0021-972X/04/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 89(4):1973–1976

doi: 10.1210/jc.2003-031545

Effect of Aldosterone and Glycyrrhetinic Acid on the Protein Expression of PAI -1 and $p22^{phox}$ in Human Mononuclear Leukocytes.

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ABSTRACT Aldosterone excess can produce heart and kidney fibrosis, which seem to be related to a direct effect of aldosterone at the level of specific receptors. We report a direct, mineralocorticoid-mediated effect on the protein expression of two markers of oxidative stress after incubation of mononuclear leukocytes with $1x10^{-8}$ M aldosterone ($p22^{phox}$/β-actin = 1.38±0.05 and PAI-1/β-actin = 1.80±0.05). The same effect was also found with $3x10^{-5}$ M glycyrrhetinic acid, the principal constituent of licorice root ($p22^{phox}$ /β-actin = 1.37±0.97 and PAI-1/β-actin = 1.80±0.04). The effect of both aldosterone and glycyrrhetinic acid is blocked by incubation with added $1x10^{-6}$ M of receptor-antagonist canrenone. Canrenone alone did not show any effect. PAI-1 related protein was also found using $4x10^{-9}$ M aldosterone. Incubations with $1x10^{-9}$ M for 3 hours as well as $1x10^{-8}$ M aldosterone for 5, 10 and 20 minutes were ineffective for both proteins. These data support the previous finding of an involvement of mononuclear leukocytes in the pathogenesis of the oxidative stress induced by hyperaldosteronism. In addition, the results confirm our previous data on a direct effect of glycyrrhetinic acid at the level of mineralocorticoid receptors.

INTRODUCTION

There is an increasing recognition of oxidative stress as a major damaging factor in a variety of human diseases. The activation of the renin-angiotensin-aldosterone system plays a pivotal role in the induction of oxidative stress (1,2). Angiotensin II is, in fact, a well-known promoter of oxidative stress via up-regulation of NADH/NADPH oxidase (3). The effects of long-term signalling pathways of angiotensin II in the cardiovascular system, which ultimately lead to cardiovascular remodelling and atherosclerosis, are determined mostly through production of reactive oxygen species (ROS) and activation of redox sensitive genes (4,5).

Evidence has recently emerged that aldosterone is an important factor in endothelial dysfunction and remodelling through direct effects in non-epithelial cells found in non-classic target tissues, such as vascular wall and heart and/or through the enhancement of angiotensin II effects (6-8). Aldosterone increases the expression and synthesis of type 1 collagen and transforming growth factor β (TGFβ) as well as plasminogen activator inhibitor-1 (PAI-1) gene expression in a variety of organs, including kidney, vasculature and heart. This strongly supports its role in fibrinolysis regulation, organ hypertrophy, fibrosis and remodelling in vascular smooth muscle, renal and myocardial cells (9-13). A limited number of reports in animal models have investigated the role of aldosterone in generating ROS and vascular injury (14,15). In addition, in a human adrenal adenoma, where aldosterone is overproduced, we showed an increased gene and protein expression of PAI-1, $p22^{phox}$, a key subunit of NADPH oxidase, and TGFβ (16).

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Received: 9/4/03. Accepted: 1/28/04.

Circulating blood cells are widely used to study *ex vivo* pathophysiological mechanisms of hypertension and remodelling. The role of various inflammatory mechanisms such as mononuclear leukocyte (MNL) infiltration in the development of hypertensive target organ damage has been stressed (15,17-20). Accumulation of peripheral MNL precedes coronary vascular remodelling which is an early feature of ventricular dysfunction that ultimately leads to heart failure (15). Aldosterone-receptor antagonism produces an amelioration of endothelial dysfunction and a reduction of NADH/NADPH oxidase activity, suggesting an important role of aldosterone in the generation of oxidants in the vasculature (19,21). It is interesting to note that an infiltration of monocytes has also been involved in kidney damage in experimental models and this effect is prevented by angiotensin converting enzyme inhibition (20).

We have also previously characterized mineralocorticoid receptors (MR) in MNL (22) where aldosterone regulates intracellular electrolytes (23) and volume (24). This type of cells is, therefore, a useful tool to investigate aldosterone-mediated actions. In particular, they permit studies on the involvement of aldosterone in the expression of markers of oxidative stress damage and cardiovascular remodelling in order to gather new information regarding these phenomena that affect vascular smooth muscle, myocardial and endothelial cells which are not as easily accessible.

The aim of our study was to evaluate *in vitro*, using human MNL from healthy subjects as models, the effect of aldosterone and glycyrrhetinic acid (GA) and their blockade with the aldosterone receptor antagonist canrenone, on levels of oxidative stress-related proteins such as $p22^{phox}$ and PAI-1.

SUBJECT AND METHODS

***Subjects*:** MNL were taken from 6 healthy volunteers, after obtaining informed consent, (4 males and 2 females age range 24-29 years) recruited from the staff of the Department of Medical and Surgical Sciences of the University of Padua. None of these subjects had taken medication for at least two

weeks and all abstained from food, alcohol and caffeine-containing drinks for at least 12 hours before the study.

***MNL preparation and incubation*:** Peripheral MNL were isolated from 50 ml EDTA anticoagulated blood by Ficoll Plaque Plus gradient (Amersham Pharmacia Biotech, Sweden) and judged to be 85% pure and functionally alive by cytofluorimetric analysis. The cells were washed 3 times in PBS, their number was counted in a Neubauer chamber and resuspended in RPMI (Serva, Italy) at a concentration of $5x10^6$ per 500 µl. An aliquot of $5x10^6$ cells was co-incubated with aldosterone alone (final concentration $1x10^{-8}$M), aldosterone $1x10^{-8}$ M plus canrenone $1x10^{-6}$M, canrenone $1x10^{-6}$M, GA $3x10^{-5}$M, GA $3x10^{-5}$M plus canrenone $1x10^{-6}$M and medium alone. Incubation was for three hours at 37°C.

We have also studied the effects of two different doses on the protein expression of two markers. An aliquot of $5x10^6$ cells from 3 healthy volunteers was co-incubated with different concentrations of aldosterone (final concentrations $1.9x10^{-9}$M and $4x10^{-9}$M), alone or with added 100 fold concentration of canrenone, or with canrenone and with medium alone. Incubation was for three hours at 37°C.

Time-course study: an aliquot of cells was incubated for 5, 10 and 20 minutes, with $1x10^{-8}$M aldosterone alone, with $1x10^{-6}$ M canrenone added, with canrenone or with medium alone.

p22^{phox} and PAI-1 protein expression (western blot): $p22^{phox}$ and PAI-1 protein expression was performed using western blot analysis. Total protein extracts were obtained by lysis of the cells with a specific buffer (HEPES 20mM, EGTA 2mM, DTT 1mM, PMSF 1mM, β-glycerophosphate 40mM, $MgCl_2$ 2.5mM, Na_3VO_4 2.0mM, aprotinin 20µg/ml, leupeptin 20µg/ml, pH 7.5). The proteins were separated by SDS-PAGE in TRIS pH 8.3. 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 glycin, 48 mM Tris base, 0.037% SDS (electrophoresis grade), 20% methanol. The membranes were incubated overnight with: primary monoclonal antibodies anti-$p22^{phox}$ diluted 1:500 (Santa Cruz Biotechnologies, Santa Cruz, USA) and anti PAI-1 (StressGen Biotechnologies, Victoria, Canada) diluted 1:500. Anti-goat IgG HRP-conjugated for $p22^{phox}$ (Santa Cruz Biotechnologies, Santa Cruz, USA) and anti-mouse IgG HRP conjugated (Santa Cruz Biotechnologies, Santa Cruz, USA) for PAI-1 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 p22^{phox} and PAI-1 protein products: $p22^{phox}$, PAI-1 and β-actin protein expression was quantified using a densitometric semiquantitative analysis using NIH image analyzer software, as previously described. The ratio of $p22^{phox}$ and PAI-1 to β-actin PCR products, expressed as pixel density, was used as indexes of $p22^{phox}$ and PAI-1 protein expression as arbitrary densitometric units (ADU).

Statistical analysis: Data were evaluated on an iMac Macintosh computer (Apple Computer, USA) using the Statview II statistical package (BrainPower Inc., USA). Data are expressed as mean ± SD and they were analyzed using the Mann-Whitney U test. Values at a 5% level or less ($p<0.05$) were considered statistically significant.

RESULTS

Fig 1 shows a representative experiment of protein expression of $p22^{phox}$ and PAI-1. In table 1 the ratios of the ADU of the two markers against β–actin are reported as mean ± SD. The mean ratio in MNL incubated with aldosterone was: 1.38±0.05 for $p22^{phox}$ and 1.80±0.05 for PAI-1; with GA it was: 1.37±0.07 for $p22^{phox}$ and 1.8±0.04 for PAI-1. The effects of aldosterone and of GA were abolished by canrenone, which suppressed both proteins. Canrenone alone did not produce any effect on the level of either protein when compared with baseline values.

Aldosterone was not effective at $1.9x10^{-9}$ M. However, at $4x10^{-9}$M for 3 hours there was a slight action of aldosterone on the protein expression of PAI-1 (baseline 0.90+0,03; aldosterone 1.3+0.02 and aldosterone + canrenone 0.87+0.03), while $p22^{phox}$ was not effective. In the time-course experiments aldosterone was ineffective in the expression of both proteins at 5-, 10- and 20-minute incubation showing no difference from baseline values.

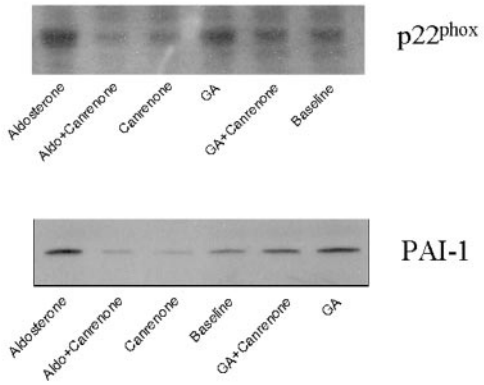

Fig.1. Western blot of $p22^{phox}$ and PAI-1 in MNL of a healthy volunteer after 3 hours of incubation with or without different substances (GA=glycyrrhetinic acid).

Table 1. Protein expression (ratio vs. β-actin of PAI-1 and $p22^{phox}$) and statistical significance after incubation with different substances.

STIMULATION	$p22^{phox}$	PAI-1	Statistical significance (p)	
Aldosterone (A)	1.38±0.05	1.80±0.05	A vs B:	p ($p22^{phox}$) = 0.0002 p (PAI-1) = 0.00003
A+ Canrenone (Can)	0.90±0.06	0.96±0.05	A vs Can:	p ($p22^{phox}$) = 0.0002 p (PAI-1) = 0.00001
Can	0.98±0.05	0.91±0.03	Can vs A:	p ($p22^{phox}$) = 0.0002 p (PAI-1) = 0.00001
Glycyrrhetinic acid (GA)	1.37±0.07	1.8±0.04	GA vs B:	p ($p22^{phox}$) = 0.001 p (PAI-1) = 0.006
GA+Can	1.16±0.06	1.5±0.05	GA vs GA+C:	p ($p22^{phox}$) = 0.008 p (PAI-1) = 0.00003
Baseline (B)	1.03±0.03	1.15±0.06		

(B vs Can, B vs A+Can, B vs GA+Can not significant for both p^{22phox} and PAI-1)

DISCUSSION

We found a direct effect of high concentrations of aldosterone on the *in vitro* protein expression in human MNL of the two markers of oxidative stress and vascular remodelling studied, i.e. PAI-1 and $p22^{phox}$. The effect of aldosterone was reversed by the addition of canrenone, a mineralocorticoid-receptor antagonist, at a concentration where this drug has been demonstrated to inhibit aldosterone binding to MR (25). It is also worthy of note that GA, at a concentration close to its affinity ratio versus aldosterone (1:3000) (25) produced the same effect, which was reversed by the same concentration of canrenone. These findings confirm our previous data demonstrating that GA not only blocks 11-hydroxysteroid dehydrogenase type 2 (11HSD 2), but also has a direct mineralocorticoid-like effect when consumed in high amounts (26).

The stimulatory effect in the levels of each protein was not evident with $1.9x10^{-9}$M of aldosterone, which is the affinity of aldosterone for mineralocorticoid receptors in MNL (22). We previously demonstrated that this concentration is able to produce the physiologic effect of aldosterone, such as the regulation of cell volume and intracellular content of sodium and potassium, which was reversed by canrenone (23,24). Higher concentrations of aldosterone were instead able to stimulate the levels of both PAI-1 and $p22^{phox}$ in all our subjects, and these findings are consistent with pathological effects of aldosterone, such as those found in primary aldosteronism which include the pro-oxidant effect.

Many studies have shown that excess aldosterone induces oxidative stress and a proinflammatory and profibrotic phenotype in several target tissues (1,19,27-29). This effect of aldosterone is probably mediated by mineralocorticoid receptors *in vivo*. The aldosterone receptor antagonist eplerenone can reduce coronary vascular inflammation and subsequent interstitial fibrosis in both an animal model and, more recently, in humans. These effects seem to be mediated by reduction of oxidative stress and attenuation of platelet aggregation (30,31). Furthermore, recent studies have highlighted the importance of enhanced oxidative stress and invading monocytes and lymphocytes in the remodelling of the kidney (20) and right and left heart (15). It has recently been demonstrated that the accumulation of monocytes is the main mechanism of cardiac fibrosis. Other studies have also shown an effect of increased sodium intake in the exacerbation of aldosterone actions at the level of non-classical target tissues.

In both our experimental model and that of Gerling (15) high concentrations of aldosterone or aldosterone-receptor agonists were used, confirming the risk for the heart and other non-classical targets. The *in vivo* implications of the MNL accumulation in heart and kidney due to high aldosterone concentrations could explain some of the results obtained in other studies evaluating the heart morphology after in vivo administration of cortisol, aldosterone or carbenoxolone (32).

The interrelations between circulating or locally produced steroids, steroid receptors, 11HSD, local lymphocyte accumulation and production of oxidative stress markers could lead to the final result of cardiac fibrosis. The results of our study clearly demonstrate that in MNL aldosterone can increase the production of both PAI-1 and $p22^{phox}$ independently of angiotensin II, which has previously been shown to mediate the effect of aldosterone (12). This direct relationship is reflected in the profibrotic effects of aldosterone in primary aldosteronism or during excessive administration of mineralocorticoids, given the fact that renin, and therefore angiotensin II, is suppressed in both these situations (33, 34).

It has been pointed out that aldosterone also possesses non-genomic effects (35). Our results clearly demonstrate that oxidative stress as reflected in the expression of PAI-1 and $p22^{phox}$ is directly linked to the binding of aldosterone to its classical receptors. In fact we found that i) the effect of aldosterone was not evident after brief incubations, ii) canrenone abolishes the effects of aldosterone and of GA, and iii) both canrenone and medium alone do not induce any protein expression.

In conclusion, we have introduced a very simple approach to study the relationship between aldosterone and oxidative stress, using an easily obtainable target tissue. Our results using this system of measuring aldosterone effects are in agreement with other more complex approaches and thereby demonstrate that MNL are a useful tool for the investigation of aldosterone effects in physiological and pathological conditions.

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Erratum

In the Table of Contents for the March 2004 issue (Volume 89, Issue 3), the article titled "Effect of aldosterone and glycyrrhetinic acid on the protein expression of PAI-1 and $p22^{phox}$ in human mononuclear leukocytes" by L. A. Calò, F. Zaghetto, E. Pagnin, P. A. Davis, P. De Mozzi, P. Sartorato, G. Martire, C. Fiore, and D. Armanini (*J Clin Endocrinol Metab* **89:**1973–1976, 2004) is incorrectly listed as beginning on page 1255. The article that actually appears on page 1255 is by L. Montanelli, A. Delbaere, C. Di Carlo, C. Nappi, G. Smits, G. Vassart, and S. Costagliola and is titled "A mutation in the follicle-stimulating hormone receptor as a cause of familial spontaneous ovarian hyperstimulation syndrome"; this article is being reprinted in its entirety, beginning on the next page. *The printer regrets the error.*