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INHIBITION OF HUMAN PLACENTAL MIXED-FUNCTION OXIDATIONS WITH CARBON MONOXIDE: REVERSAL WITH MONOCHROMATIC LIGHT

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SUMMARY

The present investigation was designed to further examine the functions of cytochrome(s) P-450 (P-450_{hpm}) present in the endoplasmic reticulum of the human placental syncitium. The antagonistic effects of androstenedione and 19-norandrostenedione with regard to respective inhibition and facilitation of the binding of carbon monoxide to the cytochrome(s) were exploited in the examination of functionality. The presence of various concentrations of 19-norandrostenedione in reaction vessels permitted carbon monoxide to inhibit the conversion of androstenedione to estrone in a concentration-dependent fashion. Optimal light-reversal of the inhibition was achieved at a wavelength of 450 nm. Optimal reversal of inhibition of placental microsomal aryl hydrocarbon hydroxylase by carbon monoxide likewise was achieved with light at a wavelength of 450 nm, thus providing definitive evidence for a role for P-450_{hpm} in placental aryl hydrocarbon hydroxylase (AHH). However, neither androstenedione nor 19-norandrostenedione affected the capacity of carbon monoxide to inhibit placental AHH. The results provided evidence for the hypothesis that at least two species of cytochrome P-450 are present in human placental endoplasmic reticulum. One species, not detectable with light spectroscopy in these experiments, appeared to function in the biotransformation of foreign hydrocarbons. The major physiologic role of the spectrally visible species appeared to be in the biosynthesis of estrogens from endogenous C-19 steroids.

INTRODUCTION

The endoplasmic reticulum of human placental syncitial cells contains only two monooxygenase systems of major scientific interest in current research. One is functional in the biosynthesis of estrogens from endogenous androgenic precursors [1] and the other in the hydroxylation of foreign carcinogenic hydrocarbons [2]. The common presence of cytochrome P-450 in placental cellular subfractions containing these enzymatic activities [3] and the specificity of steroidal binding [4–6] have suggested that the cytochrome may function as terminal oxygenase for either or both of these reactions. Traditionally, confirmation of the participation of cytochrome P-450 in a mixed function oxidation requires a demonstration that CO* will inhibit the reaction and that optimal reversal of the inhibition with monochromatic light will occur at a wavelength of 450 nm [7]. For neither of these reactions has this confirmation been demonstrated. Rates of conversion of androstenedione, 19-hydroxyandrostenedione and 19-oxoandrostenedione to estrone could not be inhibited by CO even in the presence of very high concentrations of CO and limiting oxygen concentrations (44–95% CO, 5% O_2) and high concentrations of NADPH [2, 5, 6, 8]. Hydroxylation of benzo[a]pyrene (BP) in placental microsomes reportedly may be inhibited by CO [9], but this phenomenon has not been thoroughly studied. The purpose of the present investigation was to examine critically the role of human placental microsomal cytochrome P-450 (P-450_{hpm}) in the two monooxygenase systems mentioned above. Our approach utilized the recently described antagonistic effects of 19-norandrostenedione and androstenedione with respect to their influence on the binding of CO to the placental cytochrome [10]. The results obtained provide conclusive evidence for the participation of cytochrome P-450 in the biosynthesis of

* The following abbreviations and trivial names used are: CO, carbon monoxide; BP, benzo[a]pyrene; androstenedione, 4-androstene-3,17-dione; 19-norandrostenedione, 4-estrene-3,17-dione; testosterone, 17β-hydroxy-4-androstene-3-one; 19-nortestosterone, 17β-hydroxy-4-estrene-3-one; 16α-hydroxytestosterone, 16α,17β-dihydroxy-4-androstene-3-one; 19-hydroxyandrostenedione, 19-hydroxy-4-androstene-3,17-dione; 19-oxo-androstenedione, 19-hydroxy-4-androstene-3,17-dione; norethindrone, 17α-ethinyl-17β-hydroxy-4-estrene-3-one; norethynodrel, 17α-ethinyl-17β-hydroxy-5-estrene-3-one; norgestrel, 13β-ethyl-17α-ethinyl-17β-hydroxy-4-estrene-3-one; norethandrolone, 17α-ethyl-17β-hydroxy-4-estrene-3-one; nandrolone, 17β-hydroxy-4-estrene-3-one decanoate; progesterone,4-pregnene-3,20-dione; pregnenolone, 3β-hydroxy-5-pregnene-20-one; deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; estradiol-17β, 1,3,5(10)-estratrien-3,17β-diol; estrone, 3-hydroxy-1,3,5(10)-estratrien-17-one; aminoglutethimide, α-(p-aminophenyl)-α-ethylglutarimide; metyrapone, 2-methyl-1,2-di-3-pyridyl-1-propanone; P-450_{hpm}, human placental microsomal cytochrome P-450; AHH, arylhydrocarbon hydroxylase.

estrone from androstenedione by showing that carbon monoxide will inhibit both monooxygenase systems under appropriate conditions and that optimal light reversal occurs at a wavelength of 450 nm for either reaction. Supporting evidence for the participation of a separate cytochrome in each reaction also was obtained. A report of certain prefatory aspects of this research has appeared [11].

EXPERIMENTAL PROCEDURES

Tissues. Human placentas were obtained at term from the delivery rooms of the University and Group Health Hospitals, Seattle, Washington. Microsomal fractions were prepared according to the method described previously [12] except that final protein concentrations of microsomal suspensions utilized for spectral analyses and assays of enzymatic activities were adjusted to a range of 4–25 mg/ml as determined by the method of Lowry *et al.*[12]. Hepatic microsomes were prepared from adult, male, Sprague-Dawley rats according to methods described by Mazel[14].

Chemicals. Radioactive chemicals, including [4-^{14}C]-androstenedione (58.8 Ci/mol), [4-^{14}C]-testosterone (57.1 Ci/mol), [4-^{14}C]-esterone (51.0 Ci/mol), estradiol-17β (58.0 Ci/mol) were obtained from New England Nuclear Corp., Boston, MA. Corresponding nonradioactive steroids, 19-hydroxyandrostenedione, 16α-hydroxytestosterone, 19-nortestosterone, 19-norandrostenedione, 17β-hydroxy-1,4,6-androstatrien-3-one, progesterone, pregnenolone and deoxycorticosterone, were obtained from Steraloids, Inc., Pawling, NY. Norethynodrel and norethandrolone were kindly supplied by G. D. Searle & Co., Chicago, IN; norethindrone by Parke, Davis & Co., Detroit, MI; norgestrel by Wyeth Laboratories, Philadelphia, PA, and nandrolone decanoate by Organon, Inc., West Orange, NJ. 19-Oxoandrostenedione was synthesized and purified according to methods described by Meigs and Ryan [1]. NADPH, NADH, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were obtained from Sigma Chemical Co., St. Louis, MO. Benzo[a]pyrene was purchased from Eastman Organic Chemical Co., Rochester, NY. CO was obtained from Matheson Chemical Co., Los Angeles, CA. All other chemicals and solvents utilized were reagent grade and of the highest purity commercially available.

Spectral analyses and enzyme assays. Analyses of difference spectra as well as recordings in the dual-wavelength mode were performed with a DW-2 recording spectrophotometer (American Instrument Co.). A holmium oxide filter was utilized for calibration of each spectrum. The parameters utilized for recordings of difference spectra were as follows: temperature in the cuvettes was 6°C, slit width was 3.0 nm, full scale absorbance was 0.1, recorder speed was 10 nm/s and the protein concentrations in the cuvettes were adjusted to 4 mg/ml. For recordings in the dual-wavelength mode: temperature of the microsomal suspension was maintained at 6°C, slit width was 8.0 nm, full-scale absorbance was 0.1, recording speed was 5 s/in., reference and test monochromators were set at 490 and 450 nm respectively. Microsomal preparations (2 ml) were gassed with deoxygenated CO by bubbling through the suspensions for 60 s in stoppered cuvettes. A base line was then recorded, 10 μl of NADPH (3×10^{-4} M final concentrations) were added to the sample cuvette, and recordings were initiated in split bean or dual wavelength modes. CO was deoxygenated by passing through a sintered disk in a 30 cm column of a solution containing 0.5% dithionite and 0.05% anthraquinone-3-sulfonate in 0.1 N NaOH.

Analyses of rates of formation of 3-hydroxybenzo[a]pyrene from BP in placental microsomes were performed as previously described [3]. Rates of conversion of androstenedione to estrogens in placental microsomes also were determined according to methods previously described [6].

To determine the effects of varying CO concentrations on rates of aromatization of androstenedione and hydroxylation of BP, appropriate amounts of N_2, O_2 and CO were mixed, and a continuous stream of the gas of the desired composition was passed directly over the reaction mixture in the incubation flask. This flask was sealed except for inlet and outlet gassing needles. Microsomes, buffer and cofactors were equilibrated at 37°C with the gas mixture for 15 min prior to initiation of the reaction by addition of the appropriate substrate. N_2 and CO were deoxygenated prior to entry into the gas mixing flask by the procedure described above. All incubations and preincubations were carried out with shaking in the absence of light.

Reversal of the inhibitory effects of CO (95% CO:5% O_2) on the conversion of BP to 3-hydroxy-BP or of androstenedione (in the presence of 19-norandrostenedione) to estrogens was estimated by exposing cuvettes containing appropriate incubation mixtures to light from a xenon lamp source. A spectrophotofluorometer (Hitachi, Perkin–Elmer) with monochromators accurately adjustable to various wavelengths was utilized for this purpose. The instrument was provided with attachments for the maintenance of constant temperatures (37°C) in the cuvettes. Prior to the incubation, flasks containing microsomes, substrates and phosphate buffer were equilibrated with 95% CO:5% O_2 for 60 min at 6°C in the dark. The flasks were sealed except for inlet and outlet gassing needles. After equilibrating with the gases, aliquots (2 ml) of incubation mixtures were transferred into closed cuvettes with a needle and syringe. The cuvettes then were equilibrated at 37°C for an additional 5 min before initiation of the reaction with NADPH. The cuvettes were exposed to visible light for 15 min at specific wavelengths, and reactions were terminated according to the usual procedures. Control flasks were prepared and incubated

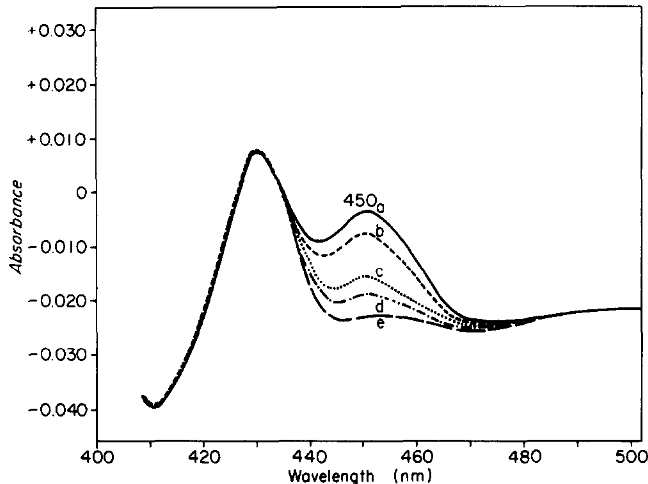

Fig. 1. CO-difference spectra of human placental microsomes associated with successive additions of increasing concentrations of androstenedione to sample and reference cuvettes. Sample and reference cuvettes both contained CO (see Experimental Procedures), and the sample cuvette contained 3 mM NADPH. Protein concentration was 4 mg/ml and androstenedione concentrations were: a, 0; b, 1.0 nM; c, 4.0 nM; d, 11.0 nM; e, 50.0 nM. Equilibrium conditions were established before the recording of each spectrum.

in an identical manner except that light was excluded from the reaction mixtures.

RESULTS

Scans of the CO-difference spectra of human placental microsomal pigments following additions of increasing concentrations of androstenedione (Fig. 1) or 19-norandrostenedione (Fig. 2) to sample cuvettes and NADPH to sample and reference cuvettes revealed that the effect of each steroid was specific for absorption at 450 nm. Additions of androstenedione consistently decreased the absorption at 450 nm relative to all other wavelengths in the spectrum and additions of 19-norandrostenedione resulted in a diametrically opposite effect. However, androstenedione produced its effects at considerable lower concentrations than 19-norandrostenedione. Titration of the opposing effects of the two steroids (Fig. 3) indicated that the effects could be balanced with androstenedione: 19-norandrostenedione concentration ratios of approximately 1:200. The ratio varied among microsomal preparations from different placentas and was as low as 1:20 in some suspensions and as high as 1:1000 in others. Reasons for this were not determined, but such variability is commonly observed in studies with placental tissues [6, 15].

Subsequent experiments with a series of steroids as well as with BP and N-2-fluorenylacetamide which are substrates for monooxygenases in placental microsomes [16], suggested (Table 1) that the capacity to displace CO from P-450_{hpm} was related not

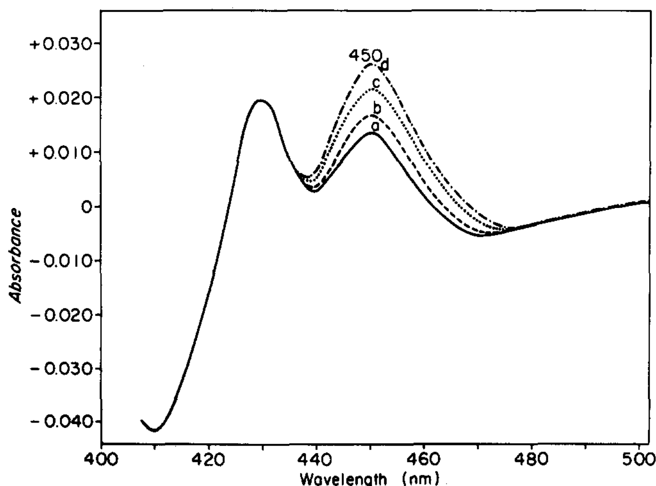

Fig. 2. Same as Fig. 1 except that 19-norandrostenedione was added to sample and reference cuvettes in increasing concentrations; a, 0; b, 0.1 μM; c, 1.0 μM; d, 0.1 mM. Equilibrium conditions were established before each recording. Higher concentrations produced no further changes. Protein concentration was 4 mg/ml.

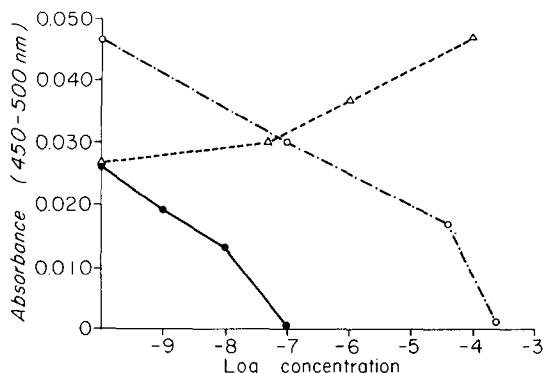

Fig. 3. Titration of the opposing spectral effects of androstenedione and 19-norandrostenedione. ——, androstenedione; - - - -, 19-norandrostenedione; -·-·-, androstenedione in the presence of 0.1 mM 19-norandrostenedione. Equilibrium conditions were established for each point. Steroids and CO were present in sample and reference cuvettes; 3 mM NADPH was present in the sample cuvette. Protein concentration was 8 mg/ml.

only to the presence of the angular methyl group at C-19 of steroid molecules but also to the substrates specificity of the aromatizing system. However, seven different 19-norsteroids exhibited the capacity to facilitate binding of CO to the cytochrome although 19-norandrostenedione displayed the greatest potency for this effect. Maximal increases in absorbance produced by 19-norandrostenedione also were 1.5–3 fold greater than those produced by other 19-norsteroids and were equivalent to those observed when steroids were absent and sodium hydrosulfite was employed as the reducing agent. It was of interest that each of the steroids that produced a measurable effect on the absorption of the CO-P-450_{hpm} complex (either increase or decrease) also exhibited measurable type I binding spectra in the same preparations.

The above results suggested that inclusion of 19-norandrostenedione in incubation flasks should allow CO to inhibit the conversion of androstenedione to estrone if P-450_{hpm} actually functioned in this reaction as previously suggested [4–6, 17]. Since 19-norandrostenedione itself is an inhibitor of the aromatizing reaction, it was necessary to examine the inhibitory effects of CO with several different concentration ratios of 19-norandrostenedione:androstenedione in the flasks. We obtained results that were very similar to those of previously described experiments [11], strongly supporting the hypothesis that P-450_{hpm} serves as the terminal oxidase for the placental conversion of androstenedione to estrone, that CO does not normally inhibit the reaction *in vitro* due to the displacement of CO from P-450_{hpm} by androstenedione and that 19-norandrostenedione antagonizes the effect of androstenedione with respect to CO and allows CO to complex with reduced P-450_{hpm} and inhibit the reaction.

As a further test of the hypothesis we examined the reversal of the CO-inhibition by monochromatic light at several wavelengths. The results of these experiments (Fig. 4) indicated that maximal reversal was obtainable with light at a wavelength of 450 nm, providing good evidence for the hypothesis. Since these and other experiments [6] suggested that the affinity of P-450_{hpm} for CO was low, we also investigated the inhibitory effect of CO with varying O_2:CO ratios (Table 2). Under the sets of reaction conditions employed, CO was a relatively ineffective inhibitor as compared with its inhibitory potency in hepatic and adrenal microsomal mixed-function oxidation

Table 1. Effect of steroids, benzo[a]pyrene and N-2-fluorenylacetamide on the binding of CO to NADPH-reduced P-450_{hpm}

Compound	Final molar concentration (μM)	Effect on absorption (450–490 nm)
Androstenedione	0.06	Decrease
19-Hydroxyandrostenedione	0.10	Decrease
19-Oxoandrostenedione	0.10	Decrease
Testosterone	0.50	Decrease
19-Norandrostenedione	30.0	Increase
19-Nortestosterone	100.0	Increase
Norethindrone	100.0	Increase
Norethynodrel	100.0	Increase
Norgestrel	100.0	Increase
Norethandrolone	100.0	Increase
Nandrolone Decanoate	100.0	Increase
1,4,6-Androstatrien-17β-hydroxy-3-one	100.0	No effect
16α-Hydroxytestosterone	100.0	No effect
Progesterone	100.0	No effect
Pregnenolonone	100.0	No effect
Deoxycorticosterone	100.0	No effect
Benzo[a]pyrene	100.0	No effect
N-2-Fluorenylacetamide	100.0	No effect

All compounds were tested in a concentration range of 10^{-8}–10^{-4} M. Concentrations listed in the table are those at which maximal effects were observed.

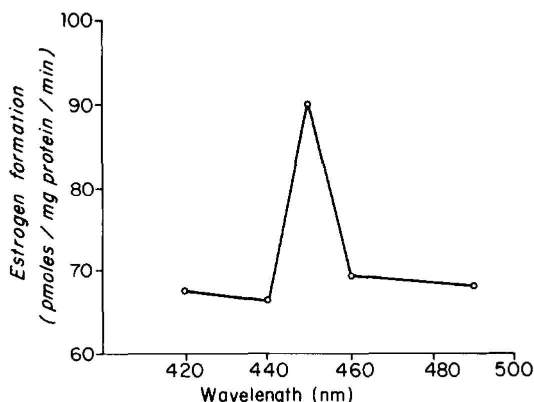

Fig. 4. Reversal of the inhibitory effect of CO on rates of conversion of androstenedione to estrogens in human placental microsomes with visible light. The concentration of 19-norandrostenedione in reaction flasks was 0.4 mM. $CO:O_2$ ratio was 19:1 (95% CO; 5% O_2). Other details are as described in Experimental Procedures.

reactions. The results also indicated that the reaction proceeded only 43% as rapidly in 20% O_2 as in 100% O_2 suggesting that the affinity of $P\text{-}450_{hpm}$ for O_2 also was quite low.

Previous experiments with CO-inhibition [9, 12] indicated that $P\text{-}450_{hpm}$ was functional in catalyzing the hydroxylation of BP via AHH in placental tissues. Assays of AHH activity under conditions in which the CO:O ratio was varied indicated an approximate 1:1 affinity of CO and O_2 for the mixed-function oxidase. This phenomenon was investigated further in order to ascertain possible relationships between the two microsomal hydroxylating systems. Additions of varying concentrations of either androstenedione or 19-norandrostenedione (10^{-4}–10^{-6} M, final concentrations) to the reaction flasks did not affect the capacity of CO to inhibit the AHH reaction, further suggesting that separate cytochromes functioned in the 3-hydroxylation of BP and the 19-hydroxylation of androstenedione. Additional support for the latter hypothesis was provided in experiments that demonstrated a lack of spectrally observable binding of BP or N-2-fluorenylacetamide to $P\text{-}450_{hpm}$ and lack of significant inhibition by BP of aromatization of androstenedione when added (10^{-4} M, final concentration) *in vitro*. Androstenedione and 19-norandrostenedione both inhibited the hydroxylation of BP but were approximately equally effective (50% inhibition at 5×10^{-4} M, final concentration) and were less effective than β-estradiol or estrone. The possibility that placental AHH activity was independent of $P\text{-}450_{hpm}$ was disproved when it was shown that maximal reversal of inhibition with CO occurred with monochromatic light at a wavelength of 450 nm (Fig. 5).

Table 2. Inhibition by CO of the conversion of androstenedione to estrone with varying concentrations of CO. Values represent means of three separate experiments with standard errors. Concentrations of androstenedione and 19-norandrostenedione in reaction flasks (including control flasks) were 2×10^{-4} M and 4×10^{-4} M respectively

Gas phase	% of control (100% O_2)	% inhibition by CO
20% O_2, 80% N_2	43 ± 5	—
20% O_2 80% CO	36 ± 3	27 ± 4
10% O_2, 90% N_2	39 ± 5	—
10% O_2, 90% CO	26 ± 7	32 ± 6
5% O_2, 95% N_2	29 ± 4	—
5% O_2, 95% CO	15 ± 3	47 ± 5

Measurements of O_2 tension (Clarke electrode) in optical cuvettes before and after bubbling CO through suspensions of placental microsomes for 1 min revealed that O_2 tensions were reduced by only 60–70% even when CO had passed through a deoxygenating system (see Experimental Procedures). Ratios of $CO:O_2$ in the cuvettes then were approximately 15:1. Experiments on the inhibition of aromatization with CO at varying $CO:O_2$ ratios (described above) suggested that at a 15:1 ratio only approximately one-half of the reduced $P\text{-}450_{hpm}$ molecules could be bound to CO (in the presence of concentrations of 19-norandrostenedione optimally effective for facilitation of CO-binding) while the other half would be bound to O_2. Spectral studies supported this idea since reduction with NADPH or NADH (which do not scavenge O_2) in the absence of steroids yielded CO-difference spectra with absorption maxima at 450 nm which were approximately half the intensity as those observed when sodium hydrosulfite (a very effective O_2 scavenger) was employed as the reducing agent. Significant differences in O_2 tension in microsomal suspensions containing androstenedione *vs* 19-norandrostenedione (10^{-4} M, final concentration) could not be detected within 30 s after the addition of the steroids. (The effect produced by androstenedione on the CO-difference spectrum is essentially immediate [10].) This result suggested that differences in effects on the CO-difference spectra could not be attributed to differential effects on O_2 uptake.

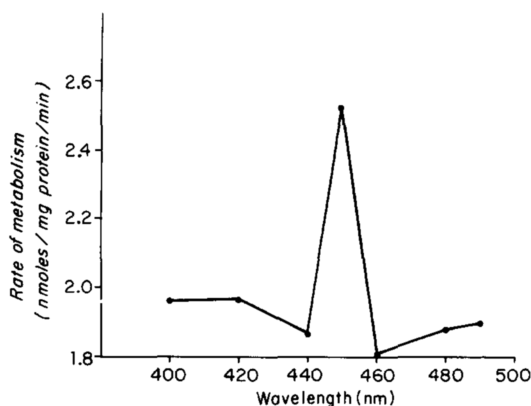

Fig. 5. Light reversal of the inhibitory effect of CO on rates of conversion of BP to hydroxylated products as determined fluorometrically (see Experimental Procedures). $CO:O_2$ ratio was 1:1 (50% CO; 50% O_2).

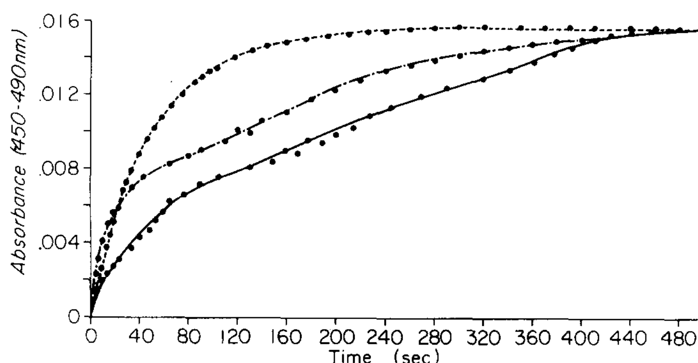

Fig. 6. Effects of androstenedione and 19-norandrostenedione on the rate of development of the absorbance difference (450–490 nm) in human placental microsomes reflecting the rate of formation of the CO-P-450_{hpm} complex. Sample and reference cuvettes both contained CO. Recordings were initiated immediately following the addition of excess sodium hydrosulfite to the sample cuvette. ——, 1.0 μM androstenedione; · · · · ·, 0.1 mM 19-norandrostenedione; –·–·–, no additional components. Protein concentrations were 4 mg/ml.

Measurements of the rates at which absorption at 450 nm increased following additions of excess sodium hydrosulfite to the cuvettes were made in the presence and absence of androstenedione or 19-norandrostenedione (Fig. 6). More rapid development of absorption in the presence of 19-norandrostenedione and less rapid development in the presence of androstenedione were observed (except for a very minor unexplained slower rate in the presence of the 19-norsteroid during the first 30 s). These experiments were consistent with the concepts that the rate of development of absorption at 450 nm of the CO-P-450_{hpm} complex was dependent upon the rate of binding of CO to the reduced cytochrome, that the rate of binding of CO is dependent on the O_2 concentration in the suspension, that the reduced, androstenedione-bound P-450_{hpm} has a higher relative affinity for O_2 than for CO as compared with the unbound form, and that the converse is true for reduced, 19-norandrostenedione-bound P-450_{hpm}.

Rates of NADPH oxidation by placental microsomes in the presence and absence of androstenedione or 19-norandrostenedione also were assessed with the DW-2 spectrophotometer utilizing dual-wavelength (340–395 nm) recording. In a typical experiment, the rate of NADPH (10^{-5} M, final concentration) oxidation in the absence of added steroids was 160 pmol/mg protein/min; in the presence of androstenedione (10^{-4} M, final concentration) the rate was 340 pmol/mg protein/min; and in the presence of 19-norandrostenedione (10^{-4} M, final concentration) the rate was 240 pmol/mg protein/min. No significant differences in rates were observed at NADPH concentrations of 3×10^{-5}–6×10^{-6} M (final concentration). Since the effects of the two steroids on CO-difference spectra were measured in the presence of 3×10^{-4} M NADPH, differential effects of the steroids on rates of NADPH oxidation could not be expected to account for the opposing effects of the steroids on the difference spectra. Previous studies [18] regarding other possible modes of NADPH degradation make it seem unlikely that differential effects of the steroids on other degradative pathways could play a role in the spectral effects.

Table 3. Inhibition of the conversion of androstenedione to estrone by various hemebinding ligands. All ligands were present in incubation flasks in final molar concentrations of 5×10^{-4} M. K_s values were determined by difference spectroscopy with the ferricytochrome. Androstenedione concentrations were 2×10^{-4} M in each experiment

Ligand	% of control	K_s value (M)
Nicotinamide	100	1.2×10^{-3}
Aniline	100	4.1×10^{-4}
Metyrapone	96	1.1×10^{-5}
Cyanophenylimidazole	84	9.8×10^{-7}
Aminoglutethimide	79	6.8×10^{-7}
Isopropylphenyl-imidazole	21	1.4×10^{-7}

In further experiments with several type II ligands it was observed that androstenedione and 19-norandrostenedione produced differential effects on binding spectra produced by aminoglutethimide, isopropylphenylimidazole, cyanophenylimidazole, nicotinamide, aniline and metyrapone in the presence and absence of reducing agents. Androstenedione, added to both sample and reference cuvettes, displaced these heme-binding ligands from P-450_{hpm}; whereas, 19-norandrostenedione facilitated their binding. At concentrations of androstenedione that displaced these type II ligands from binding, no inhibition of aromatization could be observed; however, at concentrations which did not displace the ligands, the compounds served as effective inhibitors or aromatization (Table 3). These results indicated that the effects of the steroids on ligand binding were independent of the redox state of the cytochrome and provided further evidence that steroids could displace a variety of heme-binding ligands from P-450_{hpm} by virtue of an allosteric effect produced as a result of type I binding of the steroid to the apoprotein portion of the

cytochrome. Supporting evidence for these concepts also has been provided by Bergheim *et al.*[15] who observed that metyrapone appeared to be displaced from binding to placental microsomal cytochrome P-450 by 17-α-hydroxypregnenolone. The opposing effects of androstenedione and 19-norandrostenedione on the binding of type II ligands to ferric P-450_{hpm} were concentration-dependent and were similar to those observed with binding of CO to the ferrous hemoprotein. The concentrations of the steroids required to produce the effects were related to the affinities of the ligands for P-450_{hpm} (Table 3). Highest concentrations were required for displacement of isopropylphenylimidazole and aminoglutethimide, both of which displayed K_s values of less than 10^{-6} M. No inhibition by ligands with low affinity (aniline, nicotinamide) could be observed and inhibition by ligands with intermediate affinities (metyrapone, cyanophenylimidazole) was observed only if the substrate concentrations were decreased.

DISCUSSION

The results obtained provide definitive evidence that P-450_{hpm} functions as terminal oxygenase for the mixed-function oxidative conversion of androstenedione to estrone in human placentas. There seems also little doubt that a placental P-450 is responsible for the hydroxylation of BP *via* AHH. Several lines of evidence suggest that two separate cytochromes function in these important reactions: Firstly, human placental AHH is highly inducible; specific activities are markedly increased in placentas from smokers [9, 19, 20]; whereas, the activity of the aromatizing system remains within the normal range in the same placentas [3, 21]. Differing sensitivities to inhibition by CO and estrogens, differing effects of 19-norandrostenedione on the sensitivity to inhibition by CO, lack of significant reciprocal competitive inhibition by respective substrates, lack of spectrally observable binding of BP to P-450_{hpm}, and lack of correlations between concentrations and/or spectral properties of P-450_{hpm} with placental AHH activities [3, 6, 18] also indicate the existence of two separate monooxygenase systems. Studies on binding [4–6] strongly suggest that spectrally observable P-450_{hpm} is associated primarily or exclusively with aromatization. The observation that androstenedione, testosterone, 19-hydroxyandrostenedione and 19-oxoandrostenedione (but not several other steroids) completely eliminate the absorbance of the CO-P-450_{hpm} complex [6, 10–12] also is supportive of this idea. Thus it would appear that only an extremely small or spectrally silent pool of placental P-450 functions in the hydroxylation of foreign hydrocarbons such as BP. This may be somewhat analogous to the situation in testicular mitochondria in which the oxidation reaction (side-chain oxidation of cholesterol) is much more readily verified than the presence of mitochondrial cytochrome P-450 [22].

Other studies on placental biochemical parameters provide suggestive evidence for still other forms of the cytochrome. Catalysis of N-hydroxylation of N-2-fluorenylacetamide in placental microsomes appears to occur independently of 3-hydroxylation of BP but is inhibited by CO [16]. Spectral binding studies [6] suggest that androstenedione either binds to two separate sites on the same cytochrome or to two separate cytochromes. Estradiol-17β produces type I binding spectra in some microsomal preparations but not in others [6] suggesting yet another binding site or cytochrome in those preparations. Final resolution of these questions will require rigorous purification and analyses of the cytochromes.

The previously reported lack of inhibition of the conversion of androstenedione to estrone [1, 5, 6, 8, 17] by CO seems best explicable in terms of the CO-displacing effect of androstenedione. Aromatization of 19-norsteroids [1] or of 16α-hy-droxytestosterone [23] are readily inhibited by CO but 19-norsteroids facillitate CO binding, and the latter steroid exhibited no effect (Table 1). Cases in which CO fails to inhibit the reactions involve substrates than can displace CO from binding at the concentrations utilized in the reaction systems. An alternative explanation offered by Thompson and Siiteri[5] seems somewhat less likely in light of results obtained in these experiments.

A possibility exists that, since 19-norandrostenedione inhibits the conversion of androstenedione to estrone, androstenedione could be converted to estrone *via* an alternate, CO-inhibited pathway in the presence of 19-norandrostenedione. Although no evidence is currently available to support this concept, the possibility cannot be ruled out at present. The binding specificity of the placental cytochrome [4–6] would tend to argue against such a concept since androstenedione and 19-norandrostenedione are both present in reaction flasks in high concentrations and bind with a very high affinity to the cytochrome. Under these conditions, aromatization *via* an alternative P-450-dependent pathway seems unlikely.

The mechanisms by which androstenedione and 19-norandrostenedione produce their respective effects on the CO-difference spectrum of P-450_{hpm} remain unresolved at present. From the data presented it would appear that the effect of androstenedione was not attributable to differential changes in steady-state levels of the ferrous cytochrome or to differential effects on NADPH degradation during the course of the spectral studies. The model employed by Griffin *et al.*[25] (in which camphor appears to physically displace metyrapone from binding) remains a possibility; but, if true, one must speculate that the angular 19-methyl group of androstenedione can displace CO without affecting the binding of O_2 since, otherwise, the mixed-function oxidation could not proceed. One could conceive of this possibility if CO and O_2 bound to the heme at different angles or, perhaps on opposite sides of the heme iron. Neverthe-

less, since O_2 and CO compete for binding to the iron of such hemoproteins, this idea appears to present some conceptual difficulties. It seems more reasonable to us to think in terms of an allosterically induced change in the electronic configuration at the heme site. The change, accordingly, would result in a relative increase in affinity for O_2 and a relative decrease in affinity for CO in the case of androstenedione binding. The effect produced by 19-norandrostenedione, on the other hand, could possibly be explained simply on the basis of its capacity to produce a type I difference spectrum. Compounds possessing this capacity also effect increased rates of reduction of the hepatic cytochrome [24] and may increase steady-state concentrations of the ferrous form, thereby allowing CO to complex with more cytochrome. The observation that the displacing effect of androstenedione is essentially immediate whereas several minutes are required for 19-norandrostenedione to exhibit its maximal effect [10] are consistent with these ideas. Nevertheless, considerable additional research will be required to fully elucidate the molecular mechanisms of these interesting steroidal actions.

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