Ligand dependence of cytochrome P450c17 protection against proteolytic inactivation: structural, methodological and functional implications

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Abstract Rate constants for the subtilisin-catalyzed proteolytic inactivation of cytochrome P450c17 (CYP17), the endoplasmic reticulum membrane-bound limiting enzyme of gonadal androgen synthesis, have been determined in the absence and presence of various CYP17 ligands and correlated with fractional enzyme saturation (Y). Extrapolation to $Y = 1$ reveals 15.1-, 4.0- and 7.4-fold enzyme stabilization with progesterone (substrate-type ligand), testosterone (product-type ligand) and ketoconazole (imidazole-type inhibitory ligand), respectively. Structural features of ligand accommodation can therefore be monitored by the susceptibility of target enzymes to proteolysis. It is further proposed that specific protection of a membrane protein by ligand binding during proteolytic digestion may assist in the purification of that protein. Evidence is finally presented that the gonadotropin-induced rapid CYP17 down-regulation is not promoted by an elevation of steroid hormone levels.

Key words: Proteolysis; Protein stabilization; Cytochrome P450 (CYP); Gonadotropin; Steroid; Rat testis

1. Introduction

A multitude of intracellular protein degradation mechanisms are involved in the maintenance of protein compositions in specific subcellular compartments, adaptation of protein concentration to extracellular milieus and accelerated elimination of oxidatively damaged or misfolded proteins [1–3]. In addition, there are obviously several pathways directing a protein towards degradation, for instance, by covalent post-translational modifications [1,4]. Recently, the potential role of proteolysis in the regulation of cytochrome P450 (CYP) levels has been investigated. These heme proteins catalyze monooxygenations mainly in the membranes of the smooth endoplasmic reticulum leading either to inactivating biotransformations of endo- and xenobiotics or to hydrophobic precursor transformations towards active metabolites, e.g. steroid hormones. For the hepatic isoform CYP2E1, ubiquitination has been described, especially after enzyme labilization by inactivating ligands [5,6]. The same enzyme has also been reported to be degraded by serine proteases located in the endoplasmic reticulum membranes together with their CYP substrate [7]. Hormone-induced CYP phosphorylation has also been suggested to be an initial signal facilitating CYP degradation [8,9]. On the other hand, binding of ligands obviously stabilizes several CYP isoforms and protects them against phosphorylation and/or proteolytic inactivation [6,8,9].

The present study investigates the susceptibility of cytochrome P450c17 (CYP17) towards degradation by subtilisin (a non-specific serine endopeptidase; EC 3.4.21.62) as a model protease. CYP17 (steroid 17α-monooxygenase/17α-hydroxyprogesterone aldolase; EC 1.14.99.9/4.1.2.30) is the limiting enzyme of androgen biosynthesis from gestagens in the gonads; it is a target for complex regulation by lutropin or human choriogonadotropin (hCG) and several other hormones and paracrine factors [10]. The half-life of CYP17 is shortened after hCG injection in vivo from 47 h to 6 h, possibly via activation of intracellular proteolysis [11], and it has been postulated that androgens accumulating upon gonadotropin stimulation promote CYP17 decay [12]. In view of these apparent discrepancies, a complete analysis of ligand concentration-dependent modulations of CYP17 proteolysis is therefore reported here. It will be proposed that different ligands (at saturating concentrations) decrease the conformational flexibility of this membrane protein and its susceptibility towards proteolysis with different efficiencies; this will establish a novel basis for the understanding of protein structural variations after ligand accomodation.

2. Materials and methods

Microsomal membrane suspensions were prepared by gentle homogenization of decapsulated rat testes, followed by repeated differential centrifugation exactly as described previously [13]. The final membrane pellets were rehomogenized in 250 mM sucrose, 20 mM Tris (pH 7.4) and incubated at a constant final concentration of 0.16 testis equivalents/ml corresponding to 1.2 mg protein/ml [14] at a constant temperature of 37°C. Subtilisin (Boehringer, Mannheim, Germany; 83 nkat/mg protein lyophilizate with casein as substrate at 37°C) was added at various concentrations (ranging from 3 to 50 μg/ml; see section 3 and figures). Progesterone, testosterone (both from Calbiochem, Bad Soden, Germany) or ketoconazole (a gift from Janssen, Neuss, Germany) were tested as potentially protective ligands at the concentrations specified; ligands were dissolved in methanol (maximal solvent concentration 0.3% v/v). After the incubation periods indicated (range 30–180 min), samples were rapidly cooled and analyzed immediately. Cytochrome P450c17 was measured by active-site titration [13] using either progesterone (120 000 l/(mol cm) for the maximal type I ($A_{387\ nm}-A_{420\ nm}$) difference spectrum) or ketoconazole (78 000 l/(mol cm) for the maximal type II ($A_{430\ nm}-A_{407\ nm}$) difference spectrum); back-titrations in the presence of one or the other ligand were possible since the absorption trough of the progesterone-induced difference spectrum corresponds to the isosbestic point of the ketoconazole spectrum, whereas the absorption trough of the ketoconazole difference spectrum corresponds to the isosbestic point of the progesterone spectrum. In all cases, control measurements of the dithionite-reduced+CO minus reduced spectral difference (91 000 l/(mol cm) for $A_{450\ nm}-A_{490\ nm}$) yielded identical results for CYP17 concentrations. In one experiment, the action of subtilisin on microsomal NADPH:-cytochrome reductase (EC 1.6.2.4) was investigated in the presence or absence of ketoconazole; this activity was measured by the NADPH-induced (150 μM) reduction of 30 μM cytochrome *c* (20 500 l/(mol cm) for $A_{550\ nm}-A_{600\ nm}$) [15].

For electrophoretic analyses, microsomal membranes were incu-

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bated for 1 h at 37°C with or without 40 μg subtilisin/ml and progesterone (30 μM) as a ligand. Membrane proteins were centrifuged (45 min, 150000×g, 2°C) to remove the protease and then subjected to SDS-polyacrylamide gel electrophoresis [16] (60 min) using precast (stacking gel, 5%; resolution gel, 8–18% acrylamide) gradient gels (Pharmacia, Freiburg, Germany). Molecular weight standards were from Boehringer, Mannheim, Germany. Gels were stained with Coomassie blue R250 and scanned with an Ultroscan 2202 laser densitometer (LKB, Bromma, Sweden).

An additional experiment was performed to demonstrate hormone-induced proteolytic inactivation of CYP17 and its protection by ligand binding in tissue directly. For this purpose, decapsulated rat testes were extensively washed in MEM (Minimum Essential Medium Eagle with Earle's salts; ICN, Meckenheim, Germany), carefully teased apart and then incubated in 10 ml MEM for 3 h at 33°C (the physiological scrotal temperature) with or without 10 μg/ml human choriogonadotropin (Primogonyl, Schering, Berlin, Germany); CYP17 ligands (see above) or a mixture of the protease inhibitors leupeptin (Ac-Leu-Leu-Argininal; 400 μg/ml) plus E64 (*N*-[*N*-(L-3-*trans*-carboxirane-2-carbonyl)-L-leucyl]agmatine; 20 μg/ml; both from Boehringer, Mannheim, Germany) were added. After termination of the incubation, tissue was washed again, and microsomal membranes were prepared and analyzed for CYP17 concentration as described above.

Inactivation rate constants K were derived after semi-logarithmic transformation of CYP17 concentrations versus incubation times; no significant deviation from pseudo-first order kinetics was observed. Dose-effect curves of stabilizing ligand effects were calculated after logit = $\log[x/(1-x)]$ transformations of remaining subtilisin activities versus log transformation of ligand concentrations [17].

3. Results

The microsomal membrane-bound cytochrome P450 enzyme, CYP17, is completely degradable by the non-specific protease, subtilisin, although the initial rate of inactivation is much slower than with soluble proteins such as casein. This difference may partly be due to the use of a crude CYP17 source; membrane preparations contain roughly 0.8% CYP17 related to total membrane protein. Notwithstanding, the time courses of CYP17 decay reveal the existence of a pseudo-first order process. Inactivation rate constants are proportional to the protease concentration over a 3–50 μg subtilisin lyophilizate/ml concentration range (Fig. 1A,B). In the presence of the physiological (in the rat testis) CYP17 substrate, progesterone (Fig. 1C), as well as in the presence of the broad-spectrum CYP inhibitor, ketoconazole (Fig. 1D), the pseudo-first order inactivation rate constants are significantly reduced when compared to the unprotected, ligand-free CYP17. Time courses further indicate that there is no obvious metabolism or inactivation of the ligands during incubation.

The target specificity of this effect was demonstrated by subtilisin action on NADPH:cytochrome reductase in testis microsomes. This enzyme, which has a greater portion outside the membrane than CYP17, was more rapidly inactivated (by a factor of 1.8) than CYP17 with 10 μg/ml subtilisin, but there is no protective effect of either ligand (up to 5 μM; data not shown).

The selectivity and efficiency of CYP17-ligand complex resistance against proteolytic breakdown are further realized on the basis of electrophoretic comparisons (Fig. 2). Subtilisin (40 μg, 1 h) causes nearly complete degradation of microsomal proteins into smaller peptides which probably remain inaccessible to proteolytic attack from the water phase (Fig. 2, lanes B,C; sample concentration (testis equivalents) is 4-fold higher in C,D than in sample B). In the presence of 30 μM

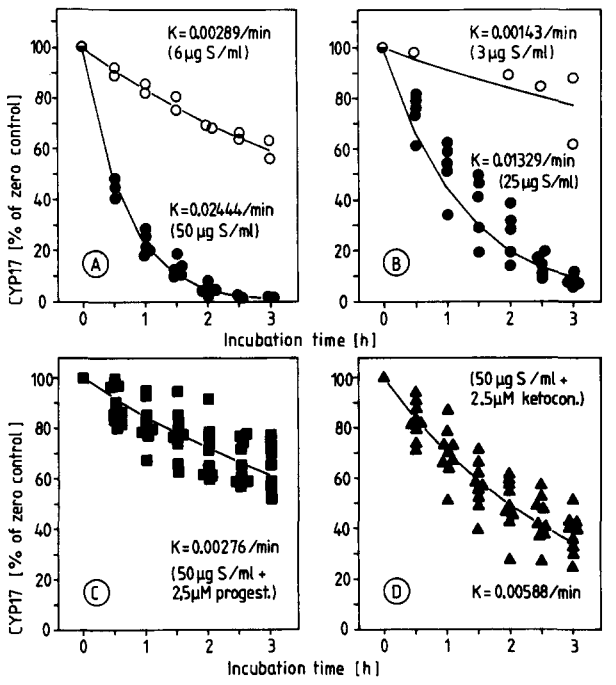

Fig. 1. Time course of cytochrome P450c17 (CYP17) inactivation in rat testis microsomes (37°C) in the presence of different subtilisin (= S) concentrations without (A,B) or with progesterone (C) or ketoconazole (D) as CYP17 ligands. Curves and inactivation rate constants K are the result of least-squares linear fitting to pseudo-first order kinetics after semi-logarithmic transformation of all individual data points.

progesterone, a single additional protein band with M_r 57400 appears (Fig. 2D), which corresponds exactly to the reported molecular mass of CYP17 (M_r 57100 [10]). A tentative correlation of protein band absorptions after densitometric scanning (based on the mean of the seven standard proteins: Fig. 2A) yields an amount of approx. 40–50 ng protein in the 57.4 kDa band. This value would correspond to about 1.0–1.2 nmol CYP17/testis equivalent; this amount is only slightly (20%) higher than that found spectroscopically in untreated rat testis microsomes. Unspecific proteolytic attack of microsomal membranes and specific ligand protection of CYP17 thus result in a significant relative enrichment of this protein.

Since the mean pseudo-first order inactivation rate constants determined in the presence of a given ligand concentration are resultants from the K values of the free and of the ligand-occupied CYP17 molecules, the fractional saturation Y has a profound effect. With 2.5 μM of either progesterone (spectral dissociation constant K_s = 0.16 μM) or ketoconazole (K_s = 0.28 μM), Y amounts to 0.94 and 0.90, respectively. After correction for this difference, the inactivation rate constants are 0.00137 min^{-1} for the CYP17-progesterone and 0.00382 min^{-1} for the CYP17-ketoconazole complexes (Y = 1), respectively, in contrast to 0.02444 min^{-1} for the free (Y = 0) CYP17. In conclusion, ligand binding to CYP17 protects this protein effectively, but not completely against proteolytic attack by subtilisin, and this process appears to vary with ligand structure.

This momentous conclusion is corroborated by analyses of the concentration dependence of the protective effect of three different CYP17 ligands, viz. progesterone, ketoconazole and

testosterone (the final product of testicular androgen biosynthesis and short-loop feedback inhibitor of CYP17; K_s = 12 μM), in the presence of two different subtilisin and one constant substrate (microsomal CYP17) concentrations (Fig. 3). The residual subtilisin activities decrease with increasing ligand concentrations in the typical fashion reflecting ligand binding to enzymes or receptors. Ligands appear to be

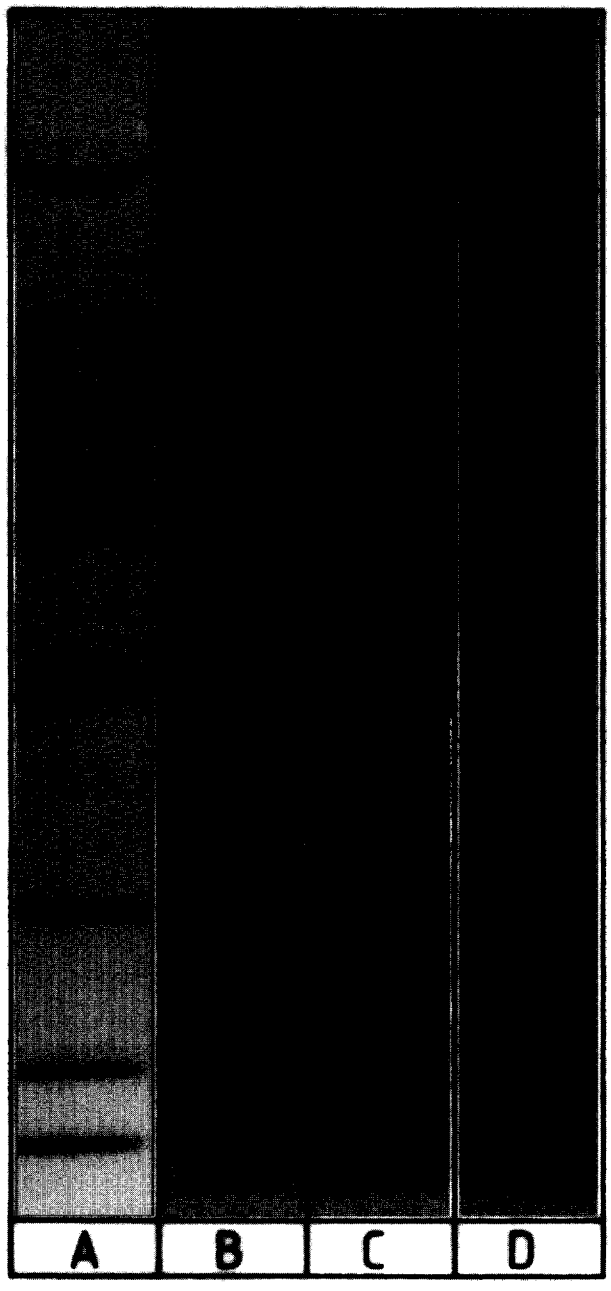

Fig. 2. Protein distribution pattern in SDS-polyacrylamide gel electrophoresis of testicular microsomal proteins before (0.001 testis equivalents/lane; B) or after subtilisin treatment (40 μg/ml, 1 h, 37°C, 0.004 testis equivalents/lane) without (C) or with 30 μM progesterone (D). Standards (300–420 ng protein/band; A) are, from top to bottom: β-galactosidase (M_r 116400), fructose-6-phosphate kinase (85200), glutamate dehydrogenase (55600), aldolase (39200), triosephosphate isomerase (26600), trypsin inhibitor (20100), and lysozyme (14300). Note the additional band appearing in lane D (vs. C) at M_r 57400.

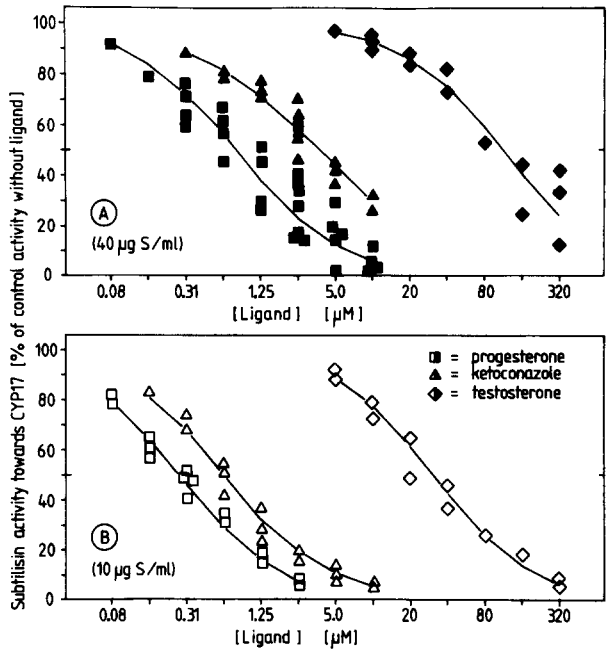

Fig. 3. Dose dependence of progesterone- (squares), ketoconazole- (triangles) or testosterone- (diamonds) induced protection of cytochrome P450c17 (CYP17) in rat testicular microsomes against proteolytic inactivation (37°C, 3 h) by two different (A,B) subtilisin (= S) concentrations. All single measurements are normalized to the corresponding control activity of subtilisin towards CYP17 without ligand. The curves are the resultants of least-squares linear fitting after logit-log transformation.

more effective with lower protease concentrations; the IC_{50} (50% inhibitory concentration) values are 0.27 vs. 0.79 μM progesterone, 0.62 vs. 3.6 μM ketoconazole and 31 vs. 110 μM testosterone, respectively, with either 10 or 40 μg subtilisin/ml assay. This difference is, however, simply a consequence of varying inactivation rate constants for $Y = 1$ with the different protease activities; it disappears if the data are transformed by plotting the actual inactivation rate constants K against the actual fractional saturations Y, followed by calculation of the ratios $K(Y = 1)$ vs. $K(Y = 0)$ for each pair of rates (Fig. 4). These ratios are independent of the protease concentration applied but are significantly lower for progesterone than for ketoconazole ($P < 0.005$) and also lower for ketoconazole than for testosterone ($P < 0.02$) (Fig. 4).

A final experiment indicates that proteolytic degradation of CYP17 may be involved in the regulation of rat testicular function. If decapsulated testes are incubated for 3 h with 10 μg/ml human choriogonadotropin, the content of CYP17 in microsomes prepared from the organs drops from 553 ± 37 (3 h control value without hCG; $\bar{x} \pm$ S.E.M.; $n = 7$) to 339 ± 31 pmol/testis equivalent ($P < 0.001$). Addition of a mixture of leupeptin and E64 to the organ incubation assays with hCG restores CYP17 to 582 ± 34 pmol/testis, indicating involvement of proteolytic activities in this hormone action. Similarly, addition of 15 μM ketoconazole also prevents CYP17 from putatively hCG-induced proteolysis (584 ± 21 pmol/testis); in the presence of 50 μM testosterone, 540 pmol CYP17/testis ($n = 2$) are found. This is a first indication that ligand protection of CYP17 against proteolytic inactivation may be a relevant process not only in artificial systems in vitro but in situ as well.

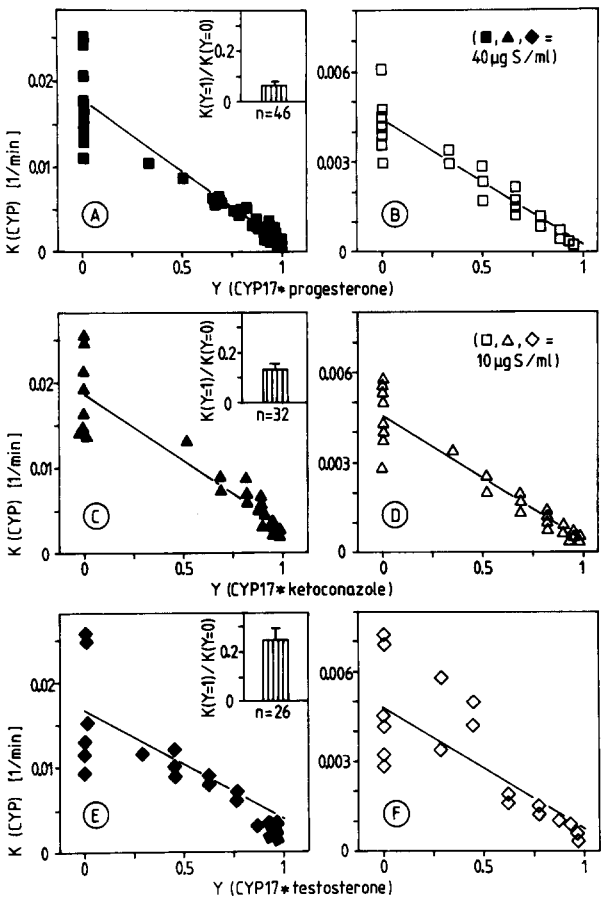

Fig. 4. Pseudo-first order inactivation rate constants (K) vs. fractional enzyme saturation (Y) plot of cytochrome P450c17 (CYP17) inactivation by two different (A,C,E vs. B,D,F) subtilisin (= S) concentrations in the presence of progesterone (A,B), ketoconazole (C,D) or testosterone (E,F). Data are identical to those presented in Fig. 3; Y values were taken from spectrophotometric titrations of ligand binding. The lines connect the mean values of $K(Y = 0)$ and of $K(Y = 1)$ obtained separately for the data sets in each of the six diagrams. For calculations of the stabilization ratios $K(Y = 1)/K(Y = 0)$, data were combined from assays with low and high subtilisin concentrations; probabilities for significant differences are given in the text.

4. Discussion

As for structural implications, the finding that cytochrome P450c17 in testicular smooth endoplasmic reticulum membranes is protected against proteolytic breakdown by several ligands is in line with previous results concerning not only certain CYP species such as 2E1 and 3A1 [6,8,9], but also a variety of other target proteins such as dihydrofolate reductase, which is protected by methotrexate [18], as well as tryptophan synthase and further pyridoxal enzymes which are more rapidly degraded in their apo form than in the holo form [19,20]. Using the novel approach to relate actual pseudo-first order inactivation rate constants to the corresponding fractional saturation of the enzyme with ligand (which is easily achieved in this case by means of spectroscopic titrations), the present study reveals that the protective potential at complete saturation depends strongly on ligand structure and accomodation by the target enzyme. Structural features of steroid hormone binding have previously been established [21]; testosterone, which lacks the D-ring side-chain with the important oxo group at C-20, is a much weaker ligand than progesterone based on the magnitude of maximal low-spin to high-spin transitions upon binding. If it is accepted that flexibility versus rigidity of certain exogenously accessible domains on a protein surface ('degrons') determines its susceptibility towards proteolytic attack [2,18], and if it is further accepted that ligands increase the rigidity of such domains [18], it can be concluded that progesterone 'fits' the CYP17 ligand-binding cavity better than testosterone with respect to reinforcement of CYP17 structure. Ketoconazole is an effective inhibitory ligand of CYP17 [22], but has originally been designed as a lanosterol mimic, blocking the lanosterol 14α-methyl demethylase reaction sequence. It is therefore comprehensible that this ligand is also less effective than progesterone in protecting CYP17 by reduction of its conformational flexibility. It is anticipated that the chosen approach may assist in improved elucidation of cytochrome P450 structures, especially if refined by the use of more specific proteases [23] and further ligands.

As for methodological implications, it is tentatively proposed that the specific retention of a membrane-associated enzyme by binding of a protective ligand under conditions where the majority of other proteins is proteolytically digested may be useful as an initial step in preparative procedures to obtain the protein in question. One remarkable advantage is the maintenance of the physiological membrane environment which has repeatedly been demonstrated to affect the function of such membrane-bound enzymes [24].

As for the functional implications of this study, it must be concluded that proteolysis is involved in the gonadotropin-induced down-regulation of rat testicular CYP17, and that this process can be antagonized by ligand binding-induced enzyme protection. Although it is improbable that steroid (progesterone as the substrate and testosterone as the final product) concentrations become high enough to be completely protective after hCG stimulation, it appears that these ligands do not labilize the CYP17 enzyme as occasionally suggested. A 'pseudo-substrate type' of steroid binding has been hypothesized, leading to an abortive activation of oxygen and oxidative damage of CYP17 [12,25]. The present results suggest that this mechanism does not account for gonadotropin-induced degradation of CYP17 in the rat testis.

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