DISPLACEMENT OF CARBON MONOXIDE FROM PLACENTAL CYTOCHROME P-450 BY STEROIDS: ANTAGONISTIC EFFECTS OF ANDROSTENEDIONE AND 19-NORANDROSTENEDIONE

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Summary: Displacement of bound carbon monoxide (CO) from NADPH- or NADH-reduced human placental microsomal cytochrome P-450 by androstenedione was reversed by 19-norandrostenedione or 19-nortestosterone. Conversely, the capacity of the 19-norsteroids to augment the formation of the CO-cytochrome complex was antagonized by androstenedione. The effects produced by each of the steroids investigated were independent of the sequence of additions of reduced pyridine nucleotides, steroids and CO, suggesting that the observed results were not due to effects on rates of enzymic reduction of cytochrome.

Introduction: The mixed-function oxidative conversion of androgenic C-19 steroids to estrogens is not inhibited by high concentrations of CO, even in the presence of limiting concentrations of O_2 and large excesses of NADPH (1,2). By contrast, aromatization of various 19-norsteroids is readily inhibited by CO (1). Several investigators (1-6) have considered the possibility that cytochrome P-450 may function as the terminal oxidase for either or both of these reactions. To date, the question is unresolved.

Reports from this laboratory (4,7,8) have indicated that androstenedione, testosterone and structurally similar C-19 steroids (but not 19-norsteroids) are capable of displacing bound CO from human placental microsomal cytochrome P-450 ($P\text{-}450_{hpm}$) thus providing a possible explanation for lack of inhibition by CO of aromatization of C-19 steroids. The displacement may occur directly as a result of allosterically induced electronic changes in the heme environment generated by the hydrophobic binding of steroid molecules to non-heme sites on the cytochrome or/and as a result of increased rates of reoxidation of the cytochrome. In this

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communication we wish to report that certain 19-norsteroids can facilitate the complexing of CO with NADPH-reduced P-450 $_{hpm}$ and, in addition, can prevent and even reverse the effects produced by C-19 steroidal androgens with respect to the capacity of the latter compounds to effect displacement of CO from ferrous $P\text{-}450_{hpm}$.

Materials and Methods: NADPH and NADH were obtained from Sigma Chemical Co., Rochester, N.Y. All steroids were obtained from Steraloids, Inc., Pawling, N.Y. Other reagents and chemicals utilized were of the highest quality commercially available.

Microsomal fractions were prepared from homogenates of human placentas delivered at term according to methods described previously (8) except that final protein concentrations of microsomal suspensions were adjusted to 8 mg/ml for dual wavelength recordings and 4 mg/ml for analysis of difference spectra in the split-beam mode. The final pH of all microsomal suspensions was 7.35.

All analyses were performed with a model DW-2 recording spectrophotometer (American Instrument Co.). The following parameters were utilized for recording in the dual wavelength mode: Temperature of the suspensions was maintained at 6°C; slit width was 8.0 nm; full-scale absorbance was 0.1; recording speed was 5 sec/inch; reference and test monochromators were set at 490 and 450 nm respectively. Samples to be analyzed were gassed with deoxygenated CO by bubbling through 2 ml of a microsomal suspension for 60 seconds in stoppered cuvettes. 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 0.1N NaOH. After verifying that no baseline changes were occurring, 10 µl of NADPH (or other reducing agents where indicated) were rapidly injected into the cuvette and recordings were initiated. For recordings of repetitive scans in the split beam mode, the temperature in the cuvette was 6°, slit width was 3.0 nm, full scale absorbance was 0.1 and the recorder speed was 10 nm/sec. Further details regarding methodology are given in the figure legends.

Results: The effects of increasing concentrations of androstenedione on

the absorbance at 450-490 nm on the CO difference spectrum measured as a function of time are illustrated in Fig. 1. Androstenedione effectively reduced the absorbance difference to zero at a concentration of 1.11×10^{-7}M. That the effect occurred specifically at 450 nm could be shown by examination of the difference spectrum (400-500 nm) as reported previously (8). Results presented in Fig. 1 suggested that the effect was independent of cytochrome P-450 reductase activity, since the decrease in absorption at 450 nm occurred immediately following addition of the steroid. Subsequent addition of 19-norandrostenedione to the cuvette resulted in increased absorbance at 450 nm relative to 490 nm. Maximal reversal of the effects of androstenedione was obtained with final concentrations of 10^{-4}M 19-norandrostenedione. The effects obtained were concentration-dependent and could be observed at androstenedione:19-norandrostenedione concentration ratios of 1:20.

Additions of 19-norandrostenedione to cuvettes containing placental micro-

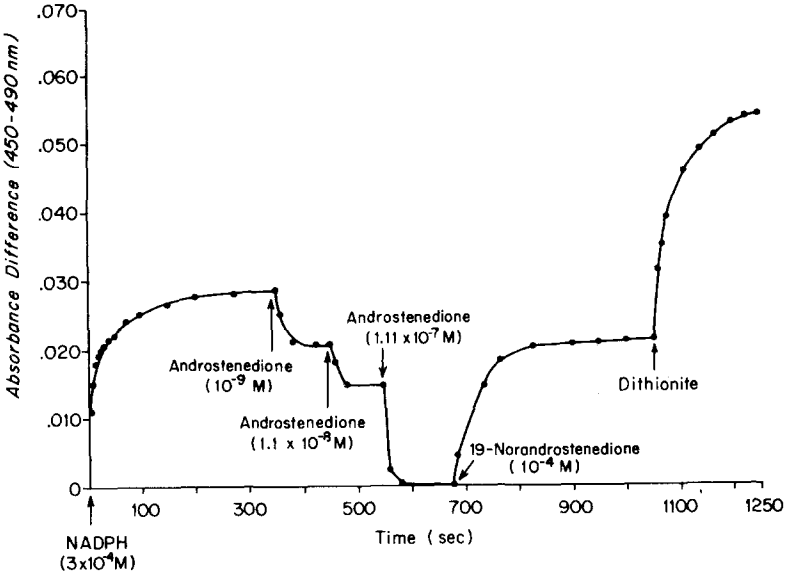

Fig. 1 Displacement of carbon monoxide from cytochrome P-450$_{hpm}$ by androstenedione and reversal by 19-norandrostenedione. Measurements were made in the dual-wavelength mode.

somes, NADPH or NADH and CO resulted in substantial increases in absorption at 450 nm relative to 490 nm (Fig. 2). This effect also could be reversed by the addition of androstenedione, although a very long period of time was required for the reversal to occur. Additions of 19-nortestosterone produced similar, although less profound, effects that also were more readily reversed (Fig. 3). The results observed following the additions of sodium hydrosulfite suggested that the steroids were affecting rates of reoxidation of the cytochrome -- androstenedione appeared to markedly accelerate, and 19-norsteroids to reduce, rates of reoxidation as compared to rates of reduction. In order to determine whether the changes in absorbance differences (450-490 nm) represented a specific increase at 450 nm, repetitive spectral scans were run utilizing 19-norandrostenedione as the 19-norsteroid. The results of this experiment are illustrated in Fig. 4. The spectrum was first allowed to stabilize (for 15 minutes) following additions of NADPH to the sample cuvette and CO to the sample and reference cuvettes. The steroid then was added rapidly to both cuvettes and scanning was begun. The re-

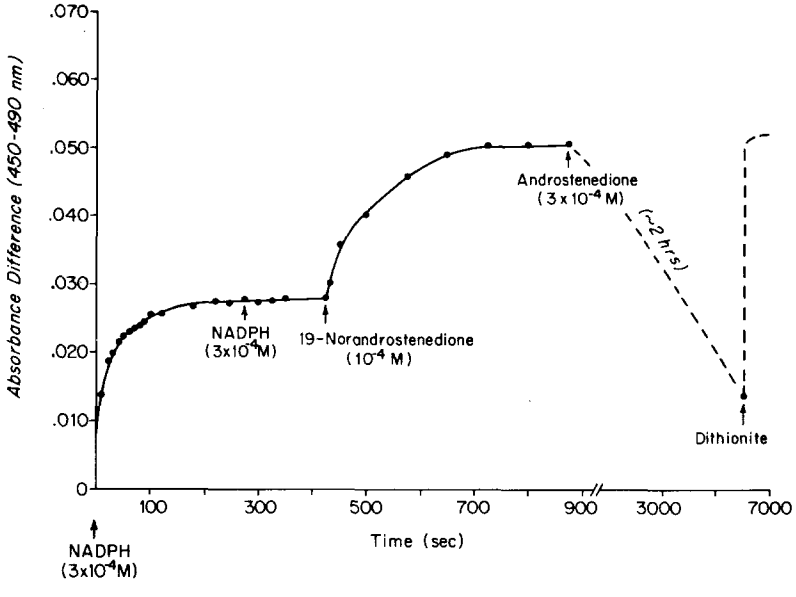

Fig. 2 Facilitation of formation of carbon monoxide-cytochrome P-450$_{hpm}$ complexes by 19-norandrostenedione and reversal by androstenedione.

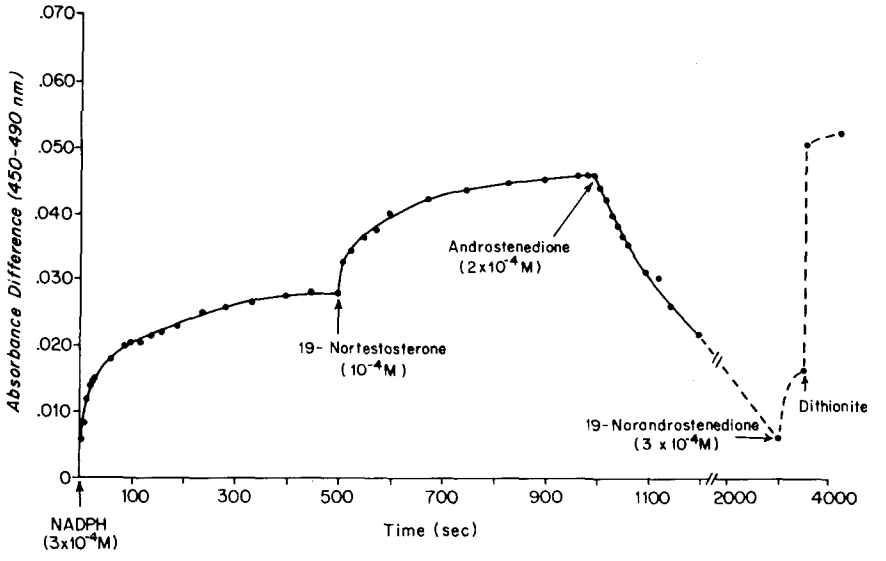

Fig. 3 Facilitation of formation of carbon monoxide-cytochrome P-450$_{hpm}$ complexes by 19-nortestosterone and reversal by androstenedione.

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sults demonstrated that the absorbance maximum at 450 nm was increasing relative to all other wavelengths in the spectrum and strongly indicated that the 19-norsteroid increased the binding of CO to the cytochrome. Similar results were obtained with 19-nortestosterone.

Discussion: Cytochrome P-450 is a collective name given to O_2-activating components of various monoxygenase systems and, like other oxygen binding hemoproteins, can attach ligand molecules at the sixth coordination position of the heme. From extensive studies on ligand binding to the sixth coordination site of hemoglobin, it is obvious that changes in the apoprotein part of the molecule can give rise to profound changes in the affinity of various heme-binding ligands for the sixth coordination site (9,10). Since androstenedione and related steroids bind to P-450$_{hpm}$ with a very high affinity and relatively high specificity (2,4, 7,11,12), it would seem probable that the capacity of androstenedione to prevent CO from binding should be related to its own ability to bind to the cytochrome. However, it is traditionally accepted (13) that if a compound can displace CO

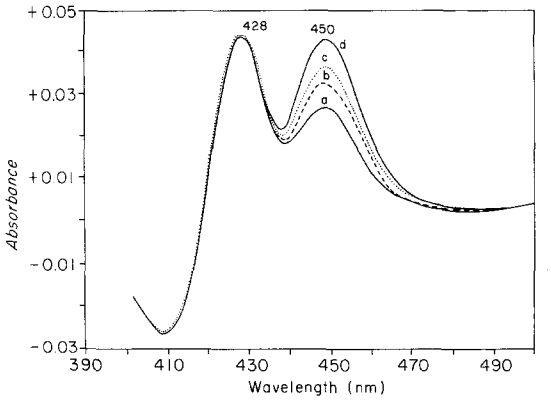

Fig. 4 Repetitive scans illustrating the effect of 19-norandrostenedione on the carbon monoxide difference spectrum. The sample was allowed to equilibrate for 15 minutes before the first scan.

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from the sixth coordination site, it must itself bind to the same site in order to do so. Nevertheless, the binding spectra produced by androstenedione are the classical "type I" spectra (2,4,7,11,12) which strongly suggest that the steroid binds to a hydrophobic site rather than the heme site. "Type I" binding also reportedly (14) facilitates P-450 reduction. Therefore, we suggest that the effect of androstenedione on binding of CO is due to an allosteric change in the apoprotein of $P\text{-}450_{hpm}$. This change, in turn, seems to increase rates of reoxidation of the cytochrome, thus resulting in a decreased steady-state concentration of the reduced form. The effect of the allosteric change upon the affinity of the reduced cytochrome for CO, however, is unknown.

An explanation for the effects of the 19-norsteroids is also difficult to formulate. These compounds also produce type I spectra when bound to $P\text{-}450_{hpm}$, albeit with lesser affinity and maximal spectral change elicitable (8,11). They reportedly compete with androstenedione with respect to binding as well as aromatization (2,15) suggesting further that these compounds bind to the same site on the cytochrome. Therefore, one must assume that the presence of the angular 19-methyl carbon on the steroid molecule can exert a critical effect on the type of allosteric change producible; i.e., its presence elicits a change which prevents complex for-

mation, and its absence is responsible for a change which promotes formation of the CO complex. Elucidation of the molecular changes occurring in conjunction with these observed events remains an exciting area of future research.

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