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Estrone Formation from Dehydroepiandrosterone in Cultured Human Breast Adipose Stromal Cells

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The metabolism of dehydroepiandrosterone (DHA) and androstenedione (A-dione) was studied in cultured human adipose stromal cells obtained from breast tissue of six premenopausal patients undergoing reduction mammoplasty. Cells were maintained in culture in the presence of 10% fetal bovine serum. Studies were carried out during the proliferative and confluent phases of culture with radiolabelled substrates (2 μCi, 10 nM). During the early phases of replication 7α-hydroxydehydroepiandrosterone (7α-OHDHA) was formed from DHA. As the cells reached confluence, the major metabolite of DHA in cells from all patients was A-dione indicating the presence of 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD). The conversion of DHA to A-dione was variable among patients when cells were confluent with 30–80% of substrate being metabolized to this product. Adipose stromal cells synthesized estrone (E_1) from DHA once A-dione formation was established. Under basal conditions E_1 was obtained in cells from three of the six patients examined with up to 36% substrate converted to this product. Dexamethasone (Dex 10^{-7} M) stimulated E_1 formation in cells from all subjects with up to 50% of substrate being converted. Parallel studies comparing the conversion of DHA with A-dione to E_1 revealed that as the cells became confluent, E_1 formation from both substrates was similar. The pattern of steroid metabolism was also examined in primary culture and in subculture. Passage 1 cells continued to form A-dione as a major metabolite of DHA, and did not revert to the pattern of metabolism found in primary cells during the early stages of replication, when 7α-hydroxylation predominated. Human adipose stromal cells actively metabolize DHA, producing 7α-OHDHA, A-dione and E_1 as principal metabolites. Changes in the circulating levels of DHA may directly influence the formation of E_1 in peripheral tissues. This source of E_1 will be modulated by factors controlling 3β-HSD and aromatase activities.

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INTRODUCTION

The adrenal steroids dehydroepiandrosterone (DHA) and dehydroepiandrosterone sulfate (DHAS) are the most abundant C_{19} steroids circulating in human plasma. The interconversion of these androgens in peripheral tissues provides a reservoir of potential precursors for metabolism to other steroids with enhanced biological activity. In females, plasma levels of DHA and DHAS reach a maximum between 20 and 30 years of age, and then progressively decline until the menopause [1]. Little information is available on the factors which regulate DHA metabolism in peripheral tissue. Diverse biological functions of DHA and DHAS have been postulated on the basis of laboratory studies in animals and epidemiological studies in humans.

Animal experiments in which DHA has been administered have consistently demonstrated effects on body weight or insulin levels. Studies in rats demonstrated that the addition of DHA to a high fat diet resulted not only in an inhibition of further weight gain, but a return to weight found in rats maintained on a normal diet [2]. The weight of the epididymal fat pads and the fat cell numbers were lower in DHA fed animals indicating that DHA prevented either the proliferation of new fat cells, or the filling of previously formed fat cells. Treatment of Zucker rats [3] and mice [4] with DHA reduced both fat cell size and number. Treatment

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with DHA was reported to decrease atherosclerosis in cholesterol fed rabbits but there was no change in cholesterol levels [5]. *In vitro*, DHA inhibits differentiation of 3T3 fibroblasts to adipocytes [6, 7] and inhibits DNA synthesis in mouse tissue [8]. DHA has also been shown to inhibit the formation of a variety of tumors in laboratory mice including spontaneous mammary tumors [9, 10]. Epidemiological studies in human populations have suggested an inverse correlation between DHA and cardiovascular mortality [11] and a relationship between DHA and DHAS levels in breast cancer in females [12–14]. The results of the breast cancer studies are influenced by the study design, but there is some agreement that postmenopausal women who developed breast cancer had elevated levels of DHA prior to the diagnosis [12] while other studies have shown low levels of DHA and DHAS in premenopausal women with breast cancer [13].

The mechanisms through which DHA and DHAS might influence these apparently diverse systems have not been elucidated. Both *in vivo* and *in vitro*, DHA inhibits glucose-6-phosphate dehydrogenase in human erythrocytes at concentrations of DHA achieved following ACTH stimulation [15]. Glucose-6-phosphate dehydrogenase from human placenta is also inhibited by DHA [16]. It has been suggested that inhibition of glucose-6-phosphate dehydrogenase may inhibit NADPH production required for lipogenesis [15] and this could affect differentiation.

We have recently shown that a major metabolite of DHA in cultured human adipose stromal cells is 7α-hydroxydehydroepiandrosterone (7α-OHDHA) [17]. Formation of 7α-OHDHA is stimulated several fold when cells are cultured in the presence of dexamethasone [18]. It was postulated that the conversion of DHA to 7α-OHDHA may influence the amount of DHA available for metabolism to A-dione. This latter conversion increases the endogenous A-dione pool potentially available for further metabolism to estrone (E_1), or to 5α-reduced androgens [19, 20].

The present study has examined changes in the metabolism of DHA by adipose stromal cells from the time of plating until they have achieved confluence and in the postconfluent state. We have found that under defined culture conditions, the pattern of metabolism in adipose stromal cells grown in primary culture changes during the phases of cell replication and confluence. DHA is metabolized primarily to 7α-OHDHA during the early stages of replication indicating the presence of 7α-hydroxylase activity. As cells approach confluence, 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) activity increases and predominates during confluence. Aromatase activity becomes evident as the amount of A-dione available as substrate increases. Conversion of A-dione to E_1 is enhanced when cells are cultured in the presence of dexamethasone (Dex), which has also been shown to stimulate the formation of 7α-OHDHA from DHA [18].

METHODS

Preparation and incubation of cells

Adipose tissue was obtained from six patients undergoing reduction mammoplasty. The age of these patients was 15, 21, 24, 36, 26, 45 for patients 1–6, respectively. The tissue from each patient was washed with Hanks balanced salt solution (HBSS), minced with scissors and incubated at 37°C with 1 mg/ml collagenase solution (Type 2) in HBSS for 30 min with constant stirring. 5 ml of collagenase solution was used per gram of tissue. After digestion, the tissue was filtered through a 250 μm nylon mesh and the filtrate was centrifuged at 800 ***g*** for 10 min. The pellet was resuspended in 10 ml lysis buffer (154 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) and after 10 min was filtered through a 30 μm nylon mesh. The filtrate was centrifuged at 800 ***g*** for 10 min and cells were washed once with HBSS. The pellet was resuspended in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (heat inactivated), penicillin G sodium (500 units/ml) and streptomycin sulfate (500 μg/ml) and transferred to 6 well plates. After 16–26 h, the attached cells were washed three times with medium, and fresh medium containing 10% FBS was added.

Steroid metabolism

The conditions of incubation were adjusted for different experiments. Incubations were carried out at 5–7 day intervals from day 4 to day 24 of culture. For studies of DHA and A-dione metabolism, medium was replaced with fresh medium containing 2 μCi ^3H substrate (10 nM). The incubation proceeded for 24 h and the conditioned medium was stored at −20°C until analyzed. Cells were removed from the plates by trypsinization with 0.05% trypsin/0.53 mM EDTA solution and counted using a hemocytometer. At the time of analysis, the medium was thawed and passed through a C_{18} SPE cartridge (between 2–10 ml medium/cartridge), and the retained steroids were eluted with methanol (5 ml per cartridge).

An aliquot of the eluate was dried under vacuum, the residue was dissolved in 70% methanol and was analyzed on HPLC using 70% methanol as the eluting solvent.

HPLC analysis

Radiolabelled steroid metabolites were analyzed by HPLC on a C_{18} Spherisorb ODS2 column (5 μm, 250×4.6 mm i.d., Jones Chromatography) and eluted isocratically with 70% methanol/H_2O. Metabolites were detected by an in-line radioactivity detector and were identified on the basis of their retention times compared to authentic standards. Quantification was carried out using [1,2,6,7-^3H]androstenedione as an external standard. The relative retention times (rrt) of the metabolites were expressed relative to the retention time of androstenedione equal to 1.

To further establish the identity of the chromatographic peaks eluting with the same retention times as the E_1 and A-dione standards, these peaks were collected and mixed with authentic ^{14}C E_1, or A-dione and 50 mg of the appropriate unlabelled steroid. Each mixture was recrystallized to constant $^3H/^{14}C$ ratios. The radioactivity in each fraction was found to be greater than 80% pure.

HPLC equipment

High performance liquid chromatographic equipment consisted of a model 6000 A pump, a model 710B automatic injector, and a model 441 UV spectrophotometer all from Waters Associates. Radiolabelled steroid metabolites were detected and quantified by an in-line Beckman model 171 radioactivity detector (Beckman Instruments, Toronto, Ontario, Canada). Data from the UV and radioactivity detectors were analyzed by computer using the MAXIMA 820 Chromatographic Software package from Waters Associates.

Non-radioactive 3-oxo-4-ene steroids and 5-ene-3β-hydroxysteroids were detected at 254 and 215 nm, respectively, on a Waters model 441 UV/VIS spectrophotometer.

Materials

Solvents obtained from BDH, Baker or Canadian Laboratory Supplies, Toronto, Canada were either American Chemical Society or HPLC grade. Radioactive steroids [1,2,6,7-^{3}H]dehydroepiandrosterone (SA, 86.6 Ci/mmol) and [1,2,6,7-^{3}H]androstenedione (SA, 86.1 Ci/mmol) were obtained from New England Nuclear, Montreal, PQ, Canada. Unlabelled steroids used as standards were purchased from Steraloids Inc., Wilton, NH, U.S.A. and Sigma Chemical Co., St Louis, MO, U.S.A. C_{18} cartridges (S.P.E. Cartridges, Scientific Products and Equipment Ltd, Concord, Ontario, Canada) were washed twice with 10 ml methanol and 10 ml H_2O before use. Tissue culture flasks and 6-well plates (Falcon) were purchased from John's Scientific, Toronto, Canada.

RESULTS

Time course of DHA metabolism during culture of adipose stromal cells

Experiments were carried out to determine the pattern of DHA metabolism by adipose stromal cells incubated with 10% FBS from plating to the time of confluence and following confluence (patient 1, Fig. 1). The main metabolites detected by HPLC were 7α-OHDHA (rrt 0.54), E_1 (0.89), A-dione (1.0) and DHA (1.21).

The formation of 7α-OHDHA predominated in the early phases of culture, reaching a maximum by day 10 (46% of substrate). Formation of A-dione was detectable by day 10 and increased 10-fold by day 15 in culture. There was an increase in the formation of E_1 between day

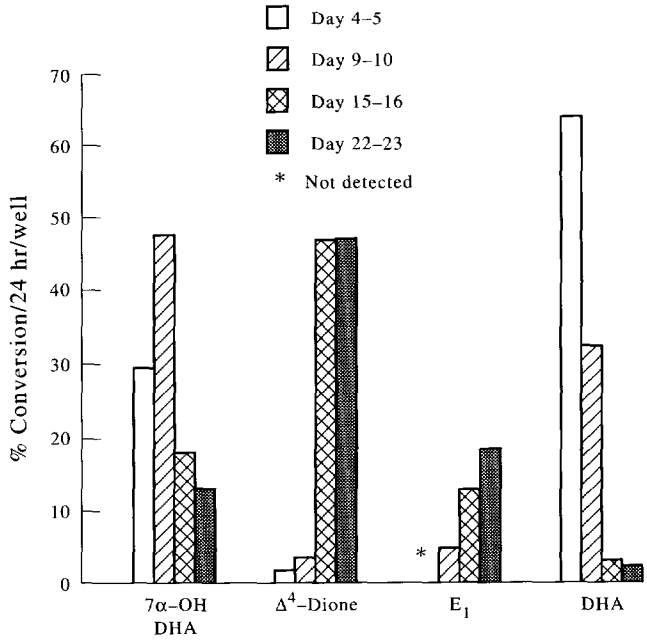

Fig. 1. The metabolism of [1,2,6,7-^{3}H]dehydroepiandrosterone (DHA) in primary cultures of human adipose stromal cells obtained from breast tissue of patient 1. Cells were cultured for 23 days with 10% fetal bovine serum and incubations with radiolabelled DHA (10 nM) were carried out for 24 h at days 4, 9, 15 and 22. Metabolites were separated by C_{18} reverse phase HPLC and were quantified using an in-line radioactivity detector and expressed as % conversion/24 h/well. 7α-Hydroxydehydroepiandrosterone (7α-OHDHA), androstenedione (A-dione), and estrone (E_1) were the main metabolites of DHA.

10 and 23 related to the increase in A-dione formation. During this same interval, there was a progressive decrease in the formation of 7α-OHDHA. Formation of several minor metabolites, one more polar than 7α-OHDHA on HPLC (rrt 0.48) and two other metabolites less polar than DHA (1.46 and 1.88) were also present. These metabolites represented between 10 and 20% of total products, although the less polar ones increased as cells became confluent.

Similar studies on two additional patients (patients 2 and 3) are shown in Fig. 2. Cell counts indicate that confluence had occurred by days 16–17 in these studies and this was consistent in other experiments carried out under similar conditions. When product formation is expressed as pg of product per 10^4 cells, there is an increase in 3β-HSD activity in these cells as they reach confluence and a fall in 7α-hydroxylase activity at or after confluence. Under basal conditions, estrone formation was not detected in patient 2 [Fig. 2(A)] but was a major product in patient 3 [Fig. 2(C)]. The metabolism of DHA in the presence of 10^{-7} M Dex was studied in each of these patients at intervals during culture [Fig. 2(B) and Fig. 2(D)]. In patient 2, Dex stimulated the formation of 7α-OHDHA 5-fold when steroid production was assessed on day 10, with a less than 2-fold increase on days 16 and 23. Addition of Dex did not increase 7α-OHDHA levels in patient 3. There was a

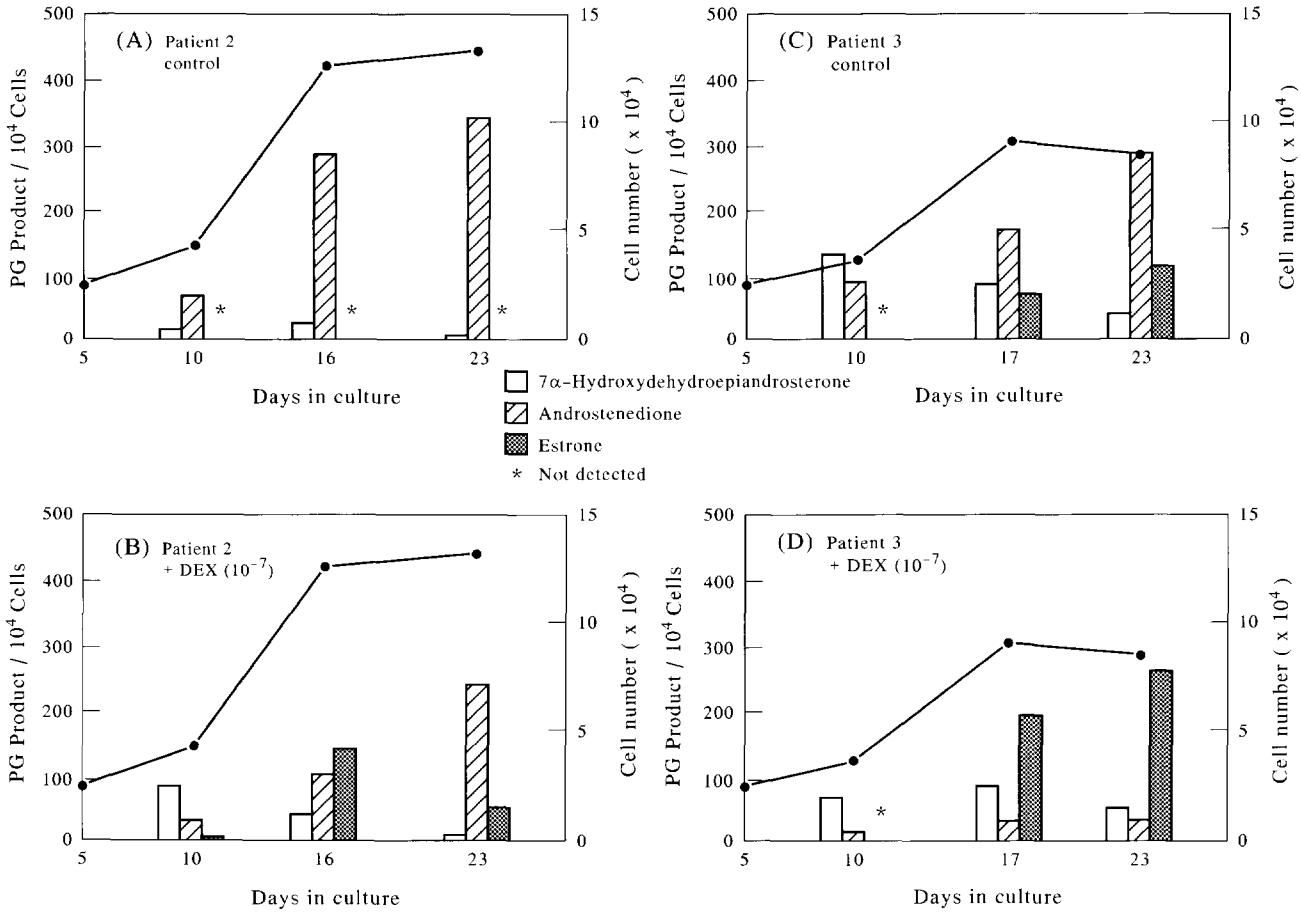

Fig. 2. The metabolism of [1,2,6,7-^{3}H]DHA in primary cultures of human adipose stromal cells obtained from patients 2 and 3. Cells were cultured with 10% fetal bovine serum for 23 days and reached confluence between days 15 and 17. Incubations with radiolabelled DHA (10 nM) were carried out for 24 h on days 9, 15, and 22. Metabolites were separated by C_{18} reverse phase HPLC and were quantified using an in-line radioactivity detector (pg/10^4 cells/24 h). A and C, cells from patients 2 and 3 without added dexamethasone. B and D, cells from patients 2 and 3 with added dexamethasone (10^{-7} M).

marked stimulation of E_1 formation by Dex in both patients and a concomitant decrease in the recovery of A-dione that was due to its conversion to E_1.

The metabolism of DHA in stromal cells from patients 4 and 5 in primary culture and during passage 1 was also examined but only data from patient 4 is outlined in Fig. 3. Cells from patient 4 were subcultured on day 12 and from patient 5 on day 14. The formation of E_1 was not detected in either of these patients under basal conditions in primary culture although A-dione was the main metabolic product. In subculture, the pattern of DHA metabolism did not revert to that seen at the start of primary culture but resembled metabolism found at the time the cells were subcultured (Fig. 3). When Dex (10^{-7} M) was added during the study of DHA metabolism, in primary culture and passage 1 cells, E_1 formation was increased several fold in both patients. The lack of E_1 formation in patients 4 and 5 under basal conditions is similar to that seen in patient 2. The findings in these patients contrast with the results in patients 1, 3 and 6 where E_1 formation was readily detectable under basal conditions.

Table 1 shows a comparison of the metabolism of DHA and A-dione in adipose stromal cells from patients 2 and 6 during culture with and without Dex. There was an approx. 10-fold increase in the conversion of DHA to A-dione between day 10 and day 15 in culture in cells from patient 2, and an 8-fold increase in patient 6. By day 10, the addition of Dex stimulated E_1 formation from added A-dione in cells from both patients indicating the presence of aromatase activity. However, basal E_1 formation from DHA was negligible by day 10. In studies on cells from patient 2, the conversion of DHA to A-dione had increased by day 15 and the formation of E_1 from DHA and A-dione was comparable under both basal and Dex stimulated conditions. The cells from patient 6 showed qualitatively similar results. This patient had very high basal aromatase activity with rapid conversion of A-dione substrate and A-dione formed from DHA to E_1 in the absence of added Dex when examined at day 15. The addition of Dex to the cells from patient 6 resulted in almost complete conversion of A-dione to E_1 by day 22, and 48% conversion of DHA to E_1.

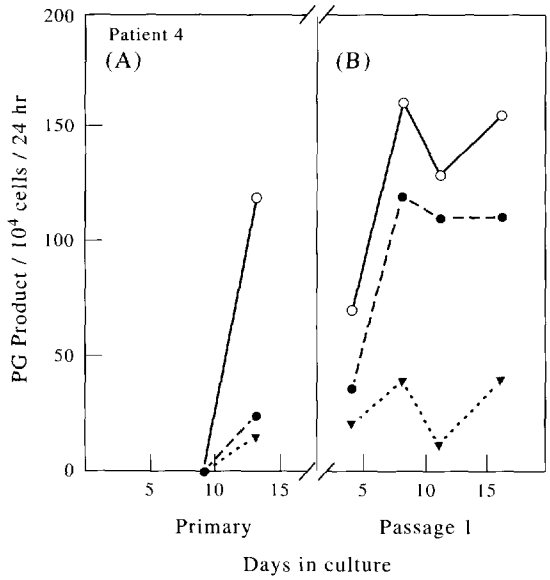

Fig. 3. The metabolism of [1,2,6,7-^{3}H]DHA in primary culture and in passage 1 cells obtained from breast tissue of patient 4. Cells were subcultured on day 12 and passage 1 cells were cultured for an additional 15 days. Incubations with radiolabelled DHA (10 nM) were carried out for 24 h; in primary cultures they were performed at days 8–9 and 12–13, and in passage 1 cells at days 3–4, 7–8, 10–11 and 15–16. Steroid metabolism was studied in the presence and absence of dexamethasone (Dex, 10^{-7} M). Metabolites were separated by C_{18} reverse phase HPLC and were quantified using an in-line radioactivity detector (pg product/10^4 cells/24 h). (A) Primary cultures of adipose stromal cells from patient 4; (B) passage 1 cells from patient 4. Androstenedione formation under basal (○) and Dex stimulated (●) conditions; estrone formation in the presence of Dex (▲). Estrone formation was not detectable under basal conditions.

DISCUSSION

Human adipose tissue has been shown to contain enzymes capable of converting androstenedione to estrone, testosterone and to 5α-reduced metabolites [20]. Recent studies have also documented formation of 7α-OHDHA from DHA [17]. Conversion of A-dione to E_1 [21], and DHA to 7α-OHDHA can both be stimulated by glucocorticoids [18] although the magnitude of the stimulation varies. The present study has demonstrated that DHA is converted to A-dione in human adipose stromal cells and that the activity of 3β-HSD, the enzyme which metabolizes DHA to A-dione, increases as the cells proliferate and achieve confluence. The activity of 3β-HSD varies from patient to patient but A-dione is always the major product when the cells are confluent with 30–80% of substrate being metabolized to this product under basal conditions.

The 3β-HSD from human adrenal and gonadal tissue has been purified, and the gene encoding its synthesis has been cloned [22]. In the rat there are four types of 3β-HSD which have similar substrate specificities but some degree of tissue specificity. Two types of 3β-HSD are expressed in the adrenal, ovary and adipose tissue while only Type IV is expressed in the kidney [22, 23]. In the human, two types of 3β-HSD have been identified but the tissue specificity has not been completely established [22]. Type I is expressed in placenta, skin and breast while Type II is expressed in the adrenals and gonads.

A variety of factors have been shown to regulate 3β-HSD in animal models. In mouse Leydig cells, 3β-HSD mRNA levels are influenced by cAMP and suppressed by endogenously produced steroids [24]. In the rat ovary, 3β-HSD mRNA is stimulated by HCG, and this stimulation is potentiated by prolactin [25]. In cultured rat Leydig cells, dexamethasone and insulin inhibited 3β-HSD levels [26]. The activity of 3β-HSD is also inhibited by basic FGF [27] and platelet derived growth factor [28] and stimulated by IGF-I [29]. Human placental 3β-HSD is inhibited by synthetic progestins, trilostane, epostane and cyanoketone [30, 31].

The present study has shown that adipose stromal cells can convert DHA to E_1. Formation of E_1 from DHA varied with tissue from different patients. Under basal conditions, conversion of DHA to E_1 could be detected in three of the six patients examined. Similar variability had been noted in earlier studies of E_1 formation from A-dione in adipose stromal cells [32]. Glucocorticoids

Table 1. The percent conversion of A-dione (10^{-8} M) to E_1 and DHA (10^{-8} M) to A-dione and E_1 by human adipose stromal cells under basal and Dex (10^{-7} M) stimulated conditions in culture

Substrate and treatment	Day 5		Day 10		Day 15		Day 22	
	E_1	A-dione	E_1	A-dione	E_1	A-dione	E_1	A-dione
Patient 2								
DHA	0	0	0	4.6	3.7	44.3	1.6	52.7
DHA and Dex	0	0	2.2	2.6	23.3	16.9	10.0	37.8
A-dione	1.0	97.7*	0.5	88.7*	1.5	81.3*	3.8	77.9*
A-dione and Dex	2.5	96.7*	17.9	69.8*	24.7	57.2*	23.0	57.1*
Patient 6								
DHA	0	0.3	0	1.2	18.3	9.8	36.5	16.8
DHA and Dex	0	0	0	1.2	23.1	5.8	48.6	3.3
A-dione	0	95.4*	0.6	81.6*	44.5	40.4*	58.1	27.0*
A-dione and Dex	0	95.4*	25.1	59.4*	74.5	13.2*	89.5	0*

*Recovered substrate.

stimulated aromatase activity in each of the cultures after the conversion of DHA to A-dione was established. The variability in E_1 formation in cells from different patients may in part be due to factors which modulate glucocorticoid activity in peripheral tissues such as glucocorticoid receptor function [33] or the activity of 11β-hydroxysteroid dehydrogenase [34].

MacDonald *et al.* have shown that the *in vivo* conversion of DHA and DHAS to products with greater biological activity is relatively low [35]. With the very large production rates of these steroids, however, small conversions can account for significant amounts of active steroids such as E_1, estradiol or testosterone. Haning *et al.* [36] in *in vivo* studies, found that the percent conversion of DHAS to E_1 averaged 0.08% in four premenopausal females with normal plasma DHAS levels and this accounted for 13–51% of the E_1 formed. Longcope *et al.* [37] found that the conversion of DHA to E_1 in postmenopausal women was 0.6% and this accounted for 48% of the estrone formed. MacDonald *et al.* [36] demonstrated that in the non-pregnant female, plasma DHA was converted to estrogen via two pathways. One pathway involved the conversion of circulating DHA through plasma A-dione to E_1. This accounted for approximately one third of the total conversion. The second pathway did not involve plasma A-dione and may have been due to the conversion of DHA to A-dione to E_1 in tissue without having the A-dione enter the plasma pool. In the present studies comparing DHA and A-dione as substrates for E_1 formation under different culture conditions, DHA was found to be an excellent substrate for E_1 formation in adipose stromal cells. This suggests that adipose tissue may be one site for the second pathway suggested by MacDonald *et al.* [36].

The activity of 7α-hydroxylase also changes during cell culture. It is highest in the early phases of proliferation, decreases as the cells reach confluence, and remains low following confluence. 7α-Hydroxylase activity is also variable in cells from different patients; in some subjects it was barely detectable and in others, over 50% of DHA was converted to 7α-hydroxylated metabolites [17, 18].

When the cells were subcultured from primary culture to passage one, the pattern of metabolism at the time of subculture was retained. This suggests that during primary culture, in the presence of FBS, there is an induction of 3β-HSD and reduction of 7α-hydroxylase that results in a form of differentiation which persists in subculture. When these cells are cultured in defined medium containing glucocorticoid and insulin in the absence of FBS, differentiation to mature adipocytes takes place [38]. The major metabolite of DHA under these conditions is 7α-OHDHA (unpublished data). It is uncertain which pattern of androgen metabolism predominates in the *in vivo* situation.

These studies demonstrate that DHA is actively metabolized in human adipose stromal cells. The metabolic products are influenced by culture conditions with 7α-OHDHA and A-dione being the primary metabolites. Glucocorticoids influence both 7α-hydroxylation and aromatization. Under appropriate culture conditions the conversion of DHA to E_1 is comparable to the conversion of A-dione to E_1. These studies suggest that adipose tissue may be a significant site for the conversion of DHA to E_1 *in vivo*. Changes in plasma levels of DHA observed both premenopausally and with aging may influence E_1 formation in peripheral tissue. This conversion of DHA to E_1 will be modulated by factors controlling 3β-HSD and aromatase activities.

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