)</td>
<td>7.4 ± 6.0</td>
<td>7.4 ± 6.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bax/Bcl-2 ratio</td>
<td>7.4 ± 6.0</td>
<td>7.4 ± 6.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eNOS activity (μmol/min/mg protein)</td>
<td>7.4 ± 6.0</td>
<td>7.4 ± 6.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eNOS expression (α units/mg protein)</td>
<td>7.4 ± 6.0</td>
<td>7.4 ± 6.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrite/Nitrate (NOx) (μmol/L)</td>
<td>7.4 ± 6.0</td>
<td>7.4 ± 6.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD.

CAD indicates coronary artery disease; eNOS, endothelial nitric oxide synthase; Bax, protein expression of Bcl-2 associated protein X; Bcl-2, protein expression of B cell lymphoma/leukemia 2; Ang II, angiotensin II; BK, bradykinin; TNF-α, tumor necrosis factor alpha; ns, not significant.

*Controls vs. CAD baseline (n = 87); Δ perindopril vs. Δ placebo: Δ is the difference between 1 year and baseline values; §insignificant values between perindopril at 1 year and controls.

Fig. 4. 4,6-diamino-2-phenylindole (DAPI)-positive endothelial cells with typical chromatin condensation.
specific B2 receptor antagonist–counteracted the beneficial effect of perindopril. Indeed, eNOS protein expression was reduced from 8.9±3.6 to 6.9±2.6 arb.units/mg protein with the addition of icatibant (P<0.01, n=12). These ex vivo results are in keeping with the ones observed in vivo on serum NOx. In fact, there was a statistically significant increase of NOx after 1 year treatment with perindopril (P<0.01); moreover, we observed lower values at baseline in CAD patients in respect to the control group (Table 2).

An example of typical apoptosis, characterized by nuclear fragmentation, as observed by fluorescence microscopy, is shown in Fig. 4. Serum from patients with CAD significantly increased the rate of apoptosis of HUVECs compared with the control group (6.8±1.9 versus 1.3±0.6% in the perindopril and control group at baseline, respectively; P<0.01) (Table 2).

Treatment for 1 year with perindopril 8 mg/day caused a 31% reduction in the rate of apoptosis (P<0.05) (Table 2). The effect of perindopril on the rate of apoptosis was reflected by the reduction in the Bax/Bcl-2 ratio (P<0.01). Also in this case, the effects of serum from treated patients were at least in part mediated by the activation of BK B2 receptors since apoptosis was increased from 5.3±1.3 to 6.8±0.8% with the addition of icatibant in the incubation medium (P<0.01, n=12).

Table 2 also shows that plasma values of Ang II from CAD patients at baseline were higher and BK lower when compared with those from the control group (both P<0.01).
At 1 year, the differences between baseline values in CAD patients and the control group were modulated by the treatment with perindopril: in the treated group, there was a recovery of Ang II/BK balance with a significant reduction in Ang II (−27%) and an increase in BK (17%) (P<0.05 for both).

Plasma values of TNF-α from CAD patients were within the normal range of our laboratory but higher with respect to the control group (P<0.01). Treatment with perindopril reduced TNF-α levels with respect to baseline values (−13%). This reduction, compared with the unchanged pattern in the placebo group, resulted in a significant difference between perindopril and placebo at 1 year (P<0.05) (Table 2).

To further investigate the role on isolated HUVEC function of the perindopril-induced changes in Ang II/BK balance and TNF-α content, the experiments reported in Fig. 5 were performed. We tested the effect of adding three incremental dosages of Ang II (Fig. 5A) and BK (Fig. 5B) and TNF-α (Fig. 5C) to the serum of control subjects on eNOS expression and % of apoptosis, respectively. The doses were chosen in order to reproduce the range of the changes observed in the CAD patients. Addition of Ang II did not induce significant changes, BK at the higher concentration up-regulated eNOS expression and reduced the rate of apoptosis (13.78±0.87 v. 10.04±2.64 arb.units/mg protein, P<0.05; 1.20±0.45 vs. 1.77±0.67%, P<0.05, respectively). TNF-α exerted an opposite action than BK on eNOS expression, which was down-regulated (8.23±1.89 vs. 10.04±2.65 arb.units/mg protein, P<0.05) and on apoptosis, which was increased (2.53±0.49 vs. 1.77±0.67%, P<0.05).

3.1. Correlations

We found no correlation between vWf either at baseline or after treatment and blood pressure at entry, degree of perindopril-induced reduction in blood pressure, or concomitant medication.

The only statistically significant correlations were BK versus eNOS activity (r=0.43; P<0.05) and protein expression (r=0.45; P<0.05). Surprisingly, TNF-α and Ang II were not statistically significantly correlated with the apoptotic rate of HUVECs. This was confirmed by a series of ad hoc in vitro experiments using anti-TNF-α antibody that only showed a trend toward apoptosis reduction that did not reach statistical significance (P=0.1).

4. Discussion

Treatment with ACE inhibitors improves outcome in patients with CAD, including patients with left ventricular (LV) dysfunction early after acute myocardial infarction [16–18], with heart failure (HF) [19,20] and asymptomatic LV dysfunction (SOLVD) [20] as well as in stable patients at higher (Heart Outcomes Prevention Evaluation [HOPE]) [21] and lower risk (EUROPA) [1].

The above effects can be attributed to:
- blood pressure reduction
- reduction in progression of LV remodeling
- a direct vascular effect.

Our data suggest that long-term (1 year) ACE inhibition with perindopril 8 mg/day exerts a direct positive effect on the vascular endothelium. In treated patients, vWf levels were reduced as well as the rate of endothelial apoptosis, while eNOS protein expression and activity improved.

At baseline, vWf, a marker of endothelial damage, was at the upper limit of the normal values and even more elevated in the diabetic patients, indicating endothelial damage [22]. Furthermore, vWf levels were correlated with prognosis, and 1 year of treatment with perindopril significantly reduced them independently from other risk factors.

The most interesting evidence of a possible beneficial effect of perindopril on the endothelium comes from the results at cellular level in which we used a previously validated methodology [23] that allows the measurement of the harder end point of endothelial function. The data suggest that serum from CAD patients is pro-apoptotic and induces a down-regulation of eNOS protein expression and activity. These effects are counteracted by 1 year of treatment with perindopril (8 mg/day). The changes in NOx pattern after treatment in the current study indicates that these pathophysiological mechanisms can occur also in vivo [9,24]. Furthermore, there was a relationship between the ex vivo effects of perindopril on eNOS and those in vivo on vWf. A causality cannot be obviously established in such context, but the correlation between the two effects is intriguing.

We have previously reported that serum from patients with acute coronary syndromes (ACS) exerts a pro-apoptotic effect on the endothelium, thus contributing to plaque instability [23]. The results of the current study are the first ones in patients with stable CAD and suggest that even during the chronic stable phases of the disease the endothelium has abnormalities.

The obvious question is: which are the biological factors present in the serum of CAD patients accounting for the reported increased apoptosis and down-regulation of eNOS protein expression and activity? To answer this question, we have measured the balance between Ang II and BK, as they are modulated by ACE and exert pro-apoptotic and anti-apoptotic effects on the endothelium, respectively [25,26]. An increase in BK levels is also expected to stimulate eNOS protein expression and activity. Treatment with perindopril resulted in vivo in a significant BK increase and Ang II reduction suggesting that in CAD patients ACE is activated and can be effectively reduced. There was no correlation between these two parameters and the rate of apoptosis or Bax and Bcl-2 levels. The only correlation was between BK and eNOS up-regulation, thus confirming previous reports in humans that showed an increase in eNOS protein expression in mammary artery from CAD.
patients with perindopril [27]. This finding is well in keeping with the NOx data which are \textit{in vivo} increased in perindopril treated subjects and with the observation that blockade of BK B2 receptors in the \textit{ex vivo} experiments counteracts the effects of chronic treatment with perindopril on eNOS protein expression.

TNF-\(\alpha\) is another well-known inducer of apoptosis and negative modulator of eNOS. In previous studies, we established the involvement of this cytokine in inducing endothelial apoptosis in patients with advanced HF and ACS [14,23]. In the current low-risk population, the increase was within the upper normal limit, and contrary to what we have previously reported in ACS and HF, TNF-\(\alpha\) was not correlated to the degree of apoptosis or to the down-regulation of eNOS.

Obviously, it is not possible to reproduce the complex serum phenotypic changes induced by 1 year treatment with perindopril by \textit{ex vivo} manipulation of single neurohumoral factor. Nevertheless, the experiments reported in Fig. 5 confirm that directly administering BK to HUVECs causes a dose-response increase of eNOS protein expression and a reduction of rate of apoptosis. On the contrary, Ang II failed to have any effect, probably because of its early degradation. TNF-\(\alpha\) at the highest concentration confirmed its negative action on endothelial function.

Several cautions should be considered when interpreting those data.

It is not possible to compare the \textit{ex vivo} changes to those obtained \textit{in vivo}. They carry only information and suggestive value.

We used HUVECs that are neonatal differentiated endothelial cells. Therefore, this model is only partially representative for the \textit{in vivo} adult endothelium. The effects of treatment with ACE inhibition on endothelial dysfunction have been measured \textit{ex vivo}. This does not necessarily imply that the same effect could occur in patients. Nevertheless, the results obtained are coherent and suggestive for protection of endothelial function in the treated CAD patients as documented by the data on NOx production and von Willebrand factor reduction.

Another limitation is that we have not measured ACE activity. The original protocol design did actually include its measurement but logistic problems affected its evaluation. However, it is noteworthy that previous data from the literature clearly report that chronic treatment with ACE inhibitors counteracts the activity of circulating ACE [28–30].

Taken together, our data suggest that CAD is linked to a certain degree of endothelial dysfunction and 1 year’s treatment with perindopril can improve it. They do not provide information on whether long-term administration is needed or whether this can be the result of an acute effect. Experimental data suggest that prolonged administration of ACE inhibitors is needed in order to modify the eNOS pattern [27,31]. This was the reason why we studied the effect of perindopril after 1 year’s administration.

The findings of increased rate of apoptosis in CAD are important as if the rate of endothelial death is not paralleled by an equal increase in regeneration, progression of atherosclerosis and plaque rupture, the two clinical end points of the EUROPA trial, are favored.

There might be other mechanisms of action of perindopril that could explain the results of the EUROPA study. While a reduction in LV remodeling was unlikely to occur in our patients as they were selected for not having HF, we cannot exclude that pressure reduction may have played a pivotal role. It is, however, relevant to consider that in the main EUROPA study the reduction in cardiovascular events (20% relative risk) was greater than that expected for the observed reduction in blood pressure (mean systolic/diastolic blood pressure reduction 5/2 mm Hg, respectively) [1] and the positive outcome occurred also in patients with normal blood pressure and was not related to the degree of blood pressure reduction achieved by the drug [32]. Furthermore, in the recently published A Coronary disease Trial Investigating Outcome with Nifedipine GITS (ACTION) and Prevention of Events with Angiotsenin-Converting Enzyme inhibition (PEACE) studies involving a similar risk population with CAD as EUROPA, the blood pressure reduction obtained with nifedipine and trandolapril was equal or higher than that found in the EUROPA study with perindopril and yet there was no documented beneficial effect on the same cardiovascular events [33,34].

5. Conclusions

The results of PERTINENT suggest that abnormal endothelial function occurs in patients with CAD and this can be counteracted with ACE inhibition with perindopril. These effects could contribute to explaining the results of the main EUROPA study.

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