J. steroid Biochem. Vol. 17. pp. 61 to 65. 1982
Printed in Great Britain.

0022-4731/82/010061-05$03.00/0

METABOLISM OF ANDROSTENEDIONE BY GUINEA-PIG PERITONEAL MACROPHAGES: SYNTHESIS OF TESTOSTERONE AND 5α-REDUCED METABOLITES

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(*Received* 27 *October* 1981)

SUMMARY

Androstenedione metabolizing enzymes present in guinea-pig peritoneal macrophages were investigated using tritium-labeled androstenedione as the substrate. We found that the metabolites of [^{3}H]-androstenedione produced by these macrophages were testosterone, 5α-androstane-3,17-dione, isoandrosterone, androsterone, 5α-dihydrotestosterone, 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol. The rates of metabolite formation remained linear as a function of time of incubation for approximately 30 min and with macrophage number up to 2×10^7 cells per ml. The formation of these metabolites is indicative that the following androstenedione metabolizing enzymes are present in guinea-pig peritoneal macrophages: 5α-reductase, 3α-hydroxysteroid oxidoreductase, 3β-hydroxysteroid oxidoreductase and 17β-hydroxysteroid oxidoreductase. It is possible, therefore, that the macrophage, *in vivo*, may play a role in the metabolism of blood-borne androstenedione to potent androgens. These hormones are important in the regulation of many biological processes, possibly including the activity of the macrophage itself.

INTRODUCTION

Macrophages perform a multitude of important functions in addition to those involved in the process of scavenging and disposing of senescent cells and foreign matter. Macrophages are essential cells for most immune responses probably because of their unique capacity to present antigen in an appropriate form to lymphocytes, and thereby are important cells in the pathogenesis of immune diseases. Moreover, macrophages can synthesize components of complement, interferon and numerous other biologically active substances, including a wide array of enzymes.

Although much is known about the macrophage and its functions, many aspects of its physiology remain unresolved, among them the steroid metabolizing enzymes present in these cells. Therefore, we initiated studies in this regard by investigating the capacity of guinea-pig peritoneal macrophages to metabolize tritium-labeled androstenedione *in vitro*. We choose to study the metabolism of this steroid by macrophages because it is known that, in extraglandular tissues, blood-borne androstenedione serves as a prehormone in the formation of androgens.

Supported, in part, by USPHS grants AG00306, AI-11851 and AM27257.

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EXPERIMENTAL

Steroid sources

[1,2,6,7-^{3}H]-Androstenedione (SA 85 Ci/mmol), [4-^{14}C]-5α-dihydrotestosterone, [4-^{14}C]-androstenedione and [4-^{14}C]-testosterone, (SA 50 mCi/mmol) were purchased from New England Nuclear. The steroids were purified by column chromatography on celite–ethylene glycol before use [1]. The following carbon-14-labeled steroids, with a specific activity of 50 mCi/mmol, were synthesized: [4-^{14}C]-5α-androstane-3,17-dione, [4-^{14}C]-androsterone, [4-^{14}C]-isoandrosterone, [4-^{14}C]-5α-androstane-3α,17β-diol and [4-^{14}C]-5α-androstane-3β,17β-diol [2]. Nonradioactive steroids were obtained from Steraloids. Silica gel (Polygram Sil G-HY) and aluminum oxide F_{254} (Merck, Type E) precoated thin-layer chromatographic plastic sheets were purchased from Brinkmann Instruments. Primuline was purchased from K & K Laboratories.

Guinea-pig peritoneal macrophages

Macrophages were obtained from peritoneal exudates induced by mineral oil in guinea-pigs [3]. In brief, sterile mineral oil (Humble Oil, Inc., 25 ml) was injected intraperitoneally into anesthesized female Hartley guinea-pigs and 4 days later the peritoneal cavities were washed out with 100 ml of physiologic saline solution; the macrophage suspensions in oil–

water were centrifuged at 300 *g* for 10 min. This washing process was repeated twice. The experiments reported here were performed using a preparation containing 95% macrophages.

Incubation procedure

Aliquots of macrophage-enriched preparations (95%) were transferred to 16 × 100 mm culture tubes and centrifuged at 300 *g* for 10 min. The supernatant fluids were discarded and the cells were resuspended in 0.5 ml of freshly prepared Eagle's minimum essential medium (MEM) containing [1,2,6,7-^{3}H]-androstenedione (0.5 μM, 21 μCi) and incubated at 37°C for varying periods of time in a humidified atmosphere of air (95%) and CO_2 (5%); in addition incubations were conducted with various numbers of cells. The reactions were terminated by chilling and by the addition of chloroform–methanol (2:1, v/v, 2 ml) followed by addition of chloroform (6 ml) and water (1 ml). After shaking for 10 min, the chloroform layer was removed and the aqueous tissue layer was reextracted as above. Control incubations, i.e. those containing [^{3}H]-androstenedione in Eagle's MEM as above but no cells, were processed in a similar manner.

Androgen metabolites: isolation and quantification

The metabolites obtained in a 4 h incubation of guinea-pig peritoneal macrophages (5×10^7) with [1,2,6,7-^{3}H]-androstenedione (0.5 μM, 21 μCi) were separated partially by column chromatography on celite–ethylene glycol [1]. The peaks of radioactivity corresponding to the metabolites and substrate were mixed with corresponding authentic steroid carriers (100 μg each) and with carbon-14-labeled steroids: [4-^{14}C]-5α-androstane-3,17-dione (1,400 c.p.m.), [4-^{14}C]-androstenedione (10,000 c.p.m.), [4-^{14}C]-androsterone (940 c.p.m.), [4-^{14}C]-5α-dihydrotestosterone (2,100 c.p.m.), [4-^{14}C]-isoandrosterone (1,500 c.p.m.), [4-^{14}C]-testosterone (4,200 c.p.m.), [4-^{14}C]-5α-androstane-3α,17β-diol (1,200 c.p.m.) and [4-^{14}C]-5α-androstane-3β,17β-diol (1,200 c.p.m.).

The metabolites were purified by consecutive TLC on either Polygram Sil G-HY or alumina plates (when indicated in the text), both as the free steroids and after acetylation, until the ^{3}H:^{14}C ratios were constant. The solvent systems used were as follows: for [^{3}H]-5α-androstane-3,17-dione: TLC I, benzene–ethyl acetate (9:1, v/v, 3 ascents); TLC II, benzene–ethyl ether (9:1, v/v, 3 ascents); TLC III, chloroform–methanol (99.3:0.7, v/v, 10 ascents); TLC IV, methylene chloride–ethyl acetate (99:1, v/v, 3 ascents). For [^{3}H]-androstenedione plus [^{3}H]-androsterone: TLC I, methylene chloride–ethyl acetate–methanol (85:15:1, by vol., 1 ascent). For [^{3}H]-androsterone, TLC II (alumina), benzene–ethanol (96:4, v/v, 2 ascents); TLC III (alumina), benzene–ethanol (98:2, v/v, 1 ascent); TLC IV, chloroform–methanol (99.3:0.7, v/v, 10 ascents). For [^{3}H]-androsterone acetate, TLC V, methylene chloride–ethyl acetate (99:1, v/v, 3 ascents). For [^{3}H]-isoandrosterone and [^{3}H]-5α-dihydrotestosterone, TLC I (alumina), benzene–ethanol (96:2, v/v, 1 ascent); TLC II (alumina), benzene–ethanol (96:4, v/v, 2 ascents); TLC III (alumina), benzene–ethanol (98:2, v/v, 1 ascent); TLC IV, chloroform–methanol (99.3:0.7, v/v, 10 ascents). For [^{3}H]-isoandrosterone acetate and [^{3}H]-5α-dihydrotestosterone acetate, TLC V, methylene chloride–ethyl acetate (99:1, v/v, 3 ascents). For [^{3}H]-testosterone, TLC I, methylene chloride–ethyl acetate–methanol (85:15:1, by vol. 1 ascent); TLC II, ethyl acetate–isooctane (1:1, v/v, 1 ascent); TLC III, chloroform–methanol (99.3:0.7, v/v, 10 ascents). For [^{3}H]-testosterone acetate, TLC IV, methylene chloride–ethyl acetate (99:1, v/v, 3 ascents). For [^{3}H]-5α-androstane-3α,17β-diol and [^{3}H]-5α-androstane-3β,17β-diol, TLC I (alumina), benzene–ethanol (96:4, v/v, 2 ascents); TLC II (alumina), chloroform–ethanol (99:1, v/v, 1 ascent); TLC III (alumina), benzene–ethanol (98:2, v/v, 3 ascents); TLC IV, chloroform–methanol (99.3:0.7, v/v, 10 ascents). For [^{3}H]-5α-androstane-3α,17β-diol diacetate and [^{3}H]-5α-androstane-3β,17β-diol diacetate, TLC V, methylene chloride–ethyl acetate (99:1, v/v, 3 ascents). Steroids were localized on the silica gel plates by the use of a water spray and on the alumina plates by the use of a primuline spray [primuline (5 mg) in acetone–water (1 liter, 2:1, v/v)] followed by drying and visualizing under ultraviolet light. After the ^{3}H:^{14}C ratios were constant, the purified metabolites or corresponding acetates were mixed with authentic steroids (40 mg) and crystallized five times. The rates of formation of the metabolites were computed from the ^{3}H:^{14}C ratios as follows:

$$\frac{[\text{metabolite } ^3\text{H}:^{14}\text{C ratio}] \times [^{14}\text{C-internal recovery standard, d.p.m.}]}{[\text{substrate SA d.p.m./pmol}] \times [\text{cell number}] \times [\text{incubation time, h}]} = \frac{\text{pmol}}{\text{cell number} \times \text{h}}.$$

After the identification of the metabolites of [^{3}H]-androstenedione produced by guinea pig macrophages, a more direct approach was used for their quantification as follows. At the end of the incubation periods, aliquots of the chloroform extracts containing approximately 100,000 c.p.m. were set aside for TLC analysis, to ascertain that depletion of substrate did not occur in the course of the incubations and to quantify the C_{19}-steroid metabolites formed; these aliquots were mixed with the following authentic carrier steroids (25 μg each): 5α-androstane-3,17-dione, androstenedione, androsterone, isoandrosterone, 5α-dihydrotestosterone, testosterone, 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol. The mixtures were taken to dryness with a stream of nitrogen, the residues were redissolved in ethyl acetate (50 μl), and the samples were spotted on Polygram Sil G-HY sheets. The chromatograms were developed using the solvent system methylene chloride–ethyl acetate–

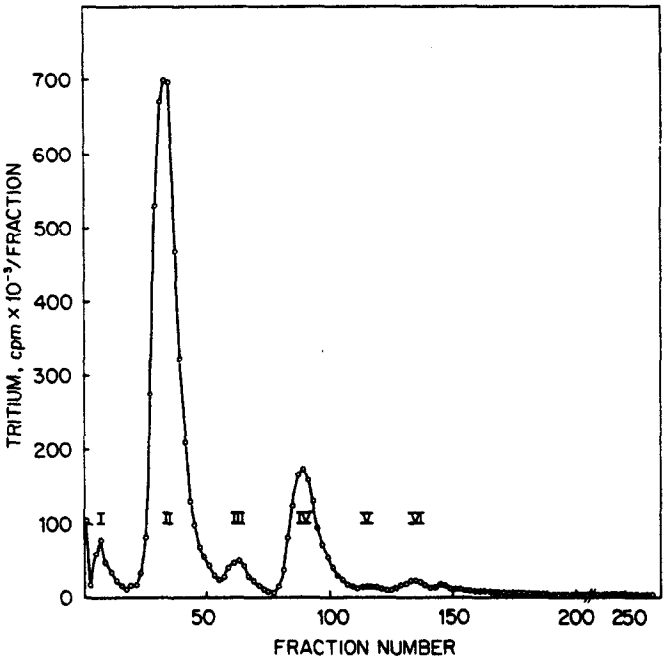

Fig. 1. Column chromatographic separation on celite–ethylene glycol of the labeled metabolites obtained from an incubation of [^{3}H]-androstenedione (0.5 μM, 21 μCi) with enriched macrophages (5×10^7) for 4 h at 37°C: 5α-androstane-3,17-dione (peak I), androstenedione plus androsterone (peak II), 5α-dihydrotestosterone plus isoandrosterone (peak III), testosterone (peak IV), 5α-androstane-3α,17β-diol (peak V) and 5α-androstane-3β,17β-diol (peak VI).

methanol (85:15:1, by vol., 1 ascent). The plates were dried in air and the carrier steroids were localized by staining with an acid spray consisting of acetic acid (100 ml), sulfuric acid (2 ml) and anisaldehyde (1 ml) followed by heating at 100°C for 20 min. The steroids were separated as follows: 5α-androstane-3,17-dione ($R_f = 0.81$); androstenedione ($R_f = 0.64$); 5α-dihydrotestosterone plus androsterone plus isoandrosterone ($R_f = 0.49$); testosterone ($R_f = 0.35$); 5α-androstane-3α,17β-diol plus 5α-androstane-3β,17β-diol ($R_f = 0.27$). The radioactivity associated with the marker steroids was expressed as a fraction of the total radioactivity recovered along the line of the developed mixture, and the corresponding fractions found in the extracts of the control experiments (without macrophages) were subtracted from the experimental samples to correct for the blank values. The fractional conversions were used to compute the rates of metabolite formation, taking into consideration the specific activity of [1,2,6,7-^{3}H]-androstenedione. Since 5α-dihydrotestosterone, androsterone and isoandrosterone, as well as the two 5α-reduced diols were not separated by TLC with the solvent system described, the rates of formation of each of these two groups of metabolites are reported together.

RESULTS

The metabolites of [^{3}H]-androstenedione produced by guinea-pig peritoneal macrophages during a 4 h incubation at 37°C were separated partially by column chromatography as illustrated in Fig. 1, and were purified further by consecutive TLC until constancy in the ^{3}H:^{14}C ratios was attained. During crystallization the ^{3}H:^{14}C ratios of the mother liquors (ML) and final crystals remained unchanged, this being a further indication that the isolated metabolites were radiochemically homogeneous (Table 1). Testosterone accounted for approximately two-thirds of the metabolites formed: the rate of testosterone formation was 32 pmol/5×10^7 macrophages $\times$ 4 h, and that of the other 5α-reduced metabolites combined was 17.2 pmol/5×10^7 macrophages $\times$ 4 h. The rates of formation of 5α-androstane-3,17-dione and isoandrosterone were similar and were approximately one fifth that of testosterone. The other 5α-reduced metabolites, 5α-dihydrotestosterone, androsterone, 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol were formed at lower rates (Table 1). When the formation rates of these metabolites were evaluated as a function of incubation time and of macrophage number, the rates remained linear with time of incubation for approximately 30 min (Fig. 2A), and with macrophage number up to approximately 1×10^7 cells per 0.5 ml (Fig. 2B). No 5β-reduced metabolites were detected.

DISCUSSION

In the present study we were able to demonstrate that several enzymes in guinea-pig peritoneal macrophages are involved in the metabolism of androstenedione. Among these, the predominant activity was the 17β-hydroxysteroid oxidoreductase (34.8 pmols of

Table 1. Characterization and quantification of tritium-labeled C_{19}-steroid metabolites isolated from an incubation of [1,2,6,7-^{3}H]androstenedione with guinea-pig peritoneal macrophages. Evidence for radiochemical homogeneity

Metabolite	^{3}H:^{14}C ratios Last TLC (After acetylation)	Crystallization ML1	ML2	ML3	Final Crystals	Metabolite formed (pmol per 5 × 10^7 macrophages per 4 h incubation)
Testosterone	560	541	527	505	576	32
5α-Androstane-3,17-dione	146	161	161	166	154	6.1
5α-Dihydrotestosterone	69	55	53	47	53	1.6
Androsterone	116	114	112	120	119	1.5
Isoandrosterone	311	332	344	311	298	6.8
5α-Androstane-3α,17β-diol	9	7	7	7	6	0.1
5α-Androstane-3β,17β-diol	60	77	65	76	63	1.1

metabolites formed per 5 × 10^7 macrophages per 4 h) which was expressed in the conversions of androstenedione to testosterone, 5α-androstane-3,17-dione to 5α-dehydrotestosterone, androsterone to 5α-androstane-3α,17β-diol, and isoandrosterone to 5α-androstane-3β,17β-diol. In addition, we detected the presence of 5α-reductase activity (17.2 pmol/5 × 10^7 macrophages × 4 h), an enzyme that catalyzed the conversion of androstenedione to 5α-androstane-3,17-dione, and testosterone to 5α-dihydrotestosterone. The other androstenedione metabolizing enzymes present in these cells were 3α-hydroxysteroid oxidoreductase (1.6 pmol/5 × 10^7 macrophages × 4 h), as indicated by the conversion of 5α-androstane-3,17-dione to androsterone, and 3β-hydroxysteroid oxidoreductase (7.9 pmol/5 × 10^7 macrophages × 4 h), as evidenced by the formation of isoandrosterone from 5α-androstane-3,17-dione. The reported rates of metabolite formation were not measured under substrate-saturation conditions since the main purpose of these experiments was to identify the androstenedione metabolizing enzymes present in guinea-pig peritoneal macrophages. Therefore, the reported rates do not represent the maximal capacity of these cells to metabolize androstenedione. There is considerably evidence that the formation of androgens by reduction of circulating androstenedione in peripheral tissues is of major physiological significance, not only as a quantitatively important source of biologically active hormones in the circulation, but also in exerting physiological effects directly within the cells in which they are formed [4]; the factors that regulate these processes in peripheral tissues, however, are poorly understood at the present time. Our findings are suggestive that peritoneal macrophages may be a site for extra-glandular conversion of androstenedione to potent androgens in the guinea-pig *in vivo*.

The role of androgens in the immune response is unclear: these hormones could affect macrophages themselves or particular subsets of lymphocytes. Mac-

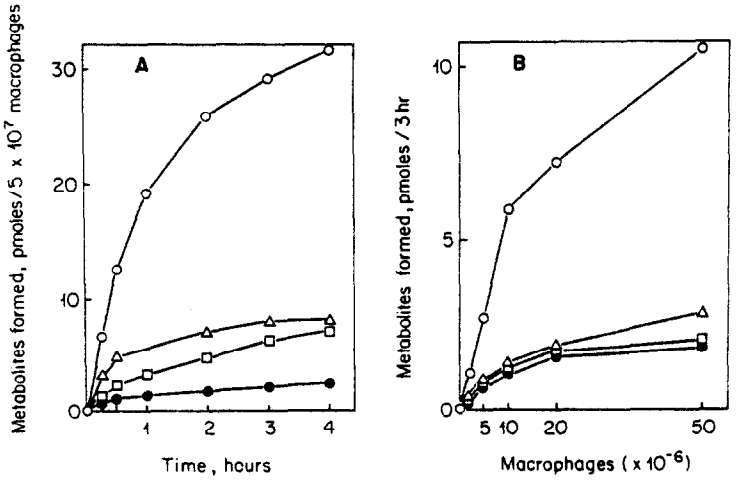

Fig. 2(A) Rates of formation of metabolites of [^{3}H]-androstenedione by guinea-pig peritoneal macrophages as a function of time of incubation. Incubations were conducted at 37°C using 5 × 10^7 macrophages and 0.5 μM (21 μCi) [1,2,6,7-^{3}H]-androstenedione: testosterone (O——O), 5α-androstane-3,17-dione (Δ——Δ), 5α-dihydrotestosterone plus isoandrosterone plus androsterone (□——□), 5α-androstane-3α,17β-diol plus 5α-androstane-3β,17β-diol (●——●). (B) Rates of formation of metabolites of [^{3}H]-androstenedione as a function of macrophage number. Incubations were conducted at 37°C for 3 h with 0.5 μM (21 μCi) [^{3}H]-androstenedione: testosterone (O——O), 5α-androstane-3,17-dione (Δ——Δ), 5α-dihydrotestosterone plus isoandrosterone plus androsterone (□——□), 5α-androstane-3α,17β-diol plus 5α-androstane-3β,17β-diol (●——●).

rophages appear to be essential for the activation of subsets of both T and B lymphocytes [5], and under defined conditions can mediate suppression [6]. It has been demonstrated that testosterone does not stimulate macrophage activity *in vivo* when administered alone, however, it potentiates strongly the stimulatory action on phagocytosis produced by 17β-estradiol [7]. Thus it is possible that macrophage enzymes engaged in metabolizing androstenedione may contribute to the complex regulatory mechanism that is operative in the immune response.

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