

RELATIONSHIP BETWEEN GLUCOCORTICOID RECEPTOR STEROID-BINDING CAPACITY AND ASSOCIATION OF THE M_r 90,000 HEAT SHOCK PROTEIN WITH THE UNLIGANDED RECEPTOR

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Summary—Treatment of rat liver cytosol with hydrogen peroxide (H₂O₂) or sodium molybdate (MoO₄²⁻) inhibits thermal inactivation of glucocorticoid receptor steroid-binding capacity at 25°C. Dithiothreitol (DTT) prevents the stabilization of receptors by H₂O₂. Heating (25°C) of immune pellets formed by immunoadsorption of L-cell murine glucocorticoid receptor complexes to protein-A-Sepharose with an anti-receptor monoclonal antibody (BuGR2) results in dissociation of the M_r 90,000 heat shock protein (hsp90) from the steroid binding protein. Such thermal-induced dissociation of hsp90 is inhibited by H₂O₂. Pretreatment of immunoadsorbed receptor complexes with the thiol derivatizing agent, methyl methane-thiosulfonate (MMTS) prevents the ability of H₂O₂ to stabilize the hsp90-receptor interaction. These data suggest a role for hsp90 in maintaining an active steroid-binding conformation of the glucocorticoid receptor.

INTRODUCTION

The 9S mammalian untransformed glucocorticoid receptor complex (M_r = 320,000) in cytosolic preparations consists of a single 4S (M_r 100,000) steroid-binding protein and at least one molecule of a M_r 90,000 non-steroid-binding heat shock protein (hsp90) [1-3]. Temperature- and salt-mediated transformation of the 9S, non-DNA-binding form of the glucocorticoid receptor to the 4S, DNA-binding form occurs concomitant with dissociation of hsp90 from the receptor complex [4-6]. Both molybdate and H₂O₂ inhibit transformation and stabilize the association of hsp90 with the receptor [5, 6]. The ability of H₂O₂ to inhibit transformation in a DTT-reversible manner suggests that H₂O₂ is acting by promoting disulfide bond formation. *In vitro* studies such as these have led to the hypothesis that the interaction of hsp90 with the glucocorticoid receptor could serve to maintain the receptor in a non-DNA-binding state. To extend this hypothesis, the present series of experiments examines the effects of H₂O₂ on the thermal inactivation of glucocorticoid-binding capacity of rat liver cytosol and on the association of hsp90 with unliganded receptors during inactivation.

EXPERIMENTAL

Chemicals

[6,7-³H]Triamcinolone acetonide (42.8 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Sodium molybdate was obtained from J. T.

Baker Co. (Phillipsburg, NJ). Radioinert dexamethasone, nonimmune mouse IgG, protein-A-Sepharose, and goat anti-mouse IgG-horseradish peroxidase conjugate were from Sigma Chemical Co. (St Louis, MO). Nitrocellulose paper (0.45 μm) and chemicals for electrophoresis were from Bio-Rad (Richmond, CA). The BuGR2 anti-glucocorticoid receptor monoclonal antibody [7] and the AC88 anti-heat shock protein monoclonal antibody [8] were kindly provided by Dr R. W. Harrison and Dr D. O. Toft, respectively.

Cell source and fractionation

Liver was obtained from 150-175g male Sprague-Dawley rats (Charles River Breeding Laboratories; Wilmington, MA) which had been adrenalectomized and maintained on 0.9% saline for 1 day prior to sacrifice. L cells were grown in monolayer culture in Dulbecco's Modified Eagle's Medium supplemented with 10% calf serum at 37°C. Rat liver and L-cell cytosols were prepared as previously described [6]. The 100,000 g supernatant (referred to as cytosol) was stored at -70°C until use.

Incubation conditions and steroid binding assay

Incubations containing cytosol and other agents were prepared as indicated in the figure legends. Additions were made from fresh stock solutions concentrated at least 20-fold. Following treatment with appropriate agents, cytosol was bound at 0°C with 10nM [³H]triamcinolone acetonide (TA) for 12 h, with or without a 1000-fold excess of radioinert dexamethasone. Steroid binding was assayed by a modification of the charcoal absorption method. Specific binding values are the average of duplicate assays, calculated by subtracting the nonspecific binding value from total binding and expressed as cpm/0.2 ml of the original undiluted cytosol.

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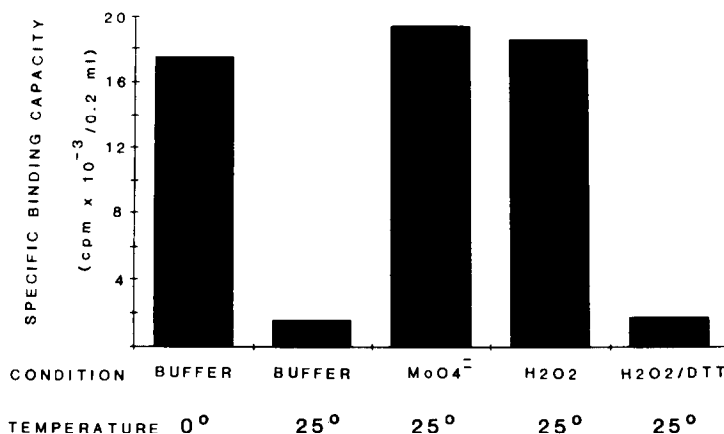


Fig. 1. H₂O₂ and MoO₄²⁻ stabilize unliganded rat liver glucocorticoid receptors at 25°C. Rat liver cytosol was treated for 10 min at 0°C with 10 mM sodium azide to inhibit catalase, followed by 10 min at 0°C with 10 mM Hepes, 10 mM MoO₄²⁻, or 20 mM H₂O₂. Cytosol was incubated for 2 h at 0°C or 25°C, and then 20 mM DTT was added at 0°C. Specific [³H]TA binding capacity was determined as described under Experimental.

Incubation with antibodies and adsorption to protein-A-Sepharose

Aliquots of L cell cytosol were mixed with an equal volume of TEG buffer [10 mM TES, 4 mM EDTA, 10% (w/v) glycerol, 50 mM NaCl, pH 7.6]. BuGR2 monoclonal antibody or nonimmune mouse IgG (1 mg/ml) was added at 4% of the final volume. The mixture was incubated for 3 h at 0°C, and then each sample was added to a 50 μl protein-A-Sepharose pellet (preequilibrated in TEG). Samples were mixed by rotation for 2 h at 4°C, and protein-A-Sepharose pellets were washed 5 times with 1 ml aliquots of TEG buffer.

Gel electrophoresis and immunoblotting

SDS-PAGE was performed in acrylamide slab gels according to Laemmli[9]. Gels were cooled to 4°C during electrophoresis. All samples were extracted from protein-A-Sepharose by boiling in SDS sample buffer containing 10% β-mercapto ethanol. M_r standards were: myosin, M_r=205,000; B-galactosidase, M_r=116,000; phosphorylase *b*, M_r=97,000; bovine serum albumin, M_r=66,000; ovalbumin, M_r=45,000; carbonic anhydrase, M_r=29,000.

RESULTS AND DISCUSSION

Having previously demonstrated that H₂O₂ resembles MoO₄²⁻ in inhibiting glucocorticoid receptor transformation by preventing temperature-dependent dissociation of hsp90 from steroid-bound receptor complexes [6], we used H₂O₂ as a tool to study the thermal inactivation of steroid-binding capacity in rat liver cytosol. As shown in Fig. 1, rat liver cytosol incubated for 2 h at 25°C loses approx. 90% of steroid-binding capacity. Thermal inactivation is prevented if MoO₄²⁻ is present during the 25°C treatment. Although we do not obtain measurable binding capacity for H₂O₂-treated cytosol in the absence of DTT, due to oxidation of the thiol(s)

required for steroid binding (data not shown), DTT addition to cytosol incubated at 25°C with H₂O₂ significantly reactivates steroid-binding capacity to 100% of the time 0 value. The stabilizing effect of H₂O₂ is prevented if DTT is present simultaneously with H₂O₂, suggesting that thiol oxidation is required for stabilization.

To investigate the mechanism by which H₂O₂ stabilizes receptors, we tested the hypothesis that H₂O₂ could be preventing dissociation of the receptor-associated protein, hsp90, analogous to the effect of H₂O₂ on inhibiting hsp90 dissociation from liganded-receptors during transformation [5]. In order to assess the effects of H₂O₂ on the association of hsp90 with unliganded receptors, L cell glucocorticoid receptors were immunoadsorbed to protein-A-Sepharose with the anti-receptor monoclonal antibody, BuGR2. These immune pellets were incubated at 25°C in the presence or absence of H₂O₂. As shown in Fig. 2 (Panel A), Western blot analysis with BuGR2 as the probe antibody reveals identical immunodetectable amounts of glucocorticoid receptor in pellets maintained at 0°C, warmed at 25°C, warmed at 25°C with H₂O₂, or treated first with the thiol blocking agent MMTS at 0°C, and then warmed at 25°C in the presence of H₂O₂. Immunoblotting with the AC88 antibody as the probe (Panel B) shows that hsp90 is lost from the immune complex upon warming at 25°C, and H₂O₂ prevents thermal-induced dissociation of hsp90. In addition, pretreatment of the pellet with MMTS prevents the ability of H₂O₂ to stabilize the hsp90-receptor interaction. The data described herein suggest that the physical interaction of hsp90 with receptors may stabilize an active steroid-binding conformation of the receptor in cytosol preparations, and that thermal-induced dissociation of hsp90 may be accompanied by a conformational change in the receptor that results in inactivation of steroid-binding capacity.

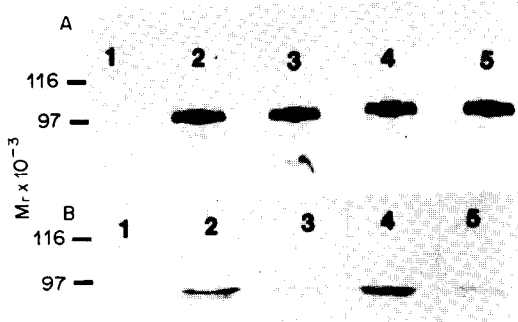


Fig. 2. Thermal-induced dissociation of hsp90 from unliganded glucocorticoid receptor complex immunoadsorbed from L-cell cytosol and stabilization of the complex by H_2O_2 . Aliquots (0.8 ml) of L-cell cytosol were immunoadsorbed with 4% nonimmune mouse IgG or 4% BuGR2 as described under Experimental. Washed protein-A-Sepharose pellets were brought up to 150 μ l with 10 mM Hepes buffer and were treated with 10 mM Hepes or 2 mM methyl methane-thiosulfonate for 30 min at 0°C, followed by 20 mM H_2O_2 or 10 mM Hepes for 1.5 h at 25°C. Protein-A-Sepharose pellets were washed 3 times with 1 ml of 10 mM Hepes. H_2O_2 (20 mM) was included in the wash buffer for samples treated with H_2O_2 . Protein-A-Sepharose-bound proteins (1/8) were analyzed by immunoblotting with BuGR2 as the probe antibody (Panel A), and 7/8 of bound-proteins with AC88 as the probe antibody (Panel B). Samples applied to each lane of the gels were: lane 1, cytosol incubated with nonimmune mouse IgG; lane 2, cytosol immunoadsorbed with BuGR2 and maintained at 0°C for 1.5 h; lane 3, cytosol immunoadsorbed with BuGR2 and warmed for 1.5 h at 25°C; lane 4, cytosol immunoadsorbed with BuGR2 and warmed for 1.5 h at 25°C with H_2O_2 ; lane 5, cytosol immunoadsorbed with BuGR2, treated with MMTS at 0°C, and warmed for 1.5 h at 25°C with H_2O_2 .

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