

Differential modulation of glucocorticoid action by FK506 in A549 cells

Jamie D. CROXTALL*¹, Mark PAUL-CLARK* and Peter Th. W. VAN HAL†

*Department of Biochemical Pharmacology, William Harvey Research Institute, Queen Mary, University of London, Charterhouse Square, London EC1M 6BQ, U.K., and †Department of Respiratory Medicine, Erasmus MC, Rotterdam, P.O. Box 2040, 3000CA, The Netherlands

Glucocorticoids inhibit the release of eicosanoid pro-inflammatory mediators. The immunosuppressant FK506 is known to enhance many aspects of glucocorticoid action. In the present study we show that FK506 (1 μ M or 10 μ M) inhibits the release of arachidonic acid and prostaglandin E₂ from A549 cells and also inhibits their proliferation. Simultaneous treatment of FK506 together with the glucocorticoids dexamethasone, methylprednisolone, fluticasone or mometasone (10 nM) enhances the growth inhibitory effect of these steroids. Furthermore, the simultaneous use of FK506 and these glucocorticoids similarly results in enhanced inhibition of arachidonic acid release. When pre-treated for 2 h, FK506 enhances glucocorticoid inhibition of COX2 (cyclo-oxygenase 2) expression. However, when administered simultaneously, FK506 blocks glucocorticoid inhibition

of COX2 expression. Nuclear uptake of glucocorticoid receptors mediated by glucocorticoids is also blocked by the simultaneous administration of FK506. These results suggest that the effect of simultaneous treatment of FK506 with glucocorticoids differs significantly from that where pre-treatment of the immunosuppressant is used. Recently, immunophilin interchange has been identified as a first step in glucocorticoid receptor activation following ligand activation. We show here that the FK51 (FK506-binding protein 51)–FK52 switch is differentially regulated by glucocorticoid and FK506 treatment strategy.

Key words: arachidonic acid, cell proliferation, FK506-binding protein 51 (FKB51), glucocorticoid receptor, glucocorticoid signalling, immunophilin.

INTRODUCTION

The unactivated GR (glucocorticoid receptor) normally resides in the cytoplasm in a heterocomplex with (Hsps) heat-shock proteins and immunophilins [1,2]. Following ligand binding, the GR is released from this complex and translocates to the nucleus where, in concert with other transcription factors, it regulates the expression of selective genes. This genomic mechanism may take up to several hours to modulate cellular functions, but is thought to account for the major actions of glucocorticoids [3]. However, we have previously described an action of glucocorticoids that involves the rapid inhibition of arachidonic acid release that is difficult to accommodate by such a mechanism [4]. In contrast with the down-regulation of COX2 (cyclo-oxygenase 2) expression, rapid inhibition of arachidonic acid release does not require nuclear translocation of GR, rather it seems the activation of Src is involved instead [4]. A similar Src-dependent mechanism mediates dexamethasone induction of apoptosis in thymocytes [5].

We have shown that a panel of clinically relevant glucocorticoids display widely differing abilities to inhibit arachidonic acid release and, in several instances, this activity is dissociated from the ability to down-regulate COX2 expression [6]. For example, methylprednisolone strongly inhibits arachidonic acid release, but inhibits COX2 expression poorly; whereas fluticasone and mometasone strongly inhibit COX2 expression, but have little effect upon arachidonic acid release. To date, the molecular mechanisms for these differences were unclear.

Hitherto, the first event in activation of GR following ligand binding was thought to be dissociation from Hsp90. However, new data has challenged this assumption where it is apparent that immunophilin interchange of FKB51 (FK506-binding protein 51) and FKB52 actually precedes melting of the GR complex from Hsp90 [7]. This is an important finding because it shows that glucocorticoids can initiate an interchange of cofactors on the GR-

bound Hsp90 template and also provides a potential mechanism of nuclear targeting for the receptor. Indeed, recent results [8] suggest that FKB52 selectively potentiates hormone-dependent genomic responses and this effect is selectively blocked by FKB51, thus providing differential control over steroid signalling events.

In the present paper we extend these findings further by showing that immunophilin interchange is not only glucocorticoid specific, but is also conditioned by the presence of the immunosuppressant FK506. Furthermore, these changes in the GR–immunophilin interaction are crucially dependent on the timing of immunosuppressant administration. Our findings may have important clinical implications in not only interpreting the apparent enhancement of glucocorticoid action by FK506, but also in determining the particular steroid selected by physicians.

EXPERIMENTAL

Cell culture

A549 cells (European Collection of Animal Cell Cultures) were maintained in continuous log-phase growth in DMEM/F-12 (Dulbecco's modified Eagle's medium/F-12) containing Phenol Red and 10% FCS (foetal calf serum) in T175 flasks (Greiner) at 37 °C, 5% CO₂/95 O₂. No antibiotics were used, since this may affect the response to FK506. The cells were not allowed to reach confluence at any time, as this diminishes their response to glucocorticoids.

Cell proliferation experiments

Subconfluent A549 cells were seeded on to 12-place multi-well plates (Falcon) at a density of 5 × 10⁴ cells/ml per well in DMEM/F-12 with 10% FCS. Following incubation overnight,

Abbreviations used: COX, cyclo-oxygenase; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; FKB51/52, FK506-binding protein 51/52; GR, glucocorticoid receptor; Hsp90, heat-shock protein 90; IL-1 β , interleukin 1 β ; PGE₂, prostaglandin E₂.

¹ To whom correspondence should be addressed (e-mail J.Croxtall@qmul.ac.uk).

the cells were washed in 2 ml of sterile PBS and 1 ml of fresh DMEM/F-12 (without Phenol Red) containing FK506 (1 μ M) alone or in simultaneous combination with various glucocorticoids (10 nM), or vehicle control added (either ethanol or DMSO). On day 2, cells were replenished with fresh experimental medium containing test glucocorticoids and FK506 or vehicle control. On day 3, the medium was removed from each well and 1 ml of PBS containing 0.05 % trypsin and 10 mM EDTA was added to each well. The dispersed cells were then counted with a Coulter Multisizer II counter. The percentage inhibition of cell proliferation for each glucocorticoid- and FK506-treated culture was calculated compared with control well. We found this experimental template best revealed the growth inhibitory effects of FK506 and glucocorticoid treatment. Trypan Blue was used to determine cell viability. The results reported are due to inhibition of cell proliferation and not cell death due to toxicity of the glucocorticoids tested.

Measurements of IL-1 β (interleukin 1 β)-stimulated PGE₂ (prostaglandin E₂) release

Subconfluent A549 cells were seeded into 12-place multi-well plates at a density of 5×10^4 cells/ml per well in DMEM/F-12 with 10 % FCS. Following incubation overnight, the cells were washed in 2 ml of sterile PBS and 1 ml of fresh DMEM/F-12 (without Phenol Red) containing FK506 (1 μ M or 10 μ M) added. After 3 h of incubation 1 ng/ml IL-1 β was added for a further 4 h. After which time, 0.5 ml of experimental medium was removed and PGE₂ was measured in the samples using an enzyme immunoassay kit.

Measurement of arachidonic acid release

Subconfluent A549 cells were seeded into 12-place multi-well plates (Falcon) at a density of 3×10^5 cells/ml per well in DMEM/F-12 with 10 % FCS and incubated overnight. ³H-labelled arachidonic acid in ethanol was evaporated to dryness under N₂ and resuspended in an appropriate volume of DMEM/F-12 (without Phenol Red), and after vortex mixing left at 37 °C for 1 h. After the cells had been washed with PBS, 9.25 KBq of [³H]arachidonic acid in 0.5 ml DMEM/F-12 (without Phenol Red or FCS) was added to each well and incubated overnight. The medium containing free [³H]arachidonic acid was then removed and the cells washed three times with 1 ml of DMEM/F-12 containing 1 mg/ml BSA. The cells thus labelled with [³H]arachidonic acid were then treated for 3 h with either FK506 (1 μ M or 10 μ M) alone or in simultaneous combination with the various glucocorticoids (10 nM) or vehicle control (either ethanol or DMSO). Then 10 nM EGF (epidermal growth factor) and 50 nM thapsigargin were added for 30 min. After incubation, 0.4 ml of medium was removed from each well for scintillation counting.

Determination of the expression of COX2

A549 cells were grown in DMEM/F-12 without serum for 24 h in T75 flasks (Greiner). The cells were then incubated with FK506 (1 μ M) alone or in simultaneous combination with the various glucocorticoids (10 nM), or vehicle control (either ethanol or DMSO), for 3 h, then 1 ng/ml IL-1 β was added for a further 4 h. The medium was aspirated and the A549 cell monolayer washed with PBS, 1 mM EDTA to remove adherent surface-bound proteins. The monolayer was dispersed with 0.05 % trypsin in PBS/10 mM EDTA. Cell pellets were snap-frozen in 3 ml of PBS/10 mM EDTA containing 1 mg/ml soya-bean trypsin

inhibitor, 0.01 % leupeptin, 1 mM PMSF and 1 mM sodium orthovanadate. Once thawed, cell lysates were clarified by centrifugation at 13 000 *g* for 20 min. Protein concentrations were measured by Bradford assay and identical concentrations were used and Western blotting carried out to assess COX2 expression.

GR nuclear localization

A549 cells were cultured in T75 flasks (Greiner) in DMEM/F-12 without serum for 24 h. The cells were then incubated with FK506 (1 μ M) alone or in simultaneous combination with the various glucocorticoids (10 nM), or vehicle control for 1 h. The medium was aspirated and the A549 cell monolayer washed with PBS, 1 mM EDTA to remove adherent surface-bound proteins. The monolayer was dispersed with 0.05 % trypsin in PBS/10 mM EDTA. Cell pellets were washed with PBS then resuspended in 0.5 ml of cold 10 mM Hepes, pH 7.9, containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol and 0.5 mM PMSF, and then allowed to swell on ice for 15 min. Following three cycles of freeze-thawing in liquid N₂, the nuclear pellet was precipitated from the cell homogenate by centrifugation at 10 000 *g* for 5 min. The nuclear pellet was resuspended in 0.2 ml of cold 20 mM Hepes, pH 7.9, containing 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and 1 mM PMSF, and vigorously rocked at 4 °C for 15 min to extract the protein component. The clarified supernatant was stored in aliquots at -70 °C. Protein equivalents were Western blotted for GR content using a polyclonal antibody (Autogen Bioclear, Calne, Wiltshire, U.K.) and detected as for COX2.

Immunophilin association with GR

A549 cells were cultured in T175 flasks (Greiner) in DMEM/F-12 without serum for 24 h. The cells were then incubated with FK506 (1 μ M) alone or in simultaneous combination with the various glucocorticoids (10 nM), or vehicle control for 10 min. The medium was aspirated and the A549 cell monolayer washed with PBS/1 mM EDTA to remove adherent surface-bound proteins. The monolayer was dispersed with 0.05 % trypsin in PBS/10 mM EDTA and cell pellets snap-frozen. Total cell lysates (100 μ l) in 0.01 M Tris/HCl, pH 8, containing 0.15 M NaCl, 0.5 % Nonidet P40 and a protease inhibitor cocktail (Boehringer-Mannheim GmbH), were pre-cleared with 20 μ l of Protein A/G-agarose (50:50 mixture; Santa Cruz Biotechnology, San Diego, CA, U.S.A.) and 5 μ l of mouse IgG (0.5 mg/ml) for 1 h at 4 °C. Protein equivalents were immunoprecipitated overnight with either an FKB51 monoclonal or an FKB52 polyclonal antibody, and the immune complex Western blotted with a polyclonal antibody to GR and the signal detected by enhanced chemiluminescence. Western blots were scanned using an Agfa Snapscan 1236S and the image composite transferred into Microsoft PowerPoint on an Apple Macintosh computer. Densitometric analysis was performed with NIH Image 1.63, and the relative band intensities reported as percentage changes within each blot. Each blot was performed three times and the S.E.M. of each densitometric measurement is presented as a percentage. The calculated values are semiquantitative and are only meant to give some relative guide to the ratio of band intensities.

Materials

IL-1 β , EGF, thapsigargin, glucocorticoids (except below) and all other general purpose, cell culture or blotting reagents were from Sigma (Poole, Dorset, U.K.). RU486 was a gift from Roussel-Uclaf (Romainville, France). Arachidonic acid was from NEN

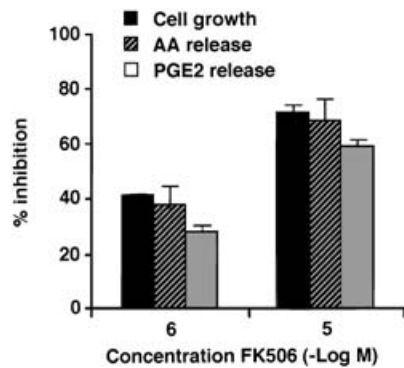


Figure 1 FK506 inhibits the proliferation of A549 cells and the release of arachidonic acid and PGE₂

Cell growth: A549 cells were incubated with either 1 μ M or 10 μ M FK506 and counted on day 3. Vehicle control wells proliferated to $232\,000 \pm 3000$ cells/well. **Arachidonic acid (AA) release:** A549 cells pre-labelled with [³H]arachidonic acid were incubated with 1 μ M or 10 μ M FK506 for 3 h prior to stimulation with 10 nM EGF and 50 nM thapsigargin for 1 h. Vehicle control wells released 4842 ± 330 dpm. **PGE₂ release:** A549 cells were incubated with 1 μ M or 10 μ M FK506 for 3 h prior to stimulation with 1 ng/ml IL-1 β for 4 h. Vehicle control wells released 200 ± 21 ng/ml PGE₂. FK506-treated wells are presented as percentage inhibition compared with vehicle controls. All experiments were performed at 37 °C. Values are the mean of 3 wells. All bars show significant inhibition compared with vehicle controls ($P < 0.05$).

Du Pont (Brussels, Belgium). Western blotting for COX2 and GR was performed using antibodies from Autogen Bioclear. Antisera for FKB51 and FKB52 were from CN Biosciences. PGE₂ enzyme immunoassay kits were from Amersham Biosciences. We thank Dr William Kreutner, Schering-Plough, Kenilworth, NJ, U.S.A., for mometasone furoate and GlaxoSmithKline, Zeist, The Netherlands, for fluticasone propionate. FK506 (Prograft) was purchased from Fujisawa, Japan.

Statistical analysis

Each experiment was performed in triplicate ($n = 3$) and each experiment is a typical example of at least three such experiments. Results were calculated as the means \pm S.E.M. and are presented as the percentage inhibition \pm S.E.M. Statistical differences were calculated using the raw data using the ANOVA test with post-analysis Bonferroni correction. A threshold value of $P < 0.05$ was taken as significant.

RESULTS

We first tested the effect of FK506 used alone upon A549 cell proliferation and pro-inflammatory mediator release (Figure 1). When treated for 3 days we found that 1 μ M FK506 inhibited A549 cell growth by 41% and 10 μ M FK506 by 71% compared with vehicle alone. Since proliferation of A549 cells is under the autocrine control of PGE₂ [9], we tested the effect of FK506 upon the release of arachidonic acid and this prostaglandin. We found that pre-treatment with FK506 for 3 h inhibited the EGF- and thapsigargin-stimulated release of arachidonic acid by $38 \pm 7\%$ at 1 μ M and $68 \pm 8\%$ at 10 μ M compared with vehicle alone. Similarly, IL-1 β (1 ng/ml)-stimulated PGE₂ release (Figure 1) was inhibited by pre-treatment with 1 μ M FK506 ($28 \pm 2\%$) and 10 μ M FK506 ($59 \pm 2\%$).

FK506 is reported to enhance many actions of glucocorticoids [10,11]. We therefore tested the combination of FK506 with

Table 1 FK506 enhances the growth inhibitory response of glucocorticoids

A549 cells were seeded at 50 000 cells/well in 12-place multi-well plates and cultured with 10 nM glucocorticoid, 1 μ M FK506 or both agents together for 3 days. Vehicle control wells proliferated to $340\,000 \pm 4000$ cells/well. Treated cultures are presented as percentage inhibition compared with vehicle control. All experiments were performed at 37 °C. Values are the mean of 3 wells. *Significant inhibition compared with vehicle controls ($P < 0.05$).

Glucocorticoid	Inhibition of cell growth (%)		
	Glucocorticoid alone (10 nM)	FK506 alone (1 μ M)	Both
Dexamethasone	41.8 \pm 0.5*	38.3 \pm 0.5*	84.4 \pm 1.0*
Methyl-prednisolone	19.0 \pm 1.0	35.5 \pm 1.5*	48.2 \pm 4.0*
Fluticasone	43.0 \pm 6.0*	30.4 \pm 3.0*	84.0 \pm 0.6*
Mometasone	47.0 \pm 0.5*	32.0 \pm 0.7*	81.0 \pm 0.5*
RU486	17.2 \pm 2.0	36.2 \pm 2.0*	35.0 \pm 4.0*

Table 2 FK506 enhances the inhibitory effect of glucocorticoids on arachidonic acid release

A549 cells pre-labelled with [³H]arachidonic acid were treated for 3 h with 10 nM glucocorticoid, 1 μ M FK506 or both agents together prior to stimulation with 10 nM EGF and 50 nM thapsigargin for 1 h. Basal release of arachidonic acid was 1118 ± 76 dpm. EGF/thapsigargin-stimulated release containing vehicle control was 4235 ± 42 dpm. Treated cultures are presented as percentage inhibition compared with vehicle control. All experiments were performed at 37 °C. Values are the mean of 3 wells. *Significant inhibition compared with vehicle controls ($P < 0.05$).

Glucocorticoid	Inhibition of arachidonic acid release (%)		
	Glucocorticoid alone (10 nM)	FK506 alone (1 μ M)	Both
Dexamethasone	40.0 \pm 7.0*	27.0 \pm 2.0*	60.0 \pm 4.0*
Methyl-prednisolone	41.0 \pm 7.0*	35.0 \pm 3.0*	66.0 \pm 7.0*
Fluticasone	17 \pm 5.0	27.0 \pm 8.0*	56.0 \pm 6.0*
Mometasone	22.0 \pm 3.0	39.0 \pm 3.0*	74.0 \pm 1.0*
RU486	0.0 \pm 4.0	27.0 \pm 5.0*	35.0 \pm 4.0*

a panel of commonly used glucocorticoids upon A549 cell growth and arachidonic acid release. Using 1 μ M FK506 and an approximate half-maximal concentration of each glucocorticoid (10 nM), we found that the combined use of these agents inhibited A549 cell proliferation to a significantly greater degree than when each agent was used alone (Table 1). No enhanced effect was seen with the GR antagonist RU486. Furthermore, the effect was specific to glucocorticoids as no enhancement was observed with progesterone, testosterone or oestrogen (results not shown).

The effect of the combined use of FK506 and glucocorticoids was more striking when we evaluated arachidonic acid release (Table 2). As we have previously shown, glucocorticoids differ widely in their ability to inhibit the release of this fatty acid [6]. For example, mometasone and fluticasone inhibit arachidonic acid release very poorly, whereas dexamethasone and methyl-prednisolone are much more effective. However, when used in simultaneous combination with FK506 all glucocorticoids tested showed a greatly enhanced inhibitory effect on arachidonic acid release. In particular, this was most apparent with mometasone and fluticasone which have no significant effect on arachidonic acid release when used alone. Again, this effect was specific to glucocorticoids only as no enhancement was seen with RU486, progesterone, testosterone or oestrogen (results not shown).

Pre-treatment with FK506 has been reported in many cases to enhance the ability of glucocorticoids to inhibit the expression of COX2 [11]. However, we show in the present study that when FK506 is used simultaneously with glucocorticoids, then the

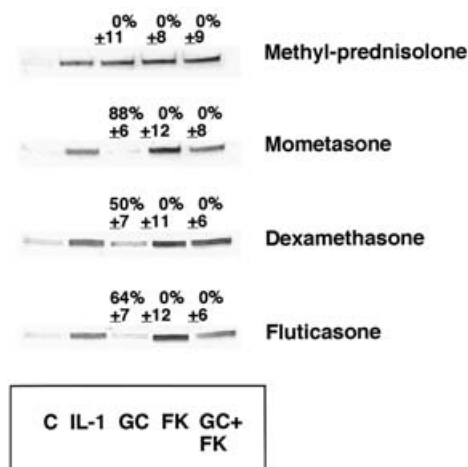


Figure 2 The simultaneous use of FK506 reverses glucocorticoid inhibition of COX2 expression

A549 cells were treated with 10 nM glucocorticoid (GC), 1 μ M FK506 (FK) or both agents together (GC + FK) for 3 h prior to stimulation with 1 ng/ml IL-1 β (IL-1) for 4 h. Samples were analysed for COX2 expression by Western blotting. Values shown are the mean of the percentage inhibition of COX2 expression compared with IL-1 β treatment alone \pm S.E.M. All experiments were performed at 37 $^{\circ}$ C and are representative of three experiments. C, control.

effect on COX2 expression is very different (Figure 2). Again, we have previously shown that different glucocorticoids vary widely in their ability to inhibit COX2 expression and, more importantly, that this ability is not correlated with the ability to inhibit arachidonic acid release [6]. Methyl-prednisolone inhibits the expression of COX2 very poorly, but dexamethasone, mometasone and fluticasone are much more effective. However, when these glucocorticoids are used simultaneously with 1 μ M FK506 for 3 h prior to treatment with IL-1 β , their ability to inhibit COX2 is significantly ($P < 0.05$) reduced. The lack of effect with methyl-prednisolone remains unchanged. This observation is seemingly in contradiction to previously reported data where FK506 enhanced the inhibitory effect of glucocorticoids on MMTV-CAT (mouse mammary tumour virus-chloramphenicol acetyltransferase) and COX2 expression [10,11]. However, the treatment strategy in these reports differs from that used in ours in that FK506 was used for 2 h prior to treatment with glucocorticoid. We found that when FK506 was used in this particular way that the ability of glucocorticoids to inhibit COX2 expression is indeed enhanced (Figure 3). The ability of methyl-prednisolone to down-regulate COX2 expression, however, remains unchanged regardless of FK506 treatment strategy.

We next examined the effect of FK506 on glucocorticoid-induced nuclear localization of GR (Figure 4). We found that 10 nM mometasone, 10 nM fluticasone and 10 nM dexamethasone enhanced the proportion of GR localizing to the nucleus of A549 cells after 1 h of treatment, whereas methyl-prednisolone was without effect. Increasing the concentration of the glucocorticoids to 100 nM produced the same result (results not shown). Similarly, 1 μ M FK506 when used alone for 1 h also resulted in a nuclear localization of GR. However, when used simultaneously, FK506 blocked nuclear uptake elicited by mometasone, fluticasone and dexamethasone. Again these observations are seemingly in contradiction to previously published data where glucocorticoids enhance nuclear uptake [7,10,11]. However, the treatment strategy used in these reports differed from ours in that FK506 was used for 2 h prior to the addition of glucocorticoid, rather than being used simultaneously.

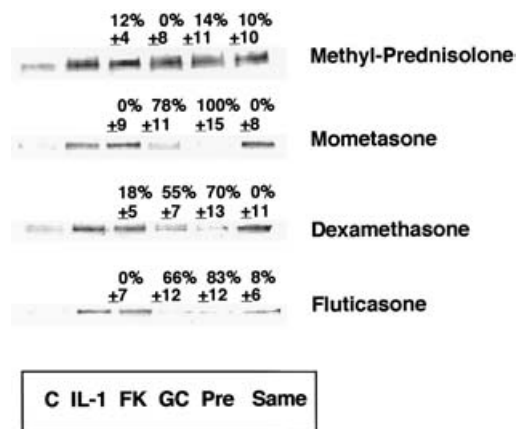


Figure 3 Pre-treatment with FK506 enhances glucocorticoid inhibition of COX2 expression

A549 cells were treated with 1 μ M FK506 (FK) for 3 h, 10 nM glucocorticoid (GC) for 3 h, both agents together (Same) for 3 h, or with FK506 for 2 h followed by glucocorticoid for 1 h (Pre) prior to stimulation with 1 ng/ml IL-1 β . Samples were analysed for COX2 expression by Western blotting. Values shown are the mean of the percentage inhibition of COX2 expression compared with IL-1 β treatment alone \pm S.E.M. All experiments were performed at 37 $^{\circ}$ C and are representative of three experiments. C, control.

Glucocorticoid-induced exchange of the immunophilin FKB51 for FKB52 has been recently shown as an early event in the activation of the GR complex [7,8]. We postulated that the differential modulation of glucocorticoid action, which appeared to depend on the timing of FK506 administration, could be explained by differential modulation of FKB51/FKB52 levels by the various glucocorticoids. We therefore examined immunophilin exchange following our treatment strategy with glucocorticoids and FK506. We were able to measure changes in immunophilin interchange following treatments as short as 10 min (Figure 4). Treatment of A549 cells with 1 μ M FK506 significantly ($P < 0.05$) increased the amount of FKB52 co-localizing with GR from $35 \pm 6\%$ to $100 \pm 17\%$. Similarly, treatment with 10 nM dexamethasone significantly ($P < 0.05$) increased the amount of FKB52 binding from $35 \pm 6\%$ to $69 \pm 7\%$. In both cases the amount of FKB51 co-localizing with GR was proportionally reduced and this was correlated with increased amounts of the receptor residing in the nucleus. Increasing the concentration of dexamethasone to 100 nM raised the proportion of FKB52 binding to $100 \pm 12\%$. Simultaneous treatment with FK506 and dexamethasone reversed nuclear uptake of GR by dexamethasone, but surprisingly the proportion of FKB52 binding the receptor remained unchanged. Perhaps even more surprising, both 10 nM fluticasone and 10 nM mometasone did not significantly induce an immunophilin switch, but nevertheless, brought about nuclear localization of GR. Increasing the concentration of these glucocorticoids to 100 nM did not change this response (results not shown). Paradoxically, 10 nM methyl-prednisolone initiated an immunophilin switch, but did not significantly induce GR nuclear uptake, and again increasing the concentration to 100 nM did not change this response (results not shown). Our results therefore appear to show that although the simultaneous use of FK506 prevents glucocorticoid-induced nuclear uptake of GR, this treatment does not result in a reversal of FKB52 binding. These results further suggest that the correlation of FKB52 binding and nuclear uptake of GR only applies when dexamethasone used alone is the ligand. Other glucocorticoids can seemingly initiate nuclear uptake independently of FKB52 association.

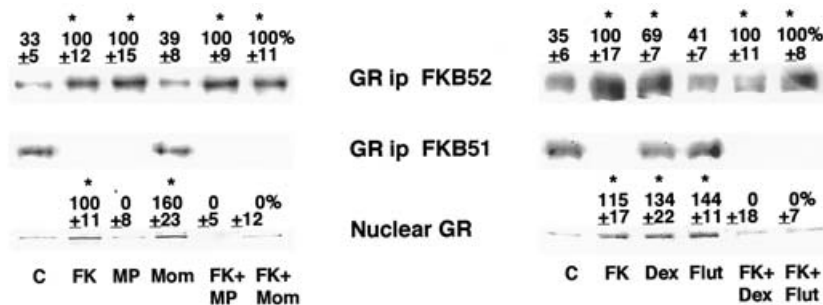


Figure 4 Differential regulation of immunophilin switching and nuclear translocation of GR by glucocorticoids and FK506

For immunophilin analysis: A549 cells were treated with 10 nM glucocorticoid, 1 μ M FK506 or both agents together for 10 min, and snap-frozen. Total cell lysates were immunoprecipitated with either anti-FKB51 or anti-FKB52 antibodies, and then Western blotted for GR content with polyclonal antisera to the receptor. Values shown are percentage FKB52 of total immunophilin associated with GR complex. For nuclear GR analysis: A549 cells were treated with 10 nM glucocorticoid, 1 μ M FK506 or both agents together for 1 h, and snap-frozen. Nuclear extracts of cell pellets were analysed for GR expression by Western blotting. Values shown are percentage increase of nuclear GR compared with control \pm S.E.M. ($n = 3$). *Significantly different from control ($P < 0.05$). All experiments were performed at 37 $^{\circ}$ C and are representative of three experiments. C, control; Dex, Dexamethasone; FK, FK506; Flut, fluticasone; Mom, mometasone; MP, methyl-prednisolone.

DISCUSSION

The effects of glucocorticoids upon nuclear targeting of GR and COX2 expression are reversed by the simultaneous use of FK506. Conversely, the effects of the same glucocorticoids upon arachidonic acid release and A549 cell growth inhibition are enhanced by the simultaneous use of FK506. These findings are seemingly in contradiction to previous reports where FK506 enhances GR translocation to the nucleus by dexamethasone and the suppression of gene expression events [7,10,11]. However, the experimental protocol used in these reports differs from that used by us in that FK506 was used for 2 h prior to the application of glucocorticoid. Clinically, FK506 is administered at the same time as the glucocorticoid and we have used a simultaneous treatment strategy in this report. Two important findings arise from this, firstly, that the timing of FK506 and glucocorticoid treatment, and, secondly, the choice of glucocorticoid may result in a qualitatively different outcome. The molecular mechanisms that underlie these differences are at the present unclear.

A differential potentiation of dexamethasone and triamcinolone acetonide transcriptional activity by FK506 has been demonstrated in mouse fibroblasts, but this was attributed to modulation of steroid uptake [12]. Recently, immunophilin switching has come to be regarded as a key molecular event in nuclear targeting of GR. Our results confirm previous observations that both FK506 and dexamethasone increase FKB52 binding and nuclear localization of GR [7,8]. Increasing the concentration of dexamethasone increases FKB52 binding and GR nuclear uptake. Other glucocorticoids elicit different degrees of FKB52 binding and this, in theory, should account for the varying ability of these steroids in nuclear targeting of GR (Figure 4). However, our observations challenge current notions of the significance of the FKB51–FKB52 switch, since methyl-prednisolone initiates an exchange but does not induce nuclear localization of GR. Similarly, both mometasone and fluticasone induce GR localization, but do not initiate an FKB51–FKB52 switch. Increasing the concentration of these glucocorticoids does not change this outcome. This suggests that our understanding of the importance of FKB52 binding to GR and its involvement in mediating nuclear localization of GR may only be valid when dexamethasone is used as a ligand. FKB51 is thought to selectively block FKB52 binding to GR and thereby regulate glucocorticoid signalling [8]. The function of FKB51 released from the GR complex following glucocorticoid binding is unknown. It may

be that this immunophilin plays a role in interacting with the signalling pathways regulated by glucocorticoids. We should also not forget that there are other regulatory co-factors, such as GRIP-1 (glucocorticoid receptor interacting protein 1) and SRC1 (steroid receptor co-activator 1), that are recruited to activated GR and link with transcription machinery. The differential recruitment of these factors by other glucocorticoids is as yet unknown, but they may also have a significant role to play.

How the timing of FK506 and glucocorticoid treatment may have such profound differences is also unknown. It might be reasonable to postulate that the kinetics of simultaneous FK506 plus glucocorticoid GR binding versus a pre-treatment strategy may have a different effect on immunophilin interchange. When used alone FK506 brings about nuclear localization of GR (Figure 4). The functional meaning of this is unclear. However, in this DNA-bound state the binding affinity of GR for glucocorticoid ligands may be increased, thus explaining the potentiation phenomenon seen in pre-treatment experimental scenarios. The glucocorticoid antagonist RU486 also brings about a melting of the GR complex and induces a nuclear localization. However, in this state GR is capped and glucocorticoid regulation of genomic effects is blocked. It would appear then that FK506 behaves in a manner contrary to glucocorticoid antagonists.

Of the glucocorticoids tested here methyl-prednisolone seemingly behaves uniquely, since it does not induce nuclear uptake of GR and does not block IL-1 β induction of COX2. This would therefore imply that this glucocorticoid elicits effects of cell growth and arachidonic acid release inhibition solely by non-genomic mechanisms. We have previously described that in A549 cells pre-treated with geldanamycin to prevent nuclear uptake of GR the inhibitory responses of dexamethasone remain unchecked [4]. It would therefore appear from this that a significant portion of glucocorticoid activity could be ascribed to non-genomic processes and this is most apparent with methyl-prednisolone.

Clearly this must have implications for interpreting the apparent enhancement of glucocorticoid action in therapeutic use. The observations described here were performed in A549 cells *in vitro*. As glucocorticoids are of major clinical relevance in the treatment of many diseases, it is important to consider how the findings could be translated to a clinical setting. The first important clinical implication from the present study is that not all glucocorticoids are the same. A great variety of different types of glucocorticoid are now available to clinicians. Which glucocorticoids are

used in specific cases is usually a matter of personal choice, since it is believed that glucocorticoids differ from each other in potency only. This study suggests that it may indeed be crucially important which glucocorticoid should be used in a specific clinical setting. This is supported by previous clinical [13–15] and cell line studies [6]. The second finding, which may have clinical implications, is that the effects of co-medication also depend on the choice of glucocorticoid. Glucocorticoids are frequently used in combination with other drugs in the treatment of patients. For example, in asthma glucocorticoids are combined with long-acting β_2 -sympaticomimetics, and it has been shown that this combination has synergic effects [16]. Clinicians now realize that different drugs may interfere with each other at the level of subcellular signalling pathways. The finding that different glucocorticoids may vary in their subcellular effects may be an important factor in the decision of which glucocorticoid to combine with another drug. Another important example of combination therapy comes from the field of transplantation. Here, glucocorticoids are often combined with calcineurin inhibitors, such as FK506 or cyclosporin A, and the results from this study may help to explain the subcellular interactions between both drugs. Furthermore, the results presented in the present study suggest that the effectiveness of combination therapy may again depend on the choice of glucocorticoid. More studies are needed to fully understand the molecular interactions of different drugs such that this may be translated into more effective combination therapies.

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