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INACTIVATION OF SOLUBLE 17β-HYDROXYSTEROID DEHYDROGENASE OF HUMAN PLACENTA BY FATTY ACIDS

C. H. BLOMQUIST*, N. J. LINDEMANN and E. Y. HAKANSON
Department of Obstetrics and Gynecology, St Paul-Ramsey Medical Center, St Paul, MN 55101 and The University of Minnesota Medical School, Minneapolis, MN 55455, U.S.A.

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Summary—The sensitivity of soluble, 17β-hydroxysteroid dehydrogenase (17β-HSD) of human placenta to inactivation by fatty acids was examined. Exposure to the unsaturated fatty acids oleic, arachidonic, linoleic and linolenic acid resulted in the loss of activity. Methyl and ethyl esters of oleic acid, the saturated fatty acid, stearic acid and prostaglandins E_2 and $F_{2\alpha}$ were without effect. Inactivation by oleic acid required the fatty acid at levels above its critical micelle concentration, 50 μM, as estimated by light-scattering. Steroid substrates and inhibitors did not protect against inactivation. NAD^+, NADH, $NADP^+$ and NADPH did protect. The concentrations of $NADP^+$, 50 μM, and NAD, 1.5 mM, necessary for complete protection were significantly greater than their respective Michaelis constants, 0.16 μM and 15.2 μM. The data suggest that soluble 17β-HSD can bind to fatty acid micelles and that the binding site(s) on the enzyme are at or near pyridine nucleotide binding sites.

INTRODUCTION

In a previous report from this laboratory [1] it was shown that exposure of guinea pig liver microsomes to unsaturated fatty acids results in a rapid loss of 17β-hydroxysteroid dehydrogenase (17β-HSD) activity. This observation, when considered with the detection of phospholipase and diacylglyceride lipase activities in decidual tissue, placenta and fetal membranes [2–5] and also the observation of increases in plasma free (nonesterified) fatty acids during the latter stages of pregnancy [6], suggests that free fatty acids could act *in vivo* to modulate 17β-HSD activity and, as a result, intracellular levels of 17β-hydroxy and 17-ketosteroids.

Fatty acids can interact with membranes and enzyme proteins in a variety of ways. In 1970, Hasselbach and Heimberg[7] demonstrated that unsaturated fatty acids could bind to sarcoplasmic reticulum. Subsequent studies have shown the effects of various fatty acids on membrane bound enzymes such as microsomal ATPase [8] and glucose-6-phosphate translocase [9]. Soluble proteins, also, can bind fatty acids by a non-covalent interaction [10, 11].

With regard to microsomal 17β-HSD activities, the mechanism of inactivation by fatty acids could be complex. Because of the natural affinity of these enzymes for hydrophobic compounds as substrates, inhibition could result from a direct binding of fatty acid at or near the active site. Alternatively, it could be a secondary effect due to the detergent-like action of fatty acids on membrane structure [9, 12]. As an approach to differentiating between these two possibilities, and to focus on an evaluation of the potential for inhibition by a direct interaction with the enzyme, we have examined the effects of fatty acids on a soluble form of 17β-HSD isolated from human placenta.

*Address for correspondence: Department of Obstetrics and Gynecology, St Paul-Ramsey Medical Center, 640 Jackson Street, St Paul, MN 55101, U.S.A.

EXPERIMENTAL

Materials

Fatty acids, fatty acid esters and Bicine (*N,N-bis*(2-hydroxyethyl)glycine) were purchased from Sigma Chemical Co., St Louis, MO. Mes(2-(*N*-morpholino)ethanesulfonic acid) was obtained from Calbiochem-Behring, La Jolla, CA. Estradiol-17β was purchased from Steraloids Inc., Wilton, NH. Pyridine nucleotides were from Pharmacia P-L Biochemicals, Milwaukee, WI. Prostaglandins E_2 and $F_{2\alpha}$ were supplied by The Upjohn Co., Kalamazoo, MI.

Preparation of 17β-HSD

Soluble 17β-HSD was purified from placental homogenates by the method of Jarabak[13], modified in this laboratory to include an ammonium sulfate, gradient solubilization step [14]. Specific activity with estradiol-17β was 0.3 to 0.5 μmol/min/mg protein. Enzyme was stored at 4°C in buffer containing 1.0 mM EDTA, 50% (v/v) glycerol and 5.0 mM potassium phosphate, pH 8.0.

17β-HSD activity

In the majority of experiments activity was measured fluorometrically at 25°C in an Aminco-Bowman Spectrophotofluorometer fitted with a con-

stant temperature cuvette chamber, as previously described [14]. One-ml reaction mixtures contained 0.1 M Bicine, pH 9.0, 1.0 mM NAD^+ and enzyme. Reaction was started by the addition of estradiol-17β in methanol. The final concentration of methanol in the assays was constant for each experiment and never exceeded 4% (v/v). NADH fluorescence at 450 nm (340 nm excitation) was recorded continuously on a Sargent–Welch Model SRG recorder and reaction velocities were estimated from the initial linear portions of the progress curves.

Because of fluorescence quenching by reduced pyridine nucleotides, a spectrophotometric assay was used in those experiments where the protective effects of NADH and NADPH were assessed. Reactant and buffer concentrations were the same as in the fluorometric assay. However, reaction volumes were increased to 3.0 ml and NADH formation was quantitated in terms of absorbance at 340 nm.

Fatty acids

Fatty acids, esters and prostaglandins were stored at −20°C as stock solutions in chloroform or methanol. For the experiments, aliquots were taken to dryness in a stream of nitrogen and resuspended in aqueous buffers by sonication (Heat Systems Ultrasonics, Model B-220, Plainview, NY).

Protein assay

Protein was quantitated by the dye binding method of Bradford [15]. Bovine serum albumin was used as the protein standard.

Light-scattering by oleic acid

Light-scattering by oleic acid at pH 7.0 and pH 9.0 was estimated on an Aminco-Bowman Spectrophotofluorometer by a procedure developed in this laboratory for estimating steroid solubilities [16].

Fluorescence polarization of NADH in the presence of oleic acid

For fluorescence polarization measurements, the Aminco-Bowman Spectrophotofluorometer was fitted with Glan prism polarizers in both the excitation and emission beams, as previously described [17]. Polarization was calculated from the equation:

$$P = \frac{I_{EE} - GI_{EB}}{I_{EE} + GI_{EB}}$$

where I_{EE} is the fluorescence emission intensity with both polarizers oriented vertically, I_{EB} that with the excitation polarizer oriented vertically and emission polarizer oriented horizontally. The grating correction factor G was calculated from the ratio of emission intensities (I_{BE}/I_{BB}) obtained with the excitation polarizer oriented horizontally and emission vertically (I_{BE}) and with both polarizers oriented horizontally (I_{BB}).

RESULTS

Fatty acids used

In our earlier studies [1] oleic, arachidonic and linoleic acids were found to be the most effective in inactivating microsomal 17β-HSD. For this reason, the majority of the inactivation experiments considered here were done with unsaturated fatty acids. Because of its stability, oleic acid was the principal fatty acid used.

Time course and concentration dependence of inactivation

The rate and extent of activity loss were dependent on the length of time of exposure and the concentration of fatty acid. No inactivation was seen with 50 μM oleic acid at either pH 7.0 or 9.0 (Fig. 1). At fatty acid concentrations above 50 μM the enzyme was more sensitive to inactivation at pH 9.0. Time-course data for inactivation at pH 9.0 in the presence of 1.0 mM oleic acid are shown in Fig. 2. Inactivation was first-order with an apparent first-order rate constant of $1.8 \times 10^{-4}\ sec^{-1}$.

Effect of various fatty acids, esters and prostaglandins

Arachidonic and oleic acids were equally effective. Linoleic acid was moderately effective and linolenic acid gave the least inactivation (Table 1).

The C_{18}, saturated fatty acid stearic acid was without effect and no inactivation was seen with the methyl and ethyl esters of oleic acid.

Exposure of 17β-HSD at pH 9.0 to 1.0 mM prostaglandin E_2 or $F_{2\alpha}$ for up to 30 min resulted in no loss of activity.

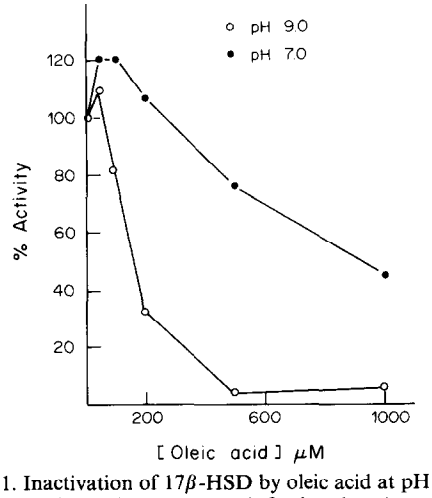

Fig. 1. Inactivation of 17β-HSD by oleic acid at pH 7.0 and 9.0. Reaction mixtures (0.5 ml final volume) containing 17β-HSD (75 μg of protein) and 0, 0.05, 0.10, 0.20, 0.50 or 1.0 mM oleic acid in 0.07 M Bicine–14% glycerol, pH 9.0, or 0.07 M Mes–14% glycerol, pH 7.0, were incubated at room temperature for 90 min. Aliquots (10 μl) were then assayed for 17β-HSD activity in reaction mixtures (1.0 ml final volume) containing 1.0 mM NAD^+ and 18.0 μM estradiol-17β in 0.17 M Bicine, pH 9.0. Activities in samples lacking oleic acid were taken as 100% (0.142 nmol·min^{-1}, pH 7.0; 0.149 nmol·min^{-1}, pH 9.0).

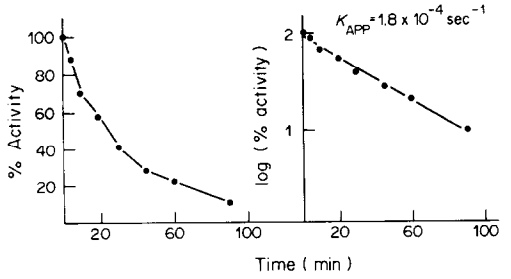

Fig. 2. Time course of oleic acid inactivation of 17β-HSD. Enzyme was incubated at room temperature with 1.0 mM oleic acid and assayed as described in the legend to Fig. 1. Activity of the control sample lacking oleic acid was 0.156 nmol·min^{-1} at 5 min and 0.147 nmol·min^{-1} at 90 min.

Reversibility of inactivation

Two approaches were taken in an attempt to reverse inactivation. The kinetic properties of microsomal 17β-HSD from guinea pig liver [18] and the 3β-HSD of human placental microsomes [19] have been shown to be reversibly altered by 2-mercaptoethanol and hydrogen peroxide. This raises the possibility that oxidations by hydroperoxides present in the fatty acid preparations could be affecting 17β-HSD activity. However, neither 2-mercaptoethanol nor dithiothreitol at 1.0 mM were able to protect against or reverse inactivation by oleic acid.

As a second approach, enzyme was exposed to oleic acid under conditions resulting in an 83% decrease in activity. Bovine serum albumin was then added to the reaction mixture to a concentration of 50 mg/ml in an attempt to adsorb the fatty acid present. However, no 17β-HSD activity was recovered when assays were done after 2, 4 and 24 h of exposure to albumin.

Protective effects of steroids and coenzymes

Steroids with a high affinity for 17β-HSD as inhibitors [14] were tested for their ability to protect against inactivation by oleic acid. Estradiol-17α, 1,3,5(10)-estratrien-3-ol, 1,3,5(10)16-estratetraen-3-ol, 1,3,5(10)-estratriene-3,16β,17β-triol and estrone did not protect against inactivation. They also did not affect the degree of protection seen in the presence of coenzyme.

Table 1. Inactivation of 17β-HSD by various fatty acids

Fatty acid	μM	% Activity
None	—	100
Oleic	100	68
	200	0
Arachidonic	200	0
Linoleic	200	42
Linolenic	200	66

17β-HSD (0.15 mg/ml) was incubated at room temperature for 30 min in 0.1 M Bicine–20% glycerol, pH 9.0, containing fatty acids as indicated. After the incubation 20-μl aliquots were taken for activity measurements as described in the legend to Fig. 1.

Table 2. Protective effects of pyridine nucleotides

Nucleotide	Concn	Activity % of control
None		18
NAD^+	2.0 mM	97
NADH	2.0 mM	38
$NADP^+$	0.2 mM	105
NADPH	0.2 mM	107

17β-HSD (0.15 mg/ml) was incubated for 3 h in 0.1 M Bicine–20% glycerol, pH 9.0, containing 200 μM oleic acid and pyridine nucleotide at the concentration indicated. The control sample, taken as 100% activity, lacked oleic acid and any pyridine nucleotide. After the incubation 10-μl aliquots were taken for the spectrophotometric assay of 17β-HSD activity.

NAD^+, NADH, $NADP^+$ and NADH were able to protect against inactivation by oleic acid (Table 2). $NADH^+$ was the least effective. $NADP^+$ and NADPH were the most effective, giving over 60% protection at 1.5 μM and essentially complete protection at 200 μM (Fig. 3A). By contrast, complete

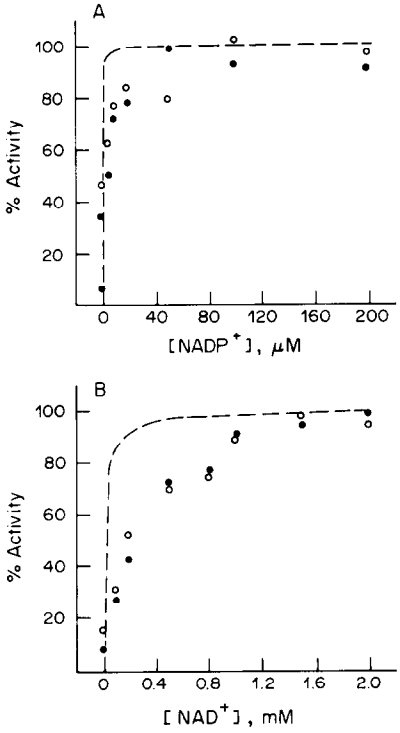

Fig. 3. Protection against inactivation by oleic acid as a function of $NADP^+$ (A) or NAD^+ (B) concentration. Reaction mixtures (0.5 ml final volume) containing 17β-HSD (75 μg of protein), 200 μM oleic acid and 0, 1.0, 5.0, 10.0, 20.0, 50.0, 100.0 or 200 μm $NADP^+$ or 0, 0.1, 0.2, 0.5, 0.8, 1.0, 1.5 or 2.0 mM NAD^+ in 0.08 M Bicine–20% glycerol, pH 9.0, were incubated at room temperature for 60 min. Aliquots (10 μl) were then taken and assayed as described in the legend to Fig. 1. Filled and unfilled circles are data from separate experiments. The dashed lines are the activities expected on the basis of coenzyme binding estimated from Michaelis constants.

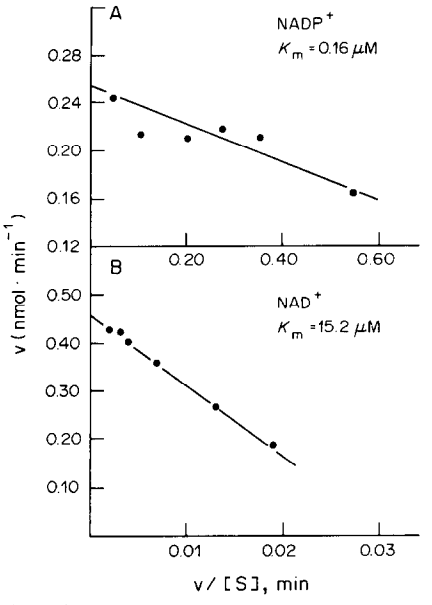

Fig. 4. Plots of v versus v/[S] for $NADP^+$(A) and NAD^+(B). Initial velocity was measured fluorometrically on the basis of NADPH or NADH fluorescence at 450 nm (340 nm excitation). Reaction mixtures (1.0 ml final volume) contained 17β-HSD (15 μg of protein), 18 μM estradiol-17β, 0.5, 1.0, 2.0, 5.0, 10.0, 15.0, or 20.0 μM $NADP^+$ or 10.0, 20.0, 50.0, 100.0, 200.0, 500.0 μM NAD^+ in 0.18 M Bicine, pH 9.0.

protection by NAD^+ required 1.5 to 2.0 mM coenzyme (Fig. 3B) and even at 2.0 mM, NADH gave only partial protection (Table 2). The dashed lines in Fig. 3 are the extent of protection expected on the basis of coenzyme binding estimated from Michaelis constants and the expression

$$v = V_{max}[S]/K_m + [S].$$

Because the concentrations of NAD^+ and $NADP^+$ necessary for complete protection were significantly greater than the Michaelis constants [20–22] and dissociation constants [20] reported in the literature, we measured the K_m-values for NAD^+ and $NADP^+$ with our 17β-HSD preparation. K_m-values, estimated from plots of v *versus* v/[S] (Fig. 4) were 15.2 μM for NAD^+ and 0.16 μM for $NADP^+$, in good agreement with previous estimates [20–22]. The highest concentrations tested were in 13-fold (NAD^+) and 31-fold ($NADP^+$) excess of K_m in each case and the highest velocities observed were 89% (NAD^+) and 95% ($NADP^+$) of V_{max} estimated graphically. Thus in neither experiment was a second, low affinity coenzyme binding site apparent in the initial velocity data. In an alternative experiment to detect a kinetically significant, low-affinity coenzyme site, 17β-HSD was assayed with 18 μM estradiol-17β in the presence of 200 μM or 2.0 mM NAD^+. Reaction velocities were 1.16 nmol·min^{-1} with 200 μM NAD and 1.25 nmol·min^{-1} with 2.0 mM NAD^+, further evidence for the absence of a kinetically significant, low affinity site for NAD^+.

Light-scattering by oleic acid

The lack of inactivation by oleic acid at concentrations below 50 μM suggested that the loss of 17β-HSD activity might be dependent upon the micellar state of the fatty acid. This was assessed by measurement of the light-scattering of 5–200 μM oleic acid at pH 7.0 and pH 9.0 (Fig. 5). Light-scattering was greater at pH 7.0. For this reason, excitation and emission slit widths were adjusted to give comparable intensities of scattered light. At pH 7.0, a sharp increase in scattered light was observed above 25 μM oleic acid. At pH 9.0, light-scattering increased sharply above 50 μM. These differences probably reflect pH-dependent changes in the ionization state of oleic acid, which would be expected to affect aggregation state and micelle formation [23]. At both pH 7.0 and 9.0, the loss of 17β-HSD activity occurred under conditions of fatty acid micelle formation.

Assessment of coenzyme and fatty acid interactions by fluorescence emission and polarization

The requirement for coenzyme concentrations significantly in excess of saturating levels, based on K_m-values, to protect against inactivation suggested the possibility that coenzyme might be binding to fatty acid micelles and as a result be unavailable for binding to 17β-HSD. That binding of NADH to enzymes significantly shifts its fluorescence emission maximum to shorter wavelengths, increases emission intensity, and also increases the yield of polarized fluorescence when polarized exciting light is used has been known for many years [24, 25]. Adsorption on non-protein solids has a similar effect [26], thus effects of this type might be expected on binding of NADH or NADPH to fatty acid micelles. When we carried out such experiments no differences could be detected

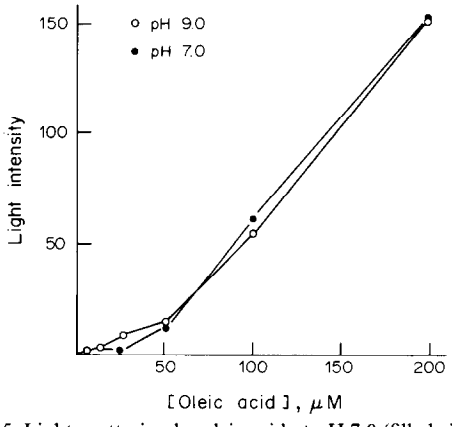

Fig. 5. Light-scattering by oleic acid at pH 7.0 (filled circles) and pH 9.0 (unfilled circles). Oleic acid (0 to 500 μM) was sonicated into 0.1 M Mes-20% glycerol, pH 7.0, or 0.1 M Bicine–20% glycerol, pH 9.0. Light-scattering at 400 nm was measured on an Aminco-Bowman spectrophotofluorometer. Excitation and emission slits were 5.0 mm and 0.6 mm at pH 9.0 and 1.5 and 0.2 mm at pH 7.0. Light intensity is in arbitrary units.

Table 3. Fluorescence polarization emission parameters for NADH and NADPH in the presence and absence of oleic acid

Nucleotide	Oleic acid (±)	Fluorescence (arbitrary units) EE	EB	BE	BB	P
NADH	–	67.3	67.6	56.1	74.2	0.137
	+	64.5	66.6	54.9	73.2	0.127
NADPH	–	57.0	56.1	45.1	61.0	0.158
	+	55.0	58.7	45.4	61.0	0.137

NADH or NADPH (10 μM) and 1.0 mM oleic acid in 0.1 M Bicine–20% glycerol, pH 9.0 (1.0 ml total volume) were incubated at 25°C in 10 × 10 mm cuvettes in an Aminco-Bowman Spectrophotofluorometer. Excitation was at 340 nm, emission at 450 nm. Polarizer orientations were: EE (excitation vertical, emission vertical), EB (excitation vertical, emission horizontal), BE (excitation horizontal, emission vertical), BB (excitation horizontal, emission horizontal).

in the fluorescence emission intensity or fluorescence polarization of 10 μM NADH or NADPH in 0.1 M Bicine–20% glycerol, pH 9.0, upon the addition of 1.0 mM oleic acid (Table 3), consistent with a lack of binding of pyridine nucleotides under these conditions.

DISCUSSION

Evidence of the possible modulation of steroidogenic enzymes by lipid soluble substances has been presented by a number of laboratories. Weiner and colleagues [27, 28] reported that human placental 5-ene,3β-hydroxysteroid hydrogenase is inhibitible by endogenous material extractable into hexane. Murphy[29], also, demonstrated that human placental 11β-hydroxysteroid dehydrogenase is susceptible to inhibition by factors extractable into lipid solvents. It was suggested [28] and evidence subsequently presented [30] that steroids could account in part for the inhibition noted. However, the potential of other lipoidal compounds to modulate activity was not precluded and data from our laboratory [1] established that microsomal 17β-HSD from guinea pig liver is susceptible to inhibition by free fatty acids *in vitro*.

The experiments reported here show that a soluble form of 17β-HSD can be inhibited by fatty acids *in vitro*. This is of interest because it demonstrates an effect of the fatty acid resulting from a direct interaction with the enzyme protein and is not an indirect result of a perturbation of membrane lipid structural domains.

The fact that inactivation required oleic acid concentrations above the critical micelle concentration is consistent with an interaction of 17β-HSD with fatty acid micelles. The increased lability of the enzyme at pH 9.0 may be an indication that the affinity for fatty acids may be modified by pH-dependent changes in the protein such as net surface charge or conformation, or both. Alternatively, pH-dependent changes in micelle structure, as suggested by the difference in the intensity of scattered light, might also affect protein binding.

The protective effects of NAD^+, $NADP^+$, NADH and NADPH suggest that the fatty acid binding site may be at or near pyridine nucleotide binding sites on the enzyme. There is, however, a major discrepancy between the concentrations of NAD^+ and $NADP^+$ required for complete protection against inactivation by oleic acid and the degree of coenzyme binding expected on the basis of Michaelis constants (Fig. 3). Our kinetic data (Fig. 4) show no evidence of a kinetically significant, low-affinity site for either NAD^+ or $NADP^+$. On the basis of a K_m of 0.16 μM for $NADP^+$, the active site would be 89% saturated at 1.0 μM nucleotide. Only 40% protection was observed under these conditions, however (Fig. 3A). Similarly, approx 95% saturation, based on a K_m of 15.2 μM would be expected at 0.2 mM NAD but only 40% protection was observed (Fig. 3B). A similar result was reported by Pons and coworkers [22] who observed that complete protection against inactivation by the alkylating agent *N*-ethylmaleimide required 0.5 mM $NADP^+$. This led them to propose the existence of low-affinity and high-affinity binding sites for coenzyme. Jarabak and Sack [22] used a fluorometric titration method to measure the binding of NADPH over the concentration range of 0.1 to 2.8 μM. Their data indicated the binding of 1 mole of NADPH per 63,800 *g* of protein. That result, when considered with the currently accepted molecular weight for 17β-HSD of 68,000 [22], is consistent with the presence of a single, high-affinity site per dimer, in agreement with the data of Pons *et al.* [22]. Studies of the binding of pyridine nucleotides at concentrations up to 2.0–5.0 mM are needed to examine the possibility of low-affinity sites on 17β-HSD in greater detail.

With regard to the possible modulation of 17β-HSD by free fatty acids *in vivo*, our data indicate that at pH 7.0 concentrations of greater than 200 μM would be required for a significant inhibition of activity. During pregnancy in the human the placenta is exposed to significant levels of free fatty acids, those which exist in nonesterified form in the plasma and within the tissue itself as well as those released as products of phospholipase A_2 or diacylglyceride lipase action on membrane phospholipids. Plasma free fatty acid levels increase from 977 ± 374 μEq/l at 33 weeks of gestation to 1255 ± 372 μEq/l at 40 weeks [6]. Free fatty acids are readily taken up by the placenta and transported to the fetus [30–32], and levels of 910 ± 570 to 990 ± 370 μEq/g fresh weight of placental tissue have been reported [33]. Thus the levels to which placental cells are exposed are consistent with the amounts needed for inhibition. However, the amounts of free fatty acid available for binding to 17β-HSD would be affected by competition with intracellular membrane phases as well as intracellular metabolism. Dancis and coworkers observed a rapid metabolism of fatty acids by placental villi [31], which obstructed measurement of fatty acid binding within the tissue. Because of this, it is

likely that 17β-HSD would have to compete with various fatty acid-metabolizing enzymes for any inhibition or modulation of its activity to occur.

In summary, we have shown that a soluble form of 17β-HSD can be inhibited by fatty acids. The mechanism of inhibition appears to involve binding of fatty acid micelles at or near sites on the enzyme protein capable of binding pyridine nucleotides. More data on the intracellular availability of free fatty acids are needed before the potential of this interaction for inhibition of 17β-HSD activity *in vivo* can be fully assessed.

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