The hypomagnesaemic action of FK506: urinary excretion of magnesium and calcium and the role of parathyroid hormone

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A B S T R A C T

A side-effect of the immunosuppressive drug FK506 (Prograf; tacrolimus) is hypomagnesaemia. We have investigated the effects of short-term (7-day) treatment of rats with FK506, using a protocol designed to indicate whether there are modifications in the renal tubular handling of magnesium and other electrolytes, or in the tissue deposition of magnesium, which may account for the hypomagnesaemia. We have also investigated whether parathyroid hormone has a role in the observed hypomagnesaemia. Two studies have been performed; in the first we administered FK506 (0.5 mg·kg\(^{-1}\) body weight·day\(^{-1}\)) or vehicle by intraperitoneal injection for 7 days, and then housed the rats in metabolic cages for the 24 h collection of urine. At the end of the metabolic cage period, the animals were anaesthetized, and blood and tissue samples were taken for analysis. In the second set of experiments the dosage regime was identical, but at the end of the treatment period the animals were anaesthetized for implantation of arterial and venous cannulae, and then received a saline (plus inulin) infusion for 6 h, during which time blood and urine samples were collected. The dose of FK506 employed did not decrease the glomerular filtration rate. FK506 elicited hypomagnesaemia in both sets of experiments, accompanied by inappropriately high fractional excretion of magnesium. There was also evidence of disruption of the normal renal reabsorption of calcium, but this did not result in hypocalcaemia. Plasma parathyroid hormone activity was not significantly different between the two groups, and there was no evidence of altered tissue content of magnesium in kidney, liver, heart, skeletal muscle or bone. The study confirms that hypomagnesaemia is a significant side-effect of FK506, even at a relatively low dose which did not decrease the glomerular filtration rate. The effect is not due to a decrease in parathyroid hormone release, or to translocation of magnesium from plasma to tissues, but does reflect decreased renal tubular magnesium (and calcium) reabsorption.

I N T R O D U C T I O N

FK506 (Prograf; tacrolimus) is a potent immunosuppressive drug, produced as a fermentation product from Streptomyces tsukubaensis. On a weight-for-weight basis, it is about 100 times more potent than cyclosporin A as an immunosuppressive agent in in vitro tests [1], and it was introduced for organ-transplant patients as a potential successor to cyclosporin A. Initial trials suggested that the adverse effects of FK506 (notably nephrotoxicity) were less severe than those elicited by cyclosporin A [2]. Nevertheless, FK506 does have adverse effects, and it shares with cyclosporin A and rapamycin the tendency to produce hypomagnesaemia. The mech-
anism of this effect is still unclear, but with all three drugs the hypomagnesaemia is associated with inappropriately high fractional excretion of magnesium [3].

The kidneys play a major role in magnesium homeostasis, and the excretion of magnesium in the urine normally reflects dietary magnesium absorption (for a review, see [4]). However, many of the details of renal magnesium reabsorption are still unclear [4]. About 70–80% of the plasma magnesium is ultrafilterable, but only approx. 15% of filtered magnesium is reabsorbed proximally, so that the proximal tubular luminal magnesium concentration increases progressively as a consequence of water absorption. The concentration of magnesium in the tubular fluid entering the loops of Henle is typically 1–1.5 mmol·L⁻¹.

Most of the filtered magnesium (60%) is reabsorbed in the loop of Henle, mainly in the cortical thick ascending limb. Although most (90%) magnesium absorption at this site is paracellular, it is reported to be influenced by many hormones, including parathyroid hormone (PTH), calcitomin, glucagon, vasopressin, insulin and steroid hormones [5]. The increased magnesium absorption elicited by these hormones occurs as a consequence of an increase in the transepithelial voltage (i.e. an increase in the lumen-positive potential), thereby increasing magnesium absorption via the paracellular pathway [6]. In the distal tubule, where transcellular magnesium absorption occurs [6], micropuncture studies have demonstrated that PTH also significantly increases magnesium transport [7].

Andoh et al. [3] have reported that many of the nephrotoxic effects of FK506 and cyclosporin A are associated with inhibition of the renal phosphatase, calcineurin. FK506 complexes with an intracellular binding protein (FKBP), and although the immunosuppressive and nephrotoxic effects of FK506 may be linked to the inhibitory effects of this complex on calcineurin [8], the hypomagnesaemic effect appears to be independent of calcineurin.

The present study was undertaken to investigate the alterations in renal electrolyte handling associated with the early hypomagnesaemic effect of FK506. We have investigated the hypomagnesaemic action of FK506, its relationship to tissue concentrations of magnesium, to calcium homeostasis and to body fluid volume, and whether alterations in plasma PTH levels are involved in compensatory or causative changes in magnesium balance induced by FK506. Receptors for PTH are present in the thick ascending limb, linked to adenylate cyclase, and PTH increases magnesium absorption at this site [9]. PTH enhances the transepithelial potential difference, but the increase in magnesium absorption induced by the hormone is greater than can be accounted for by the voltage change alone, so it is thought that the paracellular magnesium permeability is also enhanced by PTH [6]. It therefore seemed possible that a fall in the plasma PTH concentration could be responsible for the increase in fractional magnesium excretion elicited by FK506, and this too was investigated in the present study.

**MATERIALS AND METHODS**

**Animals**

Male Sprague–Dawley rats (Charles River) were housed in a light- and temperature-controlled environment, and were allowed free access to tap water and to a standard diet containing Na⁺ (0.25%), K⁺ (0.66%), Mg²⁺ (0.22%) and Ca²⁺ (0.71%). The animals were weighed and examined daily.

Each animal received, via intraperitoneal injection, either FK506 (Fujisawa Pharmaceutical Co.), 0.5 mg·kg⁻¹·day⁻¹, in 0.9% NaCl solution (delivered in a volume of 2.4 ml·kg⁻¹ body weight), or the FK506 vehicle delivered in an identical volume. This treatment continued for 7 days.

**Metabolic cage studies**

Following the final injection, each animal (control group, n = 10; FK506 group, n = 9) was transferred to an individual metabolic chamber (Metabowl; TechnoPlast) for 24 h urine collection. Free access to food and water continued.

At the end of the 24 h metabolic period, each animal was weighed and its urine sample was collected. The animals were then anaesthetized with 1 ml·kg⁻¹ body weight Sagatal (sodium pentobarbitone; 60 mg·ml⁻¹), and a carotid cannula was inserted for the removal of blood (3 ml). Further Sagatal (1 ml) was then administered to kill the animals, prior to removal of tissue samples (see below).

**Infusion studies**

At 24 h after the final FK506 (n = 8) or vehicle (n = 9) injection, the animals used for the infusion studies were anaesthetized with ether, and a tail artery and a tail vein were cannulated using flexible polypropylene tubing (PP25), containing 0.7% NaCl solution (venous line) or heparinized 0.7% NaCl solution (arterial line). Following the surgery, the animals were placed in individual Perspex restraining cages and, after recovery from the anaesthetic, each rat received a 6 h infusion (6 ml·h⁻¹) of 0.7% NaCl + 1.5% inulin solution via the venous cannula using an infusion pump (Harvard Bioscience).

The urine produced [by spontaneous voiding or mild sensory stimulation (foot stroking)] was collected, with the first 2 h being considered to be an equilibration period prior to 2 h clearance measurements. Arterial blood samples were taken at 3 h (0.4 ml), 5 h (0.4 ml) and at the end of the experiment at 6 h (5 ml). The first two blood samples were replaced with an equal volume of heparinized saline. Every blood sample was immediately...
centrifuged and the plasma was separated. At the end of the experiment, the animals were killed by intravenous administration of 1 ml of Sagatal (sodium pentabarbitalone).

**Assay procedures**

**Creatinine**
Plasma and urinary creatinine concentrations (from the animals which had been housed in metabolic cages) were determined by a colorimetric method using the picrate reaction (Diagnostics procedure no. 555; Sigma).

**Inulin**
The glomerular filtration rate (GFR) was measured as inulin clearance following determination of inulin in deproteinized plasma and urine.

**Magnesium**
Levels of magnesium in whole plasma, ultrafilterable plasma (see below) and urine were measured using a colorimetric method utilizing the interaction of magnesium with calmagite, a metallochromic dye (Sigma Diagnostics procedure no. 595).

**Calcium**
Calcium concentrations in whole plasma, ultrafilterable plasma (see below) and urine were determined by a colorimetric method based on the interaction of calcium with a chromogenic agent, o-cresolphthalein complexone (Diagnostics procedure no. 587; Sigma).

**PTH assay**
PTH was extracted from plasma prior to RIA. Radio-labelled 125I-PTH was added to each plasma sample before extraction, as a recovery marker. The extraction procedure was as follows. A C18 Sep-Pak column was equilibrated by washing with 3 ml of 90% methanol/0.5% trifluoroacetic acid (TFA) followed by 3 ml of 10% methanol/0.5% TFA. After the plasma had been loaded on to the pretreated C18 Sep-Pak column, the column was washed with 3 ml of 10% methanol/0.5% TFA followed by 3 ml of 40% methanol/0.5% TFA. The peptide was eluted with 3 ml of 90% methanol/0.5% TFA. After the plasma had been equilibrated by washing with 3 ml of 90% methanol/0.5% TFA followed by 3 ml of 40% methanol/0.5% TFA. The peptide was eluted with 3 ml of 90% methanol/0.5% TFA. After the plasma had been equilibrated by washing with 3 ml of 90% methanol/0.5% TFA followed by 3 ml of 40% methanol/0.5% TFA. The peptide was eluted with 3 ml of 90% methanol/0.5% TFA.

**Calcium and magnesium ultrafilterable fractions**
The filterable fraction of magnesium and calcium was determined using ultrafiltration membranes (Amicon-YMP). The plasma sample (0.3 ml) was pipetted into the filtration cup, which was then centrifuged at 2000 g for 45 min.

**Tissue analyses**
At the end of each metabolic cage experiment, tissue samples were obtained from kidney, liver, heart, skeletal muscle (thigh) and bone (femur). A small slice (approx. 0.1 g) was cut from each piece of tissue and weighed in a pre-weighed microtube. The samples were then dried to constant weight at 40 °C for 72 h. Concentrated nitric acid (0.2 ml) was added to each sample, which was then incubated at 40 °C for a further 48 h. Triton (0.8 ml; 0.8%) was added to each sample prior to dilution with UHQ water for magnesium atomic absorption analysis (Varian SpectrAA).

**Measurement of Na and K in urine**
This was done by flame photometry (Instrumentation Laboratory model 943).

**Statistics**
Data are expressed as means ± S.E.M. The significance of differences in corresponding periods between each experimental and control group was compared using the unpaired Student’s t-test. P values of < 0.05 were considered statistically significant.

**RESULTS**
Following treatment with FK506 or vehicle, both FK506 groups were slightly lighter (P < 0.05) than the corresponding control groups: FK506, 300.3 ± 2.8 g (n = 9) and 313.4 ± 4.4 g (n = 8); control, 309.3 ± 2.3 g (n = 10) and 326.6 ± 2.7 g (n = 9) (metabolic cage and infusion study respectively). In order to obtain similar final body weights, FK506-treated rats were heavier (P < 0.001) at the start of the treatment than were controls (the body weight of FK506-treated rats increases more slowly than that of vehicle-treated rats): FK506, 290.4 ± 1.8 g and 295.8 ± 3.0 g; control, 259.3 ± 2.5 g and 263.2 ± 2.6 g (metabolic cage and infusion study respectively). The GFR values (Figure 1) were not different between the FK506 and vehicle groups, either in the metabolic cage experiments (in which GFR was assessed as creatinine clearance; see legend to Table 1) or during the infusion experiments (where GFR was measured as inulin clearance).

Table 1 shows data from the Metabowl experiments. It can be seen that the apparent fractional excretion of magnesium was significantly (P < 0.05) increased by FK506 treatment, but this change in apparent fractional excretion occurred with no significant change in absolute magnesium excretion. Plasma magnesium and ultrafilterable plasma magnesium concentrations were reduced by FK506 (P < 0.001 for both), and this means that the filtered load of magnesium was decreased (P < 0.001), in spite of there being no change in creatinine clearance.
The metabolic cage studies also indicated that there was a tendency for the apparent fractional excretion of calcium, and absolute calcium excretion, to increase as a result of FK506 treatment, but these changes did not reach statistical significance. Plasma and ultrafilterable plasma calcium concentrations were also not different between the groups.

A final observation (Table 1) relating to the metabolic cage studies is that the FK506 group had significantly reduced sodium and potassium excretion, and a decreased sodium/potassium ratio in the urine.

The following data all relate to the saline infusion experiments. In these experiments the initial 2 h period was regarded as an equilibration period, and data are presented for the 2–4 h and 4–6 h periods of the infusion. The infusion studies reveal additional information about the nature of the renal functional changes elicited by FK506.

The plasma magnesium concentration and the ultra-
filterable magnesium concentration for the two groups are shown in Figure 2. Following the equilibration period, the plasma magnesium concentration for each group was stable over the time course of the experiment. The infusion itself lowered the plasma magnesium concentration, as demonstrated by the fact that the plasma magnesium concentration was lower in the infusion than in the non-infusion experiments, for both the control and FK506 groups.

The infusion did not affect the proportion of magnesium that was ultrafilterable in either group. However, the FK506 group showed significantly decreased values for both plasma and ultrafilterable magnesium concentrations than the control group. This decrease in ultrafilterable magnesium led to a significant ($P < 0.001$) lowering of the filtered load of magnesium (Figure 3).

The fractional excretion of magnesium was higher in the FK506 group than in the control group throughout the infusion, both when the urinary excretion of magnesium was lower than (2–4 h) and when it was not different from (4–6 h) that of the control group (Figure 3).

The plasma calcium concentration and ultrafilterable calcium concentration for the infusion experiments are shown in Figure 2. There were no significant differences between the FK506 group and the control group in either variable over the time course of the experiment. However, unlike the findings in the metabolic cage experiments, both absolute and fractional calcium excretion in the FK506 group were significantly increased compared with the control group (Figure 4) in the 4–6 h period (though not in the 2–4 h period).

Urine flow and sodium output values are shown in Figure 5. The FK506-treated animals were slower to excrete the saline load, so that, after 4 h of the infusion,
Values are means ± S.E.M. for control (open bars; n = 9) and FK506-treated (solid bars; n = 8) groups. Significant differences between control and FK506 groups: *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 5 Urine flow and urinary Na excretion in rats during 6 h of saline infusion

this group was significantly more volume expanded than the controls (0–4 h urine volume 20.7 ± 1.1 ml for the FK506 group and 26.6 ± 1.0 ml for the control group; P < 0.01). However, for the final 2 h of the infusion (4–6 h), the sodium output of the FK506 group was not different from that of the control group, and matched the sodium input.

The potassium output of the FK506 group was slightly, but not significantly, lower than that of the control group in the 2–4 h infusion period (4.69 ± 0.33 and 5.31 ± 0.29 µmol·min⁻¹ respectively); in the 4–6 h period the potassium excretion of both groups was lower than in the 2–4 h period, but the FK506 group had a slightly higher potassium excretion than the control group in this period (3.76 ± 0.20 and 3.18 ± 0.16 µmol·min⁻¹ respectively; P < 0.05). Overall, there was no difference in potassium excretion between the groups during the 2–6 h period of the infusion.

Plasma PTH concentrations were measured for each of the animals, using the terminal blood sample from the end of the infusion period. The results (FK506, 62.0 ± 14.2 ng·l⁻¹; control, 64.6 ± 13.7 ng·l⁻¹) indicate that there was no difference in PTH levels between the groups.

The measurements of magnesium concentration in tissues are shown in Table 2. There were no significant differences between control and FK506-treated rats for any of the tissues investigated.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Effects of FK506 on tissue magnesium concentrations</th>
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<td>Values are means ± S.E.M.</td>
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<tr>
<td>Concentration (µg·g⁻¹ dry tissue weight)</td>
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<tr>
<td>Tissue</td>
<td>Control (n = 10)</td>
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<tr>
<td>Kidney</td>
<td>956.8 ± 49.5</td>
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<tr>
<td>Liver</td>
<td>779.0 ± 43.3</td>
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<tr>
<td>Heart</td>
<td>1027.6 ± 62.1</td>
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<tr>
<td>Muscle</td>
<td>1290.6 ± 64.3</td>
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<tr>
<td>Bone</td>
<td>6823.9 ± 284.0</td>
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DISCUSSION

In previously published studies, FK506 treatment in experimental animals has varied with respect to daily dose, duration of treatment and method of administration (e.g. intravenous, intraperitoneal or oral). In general, to obtain comparable blood levels of FK506, oral administration requires a higher dosage than the other routes of administration, and rats require higher doses (in relation to body weight) than human subjects [10]. Doses of FK506 used in rats have been as high as 5 mg·kg⁻¹·day⁻¹ [11], but dose-dependent immunosuppression by FK506 has been demonstrated in rats with doses in the range 0.1–0.8 mg·kg⁻¹·day⁻¹ (intraperitoneal). Thus the dose used in the present study (0.5 mg·kg⁻¹·day⁻¹) is in the therapeutic range [12].

The propensity of FK506 to reduce GFR, seen in many previous studies (e.g. [12]) and shared with cyclosporin A [3], is thought to be caused by constriction of preglomerular vessels [12], possibly as a consequence of decreased NO synthesis [13]. However, the effect is dose dependent, and in the present study we chose a dose of FK506 that did not elicit changes in GFR, in order to obtain a better picture of the effect of FK506 on the tubular handling of magnesium and calcium. The finding of hypomagnesaemia without an increase in absolute magnesium excretion, seen in both the metabolic cage experiments and the infusion experiments, has been obtained by others in both rats and humans following treatment with FK506 [3,14] or cyclosporin [15,16]. The increase in the fractional excretion of magnesium elicited by FK506, which occurred in spite of a decrease in the plasma magnesium concentration and the filtered mag-
nesium load (so that one might expect a fall in the fractional excretion of magnesium as the appropriate response), suggests that renal magnesium absorption is impaired, i.e. a major effect of FK506 is a decrease in the nephron transport of magnesium.

The high fractional excretion of magnesium elicited by FK506 is inappropriate in a hypomagnesaemic state, and can certainly be said to contribute to the hypomagnesaemia. In the present study absolute magnesium excretion was not increased at day 7 of FK506 treatment, although a period of increased excretion prior to this cannot be excluded. However, if renal excretion alone does not initiate the hypomagnesaemia, the other possibilities that must be considered are decreased gastrointestinal absorption of dietary magnesium or increased movement of magnesium into body compartments other than the plasma. It has been shown that the reduced dietary intake associated with FK506 treatment in rats does not explain the hypomagnesaemia, since control rats with matched food intake maintained normal plasma magnesium [14]. However, impaired gastrointestinal absorption of magnesium with either normal or reduced dietary magnesium intake could result in hypomagnesaemia. Impaired gastrointestinal absorption of magnesium was indicated by the results of a study in which patients receiving FK506 treatment were compared with control subjects in their response to intravenously administered magnesium [17]. The FK506 patients, who were hypomagnesaemic, retained a significantly higher fraction of the administered magnesium, suggesting that at least a part of the problem for FK506 patients is maintaining adequate gastrointestinal absorption of magnesium. One of the reported renal actions of FK506 is inhibition of Na\(^+\)/K\(^+\)-ATPase activity in the medullary thick ascending limb [18]. A similar action in the intestinal epithelial cells could reduce gastrointestinal magnesium absorption.

There have been no previous reports on the effects of FK506 on tissue magnesium levels. The present findings indicate no significant changes in the magnesium concentration in any of the tissues measured. Findings with cyclosporin are equivocal. It has been reported to increase the magnesium concentration in kidney, skeletal muscle, liver and leucocytes [15,16], but there are also contradictory findings, e.g. decreased magnesium in erythrocytes and leucocytes [19,20] and no change in skeletal muscle [21]. If the hypomagnesaemia was not caused by movement of extracellular magnesium into cells, then the medium- to long-term consequence of the hypomagnesaemia is likely to be loss of magnesium from bone to the extracellular fluid. In support of this, it has been shown in rats that, following 28 days of treatment with either FK506 or cyclosporin, there was increased bone turnover with net resorption [22]. Magnesium deficiency was also indicated in renal transplant patients treated with FK506 [17]. These findings suggest that bone magnesium acts to alleviate, rather than cause, the hypomagnesaemia. It would thus appear that decreased gastrointestinal absorption of dietary magnesium in conjunction with increased fractional excretion of magnesium is the most likely cause of the hypomagnesaemia. Effects on gastrointestinal magnesium transport processes similar to those which are obviously occurring in the kidneys could account for this.

In control animals, when the urinary sodium output was increased in response to the infusion, there was also a marked increase (approx. 10-fold) in urinary calcium excretion (control group Metabowls, 31±6 nmol·min\(^{-1}\); control group 2–4 h infusion, 351±24 nmol·min\(^{-1}\)). The response of the control rats is to increase renal calcium reabsorption in the 4–6 h period. In contrast, the saline-infused FK506-treated rats did not significantly reduce their calcium loss in the final 2 h of the infusion, so that, in effect, the infusion protocol has revealed a defect in renal calcium handling following FK506 treatment. This being the case, why does hypomagnesaemia develop, but not hypocalcaemia? From the Metabowl data, it can be seen that there is decreased sodium and potassium excretion, indicating an increase in proximal tubular reabsorption (an exclusively distal increase in sodium reabsorption, which would be brought about by aldosterone, would be likely to increase potassium excretion). However, the fact that the sodium/potassium ratio is decreased may indicate that there is also a distal component of the effect. An increase in proximal sodium reabsorption will enhance calcium reabsorption at this site, but will have little effect on magnesium reabsorption, since only a small fraction of the filtered magnesium is normally reabsorbed proximally. Any impairment of more distal reabsorptive function will thus have a greater effect on magnesium balance than on calcium balance.

There have been few previous studies investigating the possibility that FK506 might disrupt the normal hormonal regulation of magnesium, and this aspect was a component of the present work. Most of the filtered magnesium is reabsorbed within the thick ascending limb of the loop of Henle (for reviews, see [4,6]). At this site magnesium reabsorption is mainly passive and paracellular, driven by the transepithelial potential difference [9]. Thus factors that could influence magnesium reabsorption at this site, and which are influenced by PTH, are the magnitude of the transepithelial potential difference and the permeability of the paracellular pathway. The results show that the plasma PTH concentration does not decrease as a consequence of FK506 treatment. Some other change in tubular function must therefore be responsible for the increased fractional excretion of magnesium. This could be a failure of the tubular response to PTH, but other mechanisms acting at this or other tubular sites cannot be excluded.

PTH also has an effect on the plasma calcium concentration (increasing it) and it thus seems likely that the
maintenance of normal plasma calcium during FK506 treatment, observed during the present study, is due at least in part to the role of PTH. It may be that the much larger reserves of calcium in the body (in the skeleton), compared with those of magnesium, makes the maintenance of plasma calcium possible in spite of the impaired reabsorption.

In conclusion, the present study has confirmed that hypomagnesaemia is a significant side-effect of FK506, even at a sub-maximal immunosuppressive dose. The findings have demonstrated that the effect is not attributable to movement of magnesium from the plasma into other tissue compartments. We have also shown that the hypomagnesaemia is not due to interference with PTH release – plasma PTH does not decrease. Further studies are needed to elucidate whether the tubular responsiveness to PTH is modified by FK506, or whether other hormones or tubular sites of magnesium re-absorption are affected by the drug.

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REFERENCES


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