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SHORT COMMUNICATION

PARTICIPATION OF A CYTOCHROME *P*450 ENZYME FROM THE 2C SUBFAMILY IN PROGESTERONE 21-HYDROXYLATION IN SHEEP LIVER

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Summary—Progesterone 21-hydroxylation in hepatic microsomes from adult male sheep is a quantitatively important metabolic pathway (0.27 ± 0.08 nmol deoxycorticosterone formed/min/mg protein; representing 13–25% of total progesterone conversion). This study was undertaken to determine whether the ovine hepatic progesterone 21-hydroxylase may be another member of the *P*450 2C subfamily, normally associated with progesterone 21-hydroxylation in rodent liver. An IgG preparation raised in rabbits against purified rat liver microsomal cytochrome *P*450 2C6 was found to recognize a single antigen (M_w 52 kDa) in sheep liver microsomes. This protein was present in sheep liver (apparent concentration 16 ± 4 ng/μg microsomal protein) representing approx. 28% of the corresponding content of *P*450 2C6 in untreated rat liver. Preincubation of the anti-*P*450 2C6 IgG with hepatic microsomes was found to decrease the rate of progesterone 21-hydroxylation to 50–80% of uninhibited control. Taken together, from these findings it is apparent that a *P*450 enzyme, most likely from the 2C subfamily, catalyses deoxycorticosterone formation from progesterone in sheep liver and that this is a quantitatively important pathway of progesterone hydroxylation in these fractions.

INTRODUCTION

Cytochromes *P*450 (*P*450) catalyse the enzymic position-specific oxidation of steroids in mammalian liver. The 21-hydroxylation of progesterone to the mineralocorticoid deoxycorticosterone occurs primarily in the adrenal cortex and is catalysed by $P450_{C21}$ [1]. However, hepatic microsomes also contain a progesterone 21-hydroxylase that is structurally dissimilar to $P450_{C21}$. In rat liver the activity appears to be catalysed by *P*450 2C6 [2] and the similar *P*450 2C5 in rabbit hepatic microsomes [3, 4]. Certain steroid hydroxylations appear to be conserved across species. For example, 6β-hydroxylation, catalysed by the quantitatively important *P*450s 3A, is a major deactivation pathway in rat [5], rabbit [6], human [7] and sheep liver [8]. It was noted previously that progesterone 21-hydroxylation represents a relatively important pathway of oxidation in ovine hepatic microsomes [8]. The present study characterized this activity and investigated further its immunochemical and functional relationship to the rat progesterone 21-hydroxylase, *P*450 2C6.

MATERIALS AND METHODS

Chemicals

[4-^{14}C]Progesterone (60 mCi/mmol) was purchased from New England Nuclear (North Ryde, NSW, Australia). Authentic progesterone metabolites were from the MRC Steroid Collection (Queen Mary's College, London, England) or Sigma Chemical Co. (St Louis, MO, U.S.A.). Other analytical reagents and solvents were obtained from Ajax Chemicals (Sydney, NSW, Australia).

Reagents for electrophoresis were from Bio-Rad (Richmond, CA, U.S.A.) or Sigma and nitrocellulose filters were obtained from Hoefer Scientific (San Francisco, CA, U.S.A.).

Preparation of hepatic microsomes

Adult male sheep livers were provided by Dr E. Lacey, CSIRO Division of Animal Health (Glebe, NSW, Australia). Microsomal fractions were prepared from these livers and from untreated rat liver according to published procedures [9]

Microsomal progesterone metabolism

Assays (0.4 ml) contained 0.15 mg microsomal protein and [^{14}C]progesterone [0.18 μCi, 50 μM; except in experiments for the determination of Michaelis constants (K_m) and maximal reaction velocities (V_{max}) of the 6β- and 21-hydroxylase pathways where the substrate concentration was varied between 5 and 200 μM] and were initiated by the addition of NADPH (1 mM final concentration). After the usual 2.5 min incubation at 37°C, the products of NADPH-mediated metabolism were extracted into chloroform and applied to TLC plates (Merck F_{254} silica gel 60). The plates were developed three times in the system toluene–ethyl acetate–acetone (10:1:1, by vol) and radioactive metabolite regions were located by autoradiography (60 h using Hyperfilm-MP, Amersham, Australia). R_f values were: progesterone 0.56, 2α-hydroxyprogesterone 0.39, 21-hydroxyprogesterone 0.32, 6β-hydroxyprogesterone 0.19 and 16α-hydroxyprogesterone 0.10. Metabolite formation was quantified by β-counting (ACS II, Amersham).

Antisera

New Zealand white rabbits were inoculated with either rat *P*450 2C6 (prepared from phenobarbital-induced rat liver essentially as described [10]) or no antigen (to obtain

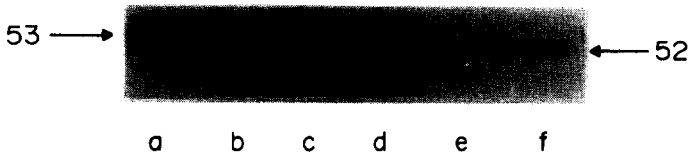

Fig. 1. *P*450 2C6 immunoreactive protein in hepatic microsomes from individual male rat (lanes a–c) and sheep (lanes d–f). Apparent molecular masses are indicated on the figure; note the approximate 1 kDa smaller mass of the anti-2C6-related polypeptide in sheep hepatic microsomes.

preimmune rabbit serum). Animals were bled via an ear vein and the serum was clotted, fractionated with ammonium sulphate, dialysed overnight and applied to DEAE-Affigel Blue (Bio-Rad). The IgG fractions eluted in the unbound fraction and protein was determined by the Lowry method [11].

SDS–Polyacrylamide gel electrophoresis and immunoblotting

SDS–Polyacrylamide gel electrophoresis of hepatic microsomal proteins from sheep and rat liver (24 μg of microsomal protein per lane) was conducted overnight as described previously [12]. Electrophoretic transfer of proteins to nitrocellulose was performed by standard methods [13] and the concentration of anti-*P*450 2C6 IgG in the wash buffer was 57 μg protein/ml. A combination of peroxidase- and ^{125}I-labelled goat anti-rabbit IgG (Amersham) was used as the second antibody according to the manufacturer's directions [14]. The nitrocellulose filters were autoradiographed for 12 h (Hyperfilm-MP) and the developed films were then scanned with a laser densitometer (LKB Ultrascan, Bromma, Sweden). Standard curves were constructed using highly purified rat *P*450 2C6.

RESULTS AND DISCUSSION

After 6β-hydroxylation, 21-hydroxylation was found to be the most significant pathway of progesterone oxidation in sheep liver microsomes (0.27 $\pm$ 0.08 nmol deoxycorticosterone formed/min/mg protein, representing 13–25% of total progesterone metabolism whereas 6β-hydroxylation represented 54–71% of total metabolism; Table 1). By comparison, in rat liver, the rate of 21-hydroxylation was similar to that in sheep liver (0.31 $\pm$ 0.09 nmol/min/mg protein), but represented only 6–9% of total progesterone hydroxylation. This is a consequence of the extensive 2α- and 16α-hydroxylation of progesterone in male rat hepatic microsomes (catalysed by the male-specific *P*450 2C11) that is not evident in sheep liver. Kinetic studies in three separate sheep hepatic fractions yielded Michaelis constants (K_m) of 20 $\pm$ 2 and 69 $\pm$ 6 μM for 21- and 6β-hydroxylation, respectively, and corresponding V_{max} values of 0.32 $\pm$ 0.12 and 3.17 $\pm$ 0.85 nmol/min/mg protein. Thus, at lower progesterone concentrations, the relative contribution of 21-hydroxylation to overall progesterone metabolism is likely to be more significant.

Table 1. Regiospecific progesterone hydroxylation in hepatic microsomes from male sheep and rat

	Hydroxyprogesterone metabolite (nmol/min/mg protein)			
Species	2α	21	6β	16α
Sheep	0.14 $\pm$ 0.01	0.27 $\pm$ 0.08	1.07 $\pm$ 0.26	0.11 $\pm$ 0.01
Rat	0.89 $\pm$ 0.11	0.31 $\pm$ 0.08	1.75 $\pm$ 0.25	1.18 $\pm$ 0.19

Data are mean $\pm$ SD of $N = 3$ individual hepatic microsomal fractions per group.

Immunochemical analysis indicated that an IgG preparation raised against rat *P*450 2C6, the enzyme considered to contribute extensively to progesterone 21-hydroxylation in rat liver, cross-reacted with an antigen present in sheep liver (Fig. 1). However, the anti-*P*450 2C6 IgG immunoreactive protein in ovine microsomes has a smaller apparent molecular mass (52 kDa) than the rat enzyme (53 kDa). The value of 53 kDa for *P*450 2C6 is consistent with other reports of similar preparations from rat liver [10]. Quantitation of anti-2C6 immunoreactive proteins in hepatic microsomes from untreated sheep, untreated rat and phenobarbital-induced rat yielded estimates of 16 $\pm$ 4, 57 $\pm$ 5 and 94 $\pm$ 11 ng/μg protein, respectively ($N = 3$ in each case). Thus the 52 kDa antigen is present in sheep liver only at 28% of the level of the 53 kDa protein in control rat liver. However, an important consideration is that the anti-rat *P*450 2C6 IgG preparation may not be fully cross-reactive with the ovine liver protein.

Subsequent studies evaluated the effect of the anti-rat *P*450 2C6 on sheep progesterone hydroxylation. Incubation of the IgG with sheep liver microsomes (10 mg IgG/mg protein) at 25°C for 40 min prior to initiation of the metabolic reaction led to a specific decrease in the rate of progesterone 21-hydroxylation (Table 2). Thus, the activity was decreased by anti-*P*450 2C6 IgG to 61% of the rate observed in the presence of the preimmune IgG. In contrast, progesterone 2α-hydroxylation was unchanged by the anti-2C6 IgG preparation and there was slight enhancement of progesterone 6β- and 16α-hydroxylation (Table 2). This preparation was found to decrease progesterone 21-hydroxylation in untreated rat hepatic microsomes to 50% of control (a ratio of 8 mg IgG/mg protein).

Studies of this type, using polyclonal antibodies raised against enzymes from one species to characterize metabolic pathways in other species, have been useful in determining the species-specificity of *P*450 proteins. An important example is the human debrisoquine 4-hydroxylase, a polymorphically distributed *P*450 now termed 2D6, which was originally identified using an antibody toward the orthologous rat protein ($P450_{UT\text{-}H}$) [15]. Similarly *P*450s 3A

Table 2. Effect of an anti-rat P450 2C6 IgG on regiospecific progesterone hydroxylation in hepatic microsomes from male sheep

	Hydroxyprogesterone metabolite (nmol/min/mg protein)			
IgG	2α	21	6β	16α
Preimmune	0.12 $\pm$ 0.04	0.31 $\pm$ 0.10	1.11 $\pm$ 0.27	0.10 $\pm$ 0.01
Anti-rat *P*450 2C6	0.13 $\pm$ 0.08	0.19 $\pm$ 0.06	1.44 $\pm$ 0.40	0.12 $\pm$ 0.02
Percent of preimmune activity	108	61	130	120

Data are mean $\pm$ SD of $N = 3$ individual hepatic microsomal fractions per group.

and 2E1 are recognized for their similarities across species [16]. The presence of catalytically similar proteins (as distinct from proteins that are merely immunochemically similar) in different species suggests a common ancestral function, perhaps prior to divergence of the species. Thus, the existence of *P*450 2C6-like proteins active in deoxycorticosterone formation in several rodent species and sheep could relate to the requirement of an enzyme system to augment adrenal mineralocorticoid synthesis [17]. Indeed, extra-adrenal progesterone 21-hydroxylation has been postulated to contribute to hypertension during pregnancy [18]. However, since the rat *P*450 2C6 enzyme also exhibits measurable activity with a range of foreign compound substrates [10] it is conceivable that the sheep enzyme identified in the present study also has a significant role in xenobiotic oxidation.

In summary, this study has provided catalytic and immunochemical evidence in support of the functional and immunochemical relatedness between rat *P*450 2C6 and a constitutive ovine *P*450. Further studies, including purification of the sheep protein and determination of its primary amino acid sequence would be necessary to ascertain its actual similarity to rat *P*450 2C6.

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