Thioredoxin: a Redox-regulating Cellular Cofactor for Glucocorticoid Hormone Action

Cross Talk between Endocrine Control of Stress Response and Cellular Antioxidant Defense System

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Abstract

Adaptation to stress evokes a variety of biological responses, including activation of the hypothalamic-pituitary-adrenal (HPA) axis and synthesis of a panel of stress-response proteins at cellular levels: for example, expression of thioredoxin (TRX) is significantly induced under oxidative conditions. Glucocorticoids, as a peripheral effector of the HPA axis, exert their actions via interaction with a ligand-inducible transcription factor glucocorticoid receptor (GR). However, how these stress responses coordinately regulate cellular metabolism is still unknown. In this study, we demonstrated that either antisense TRX expression or cellular treatment with H_2O_2 negatively modulates GR function and decreases glucocorticoid-inducible gene expression. Impaired cellular response to glucocorticoids is rescued by overexpression of TRX, most possibly through the functional replenishment of the GR. Moreover, not only the ligand binding domain but the DNA binding domain of the GR is also suggested to be a direct target of TRX.

Together, we here present evidence showing that cellular glucocorticoid responsiveness is coordinately modulated by redox state and TRX level and propose that cross talk between neuroendocrine control of stress responses and cellular antioxidant systems may be essential for mammalian adaptation processes. (*J. Clin. Invest.* 1996. 98:2469–2477.)
Key words: redox • oxidative stress • glucocorticoids • gene expression • thioredoxin

Introduction

The mammalian stress response evokes a series of neuroendocrine responses that activate the hypothalamic-pituitary-adrenal (HPA)[1] axis and the sympathetic nervous system. Coordinated interactions between stress response systems, occurring at multiple levels including the brain, pituitary gland, adrenal gland, and peripheral tissues, are required for the maintenance of homeostatic plateau (1).

Glucocorticoids, as a major peripheral effector of the HPA axis, play an essential role in reestablishing homeostatic status in every peripheral tissue in human. However, molecular mechanism for glucocorticoid-mediated attenuation of cellular stress is largely unknown (2). On the other hand, the adaptive responses also operate on various intrinsic or extrinsic forces which disturb cellular homeostasis as a part of local host-defense mechanisms at a cellular level (3). Currently, reduction/oxidation (redox) reactions are intimately involved in the control of biological processes including modulation of the function of transcription factors, e.g., AP-1 and NF-κB (4–9). Reactive oxygen intermediates (ROIs) are known to be generated as a consequence of respiratory chain reaction and as a result of stimulation by cytokines (e.g., TNF-α) and they act as a significant stressor to cells (10–12). Cells contain endogenous buffering systems against excessive production of ROIs to preserve cellular metabolism through the expression and regulation of many enzymes (3, 4). However, it is still unknown whether the peripheral adaptive responses can be specific to each stressor or if they can be generalized and nonspecific. Moreover, how these diverse stress response systems, i.e., systemic/central and peripheral/cellular, converge and orchestrate remains to be elucidated.

Glucocorticoids, on binding to the glucocorticoid receptor (GR), promote the dissociation of heat shock proteins (HSPs), and the ligand–receptor complex translocates to the nucleus and then binds to palindromic DNA sequences, called glucocorticoid response elements (GREs) (13–15). After binding to DNA, the GR differentially regulates target gene expression to produce hormone action, interacting with or without other transcription factors and coactivators/corepressors (13–19). The GR has a modular structure mainly consisting of a central DNA binding domain (DBD), nuclear localization signals, a ligand binding domain (LBD), and several transcription activation functions (13, 14). The human GR contains 20 cysteine residues, concentrated in the central region spanning the DBD and LBD (14). The cysteine residues in each domain have

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1. *Abbreviations used in this paper:* ADF, adult T cell leukemia–derived factor; CHO, Chinese hamster ovary; DBD, DNA binding domain; ECL, enhanced chemiluminescence; EMSA, electrophoretic mobility shift assay; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HPA, hypothalamic-pituitary-adrenal; HSP, heat shock protein; LBD, ligand binding domain; 2ME, 2-mercaptoethanol; NAC, *N*-acetyl-L-cysteine; PVDF, polyvinylidene fluoride; rADF, recombinant ADF; rGR DBD, recombinant DBD of the GR; ROI, reactive oxygen intermediate; TRX, thioredoxin.

been shown to be crucial for maintaining both structure and function of those domains (15, 20–22). For example, it has been shown already that conversion of sulfhydryls in the DBD to disulfides blocks GR binding to DNA cellulose (23), and that metal ions which have high affinity for thiols interfere with the DBD–DNA interaction (24).

An important constituent of cellular antioxidant buffering systems that controls the redox state of proteins is thioredoxin (TRX), a 13-kD protein that catalyzes thiol-disulfide exchange reactions (25, 26). In humans, TRX is discovered as adult T cell leukemia–derived factor (ADF) from human T lymphotropic virus type I–infected T cells (27). Aside from the activity as an autocrine growth factor for human T cell leukemia virus type I–infected T cells (28) and Epstein-Barr virus–transformed lymphocytes (29), numerous studies have shown the importance of ADF/TRX as a cellular reducing catalyst in human physiology (25, 26). The redox-active sulfhydryls of TRX are located in the highly conserved active-site sequence -Trp-Cys-Gly-Pro-Cys- (25). The pathway for the reduction of a protein disulfide by TRX entails nucleophilic attack by one of the active-site sulfhydryls to form a protein–protein disulfide followed by intramolecular displacement of the reduced target proteins with concomitant formation of oxidized TRX (25). Recently, TRX is considered to be involved in transcriptional processes: for example, NF-κB activation is inhibited, whereas AP-1 activity is induced by TRX (30, 31). Moreover, the GR in the isolated rat cytosol is shown to be stabilized and maintained in a reduced, ligand-binding form by TRX (32). However, the interplay between the GR and TRX in situ has not yet been documented.

In this study, we present evidence for the functional interaction between cellular oxidative stress, TRX, and GR in situ and indicate that cellular redox state and TRX levels are important determinants of cellular sensitivity to glucocorticoids. Thus, we propose that TRX systems may control homeostasis not only by sequestrating ROIs (33), for example, but also by fine tuning of hormonal signals.

Methods

Cell culture. CHO-pMTGR cells, which are derived from Chinese hamster ovary (CHO)-K1 cells, were engineered to express relatively high levels of human GR (300,000–500,000 molecules/cell) as described previously (34), and were kindly provided by Dr. S. Nilsson (Karo Bio, Huddinge, Sweden). The cells were maintained in Ham's F-12 medium (Gibco Laboratories, Grand Island, NY), pH 7.4, supplemented with antibiotics and 10% heat-inactivated FCS (Gibco Laboratories) in the presence of cadmium and zinc ions, 40 μM each, and 1 d before experiments the medium was replaced with fresh Ham's F-12 medium, both antibiotics, and FCS. COS-7 and HeLa cells were obtained from RIKEN Cell Bank (Tsukuba Science City, Japan) and maintained in DME (Gibco Laboratories), pH 7.4, supplemented with 10% heat-inactivated FCS and antibiotics. In all experiments, serum steroids were stripped with dextran-coated charcoal, and cells were cultured in a humidified atmosphere at 37°C with 5% CO_2.

Reagents and antibodies. Diamide, 2-mercaptoethanol (2ME), *N*-acetyl-L-cysteine (NAC), and dexamethasone were purchased from Sigma Co. (St. Louis, MO). Other chemicals were from Wako Pure Chemical (Osaka, Japan). Recombinant ADF (rADF) was produced according to the method described previously and kindly provided by Ajinomoto Co. Inc. (Basic Research Laboratory, Kawasaki, Japan) (35). Polyclonal antibody against carboxy-terminal sequence of ADF was prepared as described previously (28). Anti–human GR antibody PA1-512 was obtained from Affinity Bioreagents (Neshanic Station, NJ). All enzymes were purchased from TaKaRa Syuzo (Kyoto, Japan).

Plasmids. The expression vectors for the wild-type and mutant GR, RShGRα, I550, and Δ9-385 were described elsewhere (36) and kindly supplied by Dr. R.M. Evans (Salk Institute, La Jolla, CA). The expression plasmid for TRX, pcDSRαADF, was described previously (37). For the construction of the antisense TRX expression plasmid pASADF, pcDSRαADF was digested with BamHI and HindIII, and the fragment containing a part of TRX coding region was isolated and then subcloned into the HindIII-BamHI site of the parent pcDNA3 plasmid (38). The inverse orientation of the insert DNA was verified by sequencing. The construction of the glucocorticoid-responsive reporter construct pGRE-Luc was described previously (39). In brief, the oligonucleotides containing the GRE tandem repeat (noncoding strand, 5′-CGGATCCAGAACAGGATGTTCTAGCTACGGATCCAGAACAGGATGTTCTAGCTACG-3′), and a 122-bp fragment of the human immunodeficiency virus long terminal repeat (positions −9193 to −9071 from the transcription start site) was inserted into a pGVB luciferase expression vector (Toyo Ink, Inc., Tokyo, Japan). The β-galactosidase expression plasmid pCH110 (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) was used as an internal control for transfection efficiency.

RNA isolation and Northern blot analysis. Total RNA was isolated according to the method of acid guanidinium thiocyanate phenol chloroform extraction, and Northern blot analysis was done as described previously (39). In brief, 20 μg of total RNA was separated on a 1% formaldehyde-agarose gel, and the fractionated RNA was immobilized on a charge-modified polyvinylidene fluoride (PVDF) membrane (Immobilon-N; Millipore, Tokyo, Japan) using a pressure blot apparatus (Stratagene, La Jolla, CA), and RNA was cross-linked by means of ultraviolet irradiation. ADF cDNA probe was described elsewhere (27) and labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham International, Buckinghamshire, United Kingdom) using BcaBEST™ labeling kit (TaKaRa Syuzo) according to the manufacturer's protocol. The membrane was prehybridized with a QuikHyb™ hybridization solution (Stratagene) for 15 min at 68°C, and was hybridized with 2×10^6 cpm/ml of ^{32}P-labeled probes for 60 min at 68°C. After the membrane was washed for 15 min in 0.1% SDS with 2× SSC (0.3 M NaCl, 0.03 M sodium citrate) at 25°C, and for an additional 30 min in 0.1% SDS with 0.1× SSC at 60°C, radioactivity on the membrane was quantified with a phosphorimage analyzer (BAS2000; Fuji Photo Film, Tokyo, Japan). Autoradiography was performed usually at −80°C overnight using Amersham Hyperfilm (Amersham) and intensifying screens.

Western immunoblot analysis. Whole-cell extracts for immunoblot analysis of TRX and GR were prepared as described previously (39). In brief, cells were washed with ice-cold PBS three times and then were homogenized with a solubilizing solution containing 10 mM Tris-HCl, 10% glycerol, and 0.4 M NaCl. The homogenates were centrifuged at 100,000 *g* for 1 h at 4°C, and the resultant supernatants were used as whole-cell extracts. Protein samples were dissolved in SDS-sample buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 2% 2ME, and 0.001% bromophenol blue, and fractionated on SDS-polyacrylamide gels. After electrophoresis, proteins were electrically transferred onto a PVDF membrane (Bio-Rad Laboratories, Richmond, CA) and probed with appropriate antibodies. Antigen–antibody complexes were detected using an enhanced chemiluminescence (ECL) Western blot detection kit (Amersham) according to the manufacturer's instructions. Films were processed to densitometric analysis using an imaging densitometer (model GS-700; Bio-Rad Laboratories).

Transfection and luciferase assay. Transient transfection was performed as described previously (24). Briefly, cells were plated on plastic culture dishes (IWAKI Glass, Funabashi, Japan) to 30–50% confluency, washed with PBS three times, and medium was replaced with Opti-MEM medium (Gibco Laboratories). Plasmid cocktail was mixed with Lipofectin reagent (Gibco Laboratories) and added to the

culture. Total amount of the plasmids was kept constant (20 μg) by adding carrier plasmids (pGEM3Z unless otherwise specified; Promega Corp., Madison, WI). After 12 h of incubation, the medium was replaced with fresh Ham's F-12 medium or DME supplemented with 2% dextran-coated charcoal-stripped FCS, and the cells were further cultured in the presence or absence of various ligands for 24 h. After normalization of transfection efficiency by β-galactosidase expression, luciferase enzyme activity was determined using a luminometer (Berthold GmbH & Co. KG, Bad Wildbad, Germany) essentially as described previously (40).

Whole-cell hormone binding assay. CHOpMTGR cells were grown in 6-well flat-bottom plastic plates (IWAKI Glass) to confluence. After washing twice with PBS and once with Opti-MEM medium, the cells were incubated with 20 nM of [^{3}H]dexamethasone (35–50 Ci/mmol; DuPont/New England Nuclear Research Products, Wilmington, DE) in the presence or absence of 500 M excess of radioinert dexamethasone in triplicate, for 1 h at 37°C. 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-propanesulfonate and 1% SDS. Aliquots were added to ACS II scintillation fluid (Amersham) to determine bound radioactivity. The difference between total and nonspecific binding gives the number of specific binding sites.

Preparation of recombinant DNA binding domain of GR (rGR DBD). For the construction of the bacterial expression vector for the DBD, the DNA fragment encoding 111 amino acids (398 Methionine - 508 Serine) of the human GR was amplified by PCR with appropriate flanking sequences for enzymatic cleavage and subcloned into pMal-2 expression plasmid (New England BioLabs, Inc., Boston, MA). Subsequent to bacterial expression of the protein using *Escherichia coli,* the rGR DBD was isolated according to the manufacturer's recommendations.

Electrophoretic mobility shift assay (EMSA). EMSA for rGR DBD was carried out as described previously (24). Briefly, the GRE probe was end-labeled with [α-^{32}P]dCTP using the Klenow fragment of DNA polymerase I. The sequence of the oligonucleotide encompassing GRE is: 5′-CGAGTAGCTAGAACAGGATGTTCTGAGG-3′ (upper strand sequence). rGR DBD (30 ng protein/reaction) was incubated with 0.2 ng of ^{32}P–end-labeled GRE probe (∼ 20,000 cpm) in a 10-μl reaction mixture containing 10 mM Hepes, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.25 mM dithiothreitol, 10% glycerol, and 50 ng poly (dI-dC) (Pharmacia LKB) for 15 min on ice. Radioinert competitor DNA was included when indicated. The reaction mixture was then loaded onto a 5% nondenaturing polyacrylamide gel containing 0.25× TBE (1× TBE is composed of 89 mM Tris-base, 89 mM boric acid and 2 mM EDTA). The gels were run at 350 V for 2 h and dried. Results were visualized by autoradiography using Amersham Hyperfilm and intensifying screen. For quantitation, radioactivity of appropriate bands was counted using a BAS2000 phosphorimage analyzer.

Results

Regulation of TRX expression under oxidative stress in HeLa cells. Treatment of cells with H_2O_2 triggers generation of ROIs and expression of a number of stress-responsive genes, so that cells are able to maintain metabolic equilibrium (3, 8, 31). Since, among others, TRX is considered to play a key role when cells are exposed to oxidative stress (25, 26), we first studied the influence of treatment with H_2O_2 on both the steady state levels of mRNA and protein contents of TRX in HeLa cells. Addition of 1 mM H_2O_2 to the culture medium did not cause significant cytotoxicity as assessed by trypan blue dye exclusion assay in this study (data not shown). After treatment of HeLa cells with 1 mM H_2O_2, total cellular RNA and protein were isolated at various time points as indicated (Fig. 1). Northern blot experiments revealed that the steady state levels of TRX mRNA were significantly increased in a time-dependent fashion and peaked at 12 and 48 h (3.2- and 4-fold to control, respectively, Fig. 1 *A*). Western immunoblot experiments showed that TRX protein was also increased in a time-dependent manner and peaked at 12 and 48 h (4.5- and 2.6-fold to control, respectively, Fig. 1 *B*). These results indicate that HeLa cells may respond to oxidative stress, at least partly, via an increase in production of TRX, although critical target molecules of TRX are unknown.

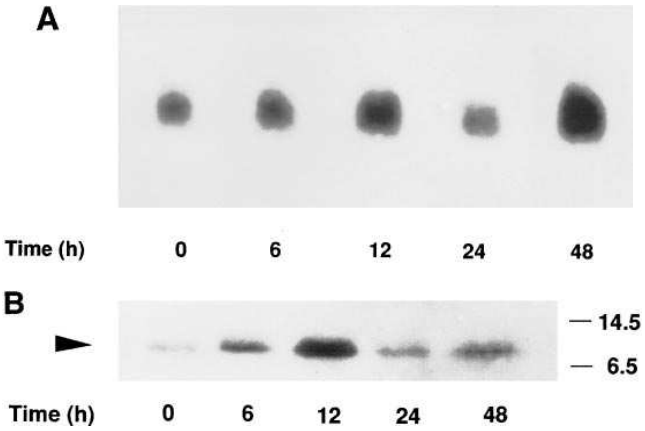

Figure 1. Induction of TRX expression under oxidative stress. HeLa cells were treated with 1 mM H_2O_2 for the indicated time periods and the induction of TRX expression was determined by Northern blot and Western immunoblot assays. (*A*) Northern blot. Total RNA was extracted, and samples of 20 μg of RNA were separated in 1% formaldehyde-agarose gel and transferred to a PVDF filter membrane. The filter was hybridized with the cDNA probe for ADF/TRX as described in Methods. Autoradiography was performed overnight at −80°C. Experiments were performed twice with identical results, and the representative autoradiogram is shown. For quantitation, a BAS2000 phosphorimage analyzer was used. (*B*) Immunoblot. Whole-cell extracts were prepared, and protein samples of 20 μg were fractionated in a 15% SDS-polyacrylamide gel and transferred to a PVDF membrane. Immunoblot analysis was performed with antibody against ADF/TRX as described in Methods. For visualization, ECL system was used. Numbers depict molecular masses determined using the molecular mass marker run in parallel (not shown). Experiments were performed twice with identical results, and the representative ECL film is shown. For quantitation, the films were processed to densitometric analysis as described in Methods. The arrow denotes the position of ADF/TRX (molecular mass is 13 kD).

In vivo complementation assay for studying the intracellular interaction between TRX and glucocorticoid hormone action. Since previous biochemical studies revealed the intimate association between TRX and the GR in vitro (32), we then asked whether TRX also interacts with the GR in situ to elucidate the interplay between peripheral limb of central stress response (i.e., glucocorticoids) and endogenous buffering system (i.e., TRX) in cells. For that purpose, we constructed the antisense TRX expression plasmid pASADF to manipulate cellular TRX levels, and cotransfected into HeLa cells with the GR expression plasmid RShGRα, and either the TRX expression plasmid pcDSRαADF or its host plasmid pcDSRα, as indicated in Fig. 2 *A*. In the absence of a synthetic glucocorticoid dexamethasone, cellular luciferase activity was not significantly influenced by expression of antisense TRX (Fig. 2 *A*,

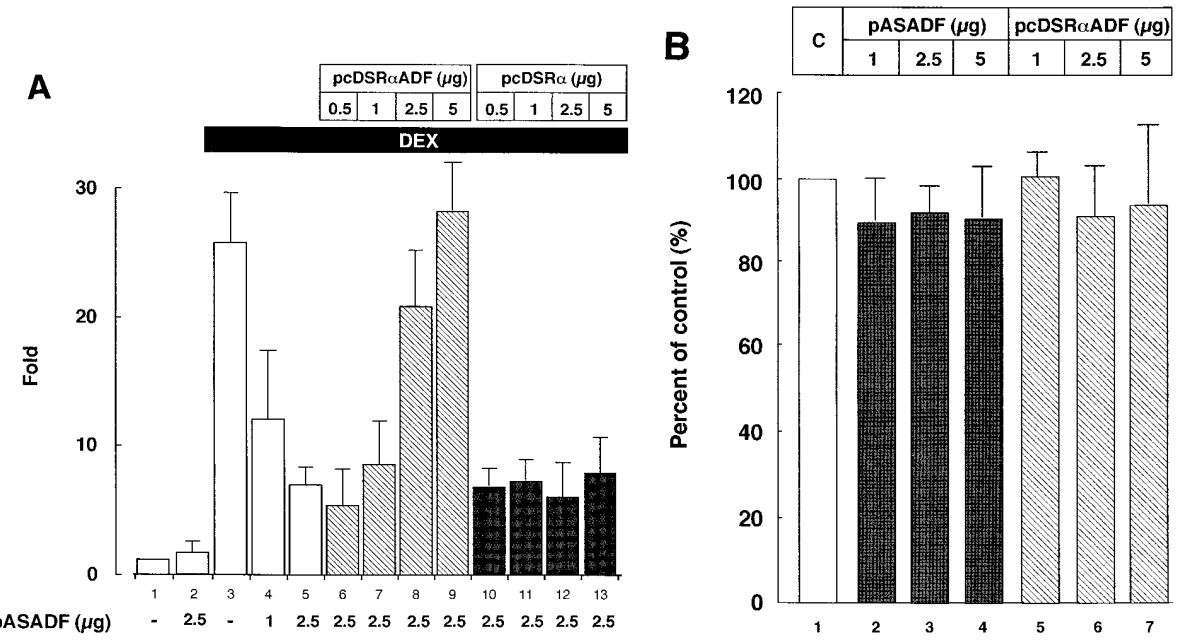

Figure 2. Regulation of glucocorticoid-mediated gene expression by cellular TRX levels. (*A*) HeLa cells were grown in 100-mm-diameter culture dishes and transfected with 10 ng of the GR expression plasmid RShGRα, 10 μg of pGRE-Luc reporter plasmid, and various amounts of the antisense TRX expression plasmid pASADF, and the TRX expression plasmid pcDSRαADF or its host vector pcDSRα as indicated. The cells were further incubated in the presence or absence of 100 nM dexamethasone (*DEX*) for 24 h, and cellular luciferase activity was determined as described in Methods. All results are expressed as fold induction compared with the cellular luciferase levels when the reporter and carrier plasmids PGEM3Z were transfected (column *1*). Three independent experiments were performed and means±SD of the results are shown. (*B*) HeLa cells were transfected with 1 μg of a control plasmid for luciferase expression, PGV-control, and various amounts of either pASADF or pcDSRαADF as indicated, and cultured for 24 h, then, cellular luciferase activity was determined as described in Methods. Expressed luciferase activity without cotransfection of either pASADF or pcDSRαADF served as control (*C*, column *1*), and results are shown as the percentage of control luciferase value. Three independent experiments were performed and means±SD of the results are shown.

columns *1* and *2*). In the presence of 100 nM dexamethasone, luciferase expression was induced by 26-fold, and antisense TRX expression repressed the hormone induction response in a dose-dependent fashion (Fig. 2 *A*, columns *3–5*), indicating that expression of the antisense TRX selectively repressed glucocorticoid-inducible gene expression without affecting basal promoter activity. Next, to confirm the contribution of TRX to glucocorticoid-mediated gene expression in vivo, we cotransfected various amounts of pcDSRαADF or its host plasmid pcDSRα with pASADF. As shown in Fig. 2 *A*, cotransfection of pcDSRαADF reversed the negative effects of the antisense TRX and restored GR-mediated transcriptional activation in a dose-dependent manner (Fig. 2 *A*, columns *6–9*). However, the antisense-mediated repression was not complemented by the transfection of pcDSRα (Fig. 2 *A*, columns *10–13*). In separate experiments, HeLa cells were transfected with the luciferase expression plasmid driven by the strong SV40 enhancer, PGV-control, with either pASADF or pcDSRαADF, and we confirmed that either antisense TRX expression or overexpression of TRX did not influence SV40 enhancer activity (Fig. 2 *B*). These results strongly suggest that cellular TRX level is one of the determinants of the transactivational function of the GR, and TRX acts as an endogenous auxiliary factor for the GR to augment hormone induction response through the GRE in situ.

Effect of treatment with H_2O_2 on glucocorticoid-mediated gene expression. Next, effect of treatment with H_2O_2 on glucocorticoid-inducible gene expression was examined. HeLa cells were transfected with pGRE-Luc, and cultured in the absence or presence of H_2O_2 and dexamethasone as indicated (Fig. 3). Treatment with 100 nM dexamethasone increased luciferase expression by eightfold, indicating that HeLa cells endogenously contain functional GR (Fig. 3, columns *1* and *5*). Addition of H_2O_2 into the culture medium decreased, without affecting basal promoter activity (columns *1–4*), this hormone induction response in a concentration-dependent manner (columns *5–8*). Since treatment of HeLa cells with H_2O_2 increases expression of TRX (Fig. 1), depletion of TRX is not likely for the explanation of the reduction of glucocorticoid responsiveness after treatment with H_2O_2. In this line, we further examined the mechanism of H_2O_2-mediated repression of glucocorticoid hormone action.

Effect of treatment with H_2O_2 and/or overexpression of TRX on the ligand binding activity of the GR. To examine which function of the GR is affected by treatment with H_2O_2 and TRX, GR-overexpressing CHOpMTGR cells, which afford sensitive measurement of GR function, were used (see Methods). CHOpMTGR cells were transfected with various amounts of TRX expression plasmid pcDSRαADF and treated with 1 mM H_2O_2 for 24 h, then cellular binding activity for tritiated dexamethasone was determined as described in Methods. As shown in Fig. 4 *A*, we found that the total number of binding sites for tritiated dexamethasone was decreased to ~ 65% of control after treatment with H_2O_2 (Fig. 4 *A*, columns *1* and *2*), and this suppression was rescued by overexpression of TRX in a dose-dependent manner (Fig. 4 *A*, columns *2–5*). Overex-

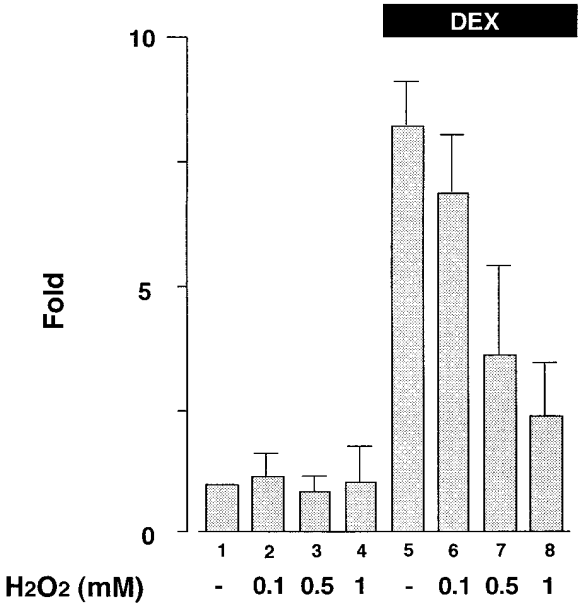

Figure 3. Effect of treatment with H_2O_2 on glucocorticoid-mediated gene expression. HeLa cells were transfected with 10 μg of the glucocorticoid-inducible reporter plasmid pGRE-Luc and cultured for 24 h in the presence of various concentrations of H_2O_2 and 100 nM dexamethasone (*DEX*) as indicated, then, cellular luciferase activity was determined as described in Methods. Results are expressed as fold induction compared with the luciferase activity in the cells which were cultured in the absence of H_2O_2 and DEX (column *1*). Means±SD of three independent experiments are shown.

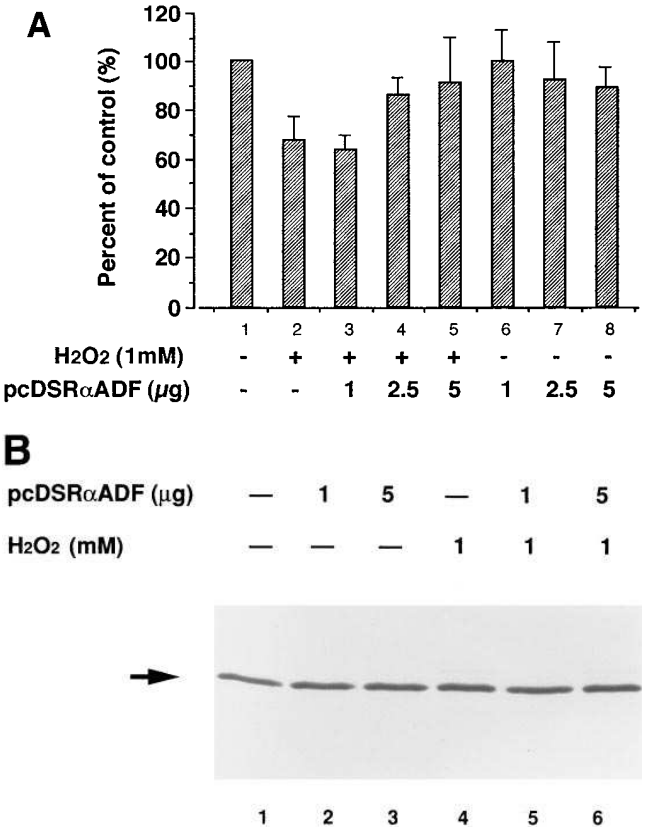

Figure 4. Effect of treatment with H_2O_2 and overexpression of TRX on the ligand binding activity of the GR in situ. CHOpMTGR cells were transfected with various amounts of the TRX expression plasmid pcDSRαADF and cultured for 24 h in the presence or absence of H_2O_2 as indicated. (*A*) The ligand binding activity was determined by whole-cell assay as described in Methods. Results are expressed as the percentage of control (column *1*) and means±SD of three independently performed experiments are presented. (*B*) Immunoblot was performed to compare the protein content of the GR. CHOpMTGR cells were transfected with pcDSRαADF and cultured in the presence of H_2O_2 as indicated for 24 h. Whole-cell extracts were prepared as described in Methods, and protein samples of 10 μg were fractionated in an 8% SDS-polyacrylamide gel, and transferred to a PVDF membrane. Immunoblot was performed with anti–human GR antibody as described in Methods. Relative molecular masses were determined by the marker run in parallel (not shown), and the arrow denotes the position of the GR (molecular mass is 94 kD).

pression of TRX did not significantly influence the ligand binding activity of the GR in the absence of H_2O_2 (Fig. 4 *A*, columns *6–8*). Western immunoblot analysis revealed that GR immunoreactivity apparently was not affected by either treatment with H_2O_2 or transfection of TRX expression plasmid (Fig. 4 *B*), indicating that the decrease in the ligand binding activity of the GR is due not to the decrease in GR protein, but to functional alteration of the GR.

Overexpression of TRX rescues the suppression of glucocorticoid-mediated gene expression by treatment with H_2O_2. Next, we studied the effect of treatment with H_2O_2 and/or overexpression of TRX on the glucocorticoid-mediated gene transcription using CHOpMTGR cells, in which TRX expression plasmid pcDSRαADF and the glucocorticoid-inducible reporter plasmid pGRE-Luc were transiently transfected as indicated (Fig. 5). Either treatment of the cells with 1 mM H_2O_2 or overexpression of TRX did not affect basal level expression of luciferase (Fig. 5, columns *2* and *3*). Addition of 100 nM dexamethasone resulted in an ~ 27-fold induction of the luciferase activity (Fig. 5, column *4*), and treatment with H_2O_2 suppressed the inductive effect of dexamethasone in a concentration-dependent manner (Fig. 5, columns *5–7*). Treatment with 1 mM H_2O_2 repressed the luciferase activity to 5.2-fold (Fig. 5, column *7*). In the presence of H_2O_2, overexpression of TRX counteracted the H_2O_2-mediated repression of hormone-inducible gene expression in a dose-dependent manner (Fig. 5, columns *8–11*). In the absence of H_2O_2, however, transient transfection of pcDSRαADF did not significantly affect the hormone induction response (Fig. 5, columns *12–15*). Western immunoblot assay revealed that GR protein levels were not significantly affected in these experiments (data not shown). Therefore, it is suggested that observed reduction in hormone-inducible gene expression after treatment with H_2O_2 is due not to alteration in GR content, but to modification of GR protein in such a way that is complemented by TRX, for example.

The negative effect of treatment with H_2O_2 appeared to be more prominent on the transactivation function of the GR when compared with those on the ligand binding activity (compare Figs. 4 *A* and 5), indicating that not only the LBD but the other domain(s) may be involved in H_2O_2-mediated repression of glucocorticoid-inducible gene expression. To address this issue, we cotransfected a panel of GR expression plasmids with pcDSRαADF and pGRE-Luc into COS-7 cells. RShGRα is a wild-type GR expression plasmid, I550 is a mutant GR-expression plasmid which is truncated at amino acid

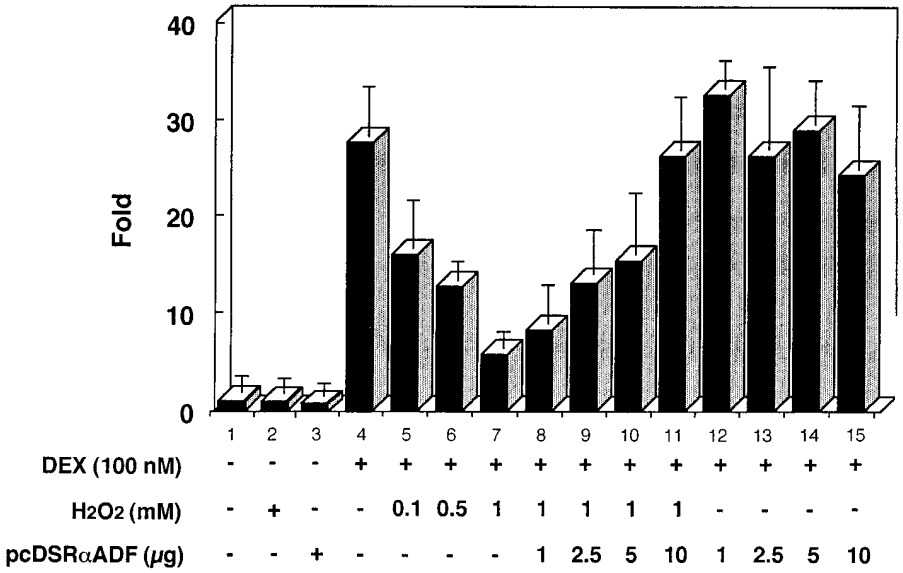

Figure 5. Repression of glucocorticoid-inducible gene activation by treatment with H_2O_2 is restored by overexpression of TRX. CHOpMTGR cells were transfected with 10 μg of the glucocorticoid-responsive reporter plasmid pGRE-Luc and various amounts of the TRX expression plasmid pcDSRαADF, then cultured in the presence or absence of various concentrations of H_2O_2 and 100 nM dexamethasone (*DEX*) for 24 h as indicated. Cellular luciferase activity was determined as described in Methods. Results are expressed as fold induction compared with the luciferase activity in the cells which were transfected with a carrier plasmid pGEM3Z and cultured in the absence of H_2O_2 and DEX (column *1*). Means±SD of three independent experiments are shown.

550 and lacks the LBD and is known to be a hormone-independent constitutive transcriptional activator, and Δ9-385 encodes another mutant GR containing an internal deletion from amino acids 9 to 385, which lacks τ1 transactivation domain and is known to be a weak ligand-dependent transcriptional activator (36). After transfection, the cells were cultured in the presence or absence of 100 nM dexamethasone and/or 1 mM H_2O_2 as indicated (Fig. 6), and cellular luciferase activity was determined as described in Methods. When wild-type GR expression plasmid RShGRα was transfected, treatment with H_2O_2 reduced hormone induction response of the luciferase expression 74- to 24-fold (Fig. 6, columns *2–4*). Cotransfection of TRX expression plasmid restored dexamethasone-mediated luciferase expression in a dose-dependent fashion as in the case of CHOpMTGR cells (Fig. 6, columns *5–7*, also see Fig. 5). When a ligand-independent transcriptional activator I550 was expressed, luciferase expression was strongly induced even in the absence of dexamethasone, and treatment with H_2O_2 decreased this induction to 40% (Fig. 6, columns *8* and *9*). Overexpression of TRX counteracted the negative effect of H_2O_2 and restored the reporter gene expression in a dose-dependent manner (Fig. 6, columns *10–12*). When a ligand-inducible weak transcriptional activator Δ9-385 was cotransfected, the effects of H_2O_2 and overexpression of TRX on the reporter gene expression were almost comparable with those when wild-type GR expression plasmid was cotransfected (Fig. 6, columns *13–18*). Thus, it is indicated that H_2O_2-mediated repression and TRX-mediated augmentation of glucocorticoid-inducible gene expression are elicited not only by the LBD, but at least by the DBD as well.

Effect of TRX on the DNA binding activity of the GR. To examine direct interaction between the DBD of the GR and TRX in vitro, we prepared rGR DBD and performed EMSA. After incubation of protein samples with the thiol oxidizing re-

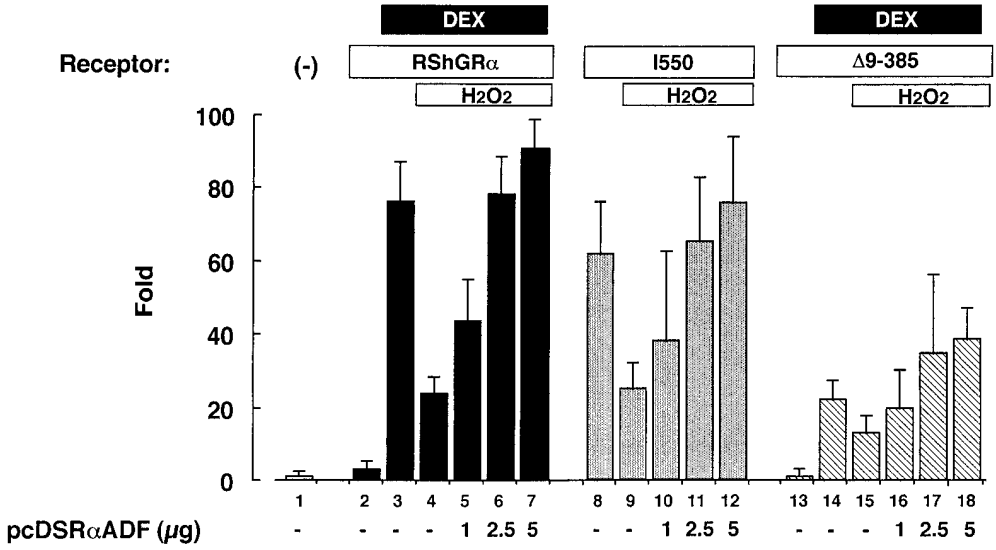

Figure 6. Domain requirement for redox regulation of the GR. COS-7 cells were transfected with 10 ng each of GR expression plasmids, RShGRα, I550, Δ9-385 (see text), 10 μg pGRE-Luc reporter plasmid, and various amounts of the TRX expression plasmid pcDSRαADF, and cultured in the presence or absence of 1 mM H_2O_2 and 100 nM dexamethasone (*DEX*) for 24 h as indicated. Cellular luciferase activity was determined as described in Methods, and results are expressed as fold induction compared with the luciferase levels in the cells without either transfection of expression plasmids or treatment with reagents (column *1*). Three independent experiments were performed and means±SD of the results are shown.

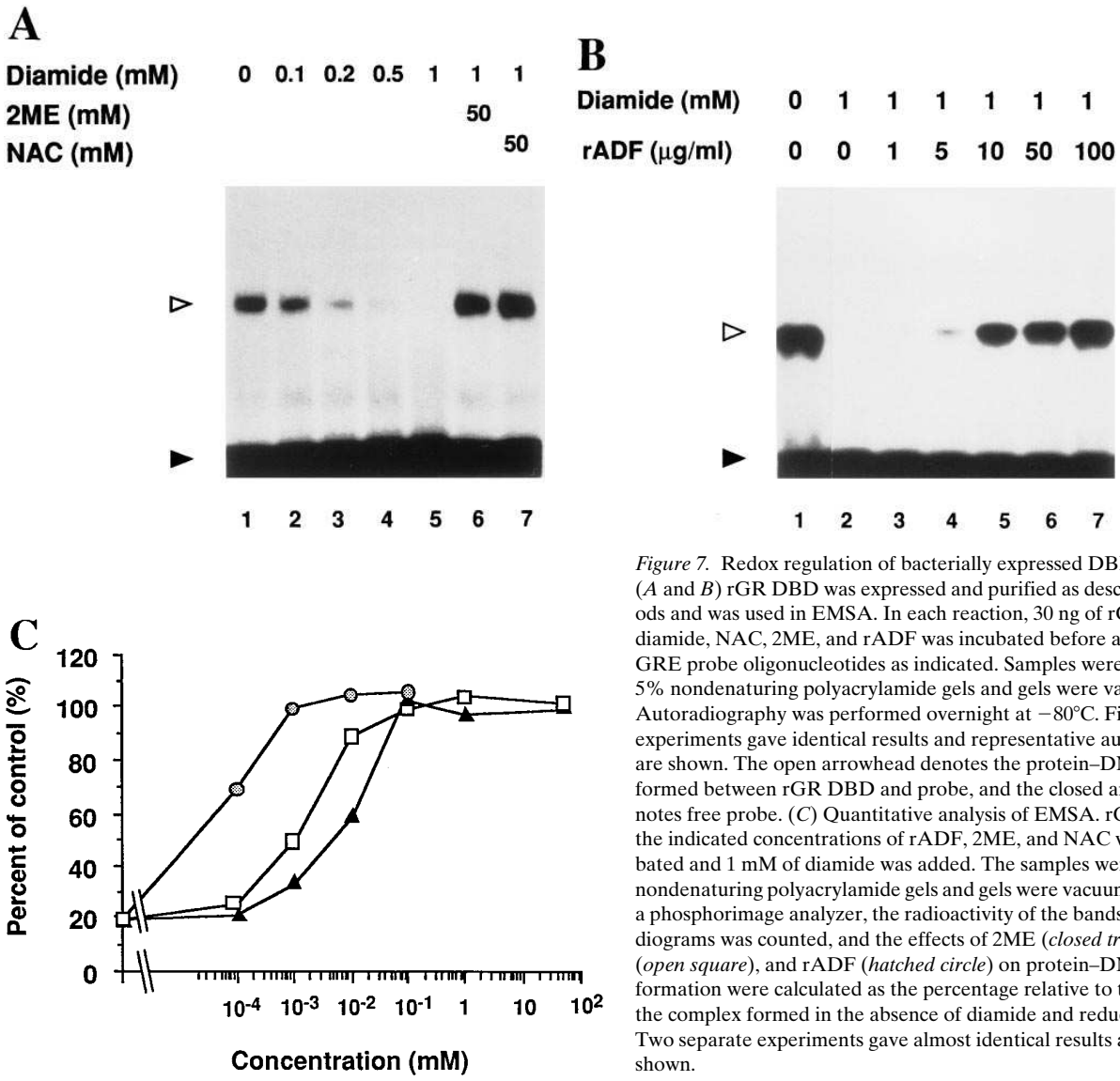

Figure 7. Redox regulation of bacterially expressed DBD of the GR. (*A* and *B*) rGR DBD was expressed and purified as described in Methods and was used in EMSA. In each reaction, 30 ng of rGR DBD and diamide, NAC, 2ME, and rADF was incubated before addition of the GRE probe oligonucleotides as indicated. Samples were analyzed on 5% nondenaturing polyacrylamide gels and gels were vacuum-dried. Autoradiography was performed overnight at −80°C. Five separate experiments gave identical results and representative autoradiograms are shown. The open arrowhead denotes the protein–DNA complex formed between rGR DBD and probe, and the closed arrowhead denotes free probe. (*C*) Quantitative analysis of EMSA. rGR DBD and the indicated concentrations of rADF, 2ME, and NAC were preincubated and 1 mM of diamide was added. The samples were run on 5% nondenaturing polyacrylamide gels and gels were vacuum-dried. Using a phosphorimage analyzer, the radioactivity of the bands of the autoradiograms was counted, and the effects of 2ME (*closed triangle*), NAC (*open square*), and rADF (*hatched circle*) on protein–DNA complex formation were calculated as the percentage relative to the amount of the complex formed in the absence of diamide and reducing agents. Two separate experiments gave almost identical results and means are shown.

agent diamide, DNA binding of rGR DBD was decreased in a concentration-dependent fashion (Fig. 7 *A*), which was restored by copresence of either 2ME or NAC (Fig. 7 *A*). rADF also squelched the negative effect of diamide on DNA binding of rGR DBD in a dose-dependent manner (Fig. 7 *B*). Quantitative analysis of the protective effects of those reducing reagents on the DNA binding activity of rGR DBD revealed that rADF is the most potent, and followed by NAC and 2ME. rADF is shown to be stronger than NAC and 2ME by ~ 200 times, when the concentrations for 50% recovery are compared (Fig. 7 *C*). After the DNA binding activity was completely restored by 2ME or NAC, rADF did not cause an additional increase in the formation of rGR DBD–GRE complex (data not shown).

Discussion

We here presented evidence showing that oxidative stress induces TRX expression, and glucocorticoid-inducible gene expression is coordinately modulated by cellular redox state and TRX level in mammalian cells. Moreover, we also indicated that redox modulation of glucocorticoid hormone action is due to thiol-mediated modification of distinct domains of the GR in situ.

We showed that treatment of HeLa cells with H_2O_2 induced TRX mRNA expression and increased TRX protein levels. Previously, we reported similar phenomena in keratinocytes and lymphoid cells (41), indicating that TRX is a ubiquitously inducible protein when cells are exposed to H_2O_2. Although TRX has been known to be involved in many cellular processes as a potent endogenous thiol-related reducing factor, critical target molecule of TRX in mammalian stress response remains largely unknown. Recently, it has been shown that expression of antisense TRX in human carcinoma cells results in reduction of cellular TRX levels and is associated with increased sensitivity to *cis*-diamminedichloroplatinum (II) and other superoxide-generating drugs, and H_2O_2 (38). Therefore, it is suggested that TRX plays an important role in cellular defense against various oxidative stresses. In this study, we further demonstrated that, through transient expression of anti-

sense TRX and overexpression of TRX, cellular TRX levels are closely associated with glucocorticoid-mediated gene expression. Since cellular treatment with H_2O_2 impairs glucocorticoid-inducible gene expression, we may speculate that the TRX system operates as an endogenous defense machinery for the GR, or glucocorticoid-mediated stress responses, against oxidative stress.

What is the molecular mechanism of TRX action on glucocorticoid-mediated gene regulation? Previous biochemical studies have revealed that TRX is required for generating the ligand-binding conformation of the GR. Antibody-mediated sequestration of either TRX or TRX reductase inhibited the ligand binding activity of the GR (32). We demonstrated that overexpression of TRX overcame the H_2O_2-mediated suppression of the ligand binding activity of the GR, thus, TRX may participate in the maintenance of the LBD function in vivo as well. On the other hand, the DBD of the GR involves cysteine cluster (see Introduction). Therefore, it is highly likely that redox manipulation affects DBD function independently from LBD function. In this line, using various GR expression plasmids, we showed clearly that not only the LBD but also the DBD is independently influenced by treatment with H_2O_2 and/or overexpression of TRX (Fig. 6). Consistently, the DNA binding activity of the rGR DBD was decreased by the thiol-oxidizing reagent diamide and replenished by rADF in vitro. The reactivity of a thiol group is determined by its accessibility and the microenvironment within the protein, including adjacent amino acids (42, 43). Both the LBD and the DBD of the GR, when they are oxidized, may have such a structure that is sterically suitable for efficient access of TRX. On the other hand, a nuclear redox factor Ref-1 rather specifically associates with the transcription factor AP-1 (44), suggesting the presence of steric preference or specificity in the interaction between the transcription factors and cellular antioxidant proteins. Thiol groups with metal associations, like the constituent of zinc finger structures, are suggested to be the strongest nucleophiles in the cell (42, 43). The other members of the nuclear receptor superfamily, which have highly conserved zinc finger structures in the DBD (13, 45), thus, could communicate with TRX as well. Recently, the solution structure of a complex of NF-κB p50 subunit and TRX was documented and specificity requirements were suggested for TRX-catalyzed disulfide bond reduction of target proteins (46). Therefore, elucidation of a mixed disulfide intermediate on the reaction pathway between the GR and TRX would definitely develop our understanding of the redox regulation of the GR.

Thus, the GR (or, steroid hormone receptors) appears to be the only transcription factor that the separate domains of the molecule (i.e., LBD and DBD) are independently redox-regulated. The DBD of the GR is postulated to be sterically hindered in untransformed unliganded GR (21 and references therein). When cells are exposed to severe oxidative stress, ligand binding, which is believed to be the initial receptor function, may be rapidly repressed and unliganded GR may harbor in the cytoplasm even in the presence of the agonistic ligands (Okamoto, K., and H. Tanaka, manuscript in preparation). On the other hand, liganded GR, in which the DBD is sterically accessible to DNA, may also be functionally downregulated via oxidative modulation of the zinc finger structure. These phenomena appear to be rationale, for example, at the inflammatory tissues where cells are believed to be exposed to severe oxidative stress (5–7), since suppression of glucocorticoid action may potentiate endogenous defense mechanisms and prevent premature termination of the cascade of inflammatory reactions for self defense. Increase in cellular TRX levels induced by oxidative stress, then, may restore the receptor activity and permit the GR to efficiently communicate with target genes. Resultant activation of antiinflammatory genes and/or repression of inflammatory genes may prevent overshoot of inflammation. This scenario would be relevant as one of the molecular mechanisms of general adaptation syndrome that was originally postulated by H. Selye (1, 2).

The harmonization of the HPA axis–mediated stress response and cellular defense system is first reported in restraint rats, in which adrenocorticotropin induced HSP70 expression in adrenal cortex (47). At the cellular level, heat shock and other metabolic stressors induce the synthesis of a set of HSPs, which interact with other cellular proteins and are considered to aid in the maintenance of cellular homeostasis. Indeed, restraint stress also induces HSP89, a rat homologue of human HSP90, which, as well as HSP70, is known to be associated with the GR in the cytoplasm (13, 14). Since HSP90 is believed to be essential for functional maintenance of the GR (48), restraint stress, through the induction of HSPs, may preserve tissue glucocorticoid sensitivity. On the other hand, we demonstrated here that increased TRX expression under oxidative stress may be important for conservation of glucocorticoid-mediated gene expression. Therefore, distinct stressors may, directly or indirectly, activate various cellular processes, some of which may converge onto eventual preservation of peripheral sensitivity to glucocorticoids.

In summary, we showed here that cellular glucocorticoid responsiveness is coordinately modulated by redox state and TRX level and we propose that cross talk between endogenous control of stress responses and cellular antioxidant defense systems may be essential for mammalian homeostatic regulation.

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