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Inhibition of mineralocorticoid and glucocorticoid receptor function by the heat shock protein 90-binding agent geldanamycin[1]

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Abstract

The effects of mineralocorticoids and glucocorticoids are mediated by the intracellular mineralocorticoid glucocorticoid receptor (MR) and glucocorticoid receptor (GR), respectively. Several studies suggest that hormone binding and, thus, receptor activation depend on the association of both MR and GR with the 90-kDa heat shock protein (hsp 90). However, there are few reports analyzing the functional relevance of this association in vivo. The present study was designed to determine how the new hsp 90-binding agent geldanamycin, which was previously shown to disrupt the formation of steroid receptor/hsp complexes, interferes with MR- and GR-mediated transactivation in intact cells. We show that geldanamycin inhibits aldosterone-dependent transactivation of a mineralocorticoid-responsive reporter genes in a concentration-dependent manner. Similar effects were observed for the dexamethasone-activated GR. However, geldanamycin did not affect transcription from a retinoic acid-dependent reporter gene. Inhibition of GR-mediated transactivation was observed both in HeLa cells expressing endogenous GR and in COS-7 cells transfected with a GRa expression vector. Binding studies indicate that geldanamycin disrupts receptor function by reducing hormone binding affinity without lowering intracellular receptor protein levels. Our data support the current model of hsp 90-dependent steroid receptor activation. Furthermore, we show for the first time that MR function also depends on the interaction with hsp 90 in intact cells. Finally, we demonstrate that the function of endogenous is thought to keep the receptor protein in an inactive, yet ligand-activable state (9–17). Ligand binding induces a conformational change in the receptor molecule, which causes it to dissociate from the hsp complex, to translocate to the cell nucleus, and, finally, to interact with specific hormone response elements in the promoter regions of hormone-responsive genes (6–8). Both MR and GR bind as homodimers to identical palindromic sequences on the target DNA, termed glucocorticoid response elements (GREs) (18). The formation of GR/MR heterodimers has also been described (19,20) and may have profound functional consequences (21). The current model of MR and GR function holds that these receptors are unable to bind their respective hormones as long as they are not associated with the hsp complex (9–17). However, experimental support for this model is mainly based on in vitro work. There are few reports analyzing the functional relevance of GR/hsp interactions in mammalian cells. In the most recent study, Whitesell et al. showed that the hspÊ90-binding agent geldanamycin can specifically disrupt GR/hsp association, thus inhibiting glucocorticoid-mediated transcriptional activation (22). MR is even less well studied in this respect. To our knowledge, there have not been any data supporting a functional role for proper MR/hsp interaction in intact cells. In this study, we show for the first time that MR function depends on the interaction with hsp 90 in intact human cells. Furthermore, we demonstrate that geldanamycin inhibits GR-mediated transcriptional activation in two human cells lines, confirming the results by Whitesell et al. and extending them to transfected as opposed to endogenous GR.

Keywords: Mineralocorticoid; Glucocorticoid; Heat shock protein

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1. Introduction

Mineralocorticoids and glucocorticoids, two major subclasses of steroid hormones, are important regulators of fluid balance and blood pressure in humans (Orth et al., 1992). In addition, glucocorticoids modulate a large number of metabolic, cardiovascular, immune, and behavioral functions (for a review see Chrousos and Gold, 1992 and Chrousos, 1995). At the cellular level, the effects of both hormones are mediated by specific intracellular receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR), respectively (Arriza et al., 1987; Hollenberg et al., 1985). These proteins belong to the phylogenetically conserved superfamily of nuclear hormone receptors (Evans, 1988; Mangelsdorf et al., 1995; Bamberger et al., 1996), which are also referred to as ligand-dependent transcription factors (Evans, 1988; Mangelsdorf et al., 1995; Bamberger et al., 1996).

In the unliganded state, both MR and GR are part of a multiprotein complex which consists of the receptor protein, two molecules of heat shock protein (hsp) 90, and several other hsps (Rafestin-Oblin et al., 1991; Caamano et al., 1993; Hutchison et al., 1993; Pratt, 1993; Smith and Toft, 1993; Czar et al., 1994; Hutchison et al., 1994). In the absence of hormone, this complex is thought to keep the receptor protein in an inactive, yet ligand-activable state (Rafestin-Oblin et al., 1991; Caamano et al., 1993; Hutchison et al., 1993; Pratt, 1993; Smith and Toft, 1993; Czar et al., 1994; Hutchison et al., 1994; Bresnick et al., 1989; Nathan and Lindquist, 1995). Ligand-binding induces a conformational change in the receptor molecule, which causes it to dissociate from the hsp complex, to translocate to the cell nucleus, and finally, to interact with specific hormone response elements in the promoter regions of hormone-responsive genes (Evans, 1988; Mangelsdorf et al., 1995; Bamberger et al., 1996). Both MR and GR bind as homodimers to identical palindromic sequences on the target DNA, termed glucocorticoid response elements (GREs) (Lombes et al., 1993). The formation of GR/MR heterodimers has also been described (Trapp et al., 1994; Liu et al., 1995) and may have profound functional consequences (Bamberger et al., 1997).

The current model of MR and GR function holds that these receptors are unable to bind their respective hormones as long as they are not associated with the hsp complex (Rafestin-Oblin et al., 1991; Caamano et al., 1993; Hutchison et al., 1993; Pratt, 1993; Smith and Toft, 1993; Czar et al., 1994; Hutchison et al., 1994; Bresnick et al., 1989; Nathan and Lindquist, 1995). However, experimental support for this model is mainly based on in vitro work. There are few reports analyzing the functional relevance of GR/hsp interactions in intact mammalian cells. In the most recent study, Whitesell et al. showed that the hsp 90-binding agent geldanamycin can specifically disrupt GR/hsp association, thus inhibiting glucocorticoid-mediated transcriptional activation (Whitesell and Cook, 1996). MR is even less well studied in this respect. To our knowledge, there have not been any data supporting a functional role for MR/hsp interaction in intact cells.

In this study, we show for the first time that MR function depends on the interaction with hsp 90 in intact human cells. Furthermore, we demonstrate that geldanamycin inhibits GR-mediated transcriptional activation in two human cell lines, confirming the results by Whitesell et al. and extending them to transfected as opposed to endogenous GR.

2. Materials and methods

2.1. Cell culture

COS-7 and HeLa cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in low glucose Dulbecco's modified Eagle's medium (Gibco BRL, Eggenstein, Germany) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and streptomycin, and L-glutamine. Cells were incubated at 37°C in an atmosphere of 5% CO_2. Twenty four hours prior to transfection, cells were removed from their culture flasks by trypsinization, resuspended in supplemented medium, and plated in 12-well plates (1.5×10^5 cells/well).

2.2. Plasmids

pMSG-luc contains the luciferase cDNA from pGEM-luc (Promega, Madison, WI) downstream of the mouse mammary tumor virus long terminal repeat in pMSG (Pharmacia). pGL3-GRE-tk81-Luc was constructed by inserting a double stranded oligonucleotide containing the GRE sequence from the human tyrosine aminotransferase promoter into a luciferase reporter vector containing a pGL3 backbone (Promega, Madison, WI) and a minimal Herpes simplex virus type 1 thymidine kinase (tk) promotor. pGL3-RARE-tk81-luc was constructed by replacing the GRE sequence in pGL3-GRE-tk81-Luc with a double stranded oligonucleotide containing the retinoic acid response element (RARE) from the human retinoic acid receptor $\beta 2$ gene promotor. pSV-β-gal (Promega, Madison, WI) encodes the β-galactosidase reporter gene linked to the constitutively active, glucocorticoid-independent SV 40 promotor and was used to control transfection efficiencies in luciferase assays. CRE-tk81-luc contains a consensus cyclic AMP response element in front of a tk81-luciferase reporter construct. pRShMR and pRShGRα contain the full length coding region of MR and GRα,

respectively, under the control of the Rous Sarcoma Virus (RSV) promotor.

2.3. Transfection and luciferase assays

Cells were transfected by the lipofection method as previously described (Bamberger et al., 1995). Nine microliters transfectant (DOTAP, Boehringer Mannheim, Mannheim, Germany) was used per well (6 μl/μg transfected DNA). Cells were transfected with 1 μg/well of the appropiate luciferase reporter plasmid, 0.4 μg/well of the expression vectors indicated, and 0.1 μg/well of the galactosidase control vector. Twenty four hours after transfection, the medium was replaced with either normal charcoal-stripped medium or charcoal-stripped medium containing geldanamycin (Drug Synthesis Branch, National Cancer Institute, Bethesda, MD). After 2 h, 10^{-7} M aldosterone and/or dexamethasone was added to the cell culture medium.

Luciferase activity in cell lysates was determined following standard protocols (Brasier et al., 1989). Eight hours after hormone treatment, cells were washed with phosphate buffered saline (PBS), trypsinized, washed, pelleted, and lysed with reporter lysis buffer (Promega, Madison, WI). After one freeze-thaw cycle, luciferase activity in the lysate was determined in a luminometer (Lumat LB 9501, Berthold, Wildbad, Germany). β-galactosidase activity was determined in the same samples with a β-galactosidase enzyme assay system (Promega, Madison, WI), following the instructions of the manufacturer. Luciferase activity was divided by galactosidase activity to normalize for transfection efficiency. All experiments were repeated at least three times in triplicates. Statistical analysis (Mann-Whitney test) was carried out using MacIntosh StatView software.

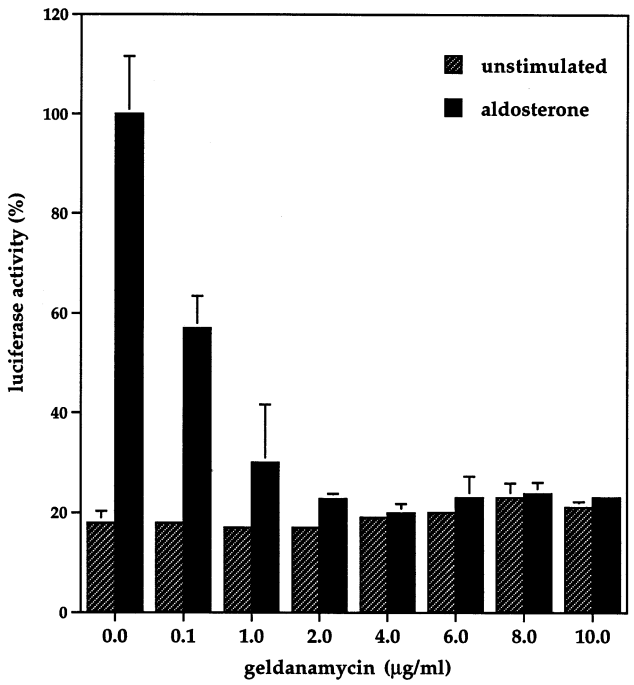

Fig. 1. Inhibition of MR-mediated gene transcription by geldanamycin. COS-7 cells were transfected with pMSG-luc (MMTV-luc) and a vector expressing the human MR. Cells were treated with aldosterone (10^{-7} M) and geldanamycin in the concentrations indicated. After 8 h, cells were lysed, and luciferase activity was determined as described in Section 2.

2.4. Hormone binding studies

COS-7 cells, which do not express functional steroid receptors, were plated in 12 well-plates (1.5×10^5 cells/well) and transfected with the MR expression vector as described above. Twenty four hours after transfection, cells were treated with 1.0 μg/well geldanamycin or with control medium, followed by 2 h incubation at 37°C. Cells were then incubated with ^{3}H-labeled aldosterone (Amersham, Arlington Heights, IL) at four concentrations (0, 1, 10, 20 nM) in the absence and presence of 500-fold excess of unlabeled hormone to determine total and non-specific binding, respectively. After a 1-h incubation at 37°C, cells were washed three times with ice-cold PBS, scraped with a 'rubber policeman', centrifuged, and resuspended in 500 μl PBS. The aliquot was transferred to scintillation vials containing 4 ml scintillation fluid, and counted in a β-scintillation counter. Specific binding was calculated by subtracting non-specific from total binding. Binding capacities were expressed as fmol/10^6 cells and the K_d in nM.

To test the influence of geldanamycin on GR hormone binding affinity, similar experiments were performed in HeLa cells, except that ^{3}H-labeled dexamethasone (Amersham, Arlington Heights, IL) was used as specific ligand.

2.5. Immunoblotting

HeLa cells were plated in six-well-plates, grown overnight, and harvested in ice-cold sample buffer 1 (50 mM Tris pH 6.8, 1% SDS, and 10% sucrose) to yield crude extracts. Protein concentrations were determined following standard protocols and using bovine serum albumin (BSA) protein standards diluted with sample buffer 1. The samples were diluted 1:1 with sample buffer 2 (50 mM Tris pH 6.8, 3% SDS, 10% sucrose, 10% β-mercaptoethanol, and 0.01% bromophenol-blue) to a final volume of 200 μl and a final protein concentration of 100 μg/ml.

Electrophoresis was performed in a 10% polyacrylamide separating gel and a 3% stacking gel as previously described (Schagger and von Jagow, 1987). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon P, Millipore, Eschborn, Germany) using the technique described by

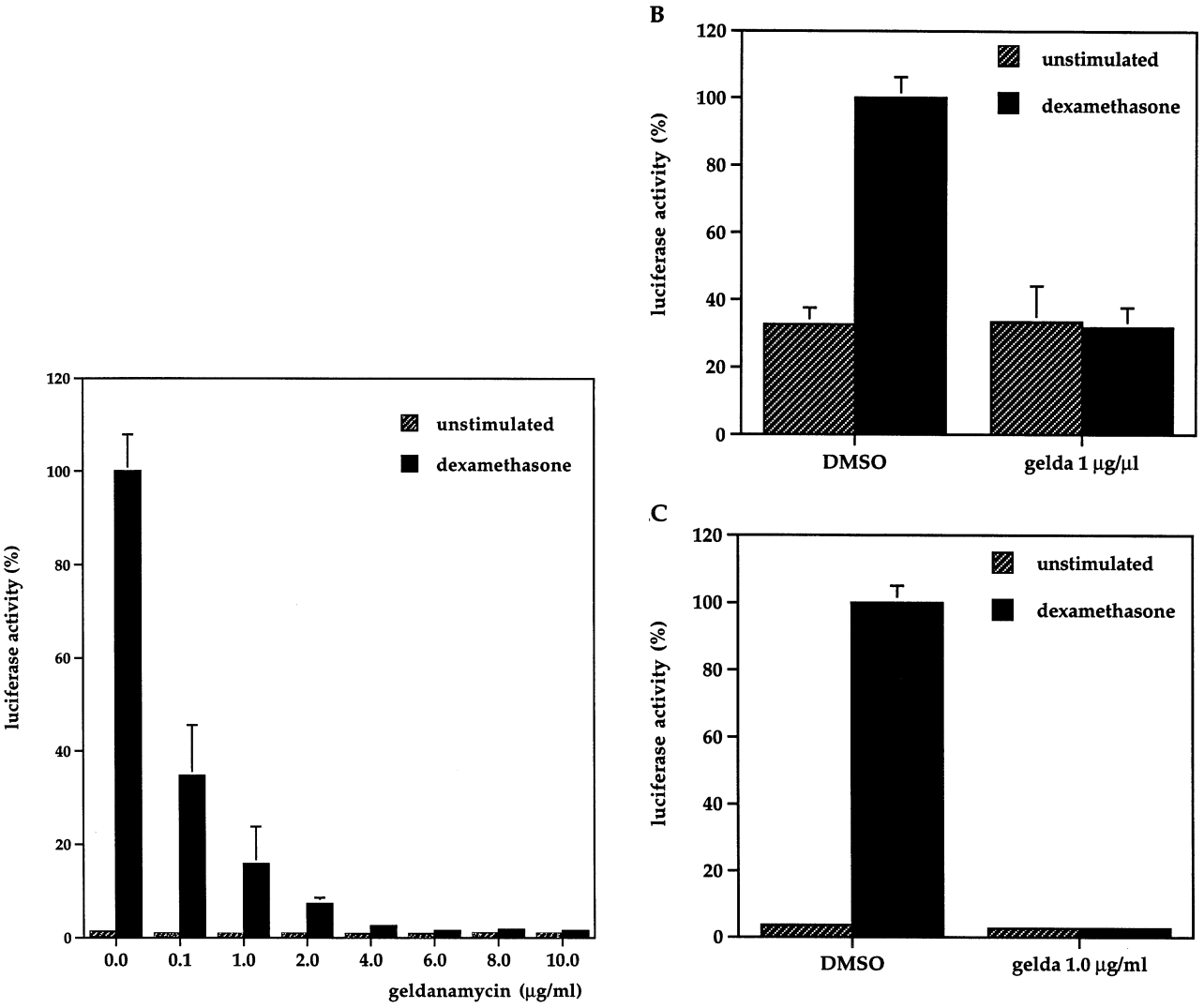

Fig. 2. Inhibition of GR-mediated gene transcription by geldanamycin. (A) COS-7 cells were transfected with pMSG-luc and a human GR expression vector. Dexamethasone (10^{-7} M) and geldanamycin were added to the cell culture medium and reporter gene activity was measured as described. (B) HeLa cells expressing endogenous GR were transfected with pMSG-luc. As with the transfected GR, hormone-induced activation of transcription is inhibited by geldanamycin. (C) Similar effects are observed with a GRE-tk81-luciferase reporter construct. DMSO, dimethylsulfoxide (dissolving agent for geldanamycin).

Towbin et al., 1979. Membranes were stained with Ponceau S dye to determine homogeneity of protein lanes. After destaining with TBS (Tris-buffered saline: 20 mM Tris/HCl, pH 7.6, 137 mM NaCl) for 5 min, membranes were incubated overnight at 4°C in blocking solution (0.1 M maleic acid, pH 7.5, 0.15 M NaCl, 0.005% Thimerosal, and 1% blocking reagent, Boehringer Mannheim, Germany).

For detection of GR, membranes were washed for 10 min in TBST (TBS plus 0.05% Tween 20) and incubated with the GR-specific polyclonal antibody GR PA1-511 (Dianova, Germany) in 9:1 TBST/blocking solution. The blots were then incubated for 1 h at room temperature, washed 3×10 min in TBST and incubated with the second antibody (goat-anti rabbit IgG-peroxidase conjugate, Sigma, Germany) for 1 h at room temperature. The second antibody was visualized by enhanced chemilumenescence (ECL) reagents (Amersham, Braunschweig, Germany) using Fuji RX 400 films.

3. Results

3.1. Inhibition of MR and GR transcriptional activity by geldanamycin

The results of the reporter gene experiments analyzing MR and GR transcriptional activity are shown in Figs. 1 and 2. In COS-7 cells transfected with pMSG-luc and the human MR expression vector, luciferase activity was significantly stimulated by aldosterone

(5.6 ± 0.6-fold, mean ± S.D.). Addition of geldanamycin inhibited MR transcriptional activity in a dose-dependent manner, with geldanamycin concentrations above 4 μg/ml leading to complete inhibition of MR function (Fig. 1). Basal promoter activity was not affected by the addition of geldanamycin, indicating that the observed effects were not due to inhibition of basal transcription factors.

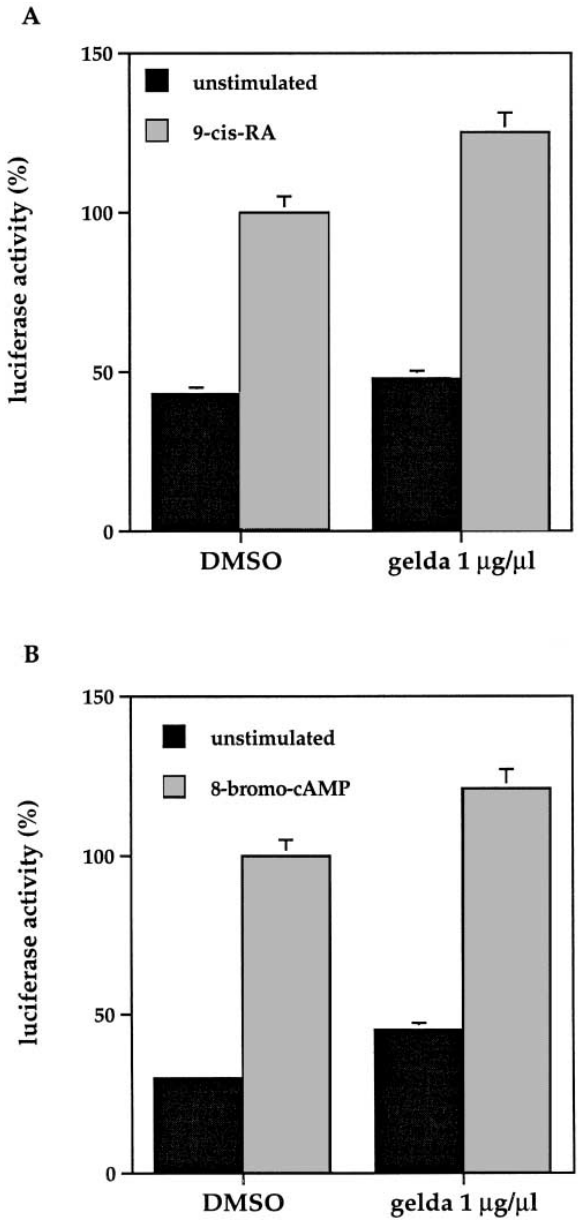

Fig. 3. (A) Geldanamycin does not inhibit retinoic acid (9-*cis*-RA)-dependent transcription. HeLa cells were transfected with a tk81-luciferase reporter gene under the control of a consensus retinoic acid response element (RARE). Treatment with 9-*cis*-RA stimulates transcription from this promoter both in the absence and presence of geldanamycin. (B) No inhibition of cAMP-dependent transcription by geldanamycin. HeLa cells were transfected with a tk81-luciferase reporter gene under the control of a consensus cAMP response element (CRE). 8-bromo-cAMP-induced stimulation of transcription is not affected by geldanamycin.

Similar effects were observed for GR (Fig. 2A). In the absence of geldanamycin, dexamethasone stimulated the MMTV promoter 76.9 ± 6.1-fold. This effect was inhibited by preincubation with geldanamycin in a dose-dependent fashion. As with the transfected receptor, the endogenous GR was also inhibited by geldanamycin. In HeLa cells, which express GR, dexamethasone-induced activation of both the pMSG-luc vector (3.1 ± 0.15-fold) and the simple pGL3-GRE-tk81-luc vector (27.1 ± 4.6-fold) was completely inhibited by 1 μg/ml geldanamycin (Fig. 2B and 2C, respectively).

To prove that the observed effects were specific for receptors which had previously been shown to bind hsp 90 in vitro, HeLa cells were transfected with a retinoic acid-dependent reporter gene. In these experiments, geldanamycin did not exert any inhibitory effect on retinoic acid-induced transcription (Fig. 3A). Similarly, a cAMP-responsive reporter gene was not inhibited by geldanamycin (Fig. 3B).

3.2. Inhibition of hormone binding by geldanamycin

To examine possible effects of geldanamycin on MR and GR hormone binding properties, we performed [^{3}H]aldosterone and [^{3}H]dexamethasone binding assays, respectively. As shown in Fig. 4A, [^{3}H]aldosterone efficiently bound to the transfected MR in COS-7 cells. In untransfected cells, specific [^{3}H]aldosterone binding was not detectable (data not shown). The calculated K_d of the transfected MR was 3 nM. In contrast, specific [^{3}H]aldosterone binding was almost completely inhibited in the presence of 1.0 μg/ml GA (Fig. 4A). Less than 1% specific binding was observed in comparison to control cells. Similar results were obtained for specific [^{3}H]dexamethasone binding to the GR in HeLa cells (Fig. 4B).

3.3. Stability of the receptor protein in the presence of geldanamycin

To examine steroid receptor protein stability in the absence of hsp 90 binding, we performed Western blot experiment in normal and geldanamycin-treated HeLa cells. As shown in Fig. 5, neither time-dependent nor geldanamycin-induced variations in GR protein levels were observed.

4. Discussion

In this study, we demonstrate that, in intact human cells, both MR- and GR-mediated transactivation depend on the unimpaired interaction between the receptor molecules and the hsp 90 complex, since inhibition

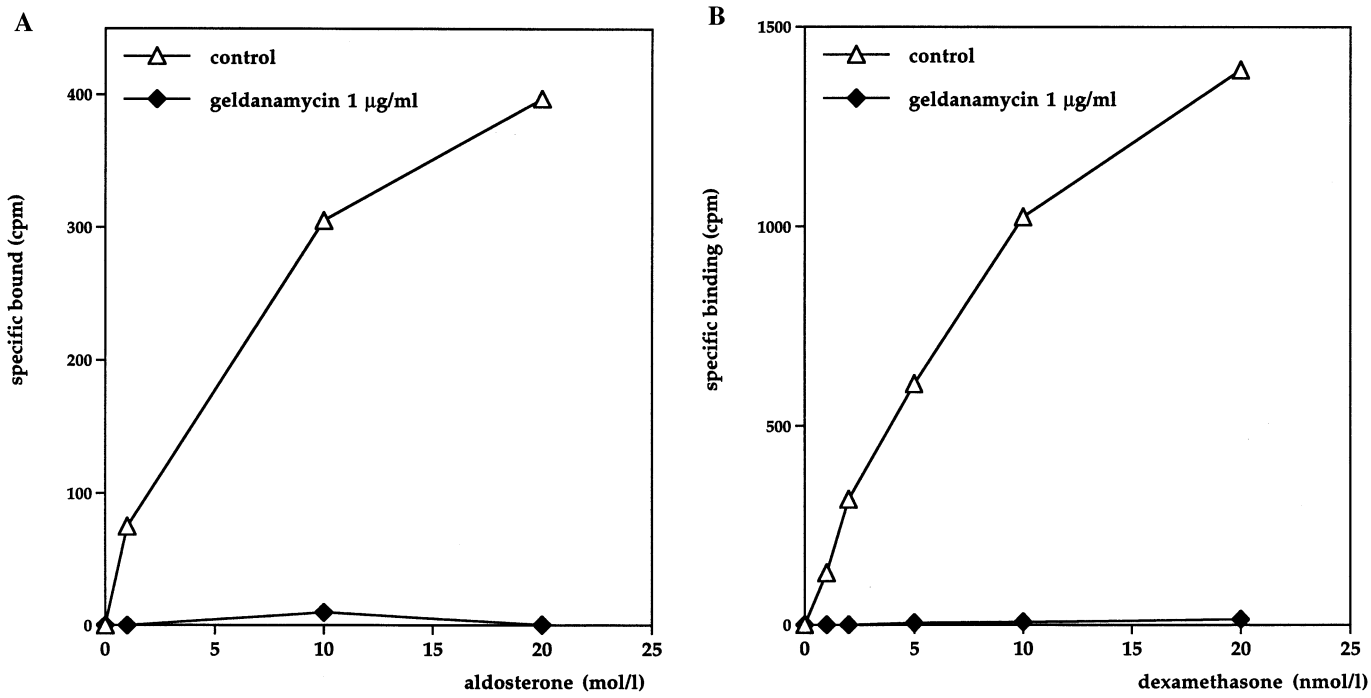

Fig. 4. Geldanamycin strongly inhibits MR and GR hormone binding affinity. (A) COS-7 cells were transfected with a MR expression vector and incubated for 2 h with ^{3}H-labeled aldosterone in the absence and presence of 500-fold excess of unlabeled hormone to determine total and non-specific binding. (B) GR hormone binding in the absence and presence of geldanamycin was analyzed using ^{3}H-labeled dexamethasone. cpm, counts per min.

of this interaction by the hsp 90-binding agent geldanamycin shifts the receptors to a nonligand-binding state, thus disrupting the hormone-induced activation cascade.

The first experimental evidence supporting the functional relevance of steroid receptor/hsp interaction came from in vitro experiments. In their fundamental study on this issue, Bresnick et al. demonstrated that the immunopurified L-cell GR was unable to bind hormone unless it was associated with hsp 90 (Bresnick et al., 1989). Subsequently, the regions required for the formation of this complex were defined in both the GR (Dalman et al., 1991) and the hsp 90 molecule (Cadepond et al., 1994; 1993). Not surprisingly, mutations in the respective parts of the hsp 90 coding region markedly reduced GR hormone binding affinity (Cadepond et al., 1994; 1993). By now, several other proteins, most notably hsp 70 and p23, were shown to be essential participants in the formation of GR/hsp 90 complexes (Hutchison et al., 1994; 1995). hsp 56, an immunophilin of the FK506- and rapamycin-binding class is also bound to this complex (Peattie et al., 1992; Radanyi et al., 1994). This molecule has not as yet been shown to be required for GR/hsp 90 association. As with GR, MR is also found associated with hsp 90. Yet, despite its physiological importance, MR is considerably less well studied and still awaits a thorough molecular dissection of its interaction with the hsp complex.

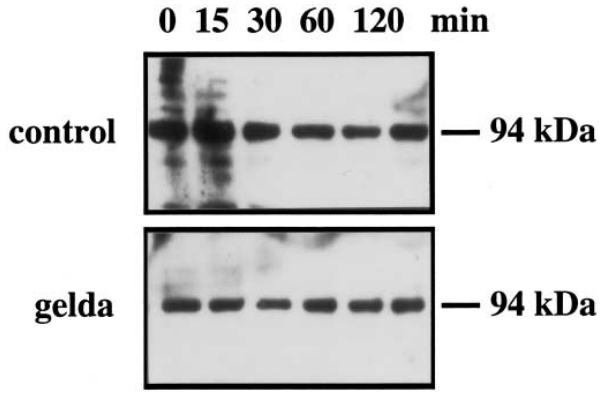

Fig. 5. Geldanamycin does not reduce GR protein levels. Protein extracts of control and geldanamycin-treated HeLa cells were analyzed by Western blot, using a polyclonal anti-GR antibody.

Support for the notion that GR/hsp 90 interactions also play an important role in maintaining hormone binding affinity in intact cells was first obtained in yeast. Picard et al. demonstrated impaired GR function in yeast mutants expressing low levels of hsp 90 (Picard, 1990). Furthermore, it was shown that yeast mutants expressing a structurally altered hsp 90 protein were unable to maintain a transfected GR in the hormone-activable state (Nathan and Lindquist, 1995; Bohen and Yamamoto, 1993). Regarding MR/hsp interactions, no comparable studies have been performed as yet.

Until recently, the results obtained in yeast had to be extrapolated to mammalian cells, since no data regarding GR/hsp interaction were available for the latter. In an elegant study, Kang et al. showed that an altered hsp 90 molecule bearing a constitutive nuclear localization signal was able to shift the GR to the nucleus (Kang et al., 1994). Their results provided the first direct proof that the association of GR and hsp 90 was not an artefact of the extraction process but actually took place in vivo. More recently, substances that specifically interfere with the association of steroid receptors and hsps have become available. In this context, geldanamycin, a benzoquinone asamycin, is of particular interest, since it has been shown to specifically bind to hsp 90 (Whitesell et al., 1994). The geldanamycin-bound hsp 90 molecule is no longer able to associate with p23 (Smith et al., 1995). Thus, an essential step in the formation of steroid receptor/hsp 90 is disrupted. As would be expected, treatment of mammalian cells with geldanamycin abrogates both progesterone receptor (PR)- and GR-mediated transactivation (Whitesell and Cook, 1996; Smith et al., 1995). Again, no such experiments were reported for MR.

In our study, we demonstrate that MR-mediated transactivation cannot be initiated in mammalian cells treated with geldanamycin. To our knowledge, this is the first study to analyze the functional relevance of MR/hsp complex formation in a whole cell system. GR-mediated transactivation was equally inhibited by geldanamycin, confirming the data published by Whitesell et al. (Whitesell and Cook, 1996). We extended these observations by showing that geldanamycin interferes with the activation of both endogenous and transfected GR. In addition, we demonstrated that a retinoic acid receptor/RARE transcriptional system was not affected by geldanamycin, indicating that the observed effects were due to specific abrogation of MR- and GR-mediated transcription and could not be accounted for by inhibition of a more common step in transcriptional activation. Hence, only receptors that had previously been shown to be associated with hsp 90 in vitro were also dependent on this interaction in vivo.

The results of our [^{3}H]aldosterone and [^{3}H]dexamethasone binding experiments indicate that geldanamycin treatment abrogates hormone binding, as would be expected from its mechanism of action described above. This is also consistent with the results published by Whitesell and Cook, 1996. However, these authors attributed the loss of hormone binding capacity at least partially to a degradation of GR protein in geldanamycin-treated cells. In contrast, we did not find any change in GR levels in geldanamycin-treated as opposed to control cells. At the present time, no obvious explanation can account for this discrepancy. It may be that the disruption of GR/hsp complexes by geldanamycin induces a conformational change in the GR molecule that makes it less recognizable for certain antibodies.

In summary, our data support the current model of hsp 90-dependent steroid receptor activation as described above. Furthermore, we show for the first time that, as with GR and PR, MR function also depends on the interaction with hsp 90 in intact cells. Finally, we demonstrate that the function of endogenous and transfected steroid receptors is equally dependent on their interaction with hsp 90.

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