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Zinc Ions Antagonize the Inhibitory Effect of Aurothiomalate on Glucocorticoid Receptor Function at Physiological Concentrations

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SUMMARY

The water-soluble gold preparation aurothiomalate, which contains gold as Au(I), is frequently prescribed for patients with rheumatoid arthritis as a disease-modifying agent. We report that aurothiomalate negatively modulates glucocorticoid hormone action; it represses the ligand- and DNA-binding activities and the transactivation function of the glucocorticoid receptor. We suggested the existence of endogenous titrating activities of Au(I) because otherwise administration of aurothiomalate to a patient with rheumatoid arthritis would be expected to result in peripheral insensitivity to glucocorticoids and worsen the patient's status. Focusing on metal ions that are present *in vivo*, we found that Zn(II) counteracts the inhibitory effect of Au(I) on glucocorticoid receptor function. This complementary effect of Zn(II) was observed at physiological concentrations. We suggest that Zn(II) preserves glucocorticoid receptor function in target tissues and maintains hormone responsiveness, even with chrysotherapy.

A growing number of reports indicate that endogenous glucocorticoids, or the HPA-axis activity, are involved in antiinflammation and immunoregulation (1). For example, compared with healthy women, patients with Sheehan's syndrome frequently develop arthritis (2, 3). Direct evidence is provided by the fact that the severity of experimental inflammation is increased in animals treated with antiglucocorticoid RU486 (Mifepristone) (4). In patients with RA, diurnal variation of the disease activity has been shown to correlate with the diurnal rhythm of serum cortisol (5). In the thymus, endogenous cortisol is now considered to play an important role in negative selection of T lymphocytes via apoptosis (6), and this suicidal pathway involves GR as a mediator of glucocorticoid action (7).

Glucocorticoid hormone exerts its action via binding to the specific and ubiquitous intracellular GR protein (8–10). GR is a member of the steroid receptor superfamily and acts as a ligand-inducible transcription factor (8–10). GR protein is composed of at least three functional domains: the ligand binding domain, the DNA binding domain, and the transactivation domain (9, 10). The DNA binding domain contains two finger motifs formed by the tetrahedral coordination of two zinc atoms by four cysteine residues each (11). The abundance of cysteine residues within the DNA binding domain (10 residues in a 60-amino acid stretch) is consistent with a critical role of these residues in tight receptor/DNA interaction (11). Genetic alterations in GR sometimes cause various extents of glucocorticoid resistance. Patients with glucocorticoid resistance are characterized biochemically by the presence of abnormal GR proteins with deletion or mutation of a critical domain or amino acid residues (Ref. 12, including references). These patients usually have compensatory elevation of the endogenous cortisol level, indicating that the HPA axis is continuously activated to complement the increased peripheral requirement for cortisol (12).

Several metal ions have been shown to affect the function of GR and other members of the steroid receptor superfamily (13–15). However, the underlying molecular mechanism remains largely unknown. Recently, heat shock and chemical stress have also been shown to influence the hormonal inducibility of glucocorticoid-regulated promoters (16). In addi-

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ABBREVIATIONS: HPA, hypothalamic-pituitary-adrenal; RA, rheumatoid arthritis; GR, glucocorticoid receptor; CHO, Chinese hamster ovary; FCS, fetal calf serum; MEM, minimal essential medium; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay; GRE, glucocorticoid response element; DTT, dithiothreitol; CAT, chloramphenicol acetyltransferase; GR DBD, DNA binding domain of glucocorticoid receptor.

tion, GR-mediated gene regulation has been shown to cross-talk with other intracellular signaling pathways that are mediated, for example, by protein kinases A (17) and C (18–20). Moreover, patients with human immunodeficiency virus-I infection often show acquired glucocorticoid resistance with unknown etiology (21). Thus, evidence is accumulating that various extracellular stimuli and/or cellular stress may cause intrinsic modification of GR and affect its function, suggesting that GR is a potential locus for the convergence of many signaling pathways distinct from that mediated by glucocorticoid hormone. Therefore, GR function is not only genetically but also nongenetically variable *in vivo*, resulting in alteration of peripheral sensitivity to glucocorticoids with considerable clinical significance.

Recently, we found that aurothiomalate, which is one of the most widely prescribed disease-modifying antirheumatic drugs for RA and contains gold as the Au(I) state (22), represses glucocorticoid hormone action *in vitro* (14, 15). Because the concentrations used in the present study are clinically achievable in blood (22), chrysotherapy might, despite its proposed antiarthritic effects (22), cause a decrease in peripheral glucocorticoid sensitivity and, at least in part, result in dysregulation of the HPA axis. Therefore, we suggested that endogenous substance(s) may counteract Au(I)-mediated repression of GR function and play an important role in homeostatic control of the HPA axis in, for example, RA patients. To investigate this, we have looked for such a substance(s) that is present *a priori* and preserves cellular glucocorticoid action. In the present study, we focused on endogenous transition metals and found that Zn(II) counteracts Au(I) so that GR function is maintained.

Materials and Methods

Reagents. Sodium aurothiomalate was obtained from Aldrich Chemical Co. (Milwaukee, WI) and used as Au(I) donor in the present study. Sodium thiomalate, malate, dexamethasone, and 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate were purchased from Sigma Chemical Co. (St. Louis, MO). $ZnCl_2$ [used as Zn(II) donor] and other chemicals were from Wako Pure Chemical (Tokyo, Japan).

Cell culture. The GR-expressing CHO cell line CHOpMTGR was originally developed and kindly provided by Dr. Stefan Nilsson, Karo Bio, Sweden (23). In brief, CHO-K1 cells were stably transfected with the full-length GR expression vector. The resultant CHOpMTGR cells express full-length GR (300,000–500,000 molecules/cell; Ref. 23). The cells were maintained in Ham's F-12 medium supplemented with antibiotics and 10% FCS. HeLa S3 cells were supplied from RIKEN Cell Bank and maintained in the MEM supplemented with 10% FCS and antibiotics. In all experiments, serum steroids were stripped from FCS with dextran-coated charcoal, and cells were cultured in a humidified atmosphere at 37° with 5% CO_2 in air.

Hormone binding assay. CHOpMTGR cells were grown in six-well flat-bottom plastic plates (IWAKI Glass, Funabashi, Japan) to confluence. The medium was replaced with Opti-MEM medium (GIBCO-BRL, Gaithersburg, MD). After washing twice with PBS and once with Opti-MEM medium, the cells were incubated with 20 nM [^{3}H]dexamethasone (35–50 Ci/mmol, DuPont/NEN Research Products, Wilmington, DE) in the presence or absence of 500-molar excess of radioinert dexamethasone in triplicate for 1 hr at 37°. The monolayer was washed three times with ice-cold PBS, and the cells were dissolved in a solution consisting of 25 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate and 1% sodium dodecyl sulfate. Aliquots were added to scintillation fluid to determine bound radioactivity. The difference between total and nonspecific binding gives the number of specific GR binding sites; and results are expressed as the number of specific binding sites for 20 nM [^{3}H]dexamethasone.

Preparation of cellular extracts and Western immunoblot analysis. CHOpMTGR cells were lysed and homogenized in TEG buffer [10 mM Tris·HCl, pH 7.4, 1 mM EDTA, 10% (v/v) glycerol, and 2 mM DTT] containing 0.1 M NaCl. After centrifugation of the homogenate at 100,000 × *g* for 45 min at 4°, the supernatant was precipitated with the use of ammonium sulfate (0.2 g/ml). The pellet was dissolved in TEG buffer without salt. Protein concentration was determined by Pierce BCA protein assay kit according to the manufacturer's guidance. Ten micrograms of protein were separated on a 7% sodium dodecyl sulfate-polyacrylamide gel and electrically blotted onto nitrocellulose filter (Bio-Rad, Richmond, CA). The GR protein bands were identified with the use of polyclonal anti-human GR rabbit antibody PA1–512 (Affinity BioReagents, Neshanic Station, NJ) and visualized by the Prot Blot Immunoblotting System (Promega, Madison, WI) using an alkaline phosphatase-conjugated second anti-rabbit goat antibody. For quantification, three independent autoradiograms were analyzed using a MasterScan densitometer (Scanalytics, Billerica, MA).

EMSA of partially purified GR. Partially purified GR was prepared from CHOpMTGR whole-cell extracts essentially as described by Cairns *et al.* (24). Briefly, whole-cell extract was prepared in the presence of molybdate and chromatographed through a phosphocellulose column. The flow-through material was applied to a DEAE-Sepharose column, and the absorbed material was eluted with 200 mM NaCl. Salt and molybdate were removed from the eluted material by chromatography on Sephadex G-25. After transformation (25°, 60 min), the receptor fraction was purified by fast protein liquid anion exchange Mono Q chromatography (Pharmacia LKB Biotechnology, Uppsala, Sweden). Fractions containing receptor were identified by ligand binding and specific DNA binding assays. These fraction containing 10–20% pure receptor were used for protein/DNA interaction experiments. Purification of the recombinant of GR DBD has been described (25).

The sequence of the synthetic oligonucleotides encompassing the GRE is 5′-CGAGTAGCTAGAACAGGATGTTCTGAGG-3′ (top strand is shown). EMSA was carried out as previously described (15). In summary, the double-stranded oligonucleotide probe was end-labeled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham, Buckinghamshire, UK) using the Klenow fragment of DNA polymerase I (TaKaRa, Kyoto, Japan). Partially purified GR or GR DBD were incubated with 0.2 ng of ^{32}P-end-labeled GRE probe (approximately 20,000 cpm) in a 20-μl reaction mixture containing 5 mM HEPES, pH 7.9, 2.5 mM EDTA, 0.25 mM DTT, 2.5 mM $MgCl_2$, 10 mM spermidine, 60 mM KCl, 10% glycerol, and 100 ng poly(dI-dC)·(dI-dC) (Pharmacia LKB Biotechnology) for 15 min on ice. When indicated, radioinert competitor oligonucleotides or anti-GR antibodies, PA1–512, and BuGR (Affinity BioReagents) were included in the reaction mixture. In immunoabsorption experiments, GR/anti-GR antibody complex was captured onto solid phase via 2 mg of protein A sepharose (Pharmacia LKB) and cleared from the reaction by brief centrifugation. The reaction mixture was loaded onto a 4% nondenaturing polyacrylamide gel containing 0.25× TBE (1× TBE consists of 0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA). Gels were run at 250 V for 2 hr and dried. Results were visualized by autoradiography.

Plasmids, transfection, and CAT assay. The plasmid GREtkCAT, which was originally named PRE-PBL7 and contains two repeats of a 23-bp GRE/PRE from the rat tyrosineaminotransferase gene (26), was obtained from Dr. B. O'Malley (Baylor College of Medicine, Houston, Texas). The construct mGREtkCAT, in which the two repeats of the GRE in GREtkCAT were replaced with two repeats of an ERE (5′-GATCCAGGTCAGGATGACCTAGCTACG-3′), was obtained from Dr. H. Oshima (Steroid Hormone Section, Laboratory of Molecular and Cellular Biology, National Institute for Diabetes and Digestive and Kidney Diseases, Bethesda, MD; Ref. 27).

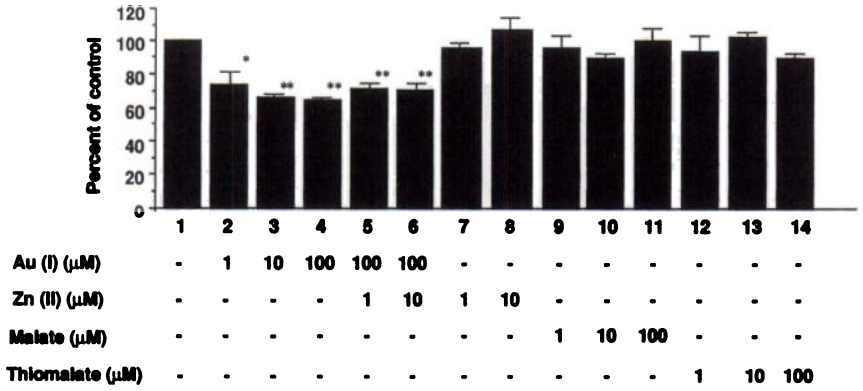

Fig. 1. Aurothiomalate represses the ligand binding activity of the GR. The GR-expressing CHO cells CHO-pMTGR were grown in Ham's F-12 medium, and the medium was replaced with Opti-MEM medium containing various metal compounds 3 hr before assay. The number of specific binding sites for 20 nM [^{3}H]dexamethasone was assayed as described in Materials and Methods. Results are expressed as percentage of control (*lane 1*) in which only ethanol vehicle was added to the medium. *, $p < 0.05$ versus control (*lane 1*), **, $p < 0.01$ versus control.

The β-galactosidase expression plasmid pCH110 (Pharmacia LKB Biotechnology) was used as an internal control for transfection efficiency. Transient transfection was performed as described previously (14, 15). Briefly, cells were plated into 10-cm-diameter plastic culture dishes (IWAKI Glass) to 30–40% confluence and washed with PBS three times, and Opti-MEM medium was added. Plasmid cocktail was mixed with 30 μl of Lipofectin reagent (GIBCO-BRL) and added to the culture. After a 12-hr incubation, the medium was replaced with Ham's F-12 medium or MEM supplemented with 10% dextran-coated charcoal-striped FCS, and the cultures were further incubated in the presence or absence of various ligands for 24 hr. After normalization for transfection efficiency, CAT enzyme activity was determined essentially as described previously (14, 15).

Statistical analysis. Levels of significance for comparisons between samples were determined using Student's *t* test distribution.

Results

Based on our previous findings and those of others that aurothiomalate, in particular, Au(I), and several other metal ions variably modify GR function (see introductory paragraphs), we focused on endogenously present transition metals as possible candidates for positive modifiers of glucocorticoid hormone action. CHOpMTGR cells express human GR at high levels (see Materials and Methods) and allow very sensitive measurement of both ligand binding and transcriptional activation functions of the receptor (23). Initially, specific binding sites for [^{3}H]dexamethasone were determined by whole-cell assays in the absence or presence of various metal ions. Aurothiomalate reduced the number of [^{3}H]dexamethasone binding sites in GR-expressing CHOpMTGR cells in a dose-dependent manner (Fig. 1). The Au(I) concentrations used here are known to be clinically attainable in blood (22). Similar concentrations of either malate or thiomalate did not influence the ligand binding capacity of GR (Fig. 1). Of the various metals tested, none, including Zn(II), significantly compensated for this Au(I)-mediated inhibition of the ligand binding activity of GR (Fig. 1 and data not shown). Western immunoblot analysis revealed that GR protein levels in cellular extracts were not significantly altered after treatment of the cells with 10 or 100 μM Au(I) (Fig. 2), indicating that the Au(I)-mediated decrease in ligand binding capacity is not due to any change in the amount of GR protein but rather to a functional alteration in the ligand binding activity of the GR protein.

In vitro DNA binding activity of GR was analyzed in EMSA. Formation of several classes of protein/DNA complexes between partially purified GR and the GRE probe was observed in our assay (Fig. 3A). Involvement of molar excess of nonlabeled GRE oligonucleotides inhibited the formation of protein/DNA complexes; however, molar excess of Sp1 oligonucleotides did not influence the complex formation, so these complexes were considered to be due to sequence-specific interaction between GRE and partially purified GR (*lanes 2–4*). The addition of neither BuGR nor human immunoglobulin, but exclusively of PA1–512, clearly supershifted the top protein/DNA complex C (*lanes 5–7*), and those complexes migrating in the lower part of the gel were not affected by the addition of those antibodies (*lanes 5–7*), indicating that the complex C was formed by the specific interaction between GR and GRE oligonucleotides. To confirm this, immunoabsorption experiments were performed. After incubation of partially purified GR with either BuGR, PA1–512, or

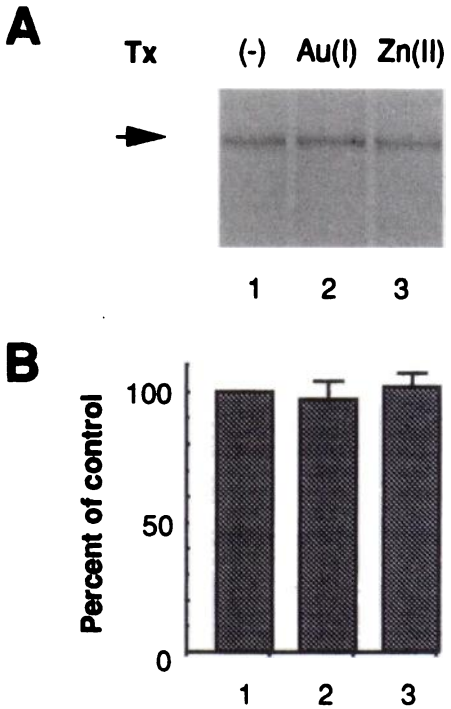

Fig. 2. Cellular GR level was not affected by treatment with Au(I) or Zn(II). CHOpMTGR cells were cultured in the absence (−) or presence of 100 μM each of aurothiomalate [Au(I)] or $ZnCl_2$ [Zn(II)] for 24 hr, and GR protein immunoreactivity in whole-cell extracts (10 μg of protein) was determined by Western immunoblot analysis using anti-human GR antibody PA1–512 as described in Materials and Methods. Experiments were repeated three times. A, Representative blot. *Tx*, treatment. B, Results from quantitative analysis using densitometry are shown as mean + standard deviation.

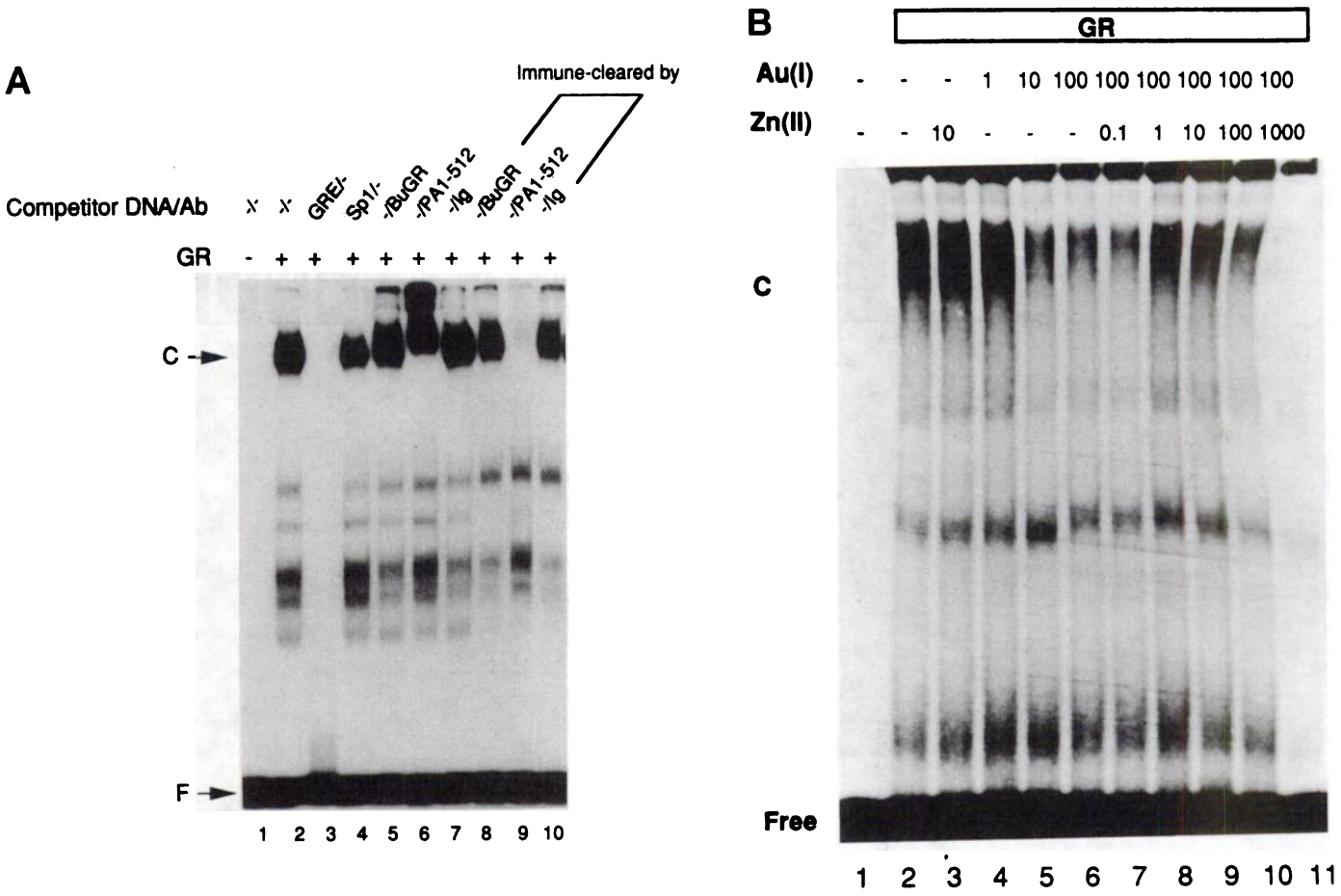

Fig. 3. Au(I)-dependent inhibition and Zn(II)-dependent restoration of DNA binding activity of the GR. A, Demonstration of GR-specific DNA binding activity. Partially purified GR (1 ng protein per lane; *lanes 2–10*) was incubated with ^{32}P-labeled GRE oligonucleotides in the absence (*lane 2*) or presence of 20-fold molar excess of specific (*lane 3*) or nonspecific (*lane 4*) competitor oligonucleotides. When indicated, either anti-GR antibody BuGR or PA1–512 or human immunoglobulin (*Ig*) was added to the reaction mixture. Immunoclearance of antigen/antibody complex was performed with protein A Sepharose in the reaction and the subsequent centrifuge. Then the formation of protein/DNA complexes was examined with EMSA. *C*, protein/DNA complexes; *F*, free probe; *Ab*, antibody. B, Partially purified GR was incubated with the indicated concentrations of aurothiomalate [Au(I)] and/or $ZnCl_2$ [Zn(II)] for 15 min on ice, followed by incubation with ^{32}P-labeled GRE oligonucleotide. Experiments were repeated five times with almost identical results; representative results are shown. *C*, Protein/DNA complexes; *Free*, free probe.

human immunoglobulin as indicated in Fig. 3A, protein A sephalose was added for immune clearance of preformed GR/antibody complexes. After centrifugation of the mixture to remove the immunoprecipitate, GRE-binding activity in the supernatant was analyzed in EMSA. Involvement of neither BuGR nor human immunoglobulin, but rather of PA1–512, clearly eliminated the formation of C1 protein/DNA complex (*lanes 8–10*). Together, we confirmed that the complex C1 was formed by the specific interaction between GR and GRE.

Using this partially purified GR, we demonstrated that aurothiomalate decreased the formation of protein/DNA complexes *in vitro* between the GR protein and the GRE (Fig. 3). This inhibitory effect of aurothiomalate was not observed when similar concentrations of malate or thiomalate were added (data not shown), indicating that the component responsible for repression of GR DNA binding is Au(I). The effective concentration of Au(I) was within therapeutic blood concentrations as suppression of GR/GRE complex formation was achieved at 10 μM and the repression was dose dependent (Fig. 3B). Notably, the addition of Zn(II) restored the sequence-specific DNA binding activity of GR in the presence of Au(I) at relatively low concentrations (i.e., 1 μM), which were within the range of physiological blood concentrations of Zn(II) (Fig. 3B and Ref. 23). At higher-than-physiological concentrations (i.e., ≥100 μM), specific complex formation between GR and GRE was rather decreased by Zn(II) (Fig. 3B). Zn(II), when added alone, did not cause any alteration in GR DNA binding activity at concentrations of 0.1–10 μM (Fig. 3B, *lane 2*, and data not shown).

The effect of aurothiomalate on cellular glucocorticoid hormone action was assessed in transient transfection experiments. After transfection of the glucocorticoid-responsive CAT reporter construct GREtkCAT or a mutant version of it, mGREtkCAT, which are driven under the control of tandem copies of GRE or ERE oligonucleotides, respectively (Ref. 27; see also Materials and Methods), CHOpMTGR cells were incubated in the presence or absence of dexamethasone, aurothiomalate, and Zn(II) (Fig. 4). Incubation with 100 nM dexamethasone produced a ~29-fold induction of CAT activity (Fig. 4A, *lane 4*), and aurothiomalate decreased the hormone induction response of the reporter gene in a concentration dependent manner (*lanes 4–7*). The inhibitory effect was not seen when cells were treated with either malate (*lanes*

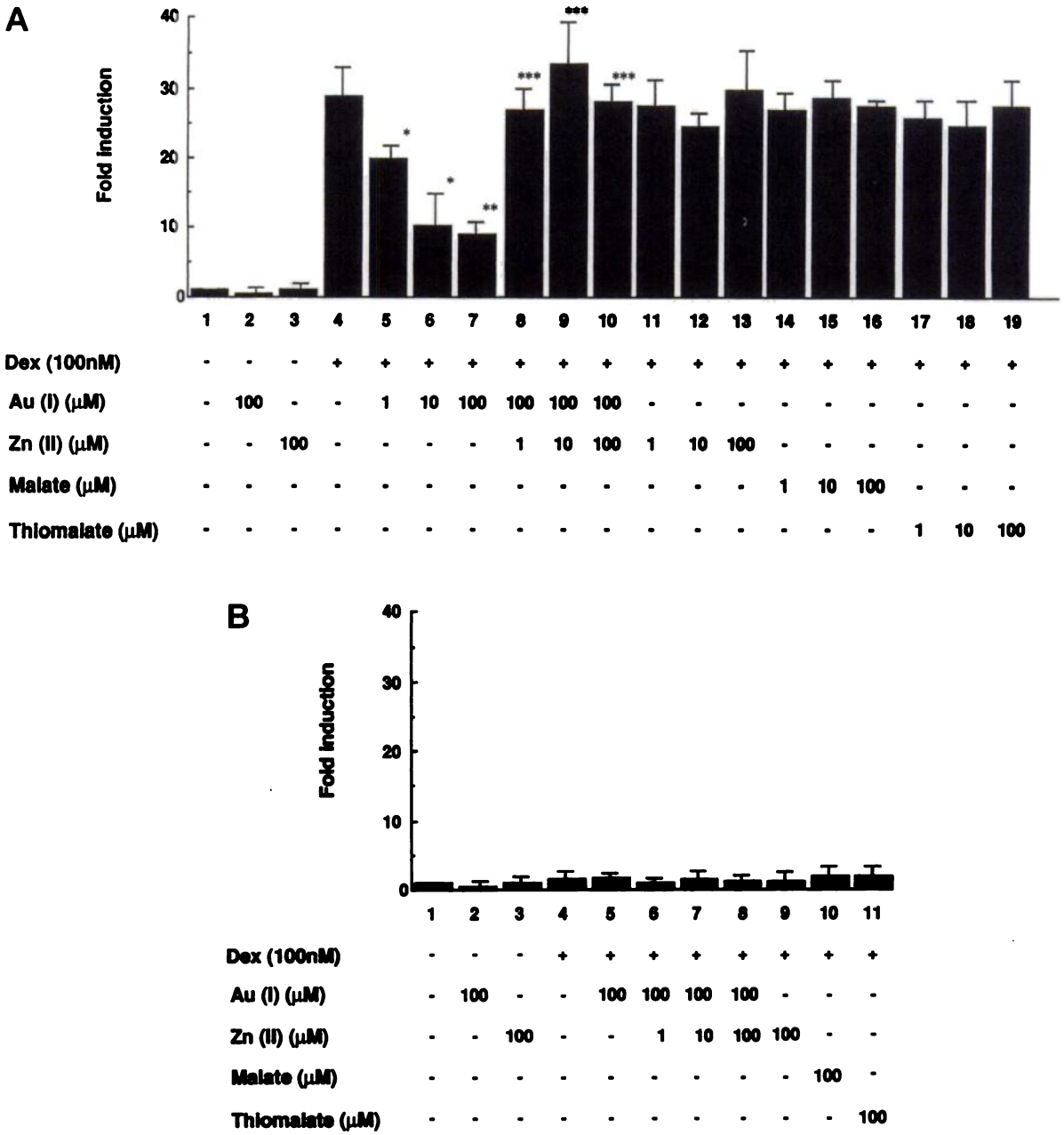

Fig. 4. Zn(II) counteracts Au(I)-mediated repression of cellular glucocorticoid responsiveness in GR-overexpressing cells. CHOpMTGR cells were transfected in duplicate with 5 μg of GREtkCAT (A) or mGREtkCAT (B) and 2 μg of the β-galactosidase expression vector pCH110 by the lipofection procedure as described in Materials and Methods. The cells were treated with dexamethasone (*Dex*), aurothiomalate [*Au(I)*], and/or $ZnCl_2$ [*Zn(II)*], and then cell extracts were prepared for CAT enzyme assay. Results are normalized to the transfection efficiency (as determined from β-galactosidase activity). For quantification, the radioactivity of each spot was counted using a BAS2000 phosphorimage analyzer. Values are given as mean and standard deviation from three experiments. A: *, $p < 0.05$ versus lane 4; **, $p < 0.01$ versus lane 4; ***, $p < 0.05$ versus lane 7.

14–16) or thiomalate (*lanes 17–19*), and is therefore caused by Au(I). In contrast, Zn(II) did not affect hormonal inducibility of the reporter gene (*lanes 11–13*, and data not shown). In the presence of Au(I), however, Zn(II) restored the hormone-dependent induction of the reporter gene (*lanes 8 and 9*). This complementation effect of Zn(II) tended to occur at physiological concentrations (23) and showed dose-dependency (*lanes 8 and 9*). However, at 100 μM, the complementation effect of Zn(II) diminished (*lane 10*). On the other hand, neither 100 μM Au(I) nor Zn(II) affected the basal level of the CAT expression (*lanes 2 and 3*). Furthermore, no significant alteration in the expression of the mGREtkCAT reporter plasmid was observed in the presence of these metals (Fig. 4B), indicating that the effects of Au(I) and Zn(II) are dependent on the GR/GRE interaction.

CHOpMTGR cells used in the present study overexpress the GR protein and are therefore suitable for quantitative analysis of the effects on GR action. To study the effect of Au(I) on GR action at physiological levels of GR protein, we used HeLa S3 cells, which are known to contain physiological levels of GR (28). The reporter plasmid pGRE-Luc was transfected into HeLa S3 cells, and the cells were cultured in the

presence or absence of dexamethasone, Au(I), and Zn(II) as indicated in Fig. 5. Treatment with dexamethasone induced the luciferase expression by 4.7-fold, and Au(I) repressed this hormone induction response to nearly basal level (*lanes 1–3*). Zn(II) counteracted the Au(I) effect and partially restored the hormone induction response (*lanes 4 and 5*). Zn(II), in the absence of Au(I), did not reveal apparent effects on either basal or hormone-induced levels of the luciferase expression (*lanes 8–11*).

To mechanistically address the inhibitory effects of Au(I) on GR function, we examined the effect of Au(I) on the GR protein in *in vitro* reconstitution experiments (Fig. 6). It has been shown that Cd(II) represses the DNA binding activity of native GR (13, 15) but restores the DNA binding activity of the metal-depleted recombinant GR DBD (29). Cd(II) restores the DNA binding activity of recombinant GR DBD at even lower concentrations than does the native metal ion Zn(II) (29). In the present study, we examined whether Au(I) could reconstitute the DNA binding activity of metal-depleted GR DBD. Recombinant GR DBD was metal depleted and Au(I) or Zn(II) was included during the reconstitution outlined in Fig. 6A (see legend). The DNA binding activity was monitored in EMSA. As shown in Fig. 6B, metal-depleted GR DBD did not bind to the GRE oligonucleotide (*lane 2*). After reconstitution in the presence of 100 to 500 μM Zn(II), protein/DNA complex formation was observed between GR DBD and the GRE probe (*lanes 3 and 4*), as previously described by Freedman *et al.* (29). Two protein/DNA complexes were formed between GR DBD and the GRE probe (Fig. 6B, *C I* and *C II*), representing monomeric GR DBD- and dimeric GR DBD-GRE complexes, respectively

Dex (nM)	0	100	100	100	100	100	0	0	100	100	100
Au(I) (μM)	0	0	100	100	100	100	100	0	0	0	0
Zn(II) (μM)	0	0	0	1	10	100	0	100	1	10	100

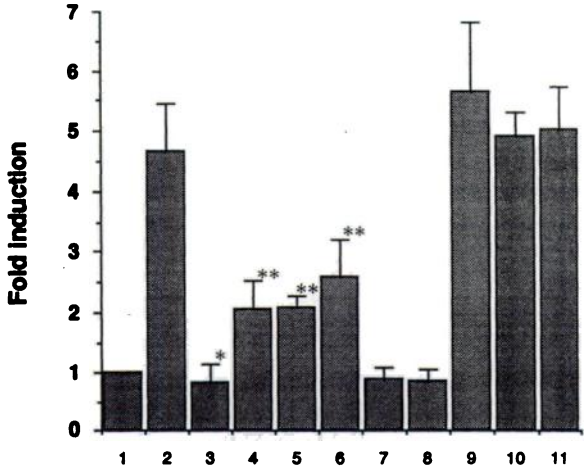

Fig. 5. Zn(II) counteracts Au(I)-mediated repression of cellular glucocorticoid responsiveness in HeLa S3 cells. HeLa S3 cells were transfected in duplicate with 5 μg of pGRE-Luc and 2 μg of the β-galactosidase expression vector pCH110 by the lipofection procedure as described in Materials and Methods. The cells were treated with dexamethasone (*Dex*), aurothiomalate [*Au(I)*], and/or $ZnCl_2$ [*Zn(II)*], and then cell extracts were prepared for luciferase and β-galactosidase assays. Results are normalized to the transfection efficiency (as determined from β-galactosidase activity). Values are given as mean and standard deviation from three independent experiments. *, $p < 0.01$ versus lane 1; **, $p < 0.05$ versus lane 3.

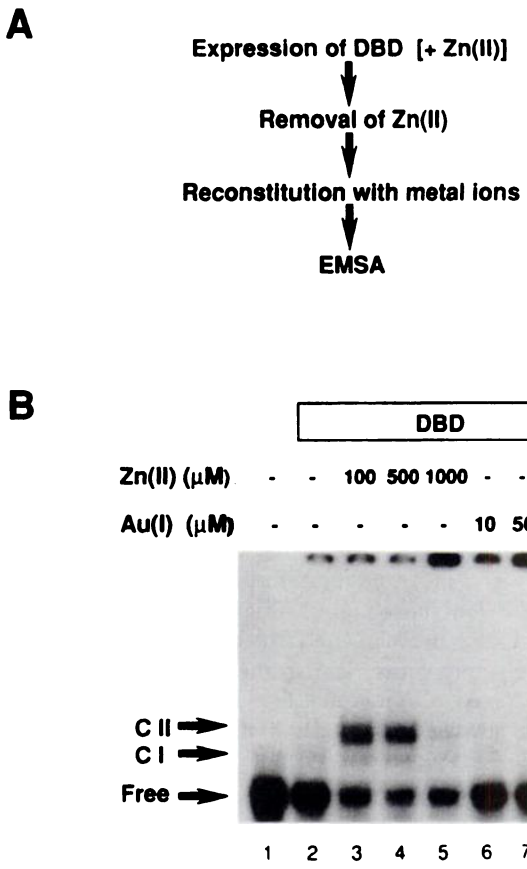

Fig. 6. *In vitro* reconstitution experiment of GR DBD. A, Schematic representation of the experimental protocol. Purified DBD was extensively dialyzed against buffer A (8 M urea, 20 mM Tris·HCl, pH 7.4, 50 mM NaCl, 10 mM EDTA, 1 mM DTT) at room temperature overnight to remove bound metal, and dialysis buffer was exchanged to buffer B (4 M urea, 20 mM Tris·HCl, pH 7.4, 50 mM NaCl, 1 mM DTT) for 4 hr at room temperature to remove Zn(II). Then, DBD was dialyzed against buffer B containing the indicated concentration of Zn(II) or Au(I) for 4 hr at room temperature. After removal of urea by extensive dialysis, DNA binding activity of DBD was analyzed with EMSA. B, EMSA of DBD. Thirty nanograms of DBD were incubated with ^{32}P-labeled GRE oligonucleotide probe for 15 min at room temperature, and formation of protein/DNA complexes was analyzed on a 5% native polyacrylamide gel. *C I and C II*, Specific protein/DNA complexes formed between DBD and GRE probe; *Free*, free probe.

(30). However, reconstitution in the presence of 10 to 100 μM Au(I) did not restore the DNA binding activity of GR DBD (*lanes 6–8*). Higher concentrations of Au(I) resulted in aggregation of GR DBD (data not shown).

In summary, aurothiomalate, in particular, Au(I), functionally antagonizes the GR and negatively modulates ligand binding, DNA binding, and transactivation functions at therapeutic concentrations. Zn(II) at physiological concentrations effectively counteracts the antiglucocorticoid effects of Au(I).

Discussion

Glucocorticoid hormone action is generally believed to be exclusively mediated by the GR, which is a ligand-inducible transcription factor (8–10). In this report, we showed that the antirheumatic drug aurothiomalate negatively modulates GR function and that Zn(II) counteracts the antiglucocorticoid action of aurothiomalate.

A large number of zinc-containing proteins have been iden-

tified, and many of them are DNA binding transcriptional regulators (31). Therefore, an appropriate concentration of zinc is very likely to be crucial for the regulation of a number of genes and to play an important role in the maintenance of homeostasis (31). Thus, cellular zinc may protect cells from potentially harmful effects of various extracellular attacks, including administration of gold compounds, through functional preservation of zinc-containing proteins, including the GR.

As summarized, several metal ions have been shown to affect the function of GR and other members of the steroid receptor superfamily. From our results and those of others (29), Au(I) may interact with GR in a manner different from that of Zn(II) or Cd(II): Au(I), in contrast to these metal ions, cannot reconstitute the DNA binding activity of metal-depleted GR DBD. Au(I) is known to bind to cysteine residues with high affinity to form linear complexes with a coordination number of 2 (33). Therefore, the inhibition of GR function by Au(I) may be ascribed to such a high affinity interaction between Au(I) and cysteine residues within the GR protein and to a subsequent failure to form a critical structural motif, especially in the DNA binding domain. Alternatively, Au(I) may cause displacement of incorporated Zn(II) from the zinc-coordinated motifs and cause structural distortion, as suggested for metallothioneins (33). Of course, we cannot rule out the possibility that Au(I) can reconstitute the DNA binding activity of GR DBD in a very narrow concentration range, which we did not detect in our *in vitro* reconstitution experiments. Biophysical analyses should be performed to define how these metal ions interact with the GR protein to cause structural and/or functional alterations. In addition to metals, a number of factors and/or cellular status have been shown to affect GR function, such as glucocorticoids (34, including references), cyclic AMP (17), protein kinase C (18–20), and cellular redox status.[1] Moreover, GR function has recently been reported to be modulated by coupling with the calcium storage protein calreticulin *in vitro* (35). Therefore, it is very important to study further how and whether Zn(II) interacts with other functional modulators of GR to regulate glucocorticoid responsiveness.

Our data demonstrate a metal-mediated modulation of GR action at physiological concentrations of GR protein. In the future, the effects of those metals on endogenous gene expression should be studied to confirm our hypothesis and to verify clinical significance of our observation. Numerous clinical observations suggest that zinc profiles may be altered in patients with, for example, RA, and plasma or serum zinc concentrations may be inversely correlated with disease activity (Ref. 36, including references). Hypoactivity of the HPA axis has been shown to operate in *de novo* onset of arthritis in Lewis rats (37), and patients with RA have been suggested to have inappropriately low HPA-axis activity (38–40). In view of our present findings, endogenous zinc appears to protect GR from, for example, gold compounds. D-Penicillamine is known to chelate zinc (41) and may contribute to a relative zinc deficiency in RA patients. Together, zinc deficiency and/or administration of aurothiomalate and/or D-penicillamine would further impair the glucocorticoid responsiveness of these patients and critically affect their endogenous anti-inflammation and/or immunosuppression control, which, at least in part, is mediated by cortisol production.

The present study indicates that the ligand binding capacity of cellular GR is significantly impaired by aurothiomalate without alteration in the net GR protein level. This effect is observed at clinically attainable concentrations of aurothiomalate (1–100 μM, Fig. 1), strongly suggesting that GR function (e.g., ligand binding activity) in circulating lymphocytes may be affected when patients are treated with aurothiomalate. Intriguingly, Schlaghecke *et al.* have reported that the number of GR in lymphocytes decreased in RA patients (42). However, GR protein level was not examined in their study (42); therefore, it remains to be clarified whether decrease in ligand binding activity of GR in RA patients is primarily due to the disease or rather to administration of certain drugs, such as aurothiomalate and D-penicillamine. Because numerous reports have shown that the GR number has close association with glucocorticoid responsiveness both *in vitro* and *in vivo* (Ref. 34, including references), net reduction in GR in target organs would result in, at least partial, insensitivity to glucocorticoids. In contrast, Schlaghecke *et al.* (43) failed to show the corresponding decrease in glucocorticoid responsiveness. Thus, to functionally characterize GR in RA patients may be extremely important.

In conclusion, GR may be a target for various extracellular stimuli and cellular stress, including drugs, and subsequently transmit the effects of these stimuli into the nucleus as GR-mediated signals. Zn(II) may, through its protection of GR from such negative regulators, play an important role in cellular homeostatic control.

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