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# DE NOVO BIOSYNTHESIS OF 6β-HYDROPEROXYANDROSTENEDIONE IN HUMAN PLACENTAL MICROSOMES

L. Tan, P. Navennec and G. Gévry

Department of Biochemistry, University of Sherbrooke Medical Faculty, Sherbrooke, Que., J1H 5N4, Canada

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*Fresh human placental microsomes were incubated at 37° C with a homogeneous mixture of tritium labeled 4-androstene-3,17-dione and unlabeled 6β-hydroperoxy-androstenedione in the presence, or absence, of various inhibitors. In all cases, radioactive 6β-hydroperoxyandrostenedione could be trapped in amounts significantly different from carefully conducted controls. The radioactive purity of the hydroperoxide formed was established by repetitive HPLC purifications whereby a constant ratio of radioactive counts versus peak area was obtained. The amount of tritiated 6-bèta-hydroperoxyandrostenedione produced was dependent both on the incubation time and on the microsomal protein concentration used. It was also found that in the complete absence of air, the unlabeled hydroperoxide could act as oxygen donor, supporting the formation of radioactive, 6-oxygenated metabolites. These findings are fully consistent with the concept that 6-bèta-hydroperoxyandrostenedione acts as an endogenous precursor in the formation of the corresponding 6β-hydroxy and 6-oxo androstene metabolites in the human placenta; a fact not previously recognized.*

The concept that peroxy or hydroperoxy derivatives of steroids can act as precursors in the biosynthesis of their hydroxy and oxo counterparts has perhaps not received proper attention in the past. Whereas in the field of prostaglandins and leucotrienes, the formation of dioxygenated intermediates and endproducts is now well-established and documented (1-3), the same can not be said for steroids. Grant (4) was in all likelyhood the first author who suggested that an 11β-hydroperoxy compound may precede the formation of the corresponding 11β-hydroxy steroid. More than a decade later Charney and Herzog (5) proposed 17α-OOH-progesterone[1] as a potential biological intermediate. However, these theoretical hypotheses, although highly original in their time, have not found general acceptance for lack of experimental proof. Recently, we have proposed in rat liver the existence of

[1] Abbreviations: 4A, 4-androstene-3,17-dione; OOH, hydroperoxy; OH, hydroxy; HPLC, high-performance liquid chromatography.

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a new pathway for the formation of 6β-hydroxy-, and 6-oxoprogesterone via the 6 β-hydroperoxide as a common intermediate (6). In this communication we present concrete experimental evidence of the de novo synthesis of radioactive 6 β -hydroperoxyandrostenedione in human placental microsomes, accompanied by formation of the corresponding 6 β -hydroxy and 6-oxo metabolites. The presence of steroidhydroperoxides in the placenta has not been reported previously.

## EXPERIMENTAL PROCEDURES

Materials. [1,2-$^3$H]4-Androstene-3,17-dione (spec. activity 41 Ci/mmol) was purchased from New England Nuclear Corp. It was purified by HPLC prior to use. Reference 4A, 6β-OH-4A and 6-oxo-4A were from Steraloids, Inc. 6 β-OOH-4A was synthesized and separated from its 6α-epimer as described earlier (7). The chemical purity of these steroids was verified by HPLC. Glucose-6-phosphate, G-6-P dehydrogenase and $NADP^+$ were from Sigma Chemical Co. All organic solvents used were freshly distilled.

Radioactivity measurements. Aliquots of the collected HPLC-fractions were mixed with a "home-made" toluene-based scintillation solution in polypropylene minivials and counted using a Beckman model LS-8000 instrument. Counting efficiency for tritium was found to be in the order of 38%.

High-Performance Liquid Chromatography. This was carried out with a system consisting of an Altex model 110 solvent delivery pump, a Chromatronix double wavelength UV-detector set at 254 nm, a Perkin-Elmer septum injector, a LKB fraction collector, a Houston Instruments potentiometric recorder, and an Alltech-CN 10 micron analytical column. The mobile phase used was a 9:1 mixture of heptane/isopropanol (HPLC-grade). Actual operating parameters are described in the legends.

Preparation of human placental microsomes. A typical batch was prepared as follows. In the cold-room, a fresh placenta, delivered at term in the University clinic, was trimmed free of umbellical cord and fetal membranes. The remaining tissue was minced, washed several times with ice-cold 1% KCl solution (pH 7.4) until the wash was no longer colored. The minced pieces were filtered over cheesecloth, their wet weight determined, and then suspended in 1.5 parts of 0.25M sucrose of pH 7.4. The suspension was cooled in an ice-bath and treated with a Polytron homogenizer, applying 10 second bursts at maximum energy 8-10 times with a 2 minute cooling period between each burst. The homogenate was centrifuged at 10,000 g for 20 minutes in a Sorvall model RC2-B instrument at 4°C. The supernatant was removed and spun at 105,000 g for 60 minutes in a Beckman L5-50 ultracentrifuge. The pellet obtained was rehomogenized in 0.05M K-phosphate buffer, pH 7.4 using a Potter-Elvehjem apparatus, and the suspension centrifuged again at the same speed for another hour. These washings were repeated once more. The final pellet was suspended in 0.05M K-phosphate buffer solution containing 20% glycerol, 1 mM DTT, 10 mM β-mercaptoethylamine.HCl, and 1.0 mM EDTA. If not immediately used for the incubations, the microsomal preparation was stored at -60° C in a polyethylene capped tube, flushed with nitrogen.

Measurement of Cytochrome P-450. Each microsomal batch was verified prior to use for its Cytochrome P-450 content according to Omura and Sato (8) by using as extinction coefficient the value of 91/cm/mM. Protein concentration was determined using bovine serum albumin as standard (9). In this way we have obtained consistently Cytochrome P-450 concentrations in the range of 16-25 pmol/mg protein.

Isotope trappings of 6 β-OOH-androstenedione. All incubations were conducted in duplicate, including the controls. The microsomal suspension was distributed evenly in the appropriate number of incubation test tubes. To each tube was added 0.5 ml of a NADPH-generating system consisting of 4 mg $NADP^+$, 6 mg of G-6-P, 2 units G-6-P dehydrogenase, and 1 mg $MgCl_2$ in the presence, or absence of inhibitors. The total volume in each tube was adjusted to 1.5 ml with K-phosphate buffer (pH 7.4). The reaction was then started by adding into each incubation tube a homogeneous mixture in ethanol of 1 microCi [$^3$H]4A and 1.5 mg of unlabeled 6β-OOH-4A. The control tubes contained the same ingredients, except that the NADPH-generating system was omitted. After 40 minutes incubation at 37°C with gentle shaking and under an atmosphere of 95% air, 5% $CO_2$, the tubes were cooled in an ice-bath, and the reaction terminated by addition of 2ml ice-cold acetone into each tube. After settling for 30 minutes, the precipitated proteins were centrifuged off. The aqueous supernatant was removed and extracted three times with 1 ml chloroform. The organic phases were pooled, washed, dried over anhydrous sodium sulfate, filtered, and evaporated overnight in a vacuum-oven at room temperature. The residue was dissolved in 100 microL dioxane and further purified by HPLC.

Incubations under exclusion of oxygen. This experiment was performed by connecting 4 incubation tubes to a high-vacuum transfer line we have constructed earlier (10). To each test tube was added 0.85 ml of a NADPH-generating system and 2.0 ml of fresh microsomal preparation (5.74 mg protein). After repeated evacuations, each time followed by the introduction of Argon, the enzymic reaction was started by the addition of 0.15 ml of a homogeneous mixture of 2.58 microCi [$^3$H]4A and 1.25 mg unlabeled 6β-OOH-4A to give a total incubation volume of 3.0 ml. The 2 control tubes contained the same components, except the unlabeled hydroperoxy steroid. Throughout the incubation, conducted at 37°C, a slightly positive Argon pressure was maintained over each tube. After 40 minutes, the reaction was terminated and the products extracted and purified as described in the previous section.

## RESULTS AND DISCUSSION

An absolute prerequisite for the isolation and identification of the various steroids generated in this study must be based on the development of a mobile phase for HPLC that would provide at least baseline resolution. Our previous experience has indicated that steroidhydroperoxides undergo intramolecular decomposition in aqueous medium when exposed to prolonged contact with an active surface. We have therefore opted for HPLC-analysis using a moderately polar cyanopropylsilyl-column and a nonprotic, isocratic mobile phase. Under these conditions, complete resolution of androstenedione and its 6-oxygenated metabolites was readily achieved (see Fig. 1).

The radioactive peaks corresponding to the retention times of authentic reference steroids were collected, dried in vacuum, and rechromatographed under the same conditions. The radioactive identity and homogeneity of each peak was thus established by repeating the HPLC-purification procedure until a constant ratio of radioactivity versus peak area was obtained, as shown in

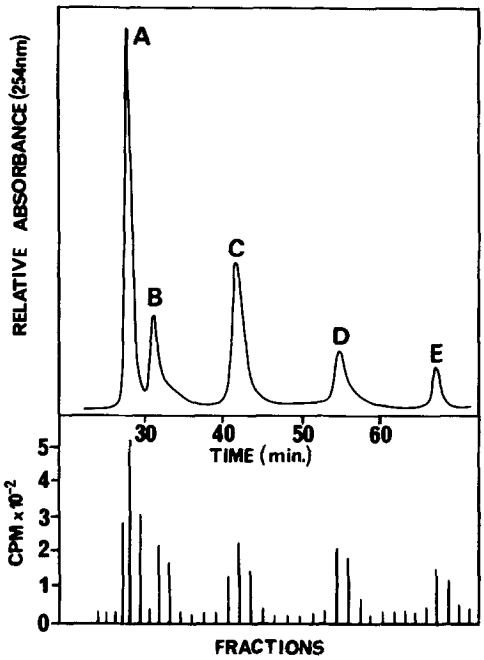


Fig.1. RADIOCHROMATOGRAM OBTAINED BY HPLC.
Freshly prepared placental microsomes were incubated at 37 °C with a mixture of [1,2-$^3$H]4-androstene-3,17-dione and unlabeled 6β-OOH-androstenedione for 40 min. After extraction with chloroform and drying, the crude reaction mixture was dissolved in dioxane and injected into an Alltech-CN analytical column using a 10% iso-propanol solution in heptane as the mobile phase. The flowrate was 0.5 ml/min. One-minute fractions were collected and aliquots thereof counted. Peak A=Androstenedione; B=6β-OH-4A; C=6β-OOH-4A; D=6-oxo-4A; E=6α-OH-4A. The net radioactive counts are the average of duplicate incubations.

Table I. The chemical identity of each peak was also ascertained by superimposing their IR-spectra over those of the corresponding reference steroid. The formation of radioactive 6 α-hydroxy-4A is noteworthy. This compound could result, either from the direct action of a 6 α-hydroxylase

TABLE I. PROOF OF RADIOACTIVE PURITY OF THE 6-OXYGENATED PRODUCTS

| | 6β-OOH-4A | | | 6β-OH-4A | | | 6-oxo-4A | | |
|---|---|---|---|---|---|---|---|---|---|
| HPLC-run# | area* | cpm | %area/cpm | area* | cpm | %area/cpm | area* | cpm | %area/cpm |
| 1 | 106 | 365 | 0.29 | 560 | 520 | 1.08 | 653 | 851 | 0.77 |
| 2 | 250 | 925 | 0.27 | 276 | 303 | 0.91 | 259 | 520 | 0.50 |
| 3 | 610 | 2875 | 0.22 | 72.2 | 234 | 0.31 | 122 | 250 | 0.49 |
| 4 | 270 | 1060 | 0.25 | 58.1 | 188 | 0.31 | | | |

Each radioactive peak collected was subjected to repetitive isocratic HPLC on an Alltech-CN column with isopropanol/heptane (1:9) as eluant at a flowrate of 0.9 ml/min. *Peak areas measured by triangulation and expressed in mm$^2$.

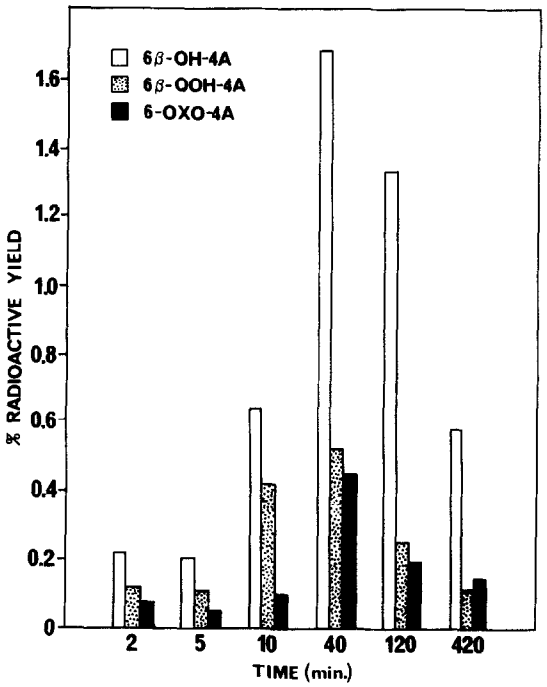


Fig.2. TIME-COURSE ANALYSIS OF THE TRITIATED METABOLITES AS RESOLVED BY HPLC. The incubations were conducted in duplicate with 15.8 mg microsomal protein, 1.03 μCi [$^3$H]-4A, 1.25 mg unlabeled 6β-OOH-4A, and a NADPH-generating system at 37°C with gentle shaking. The reaction was terminated after 2, 5, 10, 40, 120, and 420 min. The products were extracted and analyzed by HPLC as in Fig.1. Controls contained boiled microsomes.

enzyme in the placenta, or more likely from stereospecific reduction of the tritiated 6-ketoandrostenedione metabolite formed, thereby generating the thermodynamically more stable equatorial epimer at C-6. The presence of steroid oxido-reductases in placental microsomes is well-known (11,12). On the other hand, 6β-hydroxy-, and 6-ketoandrostenedione may be viewed as secondary metabolites having as a common precursor the 6β-hydroperoxy steroid. In fact, when we studied the formation of the latter 3 steroids as a function of time, we obtained the time-course curve as diagrammed in Fig. 2. The highest yields for all 3 steroids were obtained after 40 minutes of incubation; the 6β-alcohol being the most abundant product at the times measured. It should be noted that after 7 hours exposure to the microsomal preparation, the very reactive hydroperoxy steroid was still present, albeit in the lowest amount. Not surprisingly, up to 120 minutes of incubation, the 6-bèta-hydroperoxide was formed in greater quantity than the 6-ketone,

supporting our contention of a precursor-product relationship between these two steroids. We would like to point out that steroid hydroperoxides can spontaneously undergo chemical, i.e. non-enzymic, dehydration to the corresponding ketone (13). On the other hand, stereospecific reduction of the hydroperoxy intermediate to the axial, 6β-alcohol, the most abundant product formed in this study, may be due to an enzyme-dependent reaction. Of critical importance is the question of whether the conversion of [$^3$H]4-A to 6β-OOH-4A is enzyme-catalyzed or not. Because, as in all studies of this nature, the formation of artifacts due to chemical autoxidation can not technically be prevented, we have simultaneously carried out control incubations with heat-inactivated microsomes to take this fact into account. Hence, all data published in this paper are net values obtained by substraction of the initially recovered radioactive counts with the amounts caused by non-enzymic events. Furthermore, if the radioactive 6β-OOH-4A formed is of true enzymic origin, then it should be possible to decrease its biosynthesis through the action of certain enzyme inhibitors. That this turns out to be indeed the case is shown in Table II. Clearly its formation is dependent not only on the presence of molecular oxygen, but also on that of NADPH. Carbonmonoxide inhibits hydroperoxide synthesis 85% when compared to the uninhibited control. p-Hydroxymercuribenzoate, a potent inhibitor of enzymes containing free -SH groups, shows 80% inhibition. The very different values obtained under identical experimental conditions for 6-alpha-OH-4A

TABLE II. EFFECT OF INHIBITORS ON FORMATION OF 6-OXYGENATED ANDROSTENES

| | % Net radioactive yield* | | | | |
|---|---|---|---|---|---|
| Inhibitor | 6β-OOH-4A | 6β-OH-4A | 6α-OH-4A | 6-oxo-4A | Sum of 4 |
| None | 0.35(100) | 0.16(100) | 0.29(100) | 0.23(100) | 1.06(100) |
| 4 mM pHMB | 0.07(20.0) | 0.07(43.8) | 0.36(124) | 0.0 (0.0) | 0.50(47.2) |
| N atmosphere | 0.07(20.0) | 0.18(113) | 0.33(114) | 0.14(60.9) | 0.72(66.0) |
| Carbon monoxide | 0.05(14.3) | 0.11(68.8) | 0.22(75.9) | 0.01(4.4) | 0.39(36.8) |
| No NADPH | 0.03(8.6) | 0.0 (0.0) | 0.13(44.8) | 0.0(0.0) | 0.16(15.1) |

Fresh microsomes (19.5 mg/ml) were incubated with 1 μCi [$^3$H]4A and 1.5 mg unlabeled 6β-OOH-4A with, or without inhibitors added as indicated. After 40 min at 37°C, the reaction was stopped and the products extracted and analyzed by HPLC. Values in parenthesis are yields relative to the non-inhibited reaction. *Values substracted from the boiled control samples.

TABLE III. FORMATION OF [$^3$H]6β-HYDROPEROXYANDROSTENEDIONE RELATIVE TO PROTEIN CONCENTRATION

| Protein conc. (mg/ml) | % Total radioactive yield 6β-OOH-4A | Sum of 4* |
|---|---|---|
| 0.2 | 0.19 | 0.88 |
| 1.0 | 0.53 | 2.22 |
| 2.0 | 0.73 | 3.06 |
| 5.0 | 0.74 | 3.10 |

The incubation medium contained 1.0 μCi [$^3$H]Androstenedione, a NADPH-generating system, and the indicated amounts of microsomal protein. After 40 min at 37°C, the reaction was terminated, and the products analyzed by HPLC. *Incl. 6β-OH-4A, 6α-OH-4A, and 6-oxo-4A.

support our interpretation of its formation via the 6-keto steroid and catalysis by a non-oxygenase type enzyme. Table III shows a distinct correlation between 6-beta-OOH-4A synthesis and the microsomal protein concentration present in the incubation medium; again favoring the concept of an enzymic hydroperoxidation reaction. We next studied the possibility of 6 β-OOH-4A functioning not only as a substrate, but also as a potential oxygen donor under oxygen-deprived conditions. Other hydroperoxide compounds have been described earlier by several workers to be able to support biological oxidation (14,15). Table IV summarizes the results we have obtained when radioactive androstenedione was incubated under positive Argon atmosphere in the presence, or absence, of excess unlabeled 6β-OOH-4A. The significant difference of 6-oxygenated androgens formed under these two conditions, clearly indicate that the hydroperoxide can indeed replace molecular oxygen in biological oxidation reactions.

The presence of 6β-hydroxy-, and 6-ketosteroids in the human placenta (16,17), and other tissues, such as rat liver (18), adrenals (19), and fetal

TABLE IV. 6β-OOH-ANDROSTENEDIONE AS OXYGEN DONOR

| Substrate [$^3$H]4A | Total radioactive counts in cpm* (% yield in parentheses) 6β-OOH-4A | 6β-OH-4A | 6α-OH-4A | 6-oxo-4A |
|---|---|---|---|---|
| with 6β-OOH-4A | 9523 (0.21) | 14693 (0.32) | 5648 (0.12) | 4783 (0.10) |
| without 6-OOH-4A | 5355 (0.11) | 6740 (0.15) | 8280 (0.18) | 2049 (0.04) |

2.58 μCi [$^3$H]4A, 5.74 mg microsomal protein, 1.25 mg unlabeled 6β-OOH-4A, and a NADPH-generating system were incubated for 40 min at 37°C under 100% Argon atmosphere. The controls contained the identical components, except that no "cold" 6β-OOH-4A was added. The extracts were analyzed by HPLC. *Average of duplicate incubations.

blood cells (20) has been reported. They are traditionally described as products of metabolism whereby hydroxylation is viewed only as a way of rendering the parent molecule more hydrophilic, facilitating its subsequent excretion in the form of conjugates or sulfates. However, the question of whether some C-6 oxygenated steroids may exert any specific physiological function of their own is far from being settled. For instance, we have recently found that 6 β-OOH-androstenedione competitively inhibit the activity of estrogen synthetase, the major steroidal enzyme of human placental microsomes (7). As a consequence, it became imperative to establish whether we are dealing in this instance with a xenobiotic substance, or on the contrary, with an endogenous compound. Based upon the following experimental results and criteria: (a) stereospecific introduction of molecular oxygen at the sterically hindered, 6β-position, (b) dependency on the presence of NADPH, (c) the decreasing effect on its formation when inhibitors are present, and finally (d) its time-, and protein-concentration dependency, we conclude that the 6β-hydroperoxyandrostenedione we have isolated in this work, is indeed of enzymic origin and that it can therefore be regarded as an endogenous placental steroid. This means that the earlier proposed concept of the existence of steroid-oxidizing enzymes in mammalian tissues, other than mono-oxygenases of the Cytochrome P-450 type, can now no longer be ignored.

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