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Zinc Reversibly Inhibits Steroid Binding to Murine Glucocorticoid Receptor

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Previous work has demonstrated that several transition metals and their anions, including cadmium, arsenite, and selenite, can inhibit glucocorticoid binding to glucocorticoid receptors *in vitro.* In this study, we demonstrated that *in vitro* zinc can also inhibit the binding of glucocorticoids to their receptor at relatively modest concentrations (10 to 100 μM). This inhibition was demonstrated in both crude and immunopurified receptor preparations and was reversible following removal of zinc. Inhibition could also be reversed by addition of the reducing agent dithiothreitol (DTT). This suggested that zinc might be acting by interacting with the vicinal dithiols in the steroid binding region of the receptor as previously described for other transition metals and anions. The ability of a biologically important trace metal to block steroid binding suggests a role for zinc in the regulation of glucocorticoid receptor-ligand interactions and may explain the ability of zinc to block glucocorticoid-induced apoptosis. © 1997 Academic Press

The glucocorticoid receptor (GR) is a member of the steroid receptor superfamily of transcription factors (1). It is virtually ubiquitous in all mammalian tissues and is distinguished by nuclear translocation and transcriptional activation following the binding of ligand (2). Central to understanding the role of hormone binding in transformation of the GR has been the mapping and functional study of the steroid binding domain which includes the observation that GR steroid binding domain contains several clustered cysteine residues (3-5). Maintenance of these residues in a reduced state was necessary for high affinity steroid binding *in vitro* (6). In addition, steroid binding was inhibited by agents that cross linked or modified vicinal thiols, including arsenite (AsO_2^-), cadmium, selenite (SeO_2^-), and MMTS (7). These effects could be reversed by reduction of vicinal thiols with DTT or 2-ME (7,13). Site-directed mutagenesis of cysteine residues in the steroid binding domain of transfected rat GR in rat hepatoma cells suggested that Cys656 and Cys660 in the binding domain were necessary for normal steroid binding and receptor transformation (5). These results suggest that vicinal thiols in the steroid binding domain play an important role in steroid binding, and that the redox state of these residues affect binding affinity.

The ability of arsenite, cadmium and similar metals to inhibit steroid binding via cross linking of vicinal thiols have made them important tools in elucidating the mechanism of ligand binding to the GR (7,13). It is also possible that biologically relevant transition metals such as zinc might actually play a role in regulating the ligand binding activity of GR. Nevertheless, previous work had shown zinc did not prevent steroid binding in rat fibroblast cytosols, despite its atomic similarity to cadmium (7). This surprising observation led us to reexamine whether zinc could inhibit steroid-receptor binding in another cell system, namely the mouse liver GR.

MATERIALS AND METHODS

Preparation of crude liver cytosols. Livers from young normal or adrenalectomized male A/J mice (4 -12 week old) were mechanically homogenized at 4°C in hypotonic buffer containing 10 mM HEPES, 10% glycerol, 30 mM sodium molybdate, 1 mM dithiothreitol at pH 7.2. The resulting suspension was filtered, centrifuged at 5000 × g for 30 minutes at 4°C and subsequently ultracentrifuged at 100,000 × g for 60 minutes at 4°C. The resulting cytosol was then frozen and stored at −70°C. Total protein concentration was determined by Bradford protein assay (Bio-Rad).

Ligand binding assay. Ligand binding analysis was carried out as previously described (8) using 1 milligram crude liver cytosol (total protein), [6,7-^{3}H(N)]-dexamethasone ([^{3}H]DEX), 40 - 50 Ci/mmol specific activity (NEN-Dupont) at 50 nM with either unlabeled dexamethasone (DEX) at 25 μM (500-fold excess of cold competition) or buffer only in the case of no cold competitor. Unbound isotope was

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Abbreviations used: DCC, dextran-coated charcoal; DEX, dexamethasone; DTT, dithiothreitol; GR, glucocorticoid receptor; HRP, horseradish peroxidase; 2-ME, 2-mercaptoethanol.

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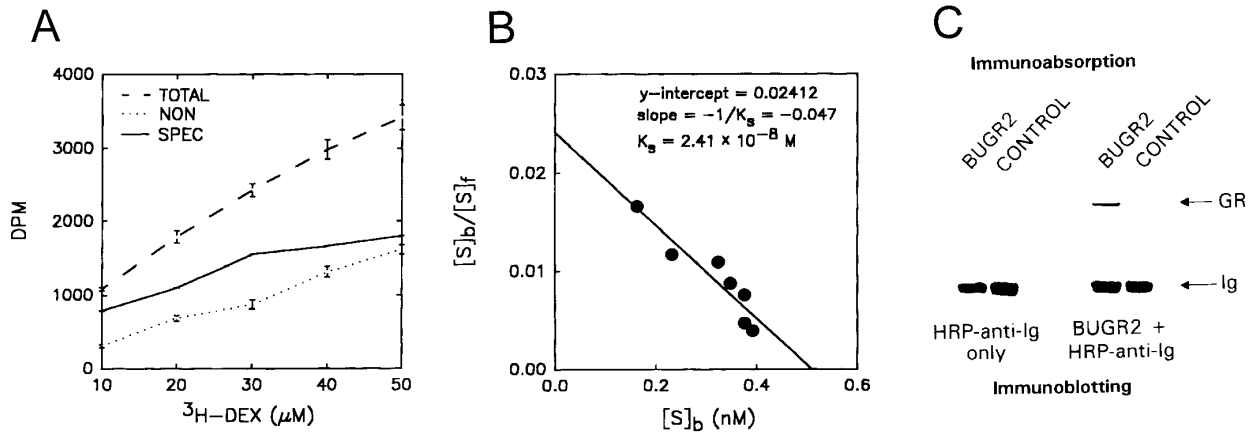

FIG. 1. **(A)** [^{3}H]DEX binding activity in mouse liver cytosol. Specific (SPEC) binding equals the difference between total (TOTAL) and non-specific (NON, with cold competitor) binding. **(B)** Eadie-Scatchard analysis of [^{3}H]DEX binding activity for mouse liver cytosol with [^{3}H]DEX concentrations ranging from 10 to 100 μM. **(C)** Western blot of immunoabsorbed GR from mouse liver cytosol. Cytosol was immunoabsorbed with BUGR2 (anti-GR) antibody or isotype-matched control and immunoblotted with BUGR2 plus HRP-conjugated secondary antibody or secondary antibody alone. GR, glucocorticoid receptor; Ig, immunoglobulin heavy chain.

removed using dextran-coated charcoal (DCC) and the remaining supernatant was analyzed for [^{3}H]DEX on a scintillation counter. Specific binding was calculated as the difference between samples containing [^{3}H]DEX alone (total binding) and samples containing 500-fold excess of cold competitor (nonspecific binding). Eadie-Scatchard analysis was carried out as previously described (11).

Zinc was added as zinc sulfate heptahydrate from a stock solution immediately prior to ligand addition. To determine whether the effects of zinc on ligand binding were reversible, the metal chelating resin Chelex 100 (Bio-Rad) was added to remove zinc following initial receptor-ligand binding. Tubes were then incubated at 4°C for 15 minutes followed by centrifugation and transfer of supernatant to new tubes. Samples were then incubated and harvested as described above.

Immunoabsorption of GR, SDS-PAGE, and Western blotting. Immunoabsorption of mouse cytosolic GR was carried out in a manner similar to that described previously (9) using the monoclonal anti-rat GR antibody BUGR2 (10) (Affinity Bioreagents, Neshanic Station, NJ, USA) and protein A-Sepharose for immunoabsorption. Immunoabsorbed receptor was denatured and electrophoresed on 8% SDS-denaturing polyacrylamide stacking minigels in SDS-Tris-glycine buffer pH 8.3. Gels were then electroblotted onto PVDF membranes in 20% methanol transfer buffer at 4°C and immunoblotted using the anti-rat GR antibody BUGR2 (10) and horseradish peroxidase-conjugated sheep-anti-mouse IgG (Amersham). Blots were then developed using luminal chemiluminescence (Amersham) and exposed to autoradiography film to detected protein bands.

To determine the effects of zinc on steroid binding to immunoabsorbed receptor, cytosolic receptor was immunoabsorbed with BUGR2 and Sepharose-protein A as described above. Aliquots of beads were then incubated with 50 nM [^{3}H]DEX with or without cold competitor and with or without zinc for 2 hours at 4°C with vigorous shaking. Pellets were then washed and solubilized with 0.4 M KSCN and counted for [^{3}H]DEX.

RESULTS

GR in mouse liver cytosol was initially evaluated both by Eadie-Scatchard analysis and by immunoblotting (FIG. 1). Liver cytosol showed specific DEX binding activity, which constituted approximately 50% of total binding at 50 nM [^{3}H]DEX, consistent with previous reports (Fig. 1A) (11). The seven-point Eadie-Scatchard analysis plot also indicated a single binding affinity, suggesting that the mouse liver cytosol contained a single DEX binding protein (Fig. 1B) (11,12). The K_s derived from the slope of the curve was 2.41×10^{-8} M, consistent with literature values for other GR (12). Liver cytosol from normal and adrenalectomized mice gave essentially identical results (data not shown). Immunoblotting for GR with the anti-GR antibody BUGR2 gave a single 97 kd band, believed to be mouse GR (Fig. 1C) (10). Prior immunoabsorption of the liver cytosols with BUGR2 depleted their ability to bind [^{3}H]-DEX, demonstrating that the [^{3}H]DEX binding activity was due to mouse GR (data not shown). Eadie-Scatchard analysis and immunoabsorption therefore confirmed that liver cytosol contained GR-ligand binding activity consistent with previous observations.

Zinc sulfate was then added to cytosols immediately prior to addition of ligand. Zinc had a profound effect on steroid binding, with an ED_{50} between 10 to 50 μM, giving nearly complete inhibition at 100 μM (Fig. 2A). Preincubation of cytosols with zinc provided no greater inhibition than when zinc was added simultaneously with steroid (data not shown). Successful immunoabsorption of GR from zinc-treated cytosols suggested that zinc did not alter the amount of soluble GR present in the cytosols (Fig. 2A, inset).

Whether zinc could also block receptor-ligand interactions in purified as well as crude GR was then determined. GR could be mobilized to protein A-Sepharose using the BUGR2 antibody without affecting its [^{3}H]-DEX binding activity (Fig. 2B). Zinc at 100 μM also inhibited over 80% of [^{3}H]DEX binding to GR immunoabsorbed to protein A-Sepharose using BUGR2 anti-

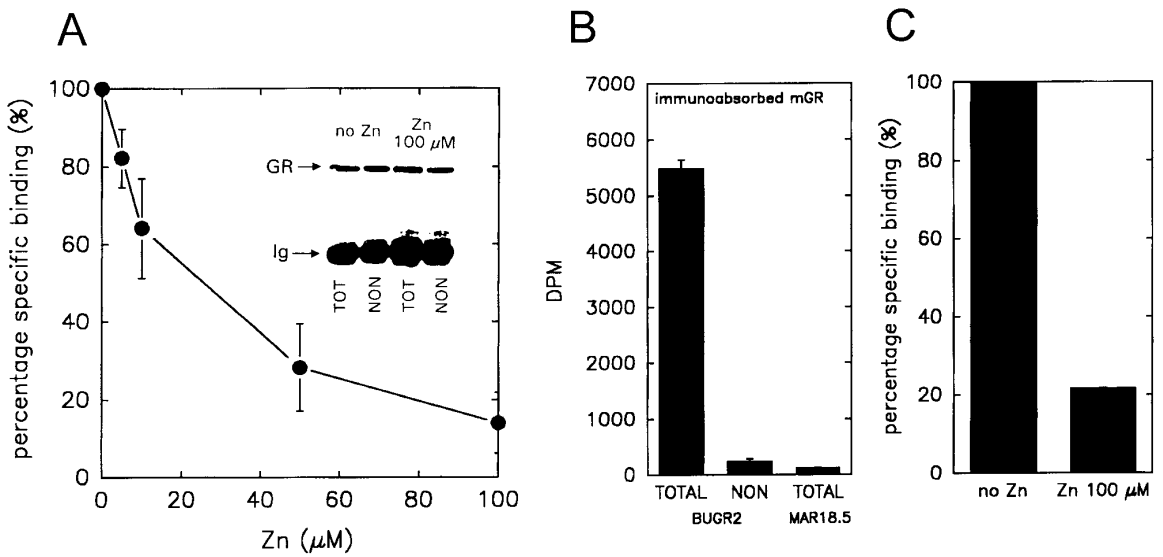

FIG. 2. **(A)** Effect of added zinc sulfate on specific [^{3}H]DEX binding activity of crude mouse liver cytosolic GR using receptor binding assay with [^{3}H]DEX at 50 nM. Data are expressed as the percentage of specific binding in the absence of added zinc. **(A, inset)** Immunoabsorption and immunoblotting for GR of above samples with zinc at 100 μM (TOT, total binding samples; NON, non-specific binding samples), showing the presence of soluble receptor. **(B)** Total (TOTAL) and non-specific (NON) [^{3}H]DEX binding activity of mouse liver cytosol GR immunoabsorbed to Sepharose protein A with BUGR2 (anti-GR antibody). Total binding with the isotype-matched control (MAR18.5) was negligible. **(C)** Effect of zinc sulfate at 100 μM on specific [^{3}H]DEX binding activity of immunoabsorbed GR as described in B. Data are expressed as the percentage of specific binding in the absence of added zinc.

body (Fig. 2C). Thus, zinc could block ligand binding to immunopurified receptor as well as crude preparations.

Although zinc inhibited receptor-ligand binding when it was added prior to or simultaneously with steroid, zinc could not dissociate preformed receptor-ligand complexes. When zinc was added two hours post-addition of ligand, it could not reverse the binding of the preformed complexes using crude cytosols (Fig. 3A). In addition, the inhibitory action of zinc on GR-ligand binding was reversible upon removal of zinc. Crude cytosolic receptor and [^{3}H]DEX were incubated with zinc as previously described. Zinc was then removed by brief incubation with Chelex 100, a metal chelating resin, followed by removal of the resin and subsequent incubation and measurement of ligand binding. Inhibition of receptor-ligand binding by zinc was almost completely reversed, being comparable to control values (Fig. 3B). Zinc-associated inhibition of receptor-ligand

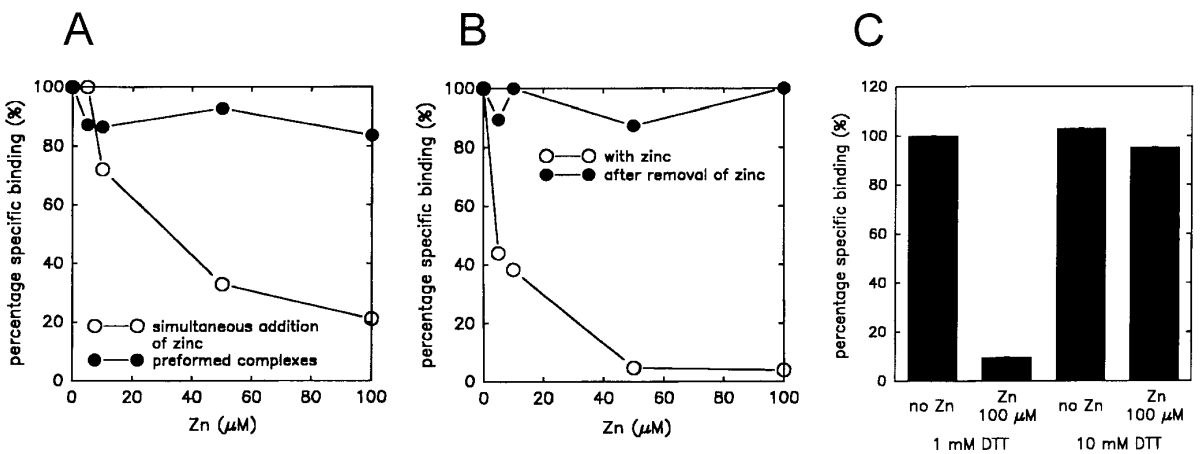

FIG. 3. **(A)** Effect of zinc on preformed [^{3}H]DEX-GR complexes using receptor binding assay. Data are expressed as the percentage of specific binding for the control (with no zinc present). **(B)** Reversibility of zinc-associated receptor-ligand binding inhibition using treatment with Chelex 100. Open circles show specific [^{3}H]DEX binding activity before removal of zinc, filled circles after removal of zinc by chelating resin. Data are expressed as the percentage of specific binding in the absence of added zinc. **(C)** Reversibility of zinc-associated specific [^{3}H]DEX-GR binding inhibition with 10 mM dithiothreitol. Data are expressed as the percentage of specific binding for the control.

binding was thus reversible for concentrations of 100 μM and less.

Reducing agents such as DTT and 2-ME can facilitate ligand binding to GR by maintaining vicinal thiols in a reduced state. DTT and 2-ME treatment can overcome the inhibitory effects of transition metals and oxyanions that cross link these thiol residues (4-7). To determine whether zinc inhibition of ligand binding to GR could be overcome by reducing agents, dithiothreitol (10 mM) were added to liver cytosols simultaneously incubated with 100 μM zinc. DTT (10 mM) completely reversed the inhibitory effects of zinc on ligand binding, suggesting that zinc may indeed be cross linking adjacent thiol residues in the steroid binding domain (Fig. 3C).

DISCUSSION

To summarize, zinc was found to inhibit receptor-ligand interactions *in vitro*, both in crude liver cytosolic and immunoabsorbed receptor. The effective concentration of zinc ranged from 10 to 100 μM, with an ED_{50} of approximately 10 to 50 μM. Treatment of crude cytosols and immunoabsorbed receptor with zinc at the above concentrations did not affect the solubility of the receptor, demonstrated by the fact that receptor could still be immunoabsorbed and detected by Western blotting following zinc treatment. Zinc was found to act prior to ligand binding in crude cytosols, and did not reverse receptor-ligand complexes once they were formed. Its inhibitory effect was also reversed when zinc was removed by chelation. Increasing the concentration of DTT was also able to reverse the inhibitory effects of zinc.

All of these experiments suggest that zinc exerted a direct biochemical effect on the mouse GR influencing its ability to bind steroid *in vitro*. In addition, the fact that zinc did not dissociate preformed complexes suggests that zinc may be acting directly within the steroid binding domain. The ability of DTT to reverse the inhibitory effect of zinc further suggests that its site of action may be amino acid residues capable of binding zinc such as cysteines, several of which are found in the steroid binding domain and are indirectly necessary for normal ligand binding and receptor activation (4,5).

In contrast to previous reports, therefore, zinc appeared to inhibit GR-ligand binding via cross linking of vicinal thiols in a similar manner to that previously reported for transition metals such as cadmium and their oxonians such as arsenite and selenite (7,13). This is not surprising given the ability of zinc and other transition metals to bind coordinately at two to four vicinal cysteines. Zinc may therefore prove to be an important tool in the characterization of the steroid binding domain, as are arsenite and cadmium. In addition, the ability of zinc to block receptor-ligand interactions at relatively low concentrations and its physiological relevance as a trace metal suggest that zinc could potentially play a role in the *in vivo* regulation of GR-ligand binding. This mechanism might also account, at least in part, for the ability of exogenously added zinc to block glucocorticoid-induced apoptosis in many cell types (14).

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