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Inhibition by aldosterone of insulin receptor mRNA levels and insulin binding in U-937 human promonocytic cells ☆

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Abstract

The effect of aldosterone on insulin receptor (IR) expression was investigated in U-937 human promonocytic cells. The putative involvement of the mineralocorticoid receptor (MR) was also analysed. Aldosterone binding assays indicated the presence of MRs with high affinity and limited capacity in these cells. RNA blot assays showed that aldosterone treatment decreased the levels of the two major IR mRNAs (11 and 8.5 kb) present in these cells in a dose- and time-dependent manner. The partial reversal of such a decrease by the mineralocorticoid antagonist spironolactone suggested that MR was involved in the process. Experiments with the RNA synthesis inhibitor actinomycin D indicated that the decrease in IR mRNA content in aldosterone-treated cells was not the result of transcript destabilisation. The inhibitory action of aldosterone was not prevented by the simultaneous presence of the protein synthesis inhibitor cycloheximide, suggesting that the reduction of IR gene expression occurs as a direct response to the action of aldosterone. Furthermore, insulin binding assays showed that aldosterone decreased IR capacity but did not alter receptor affinity. In addition, the IR turnover resulted unaltered. These results provide the first evidence for an in vitro modulation of human IR expression by aldosterone.

1. Introduction

Aldosterone, the major mineralocorticoid in humans, regulates electrolyte and water homeostasis by increasing Na^+ re-absorption and promoting K^+ and H^+ secretion in mineralocorticoid target tissues. Recently, several new activities not directly related to electrolyte transport have also been attributed to aldosterone in not classical mineralocorticoid target tissues. These include, differentiation in 3T3-L1 adipocytes [1], immune actions in human mononuclear cells [2], alterations in collagen synthesis in rat cardiac fibroblasts [3], modifications in membrane properties in rat CA1 pyramidal neurones [4], and changes in gene expression in rat hippocampus [5].

The action of aldosterone is mediated by the mineralocorticoid receptor (MR). This receptor belongs to the class I of nuclear receptors, which also includes receptors for glucocorticoids, progestins, androgens and estrogens [6]. Aldosterone binds to MR in the cytoplasm of the cell and the activated complex is then translocated into the nucleus, where it acts as a transcription factor, binding to hormone response elements in the promoter region of regulated genes. In addition to its high affinity for mineralocorticoids, MR shows nearly identical affinity for glucocorticoids [7]. Moreover, no selective mineralocorticoid responsive elements have been reported, and MRs and glucocorti-

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coid receptors appear to bind with equal affinities to common hormone response elements [8]. Furthermore MR and glucocorticoid receptors seem to act as dimers, either homodimers or heterodimers, enhancing [9] or lowering [10] transcriptional activity. In addition to transcriptional regulation, other intracellular processes can also be affected by aldosterone including mRNA stability, translational efficiency and protein turnover [11]. Nevertheless, the implication of these post-transcriptional mechanisms in the mineralocorticoid action has been scarcely studied.

Earlier in vivo and in vitro studies in our laboratory and others have demonstrated regulation of insulin receptor (IR) gene expression by glucocorticoids [12–19], progestins [20] and androgens [21]. We have also observed a tissue-specific modulation of IR gene expression in an in vivo situation of mineralocorticoid excess in rats [22]. With these antecedents the aim of the present investigation was to examine the possibility of an in vitro MR-mediated effect of aldosterone on IR expression using the U-937 human promonocytic cell line [23]. These cells represent a useful in vitro monocyte-like model for the evaluation of IR expression and its modulation by diverse agents [16,24–25]. In particular, we analyse (i) the presence of MR in U-937 cells, (ii) the effect of aldosterone on IR mRNA level and stability, and (iii) its effect on insulin binding and IR turnover in these cells.

2. Materials and methods

2.1. Cell culture and treatments

U-937 human promonocytic cells (mycoplasma-free) were grown in suspension in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 0.2% sodium bicarbonate and antibiotics in a humidified 5% CO_2 atmosphere at 37°C as previously described [19]. Aldosterone (Sigma Química, Madrid, Spain) was dissolved in absolute ethanol at 10^{-2} M and applied to the cells at final concentrations of 10^{-10}–10^{-7} M, according the experiments. Spironolactone (Sigma) was also dissolved in absolute ethanol at 10^{-2} M and applied at a final concentration of 10^{-6} M. Once treated, the cells were collected by centrifugation and washed three times with PBS buffer to remove endogenous steroids and serum proteins. The toxicity of the treatments was estimated by evaluation of permeability to trypan blue [26].

2.2. RNA extraction and blot assays

Extraction of total RNA was performed according to the method of Chomczynski and Sacchi [27]. For dot-blot assays, 10 μg RNA samples were denatured and applied onto nitrocellulose membranes (Hybond-C, Amersham, Buckinghamshire, UK) using a microfiltration apparatus (Bio-Rad, Richmond, CA, USA). For Northern blot assays, 30 μg RNA samples were denatured, electrophoresed in 1.1% agarose-formaldehyde gels containing 0.5 μg/ml ethidium bromide and blotted onto nylon membranes (Hybond-N, Amersham). Ethidium bromide staining of 28 S ribosomal RNA was routinely checked before blotting as a control of sample loading onto the gels, and after blotting as a control of RNA transfer to the membrane [25]. The RNA blots were pre-hybridised, hybridised with excess [^{32}P] labelled probes, washed under highly stringent conditions and autoradiographed [28]. When required, the relative intensity of dots or bands was measured with a laser densitometer (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA, USA). The probe used was the 4.1-kb human insulin receptor-specific EcoRI fragment of the pHIR/P12-1 clone [29], obtained through the ATCC. The fragments were labelled to approximately 10^9 cpm/μg of DNA with [α-^{32}P]dCTP (3000 Ci/mmol, Amersham) by random hexanucleotide priming.

2.3. IR mRNA stability and the novo protein synthesis

IR mRNA half-life was determined by an actinomycin D decay curve [25]. Actinomycin D (Sigma) was dissolved in absolute ethanol at 4×10^{-3} M and applied at 4×10^{-6} M for increasing time periods (2, 4, and 6 h) to both untreated cells and cells pre- treated for 24 h with aldosterone. The accumulations of total IR mRNA were measured by dot-blot assays.

De novo protein synthesis in aldosterone-treated cells was tested using cycloheximide (Sigma) [25]. This inhibitor of translation was dissolved in RPMI medium at 10^{-2} M and applied at 10^{-5} M, 30 min before the 24 h-aldosterone treatment. The IR mRNA levels were analysed by dot blot assays.

2.4. Binding assays

Aldosterone binding characteristics in these cells were assessed by a modification of the whole cell steroid binding assay of Jakob et al. [30]. In brief, 12×10^6 cells/ml were incubated with 0.5×10^{-9} M [^{3}H]aldosterone (84.4 Ci/mmol; NEN Life Science Products, Belgium) at 37°C for 2 h in the absence or presence of increasing concentrations (10^{-9} to 5×10^{-9} M) of unlabelled aldosterone. Cells were then washed and resuspended in an ice cold buffer (5×10^{-2} M Tris, pH 7.4, 0.25 M sucrose, 5×10^{-3} M $MgSO_4$) for 15 min. The suspension was treated with 1% Triton X-100 for 15 min at 4°C and centrifuged to separate the upper cytosolic fraction and the lower nuclear fraction. Both fractions were initially estimated

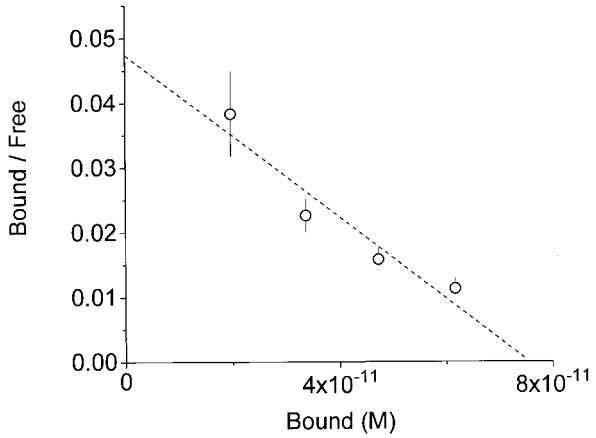

Fig. 1. Presence of mineralocorticoid receptors in U-937 cells. Scatchard analysis of the displacement of [^{3}H]aldosterone binding by increasing concentrations of unlabelled hormone (mean ± SEM).

by scintillation counting. Aldosterone binding was detected predominantly in the cytosolic fraction and consequently only this fraction was used for counting. The data were analyzed by the method of Scatchard [31], subtracting from each point the non-specific binding in the presence of 5×10^{-8} M unlabelled aldosterone.

Insulin binding assays were carried out as previously described [16,25]. 5.7×10^6 cells/ml were incubated with 0.5×10^{-10} M mono [^{125}I]insulin (112 μCi/μg; NEN Life Science Products) at 15°C for 1 h, in the absence or presence of increasing concentrations (5×10^{-11} to 1.25×10^{-8} M) of unlabelled insulin (Novo Nordisk A/S. Bagsvaerd, Denmark). Non-specific binding was determined in the presence of 2.5×10^{-7} M unlabelled insulin and subtracted from each point. The data were analysed by the method of Scatchard using the LIGAND program of Munson and Rodbard [32].

Turnover of IRs was assessed by a cycloheximide decay curve [33] in the absence or presence of 10^{-9} M aldosterone during 12, 24 and 38 h.

2.5. Statistical analysis

Unless otherwise stated, data are expressed as mean ± SEM of at least three determinations. Where appropriate, an unpaired Student's t test was also employed. The threshold for significance was set at $p < 0.05$.

3. Results

In order to gain insight upon a hypothetical aldosterone action on IR gene expression in U-937 cells, our first purpose was to identify MRs in these cells.

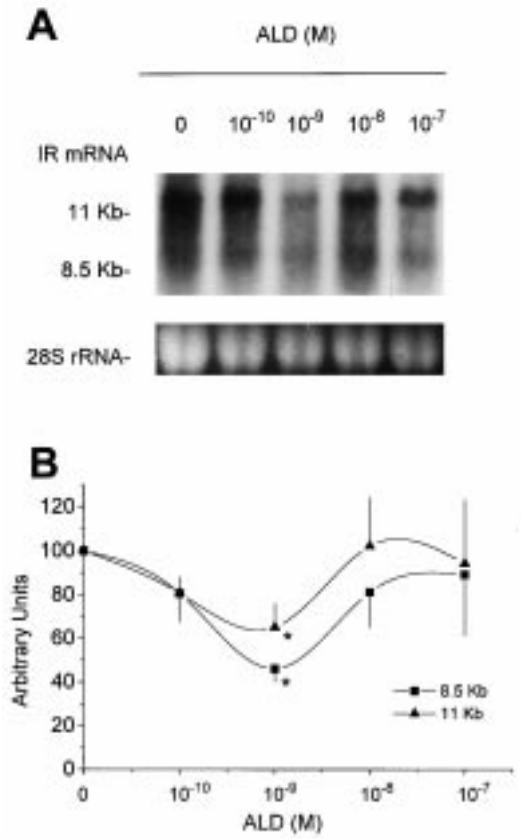

Fig. 2. Dose-dependent effect of aldosterone (ALD) on insulin receptor (IR) mRNA levels in U-937 cells. Samples of 30 μg of total RNA extracted from untreated cells (0) and cells treated for 24 h with increasing concentrations of ALD were used in Northern assays. (A) Autoradiograph of a representative dose-response experiment, showing the two major IR mRNA species. Ethidium bromide staining of 28 S ribosomal RNA in the gel is shown as a control of sample loading. (B) Densitometric readings of the total autoradiographs normalised with their respective 28 S rRNA values. IR mRNA species were quantified separately and expressed as the percentage of the value of each species in untreated cells (mean ± SEM, $^*p < 0.05$).

Fig. 1 shows the Scatchard analysis of the displacement of [^{3}H]aldosterone binding to the cells by increasing concentrations of unlabelled hormone. The plot revealed the presence of a single class of MRs of high affinity (Kd (10^{-9} M): 1.60 ± 0.17) and limited capacity (2275 ± 247 sites per cell) for the hormone. Therefore, our results indicated the presence of high affinity aldosterone binding sites in U-937 cells.

Then, we wanted to analyse possible MR-mediated effects of aldosterone on IR gene expression. Thus, U-937 cells were incubated for 24 h with increasing concentrations of this steroid (from 10^{-10} M to 10^{-7} M) and the IR mRNA levels determined by Northern blot assays (Fig. 2A and B). These cells exhibited two major IR mRNA species of approximately 11 and 8.5 kb in size, and a ratio (11 kb/8.5 kb) of 1.20 ± 0.02 in accordance with our previous findings [28]. The levels

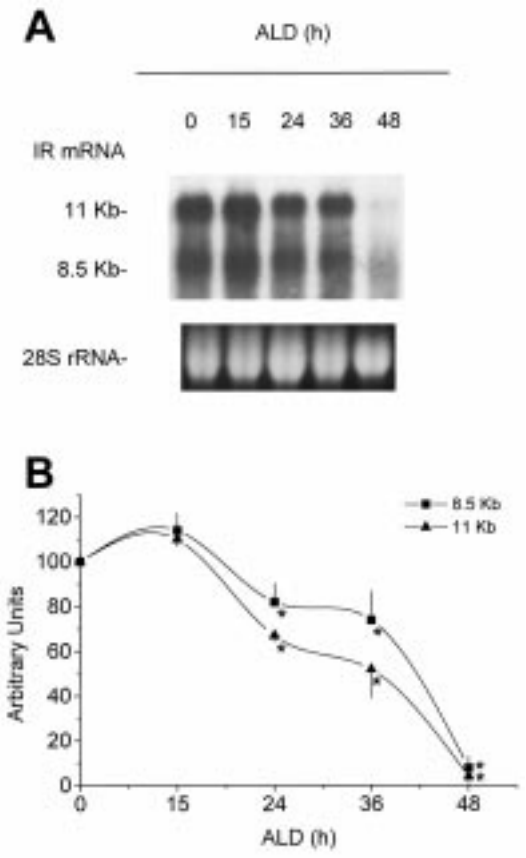

Fig. 3. Time-dependent effect of aldosterone on insulin receptor (IR) mRNA levels in U-937 cells. Total RNA extracted from untreated cells and cells treated with 10^{-9} aldosterone (ALD) for the indicated time periods were used in Northern assays. (A) Autoradiograph of a representative time-response experiment, showing the two major IR mRNA species. The respective amounts of 28 S rRNA revealed by ethidium bromide are also shown. (B) Densitometric readings of the total autoradiographs normalised with their respective 28 S rRNA values. IR mRNA species were quantified separately and expressed as the percentage of the value of each species in untreated cells (mean ± SEM, $^{*}p < 0.05$).

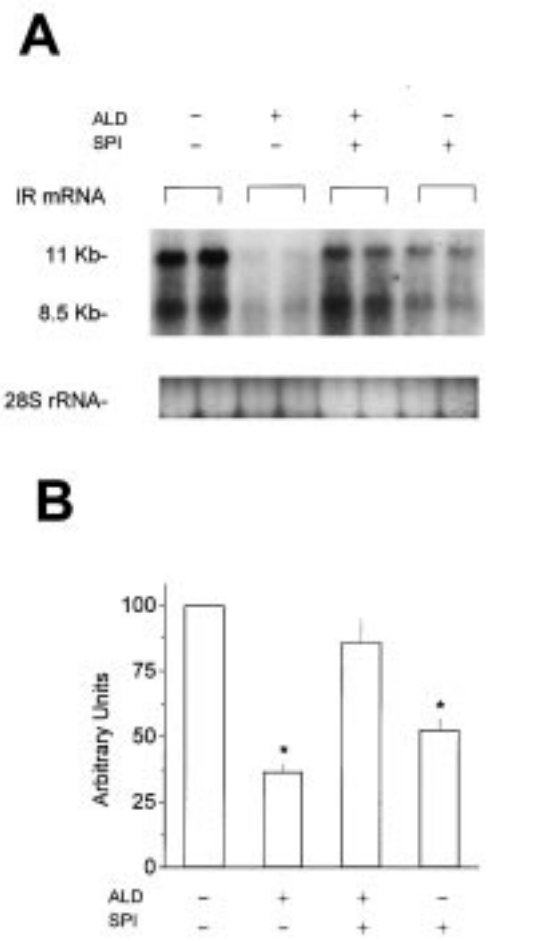

Fig. 4. Effect of spironolactone (SPI) on insulin receptor (IR) mRNA levels. Cells were incubated for 24 h in the absence or presence of 10^{-6} M spironolactone (SPI), 10^{-9} M (ALD) or both. (A) Autoradiograph of a Northern blot experiment carried out in duplicate. (B) Densitometric readings of the total autoradiographs normalised with their respective 28 S rRNA values. Total RNA (11 + 8.5 kb species) levels in untreated cells were given the arbitrary value of 100 (mean ± SEM, $^{*}p < 0.05$).

of both RNAs were decreased about 20% upon treatment with 10^{-10} M aldosterone, reaching minimum values (54% decrease) with the addition of 10^{-9} M, and returning to near normal levels at higher aldosterone concentrations (Fig. 2A and B).

To assess the time-course of aldosterone inhibition of IR mRNA levels, U-937 cells were incubated for up to 48 h with 10^{-9} M aldosterone (Fig. 3A and B). This steroid produced an inhibition in the levels of both IR mRNA species, which was first observed at 24 h of treatment (30% decrease), reaching the 100% of inhibition 48 h after addition of hormone (Fig. 3A and B). Such an inhibition was not associated with any cytotoxic action of aldosterone, since the cell viability was always greater that 95%, even after 48 h of treatment with the hormone. These results demonstrated that aldosterone regulated IR gene expression at the RNA level in a dose- and time- dependent manner in U-937 cells.

The involvement of the MR in the aldosterone-induced inhibition of IR mRNA levels was investigated by blocking the MR with the mineralocorticoid antagonist spironolactone [34] at a concentration of 10^{-6} M for 24 h (Fig. 4A and B). We observed that the combined administration of aldosterone plus spironolactone partially abolished the inhibition of IR gene expression exerted by aldosterone alone. Spironolactone, itself, also caused a decrease in IR mRNA levels but to a lesser extent than aldosterone (Fig. 4A and B). This could be due to the ability of the spironolactone to act also as a partial agonist in certain cells [35]. Thus, the MR was shown to be

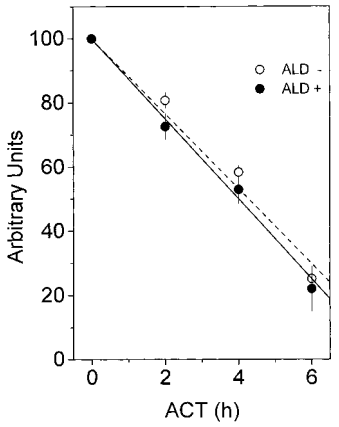

Fig. 5. Effect of aldosterone on insulin receptor (IR) mRNA stability. U-937 cells were incubated for 24 h in the absence (ALD−) or the presence (ALD+) of 10^{-9} M aldosterone, followed by the addition of 4×10^{-6} M actinomycin D (ACT) and further incubation for the times indicated. Samples of 10 μg of total RNA were used for dot-blots. The autoradiographs were analysed by densitometric scanning. RNA levels were expressed as the percentage of values at 0 h of ACT treatment, in both untreated and aldosterone-treated cells (mean ± SEM).

involved in the aldosterone-elicited decrease in IR mRNA levels in U-937 cells.

To further assess the mechanisms of the aldosterone-elicited decrease in IR mRNA levels, we determined whether this effect could be due to a decrease in transcript stability. For this purpose actinomycin D was applied for increasing time-periods to untreated cells and cells treated for 24 h with aldosterone, and the accumulation levels of total IR mRNA was determined by dot-blot assays. The results in Fig. 5, indicate that the half-life of this mRNA, which was 4.32 ± 0.07 h in untreated cells in accordance with our

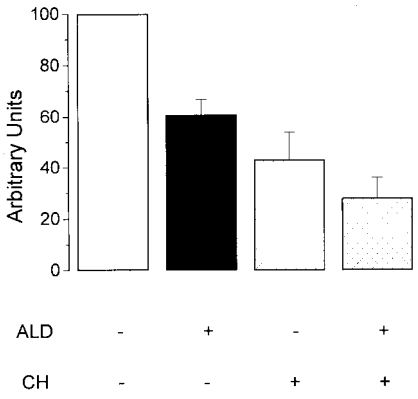

Fig. 6. Effect of cycloheximide (CH) on insulin receptor (IR) mRNA levels. Cells were pre-incubated for 30 min in the absence or presence of 10^{-5} M cycloheximide and then incubated for a further 24 h in the absence or presence of 10^{-9} aldosterone (ALD). Samples of 10 μg of total RNA were used for dot-blot. The autoradiographs were analysed by densitometric scanning. RNA levels in untreated cells were given the arbitrary value of 100 (mean ± SEM).

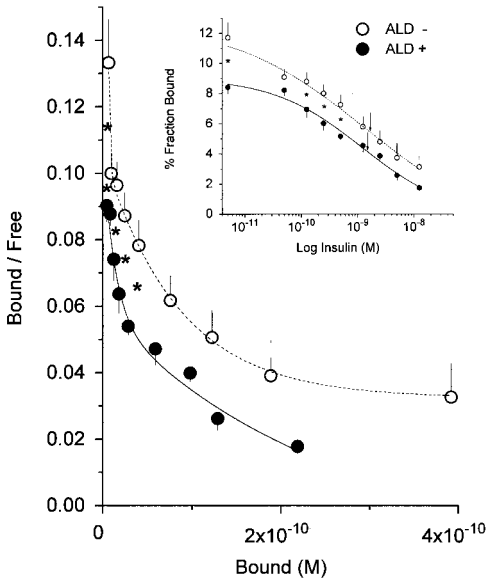

Fig. 7. Regulation by aldosterone of insulin receptors in U-937 cells. Scatchard analysis of the displacement curves of mono [^{125}I]insulin binding in untreated cells (ALD−) and cells treated for 48 h with 10^{-9} M aldosterone (ALD+). In the insert, arrows represent the corresponding amount of unlabelled insulin required to decrease maximum specific binding by 50% (IC_{50}) in each group of cells (mean ± SEM, $^{*}p < 0.05$).

previous findings [25,28], was not significantly altered: 3.97 ± 0.28 h in aldosterone-treated cells. Therefore, the aldosterone-elicited decrease in IR mRNA levels may not be explained by a reduction in transcript stability.

To determine the possible dependence on protein synthesis of the aldosterone-elicited decrease in IR gene expression, the levels of this RNA were measured by dot-blot assays in untreated cells and in cells treated for 24 h with aldosterone, in the absence or presence of the inhibitor of translation, cycloheximide (Fig. 6). In these experiments, cells treated with aldosterone in the absence of cycloheximide showed approximately a 40% reduction of IR mRNA levels. Although cycloheximide decreased the basal levels of IR mRNA, perhaps due to the long duration of the treatment [19,25], a reduction of approximately 40% of IR mRNA also occurred in the presence of cycloheximide after treatment with aldosterone. These decreases in the same range in the absence or presence of the translation inhibitor (Fig. 6) indicated that the inhibition of IR gene expression was a direct response to the action of aldosterone, not mediated by the prior induction of other gene products.

Then, we wanted to analyse whether the decrease in IR mRNA levels could lead to a decrease in insulin binding. With this aim, displacements curves of [^{125}I]insulin binding were measured in untreated and cells treated with 10^{-9} M aldosterone for 48 h. Scatchard analysis in Fig. 7 shown that aldosterone

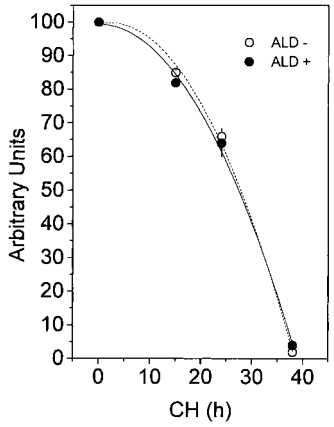

Fig. 8. Insulin receptor (IR) turnover in the presence of cycloheximide (CH). Cells were pre-incubated for 30 min with 10^{-5} M CH and then incubated for the times indicated with CH in the absence or presence of 10^{-9} M aldosterone. Maximum specific binding was then quantified and expressed as the percentage of values at 0 h of CH treatment (mean ± SEM).

treatment caused a clear reduction (34% decrease) in insulin receptor capacity (15611 ± 1791 vs 23616 ± 7227 sites per cell) although the effect was only statistically significant at unlabelled insulin concentrations below 1.25×10^{-9} M but not at higher concentrations. In addition, IR affinity, reflected by the IC_{50} value (insert in Fig. 7), was almost identical in both treated and untreated cells (Kd (10^{-9} M): 1.20 ± 0.09 vs 1.30 ± 0.17), indicating that the decrease in binding was not related to alterations in receptor affinity. Therefore, the aldosterone-provoked decrease of IR expression at the RNA level was also accompanied by a decrease in IRs at the protein level.

Inhibition of protein synthesis with cycloheximide was used to assess turnover of IRs in the presence or absence of 10^{-9} M aldosterone (Fig. 8). The decline in receptor levels gave an exponential plot as a function of time in both cases. The IR half-life was approximately 24 h in the absence or presence of the hormone. This showed that aldosterone does not directly affect IR stability.

4. Discussion

In the first part of this work, a whole cell cytoplasmic association of [^{3}H] aldosterone was used to demonstrate the presence of MRs in U-937 cells. They consisted of a single class of receptors with characteristics of affinity and capacity very similar to those detected by other authors in more classical mineralocorticoid target tissues [7]. Although presence of MRs in U-937 cells has not been previously demonstrated, other authors have reported their existence in other related cells such as human leukocytes [36,37] and mouse macrophages [38]. In addition, basal expression of MR mRNA has been recently described in cultures of human monocytes [2]. Given that the Kd value of MRs in U-937 cells is comparable to the physiological concentration of aldosterone in Man [39], it seems possible that these MRs could mediate physiological aldosterone effects, including changes in gene expression, in this human cell line.

The second part of the investigation allowed us to demonstrate that aldosterone was able to modulate the levels of the two main IR mRNAs (11 and 8.5 kb) present in U-937 cells. Inhibition of both species was shown after the addition of a concentration of 10^{-9} M of this steroid. This concentration is equivalent to the Kd value of MRs in these cells, indicating that such a reduction could be produced after aldosterone binding to MRs. However, the effect of aldosterone was biphasic because it increased IR mRNA levels at higher concentrations (10^{-8}–10^{-7} M). This probably indicates that, at high concentrations, the hormone also binds to glucocorticoid receptors, thus counteracting the MR-mediated inhibitory effect. This hypothesis, is supported by our previous data [19] indicating an increase in IR mRNA levels in U-937 cells by dexamethasone at concentrations of 10^{-8}–10^{-6} M. These concentrations are in agreement to the Kd values of glucocorticoid receptors in these cells [40]. Therefore, it is possible to speculate that the in vitro regulation of IR gene expression by aldosterone in U-937 cells involves MRs at physiological concentrations of aldosterone, but also glucocorticoid receptors at higher concentrations.

Another fact is that these findings of an in vitro reduction of IR mRNA levels together with our previous in vivo results of a tissue specific modulation of IR mRNA levels in mineralocorticoid-treated rats [22], suggest that the effect of mineralocorticoids on IR gene expression was highly tissue-specific.

The decrease in IR mRNA levels attributed to aldosterone could involve a transcriptional mechanism. We did not directly analyse transcription itself, but aldosterone has been reported to decrease the levels of other mRNAs such as rat 5HT1A receptor [41], rat neuropeptide Y [42], rat Na^+ K^+-ATPase alpha 1 [43] and human interleukin-1 receptor antagonist [2], acting basically at the transcriptional level. Although specific mineralocorticoid response elements have not been identified in the IR gene promoter, several studies have reported the presence of up to five putative glucocorticoid response elements in this promoter [44]. We are presently investigating whether these elements mediate the aldosterone-induced reduction in IR mRNA.

The mineralocorticoid antagonist spironolactone was used to study the putative involvement of MR in the aldosterone-provoked decrease of IR mRNA levels. The partial reversal of the aldosterone effect by

spironolactone strongly suggests that MR is involved in the process. The fact that the reversion was only partial could be explained by the ability of the spironolactone to act also as a partial agonist in certain cells [35].

The present results also demonstrate that aldosterone did not affect transcript stability. IR mRNA half-life was approximately 4 h in both untreated and aldosterone-treated cells. This fact indirectly supports the suggestion that the inhibition was transcriptionally regulated. In this sense we have previously observed that the synthetic steroid dexamethasone [19] did not alter IR mRNA half-life in these cells.

Our results indicate that the decrease of IR gene expression in U-937 cells took place in the presence of the protein synthesis inhibitor cycloheximide, suggesting that it occurred as a direct response to the action of the hormone not mediated by the prior induction of other gene products. In a similar manner, we have previously observed that the regulation of IR gene expression by dexamethasone in these cells was not prevented by the simultaneous presence of this protein synthesis inhibitor [19].

The third part of the investigation demonstrated that aldosterone inhibited insulin binding in U-937 cells by decreasing total receptor number (34% decrease), without altering receptor affinity. This decrease at the translational level was observed in cells treated with 10^{-9} M aldosterone for 48 h, conditions that are similar to those previously used for the detection of maximum inhibition of IR mRNA levels by this hormone. This negative regulation of IRs by aldosterone would cause a decrease in the insulin responsiveness in these cells. In this regard, we have also observed a negative regulation of insulin binding in human adipocytes from a patient with primary hyperaldosteronism and impaired glucose tolerance [45].

Finally, IR turnover seemed to be unaffected by aldosterone, since IR protein was estimated to have a half-life of approximately 24 h in both untreated and hormone-treated cells.

In conclusion, aldosterone induced an inhibition of IR mRNA levels and insulin binding in U-937 cells, and the effects were mediated by hormone binding to MR. These results represent the first demonstration of an in vitro modulation of human IR expression by aldosterone. The functional significance of this MR-mediated decrease in IR expression is currently being studied in our laboratory.

Acknowledgements

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