Cytochrome P450 17α-Hydroxylase/17,20 Lyase (CYP17) Function in Cholesterol Biosynthesis: Identification of Squalene Monoxygenase (Epoxidase) Activity Associated with CYP17 in Leydig Cells

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Cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17) is a microsomal enzyme catalyzing two distinct activities, 17α-hydroxylase and 17,20-lyase, essential for the biosynthesis of adrenal and gonadal steroids. CYP17 is a potent oxidant, it is present in liver and nonsteroidogenic tissues, and it has been suggested to have catalytic properties distinct to its function in steroid metabolism. To identify CYP17 functions distinct of its 17α-hydroxylase/17,20-lyase activity, we used MA-10 mouse tumor Leydig cells known to be defective in 17α-hydroxylase/17,20-lyase activity. A CYP17 knocked down MA-10 clone (MA-10CYP17KD) was generated by homologous recombination and its steroidogenic capacity was compared with wild-type cells (MA-10wt). Although no differences in cell morphogenic capacity was compared with wild-type cells by homologous recombination and its steroidogenic ability of cells treated with 22R-hydroxycholesterol, which suggested a defect in cholesterol biosynthesis. Incubation of MA-10CYP17KD cells with 14C-labeled squalene resulted in the formation of reduced amounts of radiolabeled cholesterol compared with MA-10wt cells. In addition, treatment of MA-10CYP17KD cells with various cholesterol substrates indicated that unlike squalene, addition of squalene epoxide, lanosterol, zymosterol, and desmosterol could rescue the hormone-induced progesterone formation. Further in vitro studies demonstrated that expression of mouse CYP17 in bacteria resulted in the expression of squalene monoxygenase activity. In conclusion, these studies suggest that CYP17, in addition to its 17α-hydroxylase/17,20-lyase activity, critical in androgen formation, also expresses a secondary activity, squalene monoxygenase (epoxidase), of a well-established enzyme involved in cholesterol biosynthesis, which may become critical under certain conditions. (Molecular Endocrinology 19: 1918–1931, 2005)
CYP17 is believed to be involved in the formation of four of the main steroid hormones, namely, testosterone, estradiol, cortisol, and DEHA (1–4). Deficiency of CYP17, normally present in both the gonads and adrenal cortex, leads to impaired production of cortisol, androgens and estrogens, with accompanying overproduction of mineralocorticoids, in particular 11-deoxycorticosterone (5). Consequently, affected females (46XX) have hypertension and absence of sexual development producing primary amenorrhea, whereas affected males (46XY) are also hypertensive and have female external genitalia (6). Despite the increased levels of corticosterone and 11-deoxycorticosterone, suppressed levels of aldosterone are observed in most patients with 17α-hydroxylase deficiency, although in some cases elevated aldosterone levels were reported (7).

CYP17 was originally thought to be present exclusively in the steroidogenic cells of the rodent gonads (1). Later on CYP17 mRNA, protein and/or activity were found in other tissues in the rat, including liver (8, 9), brain (10, 11), stomach, duodenum, and kidney (12). In humans, CYP17 was found, in addition to the gonadal tissues, in the adrenal and other nongonadal tissues (13). A tissue-wide distribution of this enzyme in other species, such as zebrafish, has been also

Fig. 1. Cholesterol Biosynthesis (A) and Steroidogenic Pathway (B)

Each arrow represents one or more enzymatic steps in cholesterol and steroid biosynthesis. The names of the participating enzymes are shown: squalene monoxygenase catalyzes the formation of squalene epoxide from squalene; P450scc (CYP11A1; mitochondrial cholesterol side-chain cleavage enzyme) mediates 20α hydroxylation, 22 hydroxylation and scission of the c20, 22 bond; 3βHSD mediates both 3βHSD and Δ5-Δ4 isomerase activities; CYP17 (microsomal 17 hydroxylase) mediates c17 hydroxylation of pregnenolone and progesterone to yield 17OH pregnenolone or 17OH progesterone, and scission of the c17, 20 bond of the respective 17-hydroxylated steroids (c17, 20 lysis reaction) to yield DHEA and androstenedione (1, 3).
shown (14). Although in rodents the role of this enzyme in the nongonadal tissues is not clear, a comparison of CYP17 protein expression and activity in immature rat liver vs. the gonads, indicated that the total amount of steroid metabolites produced by the liver CYP17 could be greater than that produced by the gonadal CYP17 (9). This finding suggested that CYP17 might play a role in synthesis of liver steroid hormones distinct to androgens and estrogens.

In agreement with its widespread localization, CYP17 is a potent oxidant, and other catalytic properties for the enzyme have been suggested (15). Although almost all of today's knowledge on CYP17 stems from work performed with the gonadal enzyme, a recent detailed analysis by Lieberman and Warne (15) of existing experimental evidence bearing on the role of 17α-hydroxylase suggests that it may be only an assumption to maintain that a C21-steroid, e.g. pregnenolone (or progesterone), is a uniquely specific substrate (15) for the enzyme. The later review together with additional experimental data indicating that cytochrome P450 enzymes may exist in multiple conformations and that each of two or more conformers may contain multiple substrate binding sites (16), and the finding that CYP17 is present in nonsteroidogenic tissues, suggested that CYP17 may serve other critical cell functions.

MA-10 cells are a clonal strain of mouse Leydig tumor cells adapted to continuous culture (17). MA-10 mouse Leydig tumor cells synthesize progesterone as the major final steroid product after stimulation by human chorionic gonadotropin (hCG) (17). Although these cells contain CYP17 mRNA (18), they are deficient in 17α-hydroxylase/17,20-lyase CYP17 activity (17, 19, 20). Thus, MA-10 Leydig tumor cells represent an ideal in vitro cell system to study the 17α-hydroxylase/17,20-lyase-independent functions of CYP17 in steroidogenesis. We report herein that in Leydig cells, CYP17, in addition to its 17α-hydroxylase/17,20-lyase activity, critical in androgen formation, is associated with the squalene monooxygenase (epoxidase) activity critical in cholesterol biosynthesis.

RESULTS

CYP17 Gene Knockdown in MA-10 Cells by Gene Targeting

A CYP17 knocked down MA-10 clone (MA-10CYP17KD) was generated by homologous recombination and its steroidogenic capacity was compared with wild-type cells (MA-10wt). Using CYP17 cDNA [Ref. 21 National Center for Biotechnology Information (NCBI) GenBank accession no. NM_007809] as a probe, we screened a mouse bacterial artificial chromosome library and isolated a clone containing CYP17 genomic DNA (120 kb). The CYP17 gene was sequenced and shown to include eight exons (Fig. 2A). The sequence of the entire CYP17 gene sequence was deposited in the NCBI GenBank (accession no. AY594330). A targeting vector was constructed with the neomycin resistance (neo) gene (2.0 kb) used to replace the entire (exons and introns, 8.2 kb) CYP17 as well as 5′ and 3′ DNA fragments (>3 kb) (Fig. 2B). The rationale for replacing the entire gene instead of interrupting one of the exons was to avoid potential recombination with sequences homologous to the remaining genomic sequences of the CYP17 gene. The targeted allele was generated by homologous recombination (Fig. 2C). The KpnI linearized targeting vector was transfected into MA-10 wild-type cells, and G418-resistant positive clones were selected. The integration of the CYP17 deletion in the MA-10 selected clones was evaluated by Southern blot (Fig. 2D). Hybridization of the genomic DNA digested with XbaI and CiaI with the designed 3′ probe resulted in the identification of a 7.0-kb fragment in the MA-10 wild-type genomic DNA and 7.0- and 3.7-kb fragments in the MA-10 cells containing a CYP17-deleted allele. The generated MA-10 CYP17 knockdown (MA-10CYP17KD) clone was maintained as a stable cell line.

CYP17 Gene Knockdown Does Not Affect Cell Morphology and Proliferation

Figure 3, A and B, demonstrates that no morphological differences were observed between the MA-10wt and MA-10CYP17KD cells. Moreover, there were no differences between the rates of MA-10wt and MA-10CYP17KD cell proliferation, examined by 5-bromo-2′-deoxyuridine (BrdU) incorporation (Fig. 3C).

CYP17 Gene Knockdown Reduces hCG-Stimulated Progesterone Formation

Immunoblot analysis using anti-CYP17 antiserum (22) demonstrated the presence of two immunoreactive proteins of 48–50 kDa and 28–30 kDa (Fig. 4). CYP17 immunoreactive protein levels were reduced in MA-10CYP17KD compared with MA-10wt cells (Fig. 4). Real-time quantitative PCR analysis indicated that CYP17 mRNA levels were reduced by 50% (P < 0.001) in MA-10CYP17KD compared with MA-10wt cells (Fig. 5A). Treatment of MA-10wt cells with increasing concentrations of hCG for 2 h resulted in a dose-dependent increase in progesterone formation (Fig. 5B). However, treatment of MA-10CYP17KD cells with increasing concentrations of hCG for 2 h resulted in 90% and 76% reduced progesterone formation in the presence of 10 and 50 ng/ml hCG, respectively (Fig. 4B), suggesting that CYP17 may play a role in progesterone biosynthesis. This reduced steroid formation by MA-10CYP17KD in response to hCG could be rescued by transfecting the cells with the pZeoSV2(−) vector containing mouse CYP17 cDNA (Fig. 5B).

MA-10wt and MA-10CYP17KD cells treated with 10 μM and 20 μM 22(R)-hydroxycholesterol for 2 h produced equal amounts of progesterone (Fig. 5C), indicating that cholesterol metabolism to progesterone was not...
affected by knocking down the CYP17 gene in MA-10 cells. Considering the critical role of cAMP-dependent protein kinase A (PKA) in mediating the effects of peptide hormones in steroidogenesis (1–4) and on CYP17 activity (23), we also examined whether PKA activity was altered in MA-10CYP17KD compared with MA-10wt cells. No difference in the hCG-stimulated PKA activity between the two cell lines was observed (data not shown).

RNA interference methodology was also used to specifically suppress CYP17 mRNA expression by complementary double-stranded RNA. Real-time quantitative PCR measurement of CYP17 mRNA in control and MA-10 cells treated with short interfering RNA (siRNA) for CYP17 (MA-10-CYP17KD cells) demonstrated suppression of CYP17 mRNA levels by 50% compared with wild-type cells ($P < 0.001$; Fig. 6A).

De Novo Synthesis of Steroids Is Reduced in MA-10CYP17KD Cells

To investigate the effect of CYP17 on the de novo synthesis of steroids by MA-10wt and MA-10CYP17KD cells, we blocked endogenous cholesterol synthesis with the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor lovastatin. Cells were then incubated with the radiolabeled cholesterol precursor $[^{3}H]$mevalonate. HPLC separation of the samples and steroid identification using standards,
indicated that the MA-10 CYP17KD cells synthesize much less pregnenolone, progesterone and DHEA (Fig. 7B), compared with MA-10wt cells (Fig. 7A). Similar results were obtained using various thin layer chromatography separation methods (data not shown).

CYP17 Is Associated with the Squalene Monoxygenase (Epoxidase) Activity

To study the role of CYP17 in cholesterol biosynthesis MA-10wt and MA-10CYP17KD cells were incubated with 14C-labeled squalene and a radiolabeled steroid co-migrating with radiolabeled cholesterol was isolated by thin-layer chromatography (TLC). In agreement with the data presented earlier (Figs. 5 and 6), radiolabeled cholesterol synthesis from squalene was 50% lower in MA-10CYP17KD compared with MA-10wt cells (Fig. 8A).

To determine which step in cholesterol biosynthesis was affected by knocking down CYP17 gene and mRNA, we added substrates of the various metabolic steps leading to cholesterol formation. A 30-μM concentration of squalene, squalene epoxide, lanosterol, zymosterol, and desmosterol were added onto MA-10wt and MA-10CYP17KD cells for 3 h followed by 2 h of treatment with saturating concentrations (50 ng/ml) of hCG. Figure 8B shows that, in contrast to squalene, addition of squalene epoxide, lanosterol, zymosterol and desmosterol rescued the hCG-induced progesterone formation in MA-10CYP17KD cells, suggesting that CYP17 was associated with squalene monoxygenase activity (formerly known as squalene epoxidase).

To demonstrate that CYP17 protein was responsible for the squalene epoxidation reaction seen, mouse CYP17 cDNA was inserted into the histidine tag containing pET15b vector (24) and transfected into TOP10 Escherichia coli cells. Isopropyl-1-thiol-β-D-galactopyranoside (IPTG, 1 mM) together with 5-aminolevulinic acid (1 mM) and riboflavin were used to induce the expression of the recombinant mouse CYP17 and examine the expression of CYP17 and squalene monoxygenase activities. Recombinant CYP17 was further purified by affinity chromatography using nickel-nitrilotriacetic acid His-bind resin. Proteins in fractions from the purification steps were separated by SDS-PAGE followed by Coomassie blue staining (Fig. 9A). Two major bands of 48–50 kDa and 28–30 kDa were eluted (fraction 4) and recognized by the CYP17 antiserum used (Fig. 9B).

Bacteria where CYP17 expression was induced as described above were used to examine their ability to metabolize radiolabeled progesterone and squalene. 3H-Progesterone was incubated with bacteria together and substrate loss was determined by TLC separation. Figure 9C shows that, as expected, mouse CYP17 catalyzes progesterone metabolism. Figure 9D shows that CYP17 expressing bacteria induce the me-
Fig. 5. Steroidogenesis in CYP17 Knockdown Cells

A, Real-time quantitative RT-PCR used to compare the content of CYP17 transcripts in MA-10wt and MA-10CYP17KD cells. The relative fold change was defined as the transcript level of CYP17 in MA-10CYP17KD cells divided by that in MA-10wt that was set as 1. Representative data (means ± SEM) from three independent experiments (each experiment performed in triplicates) are shown. B, hCG-induced progesterone formation in MA-10wt, MA-10CYP17KD, and MA-10CYP17KD-re cells. Cells were treated for 2 h with the indicated concentration of hCG. At the end of the incubation progesterone was measured in the media by RIA and cells were collected to determine protein levels. C, 22R-hydroxycholesterol-supported progesterone formation in MA-10wt and MA-10CYP17KD cells. Cells were treated for 2 h with the indicated concentration of 22R-hydroxycholesterol. At the end of the incubation progesterone was measured in the media by RIA and cells were collected to determine protein levels. Data shown are means ± SEM from an experiment performed in triplicates. Similar results were obtained in two other independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Fig. 6. Effect of CYP17 siRNA on Progesterone Formation

CYP17 siRNA was prepared and transfected into MA-10 wild-type cells as described in Materials and Methods. A, Real-time quantitative RT-PCR used to compare the content of CYP17 transcripts in MA-10wt and MA-10si-CYP17KD cells. The relative fold change was defined as the transcript level of CYP17 in MA-10si-CYP17KD cells divided by that in MA-10wt that was set as 1. Representative data (means ± SEM) from three independent experiments (each experiment performed in triplicates) are shown. B, hCG-induced progesterone formation in MA-10wt and MA-10si-CYP17KD cells. Cells were treated for 2 h with the indicated concentration of hCG. At the end of the incubation progesterone was measured in the media by RIA and cells were collected to determine protein levels. C, 22R-hydroxycholesterol-supported progesterone formation in MA-10wt and MA-10si-CYP17KD cells. Cells were treated for 2 h with the indicated concentration of 22R-hydroxycholesterol. At the end of the incubation progesterone was measured in the media by RIA and cells were collected to determine protein levels. Data shown are means ± SEM from an experiment performed in triplicates. Similar results were obtained in two other independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
tabolism of 14C radiolabeled squalene, determined as described (25), in a substrate- and protein-dependent manner. Figure 9E shows that control bacteria and bacteria transformed with control pET15b vector failed to metabolize squalene.

The Squalene Monooxygenase Activity Is Distinct to 17α-Hydroxylase/17,20-Lyase Activity Associated with CYP17

Figure 10A shows that the 17α-hydroxylase/17,20-lyase activity inhibitor SU10603 (26) inhibited in a dose-dependent manner the hCG-induced testosterone formation by normal rat Leydig cells (ANOVA; ***, P < 0.001). However, this compound failed to inhibit progesterone formation by MA-10 Leydig cells (Fig. 10B), suggesting that 17α-hydroxylase/17,20-lyase activity is distinct to the squalene monooxygenase activity associated with CYP17. These data were further confirmed in in vitro studies using bacteria expressing CYP17. Figure 10C shows that SU10603 inhibited the 17α-hydroxylase/17,20-lyase activity expressed by recombinant CYP17. However, this compound failed to inhibit the squalene monoxygen-
ase activity expressed by the recombinant enzyme in bacteria (Fig. 10D).

**DISCUSSION**

CYP17 enzyme mediates the 17α-hydroxylation of pregnenolone or progesterone to yield 17α-OH pregnenolone or 17α-OH progesterone and the cleavage of the C17,20 bonds of these compounds to yield the androgens DHEA or androstenedione in rodent gonads and androgens and corticosteroids in human gonads and adrenals (Fig. 1B). Although the function of CYP17 in gonadal and adrenal steroids is unquestionable, our interest in the function of this enzyme stemmed from our findings on the mechanisms underlying DHEA formation in brain. The levels of DHEA in brain are distinct from those in plasma, and its function as neuroactive steroid at the γ-amino-butyric acid and N-methyl-D-aspartate receptor level, as well as at other brain functions, has been well established (3, 27). We initially reported that rat tumor glioma cells, which do not contain the enzyme P450c17, were able to produce DHEA through an alternative P450c17-independent pathway (18). This pathway involved the formation of a DHEA precursor where both C-17 and C-20 are oxygenated (18). This pathway was also found in MA-10 Leydig tumor cells (18), rat brain glia (28), human brain glia cell lines (29), bovine brain (30), and human brain postmortem specimens (31), suggesting that this is not a species- and tissue-specific process. These studies led us to attempt generating a CYP17 knockout mouse model with the hope that this animal model will be able to solve the question of whether an alternative pathway of DHEA formation exists. The results from these studies indicated that CYP17 deletion caused a primary phenotype (infertility) probably not due to the anticipated androgen imbalance and a secondary phenotype (change in sexual behavior) due to the androgen imbalance (32). These results unveiled that CYP17, in addition to its role in androgen formation, is present in germ cells where it is required for fertility, an unexpected and surprising finding further suggesting that CYP17 exerts functions...
distinct to 17α-hydroxylase/17,20-lyase enzymatic activities (32).

To investigate 17α-hydroxylase/17,20-lyase-independent functions of CYP17, we used MA-10 mouse tumor Leydig cells known to be defective in 17α-hydroxylase/17,20-lyase activity (19, 20) and where we were unable to show the presence of immunoreactive CYP17 by immunocytochemistry (19). A CYP17 knocked down MA-10 clone (MA-10CYP17KD) was generated by homologous recombination and its steroidogenic capacity was compared with wild-type cells (MA-10wt). The results obtained demonstrated that the expected 3.7-kb restriction fragment was present in the MA-10CYP17KD mutant cells, indicating that the CYP17 gene was altered/interrupted. Like many permanent cell lines, MA-10 cells are polyploid. Southern blot analysis demonstrated that only part of the CYP17 DNA was interrupted because the bands found in MA-10wt cells were also present in the MA-10CYP17KD cells. Despite this partial interruption of the CYP17 gene, immunoblot analysis and real-time quantitative PCR analysis indicated that CYP17 protein and mRNA levels were reduced by approximately 50 and 75%, respectively, in MA-10CYP17KD compared with MA-10wt cells and CYP17 gene knockdown reduced by 50% the hCG-stimulated progesterone formation.

In contrast to previous studies where we failed to show by immunocytochemistry the presence of CYP17 immunoreactivity in MA-10 cells (19), immunoblot analysis of MA-10 extracts, performed with the same antiserum, demonstrated the presence of two immunoreactive proteins of approximately 30 and 50 kDa. Although CYP17 has been shown to be a 50-kDa protein, the presence of a lower molecular mass immunoreactive form in rat Leydig cells was previously shown by Hales et al. (22). The finding that expression of the histidine-tagged mouse CYP17 cDNA in bacteria followed by nickel affinity purification of the expressed proteins results in the isolation of a lower molecular mass form (30 kDa) strongly suggests that this lower molecular mass form may be an enzymatically active form of the enzyme.
of both the 50- and 30-kDa proteins recognized by the CYP17 antiserum, suggests that the 30-kDa protein is indeed encoded by the CYP17 cDNA, and it is either a degradation product or a truncated form of the 50-kDa enzyme.

There were no morphological differences and differences in the rates of cell proliferation between the MA-10wt and MA-10CYP17KD cells. It was interesting, although not surprising, that inactivation of part of the gene was associated with this dramatic reduction of the gene function. Such phenomena were previously reported in various cell types, including steroidogenic cells (33–35). It was, however, surprising the finding that MA-10CYP17KD cells produced dramatically reduced amounts of progesterone, the well-established precursor of CYP17 enzyme, in response to hCG treatment, suggesting that CYP17 may play a role in progesterone biosynthesis rather than metabolism. This reduced progesterone formation by MA-10CYP17KD cells in response to hCG could be rescued by transfecting the cells with the CYP17 cDNA, indicating that the reduction seen was indeed due to the inhibition of CYP17 expression.

Parallel studies, where CYP17 mRNA expression was suppressed by 50% using complementary silencing RNAs, led to a 50% suppression of the hCG-stimulated progesterone formation, further demonstrated that the reduced progesterone biosynthesis in response to hCG in MA-10CYP17KD cells was not due to nonspecific effects introduced by the gene targeting procedure. MA-10wt, MA-10CYP17KD, and MA-10si-CYP17KD cells treated with the hydrosoluble cholesterol 22R-hydroxycholesterol, which is able to freely cross membranes acting directly on the P450scc, produced equal amounts of progesterone, suggesting that cholesterol metabolism to progesterone was not affected by knocking down the CYP17 gene in MA-10 cells. Moreover, the finding that PKA activity was similar in all three cell lines suggested that the LH receptor-activated cAMP-dependent signal transduction pathway, critical in steroidogenesis and CYP17 activity (23) was not affected by knocking down the expression of CYP17.

Incubation of MA-10CYP17KD cells depleted of endogenous cholesterol, using the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor lovastatin, with radiolabeled mevalonate further demonstrated that MA-10CYP17KD cells synthesize much less pregnenolone and progesterone compared with MA-10wt cells. These findings suggest that CYP17 might be involved in cholesterol biosynthesis. The formation of DHEA by MA-10 cells was a surprising finding because these cells are not known to form androgens and are devoid of CYP17 activity (17–20). We previously reported that MA-10 cells could form DHEA using as a precursor a steroid oxygenated at C17 and C20 that could be a cholesterol metabolite (18). In that case, decreased cholesterol synthesis would result in reduced formation of DHEA, via a CYP17-independent pathway. The reduced biosynthesis of cholesterol in MA-10CYP17KD compared with MA-10wt cells was demonstrated by incubating the cells with radiolabeled squalene and measuring the formation of reduced amounts of radiolabeled cholesterol. Interestingly, cholesterol synthesis from squalene was 50% lower in MA-10CYP17KD compared with MA-10wt cells, in agreement with the 50% reduction of CYP17 mRNA levels in MA-10CYP17KD cells, suggesting that the dramatic decrease in progesterone formation seen in MA-10CYP17KD cells may be due to the amplification of the response to hCG. This inhibition was localized at the level of squalene monoxygenase (EC 1.14.99.7) enzyme activity (formerly known as squalene epoxidase; Ref. 36) because, in contrast to squalene, addition of squalene epoxide, lanosterol, zymosterol, and desmosterol rescued the hCG-induced progesterone formation in MA-10CYP17KD cells. To confirm these data, mouse CYP17 was expressed in bacteria that were incubated with radiolabeled progesterone or squalene, substrates of the 17α-hydroxylase/17,20-lyase and squalene monoxygenase activities, respectively. Because Escherichia coli contains both reduced nicotinamide adenine dinucleotide phosphate (NADPH)-flavodoxin reductase and flavodoxin that serve as an electron-transfer system for microsomal cytochrome P450s, including CYP17 (37), expression of mouse CYP17 in these cells resulted in progesterone metabolism. These data are in agreement with similar studies in E. coli performed using bovine and human CYP17 (37–39). The finding that expression of mouse CYP17 in E. coli could also result in the metabolism of radiolabeled squalene in a substrate- and protein-dependent manner clearly indicates that CYP17 itself expresses squalene monoxygenase enzymatic activity.

Squalene monoxygenase (epoxidase) is a flavin-requiring, noncytochrome P450 oxidase that catalyzes the conversion of squalene to (3S)-2,3-oxidosqualene (Refs. 40–42; and http://www.uky.edu/pharmacy/ps/porter), the first oxygenation step in steroid biosynthesis (42). It has been suggested that squalene monoxygenase is one of the rate-limiting steps in sterol biosynthesis, and it is a target for the development of therapeutics for hypercholesterolemia (43, 44). In neural tissue, this enzyme is critical for cholesterol synthesis used in myelin membrane formation, and it has been identified as the site of inhibition of tellurium-containing compounds that cause peripheral neuropathy (http://www.uky.edu/pharmacy/ps/porter).

Despite the observation that CYP17 expresses squalene monoxygenase activity and the fact that both proteins are strong oxidases, these two proteins are distinct. First, CYP17 is a cytochrome P450, whereas squalene monoxygenase is not (Refs. 40–42; and http://www.uky.edu/pharmacy/ps/porter) and second, a search through NCBI gene and protein banks failed to identify the presence of any sequence homology either at the nucleotide or amino acid levels, between CYP17 and squalene monoxygenase (NCBI, accession nos. NM_007809 vs. NM_009270 and NP_031835 vs. NP_033296). Moreover, using the 17α-hydroxylase/17,20-lyase enzymatic activity inhibitor SU10603, we were able to demonstrate that the squalene monoxygenase activity is distinct to 17α-hydroxylase/17,20-
lyase activity associated with CYP17. This was based on the observations that SU10603 1) inhibited the hCG-stimulated testosterone formation by normal rat Leydig cells, known to express an active CYP17 enzyme, as well as the 17α-hydroxylase/17,20-lyase activity expressed by recombinant CYP17 expressed in bacteria, and 2) failed to inhibit progesterone formation by MA-10 Leydig cells, devoid of CYP17 activity, and squalene metabolism by the recombinant enzyme. In clinical cases, deficiency of CYP17 leads to impaired production of cortisol and androgens and estrogens, with accompanying overproduction of mineralocorticoids, leading to male pseudohermaphroditism (XY individuals) and/or absence of pubertal development (6, 7). However, no peripheral neuropathy has been reported in these individuals. These observations suggest that the squalene monoxygenase activity of CYP17 is secondary to its 17α-hydroxylase/17,20-lyase activity and to the endogenous squalene monoxygenase enzyme. Nevertheless, considering the widespread distribution of the enzyme in nongonadal tissues, such as liver, and nonsteroidogenic tissues indicates that the squalene monoxygenase function of CYP17 may be involved in cholesterol formation either as a secondary or an alternative pathway. This hypothesis is supported by the findings that CYP17 might play a role in synthesis of liver steroid hormones distinct to androgens and estrogens (8). Moreover, our recent observations that CYP17 is present in germ cells, where it is required for germ cell function, sperm formation, and fertility (32), suggest that a function associated with cholesterol synthesis might be at the origin of the morphological defects seen in CYP17 sperm.

In conclusion, the studies presented herein confirm the Lieberman and Warne proposal (15) that CYP17 is a potent oxidant that has other catalytic properties distinct to its 17α-hydroxylase/17,20-lyase activity and identifies squalene monoxygenase activity, critical in cholesterol biosynthesis, as one of its associated enzymatic activities. Evidently, additional in vitro and in vivo studies are required to further validate these findings and identify their physiological significance.

MATERIALS AND METHODS

Materials

[1,2,6,7-3H(N)] progesterone (specific activity, 92 Ci/mmol) and (RS)-[5-3H] mevalonolactone (MVA; 27.8 Ci/mmol) were purchased from NEN/DuPont (Boston, MA). [1,5,9,14,20,24-14C]-squalene (200 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. and zymosterol from Steraloids Inc. (Newport, RI). Squalene epoxide was purchased from American Radiolabeled Chemicals, Inc. and zymosterol from Steraloids Inc. (Newport, RI). Organic solvents were of HPLC grade purchased from Fluka (New York, NY) and Fisher Scientific (Pittsburgh, PA).

CYP17 Gene Sequencing

Using CYP17 cDNA (21, NCBI GenBank accession no. NM007809) as a probe, a mouse bacterial artificial chromosome library was screened and a clone containing the CYP17 genomic DNA (120 kb) was isolated (Genome Systems Inc., St. Louis, MO). Nucleotide sequencing was performed using the ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA). Gel electrophoresis and computer analyses were performed at Lombardi Cancer Center sequencing facility (Georgetown University). The CYP17 gene sequence obtained was deposited in the NCBI GenBank (accession no. AY594350).

Construction of Targeting Vectors

The various isolated DNA fragments were subcloned into pCR2.1 (Invitrogen), pBlueScript (KS−) (Stratagene, La Jolla, CA) and pGT-N29 (New England Biolabs) vectors. The 3.3-kb 5’ and 3.5-kb 3’ DNA CYP17 fragments were constructed together with neomycin resistance (neo) gene. Figure 2 details the procedure used for the construction of targeting vectors.

Cell Transfection and Clone Screening

MA-10 mouse Leydig tumor cells were maintained in DMEM/F-12 medium with 5% fetal bovine serum, 2.5% horse serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 C and 4% CO2 in 95% air. The knockdown gene-targeting construct containing the neo gene as a selection marker (8.2-kb fragment of CYP17 gene replaced by 2.0 kb neo gene) was transfected into MA-10 cells by electroporation. Briefly, 40 μg of the targeting vector was linearized by KpnI and suspended together with 107 MA-10 mouse Leydig cells in 0.8 ml of DMEM/F-12 medium. Cells were electroporated at 220 V, 950 μF and 19 ms in the Gene Pulsar II System (Bio-Rad, Hercules, CA). Neomycin-resistant clones were selected in the presence of 200 μg/ml G418. Homologous recombination was identified by genomic Southern blot hybridization performed as previously described (32, 33).

Real-Time Quantitative RT-PCR

Total RNA from MA-10wt and MA-10CYP17KD cells was isolated using the RNAzol B reagent (Tel-Test, Inc., Friendswood, TX). Reverse transcription and real-time PCR were performed using the TaqMan reagent (Applied Biosystems). An Applied Biosystems Prism 7700 Sequence Detection System was used with the default thermal cycling program (95 C for 10 min followed by 40 cycles of 95 C, 15 sec, 60 C, 1 min). 18S rRNA was used as endogenous reference. The primers (in exon 5) used for Q-PCR amplification of CYP17 were designed by Primer Express (Applied Biosystems) and they were: sense: 5’-AAGGCCAGGACCAAGTGTTG-3’, antisense: 5’-CCACCGTGACAAGGATATGCT-3’.

siRNA Preparation and Transfection

siRNAs corresponding to CYP17 gene were designed as recommended by Silencer siRNA Construction Kit (Ambion Inc., Austin, TX), and the templates were chemically synthe-
sized by Biosynthesis Inc. The following pair gene sequences were used: antisense si-CYP17 oligonucleotide template: 5′-AATGCTGATATTTCTGACCTGTC-3′ and sense si-CYP17 oligonucleotide template: 5′-ATGCTGATATTTCTGACCTGTC-3′. siRNA preparation and purification were performed according to the manufacturer’s instruction (Ambion, Inc.). Transfections of siRNA for endogenous gene targeting were carried out using the Oligofectamine Reagent (Invitrogen). A 200-nM siRNA concentration was transfected into MA-10 cells. After incubation for 4 h at 37 °C in a CO2 incubator, growth medium containing three times the normal concentration of serum was added to the cells. Real-time quantitative RT-PCR, to assess CYP17 mRNA levels, and RIA to determine progesterone formation by MA-10, MA-10CYP17KD, and MA-10CYP17KD-re cells, were performed 48–72 h after transfection.

Transfection of CYP17 cDNA into MA-10CYP17KD Cells

CYP17 mouse cDNA (1.8 kb) (21) was prepared by RT-PCR using the following pair of primers: sense (upstream −295) 5′-cattacgcaagcggcggagca-3′ and antisense (downstream 1540): 5′-acgttgtgccctacttgctgtc-3′. The generated cDNA was inserted into pZeoSV2(−) (Zeocin+) (Invitrogen) and used to transfect MA-10CYP17KD cells using Lipofectamine 2000 (Invitrogen). Cells were selected by their ability to resist Zeocin (400 μg/ml media) for 7 d. Zeocin-resistant cells (MA-10CYP17KD-re) were seeded in 96-well plates and their ability to form progesterone in response to hCG was monitored by RIA.

Measurement of Progesterone Formation

MA-10, MA-10CYP17KD, and MA-10CYP17KD-re cells transfected with either CYP17 cDNA (MA-10CYP17KD-re) or CYP17 siRNA (MA-10CYP17KD-re) were washed with serum-free medium and treated with the indicated concentrations of hCG or 22(R)-hydroxycholesterol for 2 h at 37 °C. In search of the metabolic step in cholesterol synthesis affected by CYP17, 30 μM of the substrates squalene, squalene epoxide, lanosterol, zymosterol, or desmosterol were added in cultured MA-10 and MA-10CYP17KD-re cells. After a 3-h incubation at 37 °C, 50 ng/ml hCG were added for an additional 2 h treatment. Progesterone formation was measured by RIA (45).

De Novo Steroid Synthesis

Steroid synthesis experiments were performed with MA-10, MA-10CYP17KD, and MA-10CYP17KD-re cells at 60–80% confluence. To determine the ability of the cells to synthesize de novo steroids, cells were incubated at 37 °C and 4% CO2 with specific steroidogenic substrates as previously described (46), with some modification. In brief, endogenous cholesterol synthesis was blocked by incubating cells with 20 μM lovastatin, a competitive nonsteroidal inhibitor of hydroxymethylglutaryl-CoA reductase, in serum-free medium for 1 h. Cells were subsequently incubated with (RS)-[5-3H] mevalonate (MVA, 1.5 μCi/ml) and 10 μM unlabeled MVA. One hour later, media and cells were combined and homogenates were prepared on ice. Homogenates were extracted twice with 4 ml of diethyl ether/ethyl acetate (1:1, vol:vol) and evaporated. Extracts were reconstituted in 100% methanol and separated on a Beckman Gold HPLC system, using a 126 NM solvent module, 166 NM detector and a Beckman Coulter (4.6 mm × 25 cm) column (Beckman Coulter, Inc., Fullerton, CA). Fractions eluted with 50% acetonitrile were collected and radioactivity present in the fractions was measured by liquid scintillation spectrometry. Radiolabeled steroids were identified by their respective retention times compared with radiolabeled standards (retention time of MVA = 6 min, DHEA = 19 min, pregnenolone = 49 min, progesterone = 53 min). In previous experiments, steroids in these fractions were identified as pregnenolone, progesterone, and DHEA, respectively by gas chromatography-mass spectrometry (18).

Cholesterol Biosynthesis

MA-10 and MA-10CYP17KD cells were seeded into six-well plates and used at 60–80% confluency. Fresh serum-free medium was then added together with 14C-labeled squalene (0.05 μCi). After a 4-h incubation, cells and media were extracted with diethyl ether/ethyl acetate (1:1, vol:vol). The organic phase was collected and evaporated to dryness. The dried residues were resuspended in ethyl ether and run on C18 silica gel TLC plates (Whatman, Inc., Clifton, NJ) with a mobile phase of methylene chloride/ethyl acetate (97:3, vol:vol) (29). 14C-labeled standards for squalene and cholesterol were run on each plate. Fractions were collected from the plate and radioactivity was measured by liquid scintillation counting.

Immunoblot Analysis

Protein extracts from MA-10, MA-10CYP17KD, or transformed bacteria were used for immunoblot analysis performed as previously described (47). Anti-CYP17 antiserum was a gift from Drs. D. B. Hales (University of Illinois, Chicago, IL) and A. Payne (Stanford University, Stanford, CA) and anti-GAPDH antisera, used as a loading control, was from Trevigen (Gaithersburg, MD). Immunoactive proteins were detected using an enhanced chemiluminescence western blot detection kit (Amersham Biosciences).

Expression of Recombinant CYP17 in Bacteria and Purification of the Recombinant Protein

The PET system (Novagen, Madison, WI) was used to express the mouse CYP17 recombinant protein (24). The PET-15b (containing a histidine tag) vector was modified by constructing in an oligo DNA fragment (5′-CATATGCGGGCG CC TTAATTACCGCGGTCGAC ACTAGTGGATCC-3′) containing two cloning sites (NotI-SalI) between the multiple cloning sites (Ndel-BamHI). The CYP17 cDNA insert containing the full-length coding sequence as well as the NotI and SalI site extensions at the 5′ and 3′ ends was generated by PCR using the following primers: sense 5′-ATGCGCCCGGCA ATGTGGGAGACGGGTGTCATGACAACTAGTGGATCC-3′, antisense 5′-CGGTGCGACCCCTCTAGTGTCGATACCTACCAATGATCGACGCGGCCGC-3′, respectively. The CYP17 fragment was inserted into the modified PET15b vector and linearized with NotI and SalI downstream of the T7lac promoter. The CYP17 expression vector was transformed to the TOP10 E. coli bacterial strain alone or transformed with the PET15b vector. Purification of recombinant mouse CYP17 was performed using the BugBuster Ni-NTA His-Bind Purification Kit (Novagen). CYP17 protein expression was monitored by 4–20% SDS-PAGE protein separation followed either by Coomassie Blue staining (24) or electrophoresis and transferred to nitrocellulose membranes and immunoblot analyses performed as described (47).

Assay for CYP17 17α-Hydroxylase/17,20-Lyase Activity in Bacteria

Measurement of CYP17 activities in bacteria extracts was performed as described by Ehmer et al. (38) and Imai et al. (39). In brief, [3H]progesterone was added in 400 μl of 0.1 M sodium phosphate buffer (pH 7.4) containing 12.5 mM glu-
cose, 1 mM NADPH, in the presence or absence of SU10603 (10 μM), and the solution was preincubated at 37 C for 2 min. The reaction was initiated by the addition of 100 μl of thawed bacterial suspension and the samples were incubated for 45 min at 37 C. The reaction was stopped by heating the tubes at 95 C for 5 min. Steroids were extracted in 600 μl of cold ethylacetate for 5 min. After centrifugation at 3000 g, 400 μl of supernatant were removed and evaporated to dryness, and the extracted steroids were dissolved with 100 μl of acetone and separated on TLC (Whatman Silica Gel 60A). The solvent system used was chloroform:ethylacetate (80: 20). The location of separated steroids were determined by UV light (254 nm) and visualized and analyzed by phosphoimaging using the Cyclone Storage phosphor system (PerkinElmer, Boston, MA). CYP17 activity was determined by quantifying the loss of the substrate progesterone.

Assay for CYP17 Squalene Monoxygenase Activity in Bacteria

The CYP17 squalene monoxygenase activity was determined on the basis of the procedure described by Singh et al. (25) with minor modifications. In brief, 100 μl bacterial suspension, 30 μM flavin adenine dinucleotide, [14C]-squalene, and 10 μg of phosphatidylglycerol were added in 200 μl of 20 mM Tris-HCl buffer (pH 7.4) in the presence or absence of 1 mM EDTA, and in the presence or absence of SU10603 (10 μM). The reaction was initiated by the addition of 1 mM NADPH and the mixture was incubated for 60 min at 37 C. The reaction was stopped by the addition of 0.5 ml of 10% KOH in methanol and the incubation volume was brought to 1 ml with water. Samples were capped and after saponification for 60 min at 80 C the neutral lipids were extracted with 3 ml of petroleum ether. The solvent was removed under evaporative centrifugation and the lipids were resuspended in 50 μl of the petroleum ether and spotted onto silica thin-layer plates. Lipids were separated in 5% ethyl acetate in hexane and visualized and analyzed by phosphoimaging using the Cyclone Storage Phosphor screen. CYP17 squalene monoxygenase activity was determined by quantifying the loss of the substrate squalene.

Isolation of Rat Leydig Cells

Testicular interstitial cells were prepared by collagenase dissociation (48) of testes obtained from adult Sprague Dawley (300 g) rats. This preparation contained 20–30% 3βHSD-positive cells (Leydig cells). Leydig cells were further purified using discontinuous Percoll gradient centrifugation as previously described (48). The preparations obtained contained 75–85% Leydig cells as shown by the histochemical staining for 3βHSD. When purified rat Leydig cells were used, 50,000 cells per 500 μl of serum-free media were incubated in bovine serum culture tubes at 32 C. At the end of the incubation time period the cell media were saved, centrifuged at 1500 x g for 10 min, and stored at −20 C until use. Testosterone formation by the cells was determined by RIA, as previously described (45).

Cell Proliferation Assay

MA-10nt and MA-10CYP17D cells were plated in 96-well plates at a concentration of 103 cells/well. After seeding in the plate for 24, 48, and 72 h, cell proliferation was analyzed by measuring the amount of BrdU incorporation determined by the BrdU ELISA (Roche Molecular Biochemicals, Indianapolis, IN).

Miscellaneous

Cell protein content was determined according to the method of Bradford (49) using BSA as standard. Statistical analysis of the data, expressed as mean ± SEM, was performed by ANOVA followed by the Student–Newman–Keuls’s test using the Instat (version 3.0) package from GraphPad (San Diego, CA).

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