Studies on functional interaction of adrenal microsomal cytochrome P450s with NADPH-cytochrome P450 reductase

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Several cytochrome P450 enzymes are known to participate in adrenal steroidogenesis; P450 scc, the cholesterol side chain cleavage enzyme, and P450 11β, the steroid 11β-/ 18/ 19-hydroxylase, in mitochondria, and P450 C21, the corticosteroid 21-hydroxylase, P450 17α, the steroid 17α-hydroxylase/ 17-20 lyase.in microsomes (Figure 1). Microsomal cytochrome P450 catalyzes monooxygenase reactions of variety of endogenous and exogenous hydrophobic molecules with the consumption of molecular oxygen and two electrons which are supplied by NADPH-cytochrome P450 reductase (CPR). The adrenal endoplasmic reticulum contains two species of cytochrome P450, P450C21 and P450 17α. P450C21 is the single enzyme catalyzing the 21-hydroxylation for both glucocorticoids and mineralocorticoids. P450 17α catalyzes steroid 17α-hydroxylase and 17, 20 lyase activities and is essential for the synthesis of glucocorticoids (17α-hydroxylase activity) and sex steroids (17, 20 lyase activity).

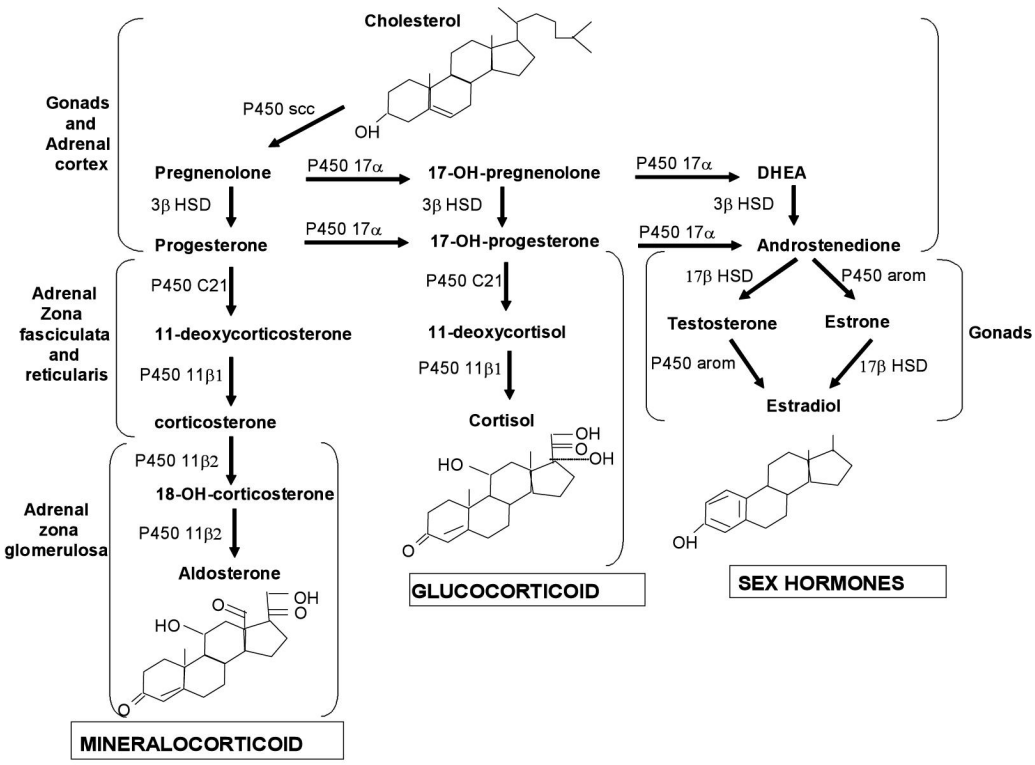

Figure 1 . The biosynthetic pathway of steroid hormones

Addison's disease (AD) is one of the adrenal dysfunction diseases. In developed countries, 80% of the cases of AD are autoimmunity in which the immune system erroneously makes antibodies against the cells of adrenal cortex and slowly destroys them. In 1994 it was shown that P450C21 antibodies in sera from patients with Addison's disease have an inhibiting effect on enzymatic activity of P450C21 in vitro. Two hypotheses for the inhibition of P450C21 enzyme activity by P450C21 autoantibodies (Abs) were examined: (a) conformational changes of P450C21 (b) disruption of electron transfer from CPR to P450C21. The effect of Abs binding on the conformation of recombinant P450C21 in yeast microsomes was studied using dithionite-reduced CO difference spectra. The effect of P450C21 Abs on electron transfer was assessed by analysis of reduction of P450C21 in yeast microsomes in the presence of CO after addition of NADPH. Binding of the Abs did not induce significant change in the P450C21 absorbance at 450 nm (native) and did not produce a detectable peak at 420 nm (denatured) in the dithionite-reduced CO difference spectra. This indicated that conformation of P450C21 around the heme was not altered. However, incubation of the P450C21 in yeast microsomes with P450C21 Abs inhibited the active complex formation between CPR and P450C21. Our observations suggested that the most likely inhibitory mechanism for P450C21 Abs to inhibit P450C21 activity is disruption of the interaction between the CPR and P450C21.

To identify the interaction regions of P450C21 with CPR, the inhibitory effects of five monoclonal antibodies (mAbs) on the activity of P450C21 were examined. The epitope regions (ER) of five mAbs on P450C21 are as follow: AA391-405 (ER1), AA406-411 (ER2), AA335-339 (ER3), AA1-142 (ER4) and AA172-280 (ER5) for mAb1, mAb2, mAb3, mAb4 and mAb5, respectively.

mAb1, mAb2 and mAb3 inhibited bindings of autoantibodies from Addison's disease patients to P450C21, while mAb4 and mAb5 had no effect on autoantibodies binding. ER2 and ER3 are parts of two distinct major epitopes recognized by autoantibodies from Addison's disease patients' sera while ER1 is a part of a minor epitope. In our experiments, mAb3 inhibited P450C21 activity showing that the ER3 was located in the interaction region of P450 to CPR. The epitope region of the mAb3 was located near or on the putative CPR binding site of the P450s.

The interaction region of adrenal microsomal P450s with CPR was confirmed by another experimental method. Another P450 in adrenal microsomes is cytochrome P450 17α which must interact with CPR in the same way as P450C21. By using the chemical modification and peptide mass finger printing the interaction region of P450 17α with CPR was investigated. The importance of lysines and arginines of P450 in the interaction with CPR have been already reported by other scientists.

Lysine residues of recombinant guinea pig P450 17α in DLPC (L-α-phosphatidyl choline dilauroyl) micelles were acetylated by acetic anhydride in the absence and presence of CPR. Acetylated P450 17α was separated from CPR by SDS-PAGE and peptides were prepared by in-gel digestion. Molecular weights of peptide fragments were determined by MALDI-TOF mass spectrometry (AXIMA-

CFR plus). Activity of acetylated P450 17α was measured in the conversion of [^{3}H] progesterone to [^{3}H] Androstenedione and [^{3}H] 17-OH-progesterone.

Dithionite-reduced CO difference spectra of P450 17α showed no peak at 420nm after acetylation in the absence or presence of CPR, showing that acetylation did not make significant conformational change around heme. Eight acetylated peptides were identified clearly with the increase of 42 m/z in the molecular mass of modified peptides in P450 17α acetylated without CPR. The acetylated positions in those eight modified peptides were detected as K58, K59, K91, K227, K234, K326, and K327. After acetylation in the presence of CPR one acetylated peptide at 1884 m/z was disappeared that has double acetylations at K326 and K327 in J-helix of P450 17α. The activity of P450 17α acetylated in the absence of CPR was decreased to 30%, but almost no inactivation was detected in P450 17α after acetylation in the presence of CPR. The protection of enzyme from inactivation shows the importance of K326 and K327 of P450 17α in interaction with CPR. Our results provided the first experimental proof for the importance of J-helix of P450 in the interaction with CPR.

In this study it was clearly shown that interruption of electron transfer from CPR to P450C21 is the most possible inhibitory mechanism for autoantibodies in AD patients to inhibit P450C21 activity. Also the comparison of the results of two different methods showed that the interaction region of microsomal P450s to CPR is located near to J-helix in the proximal site of P450.

Key words: Adrenal, microsomal P450s, CPR, interaction