The Anti-aromatase Effect of Progesterone and of its Natural Metabolites 20α- and 5α-Dihydroprogesterone in the MCF-7aro Breast Cancer Cell Line

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Abstract. *Background: Progesterone is metabolized in the normal breast mainly into 4-ene-pregnenes (e.g. 20α-dihydroprogesterone, 20αDHP) but, in contrast, in breast cancer tissue the 5α-dihydropregnanes (e.g. 5α-dihydroprogesterone, 5αDHP) are prevalent. In the present study the effect of progesterone and its main metabolites 20αDHP and 5αDHP on the aromatase activity in a stable aromatase-expressing estrogen receptor-positive human breast cancer cell line, MCF-7aro, was explored. Materials and Methods: The MCF-7aro cells were stripped of endogenous steroids and incubated with physiological concentrations of [^{3}H]-testosterone ([^{3}H]-testos: 5×10^{-9}M) alone or in the presence of progesterone, 20αDHP or 5αDHP (5×10^{-6} or 5×10^{-8}M) for 24 h at 37°C. The cellular radioactivity uptake was determined in the ethanolic supernatant and the DNA content in the remaining pellet. [^{3}H]-Estradiol (E_2), [^{3}H]-estrone (E_1) and [^{3}H]-testos were characterized by thin layer chromatography and quantified using the corresponding standard. Results: Aromatase activity was present at a high level in the MCF-7aro cells after incubation with [^{3}H]-testos when the concentration of [^{3}H]-E_2 was 3.70 pmol/mg DNA; 20αDHP at concentrations of 5×10^{-6}M or 5×10^{-8}M significantly inhibited this conversion by 50.3% and 36.5%, respectively. No significant effect was found with the metabolite 5αDHP or the parent hormone, progesterone. Conclusion: The MCF-7aro cell line shows high detectable aromatase activity. The present data indicate that the progesterone metabolite 20αDHP, found mainly in normal breast tissue, can act as an anti-aromatase agent.*

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The importance of progesterone in the development of the normal breast, as well as in the menstrual cycle, pregnancy and lactation has been well documented (1-3). This biological action is generally performed in combination with estrogens. In various organs, progesterone is largely metabolized and the metabolic transformations are important not only because the biological effect of the steroid hormone can be blocked, but also because some metabolic products can play a major role in its biological responses. As an example, in a pioneer study it was demonstrated that in the human endometrium, transformation of progesterone to 20α-dihydroprogesterone (20αDHP) can control the oxido-reductive interaction of estrone / estradiol and consequently regulate the action of this hormone (4).

In a series of studies carried out with breast tissues it has been demonstrated that progesterone is converted into various metabolic products. In normal breast tissue, the transformation is mainly to 4-pregnene derivatives, whereas in tumor tissue, 5α-pregnane derivatives are predominant (5, 6). These transformatons are indicated in Figure 1.

The cytochrome P450 aromatase complex (CYP19 gene) operates in the last step of the bioformation of estrogens by the conversion of androgen precursors. At present, anti-aromatase agents are largely used as the first-line treatment of breast cancer patients (7-10). The cytochrome P450 aromatase activity is very low in breast cancer tissue (11, 12), however, a stable hormone-dependent breast cancer cell line, MCF-aro, with high aromatase activity, has been obtained by transfection with the aromatase gene (13).

In the present study, the effects of both the 20αDHP (4-pregnene derivative) and 5α-dihydroprogesterone (5αDHP, 5α-pregnane derivative), were compared with the parent hormone progesterone in the conversion of testosterone to estradiol in MCF-7aro cells.

Materials and Methods

Chemicals. [1,2,6,7-3H(N)]-Testosterone (sp. act. 95 Ci/mmol) and [4-^{14}C]-estradiol (^{14}C-E_2) (sp. act. 57 mCi/mmol) were purchased from New England Nuclear Division (PerkinElmer Life Sciences, Courtabœuf, France). The purity of the radioisotopes was assessed by thin-layer chromatography (TLC) in the appropriate system before use. Unlabeled estrone (E_1), estradiol (E_2), progesterone and testosterone were obtained from Sigma-Aldrich Chimie (St. Quentin, Fallavier, France). The two progesterone metabolites: 5α-pregnane-3,20-dione (5αDHP) and 4-pregnen-20α-ol-3-one (20αDHP) were obtained from Steraloids (Rhode Island, USA). All the chemicals were of the highest purity available.

Cell culture. The human hormone-dependent MCF-7aro cell line was kindly provided by Dr S. Chen (Beckman Research Institute, Duarte, USA). MCF-7aro is a stable aromatase-expressing estrogen receptor positive mammary cancer cell line, prepared by aromatase cDNA transfection and G418 (neomycin) selection (13). The cells were routinely grown in Eagle's minimal essential medium (MEM) buffered with 10 mmol/l HEPES (pH 7.6), supplemented with 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% v/v fetal calf serum (FCS) (ATGC, Marne-La-Vallée, France), and incubated at 37˚C in a humidified atmosphere of 5% CO_2. The media were changed twice a week. The cells were passaged every 10-12 days and replated in 75 cm^2 flasks (ATGC) at 3×10^6 cells/flask. Three days before the experiments, the cells were transferred to MEM containing 5% steroid-depleted FCS. The FCS had been treated overnight at 4˚C with dextran-coated charcoal (DCC) (0.1-1% w/v, DCC-FCS) to remove endogenous steroids.

Isolation and quantification of [^{3}H]-estradiol from MCF-7aro cells incubated with [^{3}H]-testosterone. Cells near confluence were cultivated in MEM-DCC-FCS (10 ml) with the addition of the androgen precursor, [^{3}H]-testosterone ([^{3}H]-testos) at a physiological concentration of 5×10^{-9} mol/l, alone, or in the presence of progesterone or the progesterone metabolites 5αDHP and 20αDHP prepared in ethanol (final concentration <0.1%) at a concentration of 5×10^{-6} or 5×10^{-8} mol/l. Co-factor NAD(P)H was not added. Control cells received the ethanol vehicle only. Twenty-four hours later, the medium was removed, the cells washed twice with ice-cold HBSS (Hank's balanced salt solution, calcium/magnesium-free) and harvested with 15 ml HBSS by scraping with a rubber policeman. After centrifugation, the pellet was precipitated by 80% ethanol and the radioactivity extracted for at least 24 h at –20˚C. The cellular radioactivity uptake was determined in the ethanolic supernatant and the DNA content in the remaining pellet evaluated according to Burton (14). After 24 h incubation with the test compounds, the quantity of DNA in the flasks was not significantly different from that of control cells. After evaporation of the organic phase, the extracts were redissolved in 50 μl ethanol and the qualitative analysis and quantitative evaluations of testosterone, E_1 and E_2 were carried out after isolation by TLC on silica gel $60F_{254}$ plates (Merck, Darmstadt, Germany), which were developed with the chloroform-ethylacetate (4:1, v/v) or cyclohexane-ethylacetate (1:1, v/v) system. [^{14}C]-E_2 (5,000 dpm) was added to monitor analytical losses and unlabeled E_1, E_2 and testosterone (50 μg) were used as carriers and reference indicators. We had previously determined that the percentage of ^{14}C-E_2 recovered during the extraction process was consistently similar (92% ±4%). After visualization of the estrogens under U.V. at 254 nm, the appropriate areas were scraped off, placed in liquid scintillation vials with ethanol (0.5 ml) and allowed to extract for 1 h at least. Three ml of Opti-fluor (Packard, Rungis, France) were added and the vials analyzed for ^{3}H and ^{14}C contents with quench correction by external standardization. The quantitative evaluation of the transformation of the [^{3}H]-testos to [^{3}H]-E_2, corresponding to the aromatase activity at 24 h, was calculated as the percentage of the total radioactivity associated with the cells expressed as pmol/mg DNA.

Statistical analysis. The data were expressed as the mean±standard error of the mean (SEM) values. The Students *t*-test was used to assess the significance of the differences between means and *p*-values ≤0.05 were considered significant.

Table I. *Effect of progesterone (Prog.), 20α-dihydroprogesterone (20αDHP), and 5α-dihydroprogesterone (5αDHP) on the conversion of testosterone (Testos) to estradiol (E_2) in MCF-7aro breast cancer cells.*

	Testos±S.E.M. pmol/mg DNA	E_2±S.E.M. pmol/mg DNA	Testos/E_2
Control=			
[^{3}H]-Testos 5×10^{-9}M	1.59±0.12	3.70±0.3	0.430
+ Prog.			
5×10^{-8}M	2.41±0.18	3.41±0.26	0.705
5×10^{-6}M	2.37±0.24	3.35±0.32	0.707
+ 20αDHP			
5×10^{-8}M	3.48±0.20	2.35±0.21*	1.481
5×10^{-6}M	4.75±0.26	1.84±0.17*	2.582
+ 5αDHP			
5×10^{-8}M	1.82±0.28	3.67±0.24	0.496
5×10^{-6}M	1.43±0.19	3.89±0.23	0.368

[^{3}H]-Testos, 5×10^{-9} mol/l, was incubated with MCF-7aro breast cancer cells for 24 h at 37˚C in the absence (control) or presence of Prog., 20αDHP and 5αDHP in the range of 5×10^{-8} to 5×10^{-6} mol/l. The values of [^{3}H]-Testos. and [^{3}H]-E_2 were carried out after isolation of the hormone as indicated in Materials and Methods. The data represent the average±S.E.M. of three independent duplicate determinations. *$p<0.05$ *vs*. control control value.

Results

As reported in Table I, the MCF-7aro cells had the capacity to transform the androgenic substrate, testosterone, into the biologically active estrogen E_2. The [^{3}H]-E_2 biosynthesis was high and corresponded to 3.70±0.3 pmol/mg DNA, although no co-factor NAD(P)H was added to the cell cultures. As no [^{3}H]-E_1 was found to the MCF-7aro cells, oxidative 17β-hydroxysteroid dehydrogenase (17β-HSD) (type 2) activity appeared to be very low or absent in the present experimental conditions. When the 4-pregnene metabolite 20αDHP was incubated with the precursor [^{3}H]-testos in the MCF-7aro cell cultures, a significant dose-dependent inhibitory effect was

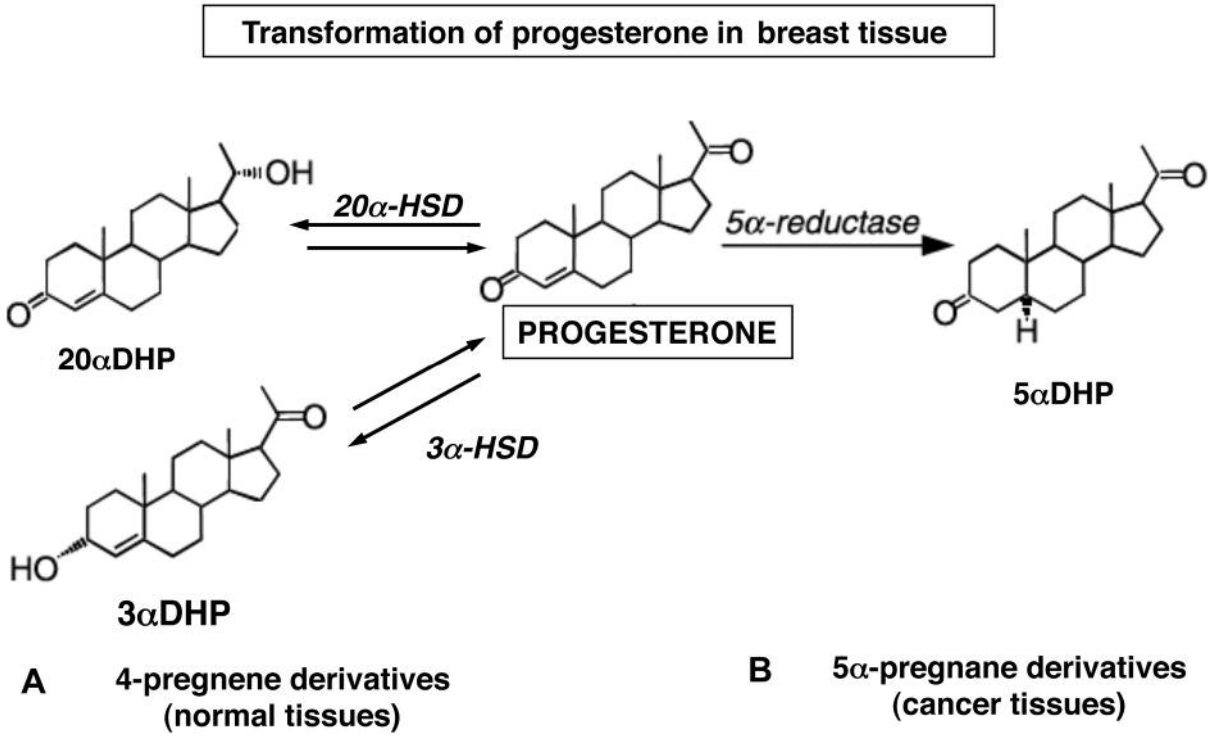

Figure 1. *Metabolic transformation of progesterone in human breast tissue. A) In normal breast tissue, progesterone is preferentially metabolized by reversible 20α- and 3α-hydroxysteroid dehydrogenase (20α-HSD and 3α-HSD, EC:1.1.1.49, AKRC1, oxido-reductase pathway) activities, leading to the 4-pregnene derivatives: 20α- and 3α-dihydroprogesterone (20αDHP, 3αDHP) respectively. These metabolites show beneficial biological activities with anti-proliferative effects in normal breast. B) In cancerous breast tissues, progesterone is preferentially metabolized by the irreversible 5α-reductase (EC:1.3.99.5, SRD5A1) pathway leading to the mean 5α-pregnane derivative: 5α-dihydroprogesterone (5αDHP). This metabolite has unfavourable biological activities with proliferative effects in tumoral breast tissues.*

observed in the conversion to E_2 (Figure 2). At the lower concentration (5×10^{-8} mol/l), 20αDHP exerted an inhibition of 36.5% on the production of [^{3}H]-E_2, while at the higher concentration (5×10^{-6} mol/l) the inhibition was 50.3%. In contrast, the 5-pregnane metabolite, 5αDHP, had no inhibitory effect at 5×10^{-8} mol/l and even showed a slight (but not significant) stimulatory effect at 5×10^{-6} mol/l (+5.2%). At both concentrations (5×10^{-8} and 5×10^{-6} mol/l) of progesterone had a weak but not statistically significant inhibitory effect (7.8% and 9.4%, respectively). This observation indicated that the conversion of progesterone to the 4-pregnene metabolites was not favored in these MCF-7aro cell culture conditions. Using non-linear regression analyses, the IC_{50} value, corresponding to the concentration of 20αDHP giving 50% inhibition of the aromatase activity *vs.* control ([^{3}H]-testo alone) was $4.56\pm0.22\times10^{-6}$ mol/l.

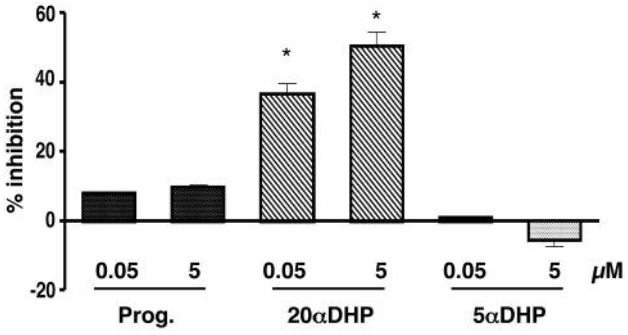

Figure 2. *Effect of progesterone (Prog.), 20α-dihydroprogesterone (20αDHP) and 5α-dihydroprogesterone (5αDHP) on the conversion of testosterone (Testos) to estradiol (E_2) in the MCF-7aro human breast cancer cell line. Preconfluent cells were incubated 24h at 37°C with a physiological concentration (5×10^{-9} mol/l) of [^{3}H]-Testos alone (control) or in the presence of Prog., 20αDHP or 5αDHP at the concentrations of 5×10^{-8} and 5×10^{-6} mol/l. Results are expressed in pmol of E_2 formed from Testos/mg DNA, and the percentage (%) of inhibition was obtained by calculating the ratio [(control–test)/control] ×100. The values are the mean±S.E.M. of duplicate determinations of three independent experiments. *$p\leq0.05$ vs. control value.*

Discussion

Recently, it has been demonstrated that in breast cancer cells or tissues, the expression of 5α-reductase mRNA is high and the conversion of progesterone to 5αDHP is favored and this

metabolite can increase the level of estrogen receptor (ER). On the other hand, 4-pregnenes are predominant in normal breast cell tissues or as the 3α- and 20α-hydroxysteroid dehydrogenase mRNA expressions are high. These 4-pregnene metabolites can decrease the level of ER (15-18). This tissue-specific localization of progesterone metabolism is very significant as it is known that 5αDHP stimulates proliferation and detachment of breast cells, thus potentially promoting mitogenesis and metastasis, whereas 20αDHP has the opposite effect by suppressing proliferation and cell migration (5, 6). The process of carcinogenesis is often accompanied by the modification of steroid metabolism, such as 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD; AKR1C3) which converts E_1 to the potent estrogen E_2 in hormone-dependent breast cancer cells, whereas the isozyme 17β-HSD type 2 preferentially transforms E_2 to the less potent E_1 in normal and hormone-independent breast cancer cells (19, 20).

The metabolism of steroid hormones represents an important feature by generating metabolites with either potentially protective or aggressive biological activities for the tissues. For instance in breast tissues, estrogens can be converted to catecholestrogen metabolites which have opposite effects. While 2-hydroxy-catecholestrogen has antiproliferative properties, others, such as 4-hydroxy and 16α-hydroxy derivatives, possess estrogenic properties and can be involved in carcinogenesis (21-26). It has been demonstrated that in breast cancer cell lines, progesterone down-regulates gene expression of catechol-*O*-methyl transferase (COMT), the enzyme which protects breast cells by detoxifying catecholestrogens (27). In this process, the two progesterone receptor isoforms, PR-A and PR-B, have opposite effects on the regulation of COMT expression: PR-A is associated with the up-regulation of COMT while PR-B with the down-regulation.

It has been reported that specific receptors, located at the plasma membrane level of breast cells (m5αDHPR and m20αDHPR) (28), bind the progesterone metabolites and can act as independent autocrine-paracrine steroid hormones for regulating, favorably or not, various physiological cell functions. Progesterone also possesses a specific membrane receptor (mPR) linked to cell signaling pathways, to initiate the non-genomic effect of the hormone, which is different from those for the metabolites (29). The modulation of m5αDHPR and m20αDHPR is dependent on the respective concentrations of 5α-pregnane and 4-pregnene derivatives. These observations could indicate that cross-talk signals can occur between membrane-initiated progesterone metabolite responses and the control of various crucial enzymatic pathways implicated in the metabolism of steroid hormones, as it is known that this type of regulation is often expressed in a cell-type-specific fashion with a tissue-specific patho-physiological outcome.

It is to be noted that dutasteride, a 5α-reductase inhibitor used in prostate cancer and benign prostate hyperplasia therapy for blocking the production of the biologically active 5α-dihydrotestosterone from testosterone, can reduce the conversion of progesterone to 5α-pregnanes by 95% (30).

In previous studies in this laboratory we have shown that E_2 can control its own bioformation by blocking the two key metabolic routes in breast cancer: the sulfatase pathway, for the conversion of estrone sulfate to E_2 in human hormone-dependent breast cancer cell lines and in breast tumor tissues (31, 32); and the aromatase pathway for the conversion of androgens to E_2 in MCF-7aro cells (33). The present study showed that the progesterone metabolites can be selectively involved in the control of aromatase activity in breast cancer cells and belong to the selective estrogen enzyme modulator (SEEM) family (34). Using the MCF-7aro breast cancer cell line which is rich in aromatase activity, it was clearly demonstrated that 20αDHP, a 4-pregnene metabolite, could act as an anti-aromatase agent, however 5αDHP, did not provoke any effect on this enzyme.

The specific action of 20αDHP on the aromatase pathway is very interesting as this compound possesses anti-proliferative properties (6). Consequently this progesterone metabolite might be involved in the control of estradiol production in the normal breast cell and might therefore be one of the multifactorial factors involved in breast carcinogenesis. In contrast the 5αDHP, mainly present in breast cancer tissue, had no effect on the aromatization of androgens. In addition it was observed that progesterone itself had very little or no effect on aromatase activity. The present information provided a further example of the importance of considering the metabolic transformation of the hormone in its biological response, which acts not only as the hormone itself, but also as a precursor for the production of active steroid hormone metabolites. It is also suggested that the utilization of 20αDHP could open new possibilities in the prevention and treatment of breast cancer.

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