Biochemical Pharmacology, Vol. 56, pp. 79–86, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00121-X

Restoration of the Glucocorticoid Receptor Function by the Phosphodiester Compound of Vitamins C and E, EPC-K1 (L-Ascorbic Acid 2-[3,4-Dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2*H*-1-benzopyran-6-yl Hydrogen Phosphate] Potassium Salt), via a Redox-dependent Mechanism

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ABSTRACT. We examined the effect of the novel antioxidant EPC-K1 (L-ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2*H*-1-benzopyran-6-yl hydrogen phosphate] potassium salt) on glucocorticoid receptor function. We used cloned CHOpMTGR cells in which human glucocorticoid receptor cDNA was stably transfected and the glucocorticoid receptor was expressed at high levels. We recently suggested that glucocorticoid-mediated gene expression is modulated via the cellular redox state [Makino *et al.*, *J Clin Invest* **98:** 2469–2477, 1996]. In the present study, this issue was clearly evidenced by the finding that cellular treatment with H_2O_2 decreased the ligand binding and transcriptional activity of the glucocorticoid receptor, and we showed that these inhibitory effects of H_2O_2 were effectively titrated by the addition of EPC-K1. Moreover, DNA-binding activity of the bacterially expressed DNA-binding domain of the glucocorticoid receptor was repressed by the thiol-oxidizing reagent diamide; EPC-K1 also counteracted this repressive effect of diamide. Thus, the redox state was indicated to influence glucocorticoid receptor function at various steps, and EPC-K1 may be useful in restoring the cellular glucocorticoid-responsiveness in oxidative conditions. BIOCHEM PHARMACOL **56**;1:79–86, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. steroid hormones; transcription; nuclear receptors; redox; inflammation

Glucocorticoids are the most widely used compounds as treatment for the control of a number of inflammatory or immune diseases and act by binding to their specific intracellular receptor, GR† [1]. The GR is a member of the nuclear receptor superfamily, and, as well as other members of the superfamily, has a modular structure consisting of a central DBD, an LBD, and several regions for transactivation [2, 3]. Moreover, the GR contains two distinct nuclear localization signals that can function when fused to heterologous proteins to target them to the nucleus in a hormone-independent and -dependent manner, respectively [4]. Upon binding hormone, a conformational change of the receptor occurs, and the ligand–receptor complex translocates to the nucleus and binds to palindromic DNA sequences, termed as GREs, exclusively as homodimers [2, 3]. After binding to DNA, the GR is believed to communicate with the basal transcriptional machinery, interacting with or without other transcription factors and coactivators/corepressors, and then differentially regulating target gene expression [2, 3, 5]. Therefore, the pharmacological action of glucocorticoid agonists and antagonists is determined by, at least, ligand-based selectivity, receptor-based selectivity, and effector site-based selectivity [6]. Moreover, we have suggested recently that post-translational modification of the GR by metal ions and the cellular redox state is another important mechanism for fine-tuning of glucocorticoid action, most likely through the reversible modification of the sulfhydryl groups of the receptor [7–9]. Sulfhydryl groups in the GR are included as part of the cysteine residues [2]; e.g. the human GR contains 20 cysteine residues, concentrated in the central region spanning the DBD and LBD [2], and several, but not all, cysteine residues in each domain have been shown to be required for essential receptor functions [10–13]. It has been demonstrated that the ligand-binding activity of the

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†*Abbreviations:* DBD, DNA binding domain; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; GR, glucocorticois receptor; GRE, glucocorticoid response element; and LBD, ligand binding domain.

Received 29 July 1997; accepted 26 February 1998.

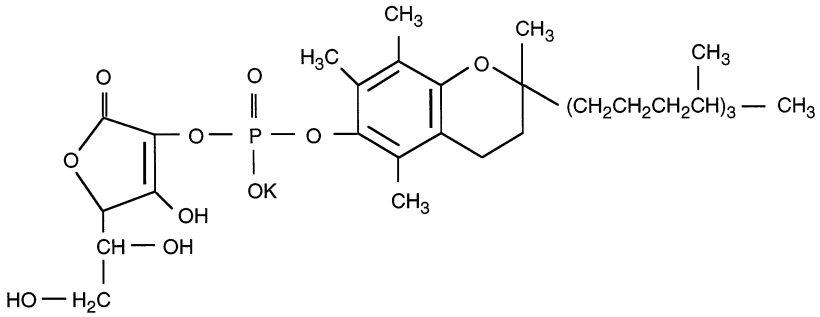

FIG. 1. Structure of L-ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2*H*-1-benzopyran-6-yl hydrogen phosphate] potassium salt (code name EPC-K1).

L-ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-*2H*-1-benzopyran-6-yl hydrogen phosphate] potassium salt

$C_{35}H_{56}O_{10}PK$: MW 706.90

GR is determined by the absence or presence of intramolecular disulfide between a vicinally spaced pair of cysteine thiol groups lying in the LBD [10]. Cysteine residues located in the DBD are components of the zinc finger structure, which is required for recognition and stable interaction with the target DNA [11]. Crystallographic analysis of the DBD–DNA complex has demonstrated that those cysteine residues are coordinating zinc atoms and have critical and direct roles in the receptor–DNA interaction [12]. Conversion of sulfhydryls in the DBD to disulfides blocks the receptor binding to DNA cellulose [13], and metal ions that have high affinity to thiol interfere with the interaction between the GR and GRE [8]. Thus, modification of the thiols in the GR, for example by antioxidants, appears to be a realistic method for pharmacological modulation of glucocorticoid action.

The novel bipolar compound EPC-K1 represents a phosphodiester linkage of α-tocopherol and ascorbic acid (Fig. 1), both of which are known to be radical scavengers [14, 15], and it has been shown to have potent hydroxyl radical-scavenging activity, as determined by electron spin resonance spectrometry [16]. Moreover, this compound inhibits lipid peroxidation and phospholipase A_2 activity *in vitro* [17]. This radical scavenging activity has prompted various clinical applications of this drug, and a number of animal studies have been conducted; for example, EPC-K1 has been shown to ameliorate tissue injury during and after cerebral ischemia [18, 19] and to show cardioprotective effects in ischemic myocardium [20].

In the present study, we confirmed that the ligand-binding activity, DNA-binding activity, and transactivation function of the GR are strictly regulated in a redox-dependent fashion, and showed that EPC-K1 potentiates GR function in oxidative conditions.

MATERIALS AND METHODS

Plasmids

The expression plasmids for wild-type and mutant GR, RShGRα, I550, and Δ9-385, were provided by Dr. R. M. Evans, Salk Institute, [21]. The glucocorticoid-responsive reporter plasmid pGRE-Luc, in which the firefly luciferase expression is driven under the control of a tandem repeat of GREs, was described elsewhere [22]. The β-galactosidase expression plasmid pCH110 (Pharmacia LKB Biotechnology) was used as an internal control for transfection efficiency.

Reagents

EPC-K1 (L-ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2*H*-1-benzopyran-6-yl hydrogen phosphate] potassium salt) was synthesized and provided by the Senju Pharmaceutical Co. Ltd. Dexamethasone was purchased from the Sigma Chemical Co. Other chemicals were from Wako Pure Chemical, unless otherwise specified.

Cell Culture

The GR-expressing Chinese hamster ovary cell line CHOpMTGR was described elsewhere and was provided by Dr. Stefan Nilsson (Karo Bio) [23]. The cells were maintained in Ham's F-12 medium (Life Technologies, Inc.) supplemented with antibiotics and 10% FBS (Life Technologies, Inc.) in the presence of cadmium and zinc ions, both at a concentration of 40 μM. One day before each experiment, the medium was replaced with Ham's F-12 medium supplemented with antibiotics and 10% FBS. COS7 cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with antibiotics and 10% FBS. In all experiments, serum steroids were stripped with dextran-coated charcoal, and cells were cultured in a humidified atmosphere at 37° with 5% CO_2.

Transfection

Cells were transiently transfected by means of a Lipofectin procedure essentially as described elsewhere [7–9].

Reporter Gene Assay

Cells were plated in 6-well plastic culture dishes to 30–50% confluency and washed with PBS three times; the medium was then replaced with Opti-MEM. A plasmid mixture containing 5 μg of pGRE-luc, with 10 ng of each GR expression plasmid when indicated, was combined with 10 μL of Lipofectin reagent and added to the culture. After 12 hr of incubation, the medium was replaced with fresh medium supplemented with 2% dextran-coated charcoal-stripped FBS, and the cells were cultured further in the presence or absence of various ligands for 24 hr. After normalization of transfection efficiency by β-galactosidase expression, luciferase assay was performed as described previously [9], and results were expressed as fold induction versus appropriate control.

Hormone-Binding Assay

CHOpMTGR cells were grown in 12-well flat-bottom plastic plates (IWAKI Glass) until confluency, and the medium was replaced with Opti-MEM. After washing twice with PBS and once with Opti-MEM, the cells were incubated with 20 nM of [^{3}H]dexamethasone (35–50 Ci/mmol, DuPont/NEN) in the presence or absence of a 500-molar excess of radioinert dexamethasone 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 of 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate and 1% SDS. Aliquots were added to scintillation fluid to determine bound radioactivity [24]. All assays were performed in triplicate, and the difference between total and nonspecific binding was considered specific and used as the GR number in this study.

Western Immunoblot Analysis

Western immunoblot analysis of the GR was performed as described previously [24]. Briefly, 10 μg of protein sample was fractionated by 10% SDS–PAGE. After electrophoresis, proteins were electrically transferred to a polyvinylidene fluoride membrane (BioRad) and probed with the anti-human GR antibody PA1-512 (Affinity Bioreagents). Antigen–antibody complexes were detected by an ECL detection system (Amersham) according to the manufacturer's instructions.

Bacterial Expression and Purification of the DBD of the GR

The recombinant protein spanning the DBD of the GR was prepared as described previously [9]. Briefly, the DNA fragment encoding 111 amino acids (398 Met–508 Ser) of the human GR was amplified by polymerase chain reaction with appropriate flanking sequences for enzymatic cleavage and subcloned into a pMal™-c2 expression plasmid (New England BioLabs, Inc.). Subsequent bacterial expression using *Escherichia coli* and the purification of the recombinant DBD of the GR were performed according to the manufacturer's recommendation.

DNA Binding Assay

EMSA was carried out as described previously [7–9]. Briefly, double-stranded oligonucleotide probes encompassing the GRE were end-labeled with [α-^{32}P]dCTP (Amersham) using the Klenow fragment of DNA polymerase I (TaKaRa). Fifteen nanograms of the recombinant DBD of the GR was incubated with 0.2 ng of ^{32}P-labeled GRE probe (approximately 20,000 cpm) in a 10-μL reaction mixture containing 10 mM of HEPES, pH 7.5, 50 mM of KCl, 0.1 mM of EDTA, 2.5 mM of $MgCl_2$, 0.25 mM of dithiothreitol, 10% glycerol, and 10 ng of poly(dI-dC) (Pharmacia LKB Biotechnology) 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 1× Tris–borate–EDTA buffer (50 mM of Tris, 50 mM of boric acid, and 1 mM of EDTA). The gels were run at 300 V for 2 hr and then dried. Results were visualized by autoradiography. The sequences of the oligonucleotides for the GRE probe and for AP-1 and NF-κB used as nonspecific competitors were the following (upper strand is shown): GRE, 5′-CGAGTAGCTAGAACAGACTGTTCTGAGG-3′ [9]; AP-1, 5′-TAAAAAAGCATGAGTCAGACACCTGAGCT-3′ [25]; and NF-κB, 5′-CTCGAGTTGAGGGGACTTTCCCAGGCG-3′ [26].

RESULTS

Effect of EPC-K1 on the Glucocorticoid-inducible Gene Expression

In the present study, using recently developed experimental systems [9], we examined the effects of EPC-K1 on GR function. First, we determined the effect of EPC-K1 on the glucocorticoid-inducible gene expression in transient transfection experiments by transfecting the glucocorticoid-responsive reporter plasmid pGRE-Luc into GR-expressing CHOpMTGR cells. Treatment of the cells with 100 nM of dexamethasone produced an approximately 170-fold induction of cellular luciferase activity (Fig. 2). Addition of H_2O_2 compromised the hormone-induced response of the reporter gene expression in a concentration-dependent fashion (Fig. 2). Although EPC-K1 alone did not affect dexamethasone-induced luciferase expression, H_2O_2-mediated repression was titrated effectively by EPC-K1, and the hormone induction response was recovered (Fig. 2). Western immunoblot experiments done in parallel revealed that GR protein levels were almost constant in the transfection assays (data not shown). Thus, glucocorticoid-inducible gene expression was modulated by the cellular redox state, and EPC-K1 efficiently preserved the transactivational function of the GR under oxidative conditions. This up-regulatory effect of EPC-K1 was decreased at high

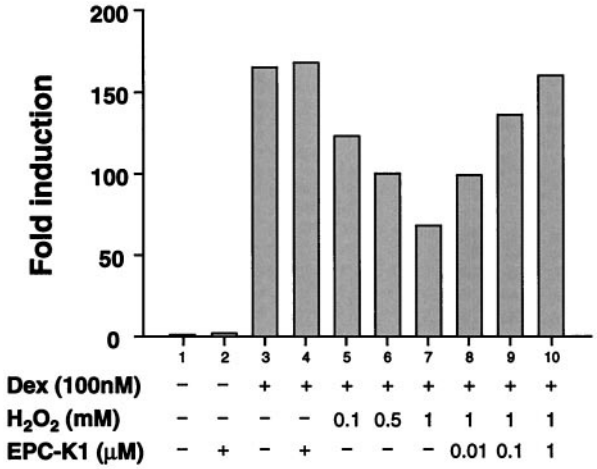

FIG. 2. Effects of EPC-K1 on the transactivational function of the GR. CHOpMTGR cells were transfected with 5 μg of pGRE-Luc and 2 μg of β-galactosidase expression vector pCH110 by the lipofection procedure described in Materials and Methods. The cells were treated with dexamethasone (Dex), H_2O_2, and EPC-K1 for 24 hr as indicated, and the cell extracts were prepared for luciferase assay. Results were normalized to the transfection efficiency (as determined from β-galactosidase activity). Two independent experiments gave almost identical results.

concentrations of EPC-K1 (e.g. 10 μM), most likely due to impairment of cell viability (data not shown).

Effect of EPC-K1 on the Ligand Binding Activity of the GR

We also examined the effect of EPC-K1 on the ligand binding activity of the GR in CHOpMTGR cells. The cells were cultured in the absence or presence of H_2O_2 and EPC-K1 for 12 hr as indicated, and the number of specific binding sites for dexamethasone was determined. We demonstrated that the ligand binding activity of the GR was not altered markedly after treatment of the cells with EPC-K1 alone, but was decreased to approximately 75% of the control level after treatment with 1 mM of H_2O_2 (Fig. 3A). Addition of EPC-K1 partially counteracted H_2O_2-mediated suppression of the ligand-binding activity (Fig. 3A), whereas cellular GR protein levels were almost unchanged during the experiments (Fig. 3B).

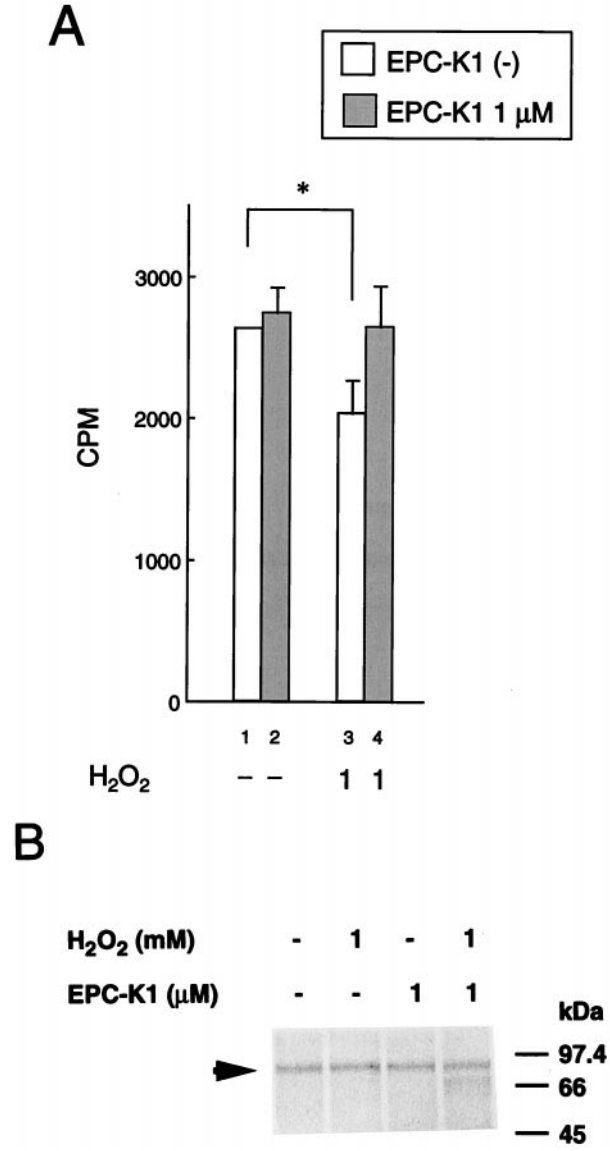

FIG. 3. Effects of EPC-K1 on ligand-binding activity and immunoreactivity of the GR. (A) CHOpMTGR cells were cultured in the absence or presence of the indicated concentration of EPC-K1 and/or 1 mM of H_2O_2 for 24 hr, and the number of specific binding sites for 20 nM of [^{3}H]dexamethasone was determined as described in Materials and Methods. The means and SD of four independent experiments are shown. Student's *t*-test was used for statistical analysis. *$P < 0.05$. (B) CHOpMTGR cells were cultured in the presence of the indicated concentrations of H_2O_2 and EPC-K1, and the protein immunoreactivity of the GR was determined in whole cell extracts (10 μg of protein) by Western immunoblot analysis using anti-human GR antibody PA1-512 as described in Materials and Methods. The arrow denotes the position of the glucocorticoid receptor, and the lines represent the position of the molecular weight markers run in parallel.

Analysis of GR-Domain Requirements for EPC-K1 Action

To test which domains of the GR mediate EPC-K1 action, we cotransfected various expression plasmids for the GR with pGRE-Luc into COS7 cells. I550, which is functionally identical to rat GR mutant VAN556 [27], is a truncated human GR mutant that lacks the LBD and acts as a ligand-independent transcriptional activator (Fig. 4A; and [21]). Δ9-385 is another human GR mutant in which the major transactivation domain is deleted and acts as a weak ligand-inducible transactivator (Fig. 4A, and [21]), just like the rat GR mutant VA407C [27]. When wild-type human GR expression plasmid RShGRα was cotransfected, both the negative effects of H_2O_2 and the positive effects of EPC-K1 were reproduced, as in the case of GR-expressing CHOpMTGR cells (Fig. 4B, also see Fig. 2). I550 stimu-

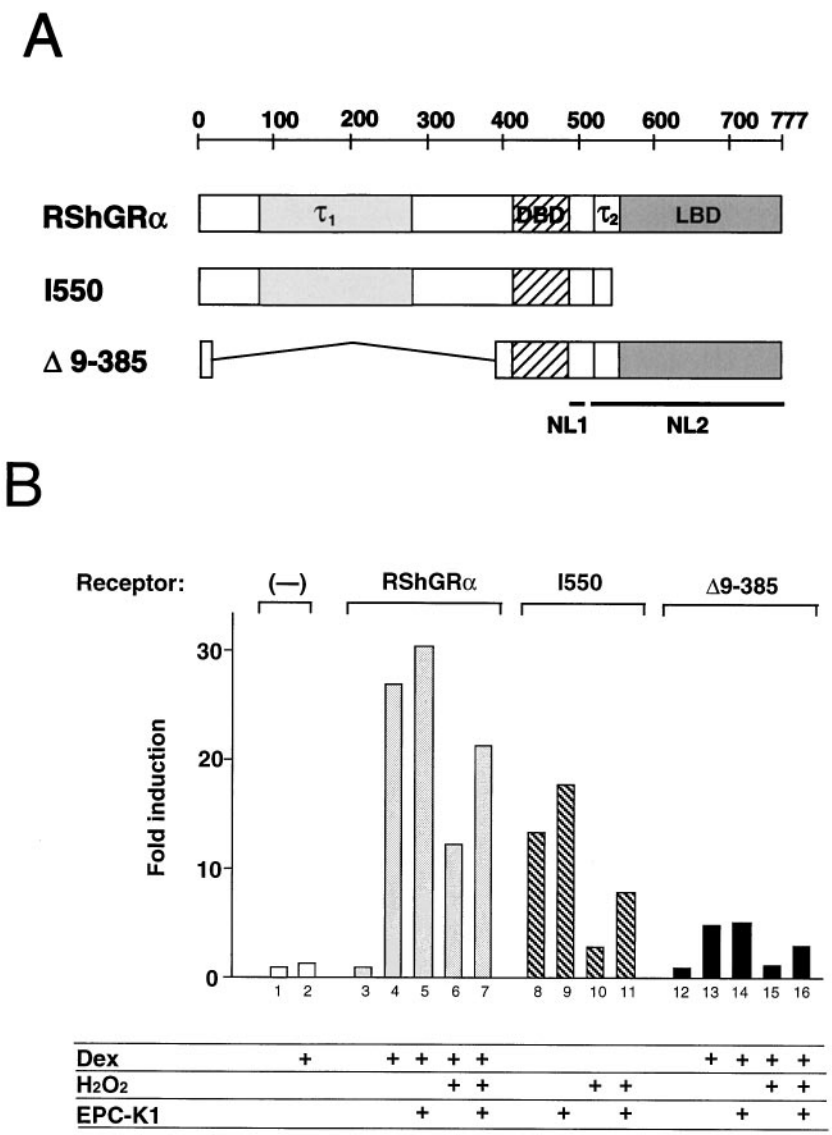

FIG. 4. Domain analysis of redox regulation of the GR and the effect of EPC-K1. (A) The expression plasmids for the GR. τ_1 and τ_2, transactivation domains; DBD, DNA binding domain; NL1 and NL2, nuclear localization signals; LBD, ligand-binding domain. (B) COS7 cells were cotransfected with each GR expression plasmid and pGRE-Luc and cultured in the absence or presence of Dex (100 nM), H_2O_2 (1 mM), and EPC-K1 (1 μM) for 24 hr as indicated. Luciferase activity was determined as described in Materials and Methods. Results are expressed as fold induction versus the luciferase activity of lane 1.

lated the reporter gene expression in the absence of the ligands, and treatment with H_2O_2 severely compromised I550 function and decreased luciferase activity by five-fold (Fig. 4B). EPC-K1 rescued I550 function and completely restored the induction of the reporter gene expression (Fig. 4B). When Δ9-385 was expressed, treatment with dexamethasone weakly induced luciferase expression, but mutually exclusive effects of H_2O_2 and EPC-K1 were observed as well (Fig. 4B). Because both the DBD and NL1 contain cysteine residues, it is possible that each or both domains could be a direct target of H_2O_2 treatment.

Effects of EPC-K1 on DNA-Binding Activity of the GR In Vitro

EMSA using the recombinant GR DBD protein showed a single class of specific DNA–protein complex formation with the oligonucleotide probe carrying the GRE (Fig. 5A). Preincubation of the recombinant GR DBD protein with a thiol oxidizing reagent, diamide, decreased the DNA binding activity, and 0.1 and 1 μM of EPC-K1 restored the formation of the complex formed between the recombinant GR DBD and target DNA sequences in a concentration-dependent manner (Fig. 5B).

DISCUSSION

It has become clear recently that eukaryotic gene expression is modulated by changes in the cellular redox state (reviewed in [28] and [29]), and this redox-dependent regulation of gene expression is now considered important in cellular homeostatic processes. Moreover, alteration in tissue and/or cellular redox state, which often affects various transcription factor functions, has close association with a variety of disease processes, e.g. inflammation [30], immune reaction [31], atherosclerosis [32, 33], diabetes mellitus [34, 35], and neoplasm [36, 37]. As well as other transcription factors, the zinc finger transcription factor GR could also be a prime target for redox regulation since the

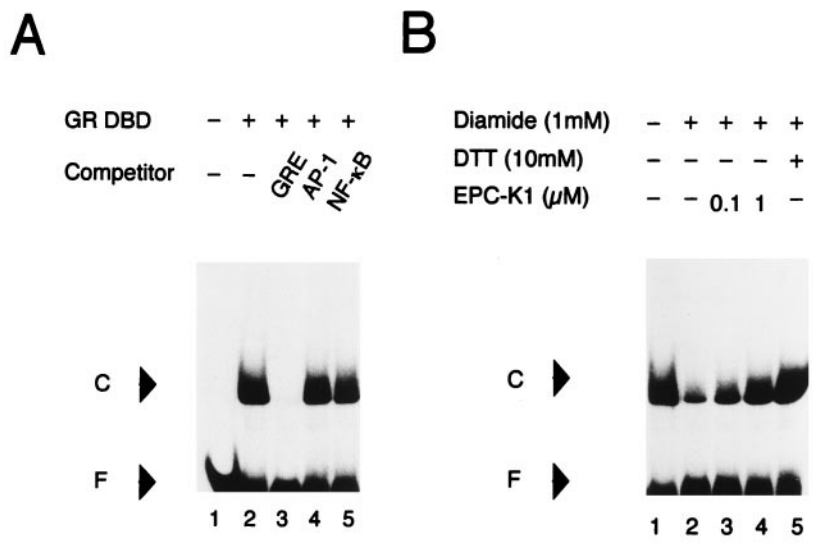

FIG. 5. Effects of EPC-K1 on DNA-binding activity of the GR *in vitro*. The recombinant DNA-binding domain of the GR (GR DBD) was incubated with ^{32}P-labeled oligonucleotide probe encompassing the GRE in the presence of the indicated concentrations of EPC-K1. A 20-fold molar excess of specific (GRE) or nonspecific (AP-1, NF-κB) competitor oligonucleotides (panel A), diamide, dithiothreitol (DTT), and EPC-K1 (panel B) were included in the reaction mixture as indicated. The formation of protein–DNA complexes was examined by EMSA as described in Materials and Methods. C, protein–DNA complex; and F, free probe.

GR contains essential cysteine residues as part of the LBD and DBD (see Introduction). Because the GR is believed to be a unique molecule to transmit anti-inflammatory action of glucocorticoids in inflammatory and/or immune-mediated disorders [1], redox regulation of the GR could be of vital importance in glucocorticoid pharmacology and clinical medicine. Here we showed that glucocorticoid-inducible gene expression is suppressed under oxidative stress conditions, and that the novel antioxidant EPC-K1 rescues this oxidant-mediated repression of the receptor function.

Ligand binding to the GR triggers the cascade of glucocorticoid-mediated signal transduction that is governed by several distinct domains of the receptor but is inactive in the absence of ligands. Thus, ligand–receptor interaction could be a prime regulatory site in redox regulation of the GR function. Because GR protein levels were almost constant during treatment with H_2O_2 and EPC-K1, the observed alteration in GR ligand-binding activity is strongly suggested to be due to functional modulation of the receptor. Vicinally spaced pairs of thiols have been shown previously to be redox-regulated in the LBD, and sulfhydryl-reducing reagents restore the ligand-binding activity [10]. Treatment of cells with H_2O_2 generates hydroxyl radicals in the cytoplasm and may interfere with the interaction between glucocorticoids and the GR, most possibly via oxidative modification of the LBD. Thus, subsequent receptor transformation and nuclear translocation may be repressed (K. Okamoto *et al.*, unpublished observations).

Because ligand binding is considered to unmask the DBD, we may consider that the DBD function could be independently controlled via oxidoreductive modification. Indeed, a zinc finger transcription factor, Sp1, has been shown to be redox-regulated [38]. It has been shown that the transactivation functions of I550 and Δ9-385 are smaller than that of wild-type GR [39] (also see Fig. 4B). In clear contrast, however, the effect of treatment with H_2O_2 was more prominent in those mutant GR than in wild-type GR (Fig. 4B), strongly indicating that at least the central DBD is one of the potential targets of oxidative modification. As well as the rat GR mutant VAN556 [27], I550 has been shown to be constitutively localized in the nucleus [40]. Thus, our findings may suggest that redox regulation of the GR occurs independently in the nuclear compartment (Y. Makino *et al.*, unpublished observations). The findings from EMSA further supported this issue, indicating the direct interaction between the DBD of the GR and EPC-K1. On the other hand, at this moment we cannot rule out the possibilities that oxidative conditions influence the dimerization and function of the N-terminal transactivation domain of the GR.

Antioxidant EPC-K1 quenches the negative effects of treatment with H_2O_2 on GR function, as an endogenous reducing catalyst, thioredoxin [9]. Because both EPC-K1 [11] and thioredoxin [9] show radical scavenging activity, both reagents may share a common mechanism in quenching generated oxygen radicals and preserving GR function. Alternatively, we may speculate that EPC-K1 replenishes endogenous cellular reducing systems, i.e. thioredoxin, and indirectly replenishes GR activity. Further experiments are needed to elucidate the molecular target of EPC-K1.

The development of drugs that restore GR function may be beneficial in achieving efficient anti-inflammatory action of glucocorticoids in patients with, for example, inflammatory diseases. EPC-K1, being a water-soluble antioxidant/radical scavenging compound, may be a potential pharmacological modifier of the GR *in situ*. On the other hand, it is also known that glucocorticoids play an important role in the maintenance of the hypothalamic-pituitary-adrenal axis and the regulation of hippocampal neurons to modulate long-term memory [41–43]. Because ischemia–reperfusion injury is known to cause significant oxidative stress in the brain [44], EPC-K1 might ameliorate brain dysfunction and/or damage after cerebrovascular accidents [19, 20]. Therefore, we conclude that EPC-K1 potentiates GR function in an oxidative state and may be useful in

treating inflammatory diseases and preserving brain function in patients with cerebrovascular diseases. Indeed, clinical trials to investigate the effect of EPC-K1 on acute vascular accidents in the brain are now in progress in Japan.

We thank Drs. R. M. Evans (Salk Institute) for plasmids, S. Nilsson (Karo Bio) for CHOpMTGR cells, and Dr. Takahiro Sakaue (Research Laboratory for Drug Discovery, Senju Pharmaceutical Co. Ltd.) for EPC-K1. This work was supported by the Japan Ministry of Education, Science, Sports and Culture, the Japan Ministry of Health and Welfare, the Japan Rheumatism Foundation, the Japan Research Foundation for Clinical Pharmacology, and the Akiyama Foundation.

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