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Short communication

FK506 (tacrolimus) increases rat α_1-acid glycoprotein expression in liver and primary cultured hepatocytes

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Abstract

FK506 (tacrolimus) (10 mg/kg, s.c., 5 days) increased rat α_1-acid glycoprotein (AGP) in serum and AGP mRNA in liver. FK506 potentiated the dexamethasone-increased AGP expression in primary cultured hepatocytes. In the luciferase promoter assay, FK506 potentiated the dexamethasone-increased promoter activity of the AGP gene in cultured rat hepatocytes, although FK506 alone had no effect on its activity. The combined effect of FK506 and dexamethasone was diminished by glucocorticoid responsive element (GRE) deletion and mutation or by an anti-glucocorticoid. These results indicated that FK506 causes the transcriptional induction of AGP, at least in part, via a glucocorticoid-mediated mechanism.

Keywords: FK506 (tacrolimus); α_1-Acid glycoprotein; Glucocorticoid; FK506-binding protein (FKBP)

1. Introduction

α_1-Acid glycoprotein (AGP) is the major transport protein for cationic drugs, endogenous ligands (progesterone), and some anionic drugs in plasma (Kremer et al., 1988). AGP contains a variable glycan chain at five glycosylation sites, which may be responsible for its role in modulating immune responses (Bennett and Schmid, 1980; Bories et al., 1990). The hepatic synthesis and secretion of AGP, as well as its glycosylation, are altered during acute inflammation (Pos et al., 1989). This could influence the binding of drugs and their biological function. AGP gene expression is increased by interleukin-1, tumor necrosis factor-α, interleukin-6, interleukin-8, interleukin-11, leukemia inhibitory factor, oncostatin M, and glucocorticoids (Richards et al., 1992; Baumann and Gauldie, 1994). *Cis*-acting regulatory elements have been identified in the rat AGP gene. These elements are responsive to interleukin-1, interleukin-6 and glucocorticoids (Prowse and Baumann, 1988; Klein et al., 1988). AGP gene expression is also increased by phenobarbital and rifampicin through mechanisms that are independent of the inflammatory pathway (Bertaux et al., 1992; Fournier et al., 1994a,b,c).

FK506 (tacrolimus) is a 23-membered macrolide with a very potent immunosuppressive action that has been marketed as an immunosuppressant for use after organ transplantation (Peters et al., 1993). In the field of autoimmune diseases, clinical trials of FK506 are in progress for the treatment of atopic dermatitis (Nakagawa et al., 1994). It is well known that FK506 exerts its various effects through FK506-binding protein (FKBP) (Schreiber, 1991). FK506 acts by forming complexes with FKBP, which can then bind to calcineurin, inhibiting its enzymatic activity and thereby preventing early gene expression such as that of interleukin-2 (Liu et al., 1991). To understand the involvement of FKBP in the regulation of AGP expression, we investigated the effect of FK506 on AGP expression in vivo and in primary cultured rat hepatocytes. Moreover, the mechanism of AGP gene expression induced by FK506 was investigated in cultured hepatocytes by promoter analysis using the 5′-flanking region of the rat AGP gene.

2. Material and methods

2.1. Materials

FK506 was a generous gift from Fujisawa Pharmaceutical (Tsukuba, Japan). Dexamethasone, RU486 (mifepristone) and rat AGP were purchased from Sigma (St. Louis,

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MO). The plasmid containing the full-length cDNA of rat AGP in its *Pst*I site was a generous gift from Dr. Heinz Baumann (Roswell Park Cancer Institute, Buffalo, NY). Human β-actin cDNA (2.0 kb) was obtained from Clontech (Palo Alto, CA). All other reagents used in this study were of analytical grade.

2.2. In vivo treatment of rats

Male Wistar rats weighing 200–250 g were treated with FK506 (10 mg/kg in 30% dimethyl sulfoxide, subcutaneously) and were killed 24 h after the last injection.

2.3. AGP quantitation in serum

Antiserum to rat AGP was prepared by immunization of rabbits using a 50/50 emulsion of antigen/Freund's complete adjuvant corresponding to antigen amounts of 0.75 mg. Following one booster injection at 3 weeks, two immunizations were performed over a period of 1 month. AGP concentrations in rat serum were measured by a radial immunodiffusion assay using rat AGP and rabbit anti-rat AGP antiserum. In a typical experiment, radial immunodiffusion plates consisting of 1% agarose in 7 mM Na_2HPO_4, 2 mM NaH_2PO_4, 145 mM NaCl, 5 mM EDTA, pH 7.0 were used with the antiserum. After authentic AGP and serum samples (5 μl) were applied to wells, the plates were allowed to stand for 48 h at room temperature. At that time, a fully developed precipitation ring was observed. The ring diameter was subsequently measured and quantified.

2.4. Northern blot analysis

Total RNA was isolated from rat liver or cultured hepatocytes and analyzed by Northern blot hybridization. RNA samples (7.5 μg) were resolved on denaturing 1% agarose gels, transferred to GeneScreen Plus nylon membranes (Amersham) by blotting, and baked for 2 h at 80°C. Membranes were prehybridized for 6 h at 42°C in 5× SSPE buffer (0.75 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA, pH 7.4), 10× Denhardt's solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin), 2% sodium dodecyl sulfate (SDS), 100 μg/ml sonicated and denatured salmon sperm DNA, and 50% formamide. Membranes were hybridized for 24 h at 42°C in the above solution containing full-length AGP cDNA (740 bp) and human β-actin cDNA (2.0 kb) probes radiolabeled by random priming with [α-^{32}P] dCTP (3000 Ci/mmol) (Amersham, UK). β-Actin was used as an internal control to ensure consistent transfer of mRNA. Membranes were then washed at room temperature with 2× SSC (0.3 M NaCl, 0.03 M sodium citrate) and 0.1% SDS. Radioactive signal intensity was quantified with a bio-imaging analyzer (BAS1000), and the results are expressed as the difference from control values.

2.5. Isolation and culture of hepatocytes

Hepatocytes were isolated from the livers of 180–230-g male Wistar rats by collagenase perfusion, and plated onto 35-mm tissue culture dishes precoated with collagen. Cultured hepatocytes were incubated in a humidified chamber maintained at 37°C under 5% CO_2/95% air with Williams' Medium E supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin, and 10 mM HEPES. The medium was changed into the serum-free medium 4 h later to remove unattached hepatocytes. Drugs were applied 18 h after plating of the hepatocytes and the culture medium was renewed every 24 h. Drugs were dissolved in dimethyl sulfoxide (for FK506) or ethanol (for dexamethasone and RU486). The final concentration of organic solvent in the culture medium was 0.1%.

2.6. Construction of reporter plasmids

A 380-bp fragment of the rat AGP gene, spanning nucleotides −360 to +20 relative to the transcription start site (Reinke and Feigelson, 1985), was amplified by polymerase chain reaction (PCR) by using rat genomic DNA as template and the following primers: 5′ primer, 5′-TACTTCCCGAGAGTGTTCATTCATGTGGACATAGA-3′, and 3′ primer, 5′-GCCCAGGAAGAGCTAACTGGTGGCTGGAGAGTGCA-3′. The PCR product was generated through 40 cycles of the following steps: denaturing at 94°C for 2 min, annealing at 60°C for 1 min, and elongation at 72°C for 1.5 min. The product was cloned into the pCRII vector using the TA Cloning Kit (Invitrogen). Then, using this clone containing rat AGP gene as a template, with 5′ primer 5′-CTCAGAGCTCCCTCAGGGGTTGATTTA-3′ and 3′ primer 5′-AATTCTCGAGGCCCAGGAAGAGCTA-3′, PCR was performed as follows: 94°C for 2 min, 55°C for 1 min, 72°C for 1.5 min, 40 cycles. The PCR fragment was cloned between the *Sac*I and *Xho*I sites upstream from the pGL2-Basic luciferase reporter plasmid (Promega). The resulting construct is referred to as pAGP(−360)Luc. A series of deletion constructs were also prepared by PCR using the 3′ primer 5′-AATTCTCGAGGCCCAGGAAGAGCTA-3′, and the 5′ primers 5′-GCAGGAGCTCGTGTCAGGGCTGGCT-3′ and 5′-TCTGGAGCTCCTGGCGACGCCCATG-3′ with the luciferase reporter plasmids pAGP(−172)Luc and pAGP(−60)Luc, respectively. pAGP(−360)Luc-M is the construct pAGP(−360)Luc with a mutated GRE site. It was prepared using a Transformer Site-Directed Mutagenesis kit (Clontech) and rAGP-mGRE as a mutation primer. It contains 5′-GCTGGCTTGAGGGAA**ag**TTT-T**aa**GCAAGACATTTCCC-3′ from positions −132 to

−95 bp with a mutated GRE site indicated in bold lowercase letters. All constructs and cloned fragments were sequenced by the dideoxy chain termination method (Sanger et al., 1977).

2.7. Transfection into primary cultured rat hepatocytes

Primary cultured rat hepatocytes were plated in standard Williams' Medium E onto 35-mm collagen-coated dishes. Six hours after plating, the culture medium was replaced with 1 ml of Opti-MEM containing a premixed complex of 8 μg of Lipofectin reagent and 1 μg of the reporter construct, in combination with 0.1 μg of pSV-βgal as a control for measuring transfection efficiency. The transfection mixture was replaced with standard Williams' Medium E 12 h later. Hepatocytes were harvested 24 h after transfection. The luciferase activity in cell extracts was measured using the Luciferase Assay system according to the manufacturer's instructions (PicaGene Luminescence Kit). β-Galactosidase activity was determined by an enzymatic assay using *o*-nitrophenyl β-D-galactopyranoside as the substrate, and absorbance was measured at 420 nm. The luciferase activity in each sample was normalized to that of β-galactosidase.

2.8. Statistical analyses

Differences among groups were assessed using Duncan's multiple range test (a non-parametric test) or Student's *t*-test, where $P < 0.05$ indicates the presence of a statistically significant difference.

3. Results

In order to analyze the effect of FK506 on AGP expression, rats were treated for 5 days with FK506 (10 mg/kg/day) (Fig. 1A). FK506 increased serum AGP levels from 20 mg/100 ml to 40–60 mg/100 ml. The maximal effect of FK506 was observed after 3 days, and this effect remained constant up to 10 days after administration. In parallel, the effect of FK506 on the AGP mRNA level in the liver of other rats was analyzed by Northern blot hybridization. FK506 (10 mg/kg, s.c.) increased the AGP mRNA level in the liver 12 h after administration (Fig. 1B).

To confirm the up-regulation of AGP by FK506 in vitro, cultured hepatocytes were incubated with FK506 and/or dexamethasone 18 h after plating and were harvested 48 h later to measure AGP mRNA. Dexamethasone (10^{-8} M) significantly increased the AGP mRNA level in cultured hepatocytes (Fig. 2A). FK506 (10^{-5} M) potentiated the AGP mRNA expression increased by dexamethasone (10^{-8} M), although FK506 alone did not increase AGP mRNA in cultured hepatocytes (Fig. 2A).

To determine whether the effect of FK506 on AGP mRNA was due to changes in transcriptional activation, we performed a promoter analysis in primary cultured rat hepatocytes (Fig. 2B). FK506 did not increase the luciferase activity of pAGP(−60)Luc, pAGP(−172)Luc, or pAGP(−360)Luc by itself, whereas FK506 potentiated the dexamethasone-induced promoter activity of pAGP(−172)Luc and pAGP(−360)Luc, both of which contain the GRE. The promoter activity of pAGP(−60)Luc

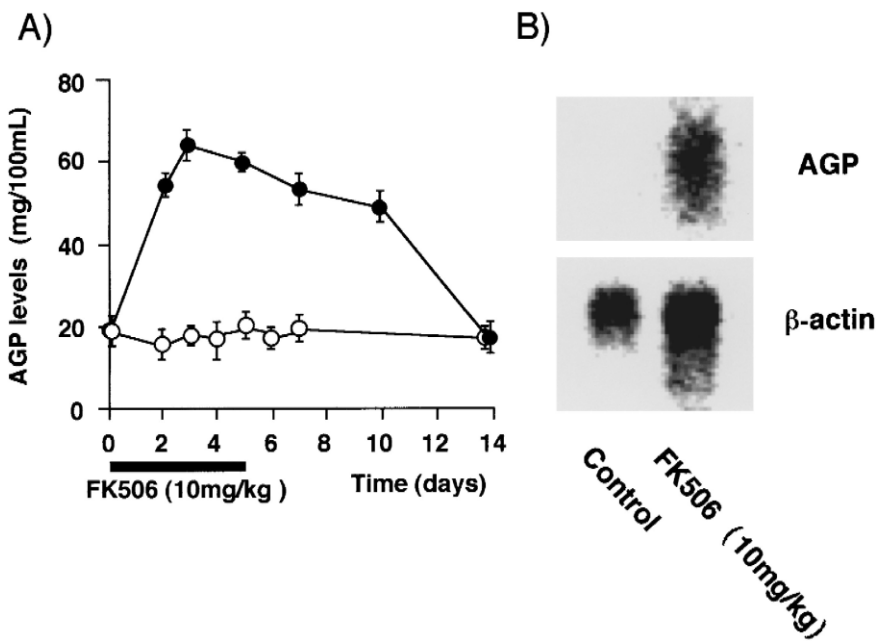

Fig. 1. (A) Effect of FK506 on AGP protein levels in rat serum. Male Wistar rats (six rats per group) were treated for 5 days subcutaneously with FK506 at doses of 10 mg/kg/day. Blood was withdrawn 24 h after the last injection and centrifuged at 3000 rpm for 10 min. The levels of AGP protein in rat serum were measured by radial immunodiffusion assay. All values are presented as the means ± standard deviation for six different rats. (B) Effect of FK506 on AGP mRNA levels in rat liver. Twenty-four hours after the last administration, the rats were killed, and the livers were dissected to prepare total RNA and to analyze AGP mRNA levels by Northern blot hybridization.

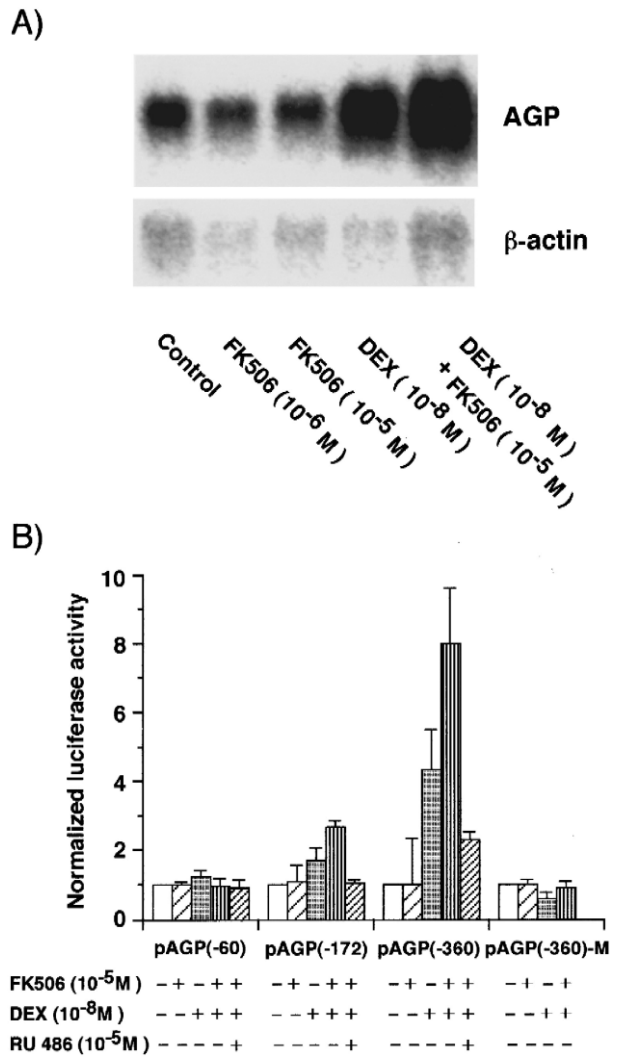

Fig. 2. (A) Effect of FK506 on AGP mRNA levels in primary cultured rat hepatocytes. Eighteen-hour-old hepatocytes were treated for 48 h with medium alone or medium containing 10^{-6} or 10^{-5} M FK506. Hepatocytes were harvested from individual dishes to isolate total RNA and AGP mRNA levels were analyzed by Northern blot hybridization. (B) Effects of FK506 and dexamethasone on luciferase activity in primary cultured rat hepatocytes transiently transfected with AGP-reporter constructs. Six hours after plating, primary cultured rat hepatocytes were transiently transfected with 1 μg of luciferase reporter plasmid, pAGP(−60)Luc, pAGP(−172)Luc, pAGP(−360)Luc, or pAGP(−360)Luc-M, plus 0.1 μg of pSV-β-galactosidase. Following transfection for 12 h, hepatocytes were treated for 24 h with medium alone or medium containing 10^{-5} M FK506, 10^{-8} M dexamethasone, or 10^{-5} M FK506 plus 10^{-8} M dexamethasone with or without 10^{-6} M RU486. Hepatocytes were harvested to measure luciferase and β-galactosidase activity. All values are expressed as the means ± standard deviation of six independent experiments and as the relative ratio of luciferase activity normalized to β-galactosidase activity in corresponding control hepatocytes. Data were analyzed using Duncan's multiple range test (a nonparametric test). Significantly different from the corresponding control: $^{*}P < 0.05$; $\#P < 0.05$ versus dexamethasone-treated group.

was not affected by dexamethasone, because the GRE is located at −121 to −107 bp in the rat AGP 5′-flanking region. In addition, the effects of dexamethasone and FK506 were blocked by a mutation of GRE (pAGP(−360)Luc-M) as well as by a glucocorticoid antagonist, RU486 (Fig. 2B). Taken together, the results suggest that the GRE plays a role in the FK506-responsive transcriptional activation of AGP.

4. Discussion

In this study, we found that FK506 increased serum in the liver in vivo AGP levels and AGP mRNA levels. An increase in AGP mRNA levels induced by FK506 in combination with glucocorticoid was observed in primary cultured rat hepatocytes. We assume that the main mechanism for the up-regulation of AGP mRNA is at the transcriptional activation level. To examine the mechanism of action of FK506 on transcription, we carried out a promoter analysis using several luciferase reporter constructs containing the rat AGP 5′-flanking region. First, although we used a rat AGP gene covering the region from −360 to +20 bp in the promoter analysis, FK506 alone had no effect on promoter activity. Next, we specifically focused on the involvement of a potential glucocorticoid response on the effect of FK506, since the region from −360 to +20 bp contains the GRE, which is located between positions −121 and −107. We found that FK506 potentiated the dexamethasone-induced promoter activity of pAGP(−172)Luc and pAGP(−360)Luc, both of which contain the GRE. The potentiation of the dexamethasone-induced transcription by FK506 was abolished in the absence of GRE sequences and in combination with the antiglucocorticoid, RU486. Since RU486 is a specific inhibitor of glucocorticoid receptor transcription activity (Moguilewsky and Philibert, 1984), it seems reasonable to conclude that the combined effect of FK506 and dexamethasone on pAGP(−360)Luc promoter activity is mediated by the glucocorticoid receptor. These findings are consistent with the observation that FK506 potentiates glucocorticoid receptor-mediated gene expression by directly binding the FKBP of the untransformed, cytosolic glucocorticoid receptor complex to increase the nuclear translocation of the glucocorticoid receptor (Ning and Sanchez, 1993). Our findings may provide insight into the pharmacodynamic effect of FK506 after organ transplantation, as well as into the regulation of AGP expression via FKBP. In a future study, we will determine whether plasma levels of AGP increase in those patients who have received FK506 treatment.

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