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Cyclosporine and bromocriptine-induced suppressions of CYP3A1/2 and CYP2C11 are not mediated by prolactin

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Abstract

The purpose of this study was to determine if the suppression of hepatic CYP3A1/2 (cytochrome *P*450 3A1/2) and CYP2C11 (cytochrome *P*450 2C11) by cyclosporine is mediated by prolactin. Male intact rats were given subcutaneous doses of either 15 mg/kg/day of cyclosporine or 1 ml/kg/day of cyclosporine vehicle concomitantly with one of the following: 500 mg/kg prolactin, 1 ml/kg prolactin vehicle, 4 mg/kg bromocriptine, or 1 ml/kg bromocriptine vehicle for 14 days. Protein expressions were measured using Western blot analysis and activities were measured using an in vitro testosterone hydroxylation assay. The administration of prolactin did not significantly alter CYP3A1/2 protein expression. Hypoprolactinemia, produced by bromocriptine, caused a significant suppression of CYP3A1/2 activity levels when bromocriptine was administered alone and in combination with cyclosporine ($P<0.001$, $P<0.05$; respectively). However, the cause of the suppression by bromocriptine was likely not the result of lowering prolactin levels. Bromocriptine administration also lowered CYP2C11 protein expression and activity, while prolactin administration had virtually no effect on CYP2C11. Chronic cyclosporine administration caused a 140% increase in prolactin area under the curve (AUC) ($P<0.001$). Bromocriptine caused a significant decline in endogenous prolactin secretion, however, concurrent cyclosporine administration did not recover these levels. Overall, while both cyclosporine and bromocriptine, separately, can significantly alter the fate of hepatic *P*450 enzymes, the suppression is likely not due to an alteration in prolactin levels.

Keywords: Cytochrome *P*450; Cyclosporine; Prolactin; Bromocriptine; Drug metabolism

1. Introduction

A connection between the neuroendocrine and the immune system has been shown to exist in various ways. Prolactin, a peptide hormone produced in the pituitary, is part of a superfamily of hormones that includes growth hormone and placental lactogens. Although the chief functions of prolactin are maintaining lactation and stimulating mammary gland growth, this lactogenic hormone has been demonstrated to be one of the critical components in maintaining immune function (Gala, 1991; Nagy and Berczi, 1978). This role in the immune system ranges from driving T-cell proliferation in murine T-cell clone L2 cells (Clevenger et al., 1990) to inducing interleukin-2 receptors on ovariectomized female rat splenic lymphocytes (Mukherjee et al., 1990).

Alterations in the level of circulating prolactin can cause immune dysfunction. When male rats were treated with ovine prolactin, their ability to reject parasitic infection was suppressed (Kelly and Dineen, 1973). Hypoprolactinemia can also result in a failed immune response. This condition can be artificially produced by the administration of bromocriptine (Nagy et al., 1983). Bromocriptine is a dopamine receptor agonist that inhibits prolactin secretion and is commonly used in the treatment of hyperprolactinemia, Parkinsonism, and acromegaly (Vance et al., 1984). Bromocriptine's effect on the endocrine system has been clearly demonstrated, however, specific studies on the effect of bromocriptine on hepatic drug metabolism have been limited.

Cyclosporine is a widely used immunosuppressant drug given to patients following organ transplantation. Cyclosporine is an undecapeptide that blocks transcription of genes for IL-2 at the mRNA level, thus inhibiting helper T-

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cell proliferation (Granelli-Piperno, 1988; Kronke et al., 1984). Although it has been well established that the relationship between cyclosporine and prolactin is antagonistic, the exact mechanism of how this antagonism takes place is not certain. At specified concentrations, cyclosporine has been shown to completely inhibit prolactin binding to lymphocytes of human mononuclear cells (Russell et al., 1985). This concept was challenged by the finding that cyclosporine inhibited the prolactin-stimulated growth of the rat lymphoma Nb-2 cell line without affecting prolactin binding to receptors (Varma and Ebner, 1988).

Within the liver, cyclosporine is almost exclusively metabolized by the CYP3A isoform family of the cytochrome *P*450 (*P*450) system in humans and animals (Kronbach et al., 1988; Maurer, 1985), although other isoforms may also be involved (Brunner et al., 1996; Prueksaritanont et al., 1993). Therefore, factors that affect the *P*450 system should also be suspected of affecting the metabolism of cyclosporine. For example, when hepatic *P*450 levels were increased in rats, the nephrotoxic effects of cyclosporine were lessened (Cunningham et al., 1985). While cyclosporine is metabolized by *P*450 enzymes, cyclosporine also has an effect on the disposition of these enzymes. Chronic treatment with moderate daily doses of cyclosporine suppresses protein levels and catalytic activity of the CYP3A2 and CYP2C11 isoforms in the rat liver (Brunner et al., 1996). Furthermore, this inhibition occurs in a time-dependent (Brunner et al., 1998) and dose-dependent (Brunner et al., 2000) manner. Since cyclosporine is not only a substrate, but also an inhibitor of CYP3A, chronic cyclosporine therapy may result in a cycle of protein suppression and accumulation of drug in the body that eventually leads to organ toxicity.

Few studies have examined the possible interrelationship between prolactin, immune suppression, and the effects on hepatic drug metabolism. The aim of our study was to determine if the suppression of hepatic CYP3A and CYP2C11 by cyclosporine is mediated by prolactin through the use of exogenous prolactin to increase circulating prolactin levels and bromocriptine to drastically reduce prolactin secretion. The results from this study will aid in the understanding of how hormonal status can affect drug metabolism in the body and attempt to untangle the complex relationship between the endocrine system, immune function, and drug metabolism.

2. Materials and methods

2.1. Materials

Unless otherwise noted, all chemicals were purchased in the highest purity available from EM Science (Gibbstown, NJ, USA). Cyclosporine was generously provided by Novartis (East Hanover, NJ, USA) 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 cyclosporine vehicle was the same commercially available olive oil. Bromocriptine was purchased in the form of 2-bromo-α-ergocryptine methanesulfonate salt (Sigma-Aldrich, St. Louis, MO, USA) and dissolved in 70% ethanol along with an equivalent weight of tartaric acid. This solution was then suspended in pure olive oil at a concentration of 4.6 mg/ml of bromocriptine with the percentage of ethanol not exceeding 12.5% of the total volume of the suspension. Purified ovine prolactin was purchased from the National Hormone and Pituitary Program under the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK, Torrance, CA, USA) and solubilized in 0.03 M $NaHCO_3$ in 0.15 M NaCl at a final pH of 8.5. Prolactin vehicle consisted of 0.03 M $NaHCO_3$ in 0.15 M NaCl. Ovine prolactin has been shown to have similar physiological function to rat prolactin when examined for its effectiveness in eliciting tyrosine kinase activity in Nb2 cells (Rillema et al., 1992) and restoration of lactation in bromocriptine-treated female rats (Hebert et al., 1993).

The anesthetic mixture consisted of a 1:1:1 (v/v/v) ratio of ketamine (100 mg/ml), xylazine (20 mg/ml), and acepromazine (10 mg/ml). Ketamine and xylazine were purchased from Sigma. Acepromazine was purchased from Fort Dodge Laboratories (Fort Dodge, LA, USA). Rat CYP3A1/2 and CYP2C11 selective polyclonal antibodies were purchased from Gentest (Woburn, MA, USA). Rat CYP3A1/2 antibody was isolated from goats that were immunized with CYP3A2 purified from rat liver. Due to the polyclonal nature of the antiserum, two indistinguishable bands (CYP3A1 and CYP3A2) are detected and are therefore collectively referred to as CYP3A1/2. The microsomal standard used for the relative quantitation of CYP3A1/2 protein was composed of phenobarbital-treated rat liver microsomes (Xenotech, Lenexa, KS, USA). Rat CYP2C11 antibody is also polyclonal and raised in goats, however the cross reactivity with CYP2C13 can be readily distinguished from CYP2C11 based on mobility. CYP2C11 standard was composed of dexamethasone-treated male rat liver microsomes (Xenotech). The horseradish peroxidase-conjugated rabbit anti-goat (secondary *P*450 antibody) was purchased from ICN Pharmaceuticals (Aurora, OH, USA). Rat prolactin enzyme immunoassay (EIA) kits were obtained from Cayman Chemical (Ann Arbor, MI, USA) and were manufactured by Spi-Bio (Massy Cedex, France).

2.2. Animals

All procedures were approved by the Institutional Animal Care and Use Committee of The University of Texas at Austin and were in accordance with the guidelines established by the National Institutes of Health for the humane treatment of animals. Eight- to nine-week-old, male Sprague–Dawley rats were purchased from Harlan Sprague–Dawley (Indianapolis, IN, USA). During the study, all rats

were kept in a 12-h light/dark cycle environment with free access to low-salt rat chow (Harlan) and deionized water. Due to the rat's resistance to nephrotoxicity during cyclosporine therapy, low-salt rat chow was given to predispose the animals to develop chronic cyclosporine nephropathy similar to that in humans (Shihab et al., 1997). Food consumption and body weight were measured daily.

Rats were randomly assigned to one of eight groups (N=6 each). The groups are as follows: +PRL/+CsA, +PRL/−CsA, −PRL/+CsA, −PRL/−CsA, +BRC/+CsA, +BRC/−CsA, −BRC/+CsA, and −BRC/−CsA (PRL=prolactin, CsA=cyclosporine, BRC=bromocriptine). Animals receiving prolactin were administered twice daily subcutaneous doses of 500 ng/g body weight of ovine prolactin. The prolactin vehicle was administered in the same fashion as prolactin at 1 ml/kg. Cyclosporine administration consisted of once daily subcutaneous injections of a 15-mg/kg dose and cyclosporine vehicle was also administered once daily at a dose of 1 ml/kg.

2.3. Blood collection

Two days prior to blood collection, jugular cannulas were implanted surgically in all animals as previously described (Waynforth and Flecknell, 1992). Twenty-four hours prior to blood collection, rats were placed into standard rodent metabolic cages for passive urine collection. Following this 24-h period, 0.1 ml blood samples were collected once every 15 min for 4 h via the indwelling jugular cannula. Plasma volume was replaced with sterile saline. After collection, the blood was allowed to clot on ice and was centrifuged at 9000×g for 5 min at 4 °C. Serum was harvested immediately and stored at −80 °C until assayed.

2.4. Microsome isolation

Upon sacrifice of the animals, livers were excised immediately. Liver microsomes were isolated with modifications of a previously described method of differential centrifugation (Coon et al., 1978) and kept at 4 °C during the entire preparation. Briefly, liver tissue was ground in three volumes of Tris chloride buffer consisting of 1 mM EDTA and 0.15 M potassium chloride using a PowerGen 700 homogenizer (Fisher Scientific, Pittsburgh, PA, USA). Samples were then centrifuged and resuspended in a Tris chloride buffer containing 1 mM EDTA and 0.2 M sodium pyrophosphate. After the final spin, the supernatant was discarded and the pellet washed and homogenized in Tris buffer containing 20% glycerol for storage. The microsomes were then stored at −80 °C until analysis.

2.5. Gel electrophoresis and immunoblot analysis

Sodium docecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described (Laemmli, 1970) using an 8% polyacrylamide separating gel. Twenty-five micrograms of protein was loaded onto the gel and transferred to nitrocellulose sheets by a prior described method (Schnier et al., 1989). After protein transfer, the nitrocellulose sheets were blocked with 3% non-fat dry milk in Tris-buffered saline (TBS) at room temperature. Detection of putative proteins was achieved with goat anti-rat IgG (in a 1:2000 dilution) that was immunoreactive to the specific P450 enzyme of interest in 3% non-fat dry milk and then rabbit anti-goat horseradish peroxidase (in a 1:2000 dilution) also in 3% non-fat dry milk at room temperature. Immune complexes for CYP3A1/2 and CYP2C11 were detected with an NEN chemilumnescence reagent kit as described by the manufacturer (New England Nuclear Life Science Products, Boston, MA, USA). Blot densities were measured using a flatbed scanner (Microtek, Hsinchu, Taiwan) and analyzed on a Dell PC computer using the Kodak 1D image analysis software, version 3.5 (Eastman Kodak, Rochester, NY, USA).

2.6. Testosterone hydroxylation assay and high-performance liquid chromatography assay

Liver samples for the testosterone hydroxylation assay were prepared as previously described (Brunner et al., 1996). In brief, 200 μg of liver microsomal protein was added to 0.02 M potassium phophate buffer (pH 7.4), regeneration system, and water up to 1 ml. The samples were then incubated with 250 μM testosterone for 3 min at 37 °C followed by the addition of glucose-6-phosphate dehydrogenase (1 unit/μl) for 15 min under the same conditions. The reaction was quenched with dichloromethane and 11α-hydroxyprogesterone (1.2 μg) was added as the internal standard. The organic phase was transferred and evaporated, then dissolved in methanol and stored at 4 °C. The testosterone/bromocriptine inhibition assay was carried out in similar fashion to the testosterone hydroxylation assay with the exception that bromocriptine was added at a concentration of 107 μM to each tube at the same time as the testosterone. The volume of bromocriptine was accounted for by subtracting an equal volume from the water component in each sample tube.

Separation and detection of testosterone and metabolites were performed on a Shimadzu high-performance liquid chromatography (HPLC) system (Shimadzu Scientific Instruments, Columbia, MD, USA). The column, a Supelcosil LC-18 (Sigma-Aldrich), was kept at a constant temperature of 40 °C and a wavelength of 238 nm was used to detect the analytes. All other conditions were identical to our previously described method (Brunner et al., 2000). Peak areas of corresponding hydroxylation metabolites were measured and compared to peak areas of the internal standard within the same run.

2.7. Rat prolactin enzyme immunoassay

The enzyme immunoassay used to determine prolactin levels was manufactured by Spi-Bio. The principle of the

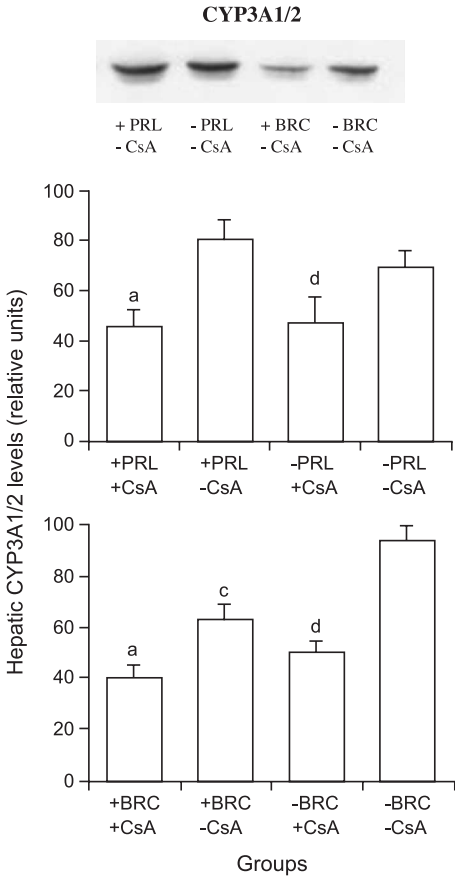

Fig. 1. Western blot analysis of hepatic CYP3A1/2 microsomal protein expression in 14-day groups. Groups were given subcutaneous doses of either 15 mg/kg cyclosporine (+CsA) or cyclosporine vehicle (−CsA) in combination with prolactin (+PRL), prolactin vehicle (−PRL), bromocriptine (+BRC), or bromocriptine vehicle (−BRC). All values are expressed as a percent density of the protein standard used for each blot. a=P<0.05 between +PRL/+CsA and +PRL/−CsA (top graph) or between +BRC/+CsA and +BRC/−CsA groups (bottom graph), c=P<0.05 between +BRC/−CsA and −BRC/−CsA groups, d=P<0.05 between −PRL/+CsA and −PRL/−CsA (top graph) or between −BRC/+CsA and −BRC/−CsA groups (bottom graph).

assay is based on the competition between unlabelled rat prolactin and acetylcholinesterase bound to a tracer. The yellow color formed by exposing the sample and acetylcholinesterase to an enzymatic substrate (and chromagen) for acetylcholinesterase was measured spectrophotometrically. The limit of detection for the assay was 0.5 ng/ml.

2.8. Data analysis

Sample densities from Western blots were compared with standard microsomal protein densities and expressed as a percentage. Prolactin area under the curve (AUC) values represent the total area under the entire secretion profile curve of each animal and then averaged for all values within each treatment group. One-way ANOVA (analysis of variance) and a priori means comparisons tests were employed using respective vehicle groups as the control with the aid of the SuperANOVA statistical program (Abacus Concepts, Berkeley, CA, USA). 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.

3. Results

3.1. Effect of cyclosporine and alterations in prolactin hormonal status on hepatic P450 protein levels

Cyclosporine administered over a 14-day period has been shown to significantly reduce CYP3A1/2 protein expression in the male rat liver (Brunner et al., 1998). Fig. 1 shows that cyclosporine given in the presence of either prolactin or prolactin vehicle resulted in a decrease in CYP3A1/2

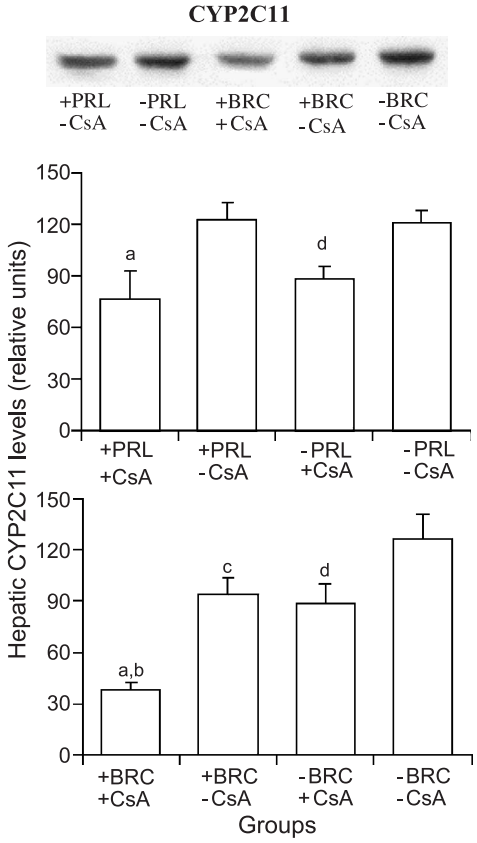

Fig. 2. Western blot analysis of hepatic CYP2C11 microsomal protein expression in 14-day groups. Groups were given subcutaneous doses of either 15 mg/kg cyclosporine or cyclosporine vehicle in combination with prolactin, prolactin vehicle, bromocriptine, or bromocriptine vehicle. All values are expressed as a percent density of the protein standard used for each blot. a=P<0.05 between +BRC/+CsA and +BRC/−CsA groups (bottom graph), b=P<0.05 between +PRL/+CsA and −PRL/+CsA (top graph) or between +BRC/+CsA and −BRC/+CsA groups (bottom graph), c=P<0.05 between +BRC/−CsA and −BRC/−CsA groups, d=P<0.05 between −PRL/+CsA and −PRL/−CsA (top graph) or between −BRC/+CsA and −BRC/−CsA groups (bottom graph).

expression ($P<0.01$; $P=0.063$, respectively) as compared with cyclosporine vehicle control groups. The administration of prolactin did not cause a notable difference in the protein levels of CYP3A1/2, either when given in combination with cyclosporine or cyclosporine vehicle. Conversely, when cyclosporine was administered concomitantly with either bromocriptine or bromocriptine vehicle, there was a significant decline in both cases ($P<0.01$; $P<0.001$, respectively). In addition, the introduction of bromocriptine, which effectively abolishes prolactin secretion, resulted in a significant decrease in the expression of CYP3A1/2 in the absence of cyclosporine ($P<0.001$). There was no difference in suppression when cyclosporine was present along with bromocriptine (+BRC/+CsA) as compared with the −BRC/+CsA group ($P=0.205$), indicating the lack of an additive suppression of CYP3A1/2 with the administration of both bromocriptine and cyclosporine. However, the addition of cyclosporine did further contribute to the existing suppression caused by bromocriptine and cyclosporine vehicle ($P<0.001$).

Suppression of CYP2C11 by chronic cyclosporine treatment has been documented before (Brunner et al., 1998) and is confirmed by our present study. However, administration of exogenous prolactin did not have a significant effect on CYP2C11 protein expression (Fig. 2). Conversely, bromocriptine and cyclosporine separately caused a significant decrease in CYP2C11 ($P<0.05$; $P<0.05$, respectively) and the combination of the two drugs caused even greater decreases in protein expression when compared to the +BRC/−CsA and −BRC/+CsA groups ($P<0.01$; $P<0.01$, respectively).

3.2. Effect of cyclosporine and alterations in prolactin hormonal status on hepatic P450 activity levels

The in vitro conversion of testosterone to 6β-hydroxytesterone (6β-OHT) is primarily catalyzed by CYP3A2 (Waxman et al., 1985; Waxman et al., 1983) and to a lesser extent by CYP3A1 (Sonderfan et al., 1987). Thus, the measure of 6β-OHT formation is a reliable indicator of CYP3A1/2 activity levels. A testosterone hydroxylation assay and subsequent HPLC analysis revealed that cyclosporine significantly impaired the formation of 6β-OHT in the liver when given in combination with prolactin vehicle ($P<0.05$) or with prolactin ($P<0.05$) (Fig. 3). The administration of prolactin did not change 6β-OHT levels,

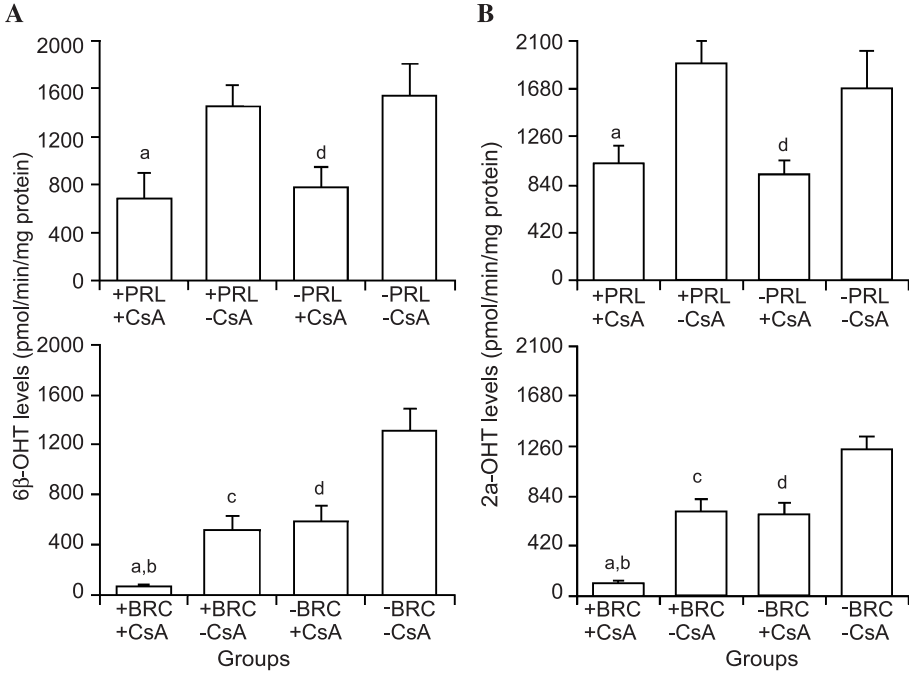

Fig. 3. Panel A: In vitro testosterone hydroxylation by hepatic microsomes following 14 days of treatment. Groups were given subcutaneous doses of either 15 mg/kg cyclosporine or cyclosporine in combination with prolactin, prolactin vehicle, bromocriptine, or bromocriptine vehicle. Graphs represent CYP3A1/2 activity in the form of 6β-hydroxytestosterone production. Units for metabolite production are pmol metabolite/min/mg microsomal protein. a=$P<0.05$ between +BRC/+CsA and +BRC/−CsA groups (bottom graph), b=$P<0.05$ between +PRL/+CsA and −PRL/+CsA (top graph) or between +BRC/+CsA and −BRC/+CsA groups (bottom graph), c=$P<0.05$ between +BRC/−CsA and −BRC/−CsA groups, d=$P<0.05$ between −PRL/+CsA and −PRL/−CsA (top graph) or between −BRC/+CsA and −BRC/−CsA groups (bottom graph). Panel B: In vitro testosterone hydroxylation by hepatic microsomes following 14 days of treatment. Groups were given subcutaneous doses of either 15 mg/kg cyclosporine or cyclosporine in combination with prolactin, prolactin vehicle, bromocriptine, or bromocriptine vehicle. Graphs represent CYP2C11 activity in the form of 2α-hydroxytestosterone production. Units for metabolite production are pmol metabolite/min/mg microsomal protein. a=$P<0.05$ between +PRL/+CsA and +PRL/−CsA (top graph) or between +BRC/+CsA and +BRC/−CsA groups (bottom graph), b=$P<0.05$ between +BRC/+CsA and −BRC/+CsA groups (bottom graph), c=$P<0.05$ between +BRC/−CsA and −BRC/−CsA groups, d=$P<0.05$ between −PRL/+CsA and −PRL/−CsA (top graph) or between −BRC/+CsA and −BRC/−CsA groups (bottom graph).

confirming the CYP3A1/2 protein measurements. However, when prolactin secretion is depleted with bromocriptine treatment, CYP3A1/2 activity was significantly lowered. This was evident in the presence ($P<0.01$) and in the absence of cyclosporine ($P<0.001$).

The formation of 2α-OHT mainly corresponds to the catalytic activity of CYP2C11 (Waxman, 1984). Cyclosporine significantly lowered CYP2C11 activity by 27% both when given in combination with prolactin or prolactin vehicle (Fig. 3). However, the effect of excess exogenous prolactin alone did not produce a significant change in CYP2C11 activity. Bromocriptine caused a decrease in CYP2C11 activity in the absence of cyclosporine, similar to protein expression results. Upon bromocriptine administration with cyclosporine, 2α-OHT levels were significantly lower than from the effects of bromocriptine or cyclosporine alone ($P<0.001$; $P<0.001$, respectively).

3.3. In vitro effect of bromocriptine on CYP3A- and CYP2C11-catalyzed testosterone hydroxylation

The observation that bromocriptine in combination with cyclosporine additively decreased CYP3A1/2 and CYP2C11 activity led us to investigate if there was a direct competition between bromocriptine and testosterone for enzyme activity in vitro causing decreased in vitro testosterone metabolite production. We found that the presence of bromocriptine suppressed the production of 6β-OHT by 35% ($P<0.05$). However, the presence of bromocriptine did not significantly alter the production of 2α-OHT in vitro ($P=0.33$) (Fig. 4).

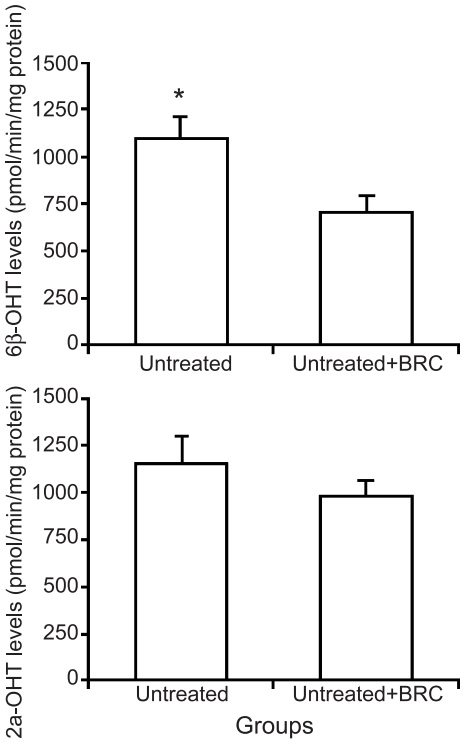

Fig. 4. Bromocriptine was co-incubated with testosterone and hepatic microsomes in the in vitro testosterone hydroxylation assay. Graphs represent CYP3A1/2 activity in the form of 6β-hydroxytestosterone production and CYP2C11 activity in the form of 2α-hydroxytestosterone production. Units for metabolite production are pmol metabolite/min/mg microsomal protein. *=$P<0.05$.

3.4. Circulating endogenous serum prolactin levels

Because prolactin secretion in the rat has been described as episodic in nature (Lafuente et al., 1996), collection of blood samples took place over a 4-h period. The serum from these samples was analyzed for prolactin and a graph of concentration versus time was generated for each animal. These graphs were used to produce area under the curve (AUC) calculations and the values were averaged within each group (Fig. 6).

Cyclosporine had a significant inductive effect on circulating prolactin levels. As shown in a previous study (Lafuente et al., 1996), chronic cyclosporine treatment resulted in an induction of rat prolactin levels. In addition, our study shows a 104% increase in AUC of circulating prolactin levels ($P<0.001$) caused by long-term cyclosporine therapy. However, when compared with cyclosporine vehicle, the introduction of cyclosporine had virtually no effect when bromocriptine was co-administered ($P=0.841$). Conversely, bromocriptine was able to significantly lower prolactin levels even in the presence of the inductive effects of cyclosporine ($P<0.001$) when compared with the administration of bromocriptine vehicle and cyclosporine. As anticipated, bromocriptine administration resulted in a decrease in endogenous circulating prolactin levels as compared with the administration of bromocriptine vehicle ($P<0.05$) as shown in representative graphs in Fig. 6.

The administration of prolactin over 14 days failed to cause an increase in rat prolactin AUC levels. This is in accordance with the previous studies showing little effect of the administration of ovine prolactin on the endogenous secretion of prolactin in ovariectomized female rats (Luquita et al., 1996).

3.5. Effect of bromocriptine alone and in combination with prolactin

In order to examine if the effects of bromocriptine on *P*450s were a direct result of depleting prolactin levels or another mechanism, we also investigated the effect of administering both bromocriptine and prolactin concomitantly. This portion of the study entailed treating two groups of animals with either bromocriptine in combination with prolactin or bromocriptine in combination with prolactin vehicle. Re-introducing prolactin to animals treated with bromocriptine served to eliminate the effect of prolactin as the cause of altered drug metabolism. In other words, the results of this experiment would help clarify if bromocriptine is truly suppressing hepatic *P*450s through the

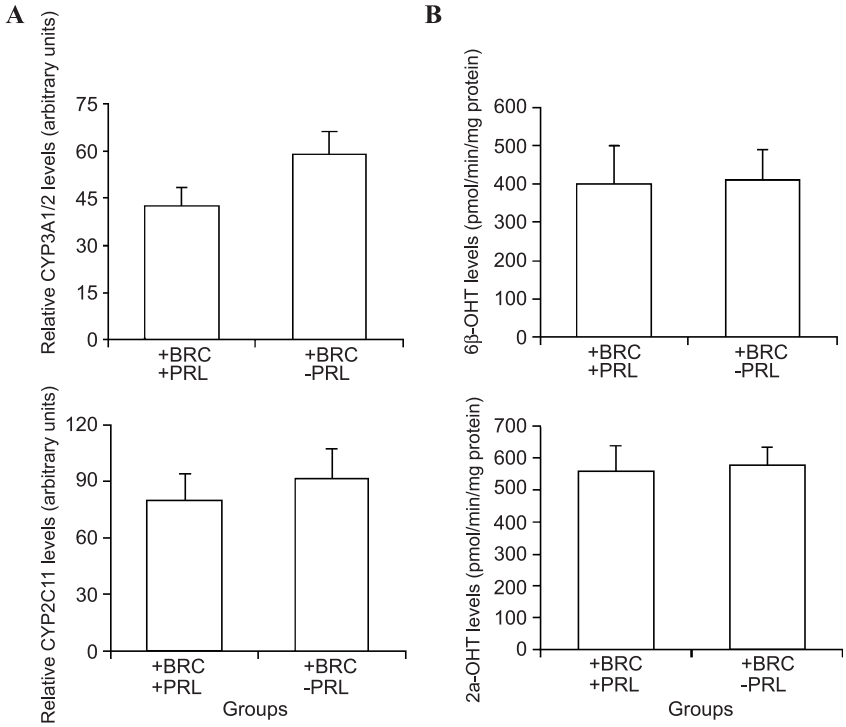

Fig. 5. Panel A: Western blot analysis of hepatic CYP3A1/2 and CYP2C11 microsomal protein expression in 14-day groups. The two groups were administered either bromocriptine with prolactin or bromocriptine with prolactin vehicle. All values are expressed as a percent density of the protein standard used for each blot. Panel B: In vitro testosterone hydroxylation by hepatic microsomes following 14 days of treatment. The two groups were administered either bromocriptine with prolactin or bromocriptine with prolactin vehicle. Graphs represent CYP3A1/2 activity in the form of 6β-hydroxytestosterone production and CYP2C11 activity in the form of 2α-hydroxytestosterone production. Units for metabolite production are pmol metabolite/min/mg microsomal protein.

reduction of prolactin or if the mechanism is independent of its effects on prolactin disposition. When bromocriptine and prolactin are given together, the hormonal status of the animal theoretically mimics a normal in vivo situation. Likewise, when bromocriptine is given with prolactin vehicle, the resultant effect is an animal with severely reduced prolactin levels due to the effect of bromocriptine.

CYP3A1/2 protein levels were slightly lower for animals treated with bromocriptine and prolactin as compared with animals treated with bromocriptine and prolactin vehicle (Fig. 5). However, the difference was not statistically significant. The production of 6β-OHT remained unchanged between the groups (Fig. 5). Similarly, CYP2C11 protein expression and activity were not significantly modified.

4. Discussion

We have shown previously that chronic cyclosporine dosing results in a demasculinization of hepatic *P*450 protein expression in rats (Brunner et al., 1996; 1998). This change in *P*450 expression was postulated to be due to an effect on growth hormone secretion. More recently, we have demonstrated that cyclosporine does not modulate these *P*450 proteins by altering circulating growth hormone levels (Lu et al., 2003). This does, however, leave the possibility that another pituitary hormone such as prolactin may play a role in the suppressive effect of cyclosporine. Due to its role in the immune system and the ability to act locally upon its release, prolactin has been rightfully viewed as a cytokine for roughly the past decade. With prolactin's immunomodulatory function in mind, we investigated this hormone's role in the suppressive action of cyclosporine on hepatic *P*450 enzymes. This concept was examined by independently supplementing and abolishing prolactin release in an in vivo system and analyzing the resultant effect on *P*450 enzymes while in the presence or absence of chronic cyclosporine therapy.

The CYP3A subfamily of *P*450 enzymes contributes significantly to the overall drug metabolism that takes place in the rat liver. Since cyclosporine is both a substrate and an inhibitor of CYP3A2, this isoform was of primary importance for this study. Results from the CYP3A1/2 protein expression data suggest that drug metabolism is influenced more by the absence of prolactin secretion rather than the super-physiological state of the hormone (Fig. 1). Previous studies involving treatment with prolactin did not directly address the effect of excess prolactin on the disposition of CYP3A1/2. We show that chronic subcutaneous administration of prolactin had no effect on CYP3A1/2 protein levels or activity. Also, prolactin did not alter the suppressive effect of cyclosporine on this enzyme.

While prolactin supplementation did not significantly alter the expression of CYP3A1/2, to fully understand the overall maintenance of hepatic drug metabolism, the effect of abolishing prolactin also needed to be investigated. When bromocriptine (an inhibitor of prolactin secretion) was administered in chronic subcutaneous doses, it not only resulted in a suppression of CYP3A1/2 protein expression and activity, but it also augmented the suppression in 6β-OHT production caused by cyclosporine. To investigate the possible cause of this additive suppression, we incubated hepatic microsomal samples from a vehicle-treated animal in the presence of both bromocriptine and testosterone as metabolic substrates. The results showed that the presence of bromocriptine at a concentration of less than one half of testosterone decreased the amount of CYP3A-catalyzed testosterone hydroxylation product, but had no significant effect on CYP2C11 activity. This result indicates that bromocriptine is competing with testosterone for *P*450 metabolism, thus lending less enzyme activity towards testosterone metabolism. Since CYP3A has been shown to oxidize bromocriptine in rat liver microsomes (Peyronneau et al., 1994), bromocriptine is likely suppressing the production of 6β-OHT through competition with testosterone for CYP3A-catalyzed metabolism. Based on this information, bromocriptine could also compete with cyclosporine for CYP3A metabolism, leading to an accumulation of cyclosporine and resulting in a further decrease in overall CYP3A expression and activity.

In order to confirm bromocriptine's effects are due to the alteration of prolactin, in two separate groups, we supplemented prolactin during bromocriptine treatment and compared those effects to a prolactin vehicle group. Our data demonstrate that supplemental doses of prolactin did not overcome the suppressive effect of bromocriptine on CYP3A1/2 or CYP2C11. This suggests that prolactin is not the mediating factor in the suppressive effect of bromocriptine. However, as only one concentration of bromocriptine and prolactin were used, it remains a possibility that the prolactin dose was not sufficient enough to overcome the effects of the sizeable dosage of bromocriptine.

The expression of CYP2C11 is obligatorily dependent on the pulsatile secretions of growth hormone in the adult male rat (Waxman et al., 1991). Without the presence of differentiable pulses and trough periods, hepatic CYP2C11 will

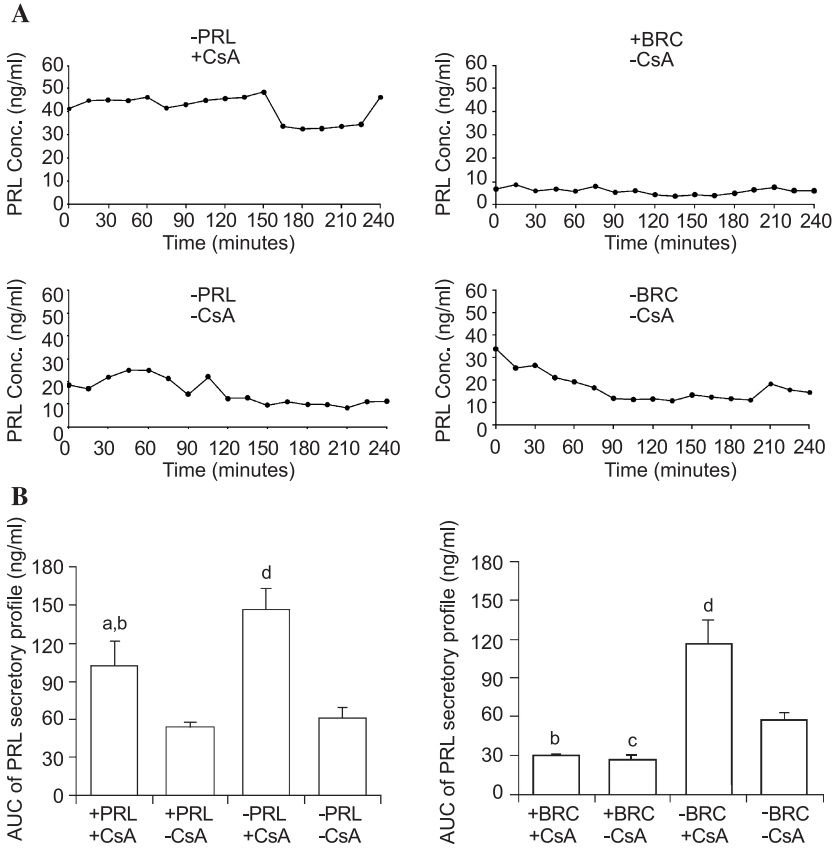

Fig. 6. Panel A: Representative graphs depicting prolactin secretion profiles of one representative animal from each of the following groups: −PRL/+CsA, −PRL/−CsA, +BRC/−CsA, −BRC/−CsA, demonstrating the inductive effect of cyclosporine on prolactin secretion and the suppressive effect of bromocriptine, respectively. Blood samples were collected every 15 min over a 4-h period. Panel B: Area under the curve calculations of prolactin secretory profiles for circulating serum prolactin in blood samples collected every 15 min over a 4-h period. Units are expressed as ng of circulating prolactin per ml of serum. a=$P<0.05$ between +PRL/+CsA and +PRL/−CsA, b=$P<0.05$ between +PRL/+CsA and −PRL/+CsA (left graph) or between +BRC/+CsA and −BRC/+CsA groups (right graph), c=$P<0.05$ between +BRC/−CsA and −BRC/−CsA groups, d=$P<0.05$ between −PRL/+CsA and −PRL/−CsA (left graph) or between −BRC/+CsA and −BRC/−CsA groups (right graph).

fail to express. Since CYP2C11 is highly dependent on the pattern of growth hormone secretion, this hormonal dependence may extend to other hormones with gender-dependent secretion, such as prolactin. The presence of excess prolactin as well as the reduction of prolactin secretion by bromocriptine suppressed CYP2C11 protein expression (Fig. 2). This effect was also observed in CYP2C11 activity from bromocriptine-treated rats (Fig. 3). As with the CYP3A1/2 data, the depletion of prolactin is more dominant in affecting CYP2C11 activity than the presence of excess prolactin. Additionally, the data from the in vitro experiments revealed that the levels of CYP2C11-catalyzed product did not change in the presence of bromocriptine. This provides evidence that the in vivo suppression of CYP2C11 activity by bromocriptine is not an artifact of competition with bromocriptine for metabolism, as is the case for CYP3A1/2. Thus, the effect on CYP2C11 initiated by bromocriptine is likely due to actual suppressive actions of bromocriptine. Although reports of the role of prolactin in drug metabolism are limited in the literature, one study did demonstrate that there were no differences in levels of CYP2C11 in hypophysectomized female rats treated with prolactin as compared with untreated hypophysectomized rats (Yamazoe et al., 1987). While the replacement of prolactin cannot induce the production of male-specific *P*450 enzymes in the female rat, we have shown that the lack of prolactin can decrease CYP2C11 in the male rat, demonstrating the importance of maintaining prolactin status in vivo. Given that the expression of CYP2C11 is strongly dependent on the levels of circulating growth hormone, it is reasonable that CYP2C11 is also particularly responsive to levels of circulating prolactin.

As anticipated, bromocriptine effectively depressed circulating prolactin levels as evidenced by the area under the curve values for prolactin concentrations in blood collected over time (Fig. 6). Circulating prolactin levels increased with the administration of cyclosporine but not when bromocriptine was given at the same time. This indicates that prolactin inhibition caused by bromocriptine dominates over the induction of prolactin secretion by cyclosporine. Others have also reported that chronic cyclosporine dosing significantly increased the circulating levels of prolactin in rats (Lafuente et al., 1996). However, the data from that particular study showed that when using an ectopic pituitary graft in conjunction with cyclosporine treatment, the levels of prolactin are lower than with the ectopic graft alone. The use of an ectopic pituitary graft often complicates the investigation of hormonal effects since the pituitary releases a host of other hormones such as growth hormone, which is known to alter CYP3A2 and CYP2C11 expression. For this reason, we chose to selectively supplement prolactin using twice daily injections to eliminate potential interferences from other pituitary hormones. Our study indicates that chronic cyclosporine treatment induces circulating prolactin levels by promoting endogenous secretion of prolactin.

Although growth hormone plays a key role in the male-specific expression of *P*450s, the secretion of prolactin does not seem to be as critical in the proper maintenance of CYP3A2 and CYP2C11 expression. Accordingly, cyclosporine-induced suppression of CYP3A1/2 and CYP2C11 is likely not mediated by prolactin. We have also shown that while bromocriptine suppresses CYP3A1/2 and CYP2C11, it is likely acting via a different mechanism than inhibiting prolactin release. This drug may have intrinsic suppressive action and may be of considerable clinical significance to further investigate bromocriptine's effects on hepatic drug metabolism.

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