Addition of the selective aldosterone receptor antagonist eplerenone to ACE inhibition in heart failure: effect on endothelial dysfunction

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Abstract

Objectives: To investigate the effects of adding the selective aldosterone receptor antagonist eplerenone to ACE inhibition on endothelium-dependent vasodilation in rats with chronic heart failure (CHF). Background: Addition of the non-selective aldosterone antagonist spironolactone to ACE-inhibitors reduces mortality and morbidity in CHF and improves endothelial vasomotor dysfunction, but is associated with considerable side-effects. Methods: Starting 10 days after extensive myocardial infarction (MI) or sham-operation, Wistar rats were treated either with placebo, the ACE inhibitor trandolapril (TR, 0.3 mg/kg body weight per day), the selective aldosterone receptor antagonist eplerenone (EPL, 100 mg/kg per day) or a combination of both for 9 weeks. Results: Maximum acetylcholine-induced, nitric oxide-dependent relaxation was significantly attenuated in aortic rings from rats with CHF compared with sham-operated animals (R55% vs. 87%). EPL alone slightly and TR significantly improved NO-mediated relaxation (CHF-EPL 66%; max CHF-TR: 78%), while treatment with both EPL and TR completely restored endothelium-dependent vasorelaxation (CHF-EPL-TR: 83%). Aortic superoxide formation was significantly increased in rats with CHF compared with sham-operated animals, but was normalised by treatment with EPL or TR-EPL. Expression of the endothelial nitric oxide synthase was decreased in CHF and normalised in all treatment groups. Conclusions: In experimental CHF, the selective aldosterone antagonist EPL reduced the increased vascular superoxide formation. Although a combination of TR and EPL normalised endothelium-dependent relaxation, ACE inhibition as a monotherapy was almost equally effective.

Keywords: Endothelial function; Myocardial infarction; Heart failure; ACE inhibitors; Aldosterone

1. Introduction

The reduced endothelium-dependent vasodilator capacity of coronary, large conductance and peripheral arteries contributes to reduced myocardial perfusion, increased peripheral vascular resistance and cardiac workload in patients with chronic heart failure (CHF) and experimental models of cardiac dysfunction [1–4]. The endothelium plays a crucial role in the control of vascular tone by releasing endothelium-derived autacoids, the most important of which is nitric oxide (NO) [5]. Decreased bioavailability of endothelium-derived NO is a major contributor to endothelial dysfunction in CHF [6]. Moreover, experimental evidence has been provided for increased vascular release of superoxide anions (O2·-) [7–11]. O2·- rapidly scavenges NO in the vascular wall and a reduction of bioactive NO may occur despite normal or even an increased NO-generation [7,12].

These pathophysiological events are modulated by different endogenous neurohumoral systems such as the renin-angiotensin system, which is markedly activated in
CHF [13,14]. Treatment with ACE inhibitors to prevent the deleterious effects of angiotensin II on the myocardium and peripheral vasculature favorably alters hemodynamics, improves symptoms, reduces overall mortality in patients with CHF, and enhances NO-dependent dilatation [14–16]. Originally, it had been assumed that ACE-inhibitors block angiotensin II-dependent adrenal aldosterone secretion, but it was shown that an ‘aldosterone escape’ occurs [17], which seems to be angiotensin-independent as even a combination of ACE inhibition and angiotensin II antagonism could not sustain aldosterone reduction chronically [18]. Increased plasma levels of aldosterone are considered to be an independent risk factor for worse outcome in patients with CHF [19]. The clinical relevance of this observation has been supported by the convincing results of the RALES study: adding the non-selective mineralocorticoid receptor antagonist spironolactone to ACE-inhibition markedly reduced overall mortality [20]. Subsequent studies demonstrated that endothelium-dependent NO-formation in CHF was improved by addition of spironolactone, and antioxidative properties of long-term treatment with spironolactone have been identified [21,22].

Although spironolactone is an effective aldosterone receptor antagonist, progestational and anti-androgenic side-effects such as gynecomastia, abnormal menstrual cycles, and impotence, limit its use. A new specific aldosterone receptor antagonist (SARA), eplerenone (EPL), is currently in development. Like spironolactone, it is a competitive antagonist of the aldosterone receptor. It is a molecule that takes advantage of replacing the 17-thioacetyl group of spironolactone with a carbomethoxy group. This confers excellent selectivity for the aldosterone receptor over other steroid receptors. In addition, this chemical substitution produces a molecule that has negligible activity at the cytochrome P-450 enzyme of endocrine organs [23].

In the present study, we investigated the effect of long-term treatment with the SARA EPL and the ACE inhibitor trandolapril (TR) either alone or in combination on endothelial function in rats with CHF after experimental myocardial infarction (MI).

2. Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1. Study protocol, myocardial infarction and hemodynamic measurements

Left coronary artery ligations were performed in adult male Wistar rats (250–300 g) as previously described [24]. Briefly, the thorax was opened under ether anesthesia, the heart exteriorised and a ligature placed around the proximal left coronary artery. On the 10th postoperative day, surviving rats were randomly allocated to placebo (Placebo), either the SARA eplerenone (EPL, 100 mg/kg body weight per day) or the ACE inhibitor trandolapril (TR, 0.3 mg/kg per day), or a combination of both. Hemodynamic studies were performed after 10 weeks under barbiturate anesthesia (pentobarbital, 30 mg/kg body weight, i.p.) and spontaneous respiration, after treatments had been withheld for 24 h before hemodynamic studies were performed in order to avoid acute treatment effects. Saline-filled catheters (PE50) were advanced from the right carotid artery into the left ventricle and connected via a three-way stopcock to a Millar micromanometer and Statham transducer. Afterwards, the transducer was withdrawn to the ascending part of the thoracic aorta and blood pressure was recorded [24].

2.2. Sample collection and determination of infarct size

After hemodynamic measurement, the heart was removed and the left ventricle was then cut into three transverse sections: apex, middle ring (3 mm), and base. From the middle ring, 5-μm-sections were cut at 100-μm-intervals and stained with picrosirius red. The boundary length of the infarcted and non-infarcted surfaces of the endocardium and the epicardium was traced with a planimeter digital image analyser and infarct size (fraction of the infarcted left ventricle) was expressed as a percentage of length; only rats with extensive infarcts (>45%) were included in the vascular reactivity studies.

2.3. Vascular reactivity studies

The descending thoracic aorta was dissected following removal of the heart and cleaned of connective tissue. The upper section (10 mm) was immediately frozen for Western blot analysis. The lower section (10 mm) was used for measurement of O₂⁻ production, while the remainder was cut into 3-mm rings which were mounted in an organ bath (FMI, Seeheim, Germany) for isometric force measurements. The rings were equilibrated for 30 min under a resting tension of 2 g in oxygenated (95% O₂, 5% CO₂) Krebs-Henseleit solution (NaCl 118 mmol/l, KCl 4.7 mmol/l, MgSO₄ 1.2 mmol/l, CaCl₂ 1.6 mmol/l, K₂HPO₄ 1.2 mmol/l, NaHCO₃ 25 mmol/l, glucose 12 mmol/l; pH 7.4, 37 °C) containing diclofenac (1 μmol/l) [25]. Rings were repeatedly contracted by KCl (with a maximum of 100 mmol/l) until reproducible responses were obtained. Thereafter, the rings were preconstricted with phenylephrine (0.3–1 μmol/l) to comparable constriction levels and the relaxant responses to cumulative doses of aceylcholine and to sodium nitroprusside were assessed [26].

2.4. Measurement of superoxide anion formation

Vascular O₂⁻ formation was measured using lucigenin-enhanced chemiluminescence [25]. The light reaction
between \( \text{O}_2^{-} \) and lucigenin (5 \( \mu \text{mol/l} \)) [27] was detected in a luminometer (Wallac, Freiburg, Germany) during incubation of rings in a HEPES-modified Krebs buffer (pH 7.40). The specific chemiluminescence-signal was expressed as counts per minute per mg dry weight of tissue (cpm/mg).

The oxidative fluorescent dye hydroethidine (HE) was used to evaluate in situ production of superoxide. HE is freely permeable to cells and in the excited presence of \( \text{O}_2^{-} \) is oxidised to ethidium bromide (EtBr), where it is trapped by intercalating with the DNA [28]. EtBr is excited at 488 nm and the emission measured at 610 nm. In cell-free assays, addition of hydrogen peroxide to HE does not significantly increase EtBr fluorescence [29,30].

Unfixed frozen ring segments were cut into 30-\( \mu \text{m} \)-thick sections and placed on a glass slide. HE (2 \( \mu \text{mol/l} \)) was topically applied to each tissue section and coverslipped. Slides were incubated in a light-protected humidified chamber at 37 °C for 30 min. Images were obtained with a Bio-Rad MRC-1024 laser scanning confocal microscope equipped with a krypton/argon laser. Aortic rings from CHF animals and control tissues were processed and imaged in parallel. Laser settings were identical for acquisition of images from CHF and control specimens. Fluorescence was detected with a 585-nm-long pass filter.

2.5. Western blot analysis

Aorta samples were homogenised in ice-cold RIPA buffer (NaCl 150 mmol/l, Tris–Cl 50 mmol/l, EDTA 5 mmol/l, Nonidet P-40 1% v/v, deoxycholate 0.5% w/v, NaF 10 mmol/l, sodium pyrophosphate 10 mmol/l, phenylmethylsulfonyl fluoride 100 mmol/l, aprotinin 2 \( \mu \text{g} / \text{ml} \), and leupeptin 2 \( \mu \text{g} / \text{ml} \)). Proteins were determined by Bradford assay. Aorta extracts (30 \( \mu \text{g} \) protein per lane) were mixed with sample loading buffer and under reducing conditions separated on 10% SDS–polyacrylamide gel. Proteins were electrotransferred overnight at 4 °C onto PVDF membrane (Immun-Blot®, Bio-Rad). The bands were detected using chemiluminescence assay (ECL+ Plus, Amersham). For detection of endothelial NO synthase (eNOS), we used a mouse monoclonal antibody (N-30020, Transduction Laboratories) diluted 1:1000.

2.6. Materials

All biochemicals were obtained from Sigma (Deisenhofen, Germany). EPL was provided by Pharmacia (Erlangen, Germany) and TR by Knoll (Ludwigshafen, Germany).

2.7. Statistics

Relaxant responses were given as percentage relaxation relative to the preconstriction level. Values are expressed as mean±S.E.M. of \( n \) experiments and statistical analysis was performed by repeated-measures two-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test. Hemodynamics, \( \text{O}_2^{-} \) formation and eNOS protein expression were analysed using one-way ANOVA with Tukey-Kramer multiple comparisons test. \( P \)-values \( <0.05 \) were considered statistically significant.

3. Results

3.1. Global parameters

Global parameters of CHF rats and sham-operated animals are shown in Table 1. Infarct sizes were comparable among the different experimental groups. Blood

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Global parameters in CHF rats 10 weeks after MI as compared with sham-operated animals</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Sham placebo</td>
</tr>
<tr>
<td>Infarct size (%)</td>
<td>16</td>
</tr>
<tr>
<td>SAP (mmHg)</td>
<td>136±4</td>
</tr>
<tr>
<td>DAP (mmHg)</td>
<td>111±4</td>
</tr>
<tr>
<td>Heart rate (min⁻¹)</td>
<td>400±9</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>5.1±0.6</td>
</tr>
<tr>
<td>dP/dtmax (mmHg/s)</td>
<td>11845±335</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>Left ventricle</td>
</tr>
<tr>
<td>Right ventricle</td>
<td>233±8</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>451±9</td>
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</tbody>
</table>

Rats were either treated with placebo, with trandolapril alone (TR, 0.3 mg/kg body weight per day), with eplerenone alone (EPL, 100 mg/kg body weight per day) or with a combination of both (TR-EPL). DAP, diastolic arterial pressure; LVEDP, left ventricular end-diastolic pressure; SAP, systolic arterial pressure.

*\( P<0.05 \), versus sham placebo.
† \( P<0.01 \), versus CHF placebo.
‡ \( P<0.05 \) versus CHF placebo.
pressures were significantly lower in all groups of rats with CHF, whereas left ventricular end-diastolic pressure was markedly elevated.

3.2. Vasodilator responses in aortic rings

Acetylcholine induced a concentration-dependent relaxation of aortic rings preconstricted with phenylephrine, which was reduced in aortae from rats with heart failure after extensive MI (Fig. 1A). Chronic treatment with EPL slightly improved endothelium-dependent relaxation, while TR significantly enhanced the acetylcholine-induced relaxation. Treatment of rats with a combination of EPL and TR led to a restoration of the endothelium-dependent relaxation in aortic rings from CHF rats (Fig. 1A).

The concentration–response curve of the endothelium-independent vasodilator sodium nitroprusside was not different between sham-operated animals and rats with heart failure (Fig. 1B). The concentration–response curve to sodium nitroprusside in aortae of rats treated with EPL alone was slightly shifted to the right, whereas endothelium-independent relaxations were similar to placebo in the other treatment groups (Fig. 1B).

3.3. Superoxide anion formation in rat aorta

\( \text{O}_2^- \) formation (lucigenin 5 \( \mu \text{M} \)) was significantly increased in aortae from rats with heart failure versus sham-operated rats, and there was a negative correlation between \( \text{O}_2^- \) generation and acetylcholine-induced endothelium-dependent relaxation (\( r^2=0.232, P<0.05 \)). \( \text{O}_2^- \) formation was reduced in aortae from EPL-treated rats, while \( \text{O}_2^- \) generation was completely normalised in all rats treated with the combination of TR and EPL (Fig. 2). Staining of aortic rings with the \( \text{O}_2^- \) sensitive fluorescent dye HE also showed a reduction of \( \text{O}_2^- \) generation in the vessel wall in animals treated with EPL or the combination therapy (Fig. 3).

3.4. Expression of eNOS in rat aorta

eNOS expression was reduced in CHF rats compared with sham-operated animals. Chronic treatment with either TR or EPL normalised the expression of eNOS (Fig. 4).

4. Discussion

In the present study, EPL normalised vascular eNOS expression and \( \text{O}_2^- \) production in rats with experimental CHF, however, only in combination with an ACE inhibitor it was able to restore the attenuated endothelium-dependent relaxation.

The impairment of endothelium-dependent, NO-mediated vasorelaxation that we observed has been repeatedly reported in patients suffering from CHF (as reviewed in Ref. [31]) and conforms with previous studies in this model of experimental CHF after MI [3,7,22,32]. The key...
Fig. 3. O_{2}^• formation in aortic rings from CHF rats 10 weeks after MI, as compared with sham-operated animals (for experimental groups see Fig. 1). Vessels were labelled with the O_{2}^• sensitive fluorescent dye HE, which produces a fluorescence when oxidised to EtBr by O_{2}^•. Data are representative for n = 4 experiments.
CHF, suppression of aldosterone production following ACE inhibition or angiotensin II antagonism is not chronically sustained even when high dosages are applied [18]. The complete normalisation of vascular $\text{O}_2^-$ generation by the SARA EPL underlines the potential role of aldosterone in stimulating vascular $\text{O}_2^-$ formation. Furthermore, in a recent study in an experimental model of atherosclerosis, EPL alone improved endothelial function and reduced NAD(P)H oxidase activity suggesting a previously unreported interaction of mineralocorticoid receptors with $\text{O}_2^-$ generating oxidases in the vasculature [42]. However, in the present study, the correlation between $\text{O}_2^-$ formation and reduction of endothelium-dependent relaxation in placebo-treated animals was only weak and the different effects of the investigated therapies suggest that the beneficial treatment effects cannot be solely ascribed to beneficial effects on the balance between NO and $\text{O}_2^-$.

In accordance with our previous findings using spironolactone [22], in the group treated with EPL we found a rightward shift of the concentration–response curve to the NO donor SNP. SNP releases NO in the vascular wall donor and is used to assess smooth muscle responsiveness. This observation may be related to a decreased activity of the soluble guanylyl cyclase induced by circulating angiotensin II [39], which is not reduced by aldosterone antagonism. The slight reduction of the sensitivity of the smooth muscle cell layer in response to the NO donor SNP may also explain the lack of a significant effect of EPL monotherapy on acetylcholine-induced relaxation despite lowering $\text{O}_2^-$ and improving eNOS expression. Therefore, the combination of an ACE inhibitor with the SARA combines the advantages of both monotherapies while antagonising each others disadvantages: the ACE inhibitor may prevent the angiotensin II-mediated desensitisation of soluble guanylyl cyclase in the EPL group and the SARA may prevent the aldosterone-mediated induction of $\text{O}_2^-$ in the TR group.

The reduction of eNOS expression in placebo-treated CHF rats, while conforming with other models of heart failure in rats [33,34], is in contrast to our previous findings in rats with chronic MI, in which an increased expression of eNOS had been observed [7]. The apparent difference may be related to the fact that only animals with extensive MI and consecutively severely depressed left-ventricular function (marked decrease in $\text{dP/dt}_{\text{max}}$) were included in the present study. This is further supported by the lower systolic pressure in the placebo group as compared with the animals in the previous study. In contrast to our observations using spironolactone, which did not significantly affect eNOS expression [22], EPL treatment resulted in a restoration of vascular eNOS expression. However it remains unclear whether this is a primary effect of the drug or may be secondary to the improvement in hemodynamics induced by EPL.

In conclusion, the selective aldosterone antagonist EPL normalised vascular eNOS expression and $\text{O}_2^-$ production.
in rats with experimental CHF. Although ACE inhibition as a monotherapy was almost equally effective, a combination of TR and EPL completely normalised endothelium-dependent relaxation. EPL may be a useful additional therapeutic option in the treatment of severe CHF.

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