Effect of canrenone and amiloride on the prooxidative effect induced by aldosterone in human mononuclear leukocytes in vitro

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ABSTRACT. Clinical studies have demonstrated that aldosterone receptor antagonists do improve the survival of patients with chronic heart diseases and in vitro studies have shown that canrenone blocks the proinflammatory effect of aldosterone in mononuclear leukocytes (MNL). The aim of the study was to compare, in the model of human MNL, the effect of potassium-sparing diuretics amiloride and canrenone, on the protein expression of p22phox, a NADPH-oxidase system subunit, that is a principal marker of production of superoxide anions. MNL were isolated from 10 informed healthy volunteers (5 males and 5 females, age range 24-36 yr) and the proteins extracted. p22phox protein expression was evaluated by Western blot and quantified using a densitometric semi-quantitative analysis. The experiments showed that aldosterone (10–8 M) enhances the protein expression of p22phox and that its effect is reversed by co-incubation with canrenone (10–4 M), while incubation with amiloride (10–4 M) reduced the prooxidative effect of aldosterone at a significantly lower extent than canrenone. Co-incubation with canrenone, amiloride, and aldosterone together produced the same effect as aldosterone plus canrenone. Incubation with cortisol (40–8 M) was not effective. These data confirm the prooxidative effect of aldosterone in MNL. The addition of aldosterone-receptor antagonist canrenone produced a higher inhibition than sodium channel blocker amiloride on the effect of aldosterone on p22phox protein expression.

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INTRODUCTION

Mineralocorticoid receptor (MR) blockers not only inhibit the volume expansion and electrolyte abnormalities produced by excess of aldosterone, but they also prevent the cardiovascular complications, due to the MR-mediated profibrotic effect of aldosterone (1-5). In a recent report, it was shown that both hypertension and diabetes increase oxidative stress in mononuclear leukocytes (MNL) and that increase of C reactive protein is related to MNL oxidative stress in these patients (6).

An invasion of monocytes, macrophages, and lymphocytes seems to be the requisite for the onset of inflammation, via secretion of cytokines and activation of the process of fibrosis within the endothelium of the affected vasculature of heart and/or of other tissues (7-9). In the atherosclerotic mouse model, eplerenone or spironolactone can block the process of atherosclerosis and suppress oxidative stress both in serum and in macrophages (10-12). A possible link between aldosterone, oxidative stress, and fibrosis is the NADPH oxidase activation by excess of aldosterone (12, 13). NADPH oxidase is the most important source of reactive oxygen species (ROS) at the level of vascular wall and fibroblasts. NADPH oxidase subunit p22phox seems a key factor in the pathogenesis of hypertension-related target damage and particularly in the mineralocorticoid hypertension, as in the 11-desoxy cortisolose plus salt intake (DOCA-salt) rat model (13).

In 1985, we characterized MR in human MNL (14) and showed that 1-h incubation of MNL with aldosterone regulates the intracellular content of sodium and potassium and the cell volume, while coincubation with canrenone blocks the effect of aldosterone (15, 16).

Subsequent studies have demonstrated that incubation of MNL in vitro with excess of aldosterone can enhance the protein expression of PAI 1 and p22phox (17).

In some clinical situations, other potassium-sparing diuretics are employed, such as triamterene or amiloride. This diuretic is widely used also in combination with hydrochlorothiazide for treatment of edema, heart insufficiency and hypertension in general. The drug counteracts the effect of aldosterone at the level of sodium channels of plasma membrane, without affecting the binding of aldosterone to MR (18). Prolonged treatment with spironolactone or amiloride leads to secondary hyperaldosteronism, but only amiloride can allow aldosterone to bind to its nuclear receptors. Several studies indicate that human lymphocytes express mRNA encoding the Na/H exchanger (NHE), which participates in the regulation of intracellular pH and cellular volume homeostasis (19-22). Treatment of macrophages or monocytes with amiloride induces an inhibition of NHE, a reduction of the intracellular pH and a stimulation of ROS synthesis (21-23).

The aim of our study was to use the model of MNL to investigate the different actions of amiloride and canrenone on the protein expression of p22phox, induced by coincubation with excess of aldosterone and to support the concept that diuretics other than aldosterone...
receptor blockers can not counteract the prooxidative effect of aldosterone.

MATERIALS AND METHODS
MNL were obtained from 10 healthy volunteers (5 males and 5 females, age range 24-36 yr) recruited from the staff of the Department of Medical and Surgical Sciences of the University of Padua. The male and female subject groups were not different for age. All the subjects were fully informed about the protocol and gave their consent to the study. None of these subjects had taken medications for at least 1 month.

MNL preparation and incubation
Peripheral MNL were isolated in plastic tubes from 50-60 ml EDTA anticoagulated blood by Ficoll Paque Plus gradient (Amersham Pharmacia Biotech, Sweden). The cells were washed 3 times in PBS, their number was counted in a Neubauer chamber and resuspended in RPMI medium (Serva, Italy) at a concentration of 5×10⁶ per 500 μl. After the washings the final preparation of MNL was free of granulocytes and contained 4-5% monocytes. The viability of the cells was 98% before and after incubation as judged by trypan blue exclusion. Aliquots of 5×10⁶ cells from 8 subjects were incubated with aldosterone alone (final concentration 10⁻⁸ M), canrenone alone (10⁻⁶ M), or co-incubated with aldosterone (10⁻⁸ M) + canrenone (10⁻⁶ M), amiloride (10⁻⁶ M) + canrenone (10⁻⁶ M), amiloride (10⁻⁶ M) + aldosterone (10⁻⁶ M), amiloride (10⁻⁶ M) + aldosterone (10⁻⁸ M) + canrenone (10⁻⁶ M). In two cases incubation was performed with medium alone, cortisol (40–8 M) and cortisol plus aldosterone. Incubations were done for 3 h at 37 C.

p22phox protein expression (Western blot)

p22phox, protein expression was performed using Western blot analysis. Total protein extracts were obtained by lysis of the cells with a specific buffer (HEPES pH 7.5, 20 mM, GTA 2 mM, DTT 1 mM, PMSF 1 mM, β-glycerophosphate 40 mM, MgCl₂ 2.5 mM, Na₃VO₄ 2.0 mM, aprotinin 20 μg/ml, leupeptin 20 μg/ml). The proteins were separated by SDS-PAGE in TRIS pH 8.3. Blots were stained with Ponceau S (Sigma-Aldrich, St Louis, USA) to confirm that protein loading was equal in all samples. Protein transfer on nitrocellulose membrane was performed using Hoefer TE 22 Mini Tank Transphor Unit (Amersham Pharmacia Biotech, Uppsala, Sweden) with the use of the following transfer buffer: 39 mM glycine, 48 mM Tris base, 0.037% SDS (electrophoresis grade), 20% methanol. The membranes were incubated overnight with primary monoclonal antibodies anti-p22phox (Santa Cruz Biotechnologies, Santa Cruz, USA), diluted 1:500. Anti-goat IgG HRP-conjugated for p22phox (Santa Cruz Biotechnologies, Santa Cruz, USA) were used as secondary antibodies. Secondary antibodies were HRP-conjugated, and immunoreactive proteins were visualized with chemiluminescence using SuperSignal WestPico Chemiluminescent Substrate (Pierce, Rockford, USA).

Evaluation of p22phox protein products

After electrophoretion, nitrocellulose membranes were stained with Ponceau S in order to confirm equal protein loading and protein transfer. Protein expression was quantified using a PCR-based densitometric semiquantitative analysis using NIH image analyzer software. The p22phox protein expression was expressed as arbitrary densitometric units (ADU).

Statistical analysis

Data are given as mean±SD. Due to the low sample numerosness and because the analytical detection method was semiquantitative, non-parametric tests were chosen to evaluate differences among treatments. To detect differences across the multiple experimental conditions, the Friedman’s test for repeated measures comparisons (non-parametric analogue of the analysis of variance) was used (24). Post-hoc comparisons were performed by using the Wilcoxon signed-rank test for paired data (25). All probability values are two-tailed; p-values <0.05 were considered statistically significant.

The statistical analysis of data was obtained using StatistiXL™ software (Kalamunda, Western Australia), run as an add-in to the Excel™ spreadsheet program.

RESULTS

Figure 1 shows a representative experiment of protein expression of p22phox in one of the subjects. Aldosterone enhances the expression of the marker and this effect is blocked by co-incubation with canrenone. Canrenone alone does not produce any effect when compared with baseline values. Amiloride alone enhances the p22phox expression, while the incubation with aldosterone plus canrenone plus amiloride shows similar results as aldosterone plus canrenone. The mean ADU values (±SD) obtained from the 8 subjects, after incubation with different drugs, are presented in Figure 2. Aldosterone increases the expression of p22phox (medium alone 259±113, aldosterone 583±231, p<0.008). The effect of aldosterone is reversed by co-incubation with canrenone (177±33, p<0.008). Amiloride alone increases the p22phox expression (507±225 vs baseline (p<0.039). The expression of p22phox after incubation with amiloride plus aldosterone (415±131, p<0.05 vs baseline) is lower than aldosterone alone (p<0.016), but not different than after incubation with amiloride alone. Co-incubation with amiloride plus aldosterone and canrenone (210±124) suppresses both the effect of amiloride (p<0.016) and of amiloride plus aldosterone (p<0.025). Finally, incubation with cortisol was not effective in modifying the protein expression of p22phox (medium alone 269±98, cortisol 273±50). Incubation with aldosterone plus cortisol increased the ADU values in the same extent as aldosterone alone.