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Metabolism of the New Immunosuppressor Cyclosporin G by Human Liver Cytochromes P450

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ABSTRACT. Cyclosporin G is a new immunosuppressor structurally similar to cyclosporin A. Although this drug is pharmacologically as active as cyclosporin A, it is less toxic, in particular at the kidney level. The aim of this work was to identify the enzyme system(s) involved in the oxidative metabolism of cyclosporin G in man: (1) in a bank of human liver microsomes ($n = 22$), cyclosporin G oxidase activity correlated significantly with cyclosporin A oxidase activity ($P < 0.0001$) and with the level of CYP3A4 ($P < 0.002$), determined by immunoblot; (2) specific inhibitors of CYP3A4, troleandomycin, and ketoconazole, inhibited cyclosporin G oxidase activity by more than 80%; (3) antiCYP3A4 antibodies specifically inhibited this activity by nearly 90%; (4) cyclosporin A was a competitive inhibitor of cyclosporin G oxidase and *vice versa*; (5). Among a battery of cDNA-expressed CYPs, only CYP3A4 was able to generate detectable amounts of metabolites of cyclosporin G and cyclosporin A with a turnover number close to that calculated from experiments with liver microsomes; (6) in human hepatocytes in culture, pretreatment of cells with rifampicin and phenobarbital, 2 inducers of CYP3A4, produced a great increase in cyclosporin G oxidase activity, while β-naphthoflavone, an inducer of CYP1As, did not. We conclude that CYP3A4 is the major enzyme involved in the oxidative metabolism of cyclosporin G in human liver. BIOCHEM PHARMACOL 51;5:591–598, 1996.

KEY WORDS. cyclosporin G; oxidative metabolism; human CYP; drug interactions; immunosuppressor

Cyclosporin G (CSG or Sandoz compound OG 37-325) is an analog of cyclosporin A (CSA) in which the α-aminobutyric acid residue has been replaced by a norvaline residue in position 2 [1, 2]. This molecule has been reported to be as efficient as CSA in the prevention of acute allograft rejection with, however, reduced renal toxic side effects [3–5]. The oxidized metabolites of CSG were found to have very low pharmacological effects [5]. The pharmacokinetic parameters of CSA and CSG were comparable in patients with renal failure [6], in spite of marked differences in blood distribution [7].

The major metabolic pathway of CSG, however, has not yet been determined at the molecular level. Yet, it is important to clarify this point because it would allow the prediction and *a posteriori* the explanation of perturbations of pharmacokinetic parameters or of pharmacological effects resulting from comedications or physiopathological stimuli [8–10]. The aim of this work was, accordingly, to identify the enzyme system(s) involved in the oxidative metabolism of CSG in humans. Using a bank of human liver microsomes characterized for the levels of several forms of cytochrome P450 (CYP) and related monoxygenase activities, CYP-specific inhibitors, antiCYP specific antibodies, cDNA-expressed CYPs and primary cultures of human hepatocytes, we demonstrate in this report that CYP3A4 is the main enzyme involved in the hepatic and possibly intestinal oxidative metabolism of CSG. A preliminary report has been published elsewhere [11].

MATERIALS AND METHODS

Drugs and Chemicals

CSG, [mebmt-β-^{3}H]CSG (specific radioactivity 6.7 mCi/mg, synthesized by Sandoz Ltd. Basel, Switzerland), metabolite GM1 of CSG (n-hydroxy-CSG, the analogue of metabolite 17 of CSA), cyclosporin A (CsA), and [mebmt-β-^{3}H]CsA (specific radioactivity 11 Ci/mmol, from Amersham, Amersham England), were generously supplied by Sandoz Ltd (East Hanover, NJ, Reuil Malmaison, France and Basel, Switzerland). In both CSG and CSA, the tritium label was incorporated at the 1-β position of amino acid 1 (N-methyl-γ-butenyl-γ-methyl-threonine). Chemical and radiochemical purity was greater than 99% as assessed by high performance liquid chromatography (HPLC). CYP inhibitors and specific substrates included: propranolol from ICI Pharma (Pontoise, France), coumarine from Fluka (Buchs, Switzerland), sulfaphenazole

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† *Abbreviations*: CYP, cytochromes P450; CSA, cyclosporine A; CSG, cyclosporine G; HPLC, high performance liquid chromatography.

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from Ciba-Geigy (Rueil-Malmaison, France), tolbutamide, quinidine, diethyldithiocarbamate, and lauric acid from Sigma (Saint Louis, MO), ketoconazole from Janssen (Boulogne, France), and triacetyloleandomycin from Pfizer (Paris, France).

Human Liver Samples

The use of human liver samples for scientific research was authorized by the French National Ethics Committee (date of last review and approval, November 1992). Clinical characteristics of some donors and patients have been given in previous papers: HL1, HL2, HL4, HL5, HL13 [8], FT1, FT4 [10], FH3, FH5, FH7, FT5, FT6, FT22, FT25, FT31 [12], 18190 and 61289 [13], FT12, FT21 and FH6 [14]. Clinical characteristics of other donors are as follows: patient FT26 was a 55-year-old woman who had undergone resection of hepatic segments 5 and 6 for an adenoma; patient FT54 was a 70-year-old woman who had undergone left lobectomy for an adenoma; patient FT60 was a 71-year-old man who had undergone resection of hepatic segments 1, 2, 3, and 4 for a metastasis of a colon cancer; subject FH68 was a 36-year-old male organ donor with amyloid neuropathy; patient FT69 was a 52-year-old man who had undergone left lobectomy for a metastasis of a gastric tumor.

Preparation and Primary Cultures of Human Hepatocytes

After washing the tissue with Eurocollins (2.05 g/L KH_2PO_4, 7.4 g/L K_2HPO_4, 1.12 g/L KCl, 0.84 g/L $NaHCO_3$, 35 g/L glucose, pH 7.3), hepatocytes were prepared and cultured as described in previous papers [10, 12–15].

Preparation of Microsomes

Microsomes were prepared either from liver samples or from cultured cells by differential centrifugation and stored as described previously [15]. Protein concentration was determined by the bicinconinic acid method (Pierce Chemical Co, Rockford IL), bovine serum albumin being used as the standard.

HPLC Analysis of CSG and CSA and of Their Metabolites

CSG and metabolites were separated and quantified by HPLC as follows. Briefly, 100 µL aliquots of 1/1 mixture of microsome supernatant, extracellular medium, or cell lysate and acetonitrile were briefly centrifuged and loaded onto a Beckman (San Ramon CA) ultra sepharose ODS column (5 µm, 4.6 mm × 25 cm) protected with a precolumn of the same phase, and eluted at 70°C with a mobile phase consisting of a mixture of water and acetonitrile (Merck, Darmstadt, Germany) whose composition by volume was programmed according to the following linear steps: (1) acetonitrile gradient from 55 to 60% from 0 to 15 min; (2) acetonitrile gradient from 60 to 70% from 15 to 25 min; (3) acetonitrile gradient from 70 to 90% from 25 to 40 min; (4) water/acetonitrile 45:55 from 40 to 45 min. Under these conditions, the elution times of CSG and metabolite GM1 (n-hydroxy-CSG) were 34.4 and 20.3 min, respectively. The radioactivity of the effluent from the HPLC column was analysed in a Radioactivity Monitor LB 506-CI (Berthold, Wildbad, Germany) after mixing in a 1/3 ratio with Zinsser scintillation cocktail (Quickszint Flow 302, Maidenhead, England). Radioactivity peaks were integrated with the aid of a computer and converted to molar concentrations of CSG and metabolites. CSG oxidase activity was expressed as the amount of total metabolites produced *versus* time. A relative uncertainty of ±15% was estimated from independent measurements in triplicate. CSA and metabolites were separated and quantitated by HPLC procedure described in a previous paper [10]. (S)-mephenytoin 4′-hydroxylase was determined as described [16].

Metabolism of CSG and CSA in Primary Cultures of Human Hepatocytes

After 96 hr of culture in the absence or in the presence of the inducer, 50 µM rifampicin, 50 µM β-naphthoflavone, or 2 mM phenobarbital, the culture medium (Ham F12 and Williams'E, 1/1 in volume, Sigma) was renewed in the absence of the inducer and in the presence of 5 µM of CSG or CSA, and of 0.5 to 1 µCi of the corresponding radiolabeled molecule. The cells were then incubated under standard culture conditions for 4 hr and 250 µL aliquots of the culture medium were collected and analyzed by HPLC for the parent molecule and metabolites, as described above. The amount of CSG or CSA and their metabolites present in the culture medium were calculated from the HPLC data and the total radioactivity recovered from the cells, including the radioactivity present in the cell pellet.

Metabolism of CSG and CSA in Human Liver Microsomes

Five hundred micrograms of liver microsomes were resuspended in 500 µL of 0.1 M potassium phosphate buffer, pH 7.4, in the presence of 2 to 50 µM CSG or of 2.5 to 10 µM CSA and of 0.5 µCi of the corresponding radiolabelled molecule. After 3-min incubation at 37°C, the reaction was initiated by the addition of 1 mM NADPH (Sigma) and quenched 30 min later (under conditions of linear kinetics) by mixing with an equal volume of acetonitrile. After centrifugation, 100 µL of supernatant was analysed by HPLC. CSG and CSA oxidase activities were expressed as pmol of total metabolites produced per mg of protein per min. In some experiments, the effect of selective CYP inhibitors or specific CYP substrates was assessed. In this case, a concentration of 5 µM of CSG, slightly lower than the K_m, was chosen so as not to saturate the enzyme; in contrast, the CYP inhibitors or substrates were used at concentrations exceeding their respective K_i or K_m to amplify the putative inhibitory effect.

Correlation Analysis of Data

Monoxygenase activities, including CSG oxidase, CSA oxidase, and (S)-mephenytoin 4′-hydroxylase, and the levels of

CYP1A2, CYP2D6, CYP2E1, and CYP3A4 obtained for each preparation of liver microsomes were compared by simple linear regression analysis for each pair of data using the Macintosh Stat View program (Abacus Concepts, Inc., Berkeley, CA).

Anti-CYP Polyclonal Antibodies

Anti-CYP polyclonal antibodies directed against rabbit CYP1A1, 1A2, 2B4, 2C3, 2E1, and 3A6 [16], monkey CYP2A [17], and human 2D6 (monoclonal, [18]) were used in this work, either in immunoinhibition or immunoquantitation experiments. Previous studies have shown that the antibodies directed against rabbit and monkey crossreact with the human orthologous forms [16, 17].

Immunoinhibition of Microsomal Monoxygenase Activities by Anti-CYP Antibodies

Human liver microsomes (1 mg/mL) were resuspended in a final volume of 500 µL in 0.1 M potassium phosphate buffer pH 7.4. They were first incubated for 20 min at room temperature in the absence or presence of various concentrations of anti-CYP antibodies (0 to 10 mg/mL) to permit antigen-antibody complex formation, and CSG was then added. The assay conditions were the same as those described above.

Immunoquantitation of CYPs in Liver Microsomes

CYP1A2, CYP2D6, CYP2E1, and CYP3A4 were quantitated by immunoblot using specific antibodies as described [16]. Purified antigens CYP3A4 [8] (1 pmol per lane) and cDNA-expressed CYP1A2, CYP2D6, and 2E1 (Gentest Co. Woburn, MA) were used on each gel as standard. Horseradish peroxidase-labelled antibodies were from Sigma. Nitrocellulose filters were from Biorad (Richmond, CA).

Assays with cDNA-Expressed Human CYPs

Microsomes from a human lymphoblastoid cell line expressing human CYP1A1, 1A2, 2A6, 2B6, 2D6, 2E1, and 3A4 (Gentest Corp., Woburn, MA) were used in this work to analyse the biotransformation of CSG. Microsomes (2 mg/mL) were resuspended and assayed as described above for liver microsomes, except that the time of incubation was 3 hr.

RESULTS

Biotransformation of CSG in Human Liver Microsomes

When CSG was incubated with human liver microsomes in the presence of NADPH, HPLC analysis of the supernatant revealed the presence of GM1 (n-hydroxy-CSG) as the major metabolite (elution time: 20.3 min) and of several other minor metabolites eluting between 7 and 20 min and between 25 and 35 min. These were not characterised further in the present work. Throughout this paper, the rate of CSG oxidation is expressed as the amount of total metabolites produced *versus* time. The range of CSG concentration varied from 2 to 50 µM and the data were analysed by Lineweaver-Burk plots. These concentrations largely exceeded the serum level of CSG in patients (0.2 µM). However, the drug is expected to concentrate in hepatocytes, so that the local concentration "seen" by the enzyme *in vivo* should be greater than 0.2 µM. In preliminary experiments, the rate of CSG oxidation was measured in 3 different preparations from our bank of human liver microsomes. These were linear with the 3 preparations used. K_m and V_{max} values for samples 61289, FT21, and FT31 were: 8.6, 7.8, and 5.8 µM, and 225, 200, and 125 pmol/mg/min, respectively. Interestingly, K_m and V_{max} values for the oxidation of CSA, determined under similar experimental conditions with samples 61289 and FT21, were similar to the data for CSG: 5 ± 2 µM and 200 ± 25 pmol/mg/min (mean ± SD), respectively. Then, 22 different preparations of human liver microsomes were used to evaluate the interindividual variability of the biotransformation of CSG (50 µM). All microsome preparations generated measurable amounts of metabolites (Fig. 1). The results revealed a fairly wide interindividual variability, the production of metabolites varying by a factor of 17 between preparations FT26 and 61289. Among the liver microsome preparations used in this study, 2 (FT6 and FH5) were shown to be deficient in CYP2D6 by Western blot, and 2 others (FT4 and FT22) were inactive for (S)-mephenytoin 4′-hydroxylation. These forms of CYP are known to be expressed polymorphically in man [19, 20]. These preparations, however, did not exhibit "anormal" CSG oxidase activity with respect to the others, suggesting that CYP2D6 and CYP2C19 are not involved to a significant extent in the biotransformation of CSG in human liver. In addition, further Western blot analysis of these preparations revealed that subjects FT6, FH5, FT4, and FT22 had "normal" levels of other CYPs, including notably CYP3A4 and 1A2.

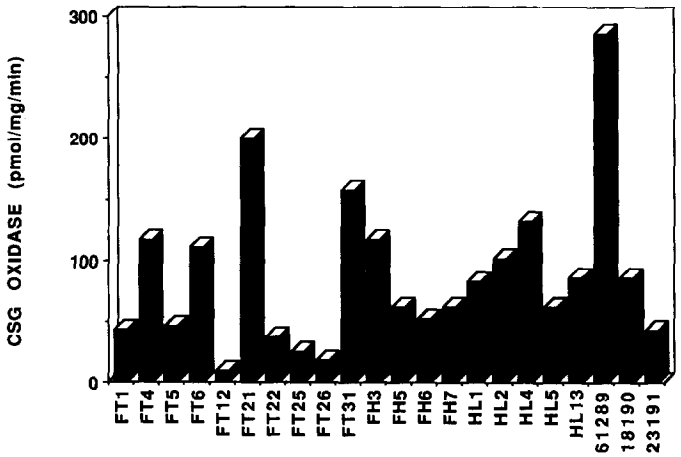

FIG. 1. Interindividual variability of the metabolism of cyclosporin G in human liver microsomes. Human liver microsomes (1 mg/mL) were incubated at 37°C in a 0.1 M potassium phosphate buffer, pH 7.4, with 50 µM CSG in the presence of 0.5 µCi of the radiolabelled compound. Thirty minutes after the reaction had been initiated with 1 mM NADPH, CSG and metabolites were analysed by HPLC. Liver microsome samples FT6 and FH5 were deficient in CYP2D6, as revealed by immunoblot. Samples FT4 and FT22 were inactive in (S)-mephenytoin 4′-hydroxylase activity.

Correlations

The preparations of microsomes used in this work were characterised for the level of several CYPs, including CYP1A2, 2D6, 2E1, and 3A4 (determined by immunoblot) and for several monoxygenase activities, including CSA oxidase (CYP3A4) and (S)-mephenytoin 4′-hydroxylase (CYP2C19). The rate of biotransformation of CSG was compared with these data by simple linear regression plots. The results are reported in Table 1, where the correlation coefficient as well as the P value are quoted for each pair of data. The rate of CSG biotransformation correlated significantly ($P < 0.002$) with the level of CYP3A4 and with CSA oxidase activity. In contrast, no correlation was observed with the other data, including the levels of CYP1A2, 2D6, 2E1, and (S)-mephenytoin 4′-hydroxylase activity.

Inhibition of CSG Oxidase Activity by Specific CYP Inhibitors and Substrates

Several compounds have been characterised as selective inhibitors of CYP forms. These include, notably, sulfaphenazole (CYP2C9) [21], quinidine (CYP2D6) [22], diethyldithiocarbamate (CYP2E1, 2A6) [23], triacetyloleandomycin, and ketoconazole (CYP3A4) [13, 24]. Propranolol has been described as a CYP1A2 inhibitor, although it might also inhibit other CYPs including CYP2D6 [25]. In addition, other molecules are known to be specifically oxidised by some CYP forms. These molecules are, thus, expected to behave as competitive inhibitors with respect to a compound metabolized by the same CYP. Coumarin (CYP2A6) [14], tolbutamide (CYP2C9) [26], (S)-mephenytoin (CYP2C19) [20], and lauric acid (CYP4A), [27] were used in this respect. The inhibitory effect of these compounds on the biotransformation of CSG is reported in Fig. 2 for preparation FT21 (similar results were obtained with samples 61289 and FT31, not shown). Clearly, ketoconazole (note that maximum inhibition was obtained with 1 μM of this compound), triacetyloleandomycin, and CSA were inhibitors. The other compounds, in contrast, did not significantly affect CSG oxidase activity. In parallel experiments, CSG was found to be a competitive inhibitor of CSA oxidase with a K_i of 7.4 μM, and CSA was found to be a competitive inhibitor of CSG oxidase with a K_i of 4.4 μM (not shown).

Immunoinhibition of CSG Oxidase Activity by Anti-CYP Antibodies

In an attempt to obtain further confirmation of the implication of CYP3A4 in the biotransformation of CSG, immunoinhibition experiments were carried out on preparation FT21. Anti-CYP3A4 and anti-CYP1A1 were used at 2 concentrations, 5 and 10 mg/mL. The specificity of these antibodies and their inhibitory effect on reactions specifically catalysed by the forms of CYP against which they are directed were tested in previous investigations [16, 17]. The results presented in Fig. 3 clearly confirm the previous observations (similar results were obtained with samples 61289 and FT31, not shown). Anti-CYP3A antibodies strongly inhibited the biotransformation of CSG and those directed against CYP1A1 had no significant effect. It must be pointed out here that, under the same experimental conditions, CSA oxidase activity was inhibited by more than 50% by these anti-CYP3A antibodies.

TABLE 1. Correlations (r and P values) between the rate of metabolism of CSG and the levels of CYPs and other monoxygenase activities in human liver microsomes ($n = 22$)

	CYPs				Activities	
	3A4	1A2	2D6	2E1	CSA	MP
r	0.644	0.032	0.038	0.324	0.828	0.229
P	0.0016	0.890	0.894	0.258	<0.0001	0.331

CSA, cyclosporin A oxidase activity; MP, (S)-mephenytoin 4′-hydroxylase.

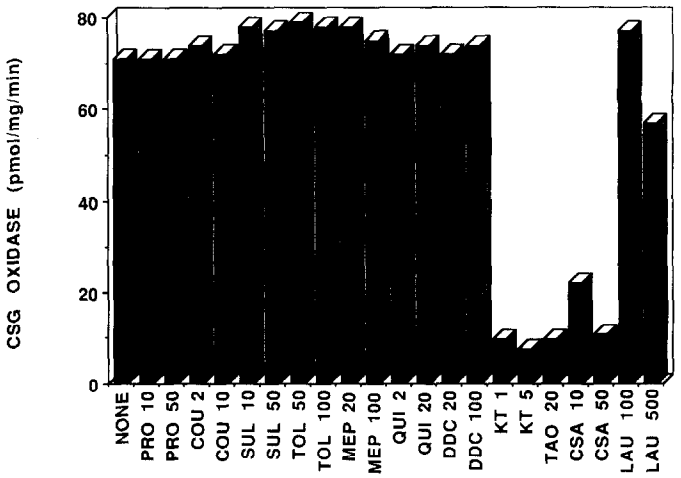

FIG. 2. Inhibition of microsomal metabolism of cyclosporin G by CYP-specific inhibitors and substrates. Human liver microsomes (1 mg/mL, sample FT21) were incubated at 37°C in a 0.1 M potassium phosphate buffer, pH 7.4, with 5 μM CSG in the presence of 0.5 μCi of the radiolabelled compound, and in the absence (none) or in the presence of (1–500 μM) CYP-specific inhibitors or substrates. Thirty minutes after the reaction had been initiated with 1 mM NADPH, CSG and metabolites were analysed by HPLC and the CSG oxidase activity is presented. Inhibitors and substrates were as follows: PRO, propranolol at 10 and 50 μM; COU, coumarin at 2 and 10 μM; SUL, sulfaphenazole at 10 and 50 μM; TOL, tolbutamide at 50 and 100 μM; MEP, (S)-mephenytoin at 20 and 100 μM; QUI, quinidine at 2 and 20 μM; DDC, diethyldithiocarbamate at 20 and 100 μM; KT, ketoconazole at 1 and 5 μM; TAO, troleandomycin at 20 μM; CSA, cyclosporin A at 10 and 50 μM; and LAU, lauric acid at 100 and 500 μM. Similar results were obtained with 61289 and FT31.

Biotransformation of CSG by cDNA-Expressed Human CYPs

The ability of several cDNA-expressed human CYPs to biotransform CSG was next investigated. For this purpose, microsomes (2 mg/mL) from a human lymphoblastoid cell line (Genetest Corp.) were incubated for 3 hr in the presence of 5 μM CSG or CSA. CYP1A1, 1A2, 2A6, 2B6, 2D6, and 2E1 were inactive. Only CYP3A4 generated detectable amounts of metabolites with both CSG and CSA, with turnover numbers of 0.1 and 0.3 min^{-1}, respectively. Because the concentration

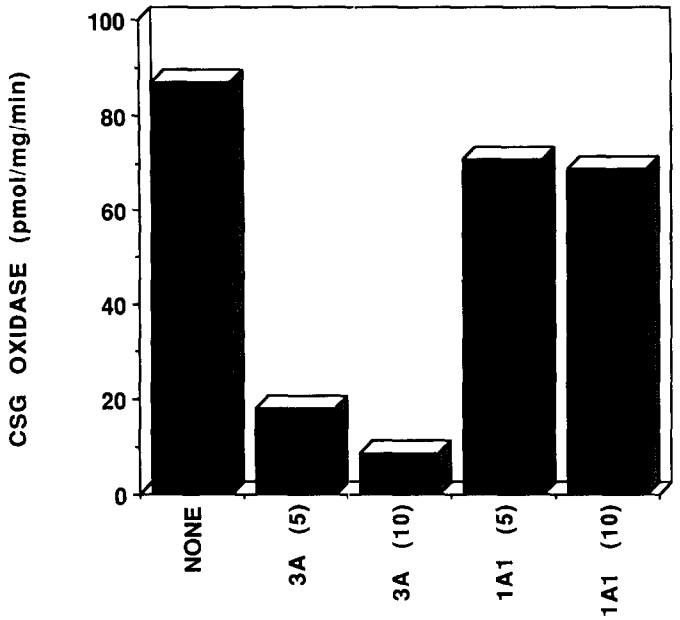

FIG. 3. **Immunoinhibition of microsomal metabolism of cyclosporin G by specific anti-CYP antibodies. Human liver microsomes (1 mg/mL, sample FT21) were incubated for 20 min at room temperature in a 0.1 M potassium phosphate buffer, pH 7.4, in the absence (none) or in the presence of (5–10 mg/mL) specific anti-CYP antibodies. Five micromolar CSG, in the presence of 0.5 μCi of the radiolabeled compound, were then added to the suspension that was incubated at 37°C. Thirty minutes after the reaction had been initiated with 1 mM NADPH, CSG and metabolites were analysed by HPLC and the CSG oxidase activity is presented as follows: 3A, anti-CYP3A6 antibodies at 5 and 10 mg/mL; 1A1, anti-CYP1A1 antibodies at 5 and 10 mg/mL. Similar results were obtained with 61289 and FT31.**

of CYP3A4 is, on average, of the order of 200 pmol/mg in human liver microsomes [28], the turnover number for CSG oxidase, calculated from the specific activity of preparation FT21 with 5 μM CSG (see Fig. 2), is 0.35 min^{-1}, in reasonable agreement with the above data.

Biotransformation of CSG in Primary Cultures of Human Hepatocytes: Effect of CYP Inducers

Two different preparations of human hepatocytes (from patients FT54 and FT60) were maintained in culture for 96 hr in the absence or in the presence of 50 μM rifampicin or 2 mM phenobarbital (inducers of CYP3A4) or of 50 μM β-naphthoflavone (inducer of CYP1A1 and 1A2) [10, 13–16]. The biotransformation of CSG (at 5 μM in the extracellular medium) was then monitored by measuring the amounts of the unchanged compound and of its metabolites in both the extra- and intracellular media after 4 hr. These experiments were repeated with CSA under identical conditions. Results are presented in Fig. 4A (for culture FT60, but similar data were obtained with culture FT54). Cells treated with rifampicin or phenobarbital produced greater amounts (4- to 5-fold) of oxidised metabolites of both CSG and CSA than untreated cells or cells treated with β-naphthoflavone. Analysis of the intracellular medium indicated that CSG metabolites did not accumulate in the cells as previously shown to occur with CSA (not shown). In parallel experiments, cells were harvested and microsomes prepared. The levels of CYP1As and CYP3A4, assessed by immunoblotting, were found to be strongly induced

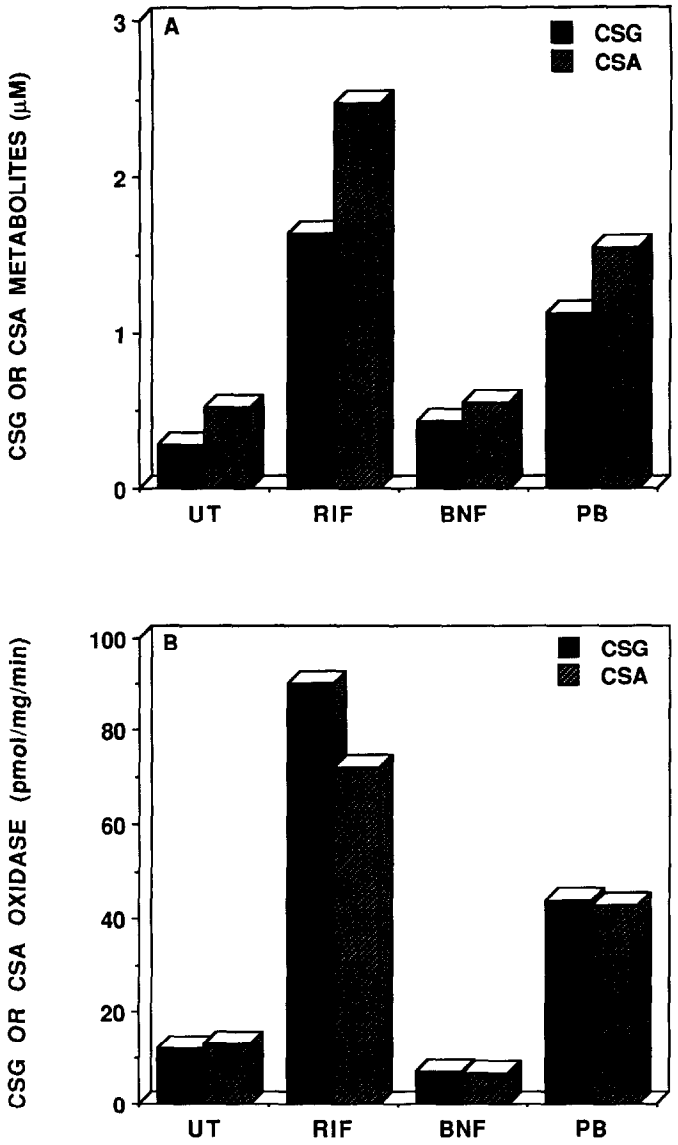

FIG. 4. **Oxidative metabolism of cyclosporins G and A in human hepatocytes in primary culture and in microsomes. Human hepatocytes from patients FT54 and FT60 were maintained in culture for 96 hr in the absence (UT) or in the presence of various inducers including 50 μM rifampicin (RIF), 50 μM β-naphthoflavone (BNF), or 2 mM phenobarbital (PB). (A) The medium was then renewed, in the absence of the inducer but in the presence of 5 μM CSG or of CSA and 0.5 μCi of the radiolabelled molecule. After a 4-hr incubation period, an aliquot of extracellular medium was collected and analyzed by HPLC. Results presented were obtained with culture FT54, but were similar with culture FT60. (B) Cells were scraped and microsomes were prepared. The metabolism of CSG and CSA was then assessed by HPLC as indicated in the legend of Fig. 1 except that, here, the concentration of cyclosporins was 5 μM. Results presented were obtained with culture FT60, but were similar with culture FT54.**

in cells treated with β-naphthoflavone and with rifampicin or phenobarbital, respectively, as expected (not shown). CSG and CSA oxidase activities measured with these microsomes (Fig. 4B) were in excellent agreement with the data on the cellular metabolism presented in Fig. 4A. These data confirm the implication of CYP3A4 in the oxidative metabolism of CSG.

Drug Interactions

Because CSG appears to be metabolized primarily by CYP3A4, like CSA, it was to be expected that both molecules would be involved in the same drug interactions. We, therefore, investigated the effects of drugs, some of which had previously been characterized as inducers or inhibitors of CSA oxidase [10–14], on the metabolism of CSG in human hepatocytes (preparations FH68 and FT69) and in human liver microsomes (sample 61289). The results for the inducers are reported in Table 2. Of 88 molecules tested in parallel on CSG and CSA, 11 compounds found to be inducers of CSA metabolism in human hepatocytes in culture were inducers of CSG oxidase, as well. The large standard variation observed on the induction of CSA oxidase activity with some drugs (rifampicin, phenobarbital, phenytoin) is due to the great number of cultures analysed and to the wide interindividual variability in the extent of CYP3A4 induction in response to these drugs. The results on 36 different drugs, some of which have been found to be inhibitors or noninhibitors of CSA oxidase, on CSG oxidase (and in parallel on CSA oxidase) are presented in Fig. 5 (see legend for the nature of the drugs and their respective K_i). These data show that there is, indeed, an excellent correlation between residual CSG and CSA oxidase activities ($r = 0.951$ with $P < 0.0001$). These data clearly confirm the conclusions drawn from the previous sections.

TABLE 2. Inducers of cyclosporin G and A oxidase activities in human hepatocytes in cultures

Drug*	CSG oxidase (in % of control)	CSA oxidase† (in % of control)
None	100	100
Azatadine	191 ± 30	199 ± 60
Carbamazepine	353 ± 90	–
Dexamethasone	385 ± 30	600 ± 400
Lansoprazole	445 ± 90	410 ± 15
Omeprazole	285 ± 30	170 ± 12
Oxomemazine	300 ± 30	220 ± 40
Phenobarbital	505 ± 50	390 ± 250
Phenytoin	404 ± 30	370 ± 230
Prednisone	240 ± 40	268 ± 30
Rifampicin	665 ± 50	450 ± 250
Virginiamycin	240 ± 60	185 ± 15

* Human hepatocytes (from patients FH68 and FT69) for CSG were maintained in culture for 96 hr in the absence (UT) or in the presence of various drugs at 50 μM, except phenobarbital (2 mM). The medium was then renewed, in the absence of the inducer but in the presence of 5 μM CSG or of CSA and 0.5 μCi of the radiolabelled molecule. After a 4-hr incubation period, an aliquot of extracellular medium was collected and analyzed by HPLC. Data presented are means ± standard deviation (standard deviation refers to the variance between different cultures of human hepatocytes).

† Data from previous reports [10–14].

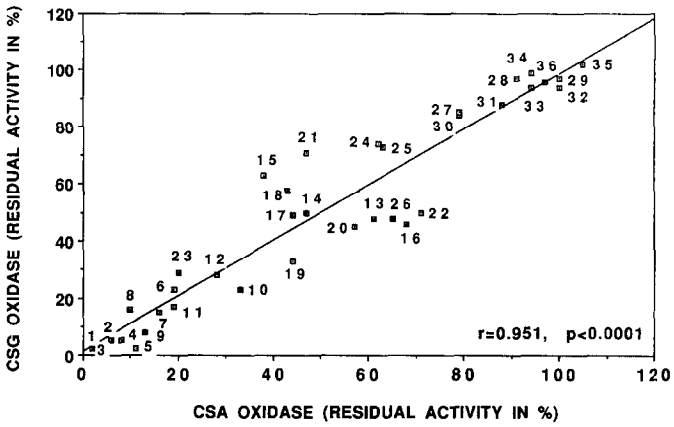

FIG. 5. Residual cyclosporin G and cyclosporin A oxidase activities after inhibition with various compounds in human liver microsomes. Human liver microsomes (1 mg/mL, sample 61289) were incubated at 37°C in a 0.1 M potassium phosphate buffer, pH 7.4, with 1 μM CSG or CSA in the presence of 0.5 μCi of the radiolabelled compound, and in the absence (100%) or in the presence of 100 μM tested drugs ($n = 36$). Thirty min after the reaction had been initiated with 1 mM NADPH, CSG or CSA and their respective metabolites were analysed by HPLC and the residual activity expressed as percentage of uninhibited activity. Residual CSG oxidase activity was plotted against residual CSA oxidase activity for each tested molecule. Each data point represents the mean of duplicate or triplicate experiments. Drug tested (K_i, in μM): 1 clotrimazole (0.1), 2 ketoconazole (0.7), 3 FK506 (4), 4 bromocriptine (8), 5 nicardipine (8), 6 nifedipine (10), 7 terfenadine (10), 8 ergotamine (12), 9 dihydroergotamine (23), 10 verapamil (24), 11 midazolam (40), 12 progesterone (45), 13 clemastine (58), 14 fluconazole (60), 15 dexamethasone (61), 16 diltiazem (63), 17 erythromycin (75), 18 glibenclamide (78), 19 virginiamycin (83), 20 dimethothiazin (85), 21 prednisolone (125), 22 omeprazole (168), 23 ethinylestradiol (172), 24 prednisone (190), 25 methylprednisolone (210), 26 lansoprazole (268), and noninhibitors ($K_i > 300$ μM): 27 griseofulvin, 28 secnidazole, 29 nystatin, 30 ketotifen, 31 mequitazine, 32 ursodeoxycholic acid, 33 indomethacin, 34 amoxicillin, 35 cefotaxin, 36 ofloxacin. Data on CSA oxidase activity were taken from previous reports [10–14].

DISCUSSION

Cyclosporin A (CSA) is widely used in the prevention of graft rejection after organ transplantation, as well as in the treatment of autoimmune disease [1, 2]. The large scale use of this drug has given rise to several important clinical concerns, the most critical being its renal toxicity [3]. The urgent need for another immunosuppressive drug with fewer side effects has led to the recent discovery of CSG [2–7]. It was, therefore, of interest to identify, at the molecular level, the enzyme system(s) involved in the biotransformation of this drug in man.

The results reported here clearly demonstrate the major implication of CYP3A4 in the hepatic metabolism of CSG. Indeed, all experimental tests were in agreement: (1) in a bank of human liver microsomes, the production of the oxidised metabolites of CSG was significantly correlated with the level of CYP3A4 determined by immunoblot and with the level of CSA oxidase, a CYP3A4 prototypical monoxygenase activity; (2) CSG oxidase was extensively inhibited by anti-CYP3A

antibodies and by the CYP3A-specific inhibitors, ketoconazole and troleandomycin; (3) CSA was a competitive inhibitor of CSG oxidase, and CSG was a competitive inhibitor of CSA oxidase; (4) among several forms of cDNA-expressed CYPs including CYP1A1, 1A2, 2A6, 2B6, 2D6, 2E1, and 3A4, only CYP3A4 generated CSG-oxidized metabolites with a turnover number close to that obtained in microsomes; (5) in human hepatocytes in culture, biotransformation of CSG was strongly accelerated by treatment of the cells with 2 specific CYP3A inducers, rifampicin and phenobarbital; (6) drugs previously characterised as inducers or inhibitors of CSA were inducers or inhibitors of CSG, as well.

The human CYP3A family has at least 3 functional genes: CYP3A4 (CYP3A3 is now believed to be an allelic variant of 3A4), CYP3A5, and CYP3A7, the products of which exhibit a high level of similarity (between 83 and 98%) in terms of their primary sequence. Clearly, CYP3A4 is the main form expressed in the adult liver and accounts for as much as 50% of total CYP proteins in some individuals. CYP3A7 is the main form expressed in the fetal liver. Shortly before birth, this gene is downregulated and CYP3A4 expression rises sharply, so that CYP3A7 is expressed at a very low level (if at all) in the adult liver. CYP3A5 is apparently expressed in a polymorphic way (in 10 to 35% of Caucasian subjects) at a relatively low level in both the fetus and adult liver. Its substrate specificity is close to that of CYP3A4 and Aoyama *et al.* [29] have shown that this form may contribute to CSA oxidase activity. It is, therefore, possible that CYP3A5 also contributes to the oxidation of CSG, especially in those individuals where CYP3A4 is expressed at a low level.

In a recent report, Turgeon et al. [30] investigated the apparent inter- and intrapatient variability in CSG metabolism *in vivo*. These authors used the erythromycin breath test to evaluate the intrahepatic level of CYP3A4 in 20 renal transplant recipients. They observed an inverse correlation between the erythromycin breath test values and the trough levels of CSG in the blood. In other words, patients with high erythromycin breath test values, reflecting a high level of CYP3A4, had lower trough levels of CSG, suggesting a high rate of elimination. They concluded that CYP3A4 was involved in the metabolism of CSG. Our results, obtained at the molecular level *in vitro*, closely confirm their conclusions drawn from *in vivo* data.

Because of the close similarity in the chemical structure of CSG and CSA, it is not surprising that the 2 drugs are metabolized by the same enzyme system, nor is it surprising that they exhibit similar kinetic parameters including K_m and V_{max}. Interestingly, it was observed by others that, although CSG and CSA exhibit marked differences in their blood distribution, their pharmacokinetic behaviours are comparable [6, 7]. This suggests that the production by CYP3A4 of oxidised metabolites of CSA or CSG is the rate-limiting step in the biotransformation and elimination of these drugs. The results presented in this report show that there is a large interindividual variability in the CSG oxidase activity in human liver microsomes. We observed a maximum 17-fold variation between patients 61289 and FT26. This has been previously observed *in vivo* by others, not only with CSA [31] but also with CSG [30]. Turgeon et al. [30] suggested that the interpatient variability in the ratio of CSG trough levels in blood to daily dose of the drug (6.6-fold between patients) was due, in part, to the contribution of the intestinal "first pass" metabolism of the drug that is being administered *per os*. Indeed, Kolars et al. [32, 33] demonstrated that CYP3A4 is expressed at a high level in the enterocytes and is able to oxidize drugs administered orally, including CSA. However, in a recent report, Lown et al. [34] found no correlation between the erythromycin breath test values and the CYP3A4 levels quantitated directly by immunoblot in enterocytes from 20 patients, indicating that the expression of CYP3A4 in the liver (reflected by the erythromycin breath test values) was not correlated with its expression in enterocytes. Because Turgeon et al. observed an inverse correlation between the erythromycin breath test values and the trough levels of CSG in the blood [29], it can be reasoned that, although there is no doubt that the metabolism of CSG occurs in enterocytes, the intestine only contributes to a minor extent in the overall metabolism of this drug in man.

A consequence of our observations is that drug interactions observed with CSA will also be observed with CSG and, presumably, to the same extent. This clearly appears from our data in Table 2 and in Fig. 5. It can be anticipated that cotreatment of patients with CSG and one or several inducers of CYP3A4 will result in an accelerated metabolism of CSG with reduced blood levels. Under these conditions, higher daily doses of CSG will be required to maintain a sufficient immunosuppressive effect. On the other hand, coadministration of CSG with inhibitors and substrates of CYP3A4 will result in a reduced metabolism of CSG, with a concomitant increased blood level of the drug. Reduction of the daily dose of CSG will be required in these cases to avoid overdosing.

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