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Research Article

FK506 sensitizes mammalian cells to high osmolarity by modulating p38 MAP kinase activation

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Abstract. The immunosuppressants tacrolimus (FK506) and cyclosporin A (CsA) have increased the survival rates in organ transplantation. Both drugs inhibit the protein phosphatase calcineurin (CaN) in activated T cells, exhibiting similar side-effects. Diabetes is observed more often in FK506 than CsA therapy, probably due to inhibition of new molecular targets other than CaN. We studied FK506 toxicity in mammalian cells. FK506, but not CsA, regulated p38 activation by osmotic stress, and decreased viability in osmostressed cells. In addition, FK506 treatment strongly increased the phosphorylation of the eukaryotic initiation factor-2α (eIF-2α) subunit. eIF-2α phosphorylation, p38 inhibition and cell lethality were relieved by addition of excess amino acids to the medium, suggesting that amino acid availability mediated FK506 toxicity. Therefore, these FK506-dependent responses could be relevant to the non-therapeutic effects of FK506 therapy.

Key words. Immunosuppressant; osmotic stress; MAP kinase; amino acid availability; translational control.

In response to enviromental stress, eukaryotic cells trigger regulatory mechanisms to increase cell survival and proliferation. Most prominent amongst these are the mitogen-activated protein kinase (MAPK) cascades. These signalling pathways, highly conserved throughout eukaryotes, are composed of a set of sequentially acting serine-threonine protein kinases, including a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK. MAPK pathways are also negatively regulated by dephosphorylation through MAPK phosphatases [1]. In mammalian cells, exposure to high osmolarity affects a family of MAPKs known as stress-activated MAPKs (SAPKs) [1]. SAPKs include the p38 and the c-jun N-terminal kinase (JNK) families. SAPK targets involved in transcriptional regulation, post-transcriptional and translational control of gene expression and cell cycle progression have been described [1, 2]. This reflects the pivotal role played by SAPKs in many aspects of eukaryotic cell physiology such as growth and development, disease and apoptosis [1].

Tacrolimus (FK506) and cyclosporin A (CsA) are immunosuppressants used to prevent allograft rejection after organ transplantation. FK506 and CsA, by complexing with the immunophilins FK506-binding protein 12 (FKBP12) and cyclophilin, respectively, block the activitiy of the calcium-calmodulin-activated protein phosphatase calcineurin [3]. Calcineurin inhibition prevents nuclear translocation of the nuclear factor of activated T cells (NF-AT) and interleukin-2 gene expression. FK506

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and CsA therapies are associated with non-therapeutic toxicities [4] and post-transplant diabetes mellitus (PTDM) is frequently observed [5]. Recent studies have confirmed the greater diabetogenic potential of FK506 versus CsA in transplant patients [5, 6] suggesting that new targets other than calcineurin might be involved in FK506 toxicity. The exact nature of these new targets has yet to be determined.

Budding yeast *Saccharomyces cerevisiae* is a valuable tool for understanding basic cellular functions conserved among simple eukaryotic systems and mammalian cells. Thus, genetic analysis in this model organism can be used to identify target(s) for drugs that are effective in both model systems but have unknown mechanisms of action [7]. FK506 has been shown to inhibit amino acid import and to induce translation of the GCN4 transcription factor mRNA in budding yeast [8]. GCN4 translation is controlled by a regulatory pathway that detects nutrient availability, called general amino acid control (GCN) [9]. Nutrient starvation conditions activate the Gcn2p protein kinase which, in turn, phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF-2*)* at serine 51 [9]. Phosphorylation of eIF-2 inhibits translation of cellular mRNAs at the initation step and favours selective translation of GCN4 mRNA mediated by four short open reading frames located in the 5′ untranslated region of GCN4 (uORFs). High levels of Gcn4p stimulate expression of genes involved in amino acid biosynthesis [10]. Phosphorylation of eIF-2α as a mediator of the translation efficiency in response to environmental conditions is conserved throughout evolution [11]. In mammals, stress conditions like nutrient deprivation, heat shock, endoplasmic reticulum (ER) stress and oxidative damage also evoke gene-specific translational control mediated by eIF-2α kinases [11].

We previously reported that FK506 increased the sensitivity of yeast cells and regulated the activity of the yeast p38 SAPK orthologue Hog1p in a Gcn2p-dependent manner [12]. Here we investigated FK506 toxicity in mammalian cells. FK506 treatment modulated p38 SAPK activation and cell viability upon osmotic stress in human HEK293T cells, and incremented phosphorylation of eIF-2α. Moreover, excess amino acids/tryptophan overcame FK506 toxic effects, suggesting that amino acid availability mediated FK506 toxicity. These effects seemed to be mediated by Hog1/p38 tyrosine phosphatases, at least in yeast cells. As alterations in translational and/or SAPK pathways are often associated with diabetes, this mechanism of FK506 action could be of significance to the secondary effects of FK506 reported in transplant patients.

Materials and methods

Mammalian cell lines, culture conditions and reagents

Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose and supplemented with 10% fetal calf serum and 1 mM L-glutamine. For preparation of DMEM containing two- or fourfold molar excess amino acids, a ×50 concentrated amino acid solution (GIBCO) was diluted in DMEM to a ×2 or ×4 final concentration, respectively. DMEM containing fourfold molar excess of tryptophan was prepared by addition of tryptophan (DIFCO) to a final concentration of 40.8 mg/l. Cells were cultured at a density of 10^6 cells/100-mm plate. For sorbitol treatment, cells were cultured with medium containing 0.5% FBS for 24 h, then the medium was removed and cells were treated with the corresponding doses of FK506 (kindly provided by Fujisawa Inc.) or CsA (Novartis) for 1 h. FK506 and CsA were prepared as stock solutions (1 mg/ml) in ethanol and serially diluted in DMEM at the desired doses. Corresponding amounts of ethanol were added in each sample to normalize solvent concentration. Cells were then stimulated with 0.5 M sorbitol. Sorbitol (Sigma) was prepared in distilled water and added to the cells at 80% confluence.

Yeast strains and growth assays

The strains used have the *W303-1a* genetic background (*MATa, ade2-11, can1-11, his3-11, leu2-3, trp1-11, ura3-1*). The *ptp2*, *ptp3* and *ptp2,3* mutants have been described elsewhere [13]. Standard methods for yeast culture and manipulations were used [14]. Mid-log yeast cultures in YPD medium (2% glucose, 2% peptone and 1% yeast extract) were tenfold serially diluted and volumes of around 3 µl were dropped with a stainless steel replicator (SIGMA) on solid plates containing 2% Bacto Agar (DIFCO) and YPD medium with or without fourfold excess tryptophan (0.4 mg/ml) (Sigma) with the corresponding doses of FK506 and/or sorbitol. Growth was recorded after 2–3 days in all cases.

Immunoblotting

To prepare protein extracts, whole-cells lysates were prepared in lysis buffer [25 mM HEPES (pH 7.5), 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM dithiothreitol, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 0.5 mM phenylmethanesulphonyl fluoride (PMSF), 1 µg/ml leupeptin, 0.5 µg/ml pepstatin and aprotinin]. For immunodetection, 20 µg of total cellular protein was subjected to SDS-polyacrylamide gel electrophoresis and transferred to PVDF (Immobilon-P; Millipore) filters. Phosphorylated forms of p38 were detected with an antiphospho-p38 antibody (Thr180/ Tyr182) from Cell Signaling Technology. Phosphorylated forms of JNK1 and JNK2 were detected with an anti-phospho JNK antibody

(Promega). Phosphorylated forms of eIF-2α were detected with anti-phospho eIF-2α antibody (Ser51) from Cell Signaling Technology. MKP1 and MKP2 levels were measured with anti-MKP1 and anti-MKP2 antibodies (Santa Cruz Biotechnology). Even loading of the gels was measured with an anti-p38 antibody (aa 341–360) (Santa Cruz Biotechnology) and an anti-eIF-2α antibody (Cell Signaling Technology). Immunocomplexes were visualized by enhanced chemiluminiscence detection (Amersham) using a goat anti-rabbit IgG-(HRP) conjugate (Bio-Rad). Figures show a representative experiment of at least two independent experiments with identical results.

FACS cytometry analysis

HEK293T cells were either pretreated with FK506 or SB203580 (Calbiochem) for 1 h, and then stimulated with 0.5 M sorbitol. Five hours later, adherent and non-adherent cells were harvested. Approximately 10^6 cells were further washed in PBS and incubated with 25 µg of propidium iodide (PI; Sigma) per millilitre. Data were plotted by using CellQuest software; 10,000 events were analysed for each sample. (Becton Dickinson Immunocytometry Systems).

Results

FK506, but not CsA, inhibits activation of the p38 SAPK by osmotic stress in mammalian cells

We previously reported that FK506 modulated osmotic stress activation of Hog1p MAPK in yeast [12]. The closest relatives of Hog1p in mammals are the p38, JNK1 and JNK2 SAPKs [1]. p38 and JNKs are activated by concomitant tyrosine and threonine phosphorylation catalysed by their respective MAPKKs [1]. Therefore, we studied FK506 effects on p38 and JNK phosphorylation in human HEK293T cells upon osmotic stress. The phosphorylation status of p38 and JNKs can be monitored by Western blot using commercially available antibodies specifically recognizing the phosphorylated (active) forms of the respective kinases. As shown in figure 1 A, FK506 treatment blocked p38 activation 30 min after sorbitol exposure in a dose-dependent manner. FK506 did not affect p38 activation by sorbitol at shorter time points (not shown), suggesting that the drug induced p38 down-regulation rather than interfering with p38 activation. JNK activation by sorbitol remained unaffected by FK506 treatment (fig. 1 A). Moreover, CsA had no effect

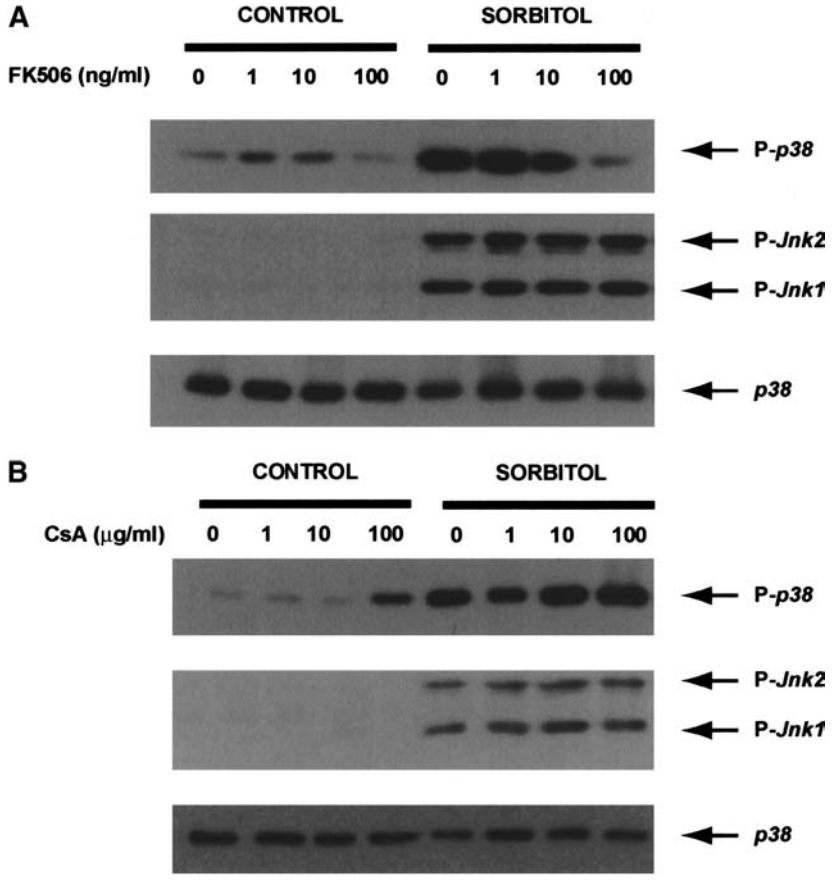

Figure 1. FK506, but not CsA, treatment inhibits p38 activation by high osmolarity in mammalian cells. HEK293T cells were treated for 1 h with increasing doses of FK506 (*A*) or CsA (*B*) as indicated, and exposed or not to 0.5 M sorbitol for 30 min. Phospho-p38, p38 and phospho-JNK1,2 levels were detected by Western blot as described in Materials and methods.

on p38 activation by osmotic stress at doses 1000-fold higher (fig. 1B). Interestingly, high doses of CsA activated p38 in the absence of osmotic stress, probably due to other effects described for equivalent CsA treaments, such as free radical generation and oxidative stress [15–17]. Taken together, these data suggest that FK506, but not CsA, was able to modulate p38 activation by osmotic stress in human cells.

Amino acid/tryptophan availability relieves the negative effect of FK506 on p38 activation

FK506 impairs yeast cell growth by inhibiting tryptophan, histidine and leucine import in strains auxotrophic for these amino acids. Accordingly, prototrophy for these amino acids/excess tryptophan alleviated the growth defect [8, 12, 18]. Moreover, tryptophan availability was shown to mediate FK506 effects on yeast Hog1p MAPK activation by osmotic stress [12]. Therefore, we explored whether the FK506 effect on p38 phosphorylation was also regulated by amino acid availability. We treated human HEK293T cells with FK506 in media containing a twofold/fourfold molar excess of amino acids (see Materials and methods) and measured activation of p38 by osmotic stress under these conditions. As shown in figure 2A, FK506 treatment did not affect p38 activation in a medium containing excess amino acids. Addition of a fourfold molar excess tryptophan to the medium relieved FK506-induced p38 inhibition in osmostressed HEK293T cells (fig. 2B). The effect was specific for tryptophan, because excess of other amino acids like leucine or histidine did not relieve the inhibition (data not shown). Overall, these results suggest that FK506 effects on p38 phosphorylation status were somehow linked to amino acid availability, and more specifically to tryptophan availability.

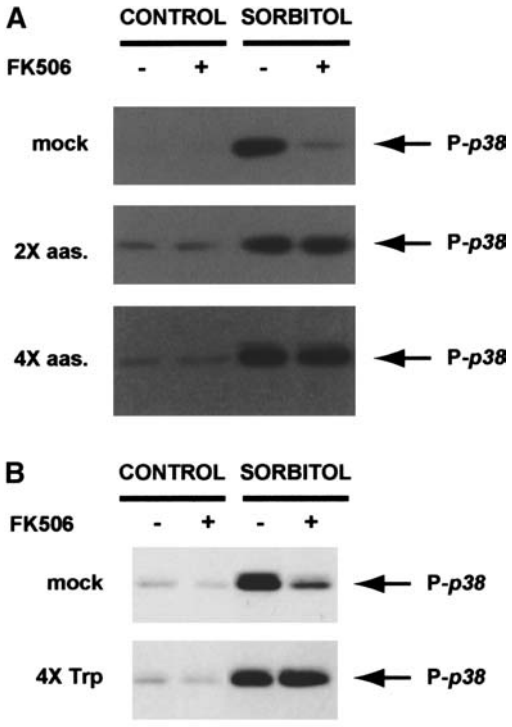

Figure 2. Excess amino acids modulate the negative effect of FK506 on p38 activation by osmotic stress. (*A*) Immunodetection of p38 phosphorylation in HEK293T cells growing in standard (mock) medium and in medium supplemented with two- (2X aas) or fourfold excess (4X aas) amino acids. Cells were pretreated (+) or not (–) with FK506 (100 ng/ml) for 1 h. Sorbitol treatment was as in figure 1. (*B*) Immunodetection of p38 phosphorylation in HEK293T cells growing in standard (mock) medium or in medium supplemented with fourfold excess tryptophan (4X Trp). Sorbitol and FK506 treatments as in (*A*).

Involvement of Hog1/p38 phosphatases in FK506 effects on p38 activity in yeast and mammalian cells

The effect of FK506 and excess tryptophan on p38 activation could be mediated by phosphatase(s) responsible for the Thr180/Tyr182 dephosphorylation of p38. We first tested this hypothesis using a genetic approach in budding yeast. FK506 modulates activation of Hog1p/p38 MAPK in yeast, leading to sensitivity to high osmolarity [12]. The major regulators of Hog1p activity are the partially redundant Ptp2p and Ptp3p protein tyrosine phosphatases [19, 20]. Thus, we analysed the growth of the single *ptp*2 and *ptp3* mutants as well as the *ptp2,3* double mutant in the presence of sorbitol and/or FK506. Disruption of *PTP2,* but not *PTP3*, substantially reduced FK506 toxicity upon osmotic shock (fig. 3A). Furthermore, excess tryptophan did not alleviate FK506 toxicity in the yeast *ptp2,3* double mutant. These results indicate that FK506 and excess tryptophan effects in yeast were somehow mediated by Ptp2p and Ptp3p phosphatases. MAPK inactivation in mammalian cells is mediated by dual-specificity MAPK phosphatases (MKPs) which are induced by enviromental stress and growth factors [21–23]. Two prevalent isoforms of MKPs, MKP1 and MKP2, are present in a wide variety of cell types [22]. Thus, we tested whether FK506 treatment modulated the expression of the MKP1 and MKP2 phosphatases in HEK293T cells under osmotic shock. We could not detect any increase in MKP1 and MPK2 levels at any FK506 dose tested (fig. 3B) even after long exposure of the filters during Western blot procedures (not shown). Furthermore, pre-incubation of cells with the tyrosine phosphatase inhibitor orthovanadate did not relieve down-regulation of p38 activation by FK506 (not shown). Taken together, these data suggest that the FK506 effects on p38 in mammalian cells were somehow mediated by p38 regulators other than tyrosine phosphatases and the MKP1,2 phosphatases.

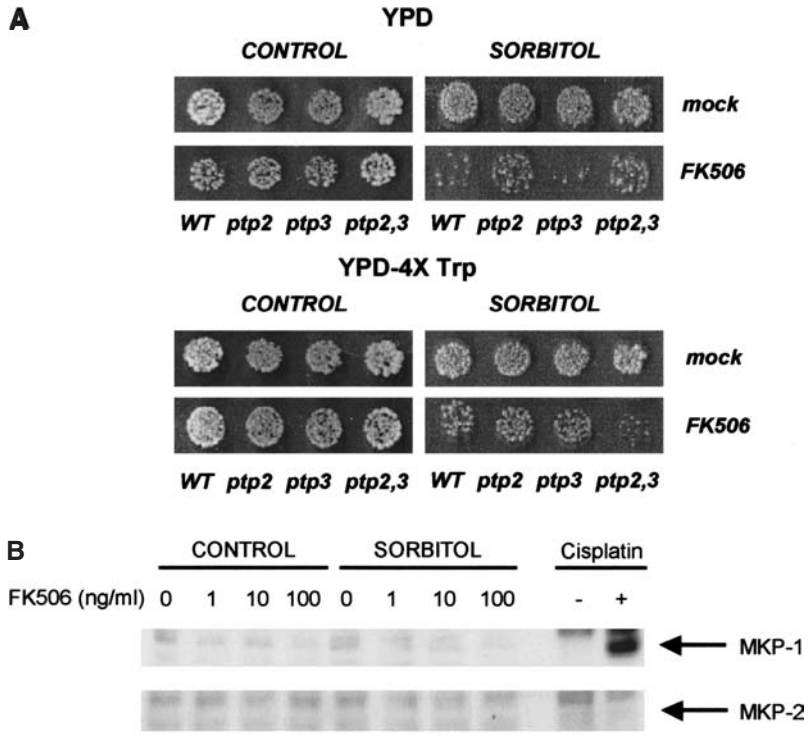

Figure 3. Role of Hog1p/p38 phosphatases in FK506 toxicity in yeast and mammalian cells. (*A*) Growth of WT, *ptp2*, *ptp3* and *ptp2,3* yeast strains on YPD plates containing FK506 (50 µg/ml) and/or 1 M sorbitol and supplemented (YPD-4X Trp) or not (YPD) with fourfold excess tryptophan. (*B*) Immunodetection of MKP1 and MKP2 levels in HEK293T cells treated for 1 h with the indicated doses of FK506. Induction of MKP1 was confirmed by treatment with 10 µg/ml of the DNA damage agent cisplatin. Sorbitol treatment was as in figure 1.

FK506 treatment increases phosphorylation of eIF-2α in an extracellular tryptophan-dependent manner

Nutrient deprivation in yeast and mammals inhibits translation initiation by activation of the Gcn2p protein kinase which phosphorylates the α subunit of eukaryotic initiation factor eIF-2 [11]. As FK506 treatment increased Gcn2p-dependent phosphorylation of eIF-2α in budding yeast [8, 12, 18], we analysed the eIF-2α phosphorylation status in HEK293T cells treated with FK506 in the absence of osmotic stress. FK506 increased eIF-2α phosphorylation in a dose-dependent manner (fig. 4A). We then investigated whether tryptophan availability modulated the increase in eIF-2α phosphorylation after FK506 addition. As shown in figure 4B, pre-incubation of cells with excess tryptophan overcame the FK506-dependent increase in phospho-eIF-2α levels. Sorbitol treatment stimulated eIF-2α phosphorylation as described elsewhere [24–26], but it remained unaffected by excess tryptophan. Therefore, FK506 treatment activated the translational control pathway in human cells and tryptophan availability was somehow involved in this process.

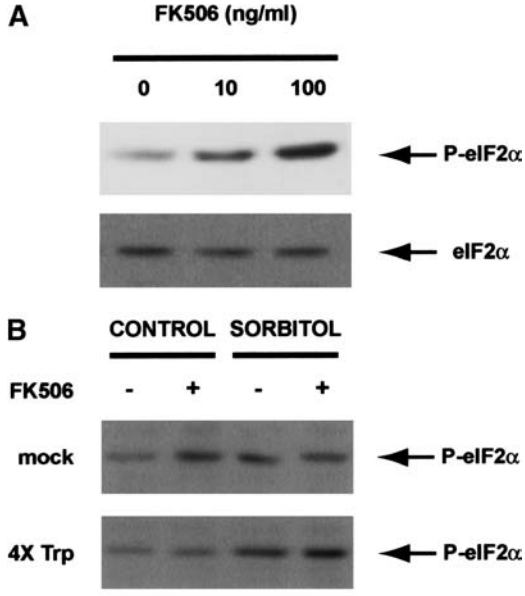

Figure 4. FK506 treatment increases phosphorylation of eIF-2α in an extracellular tryptophan-dependent manner. (*A*) Analysis of eIF-2α phosphorylation in HEK293T cells treated for 1 h with the indicated doses of FK506. (*B*) Immunodetection of eIF-2α phosphorylation in HEK293 cells growing in standard (mock) medium or in medium with fourfold excess tryptophan (4X Trp). FK506 and sorbitol treatments were as in figure 2.

Inhibition of p38 by FK506 sensitizes mammalian cells to sorbitol-induced cell death

In mammalian cells, many osmotic stress-induced responses require activation of p38 [1, 27, 28]. Thus, we tested whether lack of activation of p38 by FK506 treatment affected cell viability under osmotic stress by flow cytometry. FK506 treatment dramatically increased the number of apoptotic and necrotic cells in hypertonic medium (fig. 5). Interestingly, excess amino acids abolished both apoptosis and necrosis elicited by FK506 treatment (fig. 5). Cells were also treated with the specific inhibitor SB203580 at doses not affecting JNK activation [29], and then exposed to hypertonic medium. The eIF-2α phosphorylation status was not affected by SB203580 under these conditions (not shown). As expected, SB203580 increased cell death to the same extent as FK506 (fig. 5). However, SB203580 induced massive cell death in excess amino acid-containing medium even in the absence of osmotic stress (not shown). This was expected, as SB203580 treatment greatly enhances cell sensitivity to mild osmotic stresses like that produced by adding fourfold excess amino acids. Therefore, these data suggest that FK506-induced p38 inhibition was responsible for the observed cell death.

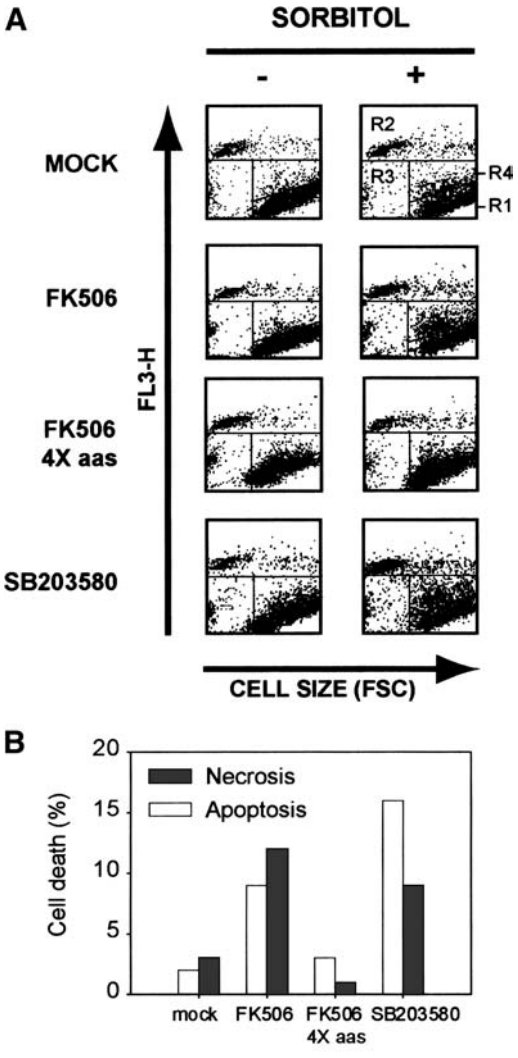

Figure 5. FK506 treatment decreases cell viability upon osmotic shock in an extracellular amino acid-dependent manner. (*A*) Flow cytometry analysis of HEK293T cells grown in standard medium or in medium supplemented with fourfold excess amino acids (4X aas) and treated or not with FK506 100 ng/ml or SB203580 10 µM for 1 h. Cells were exposed to 0.5 M sorbitol and harvested 5 h later. (*B*) Apoptotic (□) and necrotic (■) cells were quantitated as described in Materials and methods. R1, viable cells; R2 + R3, apoptotic cells; R4, necrotic cells.

Discussion

In this report, we have studied FK506 toxicity in human cells. FK506 treatment induced phosphorylation of the eukaryotic initiation factor eIF-2α subunit in HEK293T cells, and compromised p38 activation and cell viability upon osmotic shock. Excess amino acids/tryptophan relieved FK506 toxic effects, suggesting that amino acid availability, and more specifically tryptophan availability, was somehow linked to FK506 toxicity. These findings (i) highlight the existence of new FK506-specific targets and (ii) suggest a functional link between the p38 and translational control pathways.

FK506 has been shown to induce sustained tryptophan deprivation in budding yeast [8, 12, 18]. The present data suggest that FK506 might also induce tryptophan deprivation in mammalian cells: (i) FK506 treatment increased eIF-2α phosphorylation, which responds to a deficiency of essential amino acids in mammalian cells [30–32] and (ii) excess tryptophan abolished FK506 toxicity in HEK293T cells. How FK506 might induce amino acid starvation in mammalian cells remains to be determined. In yeast, this has been proposed to involve perturbations of plasma membrane architecture [8, 12, 18]. Therefore, FK506 treatment might also induce similar perturbations in mammalian cells. As amino acids regulate signalling pathways and gene expression [31], FK506-induced amino acid deficiency would interfere with cellular responses to stress conditions. Furthermore, considering that amino acids play important roles in insulin secretion and glucose homeostasis [30, 33], the effect of FK506 on amino acid availability would also contribute to FK506-induced diabetes.

FK506 treatment increased eIF-2α phosphorylation at serine 51 in HEK293T cells. This phosphorylation event is mediated by at least four protein kinases: PKR (double-stranded RNA-activated protein kinase), HRI (heme-regulated inhibitor), PERK (PKR-like endoplasmic reticulum-associated protein kinase) and GCN2 (general control non-derepressing kinase) [11]. eIF-2α kinases are activated in response to a variety of stress conditions including viral infection, ER stress, nutrient deprivation,

DNA damage, exposure to heat and high osmolarity [11, 24]. As FK506 toxicity was modulated by amino acid availability, and amino acid deprivation in mammals triggers GCN2-dependent phosphorylation of eIF-2α [30], the most plausible candidate mediating phosphorylation of eIF-2α by FK506 would be GCN2. The identification of the specific eIF-2α kinase(s) activated by FK506 treatment is currently underway. In yeast, Gcn2p is activated by binding to uncharged tRNAs, which accumulate in amino acid-starved cells [9]. However, other activation mechanisms not involving sensing of uncharged tRNAs have also been described [10]. High osmolarity was shown to inhibit translation initiation in yeast through a novel pathway not involving amino acid sensing [34]. Accordingly, sorbitol treatment stimulated eIF-2α phosphorylation in HEK293T cells (fig. 4B) which remained unaffected by excess tryptophan, suggesting that a similar mechanism could be operating in mammalian cells.

FK506 abolished p38 activation upon osmotic shock in a dose-dependent manner and incubations with 1–100 µg/ml CsA did not affect p38 activation by sorbitol. As calcineurin signalling is fully prevented by 5 µg/ml CsA in cell-based assays [35] and JNKs remained unaffected by FK506/CsA, these data indicate that (i) calcineurin is not involved in FK506-dependent p38 down-regulation and (ii) it might be mediated by a specific regulator of p38 function. This was the case in yeast, where deletion of Ptp2p, a negative regulator of Hog1/p38 [19, 20], relieved FK506 toxicity. The protective effect of tryptophan in yeast would then be explained by its ability to regulate Hog1p/p38 phosphatase function. Accordingly, excess tryptophan did not relieve FK506 toxicity in a yeast *ptp2,3* double mutant. However, FK506 treatment did not induce expression of the p38 phosphatases MKP1,2 in human cells. As MKP1,2 are labile proteins [36] and their function is mainly regulated at the transcriptional level [21–23], this finding would exclude MKP1,2 as mediators of FK506 toxic effects on p38. Moreover, treatment with the unspecific tyrosine phosphatase inhibitor orthovanadate did not affect FK506-induced down-regulation of p38 activity. This apparent discrepancy observed between yeast and mammalian cells would be explained by the different regulatory mechanisms of MAPK signalling operating in both model systems [2]. Alternatively, p38 regulators other than tyrosine phosphatases and MKP1,2 would be involved, such as serine-threonine phosphatases like PP2A [37, 38]. Additionally, p38 activation could be controlled directly or indirectly via eIF-2α phosphorylation and/or eIF-2α kinases, respectively, as described elsewhere [12, 39, 40]. Further experiments are required to discriminate between these possibilities.

All the FK506 responses described above occurred at therapeutic concentrations [41], suggesting that modulation of p38 and eIF-2α phosphorylation by the drug might contribute to immunosuppression. Indeed, FK506 treatment was shown to be relevant for suppression of allograft rejection by preventing p38 activation, in a rat model of small bowel transplantion [42]. Moreover, FK506 inhibited p38 activation in Jurkat cells as well as calcineurin, both being required for efficient immunosuppression [43], and tryptophan deprivation sensitized T cells to apoptosis prior to cell division [44]. On the other hand, patients treated with FK506 presented diminished insulin secretion [5, 6] and FK506 is known to interfere with insulin secretion in pancreatic β cells [45]. Insulin synthesis is regulated primarily at the translational level and the involvement of translational control in diabetes has been recently underscored [46–48]. Thus, regulation of translation initiation by FK506-induced eIF-2α phosphorylation could contribute to FK506-induced diabetes. Modulation of p38 activity by FK506 treatment could also be considered. p38 activation controls glucose-induced insulin gene expression in pancreatic β cells [49, 50] and insulin-dependent glucose uptake in muscle and adipose tissue cells [51]. As alterations in translational control and p38 signalling pathways can contribute to human disease [1, 52, 53], of interest would be to determine whether variations at one or more loci that regulate eIF-2α and p38 phosphorylation are important factors predisposing to FK506 toxicity in transplant patients under FK506-based therapies.

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