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The increase in urinary excretion of 6β-hydroxycortisol as a marker of human hepatic cytochrome P450IIIA induction

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1 Urinary excretion of 6β-hydroxycortisol, hepatic microsomal cortisol 6β-hydroxylase and the specific content of several forms of cytochrome P450 were measured in 8 to 14 patients before and after treatment with rifampicin (600 mg orally per day for 4 days).
2 Rifampicin treatment produced an average five fold increase in daily excretion of urinary 6β-hydroxycortisol.
3 Cortisol 6β-hydroxylase activity increased from 15 ± 6 pmol min^{-1} mg^{-1} in organ donors (considered as 'control subjects') to 87 ± 31 pmol min^{-1} mg^{-1} in rifampicin treated patients.
4 Among three forms of human P450 (P450IA, IIC and IIIA), (1), (2), measured by Western blots, only P450IIIA was significantly induced by the antibiotic.
5 Only antibodies against P450IIIA selectively inhibited cortisol 6β-hydroxylase in human liver microsomes.
6 Cortisol 6β-hydroxylase was correlated with P450IIIA specific content.
7 The urinary level of 6β-hydroxycortisol correlated with liver microsomal cortisol 6β-hydroxylase and P450IIIA specific content.
8 We conclude that P450IIIA is predominantly responsible for cortisol 6β-hydroxylase activity in human liver microsomes and that urinary 6β-hydroxycortisol is a marker of the induction of this cytochrome P450.

Keywords cytochrome P450 human liver induction 6β-hydroxycortisol rifampicin

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(1) P450: liver microsomal cytochrome P450.
(2) The recently recommended nomenclature of cytochromes P450 (Nebert *et al.*, 1989) is used throughout this paper:

The P450IIIA subfamily shown in this paper to be predominantly involved in cortisol 6β-hydroxylation contains two members in human, P450IIIA3 and IIIA4 also referred to, in previous papers, as P450 HLp and P450 NF (nifedipine oxidase), respectively. P450IIIA6 or P450 3c is the rabbit ortholog of human P450 NF.

The P450IIC subfamily, another major isozyme in human liver, has three members, IIC9 and IIC10, also called P450 MP or mephenytoin hydroxylase, and IIC8, an immuno-related member without mephenytoin hydroxylase activity.

The P450IID subfamily is represented in man by P450IID6 or P450 DB or debrisoquine hydroxylase.

The P450IA subfamily has two members, IA1 and IA2. IA2 is also referred to as P450 PA or phenacetin deethylase.

Introduction

Cytochromes P450 (P450) located in the hepatic endoplasmic reticulum are monooxygenases involved in xenobiotic catabolism. They are responsible for the oxidation of various substances such as drugs, environmental substances—including procarcinogens—as well as endogenous compounds. The inducibility of some P450s, observed after exposure to different drugs and chemicals is a main feature of these systems (Distlerath & Guengerich, 1988). Investigations carried out with laboratory animals have revealed the existence of a multigenic superfamily (Nebert & Gonzalez, 1987; Nebert *et al.*, 1989) and made possible a classification of the inducers into five different groups, according to the form(s) of P450 specifically induced: (a) polycyclic aromatic hydrocarbons (P450IA); (b) phenobarbitone (P450IIB); (c) ethanol, acetone, benzene, imidazole and derivatives (P450IIE); (d) glucocorticoids and macrolide antibiotics (P450IIIA); (e) clofibrate (P450IVA). In human liver several forms of P450 have been characterized by protein purification and/or at the gene level by cloning the corresponding cDNA. They include P450IA1, P450IA2 (Jaiswal *et al.*, 1985a,b; Kawajiri *et al.*, 1986; Quattrochi *et al.*, 1986; Distlerath *et al.*, 1985), P450IIA3 (Philips *et al.*, 1985), P450IIB6 (Miles *et al.*, 1988), P450IIC8, IIC9, IIC10 (Okino *et al.*, 1987; Ged *et al.*, 1988; Shimada *et al.*, 1986; Umbenhauer *et al.*, 1987), P450IID6 (Distlerath *et al.*, 1985; Gonzalez *et al.* 1988), P450IIE1 (Wrighton *et al.*, 1987; Song *et al.*, 1986), P450IIIA3, IIIA4 (Watkins *et al.*, 1985; Molowa *et al.*, 1986; Guengerich *et al.*, 1986; Beaune *et al.*, 1986b). The inducibility of P450IIIA3 and P450IIE1, in human, has only been recently demonstrated (Watkins *et al.*, 1985; Molowa *et al.*, 1986; Wrighton *et al.*, 1986).

Primarily, cytochromes P450 contribute to hepatic detoxication. Nevertheless, these enzymes can be involved in drug and xenobiotic-induced hepatic and extra-hepatic toxicity or cancers as well as in drug interactions (Distlerath & Guengerich, 1988). The role played by induction in these processes is crucial since it can dramatically affect the P450 isozymic pattern thus exaggerating or decreasing the incidence of toxic reactions (Park & Breckenridge, 1981). From the physician's point of view, prevention of adverse drug effects is a major concern. However, except for special instances, where liver biopsies can be obtained to allow direct enzyme measurement, no routine examination is available to evaluate the level of the various P450 forms and other enzymatic systems. For this purpose, *in vivo* kinetic studies using model drugs or endogenous substrates have been developed to assess the drug metabolizing capacity of human liver (Park, 1982; Beaune & Guengerich, 1988).

Among these methods, the measurement of cortisol metabolism to its 6β-hydroxy-derivative has been proposed as a clinical test to detect enzyme induction (Park, 1981; Saenger *et al.*, 1981; Saenger, 1983). This reaction was shown to be catalyzed by P450 enzymes and 6β-hydroxycortisol can be measured in urine where it is excreted unconjugated (Ohnhaus & Park, 1979). This is a minor pathway in cortisol metabolism but represents a sensitive target for inducers (Moreland *et al.*, 1982; Perucca *et al.*, 1988; Roots *et al.*, 1979). Furthermore, no drug administration is necessary since cortisol is an endogenous steroid. Several criticisms have been raised against this test: first, no correlation between *in vivo* and *in vitro* data characterizing 6β-hydroxycortisol production has been provided; second, contradictory results have been published on its sensitivity to different inducers (Park, 1981), and third it has not been related to any specific form of human liver P450. This last argument was the starting point of this study in which we had the opportunity to gather both *in vivo* and *in vitro* data on the biotransformation of cortisol to its 6β-hydroxy-derivative.

Since basal values of urinary 6β-hydroxycortisol are not easy to use in interindividual studies, we chose to work with a classical inducer of 6β-hydroxycortisol excretion namely, rifampicin (Ohnhaus & Park, 1979; Perucca *et al.*, 1988; Roots *et al.*, 1979), to characterize the main P450 involved in the induction process.

Urinary 6β-hydroxycortisol was measured by h.p.l.c. in 14 patients (11 men, 3 women) before and after treatment with rifampicin. In the same patients, when a liver biopsy was available, various P450 forms were measured both by their catalytic activity towards model substrates and by immunoblots using specific anti-P450 antibodies. Erythromycin demethylase and benzphetamine demethylase were chosen as markers of P450IIIA, benzo(a)pyrene hydroxylase as a marker of P450IIC and ethoxyresorufin deethylase as a marker of the P450IA subfamily (Kremers *et al.*, 1981; Dalet *et al.*, 1988). In this way we were able to compare in the same patient 6β-hydroxycortisol production *in vivo*, on the one hand, with cortisol 6β-hydroxylase activity and specific content of P450 isozymes in liver microsomes measured *in vitro*, on the other. Moreover, *in vitro* data were compared with those obtained from liver

samples of organ donors considered as 'control subjects'. Our results provide new evidence that urinary 6β-hydroxycortisol increase could be a specific marker of cytochrome P450IIIA induction.

Methods

Materials

[^{3}H]-benzo(a)pyrene (70 Ci mmol^{-1}), 1,2[^{3}H]-cortisol (40 Ci mmol^{-1}) and 1,2[^{3}H]-6β-hydroxcortisol (60 Ci mmol^{-1}) were from Amersham International (Amersham, England). Cortisol was from Sigma (Saint Louis, Mo, USA), 6β-hydroxycortisol from Steraloids (Wilton, NH, USA), erythromycin from Roussel (Paris, France), ethoxyresorufin and resorufin from Pierce (Rockford, Il, USA). Electrophoresis products were purchased from Serva (Heidelberg, FRG) or Biorad (Richmond, Ca, USA). Peroxidase-conjugated immunoglobulins were from Dako (Copenhagen, Denmark) and nitrocellulose from Schleicher and Schull (Dassel, FRG). SPE C18 columns (500 mg) for 6β-hydroxycortisol extraction were from Baker (Phillipsburg, NJ, USA). Zorbax Sil, h.p.l.c. column (4.6 × 25 mm) was from Dupont de Nemours (Wilmington, DE, USA). H.p.l.c. pumps and detectors were from Waters (Wilford, MA, USA). All other reagents were from Boerhinger (Mannheim, FRG), Merck (Darmstadt, FRG) or Prolabo (Paris, France).

Organ donors and patients

Liver specimens from kidney transplant donors (HL2, HL4, HL5, HL13, HL14, HL15, HL16) were obtained at La Timone Hospital (Marseille, France), under conditions reported in a previous paper (Combalbert *et al.*, 1989). These conditions have been shown to preserve monooxygenase activities as well as in biopsy samples (Kremers *et al.*, 1981).

Fourteen patients (GAT, HAM, JAR, ETI, PRU, BUI, TOU, RAM, NAN, GOR, MAR, POU, MOU and MEC) were included in the study; the protocol was approved by Saint-Eloi Hospital Ethics Committee (Montpellier, France). Samples were labelled 1 and 2 referring to before and after rifampicin treatment, respectively. All patients stayed in the hospital at least 1 week before entering the protocol and received no medication over this period, except for the treatment with rifampicin. None of them had received a known inducer drug before the beginning of the study. Rifampicin was given as a test drug and not for any clinical indication, (5 days is the usual period tested to observe induction). Twenty-four hour urine collection (from 08.00 h–08.00 h) was made on day 1 then rifampicin was given orally, 600 mg day^{-1} for 4 days and on day 5, 24 h urine collection was repeated. On day 1, eleven patients (GAT, HAM, JAR, ETI, PRU, BUI, TOU, RAM, NAN, GOR, MAR) had a laparoscopic examination as part of an extensive check-up and a needle liver biopsy was collected for histologic examination; the remaining tissue was used to prepare microsomes. On day 5, 11 patients underwent surgery for abdominal tumours (GAT, HAM, JAR, ETI, PRU, BUI, TOU, GOR, MAR, POU, MOU) and a wedge liver biopsy was obtained. All patients had normal liver histology and functions except BUI (granuloma) and RAM (centrilobular steatosis). Data on age, sex, cause of death (organ donors) or diagnosis are reported in Table 1. Organ donors had received, for a very short time, normal intensive care treatments known to have no measurable effect on P450 dependent monooxygenase activities (Kremers *et al.*, 1981).

Table 1 Organs donors and patient characteristics

Organ donor or patient	*Sex*	*Age (years)*	*Cause of death or diagnosis*
HL2	M	30	Brain damage, TA*
HL4	M	40	Cerebral haemorrhage
HL5	M	34	Brain damage, TA
HL13	M	23	Brain damage, TA
HL14	M	56	Gun shot wound
HL15	M	19	Brain damage, TA
HL16	M	20	Brain damage, TA
GAT	F	76	Colon cancer
HAM	M	—	—
JAR	F	61	Pancreatitis
ETI	M	70	Stomach cancer
PRU	M	58	Colon cancer
BUI	M	60	Colon cancer
TOU	F	69	Stomach cancer
RAM	M	68	Colon cancer
NAN	M	75	Stomach cancer
GOR	M	—	—
MAR	M	—	—
POU	M	69	Colon cancer
MOU	M	84	Colon cancer
MEC	M	26	Colon cancer

TA = traffic accident.

Preparation of microsomes

Microsomes were prepared from fresh liver specimens by differential ultracentrifugation

and stored at −70° C in 0.1 M potassium phosphate buffer pH 7.4, 20% v/v glycerol, 1 mM EDTA, 0.1 mM DTT (Kremers *et al.*, 1981). Protein concentration was measured with the BCA protein assay reagent from Pierce (Rockford, IL, USA) or according to Lowry *et al.* (1951), bovine serum albumin being used as a standard in either case.

Cytochromes P450 and antibodies

Human cytochromes P450 NF (P450IIIA3, IIIA4), and P450 MP (P450IIC8, IIC9, IIC10) were purified as described by Wang *et al.* (1983), Shimada *et al.* (1986) and Guengerich *et al.* (1986). Monoclonal (anti P450 NF) or polyclonal antibodies (anti P450 NF and MP) were produced in mice or in New Zealand white rabbits as described by Le Provost *et al.* (1981) and Beaune *et al.* (1985). Rabbit P450 3c (P450-IIIA6), the orthologous form of human P450 NF, and rabbit P450 LM4 (P450IA2) and the corresponding antibodies were prepared and purified as described by Bonfils *et al.* (1985) Haugen and Coon (1976) and Daujat *et al.* (1987). When necessary, immunoglobulins were further purified by ammonium sulphate precipitation and chromatography on DEAE cellulose (Le Provost *et al.*, 1981). On 'Western blots' each antibody recognized in human liver microsomes a single band which comigrated with the corresponding pure antigen. Although these polyclonal antibodies do not distinguish between some isozymes belonging to the same P450 subfamily, (i.e. IIC8, 9, or 10), they do discriminate without cross-reaction between members of different subfamilies such as IIIA, IIC, and IA, To test for antibody specificity human P450 NF, human P450 MP, and rabbit P450 LM4 antigens were used.

Immunoblot analysis

Immunoblot analysis of liver microsomes was carried out as described elsewhere (Daujat *et al.*, 1987; Guengerich *et al.*, 1982a). Samples of protein (20 μg) were separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred electrophoretically to a nitrocellulose filter. After an overnight incubation at room temperature in PBS containing 3% bovine serum albumin and 10% fetal calf serum, the filter was sequentially treated for 1 h, in the same solution, with anti-P450 antibody (0.1 mg ml^{-1}) and then with horseradish peroxidase-labeled second antibody. Extensive washings in PBS were carried out after each inbucation step. Finally, the blot was developed colorimetrically either with diaminobenzidine or 4-chloro-1-naphthol and H_2O_2. The relative concentration of the P450 forms investigated was determined from densitometric analysis of the blots with a dual wavelength Shimadzu Scanner working in the reflection mode. The integrated peak area relative to the various microsomal preparations was converted to arbitrary units by reference to a standard (1 pmol) concentration of authentic purified antigen (as described above).

Cortisol 6β-hydroxylase

Cortisol 6β-hydroxylase activity was determined by measuring by h.p.l.c. the amount of 6β-hydroxycortisol produced in a 500 μl incubation medium containing 10^{-4} M 1,2[^{3}H]-cortisol (100 mCi $mmol^{-1}$), 0.3 mM NADP, 5 mM glucose-6 phosphate, 5 mM $MgCl_2$, 30 mM sodium phosphate buffer pH 7.4, 0.5 mg microsomal protein. The reaction was started with 1 u glucose 6-phosphate dehydrogenase, allowed to proceed for 30 min at 37° C and stopped by chilling in ice. The medium was immediately extracted with ethyl acetate (2 ml, twice) and dried over anhydrous sodium sulphate; the ethyl acetate phase was evaporated to dryness under a stream of nitrogen. Extraction yields for cortisol and 6β-hydroxycortisol were checked separately with radioactive standards and were greater than 95% for both cortisol and 6β-hydroxycortisol. The extract was then analyzed by h.p.l.c. to separate cortisol and 6β-hydroxycortisol. The silica column was eluted isocratically with a mobile phase consisting of hexane : dichloromethane : ethanol (470 : 410 : 65, v : v : v). The flow rate was 2 ml min^{-1}. The absorbance was monitored at 240 nm (6β-hydroxycortisol maximal absorption); retention times were 8 min and 17 min for cortisol and 6β-hydroxycortisol, respectively. As shown in Figure 1, 6β-hydroxycortisol was the main labelled compound separated from cortisol; no other significant hydroxylation product was detectable even after overloading the column and the peak eluting just before cortisol was very likely a reduced product. No 6β-hydroxycortisol was produced when the incubation was performed in the absence of either liver microsomes or NADPH generating system; this negative control was performed for each sample. Cortisol and 6β-hydroxycortisol were collected and measured by liquid scintillation spectrometry: the first fraction represented the total elution volume occurring before the 6β-hydroxycortisol peak, then 2 ml fractions were separately collected during the elution of 6β-hydroxycortisol itself. The ratio of 6β-hydroxy-

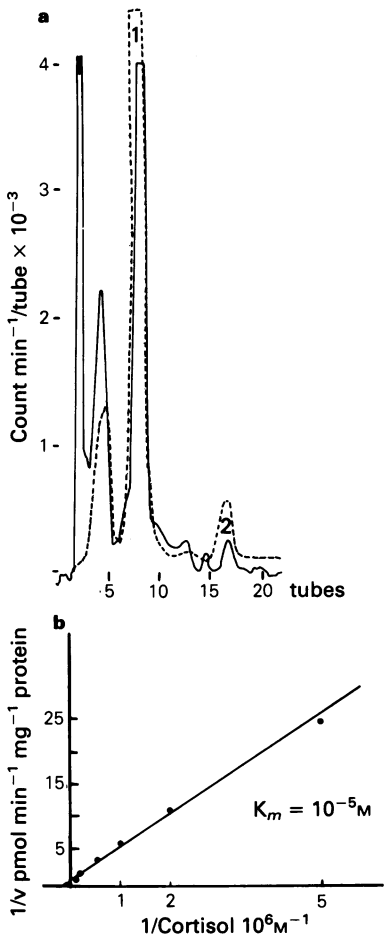

Figure 1 H.p.l.c. separation of cortisol and 6β-hydroxycortisol produced by human liver microsomes.

(a) Human liver microsomes (0.5 mg in 0.5 ml) were incubated at 37° C, under conditions described in Materials and Methods, in the presence of 10^{-4} M 1,2[^{3}H]-cortisol and a NADPH generating system. After 30 min, the reaction medium was extracted and loaded onto the column. Full line: absorbance at 240 nm. Dashed line: radioactivity.

Peak 1 = cortisol. Peak 2 = 6β-hydroxycortisol.

(b) Double reciprocal plot of cortisol 6β-hydroxylase activity against cortisol concentration. Cortisol concentration varied from 10^{-4} to 2×10^{-7} M.

cortisol radioactivity to the total radioactivity of the samples was determined. The enzymatic activity was expressed as pmol of 6β-hydroxycortisol produced per min per mg microsomal protein or per nmol of cytochrome P450. The K_m was determined by varying the amount of substrate between 10^{-4} and 2×10^{-7} M (Figure 1b). Linearity of 6β-hydroxycortisol production was checked by varying protein concentration (0.1 to 1 mg) and time (0 to 60 min).

Other monooxygenase assays

Liver microsomes were diluted to 1 or 0.5 mg ml^{-1} in 0.1 M potassium phosphate buffer pH 7.4 and appropriate aliquots of substrate solution either in buffer or in dimethylsulphoxide were added to the suspension; the concentration of DMSO, employed only for ethoxyresorufin deethylase activity was less than 0.5%, as usual in assays for this activity. The reaction was initiated at 37° C by addition of 1 mM NADPH. Erythromycin and benzphetamine demethylases were evaluated as described by Kremers *et al.* (1981) and Bonfils *et al.* (1985); the substrate concentration was 1 mM. Ethoxyresorufin deethylation was determined spectrofluorometrically according to the method of Burke & Mayer (1974), resorufin being used as a standard. In this assay the protein concentration was reduced by 0.25 mg ml^{-1} and the substrate concentration was 5 μM. Benzo(a)pyrene hydroxylase was determined according to the radiometric assay of Van Cantfort *et al.* (1978). In all monooxygenase assays, the turnover (nmol or pmol of product formed per mg of protein per min) was evaluated from the initial linear portion of the kinetic plots.

The inhibition of monooxygenase activities by anti-P450 antibodies

Immunoglobulins directed against human P450 NF and MP were preincubated with liver microsomes for 20 min at room temperature. The ratio between immunoglobulin and P450 varied from 1 to 7 mg $nmol^{-1}$. In some experiments preimmune immunoglobulins were used as controls. Monooxygenase assays were then carried out as described above.

Urinary 6β-hydroxycortisol analysis

A 10 to 20 ml aliquot of 24 h urine collected from each patient (stored at −20° C until processing) was extracted on a Baker C18 disposable column and then analyzed by h.p.l.c. Samples were eluted from the cartridge with 4 ml of 40% v/v methanol and extracted twice with 2 volumes of ethyl acetate; the extracts were washed with 0.1 volume of 0.25 N NaOH before evaporation to dryness under a stream of nitrogen; the residue was dissolved in 200 μl of methanol and 20 μl were analyzed by h.p.l.c. We used the same h.p.l.c. system as for the

assay described by Roots *et al.* (1979) and the mobile phase contained hexane : dichloromethane : ethanol : water (470 : 410 : 125 : 12 v/v/v/v), the flow rate was 2 ml min^{-1} and detection was performed at 240 nm; 6β-hydroxycortisol retention time was between 15 and 20 min. No convenient internal standard could be used because of interfering endogenous compounds. Thus 1,2[^{3}H]-6β-hydroxycortisol was used to evaluate the extraction yield which was usually about 75%. Our detection limit was 50 μg l^{-1} and on 20 repetitive assays of the same urine sample, the coefficient of variation was 10%. Various h.p.l.c. systems, as well as immunoassays, have been described for this measurement (Dumont *et al.*, 1984; Nakamura & Yakata, 1985; Hosoda *et al.*, 1981; Kishida & Fukushima, 1983; Nahoul *et al.*, 1982; Park, 1978; Zhiri *et al.*, 1986; Desage *et al.*, 1987). The h.p.l.c. method we used was validated on the basis of the following points: (1) Our data for healthy adults were in good agreement with previously published values (Dumont *et al.*, 1984; Nakamura & Yakata, 1985; Hosoda *et al.*, 1981; Kishida & Fukushima, 1983; Nahoul *et al.*, 1982; Park, 1978; Zhiri *et al.*, 1986; Desage *et al.*, 1987). (2) In addition we had the opportunity, in a separate study, to perform both our h.p.l.c. assay and an immunoassay developed by Zhiri *et al.* (1986) on the same samples; an excellent correlation was found ($r = 0.954$, $P < 0.001$, $n = 20$) although the two methods are totally different and differ in both specificity and sensitivity. Some samples of the 6β-hydroxycortisol related peak were collected during the h.p.l.c. run and the structure was confirmed by mass spectrometry for both the urinary assay and the enzymatic assay for cortisol 6β-hydroxylase activity. This h.p.l.c. assay could not separate 6α and 6β isomers, but RIA assays cannot discriminate between them either. Earlier analyses by paper chromatography showed that 6α-hydroxycortisol does not represent more than 10% of total 6-hydroxycortisol.

Free urinary cortisol analysis

In order to eliminate interference due to daily and interindividual variations in cortisol, we also measured free urinary cortisol and calculated the ratio 6β-hydroxycortisol/free cortisol for every patient. Urine (1 ml) was extracted with 30 ml of dichloromethane in the presence of 3 ml of 0.1 N NaOH. The organic phase was then dried over sodium sulphate and evaporated to dryness under a stream of nitrogen. The extract was dissolved in 100 μl of ethanol and analyzed for cortisol using a competitive radioassay described elsewhere (Murphy, 1968; Bressot *et al.*, 1987).

Statistical analysis

Linear regression analysis and Student's *t*-test were used for correlation studies.

Results

Effect of rifampicin treatment on the urinary extraction of 6β-hydroxycortisol

Data obtained in our 14 patients are shown in Table 2. During rifampicin treatment we observed an average five fold increase in 6β-hydroxycortisol daily excretion (extremes were 2 and 29 fold) and a three fold increase in 6β-hydroxycortisol/free cortisol ratio (extremes were 1.5 and 21 fold). In contrast, free urinary cortisol was not significantly modified by rifampicin treatment. These results, which are in close agreement with those reported previously (Roots *et al.*, 1979; Zhiri *et al.*, 1986; Desage *et al.*, 1987) constituted the first step of our work. Following this *in vivo* study we next focussed on enzymatic assays and cytochromes P450 measurement in microsomes prepared from liver biopsies.

The effect of rifampicin treatment on hepatic microsomal cortisol 6β-hydroxylase and other monooxygenase activities

In order to determine whether the increase in urinary excretion of 6β-hydroxycortisol following rifampicin treatment was related to an increase in its production by hepatic microsomes, a radioassay was developed for cortisol 6β-hydroxylase. A typical h.p.l.c. elution profile of the extract from the incubation medium is shown in Figure 1a. All data on cortisol 6β-hydroxylase were obtained under standard conditions (30 min incubation, 1 mg ml^{-1} protein) insuring linearity of the kinetics; the final concentration of cortisol was 10^{-4} M ($10 \times K_m$). Cortisol 6β-hydroxylase activity was measured in liver microsomes prepared from rifampicin treated patients (surgical biopsies, day 5) and from organ donors ('control subjects'). This activity could not be measured in microsomes from needle liver biopsy of patients before rifampicin treatment, because the amount of material was insufficient. The data reported in Table 3 indicate that cortisol 6β-hydroxylase activity in liver microsomes increased from 15 ± 6 in organ

Table 2 Urinary cortisol and 6β-hydroxycortisol in organ donors and patients before and after treatment with rifampicin

Subjects	*6β-OH cortisol*	*Cortisol*	*Ratio*[c]
GAT1	1520	339	4.5
GAT2	6140	149	41.2
HAM1	215	22	9.8
HAM2	450	31	14.5
JAR1	300	47	6.4
JAR2	875	76	11.5
ETI1	330	60	5.5
ETI2	770	54	14.3
PRU1	404	27	15.0
PRU2	1655	66	25.0
BUI1	370	23	11.2
BUI2	5156	133	38.8
TOU1	50	29	1.7
TOU2	1460	40	36.5
RAM1	161	19	8.5
RAM2	989	33	30.0
NAN1	373	72	5.2
NAN2	—	—	—
GOR1	148	17	8.7
GOR2	1727	44	39.0
MAR1	93	23	4.0
MAR2	1235	—	—
POU1	321	21	15.3
POU2	1016	36	28.2
MOU1	348	28	12.4
MOU2	802	41	19.6
MEC1	721	75	9.6
MEC2	2526	150	16.8
Patients UT mean ± s.d.	382 ± 367	57 ± 83	8.4 ± 4.1
(*n*)	(14)	(14)	(14)
Patients RIF mean ± s.d.	1907 ± 1755[b]	71 ± 44[b]	26.5 ± 11.0[b]
(*n*)	(13)	(12)	(12)

Twenty-four hour urine was collected from patients before (1,UT) and after (2,RIF) treatment with rifampicin and analyzed for free cortisol and 6β-hydroxycortisol (expressed in μg 24 h^{-1}). The ratio (c), 6β-hydroxycortisol/free cortisol is also presented for each patient. (a): different from donors ($P < 0.005$); (b): rifampicin-treated patients different from untreated patients ($P < 0.005$).

donors to 87 ± 31 pmol min^{-1} mg^{-1} in rifampicin treated patients ($P < 0.001$). These results clearly indicated that the increase in 6β-hydroxycortisol urinary excretion after rifampicin treatment was accompanied by an increase in liver microsomal cortisol 6β-hydroxylase. In order to identify the form of P450 involved in this process, further monooxygenase activities were assayed: erythromycin *N*-demethylase was measured to characterize P450IIIA while ethoxyresorufin *O*-deethylase was tested to characterize a different P450 subfamily (P450IA) (Watkins *et al.*, 1985; Beaune *et al.*, 1986b; Guengerich *et al.*, 1982b). The former was increased five fold ($P < 0.001$) on average—the same as for cortisol 6β-hydroxylase—while the second was not modified by rifampicin treatment (Table 3). According to these data P450IIIA (P450 NF) appeared as a reasonable candidate to support cortisol 6β-hydroxylase activity. In order to obtain further evidence in favour of this hypothesis, the effect of rifampicin on various forms of P450, measured by immunoblots, was investigated.

The effect of rifampicin treatment on the hepatic level of various isozymes of P450

Liver microsomes from organ donors and patients before and after rifampicin treatment, when material was available, were compared from immunoblots developed with specific antibodies directed against either rabbit P450 LM4 (IA2) (Daujat *et al.*, 1987), human P450 MP (IIC) (Shimada *et al.*, 1986), human P450 NF (IIIA) (Guengerich *et al.*, 1986) or rabbit P450 3c (IIIA3) (Bonfils *et al.*, 1985; Daujat *et al.*, 1987). Results are presented in Figure 2 and in Table 3. The level of P450IIIA was significantly increased in liver microsomes from rifampicin-treated patients as compared with untreated patients or organ donors. In contrast, the levels of two P450s from different subfamilies, P450IA and IIC, were not affected by the treatment. P450 IIIA, as well as P450IA and IIC, was present at a lower level in patient liver microsomes in comparison with organ donor liver microsomes. The reason for this difference is not clear but could result from the different nutritional and environmental status prevailing for patients admitted to hospital and for organ donors. In addition, P450IIIA immunoquantitation was performed using two different antibodies: a monoclonal antibody directed against human P450 NF (Le Provost *et al.*, 1981) and a polyclonal antibody directed against P450 3c, the rabbit ortholog of P450 NF (Dalet *et al.*, 1988). Data obtained with both antibodies were well correlated ($r = 0.830$, $P < 0.001$), and reinforced our confidence in the immunoquantitation of this isozyme. In order to elicit further arguments in favour of the close relation between P450 NF and 6β-hydroxycortisol production, additional analyses were carried out: immunoinhibition and correlations.

Table 3 Liver microsomal P450 isoenzymes specific content and monooxygenase activities before and after treatment with rifampicin

	P450 isozymes			*Monooxygenase activities*		
Subjects	*IA*	*IIC*	*IIIA*	*CORT*	*ERYT*	*EROD*
HL2	533	39	70	21	1000	28
HL4	590	19	83	21	620	18
HL5	110	25	40	16	370	7
HL13	664	23	83	20	230	46
HL14	390	40	83	8	1280	24
HL15	792	53	65	12	200	64
HL16	660	24	73	6	180	45
GAT1	20	—	34	—	—	—
GAT2	18	—	156	—	4300	2
HAM1	—	—	5	—	—	—
HAM2	—	—	150	—	—	—
JAR1	—	—	15	—	—	—
JAR2	—	—	102	—	2200	36
ETI1	105	18	30	—	—	—
ETI2	148	22	112	102	2180	20
PRU1	152	19	65	—	—	—
PRU2	91	5	170	51	2760	17
BUI1	—	16	31	—	—	—
BUI2	—	12	158	104	—	—
TOU1	142	11	34	—	—	—
TOU2	22	—	190	100	3190	4
RAM1	—	—	59	—	—	—
RAM2	—	—	—	—	—	—
NAN1	—	—	37	—	—	—
NAN2	—	—	—	—	—	—
GOR1	84	—	37	—	—	—
GOR2	74	—	180	107	3780	19
MAR1	—	—	18	—	—	—
MAR2	—	38	138	130	1950	13
POU1	—	—	—	—	—	—
POU2	—	14	210	56	—	—
MOU1	—	—	—	—	—	—
MOU2	—	16	210	48	—	—
MEC1	—	—	—	—	—	—
MEC2	—	—	—	—	—	—
Donors mean ± s.d.	534 ± 224	32 ± 12	71 ± 15	15 ± 6	554 ± 435	33 ± 19
(*n*)	(7)	(7)	(7)	(7)	(7)	(7)
Patients UT mean ± s.d.	100 ± 47[a]	16 ± 3[a]	33 ± 17[a]	—	—	—
(*n*)	(6)	(4)	(11)			
Patients RIF mean ± s.d.	70 ± 48[a]	18 ± 10[a]	161 ± 35[a,b]	87 ± 31[a]	2908 ± 890[a]	16 ± 11[a]
(*n*)	(6)	(6)	(11)	(8)	(7)	(7)

Microsomes, prepared from whole liver or hepatic biopsies, were analyzed by immunoblotting for their content in P450 isozymes (expressed in arbitrary unit mg^{-1}) and assayed for monooxygenase activities (expressed in pmol mg^{-1} min^{-1}) including cortisol 6β-hydroxylase (CORT), erythromycin demethylase (ERYT) and ethoxyresorufin *O*-deethylase (EROD). (a): different from donors ($P < 0.005$); (b): rifampicin-treated patients different from untreated patients ($P < 0.005$).

(Some of the data on P450IIIA, ERYT and EROD were taken from Combalbert *et al.*, 1989).

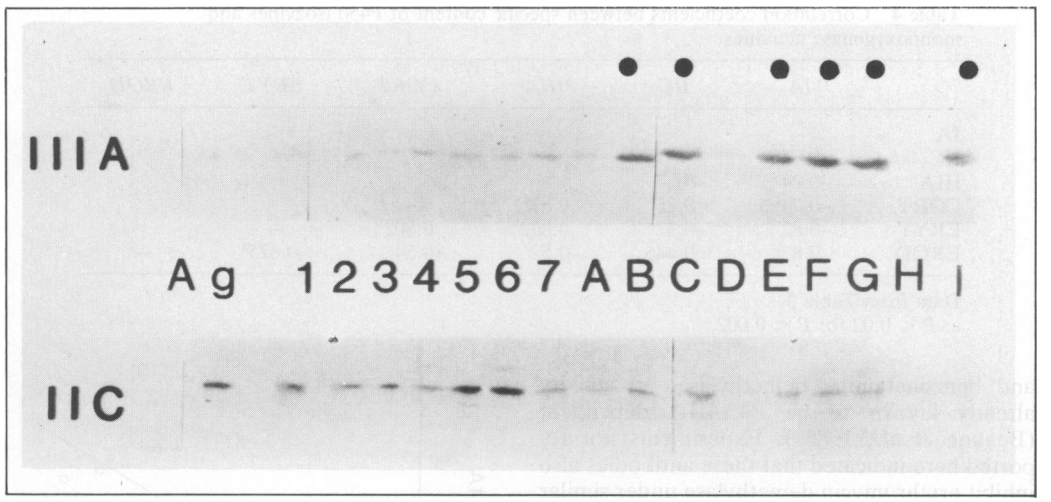

Figure 2 Immunoblot analysis of human liver microsomes using anti-P450 antibodies.
Human liver microsomes (20 μg) were submitted to electrophoresis on a 10% polyacrylamide gel and transferred electrophoretically to nitrocellulose filters. The filters were then incubated under conditions described in Materials and Methods in the presence of various anti-P450 antibodies. The upper blot was obtained with a monoclonal antibody against P450IIIA (P450 NF) (working dilution 1/200). The lower blot was obtained with a polyclonal antibody against P450IIC (P450 MP) (working dilution 1/200). Ag: pure P450IIIA or IIC. Lanes 1 to 7 refer to liver microsomes from organ donors HL2, HL4, HL5, HL13, HL14, HL15, HL16, respectively. Biopsies: A, patient ETI1; B, ET12; C, MAR2; D, BUI1; E, BU12, F, MOU2; G, POU2, H, PRU1; I, PRU2. Dots indicate samples after rifampicin treatment.

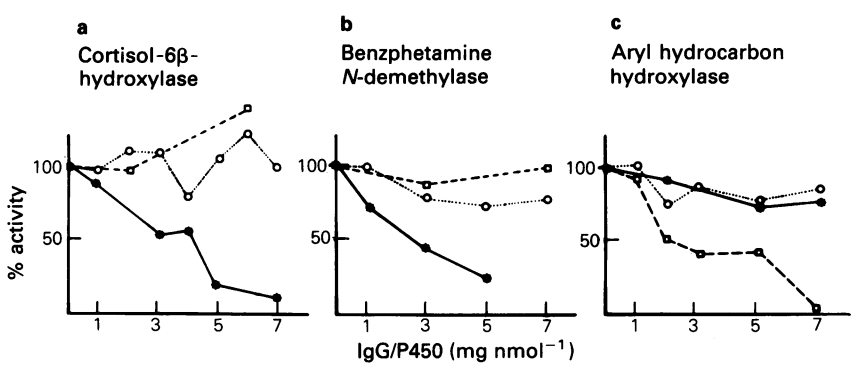

Figure 3 Inhibition of monooxygenase activities in human liver microsomes by anti-P450 antibodies.
Human liver microsomes from kidney transplantation donors were incubated for 20 min at room temperature in the presence of increasing amounts of preimmune IgG or of anti P450 antibodies. Monooxygenase activities were then assayed as usual. The uninhibited activities were: 48 pmol mg^{-1} min^{-1} for cortisol 6β-hydroxylase, 2.2 nmol mg^{-1} min^{-1} for benzphetamine demethylase and 215 pmol mg^{-1} min^{-1} for aryl hydrocarbon hydroxylase. ○: preimmune IgG; ⋆: anti P450IIIA antibody; □: anti P450IIC antibody.

Relationship between microsomal hepatic cortisol 6β-hydroxylase and P450IIIA specific content

Immunoinhibition Cortisol 6β-hydroxylase and two other classical monooxygenase activities were measured in organ donor liver microsomes in the presence of non-immune IgG or of antibodies specifically directed against human P450s, as shown in Figure 3. The amount of liver microsomes from rifampicin-treated patients was not sufficient to perform immunoinhibition experiments. These curves were obtained from one sample but the same pattern was obtained in two other livers. Clearly, antibodies against P450IIIA inhibited both cortisol 6β-hydroxylase

Table 4 Correlation coefficients between specific content of P450 isozymes and monooxygenase activities

	IA	*IIC*	*IIIA*	*CORT*	*ERYT*	*EROD*
IA	–					
IIC	0.63[a]	–				
IIIA	–0.24	–0.27	–			
CORT	–0.74	–0.25	0.63[a]	–		
ERYT	–0.82	–0.37	0.892[b]	0.802[b]	–	
EROD	0.87[b]	0.446	–0.52	–0.54	–0.625[a]	–

Data from Table 3.
a: $P < 0.02$, b: $P < 0.002$.

and benzphetamine demethylase, an activity already known to be P450IIIA dependent (Beaune *et al.*, 1986a). Experiments not reported here indicated that these antibodies also inhibit erythromycin demethylase under similar conditions. In contrast anti-P450IIIA antibodies did not inhibit benzo(a)pyrene hydroxylase an activity which is known to be dependent, at least in part, upon P450IIC (Beaune *et al.*, 1986a). On the other hand, antibodies directed against P450 MP inhibited neither cortisol 6β-hydroxylase nor benzphetamine demethylase, while they inhibited benzo(a)pyrene hydroxylase.

Correlations Table 4 presents the correlations obtained between three pairs of data. Cortisol 6β-hydroxylase was correlated with P450IIIA level in seven organ donors and eight rifampicin-treated patients ($r = 0.63$, $P < 0.02$). Erythromycin demethylase was closely correlated with cortisol 6β-hydroxylase ($r = 0.802$, $P < 0.002$), and with P450IIIA level as well ($r = 0.89$, $P < 0.002$). In contrast, cortisol 6β-hydroxylase was not correlated with P450IA, P450IIC or ethoxyresorufin deethylase, an activity dependent on P450IA subfamily (note that P450IA level also correlated with ethoxyresorufin deethylase ($r = 0.87$)).

These results confirmed the previous series of observations and strengthened the conclusion that P450IIIA is predominantly responsible for cortisol 6β-hydroxylase activity. As a final step we tried to correlate *in vivo* (urinary excretion) and *in vitro* (P450 isozyme level and cortisol 6β-hydroxylase activity) data characterizing 6β-hydroxycortisol production.

Relationship between the excretion of urinary 6β-hydroxycortisol, cortisol 6β-hydroxylase and the hepatic level of P450IIIA

Results reported in Tables 2 and 3 illustrate a concomitant increase in the 6β-hydroxycortisol/free cortisol urinary ratio, the hepatic P450IIIA level and the level of cortisol 6β-hydroxylase activity after rifampicin treatment. The plot shown in Figure 4 emphasizes the close correlation between 6β-hydroxycortisol/free cortisol urinary ratio and the hepatic level of P450IIIA ($r = 0.83$, $P < 0.001$), in agreement with the conclusions drawn from the previous sections. The correlation obtained between cortisol 6β-hydroxylase and 6β-hydroxycortisol urinary excretion was lower ($r = 0.47$, because of the small number of points (eight patients).

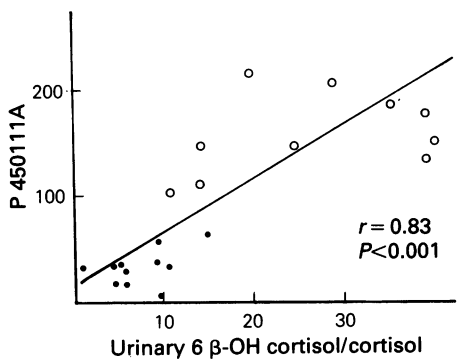

Figure 4 Correlation between P450IIIA level and 6β-hydroxycortisol excretion.
P450IIIA specific content in liver microsomes from patients before (•) and after (○) treatment with rifampicin is plotted against the corresponding 6β-hydroxycortisol/free cortisol urinary ratio. The line was obtained using linear regression analysis.

Discussion

It has been demonstrated previously that steroid hormones are hydroxylated by different forms of cytochrome P450 from liver microsomes in a regio and stereospecific manner. Progesterone and testosterone, the most widely investigated molecules in this respect, are hydroxylated at

several sites including positions 2α, 2β, 6β, 7α, 15α, 16α, 16β and 21. Of special relevance to the work presented in this paper is the finding that in rat, rabbit and man cytochrome P450 forms of the IIIA subfamily appear to be involved predominantly in the 6β-hydroxylation of progesterone, testosterone and androstenedione (Waxman *et al.*, 1988). In human liver microsomes hydroxylation of steroids at position 6β appears to be quantitatively the most important (Kremers *et al.*, 1981; Waxman *et al.*, 1988). P450IIIA is one of the most abundant forms of P450 in man (Cresteil *et al.*, 1985; Guengerich *et al.*, 1986; Kremers *et al.*, 1981), and we found that P450IIIA had a high affinity for cortisol (Figure 1).

Our results suggest that like other steroid hormones, cortisol is regiospecifically hydroxylated at position 6β by P450IIIA4—or a closely related form belonging to the same subfamily—in human liver microsomes. This conclusion follows from the following observations: (1) Rifampicin, a specific inducer of P450IIIA and of erythromycin demethylase in human liver microsomes (Combalbert *et al.*, 1989), strongly induced cortisol 6β-hydroxylase. (2) In liver microsomes from both organ donors and rifampicin-treated patients, cortisol 6β-hydroxylase activity correlated with P450IIIA level, determined immunologically with two different antibodies, as well as with erythromycin demethylase, a P450IIIA-dependent activity (Watkins *et al.*, 1985; Bonfils *et al.*, 1985); in contrast cortisol 6β-hydroxylase did not correlate with either ethoxyresorufin deethylase, a P450IA dependent activity (Guengerich *et al.*, 1982b), or P450IA or P450IIC specific content. (3) Antibodies directed against P450IIIA selectively inhibited cortisol 6β-hydroxylase.

The comparative studies carried out between both hepatic microsomal cortisol 6β-hydroxylase and P450IIIA specific content, on the one hand, and 6β-hydroxycortisol urinary level, on the other hand, in the same patients before and after rifampicin treatment, revealed a close correlation between these *in vitro* and *in vivo* parameters. Therefore we suggest that the urinary level of 6β-hydroxycortisol should no longer be considered as an unspecific marker of enzyme induction, but stand as a specific marker of P450IIIA *induction* in human liver. This finding is consistent with early papers reporting that 6β-hydroxycortisol urinary excretion is not associated with debrisoquine polymorphism (Park *et al.*, 1982) which is related to a genetic defect affecting P450IID6 gene expression (Gonzalez *et al.*, 1988) and is not affected by polycyclic aromatic hydrocarbons (P450IA1 and P450IA2 inducers) produced by cigarette smoking (Vestal *et al.*, 1987). On the other hand, anticonvulsants known to be inducers of the P450IIIA subfamily *in vivo* in laboratory animals (phenobarbitone) or in human hepatocyte cultures (phenobarbitone, carbamazepine, phenytoin, P. Maurel, unpublished observations) can also increase 6β-hydroxycortisol urinary excretion *in vivo* in man (Park, 1981; Ohnhaus & Park, 1979; Moreland *et al.*, 1982; Zhiri *et al.*, 1986).

The results shown in Figure 4 revealed a good correlation between urinary 6β-hydroxycortisol and P450IIIA specific content when data from untreated and rifampicin treated patients were analyzed together. However, the correlation was weaker when applied separately to each group of patients. This suggests that urinary 6β-hydroxycortisol is not the best parameter to evaluate the absolute hepatic level of P450IIIA in random samples. Indeed this limitation has already been emphasized (Park, 1981) and different factors such as thyroid and adrenal status, hypertension and liver diseases appear to influence 6β-hydroxycortisol excretion without affecting its metabolism (Park, 1981; Zhiri *et al.*, 1987). According to Katz *et al.* (1962), 6β-hydroxycortisol can also be produced locally in adrenals in addition to the liver.

In spite of this limitation which prevents its use in interindividual analysis, we found the 6β-hydroxycortisol/free cortisol urinary ratio to be a sensitive and specific index when looking for P450IIIA specific content modifications in response to a change in environmental conditions, in a given individual. In this respect, the measurement of urinary 6β-hydroxycortisol represents a useful tool for physicians. Thus, in man P450IIIA appears to be expressed and inducible in both sexes; it is involved in the metabolism of a large number of drugs including calcium channel blockers (nifedipine) (Guengerich *et al.*, 1986), antibiotics (troleandomycin, erythromycin) (Watkins *et al.*, 1985; Combalbert *et al.*, 1989), hypnotics (midazolam) (Fabre *et al.*, 1988), immunosuppressors (cyclosporin A) (Combalbert *et al.*, 1989), oral contraceptives (ethynylestradiol) (Guengerich, 1988); and it is inducible by several structurally unrelated compounds including antibiotics (rifampicin, troleandomycin, erythromycin, sulphamidine), synthetic steroids (dexamethasone), and anticonvulsants (phenytoin, phenobarbitone, carbamazepine) (Watkins *et al.*, 1985; Combalbert *et al.*, 1989; P. Maurel, unpublished observations). Since the hepatic level of P450IIIA may be increased by a number of drugs, daily evaluation of 6β-hydroxycortisol/free cortisol

urinary ratio should indicate rapidly whether induction of P450IIIA is occurring, thus allowing the prevention of adverse drug effects. By contrast, a decrease in 6β-hydroxycortisol production would suggest either that the drug decreases the expression of P450IIIA or that it is specifically metabolized by this form of P450 and, accordingly, competes with cortisol or, alternatively, that it is an inhibitor of P450IIIA. It should be mentioned in this respect that cimetidine, a known inhibitor of P450s, decreases 6β-hydroxycortisol excretion in man.

Heinemeyer *et al.* (1986) reported that barbiturates failed to increase urinary 6β-hydroxycortisol excretion in intensive care patients. Unfortunately the authors did not indicate the drugs administered simultaneously to these patients nor the dosage of the barbiturates. In the absence of a direct determination of P450IIIA hepatic level, two suggestions can be made when considering these data in the light of our results. The lack of increase in 6β-hydroxycortisol urinary excretion in response to barbiturates could result either from the absence of P450IIIA induction due to the special conditions (high stress, nutrition, artificial ventilation) to which these patients are exposed or from the inhibitory effect (direct binding or substrate competition at the P450IIIA active site) of some of the associated drugs on cortisol 6β-hydroxylase activity.

Several genetic polymorphisms of drug oxidation have been described recently, in man, including, as prototype drugs, debrisoquine and mephenytoin (Distelrath *et al.*, 1985; Ged *et al.*, 1988; Gonzalez *et al.*, 1988). With regard to human P450IIIA, the polymorphism exhibited at the level of enzyme activity (nifedipine oxidase) (Kleinbloesem *et al.*, 1984) could actually reflect some variation in exposure to environmental inducers. In this respect, an evaluation of the increase in 6β-hydroxycortisol/free cortisol urinary ratio after the administration of a standard dose of any convenient P450IIIA inducer might be a useful tool in a large population study designed to determine whether P450IIIA inducibility is genetically controlled.

In conclusion, we have demonstrated a clear relationship between the increase in 6β-hydroxycortisol urinary excretion and P450IIIA induction by rifampicin; that urinary 6β-hydroxycortisol is an excellent marker of P450IIIA induction alongside available methods for evaluating P450IIC and P450IID; and that our investigation combining both *in vivo* and *in vitro* approaches appears to be of great value in characterizing human P450IIIA. This type of study should be applied to other forms of P450 in order to validate newly described *in vivo* tests.

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