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Transcriptional suppression of the adrenal cortical peripheral-type benzodiazepine receptor gene and inhibition of steroid synthesis by ginkgolide B☆

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Abstract

Treatment of rats and adrenocortical cells with ginkgolide B (GKB), a purified component of *Ginkgo biloba* leaf extracts, reduces the mRNA, protein, and ligand-binding levels of the adrenal peripheral-type benzodiazepine receptor (PBR), a mitochondrial cholesterol-binding protein, leading to decreased corticosteroid synthesis. In the Y1 adrenocortical cell line, GKB reduced both PBR levels and cyclic AMP-induced steroid formation. In these cells, GKB, but not various steroids and vitamins, reduced the expression of a reporter gene driven by the DNA sequence −624/−513 relative to the transcription start site of the *PBR* encoding gene. GKB treatment did not affect the *SV40* promoter and increased the cytochrome P450 17α-hydroxylase gene promoter driven expression of the reporter gene. Electrophoretic mobility shift assays (EMSAs) indicated the presence of a functional transcriptional element bound to the −624/−513 DNA fragment. This GKB-induced inhibition of PBR was mediated by an interaction with a transcription factor that binds to the −636/−616 *PBR*-promoter region. Deletion or mutation of this sequence eliminated the DNA–protein interaction and the inhibitory effect of GKB on *PBR* gene transcription. This DNA-binding protein could be detected in nuclear extracts of rat brain, liver, and testis, but not kidney. It is also present in the human adrenal glands. However, the inhibitory effect following GKB treatment could be seen only in the adrenal glands. These results demonstrate that the GKB-activated inhibition of glucocorticoid production is due to a specific transcriptional suppression of the adrenal *PBR* gene and suggest that GKB might serve as a pharmacological tool to control excess glucocorticoid formation.

Keywords: Adrenal cortex; Peripheral-type benzodiazepine receptor (PBR); *PBR* gene promoter; Ginkgolide B; Gene transcription; Steroid biosynthesis

1. Introduction

The PBR was originally discovered as an alternative binding site for the benzodiazepine diazepam [1]. However, in addition to benzodiazepines, the PBR also binds other classes of organic compounds with high affinity, such as the isoquinoline PK 11195 [1-(2-chlorophenyl)-*N*-(1-methyl-propyl)3-isoquinolinecarboxamide] [1]. The PBR is a multimeric receptor composed of an 18-kDa receptor protein and a 30-kDa VDAC [2,3]. The 18-kDa subunit is thought to be responsible for the binding of isoquinolines, while both subunits are required for the binding of benzodiazepines [3].

Although PBR is widely distributed in the body, it is abundant in all steroidogenic tissues [1], where it is primarily located on the outer mitochondrial membrane [4]. Detailed studies have demonstrated that PBR ligand-induced stimulation of pregnenolone synthesis was due to PBR-mediated translocation of cholesterol from the outer

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Abbreviations: CAT, chloramphenicol transferase; *Cyp17*, cytochrome P450 17α-hydroxylase; dbcAMP, N^6,2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate; DTT, dithiothreitol; EGb 761, standardized *Ginkgo biloba* leaf extract; EMSA, electrophoretic mobility shift assay; βGal, β-galactosidase; GKA, purified ginkgolide A; GKB, purified ginkgolide B; PBR, peripheral-type benzodiazepine receptor; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; and VDAC, voltage-dependent anion channel protein.

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to the inner mitochondrial membrane [5,6]. Moreover, PBR has been shown to participate in hormone-induced cholesterol transport in adrenocortical and Leydig cells [7]. In addition, targeted disruption of the *PBR* gene in Leydig cells resulted in the arrest of cholesterol transport into mitochondria, thereby blocking steroid formation; transfection of the mutant cells with PBR cDNA rescued steroidogenesis [8]. More recently, the 18-kDa PBR protein was shown to be a high-affinity cholesterol-binding protein [9,10], probably playing a more general role in intracellular cholesterol compartmentalization [11], including cholesterol transfer into the mitochondria in steroidogenic cells.

In the search for a pharmacological tool to modulate PBR expression and glucocorticoid biosynthesis, we examined the effect of the *Ginkgo biloba* extract EGb 761 and its isolated components, ginkgolides. EGb 761 has been described for its beneficial effects on memory, vigilance, cognitive functions associated with aging, dementia, and the ability to cope with daily stressors [12]. The anti-stress action of EGb 761 differs from that of classical anxiolytics or antidepressants [13]. Because of the well-known pathogenic potential of glucocorticoid excess on the hippocampus [14,15], we examined whether EGb 761 and its bioactive terpene trilactones, GKA and GKB, exert their beneficial effects by regulating glucocorticoid levels. Treatment of adult rats with EGb 761, GKA, or GKB decreases serum corticosterone levels [16]. We demonstrated that EGb 761 induced a 50% decrease of the 18-kDa PBR protein and mRNA expression, accompanied by a similar decrease in the number of adrenal PBR ligand-binding sites. No changes occurred in either renal or testicular PBR ligand-binding characteristics or protein expression. Analysis of metabolically radiolabeled proteins obtained from adult rat adrenocortical cells and treated *ex vivo* with EGb 761 or GKB confirmed the reduction of the 18-kDa protein levels identified as PBR when compared with those of untreated rats. Corticosterone production was reduced by 80% in these cells in response to adrenocorticotropic hormone (ACTH) [17]. Similarly, treatment of the H-295 human adrenal cells with GKB resulted in reduced production of cortisol levels in response to cAMP [16]. These findings suggest that GKA and GKB, components of EGb 761, exert specific effects on adrenocortical cells by inhibiting PBR mRNA and protein expression, thus, limiting the amount of mitochondrial cholesterol available to the P450scc for corticosteroid synthesis. Moreover, GKB treatment reduced the ACTH-stimulated corticosteroid production without affecting basal glucocorticoid and aldosterone formation.

The rat and human *PBR* genes have been cloned [18,19]. The *PBR* gene has four exons, and the first intron constitutes the largest portion of the gene. The first exon and intron are part of the 5′ untranslated region. The *PBR* gene is lacking the TATA box, but contains multiple consensus sequences for the transcription factor Sp1 and three unidentified transcriptional regulatory elements [20]. Two were characterized as positive and one as negative-acting elements. Considering that GKB is the only known pharmacological tool regulating PBR levels and corticosteroid synthesis *in vitro* and *in vivo*, this study sought to elucidate the mechanism(s) underlying this regulation of PBR mRNA levels.

2. Materials and methods

2.1. Materials

The purified component of the *Ginkgo biloba* standardized extract EGb 761, GKB (BN 52021), was provided by the Institut Henri Beaufour-IPSEN. [1,2-^{3}H(N)]20α-Hydroxypregn-4-ene-3-one (46.7 Ci/mmol) and [*N-methyl*-^{3}H]PK 11195 (85 Ci/mmol) were purchased from DuPont NEN. Anti-20α-dihydroprogesterone antiserum was purchased from Endocrine Sciences. dbcAMP, vitamins A (retinol), D (ergocalciferol), and E (α-tocopherol), as well as testosterone and corticosterone were obtained from the Sigma Chemical Co. PBS (pH 7.2) and cell culture supplies were purchased from GIBCO. Fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Biofluids Inc. Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F12) was supplied by Irvine Scientific. Cell culture plasticware was from Corning. Electrophoresis reagents and materials were obtained from Bio-Rad. Glass fiber GF/C filters were supplied by Whatman. All other chemicals of analytical quality were obtained from various commercial sources.

2.2. Animal protocol

Experiments were performed on male 80-day-old Sprague–Dawley rats obtained from the Charles River Breeding Laboratories. The animals were housed in groups of three at the Georgetown University Research Resources Facility under controlled light and temperature conditions. Treatment with GKB was started 5 days after their arrival at the facility in order to allow for proper acclimatization. Saline (0.09%) and GKB (2 mg/kg) were administered i.p. daily at 10:00 a.m. for 8 days. The animals were killed 24 hr after receiving the last dose. Adrenals, brain, kidneys, liver, and testis were collected from both groups, snap-frozen in dry-ice, and stored at −70°. The animal protocol was approved by the Georgetown University Animal Committee (GUACUC).

2.3. Human tissues

Human adrenals from 11- and 46-year-old males were obtained from the Maryland Brain and Tissue Bank at the University of Maryland in Baltimore. The protocol for the use of human tissue was approved by the Georgetown University Institutional Review Board.

2.4. Cell culture

Mouse Y1 adrenocortical cells are steroidogenic and exhibit high levels of mitochondrial PBR [21]. The cells were cultured in DMEM/F12 (1:1, v/v) medium containing 10% FBS and were treated with GKB for 24 hr versus the untreated control. Cells were stimulated for 24 hr with dbcAMP (1 mM). The steroidogenic index was determined by the amount of 20α-dihydroprogesterone secreted as measured by radioimmunoassay (RIA) [6].

2.5. Radioligand-binding assays

Mitochondria were prepared from Y1 adrenocortical cells, both untreated and treated with GKB for 24 hr [6]. Mitochondrial proteins (50 μg) were resuspended in PBS and [^{3}H]PK 11195. Binding studies were performed at 4° in a final incubation volume of 0.3 mL, using the radioligand in increasing concentrations ranging from 0.019 to 20 nM; a 200-fold excess of unlabeled ligand was used to determine non-specific binding [16]. The maximal binding capacity (B_{max}) and dissociation constant (K_d) were assessed by Scatchard plot analysis using the LIGAND program [22].

2.6. Transient transfection and determination of CAT reporter gene activity

Y1 cells were transfected with a construct containing a 1255-bp fragment, consisting of 36 bp of the first exon and the 1219-bp upstream sequence, of the *PBR* gene promoter. The transcriptional starting point associated with the first 36 bp of exon 1 was kept intact, thereby allowing the transcription within the pCAT-Basic plasmid (Stratagene). The consensus sequence (3 GC boxes) for the Sp1 transcription factor was maintained within the −88/+36 region. The plasmid containing the different constructs used in this study was obtained from Dr. K.E. Krueger (Georgetown University). Transfection by means of calcium phosphate co-precipitation [23] was carried out for 12 hr at 37° and 3% CO_2. The cells were cultured in 100-mm plates and used at a confluency not exceeding 10–15%. Each plate was transfected with 20 μg of plasmid DNA containing the requisite construct, together with 5 μg of the internal control plasmid pSV-βGal (*lacZ*) (Promega). The *lacZ* gene was used as an internal standard to assess the transfection efficiency. The transfection was performed according to the protocol of the manufacturer (Stratagene). Following transfection, the cells were washed with sterile PBS. Fresh 10% FBS-containing medium was added, and the cells were incubated for 20 hr to allow recovery and expression of the foreign DNA. The cells were then treated with 300 ng/mL of GKB or various vitamins (A, D, and E) and steroids (corticosterone and testosterone) and incubated for 12 hr, after which they were washed twice with PBS supplemented with 1 mM PMSF, scraped, and harvested in a small volume. Y1 cells were also transiently transfected with the pCAT plasmid containing the *PBR* gene promoter insert bearing two deletion mutations (−626/−616; −636/−616). This was performed by Retrogen Laboratories. In brief, the gene was modified using the PCR amplification method in conjunction with Retrogen Laboratories proprietary protocols. Both strands of the gene in the region of the mutation sites were sequenced using an ABI 377 automated sequencer. The autoassembler program was used to compare the sequences obtained. The analysis showed 100% homology to the known *PBR* gene sequence. In separate experiments, Y1 cells were transfected with the 4500-bp promoter of the *Cyp17* gene fused with the *CAT* reporter gene [24]. In these experiments, cells were transfected with 100 mg plasmid DNA by electropermeabilization carried out using Gene Pulser® II (Bio-Rad Laboratories) at 240 V and 900 μF. After a period of 36 hr, the cells were treated with GKB as described above. dbcAMP (1 mM) was added for 12 hr to stimulate the cells transfected with *Cyp17* gene promoter. The CAT ELISA was performed on crude cellular lysates according to the protocols provided by the manufacturers (5′ → 3′, Inc. and Roche Molecular Biochemicals). βGal quantification was assessed according to the protocol of Promega based on a spectrophotometric method. The relative CAT activity was calculated by dividing the total CAT activity per dish by the total βGal activity per dish. In addition, promoterless pCAT-Basic and pCAT-control vectors were used to estimate CAT specific activities. The experiments were repeated at least five times, each time in quadruplicate, and results represent the means ± SEM (N = 20–25).

2.7. Preparation of nuclear extracts

The method developed by Andrews and Faller [25] based on the classical protocol published by Dignam *et al.* [26] was followed to prepare nuclear extracts from Y1 cells. In brief, 150-mm culture plates either were treated for 12 hr with GKB in the presence of serum, or the cells were first deprived of serum for 20 hr and then induced for 2 hr with the drug. Y1 adrenocortical cells, untreated or treated with GKB in the presence or absence of serum, were subjected to hypotonic lysis followed by high salt extraction. Each plate was rinsed with PBS and harvested in 600 μL of cold buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF at pH 7.9). The cells were incubated on ice for 10 min to allow swelling, then mixed, and centrifuged for 10 sec. The pellets were each resuspended in 50 μL of buffer C (10 mM HEPES, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF at pH 7.9) and incubated on ice for 20 min. Following centrifugation for 2 min, the supernatants were recovered, aliquoted, and immediately frozen on dry ice. Aliquots were stored at −80° until further use.

A different protocol was used for the preparation of nuclear extracts from rat tissues and human adrenal glands.

Rat tissues were collected as described above. Adrenal glands from an 11-year-old and a 46-year-old male were provided by the University of Maryland Brain and Tissue Bank. Nuclear proteins were prepared according to the method described by Blough and colleagues [27]. In brief, tissues (200 mg) were minced in 35 mL of ice-cold buffer A (10 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 5 mM KCl, 0.1 mM EDTA, pH 8, and 0.1% Triton® X-100), supplemented with 1 mM DTT, 0.1 mM PMSF, aprotinin (2 μg/mL), and leupeptin (2 μg/mL), and homogenized using an Ultra Turrax. Samples were centrifuged for 5 min at 3000 *g* in a swinging rotor at 4°. The individual pellets were resuspended in 0.5 mL of ice-cold buffer B (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 25% glycerol, 500 mM NaCl, 0.2 mM EDTA, pH 8) supplemented with 0.5 mM DTT, 0.2 mM PMSF, aprotinin (2 μg/mL), and leupeptin (2 μg/mL). After 30 min of incubation on ice with intermittent mixing of the suspensions, the samples were centrifuged for 5 min at 3000 *g* at 4°. The supernatants were recovered and transferred to 4-mL Ultrafree® filter units with a 5000 nominal molecular weight limit (Millipore). An equal volume of buffer C was added (20 mM HEPES, pH 7.9, 2 mM $MgCl_2$, 40 mM KCl, 10% glycerol) supplemented with the anti-protease mixture. The samples were subjected to centrifugation at 4500 *g* at 4° in a fixed-angle rotor for 30 min. Following the addition of 0.5 mL of buffer C, the samples were centrifuged again. The nuclear protein mixtures were aliquoted and stored at −80°.

2.8. DNA-binding activity as monitored by EMSA

Double-stranded oligonucleotides were end-labeled with [γ-^{32}P]ATP (10 μCi/μL) using T4 polynucleotide kinase (10 U). The mixture was incubated for 30 min at 37° and then applied to a G-25 prepacked column to purify the labeled specific probe. In 20 μL of final mixture, nuclear extracts (15 μg) were incubated with [poly(dI-dC)·poly(dI-dC)] (2 μg) and labeled DNA-probe (1 ng, 1×10^5 cpm) in 50 mM Tris–HCl, pH 7.9, supplemented with 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 20% glycerol. The incubation was carried out for 30 min at room temperature. For competition assays, the unlabeled probe was added to the mixture mentioned above at 100-fold excess [20]. DNA–protein complexes were analyzed by electrophoresis using either 4% polyacrylamide gels (38:2 acrylamide:bisacrylamide) or gradient gels, ranging from 4 to 15%. TBE buffer (0.25×) was used for electroelution (1× TBE: 50 mM Tris-borate, pH 8.3, 1 mM EDTA). Electrophoresis, using 10× 10 cm or 20× 20 cm gels (Novex), was performed for 3 or 6 hr, respectively, at 4°. Gels were then exposed to X-ray film.

2.9. PCR and generation of the different double-stranded oligonucleotides

We generated four pairs of oligonucleotides of different size within the −624/−513 region (−656/−500 including the primers) labeled A and B for forward and reverse primers, respectively:

A, 5′-CCTGGGAGTTGTGGTGTAC-3′	B, 5′-CACGGATCCCGGAATGATG-3′
A, 5′-TTGTGGTGTACAGTTATAATCC-3′	B, 5′-CACGGATCCCGGAATGAT-3′
A, 5′-TTGTGGTGTACAGTTATAATCC-3′	B, 5′-CCTTCTCTCCAACTCCCA-3′
A, 5′-AACCCTGGGAGTTGTGGTGTAC-3′	B, 5′-CTTCTAACTTTGTCCGAAGAATCG-3′

PCR was performed to amplify these fragments according to established conditions (94°/45 sec, 55°/45 sec for 30 cycles, followed by 72°/1 min). Amplified fragments were recovered and purified using the Quiaquick gel extraction kit (Quiagen). The identity of the generated PCR products was confirmed by automatic sequencing carried out using the ABI Prism Dye Terminator Cycle Sequencing ready reaction kit (Perkin Elmer). DNA sequencing was performed at the Lombardi Cancer Center Sequencing Core Facility (Georgetown University Medical Center).

2.10. Synthesis of the 20-bp oligonucleotides

Six double-stranded oligonucleotides of 20 bp each, spanning the −656/−586 DNA area were synthesized with a successive 10-bp overlap sequence (Bio-Synthesis, Inc.):

(**1**) 5′-ACCCTGGGAGTTGTGGTGTA-3′;
(**2**) 5′-TTGTGGTGTACAGTTATAAT-3′;
(**3**) 5′-CAGTTATAATCCCAGGGCCC-3′;
(**4**) 5′-CCCAGGGCCCAGAAGATTGA-3′;
(**5**) 5′-AGAAGATTGAAACAGGCTTC-3′;
(**6**) 5′-AACAGGCTTCTTAGCCAGCC-3′

These oligonucleotides were used as probes for the gel-shift assay using nuclear extracts prepared from Y1 cells, treated with or without GKB. Incubation conditions were identical to those described above. The specificity of the DNA–protein interaction was assessed by using scrambled (S) and mutated (M) nucleotide sequences of probe number 3.

S1, 5′-**TCGACTGACA**CCCAGGGCCC-3′;	S2, 5′-CAGTTATAAT**TCGACTGACA**-3′;
M1, 5′-CAG**CG**ATAATCCCAGGGCCC-3′;	M2, 5′-CAGTTA**AGC**TCCCAGGGCCC-3′

2.11. Protein measurements

Microgram amounts of proteins were quantified by the dye-binding assay of Bradford [28], using bovine serum albumin as the standard.

2.12. Statistics

The values shown represent the results from two to six independent experiments. Statistical analysis was performed by ANOVA using the Instat 3.00 package from GraphPad Software, Inc.

3. Results

Mouse Y1 adrenocortical cells were chosen for this study as they exhibit hormone-induced steroidogenic activity, high PBR levels (B_{max} of 11 pmol/mg protein), and have been used successfully by many investigators to study the transcriptional regulation of genes involved in the steroidogenic pathway. Treatment of Y1 cells with GKB (300 ng/mL) for 24 hr resulted in a 56% decrease of B_{max} values for [^{3}H]PK 11195 binding and had a minor effect on receptor affinity (Table 1). This inhibition was also seen following treatment for 12 hr (data not shown). To examine the effect of GKB on Y1 steroid formation, we treated the cells with 300 ng/mL of GKB for 24 hr in the absence of serum. The choice of concentration was based on dose–response studies ([16], and data not shown). Addition of the hydrosoluble analogue of cAMP, dbcAMP, induced a 20-fold increase in 20α-dihydroprogesterone production (Table 1). Treatment of the cells with GKB resulted in a 47% inhibition of dbcAMP-induced steroidogenesis. These results are in agreement with our previously published data on the effect of GKB on rat and human adrenocortical cells [16].

Based on our previous studies showing that GKB reduced PBR protein and mRNA expression [16] and preliminary studies indicating that PBR mRNA stability was not affected by GKB (data not shown), we examined the effect of GKB on the transcription of the *PBR* gene. A series of transient transfection experiments were carried out using different size fragments within the *PBR* gene promoter region subcloned into a CAT reporter plasmid. CAT values were corrected for transfection efficiency using the pSV-βGal control plasmid as the standard. Promoterless and enhancer-containing vectors were used as internal controls. The promoterless vector expressed very low amounts of CAT as determined by ELISA, while the enhancer-containing vector showed 28 times higher CAT activity. The −1219/+36 insert-containing vector expressed six times higher CAT activity compared to the promoterless vector, which represents the yield expected for a promoter with characteristics of a housekeeping gene (Fig. 1). The concentration of 300 ng/mL of GKB was chosen to examine the effect of GKB on *PBR* gene transcription based on concentration–response studies (data not shown). GKB inhibited by 50% reporter gene expression in the presence of the long fragment −1219/+36 (Fig. 1). These findings are consistent with GKB-induced inhibition of steroid synthesis by Y1 cells (Table 1). To identify the regulatory elements that mediate *PBR* gene transcription, a series of deletion constructs were used (Fig. 1). The transfections were performed simultaneously with control and GKB-treated Y1 cells. The results obtained clearly showed that there is a GKB-activated regulatory element in the −624/−513 *PBR*-promoter area. In another set of experiments, we determined the specificity of the inhibitory action of GKB on rat *PBR* gene expression by treating the transfected Y1 cells with equal amounts (300 ng/mL) of vitamins A, D, and E, corticosterone, testosterone, and GKB (Fig. 2). The choice of the vitamins and steroids tested was based on their common mechanism of action through well-defined nuclear transcription factors/receptors. The results showed that, in contrast to GKB, the various vitamins and steroids had no significant effect on *PBR* gene expression determined using the −1219/+36 and the other indicated *PBR* gene promoter fragments (Fig. 2). None of these compounds affected −513/+36 *PBR* gene transcription, in agreement with the data shown in Fig. 1.

To assess the specificity of the GKB-induced inhibition of *PBR* gene transcription, we examined its effect on two other gene promoters, the promoter of the *Cyp17* steroidogenic enzyme gene and the simian virus (*SV40*) promoter (Fig. 3). As expected, dbcAMP stimulated CAT activity in Y1 cells transfected with the *Cyp17*gene promoter in front of the *CAT* reporter gene (Fig. 3). Treatment of cells with 300 ng/mL of GKB further enhanced the dbcAMP-induced CAT activity, suggesting that GKB, while inhibiting *PBR* gene transcription, might act as a transactivator for other genes (Fig. 3). GKB had no effect on the CAT activity driven by *SV40* gene promoter (Fig. 3).

To determine whether there are protein-binding elements in the region examined by deletion analysis of PBR-promoter-containing constructs, we carried out

Table 1
[^{3}H]PK 11195 binding characteristics and dbcAMP-induced 20α-dihydroprogesterone synthesis in Y1 adrenocortical cells untreated or treated with GKB

Treatment	B_{max} (pmol/mg protein)	K_d (nM)	20α-Dihydroprogesterone (ng/mg protein)
Control	11 ± 0.12	6.7 ± 0.2	228 ± 2.8
GKB	4.8 ± 0.22*	4.3 ± 0.5	121 ± 3.0*

Cells were treated with 300 ng/mL of GKB for 24 hr, and then were collected and processed for PBR ligand-binding studies using PK 11195 as the radiolabeled ligand. In a separate experiment, cells were treated for 24 hr with GKB (300 ng/mL) and then were stimulated with 1 mM dbcAMP for 24 hr. Medium was collected, and 20α-dihydroprogesterone levels were determined by RIA. Basal values for 20α-dihydroprogesterone were 13 ± 4 ng/mg protein. Results are expressed as means ± SEM (N = 3).

* Statistically significant vs control, P<0.001.

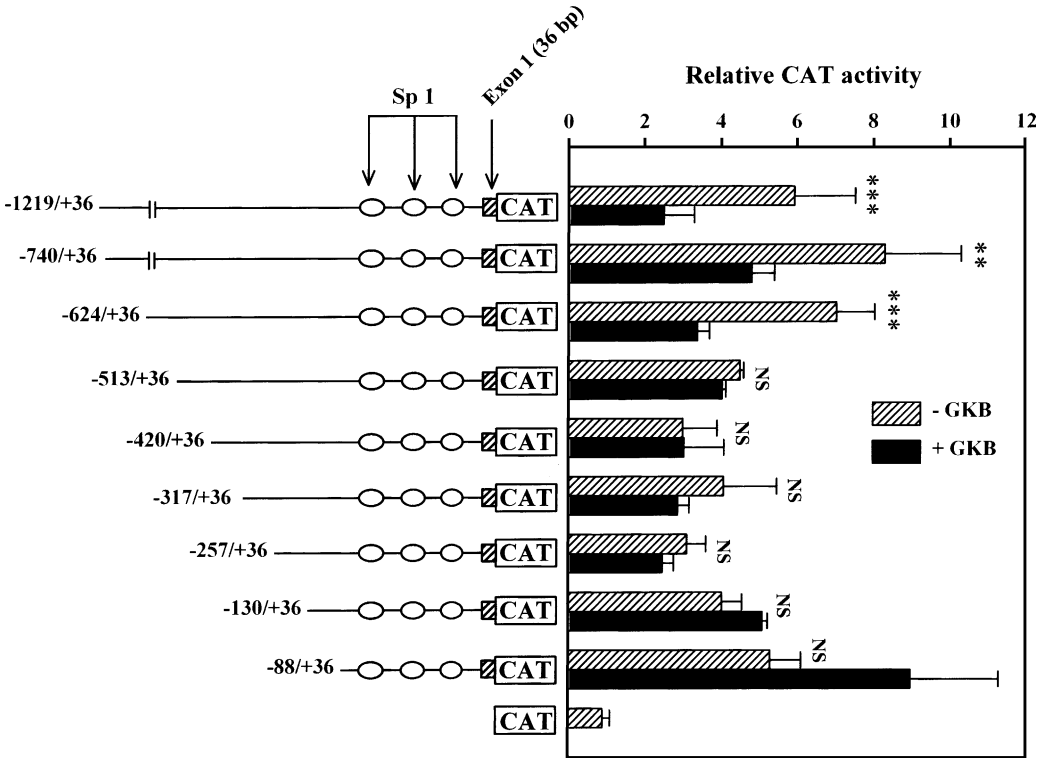

Fig. 1. Deletion analysis of the *PBR* gene promoter/pCAT vector and modulation of *CAT* reporter gene activity by GKB. The diagram represents the different extents of *PBR* gene promoter sequence present in each construct in pCAT-Basic. The shaded box in the promoter area represents exon 1 and the three gray circles indicate the consensus Sp1-binding sites. All data are represented as values relative to the βGal expression. The transfected cells were treated with or without 300 ng/mL of GKB. Results shown are means ± SEM from five independent experiments, each performed in quadruplicate. $^{***}P < 0.001$, and $^{**}P < 0.01$ versus + GKB. NS, not statistically significant.

EMSAs using crude nuclear extracts from Y1 cells untreated or treated with 300 ng/mL of GKB. Nuclear proteins were incubated with the DNA sequence of interest. We initially generated DNA fragments, using selected primers for PCR amplification, for the −1219/+36, −624/+36, and −624/−513 DNA inserts. The gel-retardation obtained with the first two fragments yielded poor resolution in a 4% acrylamide gel due to the large size of the probe (data not shown). However, using the small probe −624/−513 we observed a shift in DNA mobility, suggesting the presence of prominent regulatory elements. This effect was induced by either 2 or 12 hr of treatment with GKB, indicating that the nuclear protein(s) affected by the treatment might already be present in the cell. Two DNA–protein complexes were visualized as two shifted bands. Treatment of the Y1 cells with GKB dramatically reduced the formation of one complex (>80% decrease compared with the control) (Fig. 4). To examine the specificity of DNA–protein binding in that region of the DNA sequence, competition studies were performed using a radiolabeled probe and a 100-fold molar excess ratio of unlabeled probe. Figure 4 shows that the protein–DNA complexes seen were indeed due to specific protein–DNA interactions.

We further investigated the region spanning from −624 to −513 to delineate the exact sequence involved in the DNA–protein interactions. Three fragments of different sizes (146, 100, and 66 bp) were generated by PCR (the primers were designed around the area −656/−500). The fidelity of the PCR was confirmed by sequencing the products obtained. Because of the difference in the size of the probes, the complexes migrated to different distances on the gel. However, it was clear that GKB reduced the DNA–protein interaction of all three fragments, and this effect was more pronounced using the 66-bp probe, spanning from −656 to −590, which migrated the fastest (Fig. 5). Thus, we focused on the 66-bp probe where we observed the greatest inhibitory effect. Six fragments of 20 bp each, covering the region spanning from −656 to −586, were synthesized in a manner to create 10-bp overlapping sequences between fragments. EMSAs were carried out to investigate which oligonucleotide exhibits maximum binding. Figure 6 shows that among the six oligonucleotides, probe 3 (−616/−636) revealed a shifted band that was reduced by treatment with GKB. Probes 2 and 4 also formed the same DNA–protein complexes but with less intensity. Since the probes were overlapping by 10 bp, probes 2 and 4 showed the same GKB-induced inhibition of the DNA–protein interaction, but to a lesser extent. Two DNA–protein complexes were distinguishable using probe 3, the upper band being more affected by the treatment than the lower one. In probe 2, only the lower band was present, whereas in probe 4 only the upper band

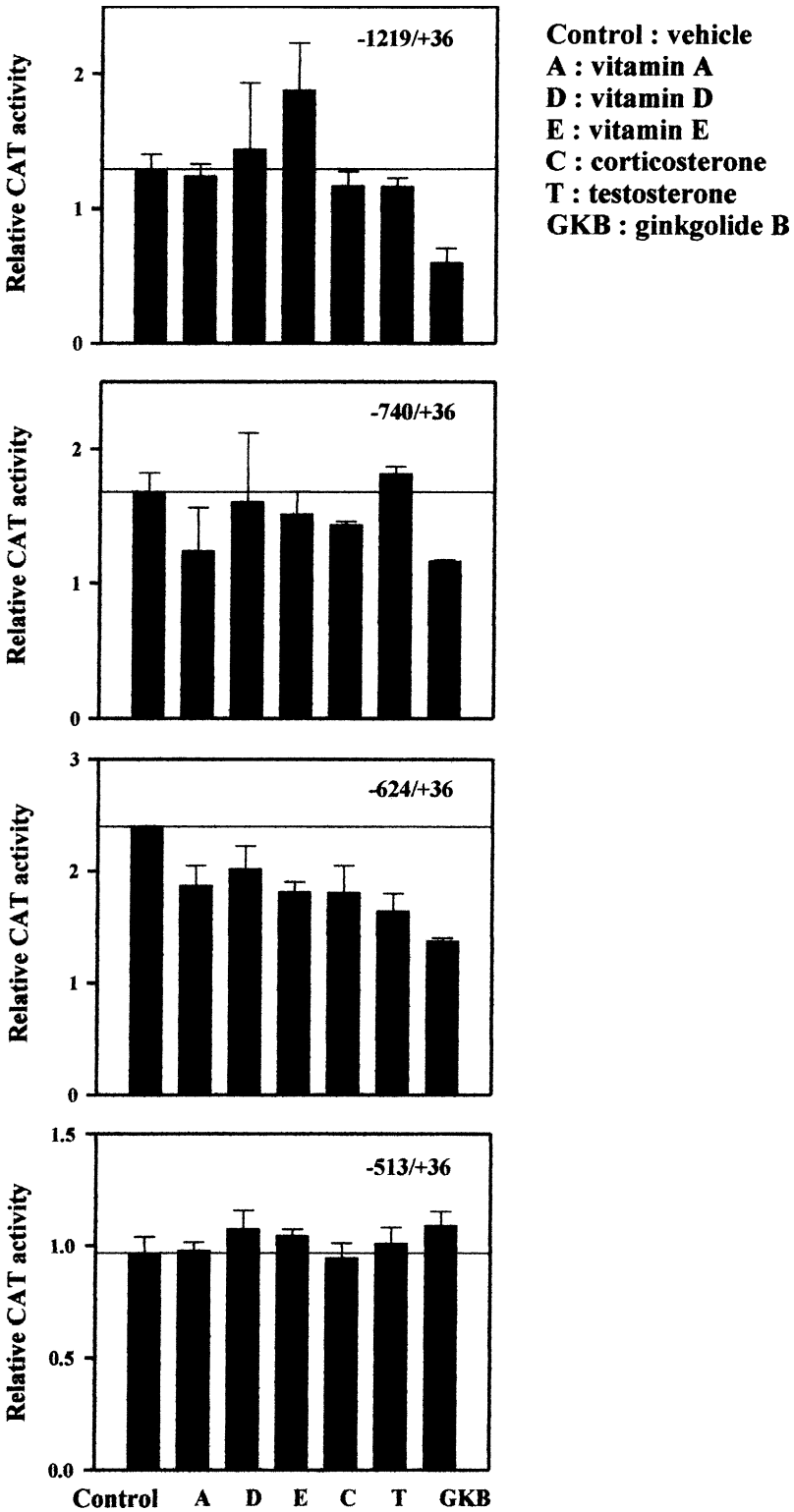

Fig. 2. Comparison of the effects of GKB, vitamins, and steroids on transfected Y1 adrenocortical cells treated for 12 hr using four *PBR* gene promoter/pCAT constructs. Concentrations equivalent to 300 ng/mL of GKB, vitamins A, D, and E, corticosterone, and testosterone were used to treat Y1 cells, transfected with the construct containing various fragments of *PBR* gene promoter in pCAT, for 12 hr. Four different fragments of the *PBR*-promoter/pCAT vector were used as indicated. Data are represented relative to βGal expression. Values are means ± SEM from three independent experiments, each performed in triplicate.

was visible. These data suggest that the 20-bp sequence as a whole was important for the DNA–protein interaction.

To further investigate the specificity of the DNA–protein interactions, scrambled and mutated oligonucleotides, based on the sequence of probe 3, were synthesized and incubated with nuclear extracts from GKB-treated and untreated control Y1 adrenocortical cells. Gel-retardation data showed that the complexes of interest were not present

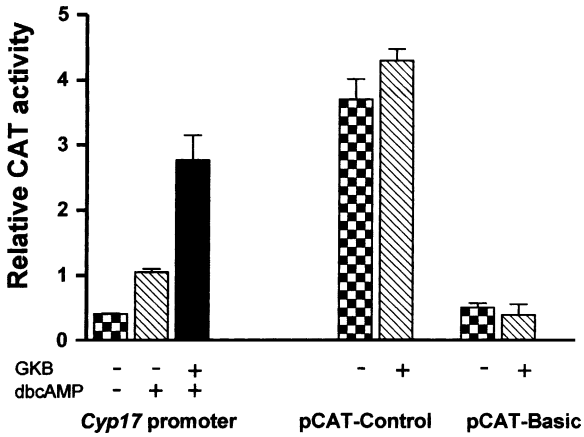

Fig. 3. Specificity of GKB-induced regulation of PBR expression as compared to SV40/pCAT-Basic, SV40 enhancer/pCAT-control, and the *Cyp17* gene promoter. Y1 cells were electroporated and treated with 300 ng/mL of GKB for 12 hr. Cells were transfected with *Cyp17* gene promoter in pCAT vector and treated with or without dbcAMP (1 mM) for 12 hr. βGal co-transfection was carried out, and the values are expressed as relative to CAT activity. Values represent means ± SEM from two independent experiments, each performed six times (N = 12).

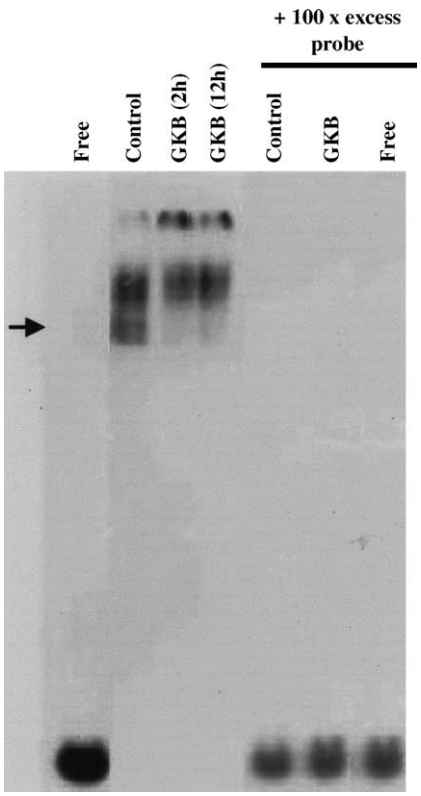

Fig. 4. Effect of GKB on the electrophoretic mobility shift characteristics of Y1 adrenocortical cells using the *PBR*-promoter sequence spanning from −624 to −513. Y1 cells were treated with or without 300 ng/mL of GKB for either 2 or 12 hr. A 100-fold excess probe was used to show the specificity of the DNA–protein interaction. Upon treatment, shifted bands can be distinguished on that panel (as indicated by an arrow). While the intensity of the upper band was reduced markedly by GKB treatment, the lower band exhibited a stronger inhibition. Free refers to free probe. Results shown are representative of three independent experiments.

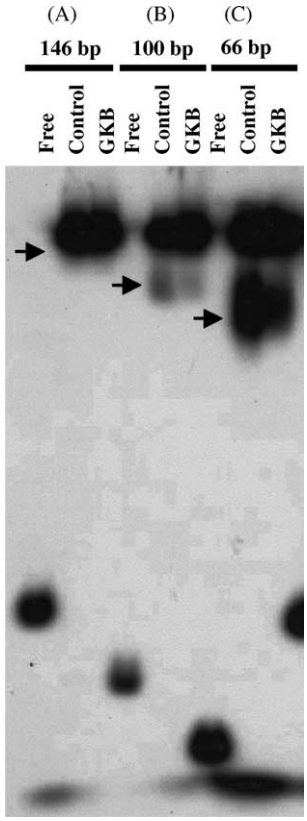

Fig. 5. GKB effect on Y1 nuclear protein interaction with different fragments designed from the *PBR*-promoter sequence −624/−513. Y1 nuclear extracts were incubated with different probes spanning from −646 to −500 covering 146 bp (A), from −646 to −546 covering 100 bp (B), and from −656 to −590 covering 66 bp (C) of the *PBR* gene promoter sequence. Because of its size, probe A did not fractionate on that gel system (4% polyacrylamide). Probes B and C show electrophoretic gel-retardation bands (arrows) that were reduced in intensity upon treatment. Results shown are representative of three independent experiments.

when using these modified probes (Fig. 7), further demonstrating the specificity of the DNA–protein complexes formed. Once the DNA fragment involved in the negative regulation of the 1255 bp *PBR* gene promoter was delineated, we transiently transfected Y1 cells with the *PBR* promoter lacking the region corresponding to either half (10 bp) or full-length probe 3 (20 bp), in order to validate the electrophoresis mobility shift studies in a cellular setting. Figure 8 shows that the inhibitory effect of GKB on the *PBR* gene promoter driven CAT reporter expression was absent when the 20 bp −636/−616 nucleotide sequence was deleted. We observed, however, an increase in CAT reporter activity, when only the 10-bp fragment was deleted. These findings might explain the presence of the two shifted bands seen by EMSA (see Fig. 4) and indicate that two distinct transcription factors may be involved in the regulation of the *PBR* gene transcription, with at least one of them directly affected by GKB.

We previously reported that GKB affected specifically adrenal PBR expression [16]. To examine the tissue specificity of the transcriptional factor(s) controlling *PBR*

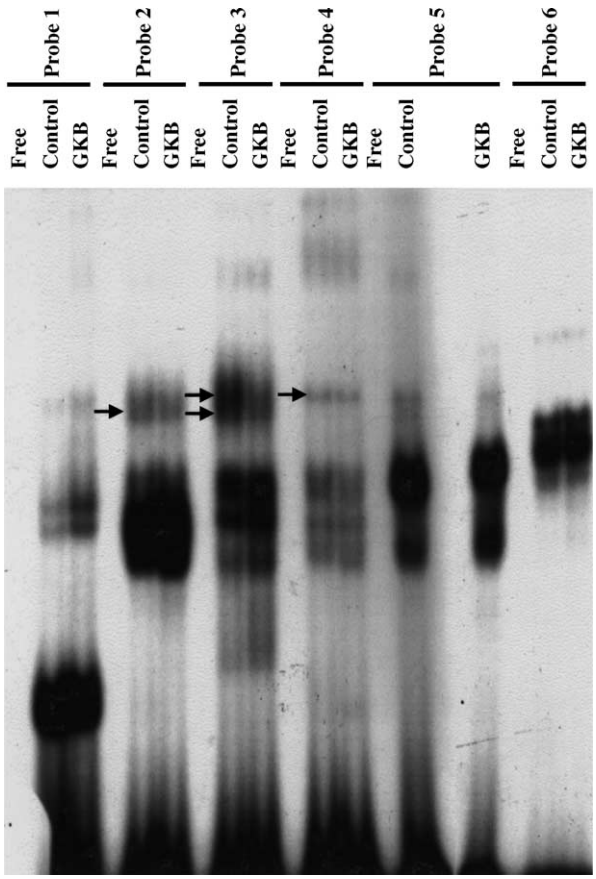

Fig. 6. Localization of the DNA-response elements and inhibition of DNA–protein interaction by GKB. The sequence spanning between −656 and −586 was divided into six distinct oligonucleotides of 20 bp each (with 10-bp overlapping sequences), and each oligonucleotide was examined for its ability to bind Y1 nuclear proteins by EMSA. All showed shifted bands. DNA–protein complexes affected by the GKB treatment were seen using probe 3. Using a 4–15% gradient polyacrylamide gel, two distinct bands can be seen, indicated by arrows. Data obtained with probes 2 and 4 show the presence of either the upper or the lower band. Free refers to free probe. Results shown are representative of four independent experiments.

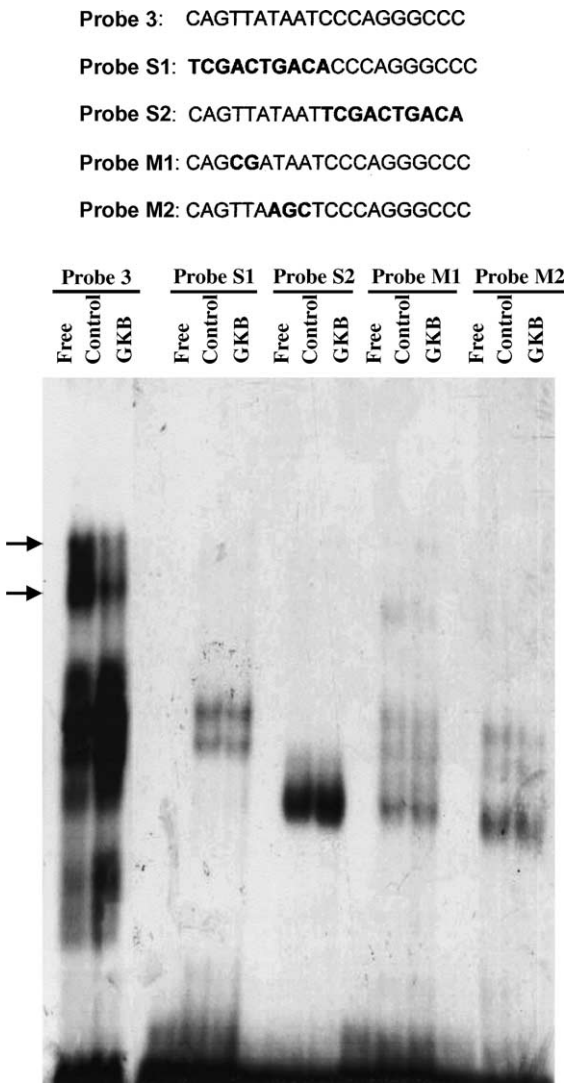

Fig. 7. DNA–protein specific interactions in the *PBR*-promoter sequence −636/−616. Probe 3 (−636/−616) was used to carry out this experiment. The specificity of this DNA sequence to interact with the nuclear protein in a GKB-dependent manner was confirmed by using either scrambled or mutated probes as shown in the figure and detailed in "Section 2" (bold characters). Although other DNA–protein complexes were observed in the mutated and scrambled fragments, the complexes that were present and affected by the GKB treatment disappeared. Results shown are representative of two independent experiments.

gene transcription, we treated adult male rats *in vivo* for 8 days with GKB (2 mg/kg, i.p.) as previously described [16]. At the end of the treatment, adrenals, brain, kidneys, liver, and testes were collected. Nuclei were isolated from these tissues, and EMSAs were performed using probe 3 (−636/−616). The transcription factor of interest was present in the adrenal glands, where a dramatic decrease due to GKB treatment was observed. This factor was also present in brain, liver, and testis, but no significant GKB effect could be seen. In the kidney, no DNA–protein complex of relevant size was found (Fig. 9).

Because we previously reported that GKB also affects human adrenal cell cortisol production [16], we examined the presence of this GKB-activated transcription factor in human adrenal tissues. Nuclear proteins from young and adult male adrenal postmortem specimens were isolated and examined for their ability to interact with probe 3 (−636/−616). The results obtained clearly indicated that human adrenals contain high levels of this GKB-activated transcription factor (Fig. 9).

4. Discussion

Glucocorticoid excess is detrimental to the immune, endocrine, and neural systems [29]; more specifically, it may cause hippocampal dysfunction leading to cognitive impairment in humans [30]. Thus, control of excessive glucocorticoid synthesis may offer a means of controlling the chronic negative effects of stress and preventing glucocorticoid-related diseases. Many investigators have reported the beneficial effects of *Ginkgo biloba* extracts

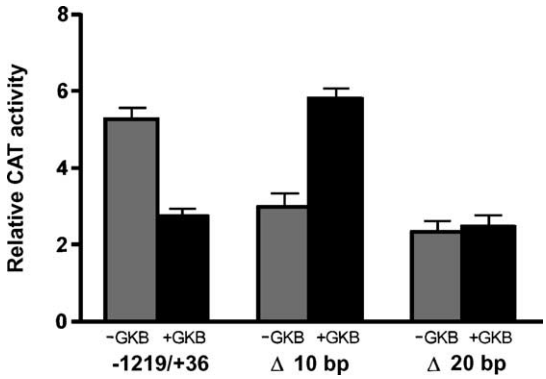

Fig. 8. Deletion mutation analysis of the −636/−616 fragment and its importance in GKB-induced inhibition of *PBR* gene transcription. Y1 cells were transfected with the 1255 bp *PBR*-promoter/pCAT vector from which either 10 bp (−636/−626; Δ 10 bp) or 20 bp (−636/−616; Δ 20 bp) were deleted. The cells were then treated with 300 ng/mL of GKB for 12 hr. GKB inhibited −1219/+36 *PBR* gene transcription ($P < 0.001$). There was an increase of CAT activity when the Δ 10 bp plasmid was used, and no effect of the GKB treatment was observed when the entire 20-bp fragment was deleted. Values represent means ± SEM from two independent experiments, each performed six times (N = 12).

related to stress, aging, and neurodegenerative diseases. Due to its neuroprotective and antioxidant properties, *Ginkgo biloba* has been shown to exert a preventive effect with regard to Alzheimer's disease [12,31,32]. Many of these properties could be attributed to the regulatory effect of *Ginkgo biloba* on glucocorticoid levels [15,33]. In previous studies, we showed the negative regulation of PBR expression in adult male rats chronically treated with either EGb 761 or GKB [16]. Among the rat tissues screened, the inhibitory effect was confined to the adrenal cortex, showing specific action of GKB on the fasciculata-reticularis compartment but not on the glomerulosa. This was supported by the fact that although circulating corticosterone levels were decreased, aldosterone levels remained unchanged. These findings indicated a beneficial role of EGb 761 and GKB in the case of excess glucocorticoid synthesis. Interestingly, EGb 761 and its ginkgolides A and B did not affect basal corticosterone production stimulated by physiological ACTH levels.

We previously reported a 65% reduction of PBR mRNA levels by GKB [16]. The present study investigated the regulation of the *PBR* gene transcription by GKB. Although the *PBR* gene has features suggesting that it is a member of the "housekeeping" gene family and as such remains always turned on, a complex but unknown regulation of *PBR* gene expression exists. Analysis of the promoter sequence −1219/+36 of the *PBR* gene revealed three regulatory elements, two positive elements located at −624/−420 and −51/−33, and a negative one at −267/−249 [20]. Our data support these findings. When the transfected Y1 adrenocortical cells were treated with GKB, the expression of CAT activity decreased by more than 50%, especially when −1219/−513 fragments were used. Interestingly, our present studies demonstrate an inhibitory effect of GKB on the *PBR* gene fragment containing the positive-acting regulatory element, previously described by Oberto *et al.* [20] (Fig. 1).

Nuclear hormone receptors are involved in the regulation of the expression of genes responsive to steroids and other metabolites, such as retinoids, vitamin D, thyroid hormones, fatty acids, and peroxisome proliferators [34]. To investigate whether *PBR* gene transcription was influenced by nuclear hormones or modulated in a specific manner by GKB, we tested the effects of vitamins A, D, and E, corticosterone, and testosterone. No significant difference was observed when the cells were treated with these various vitamins and steroids. However, GKB induced a significant decrease of relative CAT activity

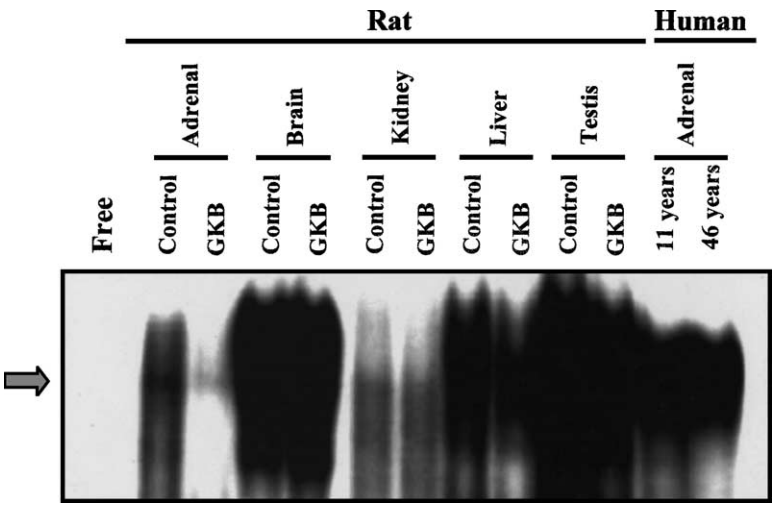

Fig. 9. Detection of the transcription factor in different adult rat tissues and human adrenals. Nuclear extracts were prepared from various tissues collected from adult rats treated for 8 days. Nuclear extracts were also prepared from human adrenals. The experiment was carried out using radiolabeled probe 3 (−636/−616). The nuclear protein binding to the −636/−616 probe is indicated by an arrow on the left. Results shown are representative of two independent experiments.

(>50%), corresponding to an inhibition of *PBR* gene expression. Once again, the region spanning from −624 to −513 showed the strongest inhibitory effect, suggesting that the DNA-response elements were present in this 112-bp sequence. The process by which the GKB effect occurs might be different from what is currently known about hormone nuclear receptors: GKB might interact with a transcription factor which binds to that particular sequence of the *PBR* gene, thereby triggering conformational changes of this factor resulting in a dissociation from its DNA-binding site in a manner similar to that described for peroxisome proliferators [35]. The GKB induction of reporter gene activity driven by *Cyp17* gene promoter and the absence of an effect of GKB on *SV40* gene promoter demonstrate the specificity of the GKB-activated inhibition of *PBR* gene transcription. This would make GKB-induced suppression of *PBR* gene transcription a specific rather than a global phenomenon.

EMSA, using the PCR-generated oligonucleotide −654/−499, revealed the presence of DNA–protein complexes, which were decreased by 50–60% upon treatment with GKB. The fact that the effect of GKB could be seen as early as 2 hr after initiation of treatment suggests that it does not require novel protein synthesis and that it is likely mediated via a direct interaction of GKB with a pre-existing protein. Moreover, the binding was specifically competed by excess probe. Smaller probes covering that region of the promoter were synthesized (100 and 66 bp), and EMSA showed a more pronounced binding with the 66-bp probe (−656/−590). In all cases, the inhibition of the binding was maintained in the samples treated with GKB. These data suggest that GKB either inhibits a transcription factor or prevents its interaction with the positive-regulatory element on the DNA sequence, thus, inhibiting *PBR* gene transcription. EMSA carried out with 20-bp probes resulted in shifted complexes, and significant inhibition of DNA–protein complex formation by GKB treatment was observed with probe 3 (−636/−616). Probes 2 and 4 revealed this inhibition to a much lesser degree, which we attribute to the 10-bp overlaps of the probes. We could distinguish two bands with probe 3: the upper band being more affected by the treatment than the lower one. Moreover, the fact that only the lower band was visible in probe 2 while the upper band was detectable in probe 4 suggests that the 20 bp as a whole are essential to the DNA–protein interaction. Indeed, deletion mutation studies of the 10 (−636/−626) or 20 (−636/−616) bp fragments on the 1255 bp *PBR* gene promoter carried out in transfected Y1 cells confirmed the importance of this DNA-response element in *PBR* gene transcription by GKB. When scrambling the first or last 10 bp of the oligonucleotide probe 3, the gel-retardation profile was lost. Similarly, site-directed mutagenesis performed by replacing 2 bp (TT) in the original probe 3 (CAG**TT**ATAAT by CAG**CG**ATAAT) also resulted in an inhibition of the DNA–protein interaction, suggesting that these 2 bp were necessary for the binding of the transcription factor to this regulatory element. It should be noted that although other DNA–protein complexes were formed, they remained present upon incubation with either scrambled or mutated probes, indicating that their interaction with the *PBR* gene promoter fragment was less specific.

To validate our findings, we used nuclear proteins obtained from adult rats treated *in vivo* with GKB. The shifted bands were observed in all screened tissues. However, the inhibitory effect of the treatment was found only in the adrenal gland. These results are in agreement with our previous data [16], showing that the GKB inhibitory effect on the 18-kDa PBR protein was confined to the adrenal gland.

Moreover, we demonstrated that the −636/−616 probe interacts with a protein of presumably similar size (considering the migration pattern) found in nuclear extracts prepared from young and adult human adrenals. This is in agreement with our previous data using the H295R human adrenal tumor cell line [16]. These data suggest that a similar regulatory mechanism of PBR and glucocorticoid synthesis by ginkgolides and EGb 761 may exist in both humans and rodents. Considering the bioavailability of ginkgolides after oral administration of the total EGb 761 preparation, the concentration of GKB used in the present experiments (300 ng/mL) is of the same magnitude as that found in the serum of rats treated with 50–100 mg/kg of EGb 761 [36] and in that of humans treated with 240 mg/day of EGb 761.[1] Interestingly, 120–240 mg/day are the doses used to stabilize the disease progression in Alzheimer's patients [31,37–39]. Thus, ginkgolide-induced regulation of PBR expression and glucocorticoid synthesis may be one of the elements involved in the neuroprotective effects of EGb 761 and may provide a tool for future pharmacological intervention towards control of glucocorticoid excess [15] linked to neurodegeneration in humans.

Based on the current knowledge on (i) transcriptional gene regulation, (ii) ligand-dependent activation of nuclear receptors, and (iii) our own data, the following hypothesis can be proposed: within the region spanning from −636 to −616 there is a positive-acting regulatory element. GKB inhibits the binding of the activating transcription factor to DNA by either changing the receptor conformation or by creating allosteric modifications. Figure 10 shows a diagram of the proposed mechanism. In this scheme, GAR represents the GKB-Activated Receptor, which is the transcription factor positively regulating PBR expression under basal steady-state conditions. Treatment of the cells with GKB and the subsequent interaction of GKB with GAR result in the dissociation of GAR from the GAR-Responsive DNA Element (GARE), leading to inhibition of PBR transcription.

In conclusion, the present study shows the regulation of gene transcription by a compound of plant origin leading to changes in endocrine function. This finding may have a major impact on our understanding of the mechanism of

[1] Biber A and Drieu K, unpublished data.

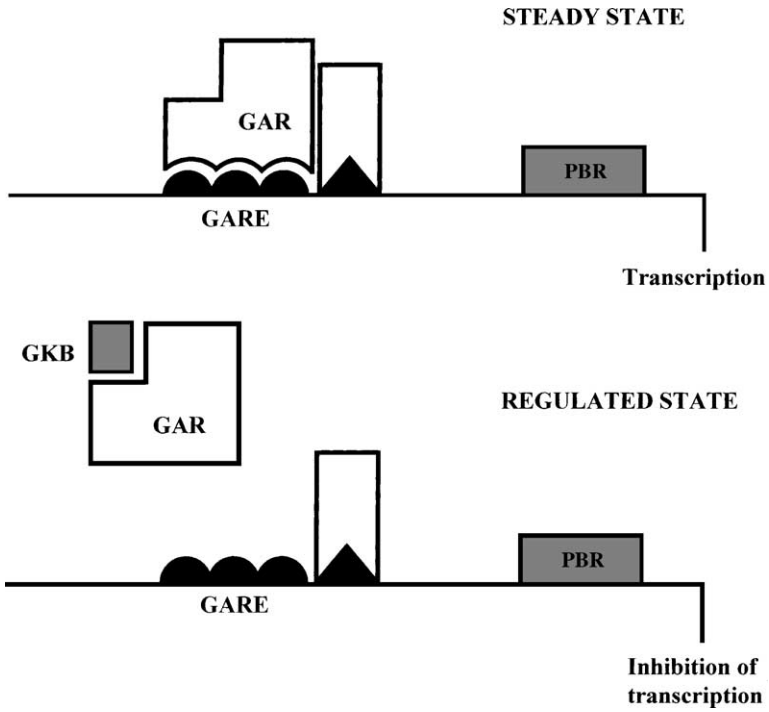

Fig. 10. Diagram illustrating the GKB-induced transcriptional suppression of the *PBR* gene. Under steady-state conditions, a DNA-binding protein interacts with the −636/−616 DNA promoter area of the *PBR* gene driving *PBR* gene transcription in a positive manner. GKB, either directly or indirectly, interacts with this transcription factor, named GKB-Activated Receptor (GAR). The −636/−616 DNA *PBR*-promoter area is named GARE for GAR-Response Element. The rectangular box corresponds to the second band seen in the gel-retardation assays, which is less affected by the GKB treatment. Both proteins might be necessary to initiate *PRB* gene transcription. Upon treatment with GKB (regulated state), the compound causes the dissociation of GAR from GARE, resulting in an inhibition of *PBR* gene transcription.

action of *Ginkgo biloba* and may lead to the development of novel pharmacological tools. The identification and characterization of the GAR transcriptional regulatory protein are under investigation.

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