Suppression of Hepatic CYP3A1/2 and CYP2C11 by Cyclosporine Is Not Mediated by Altering Growth Hormone Levels

SHIRLEY K. LU, SHELLIE M. CALLAHAN, and LANE J. BRUNNER

Pharmaceutics Division, College of Pharmacy, The University of Texas at Austin, Austin, Texas

Received September 21, 2002; accepted January 03, 2003

ABSTRACT

Cyclosporine (CsA) suppresses drug metabolism by decreasing cytochrome P450 (P450) enzyme levels in rat liver. Growth hormone (GH) is known to pretranslationally regulate P450 expression. Thus, the suppression of P450 by CsA may involve GH as an intermediate. To address this question, we examined the effects of administering exogenous GH via twice daily subcutaneous injections and in conjunction with chronic subcutaneous CsA administration for 14 days on hepatic P450 expression. CsA alone decreased CYP3A1/2 and CYP2C11 significantly, in a manner similar to that previously found. When administered in the absence of CsA, GH also suppressed CYP3A1/2 and CYP2C11 protein levels as compared with GH vehicle. In the presence of CsA, GH did not cause further suppression of either CYP3A1/2 or CYP2C11 expression when compared with CsA treatment with GH vehicle. Testosterone in vitro catalytic assays confirmed that CsA and GH separately cause significant decreases in activity levels. Also, the concomitant administration of GH and CsA caused lowered production of 16α-, 2α-, 6β-, and 2β-hydroxytestosterone as compared with the administration of GH with CsA vehicle and as compared with the administration of GH vehicle with CsA. This study shows that GH is a dominating factor over CsA in determining hepatic P450 expression and activity. In addition, CsA does not seem to alter GH levels as a mediating event in suppressing P450 expression and activity. Since CsA given in combination with GH further suppressed P450 activity as compared with CsA given in combination with vehicle, this suggests that changes in hormonal status are likely to be one of the many factors that is responsible for the lack of a clear association between cyclosporine dosing and markers of toxicity.

Immune suppression has been an effective avenue of treatment for several conditions including preventing organ transplantation rejection and autoimmune disease. One potent immunosuppressive drug, cyclosporine (CsA), a cyclic undecapeptide of fungal origin, is often used as the drug of choice following organ transplantation. CsA is mainly used for the prevention of allograft rejection and for the prevention of graft-versus-host disease following a bone marrow transplant, as well as for the treatment of arthritis (Tugwell et al., 1987). Although CsA is known to affect interleukins 3 and 4 (IL-3, IL-4), tumor necrosis factor-α, and B cells, it primarily imparts its immunosuppressive action by preventing IL-2 synthesis from activated T cells (Bunjes et al., 1981).

Despite the effectiveness of CsA in suppressing the immune response, there exist several potentially harmful side effects, including nephrotoxicity, hepatotoxicity, and hypertension (Borel, 1990). Since CsA is not only a substrate, but also an inhibitor of CYP3A2, it can modify hepatic drug metabolism in rats following chronic therapy (Brunner et al., 1996). CsA suppresses cytochrome P450 (P450) protein expression, which could then hinder further metabolism of CsA (Cunningham et al., 1985; Brunner et al., 1996). This cycle results in an accumulation of CsA, ultimately leading to organ toxicity. More specifically, it was found that CsA suppressed hepatic CYP3A1/2 and 2C11 in a time-dependent and dose-dependent manner in animals (Brunner et al., 1998, 2000). Although this phenomenon has been readily demonstrated in the rat, CsA-induced changes in hepatic metabolism have yet to be identified clearly in humans.

Pituitary hormones are known to play a significant role in P450 expression in mammals. Unlike other pituitary hormones, growth hormone (GH) lacks specific target sites. Be-
cause of this, GH can exert a wide range of physiological and metabolic effects on target tissues. GH alters drug metabolism in the liver by influencing the regulation of P450 expression. From as early as 1973, the regulation of hepatic drug metabolism enzymes in the rat was shown to be dependent on the amount of GH present in vivo (Wilson, 1973). In determining sex-specific expressions of drug-metabolizing enzymes, the pattern of GH secretion is more relevant than the actual amount of GH present. When GH is secreted in a pulsatile fashion, the male-specific P450 isofrom CYP2C11, along with its corresponding steroid 16α- and 2α-hydroxylase activities, predominates (Morgan et al., 1985; Waxman et al., 1991). Other P450 isofroms including CYP3A1/2, CYP2A2, and CYP2C13 are also male-specific but may not necessarily depend directly on intermittent GH pulses (Waxman et al., 1988, 1995). On the other hand, if GH is secreted in a continuous pattern, this is indicative of a female secretion pattern. In this case, CYP2C12 prevails, while levels of CYP2C11 and CYP3A1/2 are low to nonexistent (MacGeoch et al., 1985). Since P450 enzymes account for the majority of drug metabolism that takes place in the liver, any alteration of these enzyme levels could have a profound impact on the extent of drug metabolism and possibly lead to adverse pharmacological effects.

The purpose of this study was to investigate the role of GH in the modulation of drug-metabolizing enzymes by CsA. This could provide additional insight into the role of hormones in the regulation of drug metabolism and provide a clearer understanding of the physiological interactions that mediate the effects of CsA on P450 enzymes.

**Materials and Methods**

**Materials.** CsA was generously provided by Novartis (East Hanover, NJ) in the form of Sandimmune oral solution. The original dosage form was diluted in commercially available olive oil and stored in amber bottles to limit light exposure. The CsA vehicle was generously provided by the National Hormone and Pituitary Program. CsA was stored in amber bottles to limit light exposure. The CsA vehicle was administered twice-daily subcutaneous doses of 120 ng/g body weight of rat GH according to previous reports of successful supplementation of GH (Waxman et al., 1991). The GH vehicle was administered in the same fashion as GH at 1 ml/kg. CsA administration consisted of once-daily subcutaneous injections of a 15 mg/kg dose, and CsA vehicle was also administered once daily at a concentration of 1 ml/kg.

**Blood Collection.** On the second to last day of dosing, all animals underwent jugular cannula implantation surgery to allow for passive blood collection according to the method of Waynforth and Flecknell (1992). On the last day of dosing for each group, rats were placed into standard rodent metabolic cages for urine collection. Following this, 24-h period, 0.1-ml blood samples were collected once every 15 min for a 6-h period via the indwelling jugular cannula. After collection, the blood was allowed to clot on ice and was centrifuged at 9000 rpm for 5 min at 4°C. Serum was harvested immediately after each spin and stored at −80°C until the time of assay.

**Liver Micronsome Isolation.** Upon sacrifice of animals, the liver was immediately excised. Liver microsomal isolation was achieved with the use of a previously described method of differential centrifugation (Coon et al., 1978) and kept at 4°C during the entire preparation. Liver tissue was ground in 3 volumes of Tris chloride buffer consisting of 0.1 M EDTA and 0.15 M potassium chloride using a PowerGen 700 homogenizer (Fisher Scientific Co., Pittsburgh, PA). Samples were then centrifuged at 9,000 rpm for 20 min at 4°C. The supernatant was collected and centrifuged at 37,000 rpm for 17 min at 4°C. The supernatant was discarded and the pellet resuspended in sodium pyrophosphate buffer containing 0.1 M EDTA at pH 7.4. The suspension was then homogenized and afterward centrifuged at 37,000 rpm for another 17 min at 4°C. The supernatant was discarded and the pellet washed and homogenized in Tris buffer containing 10% glycerol for storage. The microsomes were then stored at −80°C until analysis.

**Gel Electrophoresis and Immunoblot Analysis.** Gel electrophoresis was performed using an SDS-polyacrylamide gel electrophoresis 8% polyacrylamide separating gel as previously described (Laemmli, 1970). Protein on the gel was then transferred to nitrocellulose sheets by a prior described method (Schnier et al., 1989). After transfer of proteins, the nitrocellulose sheets were blocked with 3% nonfat dry milk (NFDM) in Tris-bUFFERED saline (TBS) at room temperature. Detection of putative proteins was achieved with goat anti-rat IgG (in a 1:2000 dilution) immunoreactive to the specific P450 enzyme of interest in 3% NFDM and then rabbit anti-goat horseradish peroxidase (in a 1:2000 dilution), also in 3% NFDM at room temperature. Transitional washes using TBS and 0.05% Tween 20 in TBS were done according to previously described procedures (Schnier et al., 1989). Immune complexes for CYP3A1/2 and CYP2C12 were detected with a PerkinElmer chemiluminescence reagent kit as described by the manufacturer (Perkin Elmer Life Sciences, Boston, MA). Biotinylated secondary antibody was added to the nitrocellulose sheet, followed by a horseradish peroxidase conjugated streptavidin reagent. Chemiluminescent substrate was then added and the sheets were exposed to X-ray film. The positive bands were then developed with Kodak X-OMAT X-ray film and Kodak Rapid film. The resulting films were then scanned with a computer using the Kodak 1D image analysis software, version 3.5 (Eastman Kodak, Rochester, NY).

**Testosterone Hydroxylation Assay and HPLC assay.** Liver samples for the testosterone hydroxylation assay were performed as previously described (Brunner et al., 1996). In brief, 200 μg of liver...
data were analyzed using the SuperANOVA statistical program (Abacus Concepts, Inc., Berkeley, CA). Data are presented as mean ± standard error. When the probability of chance explaining the results was reduced to less than 5% (p < 0.05), the differences were then considered to be statistically significant.

Results

Effect of Concomitant Administration of rGH and CsA on Hepatic P450 Levels. Figure 1 represents hepatic CYP3A1/2 and CYP2C11 protein expression after 14 days of dosing as determined by Western blotting. The results from rats treated for 14 days revealed a significant decrease in CYP3A1/2 and CYP2C11 levels in the CsA group as compared with the vehicle control group (p = 0.04; p = 0.004, respectively), which is in agreement with previous reports from our laboratory (Brunner et al., 2000). Similarly, when CsA was administered in combination with GH vehicle, both isoforms were markedly depressed when compared with GH vehicle/CsA vehicle treatment. When exogenous GH was given with CsA, levels of CYP3A1/2 and CYP2C11 were not suppressed as compared with the GH/CsA vehicle group.

Fig. 1. Western blot analysis of hepatic CYP3A1/2 and CYP2C11 microsomal protein expression in 14-day groups. Groups were given subcutaneous doses of either 15 mg/kg CsA or CsA vehicle alone or a combination of GH, GH vehicle, CsA, and CsA vehicle. All values are expressed as percentage density of the protein standard used for each blot. a, p < 0.05 between C and CV groups; d, p < 0.05 between G/C and GV/CV groups; e, p < 0.05 between GV/C and GV/CV groups.
Additionally, when administered concomitantly with CsA vehicle, excess GH caused a significant decrease in CYP3A1/2 and CYP2C11 expression ($p = 0.01; p = 0.007$, respectively). Figure 2 depicts a representative Western blot to illustrate the relative differences in densities between the aforementioned groups.

**Effect of Concomitant Administration of rGH and CsA on Hepatic P450 Activity Measured by Testosterone Hydroxylation Assay and HPLC.** The formation of 6β-hydroxytestosterone (6β-OHT) correlates primarily with the activity of CYP3A2 (Waxman et al., 1983, 1985) and, to a lesser extent, with CYP3A1 (Sonderfan et al., 1987). HPLC analysis following testosterone hydroxylation assay of liver microsomes showed a significant decrease in the production of 6β-OHT ($p = 0.0001$) in the group receiving CsA only as compared with the group receiving CsA vehicle only, indicating a reduction in the overall activity of CYP3A1/2 (Fig. 3). This finding supports the suppression in protein levels found with Western blotting. Significantly lower quantities of 6β-OHT were detected in all groups administered GH (GH/CsA V, GH/CsA) versus the respective GH vehicle groups ($p = 0.026; p = 0.0001$), thus confirming that superphysiological expression of GH decreases CYP3A1/2 activity.

The formation of 16α-OHT and 2α-OHT corresponds to the activity of CYP2C11 (Cheng and Schenkman, 1983; Waxman, 1984). Since the pattern of results was nearly identical for the formation of these two metabolites, only the results for 2α-OHT are represented in graphical form. After 14 days of treatment with CsA, activity levels of CYP2C11 were lowered significantly as compared with CsA vehicle treatment. CsA treatment alone and in conjunction with GH vehicle also dramatically decreased the formation of 2α-OHT ($p = 0.0001; p = 0.0001$, respectively), as well as 16α-OHT ($p = 0.0001; p = 0.0001$, respectively). Interestingly, administration of GH (as compared with GH vehicle) significantly lowered the formations of 2α- and 16α-OHT, irrespective of the concomitant drug (CsA or CsA vehicle).

**Effect of Concomitant Administration of rGH and CsA on Renal Function Parameters.** Urine volume collected over the 24-h period was compared as a measure of kidney function (Table 1). CsA-treated rats had a nearly 2-fold greater volume of urine output as compared with the CsA vehicle-treated rats ($p = 0.02$). Similarly, the CsA-treated rats given GH vehicle had a 2.3-fold increase in urine volume when compared with CsA-treated rats given GH vehicle. However, when CsA-treated rats were administered GH, the urine flow volume was significantly decreased from the CsA/GH vehicle group ($p = 0.038$).

Serum creatinine levels and creatinine clearance rates were analyzed as an estimation of glomerular filtration rate (Table 1). Serum creatinine was only slightly higher in the GH/CsA group as compared with the GH/CsA vehicle group ($p = 0.05$). No other differences in serum creatinine levels were detected for any of the other groups. In addition, no significant differences in creatinine clearance, due to administration of either GH or CsA, were detected.

**Circulating Serum Growth Hormone Levels.** Figure 4 shows the area under the curve (AUC) for all groups. The groups that were administered two injections a day (regardless of the agents injected) generally had higher AUCs due to the combination of both higher frequency of pulses and a higher basal level of secretion. No statistically significant

**Fig. 2.** Representative blots for liver 3A1/2 and 2C11 protein expression, respectively. One representative animal was selected from each group for the densitometry figure. From left to right, the groups are: 14C, 14CV, 14G/C, 14G/CV, 14GV/C, and 14GV/CV.

**Fig. 3.** In vitro testosterone hydroxylation by hepatic microsomes following 14 days of treatment. Groups were given subcutaneous doses of either 15 mg/kg CsA or CsA vehicle alone, or a combination of GH, GH vehicle, CsA, and CsA vehicle. The top graph represents 6β-hydroxylase activity; the bottom graph represents 2α-hydroxylase activity. Units for metabolite production are pmol metabolite/min/mg microsomal protein. $a, p < 0.05$ between C and CV groups; $c, p < 0.05$ between G/C and GV/C groups; $d, p < 0.05$ between G/CV and GV/CV groups; $e, p < 0.05$ between GVC and GV/CV groups.
differences were observed between respective treatment groups. Representative 6-h GH secretion profiles from each group are presented in Fig. 5. GH release profile parameters including mean peak amplitude, mean peak number, and mean peak duration are shown in Table 2. No statistically significant differences were detected between treatment groups and their respective controls.

CsA Blood Levels. The concentration of CsA in blood for animals administered GH and CsA was slightly higher than, but not statistically different from that in animals injected with GH vehicle and CsA (p = 0.175). This was the only comparison performed since no other comparisons were useful from a statistical standpoint.

**Discussion**

The primary aim of the present study was to determine whether GH is the main intermediate through which the chronic suppression of P450 by CsA occurs in the liver of rats. With the pituitary intact, the GH secretion profile is still present, and suppression of normal levels of P450 enzymes can be readily detected. The relationship between CsA, GH, and P450 enzymes was examined by introducing exogenous GH to intact male rats while concomitantly administering CsA. Select P450 isoform expressions and activities were analyzed as indicators of drug metabolism.

Because CYP3A1/2 contributes significantly to the metabolism of numerous xenobiotics (including CsA) that takes place in the rat liver, and CsA is known to suppress this isoform, the expression and behavior of this isoform were of chief importance for this study. Previous studies show that when exogenous GH is introduced to intact male rats, CYP3A2 is significantly suppressed (Kawai et al., 2000, 2001). Our study confirmed that 14-day treatment with GH suppresses CYP3A1/2 protein expression. However, when CsA was administered in combination with GH, GH did not cause a significant suppression of CYP3A1/2 protein as compared with concomitant administration of CsA with GH vehicle. This masking effect was also evident when CsA failed to cause suppression when administered with GH and compared with administration with GH vehicle. Also, all groups receiving two injections per day experienced an overall decline in CYP3A1/2 protein expression, irrespective of the drug. This finding corresponds with the trend in GH levels represented by AUC calculations in this study. GH levels for groups receiving two injections were generally higher than in groups receiving only one. This could partly be due to the stress involved with the injection and is therefore unrelated to the drug administered. A previous study revealed a significant increase in GH following 10 to 11 days of only saline injection in adult male rats (Kant et al., 1983).

Since CYP3A1/2 catalyzes the production of both 6β-OHT and 2β-OHT, the quantities of both derivatives were measured to signify CYP3A1/2 activity. We showed that after 14 days of dosing, exogenous rat GH administered to normal rats suppressed both 6β- and 2β-OHT production. This result was evident despite coadministration with either CsA or CsA vehicle. Similarly, a previous study has reported depression of 6β-OHT levels after 7 days in intact male rats given GH in both intermittent pulses as well as in a continuous infusion (Yamazoe et al., 1986). Because the continuous presence of GH has been shown to decrease CYP3A2 activity in male rats and the long-term intermittent pulses used for this study resulted in a decrease in the activity of CYP3A1/2, this indicates that CYP3A1/2 may be responsive to an overall chronic increase in circulating GH levels. The data also show that GH and CsA have an additive suppressive effect on CYP3A1/2 activity as compared with CsA’s suppressive effects alone. Since CYP3A2 is the main isoform responsible for the metabolism of CsA, the considerable decline in CYP3A1/2 activity as a result of CsA and GH combined will likely lead to higher concentrations of CsA in circulation, thus leading to increased incidence of organ toxicity.

CYP2C11 is a sex-specific isoform whose expression is most directly regulated by the pulsatile secretions of the male GH pattern (Waxman et al., 1991; Legraverend et al., 1992). This concept was further validated when pulsatile secretions of GH via an external syringe pump as well as subcutaneous injections replaced the male-pattern GH secretions in hypophysectomized male rats and resulted in an increase in CYP2C11 mRNA expression, as well as 2α-OHT activity (Waxman et al., 1991). Although twice-daily subcutaneous injections of GH are known to increase CYP2C11 to
normal levels in hypophysectomized rats, we show that administration of GH in the same fashion to intact rats causes a decrease in CYP2C11. GH administration (with CsA vehicle) significantly reduced CYP2C11 expression when compared with the corresponding GH vehicle group. Nearly the same result has also been obtained by other researchers using the same route of administration and dose, and a comparable study period of 12 days (Kawai et al., 2001). This could indicate that exogenous GH, in addition to endogenously secreted GH, is causing the animals to continually have GH present in circulation, effectively increasing the basal level of GH. The immediate downstream consequence of the substantially higher concentration of GH in plasma may be that the GH receptor is not functioning in the correct capacity to initiate the Janus tryosine kinase/signal transducer and activator of transcription pathway. This notion is supported by the GH receptor dimerization theory, whereby GH receptors only function to trigger the intracellular signal transduction necessary for initiation of P450 protein gene transcription when they are bound to one GH molecule and then dimerize with another GH receptor molecule (Fuh et al., 1992). Therefore, excess GH in circulation may lead to a 1:1 ratio of binding and would not allow for the 1:2 ratio of GH/GH receptor necessary for initiation of action by GH.

A significant decrease in the production of 2α-OHT and 16α-OHT was exhibited with the administration of CsA alone as compared with the administration of CsA vehicle alone. This is consistent with previous results showing a suppression of CYP2C11 protein expression and enzyme activity as a result of chronic CsA administration (Brunner et al., 1996).

### TABLE 2
GH release profile parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CsA</th>
<th>CsA V</th>
<th>GH/CsA</th>
<th>GH/CsA V</th>
<th>GH V/CsA</th>
<th>GH V/CsA V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean peak amplitude</td>
<td>83.4 ± 35.5</td>
<td>90.6 ± 35.1</td>
<td>173.0 ± 38.1</td>
<td>152.3 ± 17.1</td>
<td>127.3 ± 30</td>
<td>178.9 ± 30.5</td>
</tr>
<tr>
<td>Mean peak number</td>
<td>4.8 ± 0.6</td>
<td>5.0 ± 0.4</td>
<td>5.4 ± 0.5</td>
<td>5.0</td>
<td>5.7 ± 0.5</td>
<td>5.7 ± 0.6</td>
</tr>
<tr>
<td>Mean peak duration</td>
<td>31.0 ± 8.1</td>
<td>64.3 ± 2.9</td>
<td>58.4 ± 3.6</td>
<td>66.8 ± 3.1</td>
<td>51.7 ± 2.2</td>
<td>63.7 ± 9.8</td>
</tr>
</tbody>
</table>

Fig. 5. Representative graphs depicting GH secretion profiles of aforementioned groups over a 6-h period. Blood samples were collected every 15 min.
istration of CYP in combination with GH resulted in a greater suppression of CYP2C11 activity than when either agent was administered with vehicle. Although this is in slight discordance with protein levels, the trend appears similar, although the results were not statistically significant for the protein expression data. Considering that CYP2C11 is the predominant P450 enzyme present in male rat liver, the additive suppressive effect of combined GH and CsA therapy on CYP2C11 activity may have profound consequences on drug metabolism overall.

GH secretion profile parameters were not significantly altered due to CsA treatment, which revealed that chronic CsA treatment does not alter the level of GH in circulation. Since modifying the levels of GH can lead to a change in CYP3A2 and CYP2C11 levels, CsA does not seem to be affecting P450 expression by modulating GH levels. GH may still mediate the suppression of P450 enzymes by CsA at the receptor-binding or signal transduction level. Conversely, GH did not have an effect on detectable CsA concentrations in blood either. Because the combined administration of CsA and GH produced significantly lower CYP3A2 activity than when CsA was given with vehicle, we expected to see a considerably higher concentration of CsA in blood as a result of decreased CsA metabolism by CYP3A2. However, only a slight elevation was observed. This may be due to CsA being sequestered into organs, including the liver, kidney, and small intestine, thus preventing detection in blood.

The data also suggest that changes in hormonal status are likely to be one of the many factors that is responsible for the lack of a clear association between ciclosporine dosing and markers of toxicity. Hormonal status can be altered by either manipulating the amount of hormone in circulation or the subsequent signaling action of the hormone. Modulation of GH action can occur by one or more of several possible methods including a modification of the binding capacity of circulating GH, alteration of GH receptor quantity or binding capability and/or availability, alteration of Janus tyrosine kinase-2 action, or modification of any one of the GH-activated intracellular signaling pathways (Frank et al., 2000). According to this rationale, an alteration anywhere along the GH activation pathway could affect intracellular signaling and, eventually, the downstream gene regulation of P450 protein production. Future studies are warranted to investigate GH binding and the signal transduction mechanism behind the possible function of GH in the alteration of P450 expression and activity by CsA.

Acknowledgments

We thank Dr. Eve Van Cauter and Rachel Leproult for graciously providing a copy of the ULTRA analysis software program.

References


Address correspondence to: Dr. Lane J. Brunner, College of Pharmacy, PPH 4.214E, The University of Texas at Austin, Austin, TX 78712-1074. E-mail: lane.brunner@mail.utexas.edu