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Original Article

Mineralocorticoid receptor is involved in the aldosterone pathway in human red blood cells

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Abstract: We have recently demonstrated that excessive aldosterone (Aldo) secretion in primary aldosteronism (PA) is associated with red blood cells (RBC) senescence. These alterations were prevented/inhibited by cortisol (Cort) or canrenone (Can) raising the hypothesis that Aldo effects in RBC may be mediated by mineralocorticoid receptor (MR), though to date MR has never been demonstrated in human RBC. The aim of this multicenter comparative study was to investigate whether Aldo effects were mediated by MR in these a-nucleated cells. We included 12 healthy controls (HC) and 22 patients with PA. MR presence and activation were evaluated in RBC cytosol by glycerol gradient sedimentation, Western blotting, immuno-precipitation and radioimmunoassay. We demonstrated that RBC contained cytosolic MR, aggregated with HSP90 and other proteins to form multiprotein complex. Aldo induced MR to release from the complex and to form MR dimers which were quickly proteolyzed. Cort induced MR release but not dimers formation while Can was not able to induce MR release. In addition, RBC cytosol from PA patients contained significantly higher amounts of both MR fragments ($p<0.0001$) and Aldo ($p<0.0001$) concentrations. In conclusion, in RBC a genomic-like Aldo pathway is proposed involving MR activation, dimerization and proteolysis, but lacking nuclear transcription. In addition, dimers proteolysis may ensure a sort of Aldo scavenging from circulation by entrapping Aldo in MR fragments.

Keywords: Mineralocorticoid receptor, HSP-90, primary aldosteronism, human red blood cells, cortisol, cellular senescence

Introduction

Aldosterone (Aldo) regulates fluid and electrolyte homeostasis by stimulating gene expression of serum-glucocorticoid-inducible kinase 1 (SGK1) and epithelial sodium channel (ENaC) [1], involved in Na^+ and K^+ regulation and reabsorption in renal tissues. Although normal concentrations of Aldo mediate physiological functions, high plasma levels contribute to progressive organ damage to the heart [2-4], vasculature [5] and kidneys [6-8], promoting myocardial [9-11] and vascular fibrosis [12], oxidative stress [13] and perivascular inflammation [14]. Aldo exerts its effects via the mineralocorticoid receptor (MR) which belongs to the nuclear receptor superfamily and functions as a ligand-dependent transcription factor [15]. MR is constituted by structural and functional domains including the N-terminal domain (NTD), DNA-binding (DBD) and C-terminal ligand binding domains (LBD) [16]. This receptor forms cytoplasmic hetero-complexes with a range of chaperone proteins, including the 90 kDa heat shock protein (HSP90), which plays a crucial role in the subsequent ligand binding affinity of MR [16]. The Aldo-induced MR activation has been extensively studied in the epithelial renal tissues, but has also been described in non-epithelial cells such as cardiomyocytes [17], endothelial cells [18], vascular smooth muscle cells [19], neurons [20] and mononuclear leucocytes [21], confirming the multiplicity of tissues and responses involved in Aldo signaling.

Red blood cells (RBC) are a-nucleated cells particularly sensitive to oxidative stress and circulate in direct contact with plasma steroids. We recently reported that in patients with primary aldosteronism (PA) RBC membranes were char-

acterized by a particular rearrangement of band 3 protein leading to increased aggregate formation and immunoglobulin IgG binding [22], potential marker for senescence [23-26]; both aggregation and IgG binding were triggered by Aldo in *in vitro* experiments. Interestingly, the effect of Aldo was prevented by co-addition of cortisol (Cort) or canrenone (Can), thus allowing the hypothesis that MR may be involved in Aldo induced band 3 alterations [22], although the presence of the MR in RBC has not been demonstrated yet.

In this study we investigated the presence and mechanism of action of classic MR in human RBC incubated with Aldo, Can or Cort, alone or in association. To better characterize the physiological significance of a potential MR-mediated Aldo signaling in these a-nucleated cells, we analysed cytosol from PA patients and healthy controls (HC), comparing both Aldo and MR contents.

Materials and methods

Materials

Antibody anti-MR (MCR H-300), raised against aminoacids 1-300 of the C-terminal region of MR and anti-HSP90 were purchased from Santa-Cruz; anti-MR (MA 1-620), produced with aldosterone-3 as immunogen, came from Thermo Scientific (Rockford, IL, USA), and antibodies rMR (1-18) 1D5 and rMR (1-18) 6G1, both recognizing the N-terminus of human MR, were kindly provided by Professor Celso Gomez-Sanchez, University of Mississippi Medical Center [27].

Anti-carbonic anhydrase 2 sheep polyclonal antibody was obtained from Abcam (Cambridge, United Kingdom); the protease inhibitor cocktail came from Calbiochem (Darmstadt, Germany) and protein prestained standards from Bio-Rad (Milan, Italy). Secondary antibodies conjugated with horseradish peroxidase (HRP) came from BioRad Laboratories (Hercules, CA, USA). Molecular weight markers and other reagents were purchased from Sigma (Milan, Italy).

Treatment of RBC

The Ethics Committee for Research and Clinical Trials of our University was notified and all participants gave their informed written consent prior to sample collection, according to the Declaration of Helsinki.

Twenty-two untreated patients affected by PA by serum Aldo levels (*intra-* and *inter-assay* variations 5.3% and 7.0%), aldosterone/renin ratio (ARR) and plasma renin activity (PRA) (Supplementary Table 1) and 12 (6 males and 6 females) healthy volunteers as control (HC) were enrolled at the Department of Medicine-Endocrinology of the University of Padova, Italy as previously described [22].

RBC were separately pelleted at 3750×*g* for 3 min and packed RBC were washed three times at 3750×*g* for 3 min in 5 volumes of Dulbecco's Phosphate Buffered Saline (D-PBS).

For *in vitro* treatment, only blood from volunteers was utilized. After centrifugation for 3 min at 3750×*g*, plasma, not contaminated by leucocytes, was further centrifuged at 1000×g for 20 min to obtain platelet poor plasma (PPP) [22]. In order to remove small molecular weight macromolecules such as steroid and peptide hormones, PPP was mixed with dextran-coated charcoal (1:1) and the Charcoal-stripped (CS-PPP) plasma was obtained [22]. The following day fresh RBC were obtained by the same volunteers who underwent a second blood sampling and RBC were purified as described [22].

Packed cells were pre-incubated at 37°C for 1 h with CS-PPP at 20% hematocrit in absence (C) or presence of 5 nM aldosterone (Aldo) with or without 1 μM canrenone (Can) or 5 μM cortisol (Cort) for 1 h at 37°C and recovered by centrifuging at 800×*g* for 3 min.

An aliquot of each sample was successively hemolysed in 30 volumes of hypotonic buffer (5 mM sodium phosphate, pH 8, 0.02% sodium azide, 1 mM sodium orthovanadate, and a protease inhibitor cocktail), membranes were separated from the cytosol by centrifugation (16100×*g* for 20 min). Both cytosol and membranes were submitted to Western blotting and immunostained with the appropriate antibody, then with anti-carbonic anhydrase as a cytosol loading control.

Glycerol gradient sedimentation

Aliquots of 300 ml of cytosol were loaded on a 3.9 ml glycerol (10%-40%) linear gradient in 25

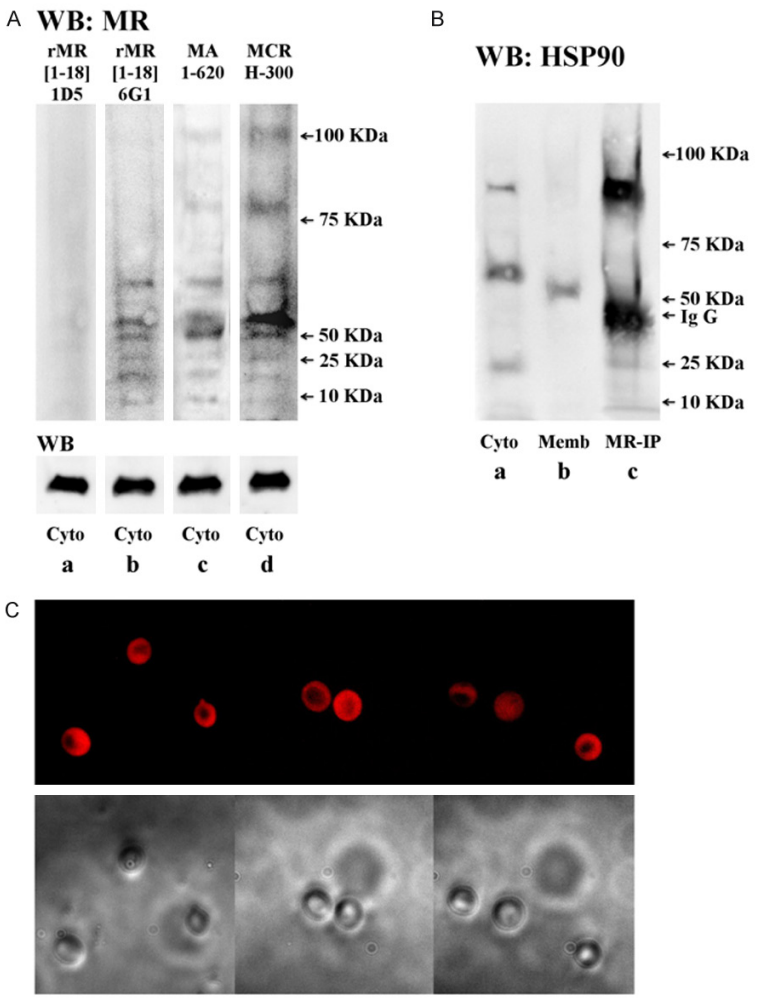

Figure 1. A. Untreated RBC were hemolysed and 60 µl of diluted cytosol were analysed by Western blotting and immuno-detected with rMR (1-18) 1D5 (lane a), rMR (1-18) 6G1 (lane b), anti-MR (MA 1-620) (lane c) or anti-MR (MCR H-300) (lane d). Blots were re-immunostained with anti-carbonic anhydrase (CA) as a loading control. B. Western blotting with anti HSP90 immunodetection of 60 µl of diluted cytosol (lane a), 10 µg of membranes (lane b) and 1.5 ml diluted cytosol immuno-precipitated with anti-MR (MCR H-300) (lane c). C. Untreated RBC fixed with 2% (w/v) paraformaldehyde in PBS, incubated with anti-MR (MCR H-300) and then stained with anti-rabbit IgG-TRITC conjugate. Fluorescence was detected with the UltraView LCI confocal system (Perkin Elmer, Waltham, MA, USA). Corresponding phase contrast images are shown in the bottom panel.

mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4, 1 mM ethylenediaminetetraacetic acid. The tubes were centrifuged 18 hours at 100 000×g in an SW60Ti rotor (Beckman Coulter, Fullerton, CA) at 4°C and fractionated from the top into 18 fractions, and subjected to Western blotting analysis under denaturing conditions. Sedimentation markers run in parallel was formed by albumin (66 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa), apoferritin (443 kDa).

Aldo detection in RBC cytosol

Packed RBC from HC, PA and *in vitro* treatments were hemolysed in 10 volumes of hypotonic buffer and cytosol, recovered as described above, were analyzed for Aldo content by radioimmunoassay (RIA) (intra- and interassay variations of 5.3% and 7.0%), using commercially available kits (DiaSorin Inc. Stillwater, MN, USA). Data were successively multiplied by 10 to take into account the dilution factor and expressed as ng/dl packed cells.

Immunoprecipitation (IP)

When MR was immuno-precipitated by anti-MR (MCR H-300) from untreated diluted cytosol (1.4 ml) or gradient fractions, 1 µl of antibody was added overnight at 4°C in agitation and recovered by protein A Sepharose addition. MR-IPs were analysed by Western blotting with the appropriate antibody.

Statistical analysis

Statistical analysis was performed by SPSS software (Chicago, IL) for Windows version 19, applying parametric and non-parametric tests when appropriate. Continuous variables were expressed as absolute numbers, average ± standard deviation, and analyzed by one-way ANOVA test. Statistical significance was defined as p values <0.05 (two-tailed).

Results

Untreated human RBC membrane and citosol were analysed by Western blotting and immuno-detections with different anti-MR antibodies

were compared (**Figure 1A**). Anti-rMR (1-18) 6G1, MR (MA 1-620) [26] and anti-MR (MCR H-300) antibodies recognized quite similarly bands at 100, 80, 50-53 and additional bands at 20-25 kDa in cytosol, but MCR H-300 was the most appropriate because of its high sensitiveness (lane d). Only negligible bands were detected with anti-rMR (1-18) 1D5 (lane a). Corresponding membranes showed no trace of MR with any of the antibodies (data not shown) (**Figure 1**).

RBC immunocytochemistry analysis with anti-MR (MCR H-300) antibodies revealed homogeneous diffuse fluorescence, confirming the presence of the receptor within the cell (C).

Because aggregation of steroid receptors in multiprotein complexes generally involves the chaperone protein HSP90 [28], we analysed its potential presence in the cytosol of human RBC. Immuno-blotting with anti-HSP90 antibodies confirmed that HSP90 was present in both cytosol (B, lane a) and, although partially proteolysed, also in membranes (lane b). Interestingly, anti-MR IP also exhibited a consistent band of anti-HSP90, co-immuno-precipitating with MR (lane c).

MR activation by Aldo

To characterize the effect of Aldo while avoiding the potential interference of other circulating steroid hormones, plasma was treated overnight with charcoal, which can bind and strip steroids and other small molecules [22]. RBC were then pre-incubated for 1 h at 37°C in charcoal-stripped (CS-PPP) plasma with 5 nM Aldo, 5 μM Cort, 1 μM Can, alone or in association (Aldo and Cort, or Aldo and Can). After RBC hemolysis, cytosol was further processed by glycerol gradient sedimentation, and sedimentation profiles were analysed by Western blotting to ascertain whether MR was present as a monomer or a dimer/polymer (**Figure 2**). In the control C, RBC cytosol immunodetection with anti-MR (MCR H-300) showed that MR was stratified into a large number of glycerol fractions, from 3 to 10, i.e., over a wide range of molecular weights, probably due to its interaction with other proteins. In addition, within each fraction, MR itself was present as different isoforms, simplified in two main regions: one, at 20-25 kDa (B) involved two bands which were more evident in fractions 4-6 (corresponding to glycerol sedimentation weight ranging from about 80 to 130 kDa); and the other involving bands from 50 to 80 kDa (A), visible only from fraction 5 to 10 (corresponding to the 100-400 kDa range of sedimentation weights). In fractions 5-7 only bands of 50-53 kDa were well distinguishable; in fractions 8-10, a new band at about 60 kDa appeared but those at 50-53 kDa disappeared (A, sample C).

The addition of 5 nM Aldo induced net rearrangement of the band at 60 kDa, which almost disappeared from fractions 8-10, whereas the 50-53 kDa band increased in fractions 4-9 (**Figure 2A** sample C compared with sample Aldo).

Concomitantly, Aldo also induced the appearance of the 80 kDa isoform in fractions 5-7 and shifting of the 20-25-kDa bands from fractions 4-6 in sample C to fractions 3-6 in sample Aldo (**Figure 2B**).

Surprisingly, when the same fractions were immuno-detected with anti-MR (MA 1-620) antibody produced with aldosterone-3 as immunogen, MR was detected only in fractions 6-10, whereas no trace was appreciable in the other fractions, in either C or Aldo samples (Supplementary Figure 1). This discrepancy with MR (MCR H-300) detections (**Figure 2**) suggests that Aldo-MR binding is not reversible since Aldo remains entrapped in the MR pocket even after MR proteolysis and the complex Aldo-MR is no longer detectable by MA 1-620 antibody, as indicated by the corresponding datasheet (**Figure 2**).

Densitometrical analysis of the bands in the 50-80 kDa region (A diagram) of C sample peaked in fractions 7-10. The same MR isoforms as in the Aldo sample (dotted lines) were shifted to fractions 5-8, whereas Cort did not induce any change in the location of the MR peak which remained similar to that of controls. These results showed that Aldo treatment induced MR to break away from the multiprotein complex, and MR dimers were formed, as indicated by the appearance of 80-kDa bands in the region of 90-170 kDa range of the gradient. In addition, the increase in low molecular weight bands (20-25 kDa) in fractions 3-6 (B) showed the degradation of MR (again demon-

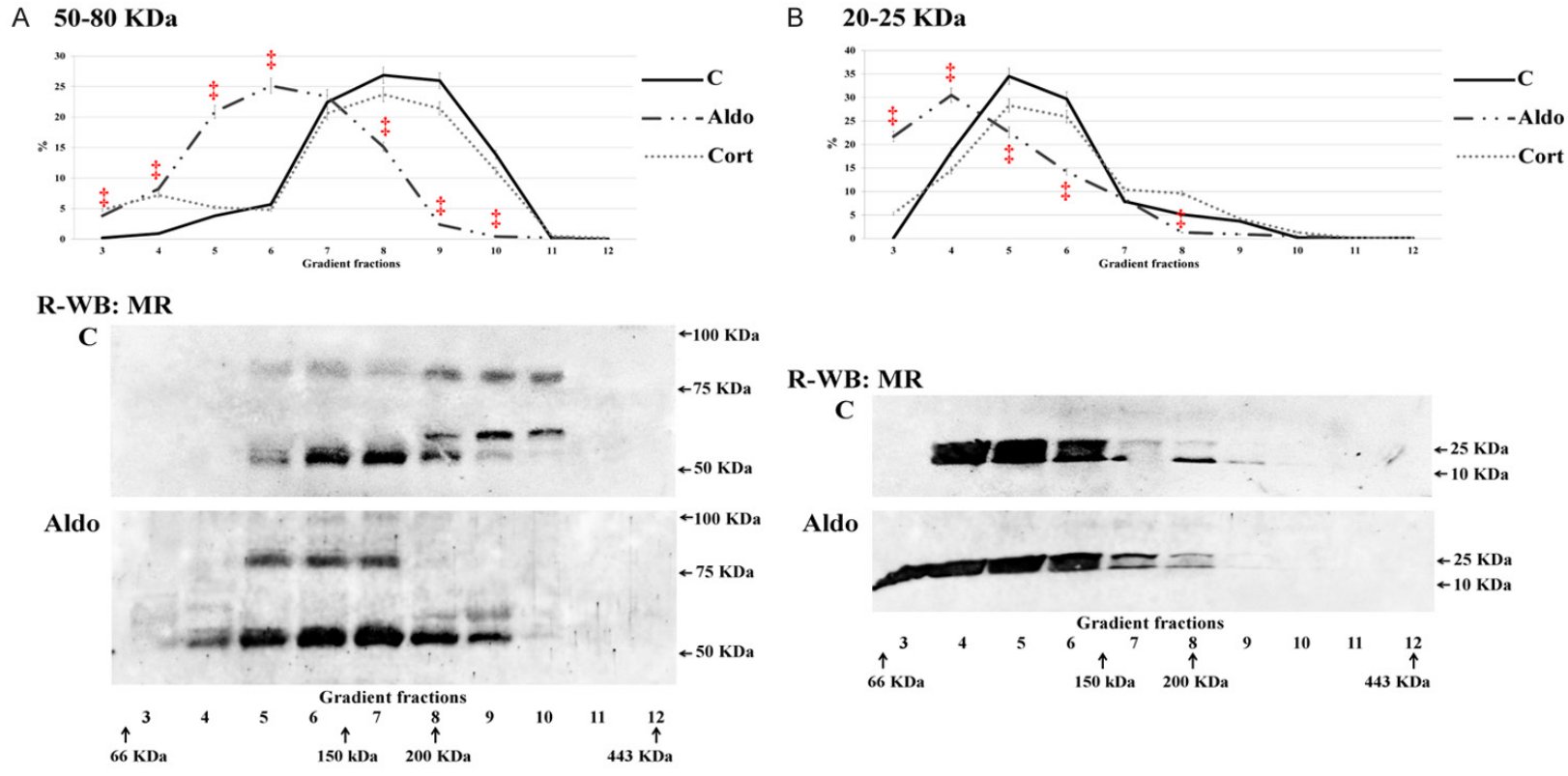

Figure 2. RBC from 12 healthy volunteers, purified as described in Methods, were incubated at 37°C for 1h in absence (C) or presence of 5 nM aldosterone (Aldo) or 5 μM cortisol (Cort) in charcoal-stripped plasma to remove small molecular weight macromolecules such as steroid and peptide hormones. Incubated RBC were hemolysed and 300 μl of diluted cytosol was loaded on top of a linear glycerol gradient and centrifuged for 18 hours at 100 000×*g*. Eighteen fractions (200 μL each) were collected from top, analysed by Western blotting and immuno-detected with anti-MR antibody (MCR H-300) raised against aminoacids 1-300. (A and B) Bands corresponding to 50-80- and 20-25-kDa regions of gels. Data are means ± SD of duplicate measurements from 8 healthy volunteers. Densitometric analysis of anti-MR Western blots of 50-80- and 20-25-kDa bands (A and B, top panels) were quantified; data are expressed as % of total amount of 50-80- or 20-25-kDa bands, respectively, in all fractions, according to $MR\text{-}bands_{fraction}/MR\text{-}bands_{total}$. Statistical analysis showed significant difference at peak values between control and Aldo conditions (‡$p<0.0001$, ANOVA, comparison C *vs* Aldo). Arrows at the bottom of panels: position of molecular weight standards on glycerol gradients, albumin (66 kDa), alcohol dehydrogenase (150 kDa), b-amylase (200 kDa) and apoferritin (443 kDa), to estimate molecular weight of protein complexes on parallel gradient runs.

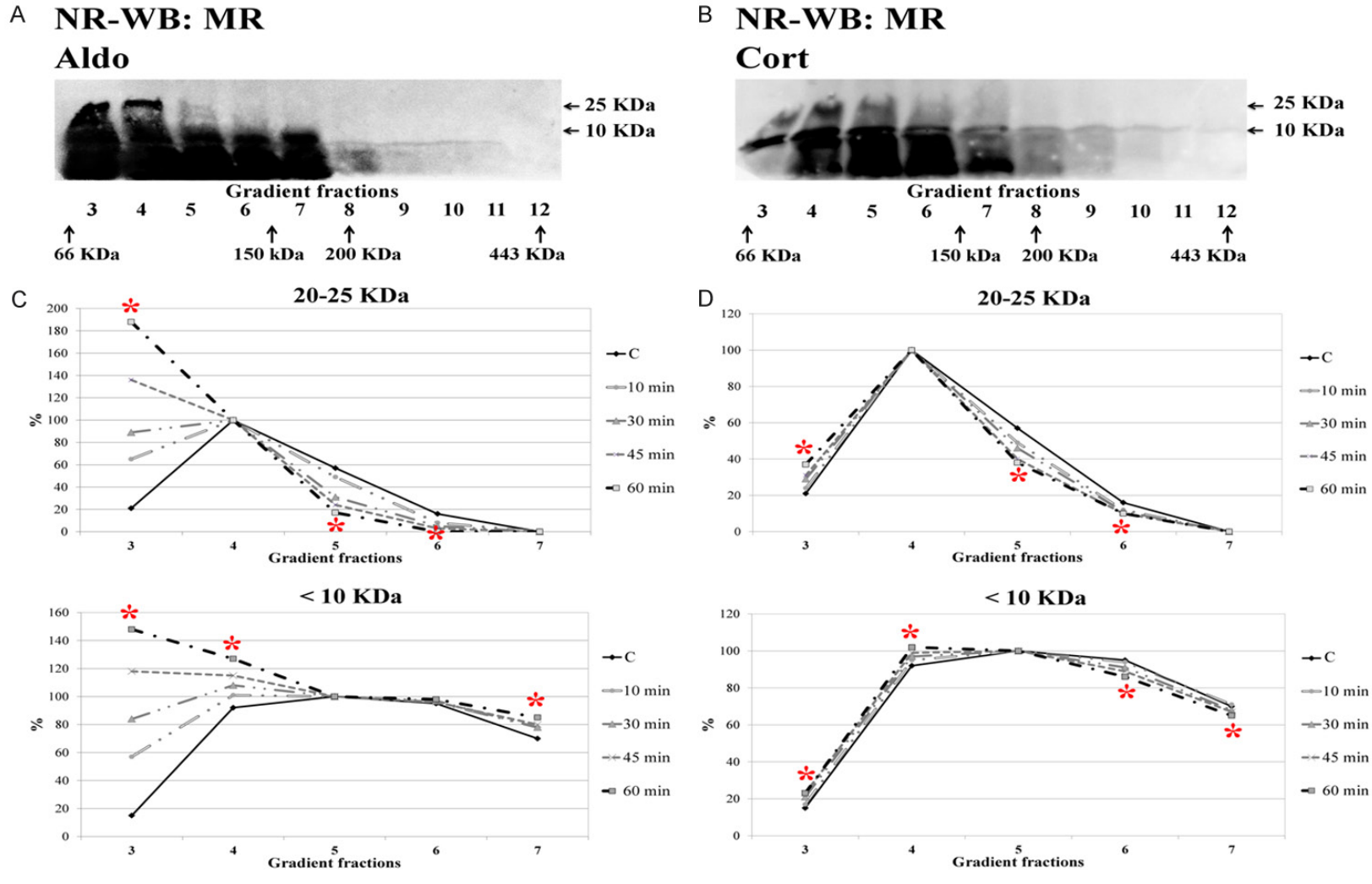

Figure 3. RBC from 12 healthy volunteers, incubated at 37 °C for increasing times (10, 30, 45, 60 min) in absence (C) or presence of 5 nM aldosterone (Aldo) or 5 μM cortisol (Cort), were hemolysed and 300 μl of diluted cytosol were loaded on top of a linear glycerol gradient and centrifuged for 18 hours at 100 000×*g*. Fractions (200 μL each) from top were analysed by Western blotting in non-reducing conditions and immune-detected with anti-MR antibody. (A and B) Bands corresponding to 20-25 and <10 kDa regions of gels of samples Aldo and Cort incubated for 60 min. (C and D) Values from samples incubated for increasing times with Aldo and Cort. Bands were densitometrically analysed and compared with corresponding controls: highest band in (C) for each molecular weight region was taken as comparison unit. Same fraction as in (C) containing highest band was considered as comparison in other samples. *Values significantly different between C and 60-min curves (ANOVA, $p<0.001$).

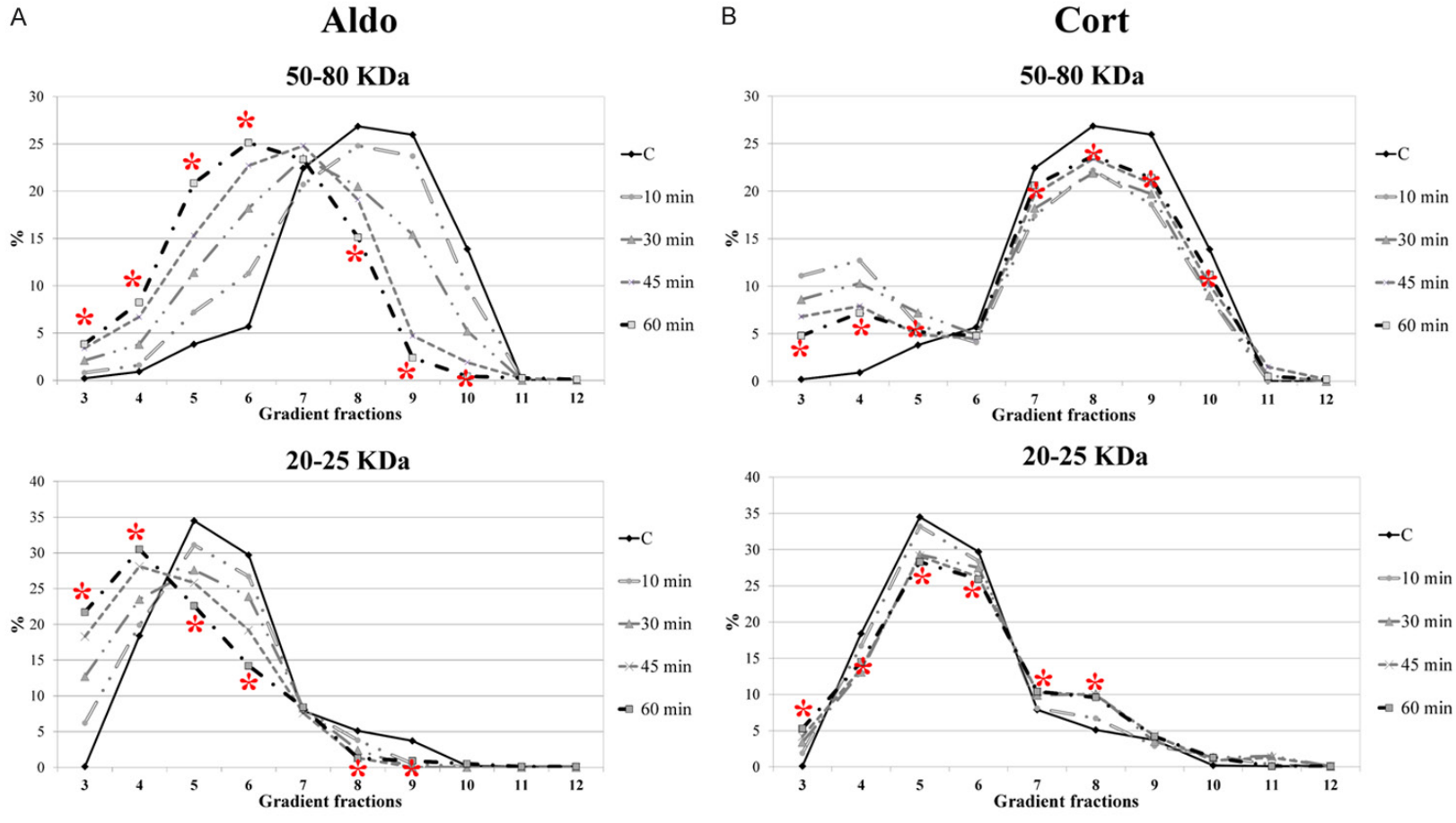

Figure 4. Isolated RBC at 20% hematocrit were incubated at 37 °C for increasing times (10, 30, 45, 60 min) in presence or absence (C) of 5 nM aldosterone (Aldo) or 5 µM cortisol (Cort). After hemolysis 300 µl of diluted cytosol was loaded on top of a linear glycerol gradient and centrifuged for 18 hours at 100 000×g. Fractions (200 µl each) from top were analyzed by Western blotting in reducing conditions and immuno-revealed with anti-MR antibody. A and B: Bands corresponding to the 50-80 and 20-25 kDa regions of gels, of samples Aldo and Cort, respectively. *Significantly different between C and 60 min curves (ANOVA, $p<0.001$).

strated by a shift of peak position) triggered by treatment with Aldo, but not by Cort.

The addition of Can, either alone or in association with Aldo, did not alter the MR situation, which was similar to that of controls (Supplementary Figure 2). Similar results were obtained when RBC were co-treated with Aldo and Cort (data not shown).

When Western blotted glycerol fractions were immuno-detected with anti-HSP90 antibodies, a major band at 90 kDa and a proteolysed isoform of about 60 kDa were mainly sedimented in fractions 6-10 (Supplementary Figure 3). Other treatments did not induce appreciable movements of trend-lines compared with controls (data not shown).

Effect of Aldo or Cort on MR fragmentation

The presence of low molecular bands (20-25 kDa) raised the possibility that a proteolytic process regulates the MR pathway. Low weight MR bands were collected in a large number of fractions (3-9), suggesting that the 20-25 kDa isoforms were both part of multi-complex proteins (fractions 8-10) or derived from a single monomer of MR (fractions 3-5). In addition, a band of less than 10 kDa was found in C samples from fractions 4-7, suggesting the occurrence of further proteolytic digestion mainly in the monomeric and dimeric forms of MR (data not shown).

Glycerol gradient fractions were thus analysed by Western blotting in non-reducing conditions to highlight any fragments broken away from the multi-protein complex. **Figure 3** compares the anti-MR patterns obtained with non-reducing (NR) Western blotting of Aldo- and Cort-treated samples. Anti-MR bands at 50-60 kDa, present in fractions 5-10 of reducing (R)-WB (**Figure 2A** and **2B**), were no longer detected, whereas two sets of bands were evidenced. The first set showed a band at high molecular weight (HMW) which did not change in any of the conditions (Supplementary Figure 4) and the second was composed by bands corresponding to 20-25 and <10 kDa bands (**Figure 3A** and **3B**). Aldo induced the formation of bands in the region ≤ 25 kDa in fractions 3-4 and made the bands <10 kDa to shift to fractions 3-8. Cort did not affect the number of low-weight bands which remained at the level of controls B) (**Figure 3**).

The intriguing involvement of a proteolytic process degrading MR after addition of Aldo but not of Cort was further investigated in experiments carried out at increasing incubation times.

Samples were treated in the absence (C) or presence of Aldo or Cort for 10, 30, 45 and 60 min and cytosols were analysed by glycerol gradient sedimentation. In the NR-WB of the resulting fractions MR bands corresponding to 20-25 and <10 kDa were densitometrically evaluated, separately counted and data reported in C (Aldo) and D (Cort). Aldo addition resulted in time-dependent MR fragmentation starting from the first 10 min of incubation in both 20-25 and <10 kDa regions. Interestingly, in the Cort-treated samples, MR only showed a slight shift to fraction 3 of the proteolytic fragments. When bands at 50-80 kDa were examined in R-WB (**Figure 4**), Cort induced a light shifting to fractions 3 and 4 in the first 10 min but, after 60 min MR bands were recovered in the same fractions as the control, as if re-adjustment of Cort-MR were compatible with re-aggregation of MR in the multi-protein complex. Conversely, Aldo effect confirmed a time-dependent shift to the first fractions.

MR association in multiprotein complexes

Further study on MR-multiprotein complex dissociation was carried out. Cytosol of untreated RBC was gradiented and fractions 8-10 of the sedimented layers were pooled. In these fractions, MR was part of the multi-protein complex and present in the highest isoforms (**Figure 2A**). The pooled fractions were then divided into five aliquots and treated at 0°C for 30 min in the absence (controls) (**Figure 5A**, lane a) or presence of 5 μM Cort (lane b), 1 μM Can (lane c), 2 or 5 nM Aldo (lanes d and e) in order to allow the ligands to bind to the receptor, which was then immuno-precipitated by the addition of anti-MR. MR-immuno-precipitates (IP) from control- and Can-treated aliquots only showed traces of MR (lanes a, c), as revealed by WB with anti-MR antibody (MCR H-300). In the Cort-treated aliquot, the MR band was more than three times that of the control (+315% lane b *vs* lane a, $p<0.0001$) whereas Aldo treatment induced a dose-dependent decrease in the MR-IP band compared to control (-25 and -55%, lanes d and e, respectively, *vs* lane a, $p<0.0001$). To better characterize the complex ligand-receptor, supernatant recovered from

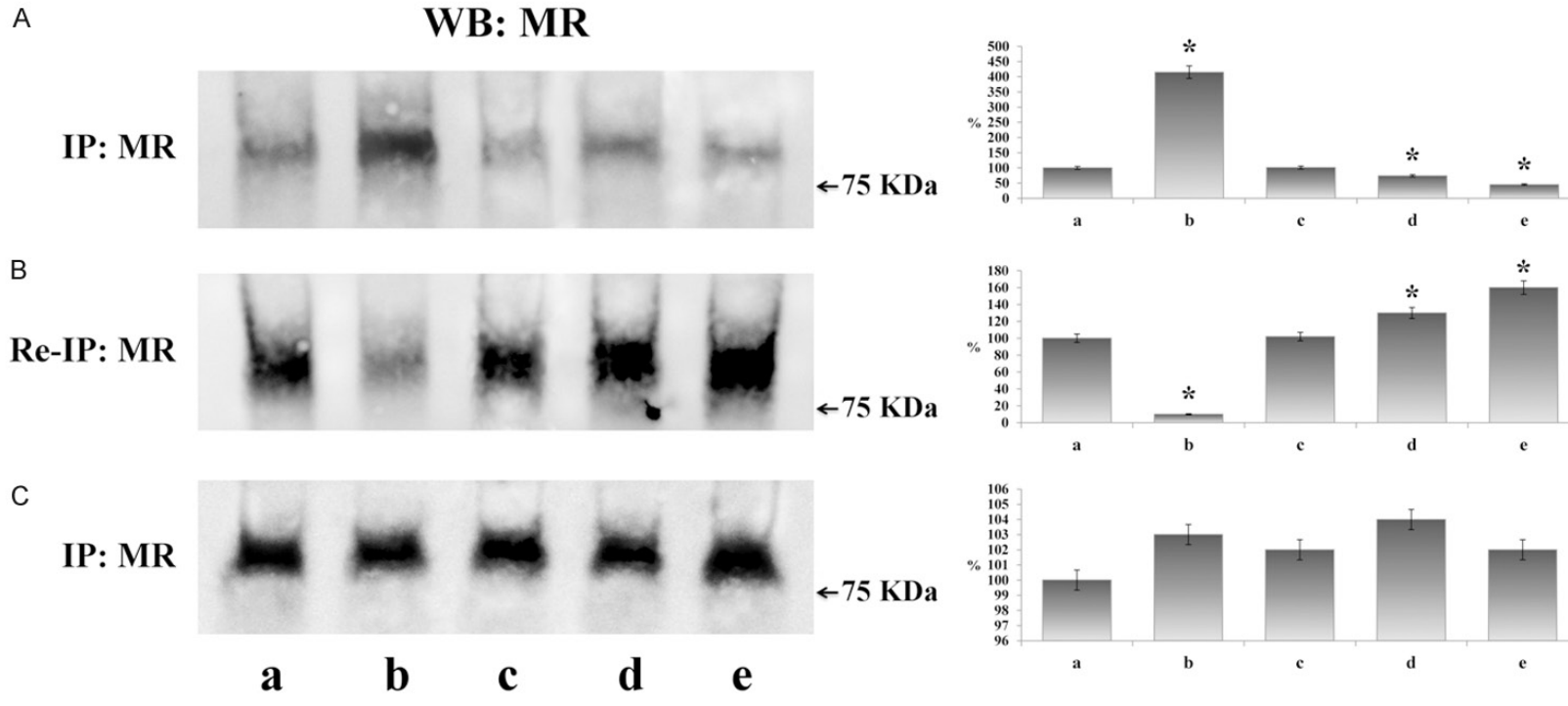

Figure 5. Fractions 8-10 from the C sample glycerol gradients were collected, divided into 5 aliquots and treated at 0°C for 30 min in absence (control) (lane a) or presence of 5 μM Cort (lane b), 1 μM Can (lane c), 2 or 5 nM Aldo (lanes d and e). A: MR was then immunoprecipitated by anti-MR (MCR H-300) and MR-IPs were analysed by Western blotting with anti-MR antibody. B: Supernatants recovered after MR-IP as described above were re-immunoprecipitated in presence of 5 mM dithiothreitol (DTT) and MR-IPs were analysed by Western blotting with anti-MR antibody. C: Fractions 8-10 from C sample glycerol gradients were collected, divided into 5 aliquots and treated at 0°C for 30 min with Gelda in absence (control) (lane a) or presence of 5 mM Cort (lane b), 1 μM Can (lane c), 2 or 5 μM Aldo (lanes d and e). MR was then immunoprecipitated by anti-MR (MCR H-300) and MR-IPs were analysed by Western blotting with anti-MR antibody. Bands were densitometrically analysed and reported on the right of the corresponding panel. *p<0.0001 *vs* control (lane a).

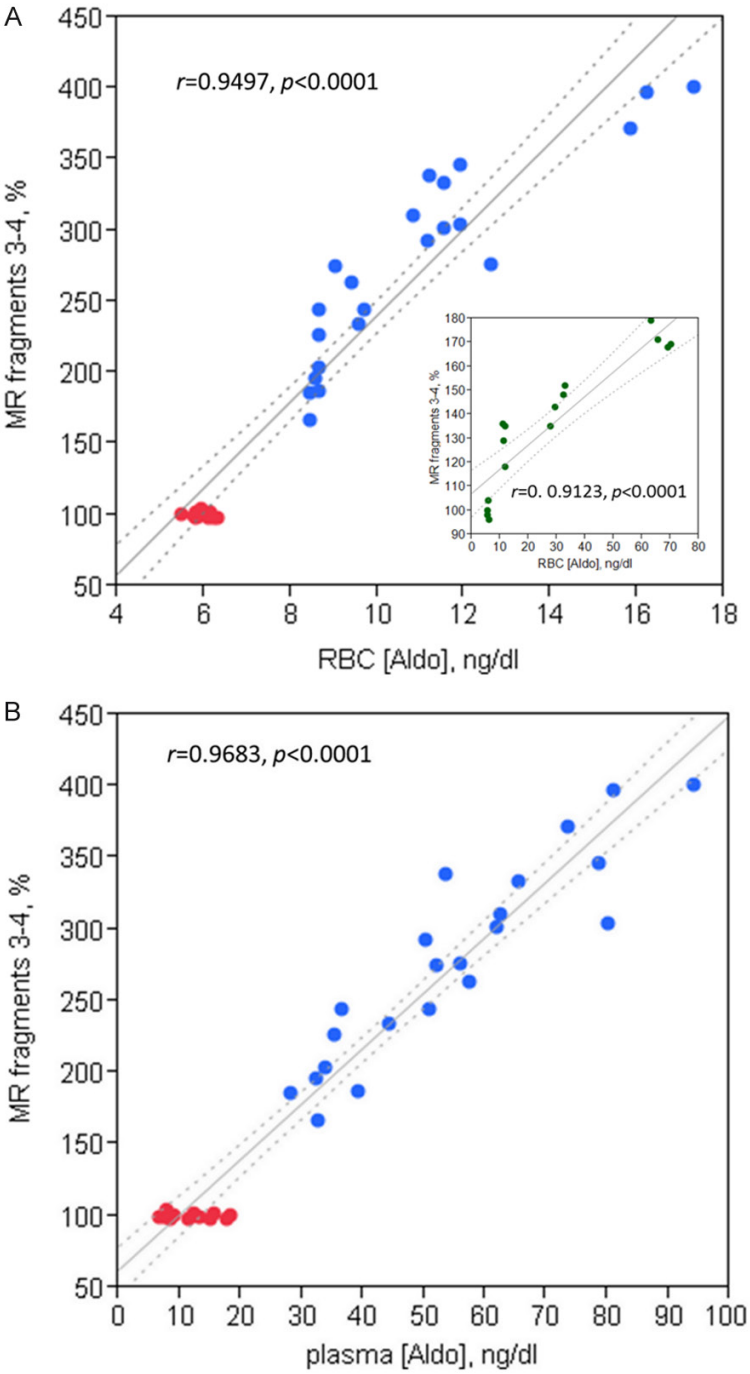

Figure 6. (A) Correlation between MR fragments in fractions 3-4 and RBC cytosol Aldo content. Red circles represent HC subjects, blue circles are PA patients. Inset: correlation between the two parameters after *in vitro* treatment. Isolated RBC from 4 HC at 20% hematocrit were incubated at 37°C for 1 h in absence (C) or presence of 2, 5 and 10 nM Aldo. (B) Correlation between MR fragments in fractions 3-4 and plasma Aldo content. Red circles represent HC subjects, blue circles are PA patients. MR fragments: diluted cytosol (300 µl) from HC (n=12), PA patients (n=22) and *in vitro* treatment (n=4), was loaded in a linear glycerol gradient and centrifuged for 18 hours at 100 000×*g*. Fractions 3 and 4 (200 µL each) from top were analysed by Western blotting in non-reducing conditions and immune-detected with anti-MR antibody. RBC [Aldo] and plasma [Aldo]: diluted cytosol (100 µl) and plasma (100 µl) were analysed by RIA as described in Methods. Continuous lines are best fit linear regression among data points; dotted lines represent the 95% confidence limits. The Pearson's correlation coefficient *r* is indicated, as well as the *p* value.

the previous IP were re-immuno-precipitated (Re-IP) in the presence of DTT, a reducing agent used to stress any possible redox modulated intramolecular interaction, and immuno-detected with anti-MR (B: Re-IP). The amount of MR recovered from Aldo-treated samples increased greatly compared with that recovered from the control (+30 and +60% lanes d and e, *vs* lane a, $p<0.0001$), whereas from the Cor-treated sample the Re-IP MR band was -90% (lane b; $p<0.0001$) compared with controls. Re-IP from Can-treated samples yielded the same band as controls (lane c *vs* lane a). (**Figure 5**).

DTT induced increase in further MR re-immuno-precipitations from almost all samples, except Cort-treated ones, may be due to the fact that the N terminal (1-300) region of MR, recognized by the antibody, would be involved in dimer/complex formation, thus preventing MR immuno-precipitation. In fact, in Aldo-treated samples Aldo onduced MR dimer formation, as shown by the shifting of the MR-recovering curve in the glycerol gradient sedimentation (**Figure 2**), involving the N-terminal region of both receptors. This reduced the possibility for the antibody to correctly immuno-precipitate MR. Moreover, Cort treatment induced MR release from the complex but

Table 1. MR fragments and RBC Aldo concentrations after *in vitro* treatment. Isolated RBC from 4 healthy controls (HC) at 20% hematocrit were incubated at 37°C for 1 h in absence (C) or presence of 2, 5 and 10 nM aldosterone (Aldo), 1 μM canrenone (Can), 5 μM cortisol (Cort) and 5 nM Aldo plus 1 μM Can (Aldo 5 nM+Can) or 5 nM Aldo plus 5 μM Cort (Aldo 5 nM+Cort). Diluted cytosol, obtained as described in Methods, was separately analysed for MR fragments and Aldo content. MR fragments: diluted cytosol (300 μl) was loaded in a linear glycerol gradient and centrifuged for 18 hours at 100 000×g. Fractions 3 and 4 (200 μL each) from top were analysed by Western blotting in non-reducing conditions and immune-detected with anti-MR antibody. RBC [Aldo]: diluted cytosol (100 μl) was analysed by RIA as described in Methods

In vitro assay	MR fragments in fraction 3-4%	RBC [Aldo] ng/dl
C	100±3	5.8±0.2
Aldo 2 nM	130±8[a]	11.6±0.5[a]
Aldo 5 nM	145±7[a]	30.7±2.5[a]
Aldo 10 nM	172±5[a]	67.1±3.2[a]
Can	104±1[b]	5.7±0.2[b]
Aldo 5 nM+Can	103±3[c]	6.2±0.3[c]
Cort	102±6[b]	5.9±0.3[b]
Aldo 5 nM+Cort	103±3[c]	6.3±0.3[c]

Data are the mean ± SD of four separate determinations. Statistical analysis was performed using ANOVA followed by Tukey-Kramer *post hoc* test. [a]$p<0.0001$, comparison *versus* C; [b]p not significant, comparison *versus* C; [c]$p<0.0001$, comparison *versus* Aldo 5 nM.

not dimer formation, thus making the MR-N-terminus recognizable by the antibody. Reducing conditions might dissociate MR from both dimer and complex, as indicated by the increase in the MR-band in Re-IP assays of controls and Aldo-treated samples (**Figure 5B**, lanes a, d, e). Can did not alter the structure of the MR-complex, which remained at control level.

Further confirmation was obtained by the addition of Geldanamycin (Gelda), an apoptotic compound which inhibits HSP90 and disrupts the cytosol-multiprotein complex [29]. In the presence of Gelda, Aldo-induced dimer formation was prevented, as demonstrated by complete recovery of MR from all samples (C, lanes a-e) and the absence of MR in the Re-IP assay in reducing conditions (data not shown).

MR proteolytic fragments and Aldo contents in PA and HC RBC cytosol

To validate if Aldo was effectively entrapped in RBC cytosol, Aldo contents by RIA in PA and HC RBC were compared. Results showed that PA RBC contained higher Aldo concentrations compared to HC group (10.89±2.64 ng/dl in PA *vs* 5.96±0.23 ng/dl in HC, $p<0.0001$) (**Figure 6A**). This fact was paralleled by a significantly higher Aldo concentration found in plasma of PA, in comparison to HC ($p<0.0001$, **Figure 6A**). In addition, cytosol from PA group was also subjected to glycerol gradient sedimentation, and fractions were investigated for MR content. MR sedimentation pattern was almost completely identical to that of HC group, but a net increase of MR fragments was immuno-detected in fractions 3-4 (277.77±69.33% in PA vs 100.42± 1.98% in HC, $p<0.0001$) (**Figure 6A**). The strict correlation among RBC cytosol Aldo content or plasma Aldo concentrations and % of MR fragments content in fractions 3-4 is shown in **Figure 6B** (see also **Table 1** for details) (**Figure 6**).

Discussion

MR activity has long been related to the genomic response occurring after Aldo addition and involving Aldo-MR shifting to the nucleus to regulate gene transcription. Recently, growing evidences of an early non-genomic response have been related to Aldo direct effects on protein kinases, Na^+/H^+ exchangers and NADPH oxidase [30, 31]. Here we demonstrate that, in human RBC, Aldo can trigger MR response similar to that involved in the genomic pathway but lacking nuclear transcription. In addition, we show that in human RBC the effector utilized (Aldo, Cort or Can) can differently modulate MR activity. In non-activated conditions, MR is mainly complexed in the cytosol in a multiprotein aggregate, which ensures it has the correct conformation for binding effectors. Aldo binds to MR, causes it to break away from the complex, as shown by the shifting of the molecular weight of MR in the glycerol gradient, and induces MR dimer formation (**Figure 7**). Aldo-MR dimers have a short life-span, as shown by the time-dependent increase of MR low molecular weight proteolytic fragments (**Figures 3** and **7**) thus ensuring signal shutdown and sequestration of Aldo in the proteolysed fragments (Supplementary Figure 1). This is further

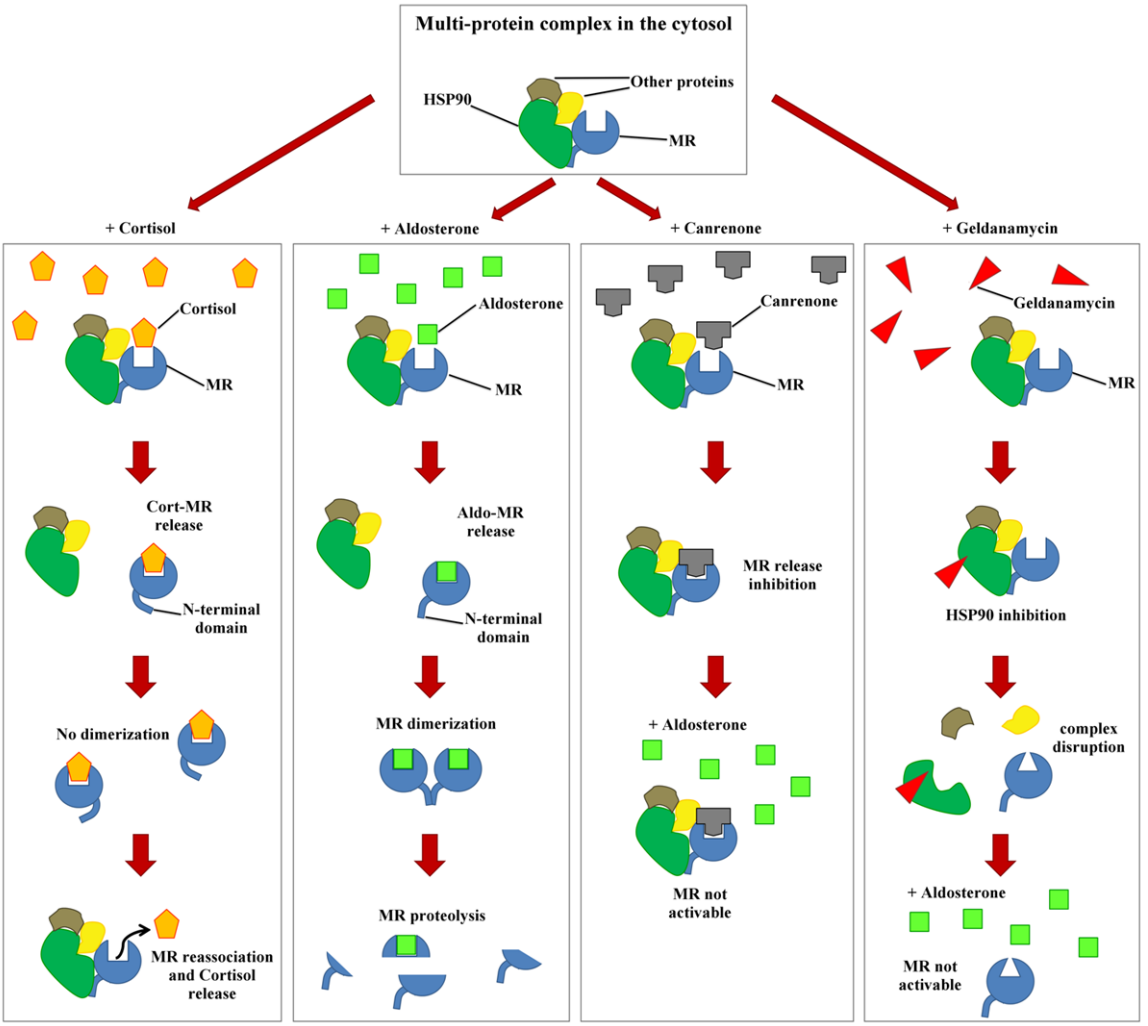

Figure 7. MR activation in human RBC. Aldo induces MR to break away from the complex and form dimers. Cort induces MR release but not dimerization and successive re-association which prevents MR degradation. Can binds to MR but prevent MR release. GA, HSP90 inhibitor, disrupts the complex and releases chaperoned proteins thus preventing Aldo-MR binding. The multi-protein complex is fundamental in maintaining MR in the correct conformation for later Aldo binding.

supported by the findings that in PA RBC cytosol both MR fragmentation and Aldo content are significantly higher than in HC RBC and a strict correlation was found among these parameters (**Figure 6**).

Beside the essential role of Aldo in regulating hydro/saline homeostasis, recent studies have emphasized its involvement in the occurrence of inflammatory status, both local [31] and general [32]. Aldo promotes tissue inflammation, leading to fibrosis and tissue remodeling in the heart, vascular system, and kidney [6-8]. It also contributes to generalized inflammatory status by activating circulating mononuclear cells, as shown by the Aldo-induced expression of PAI1 and p22phox [32] as well as NADPH-oxidase dependent oxidative species production [31]. We can argue that proteolytic events degrading MR in the presence of Aldo would interrupt Aldo-MR-mediated signaling, thus preventing further inflammation related events. In human RBC, Aldo has been demonstrated to induce dose- and time-dependent membrane alterations, strictly related to inflammatory status, leading to IgG membrane binding [22] and early RBC removal from circulation. However, as Aldo-MR binding is quite irreversible, MR degradation does not release Aldo from MR. In this way, once bound, Aldo remains sequestered

within RBC, resulting in a substantial aid for complete hormone removal from circulation, thus preventing dangerous Aldo-mediated signaling in both circulating and tissue cells [22].

When Aldo is replaced by Cort, MR shifted slightly from the gradient area of the MR complex in the early 30 min of incubation, after which MR reconstituted its initial complex (**Figures 4** and **7**). This is in line with the fact that Cort did not induce alterations in RBC membranes and, in addition, when added with Aldo, prevented Aldo-mediated alterations [22] by competing for the same ligand binding.

Can, by an irreversible binding to MR which blocks Aldo bond, did not cause any alteration in the complex, but completely avoided Aldo-induced disaggregation (Supplementary Figure 2) and alterations in membranes [22].

Interestingly, in both Aldo-MR dimer and MR-complex, MR binding is regulated by redox conditions, as shown by the differences in the immunoprecipitation assays due to reducing or non-reducing conditions (compare **Figures 2** and **3**). In addition, the observation that the anti-MR antibody used for the immunoprecipitation, recognizing the N-terminal region (aa. 1-300) of the MR, did not bind to MR when dimerised or complexed, suggests that this region is involved in dimer/complex formation (**Figure 5**). This was further confirmed by the fact that Cort induces MR release but not dimerization. In this way, the N-terminal region of Cort-MR remains free: a) to be recognized by the antibody; b) to re-associate with the multi-protein complex (**Figure 4**), by releasing Cort, thus maintaining the correct conformation for potential later Aldo binding. In addition, MR re-association prevents MR degradation, which was only slightly higher than in controls, thus contributing to MR reserve maintenance.

As determinant part of the complex, the chaperone protein HSP90 has a central role in the MR activation. Gelda, which binds to the ATP binding site of HSP90 N-terminal domain and inhibits the chaperone activity of the HSP90 [29], succeeded in releasing MR from the complex, but prevented Aldo-induced MR dimerization, thus confirming that the multi-protein complex is fundamental in maintaining MR in the correct conformation for later Aldo binding.

HSP90 is known to be involved in cell proteostasis by chaperoning native proteins into multi-protein complex to prevent misfolding and turn over. HSP90 associates with a number of signaling proteins [28] ensuring their stability and correct functioning in the regulation of the programmed cell death. The involvement of cytosolic HSP90-multiprotein complex in the development of cell patho-physiology has been recently assessed in B-cell chronic lymphocytic leukemia where HSP90 association with Lyn's dysregulated expression, activity, and localization supported abnormal cell survival by inhibiting an early player of apoptotic signaling [33, 34] (**Figure 7**).

In conclusion, our data demonstrate for the first time, that in human RBC a genomic-like Aldo signaling involves MR activation, dimerization and proteolysis but avoids gene transcription. This MR-mediated response involves ligand specificity, with Aldo inducing MR activation leading to RBC membrane alterations and IgG binding [22] and Cort preventing MR activation. This different ligand-dependent recruitment and activation pathways of MR has not yet been demonstrated in other tissues where MR specificity for Aldo against Cort was tightly related to the expression of 11-beta-hydroxysteroid dehydrogenase 2, which transforms Cort to cortisone thus preventing MR inappropriate activation [35]. The evidence that Aldo-induced activation/dimerization is regulated by oxide-reductive conditions and HSP90-chaperoned complex integrity may be crucial for the choice of a therapy in patients with high plasma concentration of Aldo. A new perspective in the treatment of this disease may also arise from the consideration that dimers proteolysis may ensure a sort of Aldo scavenging from circulation by entrapping Aldo in MR fragments.

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Disclosure of conflict of interest

None.

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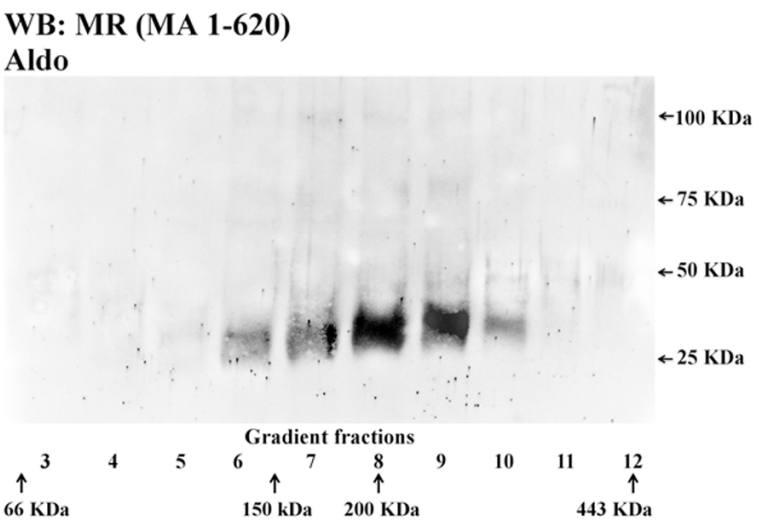
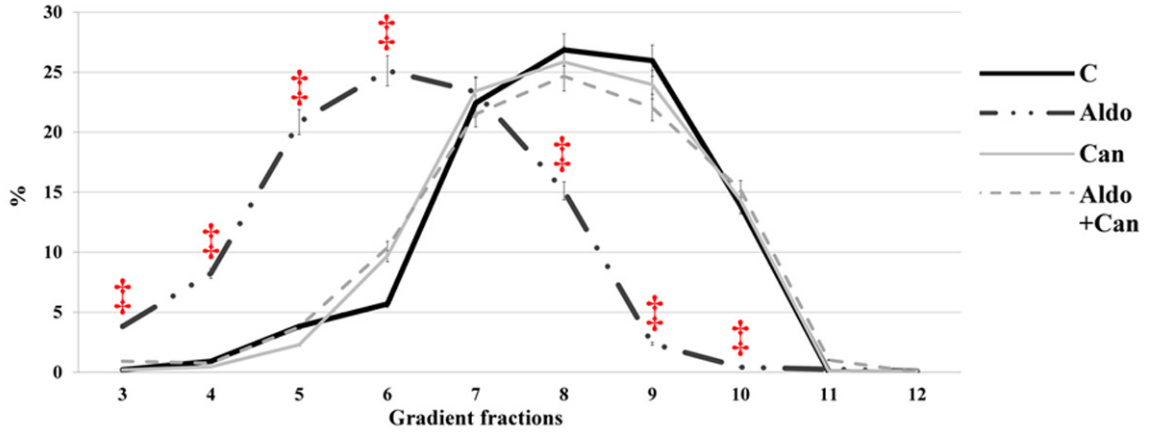
Supplementary Table 1. Baseline clinical characteristics and biochemical parameters of PA patients. Data are means ± SD

Parameter	Normal range	PA Patients (n=22)
Age (yr)	-	51.3±12.5
BMI (kg/m²)	-	27.8±3.1
Blood pressure (mmHg)		
Systolic	<120	153.4±14.9
Diastolic	<80	98.3±15.4
Sodium (mEq/L)	135-145	143.1±1.9
Potassium (mEq/L)	3.5-4.5	3.4±0.3
Aldosterone (ng/dL)	3.5-30	54.4±18.6
PRA (μg/L/h)	1.3-5.2	0.6±0.3
ARR	<30	124.2±65.1

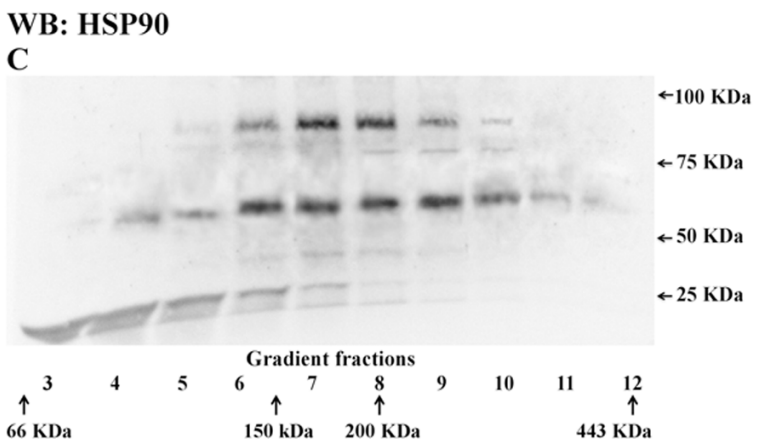
None of the patients were affected by chronic inflammatory disease. At study entry, blood samples were collected from each subject to measure sodium, potassium, serum Aldo and PRA in the upright position.



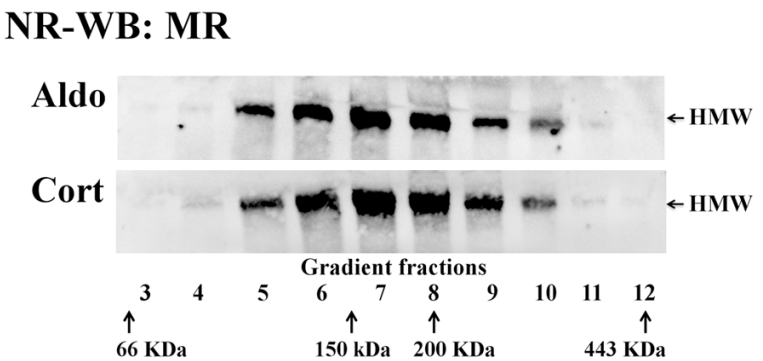
Supplementary Figure 1. Isolated RBC at 20% hematocrit were incubated at 37 °C for 1 h in presence of 5 nM Aldo (Aldo) in charcoal stripped plasma (CS-PPP) and hemolysed as described in Methods. Diluted cytosol (300 μl) was loaded on top of a linear glycerol gradient and centrifuged for 18 hours at 100 000×g. Eighteen fractions (200 μl each) were collected from top and analyzed by Western blotting and immune-revealed with anti-MR (MA 1-620) antibody produced with aldosterone-3 as immunogen (Thermo Scientific, Rockford, IL, USA).



Supplementary Figure 2. Isolated RBC at 20% hematocrit were incubated at 37 °C for 1 h in presence or absence (C) of 5 nM aldosterone (Aldo) or 1 µM canrenone (Can) and 5 nM Aldo plus 1 µM Can (Aldo+Can) in CS-PPP in order to remove small molecular weight macromolecules such as steroid and peptide hormones. Incubated RBC underwent hemolysis and 300 µl of diluted cytosol were loaded on top of a linear glycerol gradient and centrifuged for 18 hours at 100 000×*g*. Eighteen fractions (200 µL each) were collected from top and analyzed by Western blotting and immune-revealed with anti-MR antibody (MCR H-300), raised against aminoacids 1-300 (Santa-Cruz). Densitometric analysis of the anti-MR western blots 50-80 kDa bands were quantified and data were expressed as % of the total amount of 50-80 kDa bands, respectively, in all fractions, according to the calculation: $MR\text{-}bands_{fraction}$/ $MR\text{-}bands_{total}$ Statistical analysis indicated a significant difference at peak values between C and Aldo conditions. The figure is representative of 12 experiments performed in duplicate. Data are mean ± SD. ‡ $p<0.0001$, ANOVA, comparison C *vs* Aldo.



Supplementary Figure 3. Isolated RBC at 20% hematocrit were incubated at 37 °C for 1 h in absence of effectors (C) in CS-PPP and hemolysed. Diluted cytosol (300 µl) was loaded on top of a linear glycerol gradient and centrifuged for 18 hours at 100 000×*g*. Eighteen fractions (200 µl each) were collected from top and analyzed by Western blotting and immune-revealed with anti-HSP90 antibody (Santa-Cruz).



Supplementary Figure 4. RBC from 12 healthy volunteers, incubated at 37 °C for increasing times (10, 30, 45, 60 min) in absence (C) or presence of 5 nM aldosterone (Aldo) or 5 µM cortisol (Cort), were hemolysed and 300 µl of diluted cytosol were loaded on top of a linear glycerol gradient and centrifuged for 18 hours at 100 000×*g*. Fractions (200 µL each) from top were analysed by Western blotting in non-reducing conditions and immune-detected with anti-MR antibody. The pattern shows bands corresponding to high molecular weight (HMW) region of samples Aldo and Cort incubated for 60 min.