THE INHIBITION OF 16α-HYDROXYTESTOSTERONE AROMATIZATION BY CARBON MONOXIDE

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Received January 23,1975

SUMMARY

The aromatization of 16α-hydroxytestosterone[1] by human placental microsomal preparations is inhibited by carbon monoxide. At a $CO:O_2$ ratio of 19:1, the extent of inhibition was 45-60%. In the same experiments, CO inhibition of androstenedione aromatization was negligible (0-7.8%). In contrast to 19-nortestosterone, a poor substrate whose aromatization is inhibited by CO, the rates of aromatization of 16α-hydroxytestosterone and androstenedione are similar. It is possible that different cytochrome P-450 species or binding sites are involved in the aromatization of different substrates.

INTRODUCTION

Estriol, the major estrogen of pregnancy, is synthesized in the human placenta through the aromatization of 16α-hydroxylated androgens (1). Though the production of estriol in the placenta is quantitatively more important than the synthesis of other estrogens, little is known about the enzyme system involved.

The microsomal aromatization of androstenedione has been presented in far more detail. It involves a complex mixed function oxidation (2) utilizing three moles of NADPH and O_2 for each mole of estrone produced (3). As was first demonstrated for the adrenal 21-hydroxylase (4), most if not all steroid mixed-function oxidases contain the NADPH-cytochrome P-450 system. The most widely used basis for cytochrome P-450 involvement has

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[1] Trivial names: 16α-hydroxytestosterone = 16α-hydroxyandrost-4-en-17β-01-3-one; androstenedione = androst-4-en-3,17-dione; 19-nortestosterone = 17β-hydroxyestr-4-en-3-one.

been the demonstration of carbon monoxide inhibition of enzyme activity. However, while cytochrome P-450 has been shown to be present in human placental microsomes (5), microsomal aromatization of androstenedione, 19-hydroxyandrostenedione, and 19-oxoandrostenedione has been shown by Meigs and Ryan (5, 6) and others (7) to be insensitive to carbon monoxide. In comparison, the aromatization of 19-nortestosterone, a steroid which does not appear to be a natural substrate or intermediate of aromatization in the placenta, is inhibited by carbon monoxide (6).

In the light of the carbon monoxide inhibition studies which have been published and since estriol is the predominant estrogen synthesized by the placenta, it is of interest to determine whether its synthesis from 16α-hydroxytestosterone is sensitive to carbon monoxide.

METHODS

Androstenedione-4-^{14}C (New England Nuclear Corp.) and 19-nortestosterone-4-^{14}C (Amersham-Searle, Inc.) were purified on receipt by paper chromatography. Non-radioactive steroids were purchased from Steraloids, Inc.

Preparation and incubation. Microsomes were prepared from freshly delivered human term placentas. Tissue was homogenized in 3 parts 0.25 M sucrose containing 0.01 M Tris-HCl, pH 7.2 at 25°. The 15,000g supernatant was centrifuged at 100,000g for 1 hr, and washed once with 0.15 M KCl. Aromatase incubations contained freshly prepared microsomes (7-10 mg protein) and 70μM steroid substrate, 1% (v/v) ethanol, 12 mM glucose 6-phosphate, 2 mM $NADP^+$, 2 I.U. per ml glucose 6-phosphate dehydrogenase and 50 mM phosphate buffer, pH 7.0, in a total volume of 5 ml. Incubation flasks were preincubated for 10 min at 37°. The incubation, begun with the addition of microsomal protein, ran for up to 30 min at 37° and was terminated by the addition of methanol to a final methanol concentration of 70%.

Gassing. Mixtures of 95% N_2 - 5% O_2 and 95% CO - 5% O_2 were obtained using a gas proportioner (Matheson Gas Co., E. Rutherford, N.J.). All incubation flasks were equilibrated with the gas phase for 15 min prior to

addition of microsomal protein. Gassing was continuous throughout the pre-incubation and incubation.

^{14}C estrogen assay. The 70% methanol extract was extracted with ethyl acetate, washed and subjected to phenolic separation as described by Nimrod et al. (8). The phenolic separation was repeated and the radioactivity recovered regarded as the total estrogens. Paper chromatographic analysis revealed that more than 90% of the radioactivity resided in the estrone and estradiol zones.

Unlabeled estrogen assay. The washed ethyl acetate extract, obtained as above, was subjected to radioimmunoassay. Incubations containing 16α-hydroxytestosterone, in which estriol was synthesized, were assayed using an estriol-specific antibody, kindly provided by Dr. David Watson of the Worcester Foundation for Experimental Biology. Cross-reactivity with 16α-hydroxytestosterone and estrone was <0.1% and with estradiol-17β, 0.22%. Androstenedione incubations were assayed using a non-specific estrogen antibody provided by Dr. Dan Tulchinsky, Harvard Medical School. Estrone and estradiol-17β gave nearly identical standard curves, with androstenedione cross-reactivity <0.1%.

RESULTS

The ability of CO to inhibit the microsomal aromatization of various substrates was tested (Table 1). In accordance with the safety guidelines recommended by Matheson Gas Co., a 19:1 ratio of $CO:O_2$ was used with the same ratio of $N_2:O_2$ serving as the control. In most cases, the rate of aromatization in air was tested as well.

In three different experiments, CO appeared to markedly inhibit the aromatization of 16α-hydroxytestosterone to estriol (45-60%), while causing little or no change in androstenedione aromatization performed simultaneously. As an added control, 19-nortestosterone aromatization, which involves one rather than three hydroxylations and which has been shown previously to be CO-sensitive (6), was examined (Table 1). CO inhibited 19-nortestosterone

Table 1. Effect of Carbon Monoxide on Aromatization

Preparation	Substrate	Estrogen Formation* (pmoles/min/mg prot.) In Air	In 95% N_2-5% O_2	In 95% CO-5% O_2	% Of Control $CO\text{-}O_2/N_2\text{-}O_2$ x 100
4-21	16α-Hydroxytesto	177.0	106.9	59.1	55.3
4-22	16α-Hydroxytesto	193.8	90.2	35.0	38.8
4-22	Androstenedione	-	97.1	89.5	92.2
5-1	16α-Hydroxytesto	105.0	88.7	43.0	48.5
5-1	Androstenedione	130.0	84.4	87.9	104.1
3-4	Androstenedione-4-^{14}C	110.5	67.1	65.0	96.9
4-1	Androstenedione-4-^{14}C	140.1	100.1	93.7	93.6
4-2	Androstenedione-4-^{14}C	-	88.9	87.7	98.6
4-2	19-Nortesto-4-^{14}C	-	18.3	7.3	39.9

* When androstenedione or 19-nortestosterone was substrate, estrone plus estradiol-17β was measured, when 16α-hydroxytestosterone was substrate, estriol was measured.

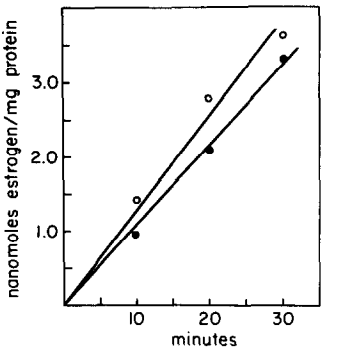

Figure 1. The conversion of 16α-hydroxytestosterone to estriol (●) and androstenedione to estrone plus estradiol-17β (○) as a function of time. Conditions were as described in Methods. 9.1 mg of microsomal protein was used per incubation.

aromatization by 60% while, in the same preparation, the aromatization of androstenedione was not significantly affected.

It should be noted that under uninhibited conditions the rate of 19-nortestosterone aromatization proceeds at no more than one-fifth that of androstenedione. In contradistinction, the rate of 16α-hydroxytestosterone aromatization, which is also CO-sensitive, is similar to that of androstenedione, both rates being essentially linear for at least 30 minutes (Figure 1).

DISCUSSION

While the placental aromatization of androstenedione is not inhibited by carbon monoxide, recent studies by Thompson and Siiteri (7) supply convincing evidence for the participation of cytochrome P-450 in this complex mixed-function oxidation based on results with inhibitors of other mixed-function oxidases, an antibody preparation specific for NADPH-cytochrome c reductase, and substrate binding spectra.

It now appears that the aromatization of 16α-hydroxytestosterone, a natural substrate for human placental aromatase presumably requiring three moles of NADPH and O_2 for each mole of estriol synthesized, is inhibited by CO. In addition, 16α-hydroxytestosterone exhibits a typical type I binding spectrum with human placental microsomes (unpublished) as do all aromatizable androgens which have been tested (7). These results lend further support

to the participation of cytochrome P-450 in placental aromatization.

It is not clear, though, whether androstenedione and 16α-hydroxytestosterone utilize the same aromatase enzyme or binding site. The difference in their CO sensitivities is puzzling since both steroids appear to be equally good substrates, exhibiting similar rates of aromatization. In this regard, Preumont et al. (9) have found that human ovarian aromatase cannot utilize 16α-hydroxytestosterone even though androstenedione is a good substrate for ovarian aromatization. It is possible that either the human placenta and ovary contain different aromatases or the aromatase which utilizes 16α-hydroxytestosterone is unique to the placenta.

ACKNOWLEDGEMENT

We thank Ms. Roberta Todd for setting up the radioimmunoassays and for her excellent technical assistance and Ms. Elizabeth Clifford for typing the manuscript.

This work was supported by Rockefeller Foundation Grant RF 72077, U.S. Public Health Service HD07923-02, and a postdoctoral fellowship from Rockefeller Foundation to J.A.C.

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