

Increased extracellular local levels of estradiol in normal breast *in vivo* during the luteal phase of the menstrual cycle

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Abstract

Estrogen exposure is a major risk factor for breast cancer. Tissue estrogen originates from the ovaries but a significant portion is also produced by enzyme activity locally in the breast itself. How these enzymes are regulated is not fully understood. The extracellular space, where the metabolic exchange and cell interactions take place, reflects the environment that surrounds the epithelium but there has been no previous study of hormone concentrations in this compartment. In the present study microdialysis was used to measure extracellular estrogen concentrations in breast tissue and abdominal subcutaneous fat in 12 healthy women *in vivo*. It was found that women with high plasma progesterone levels had significant increased levels of

estradiol in breast tissue compared with fat tissue (breast tissue 168 ± 6 pM; subcutaneous fat, 154 ± 5 pM; $P < 0.05$), whereas women with low plasma progesterone exhibited no difference. Moreover, there was a significant correlation between local breast tissue estradiol and plasma progesterone levels ($r = 0.709$, $P < 0.01$). There was no difference in estrone sulphate in breast and fat tissue regardless of progesterone levels. Estrone was not detectable. The results in this study suggest that progesterone may be one regulator in the local conversion of estrogen precursors into potent estradiol in normal breast tissue.

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Introduction

Estrogen is a key regulator of normal breast physiology as well as a major risk factor for breast carcinogenesis (Anderson *et al.* 1982, Rossouw *et al.* 2002, Beral 2003). Several studies have suggested that the concentrations of estrogen in the breast not only originate from the ovaries but that the breast itself produces a significant portion of tissue estrogens (Geisler 2003). For example, in breast cancer tissue as well as in nipple aspirate from normal breast, tissue estrogen levels have been found to be significantly higher than plasma levels (Fishman *et al.* 1977, Pasqualini *et al.* 1996, Chatterton *et al.* 2003). All enzymes necessary for estrogen synthesis – aromatase, steroid sulphatase and 17β -hydroxysteroid dehydrogenase (17β -HSD) – have been shown to be present in breast tissue (Hawkins *et al.* 1985, Poutanen *et al.* 1995, Zhou *et al.* 1997, Chetrite *et al.* 2000, Chen *et al.* 2002). The breast contains epithelial cells, fibroblasts and fat cells and all these various tissues express enzymes and contribute to the synthesis and conversion of estrogens (Hawkins *et al.* 1985, Poutanen *et al.* 1995, Zhou *et al.* 1997, Chetrite *et al.* 2000, Chen *et al.* 2002).

Although several enzymes involved in estrogen biosynthesis are characterized, the knowledge of tissue-specific expression is limited. Studies of enzyme expression do not,

however, give the end result we are interested in, namely the tissue levels of the hormones. Moreover, most studies have not combined the analysis of the expression pattern of these enzymes with determinations of hormone levels and the few studies that exist have not found any consistent relationship between hormone levels and enzyme expression (van Landeghem *et al.* 1985, Vermeulen *et al.* 1986b). In previous studies of tissue concentrations of hormones, homogenate from surgical biopsies have been used. This approach has several methodological problems summarized in a recent review (Geisler 2003). In addition to the technical problems with tissue homogenate the results obtained by this technique will almost exclusively reflect intracellular levels of the hormones. The extracellular space is, however, the environment that surrounds the epithelial cells and the compartment where the metabolic exchange and cell interaction take place. Since the sources of estrogen are both systemic and local, the hormone concentrations in the extracellular compartment will reflect the total sum of hormones from various origins. Hence, a direct measurement of tissue estrogen levels in the target organ *in vivo* is needed. Microdialysis is a technique that mimics a blood vessel and with this technique it is possible to collect molecules from the interstitial space in a specific tissue or organ *in situ*. In this study microdialysis was used to determine the local levels

of estrogens in normal human breast tissue and abdominal subcutaneous fat in healthy women with a history of regular menstrual cycles.

Materials and Methods

Subjects

12 healthy pre-menopausal women, aged 22–34 years, participated in the study. All women were free of medication and had not taken medication containing sex steroids for more than 3 months. All women had a history of regular menstrual cycles (cycle length, 27–34 days). They had had no previous breast disease or surgery and the breasts were normal on clinical examination. The local ethical committee approved the study and all women gave informed consent.

Microdialysis device

A microdialysis catheter (CMA 65; CMA/Microdialysis AB, Solna, Sweden), which consists of a tubular dialysis membrane (10 mm long \times 0.52 mm in diameter; 100 000 Da cut-off) glued to the end of a double-lumen tube (80 mm long \times 0.8 mm in diameter), was used. The catheters were inserted guided by a catheter for intravenous use (Venflon 1.4 mm; BOC Ohmeda AB, Helsingborg, Sweden). The catheters were connected to a microinfusion pump (CMA 107; CMA/Microdialysis AB) and perfused with 154 mM NaCl and 40 g/l dextran-70, at a perfusion rate of 0.5 μ l/min. The solution entered the catheter through the outer tube and left it through the inner tube, from which it was collected. After a 30-min equilibration period, the outgoing perfusate was collected and stored at -70°C for subsequent analysis.

Experimental design

Microdialysis was performed in the luteal phase in eight women and in the follicular phase in four women. Blood samples were collected from the women at each occasion to determine estradiol and progesterone to confirm the menstrual phases (estradiol and progesterone ELISA kits; DRG Instruments GmbH, Marburg, Germany).

Mepivacaine (5 mg/ml) was administered intracutaneously as a local anaesthetic prior to the insertion of the microdialysis catheters. One microdialysis catheter was placed in the upper lateral quadrant of the left breast and directed towards the nipple as previously described (Dabrosin *et al.* 1997a, 1997b, Dabrosin 2001, 2003). To be able to compare tissue levels collected by the microdialysis technique a reference catheter was placed in abdominal subcutaneous fat lateral to the umbilical region.

Microdialysis is a technique that allows continuous sampling of the extracellular space by passive diffusion of

substances over a semi-permeable membrane. The recovery – that is, the amount of substances that diffuse into the perfusion fluid – depends on the membrane properties, the flow rate and the size of the compound of interest (Ungerstedt 1991). Diffusion of low-molecular-mass substances over the dialysis membrane is almost complete at low flow rates using a 30 mm-long dialysis membrane (Rosdahl *et al.* 1998). However, for larger molecules the recovery over the membrane decreases and the measured levels in the microdialysis sample will not be absolute concentrations in the tissue. One way of estimating the recovery is to put the catheter into a test tube with a solution containing the molecule of interest and to measure the amount of substance leaving the catheter. This *in vitro* recovery can only be an estimate of the *in vivo* recovery since other factors such as tissue pressure and temperature will affect the diffusion of substances. In the case of sex steroids there is an almost complete recovery *in vitro* of the compound due to the low molecular mass of approximately 300 Da. However, *in vivo* 97–99% of these steroids are protein-bound and the whole complex will diffuse over the membrane. To estimate the recovery of estrogens, plasma was used as the solution to put the catheter in *in vitro* instead of using the hormones dissolved in a solution. With this approach the *in vitro* recovery could be measured of both protein-bound and free estrogens. The *in vitro* recovery of estradiol was $69 \pm 1.3\%$, and the recovery of estrone and estrone sulphate were close to 0. This illustrates the complexity of the system and that the *in vitro* recovery can only be a rough estimate of the *in vivo* recovery since estrone sulphate was detectable in all microdialysis samples collected *in vivo*. In this case the main interest was to compare the levels in breast and fat tissue in the same individual and not to calculate the absolute tissue levels. Therefore all microdialysis values are given as original raw data without any re-calculations.

Estradiol, estrone and estrone sulphate determinations

Plasma was collected on each occasion using a glass tube containing 3.8% sodium citrate as an anticoagulant, and were centrifuged and frozen at -70°C within 20 min of collection.

Microdialysate and plasma samples were analyzed for estradiol using a commercial quantitative immunoassay kit (DRG Instruments). According to the manufacturer the sensitivity of this kit is 16.8 pM and intra-assay variation is 5%.

Estrone and estrone sulphate were analyzed using RIA (Diagnostic System Laboratories, Webster, TX, USA). According to the manufacturer the estrone kit has a sensitivity of 4.44 pM and an intra-assay variation of 5% and the estrone sulphate kit has a sensitivity of 0.028 nM and an intra-assay variation of 4.6%. All kits were designed for a non-extracting procedure, which is an advantage when analysing the very low (microliter) volumes that are

generated during microdialysis. When designing a microdialysis experiment with an adequate recovery over the microdialysis membrane a flow rate of approximately 0.5 µl/min is required (Rosdahl *et al.* 1998). An extraction procedure usually requires a milliliter sample volume, which is not achievable with this approach. The immunoassays developed and validated (Ranadive *et al.* 1998, Kraemer *et al.* 2003) for estrogen determinations provide therefore a possibility for measurements of estrogens in microdialysis samples collected directly from a tissue *in situ*. The assays used in the present study were validated against the immunoassays used for routine patient samples at the Laboratory for Clinical Chemistry at Linköping University Hospital. Moreover, quality-control samples and known plasma samples were included in each assay run and calculation of the results was performed using standard curves generated in each run. By this approach the intra-assay variations given by the manufacturer were confirmed in my laboratory. To avoid an inter-assay variation all samples were analysed in the same run. The samples were assayed in duplicate, and the mean of the two values calculated and presented in the Results section.

Statistics

Data are expressed as means ± S.E.M. Student's *t*-test for paired observations and simple linear regression were used as appropriate. $P < 0.05$ was considered statistically significant. The study comprised 12 women and all women served as their own control to improve the power of the study.

Results

Characteristics

Plasma progesterone levels were used to verify the menstrual phase of the cycle. The normal progesterone levels in the luteal phase ranged from 13 to 79 nM. Eight of the 12 women had progesterone levels above the cut-off limit, ranging from 24 to 43 nM. The other four had progesterone levels between 1.1 and 10 nM. The characteristics of the women are summarized in Table 1.

Elevated breast tissue estradiol in women with high plasma progesterone

In women with elevated progesterone levels, the breast tissue levels of estradiol were significantly higher than estradiol in subcutaneous fat: breast tissue estradiol was 168 ± 6 pM and that of subcutaneous fat was 154 ± 5 pM ($P < 0.05$; Fig. 1A). Three out of four women with low plasma progesterone had lower levels of estradiol in breast tissue compared with fat but there was no significant difference by these four women since one had higher

Table 1 Hormone levels in plasma of 12 pre-menopausal women

Subject	Estradiol (pM)	Progesterone (nM)	Estrone sulphate (nM)	Estrone (pM)
1	262	43	8.2	165
2	213	24	6.4	166
3	251	43	6.5	149
4	337	32	10	405
5	205	40	7.8	184
6	268	37	7.7	166
7	214	40	8.5	150
8	387	31	5.1	182
9	247	1.4	11	219
10	331	1.1	6.6	172
11	225	1.2	6.0	172
12	181	10	5.4	111

levels in breast compared with fat ($P > 0.05$; Fig. 1B). This result should, however, be interpreted with care due to the low number of women in this group. There was a wide range of tissue levels between the individual women; therefore, breast tissue estradiol was calculated as a percentage of fat estradiol in the same individual. A significant correlation was found between the amount of breast tissue estradiol/fat tissue estradiol and plasma progesterone levels ($P < 0.01$; Fig. 2).

There were no differences between estrone sulphate levels regardless of plasma progesterone (breast tissue, 0.054 ± 0.018 nM; fat, 0.222 ± 0.129 nM; $P = 0.4$). Estrone was not detectable in the microdialysis samples.

Discussion

This study showed that women with high plasma progesterone levels, verifying the mid-luteal phase of the menstrual cycle, had significantly higher extracellular levels of estradiol in breast tissue *in vivo* compared with subcutaneous fat. In women with low progesterone levels, representing non-ovulating women or women in the follicular phase, there was no difference in estradiol levels between breast tissue and subcutaneous fat. Plasma progesterone correlated significantly with local breast tissue estradiol. Together these results suggest that progesterone affects the local conversion of estrogens in normal breast tissue. Moreover, this study introduces microdialysis for measurement of local tissue levels of estrogens.

The 17β-HSD enzymes catalyze the interconversion of low-activity estrogens such as estrone and high-activity estrogens such as estradiol. At least 10 isoenzymes of 17β-HSD have been characterized, which differ in their reductive and oxidative catalyzing capacities (Adamski & Jakob 2001, Geisler 2003). The isoenzyme 17β-HSD type 1 seems to be responsible for the conversion of estrone to

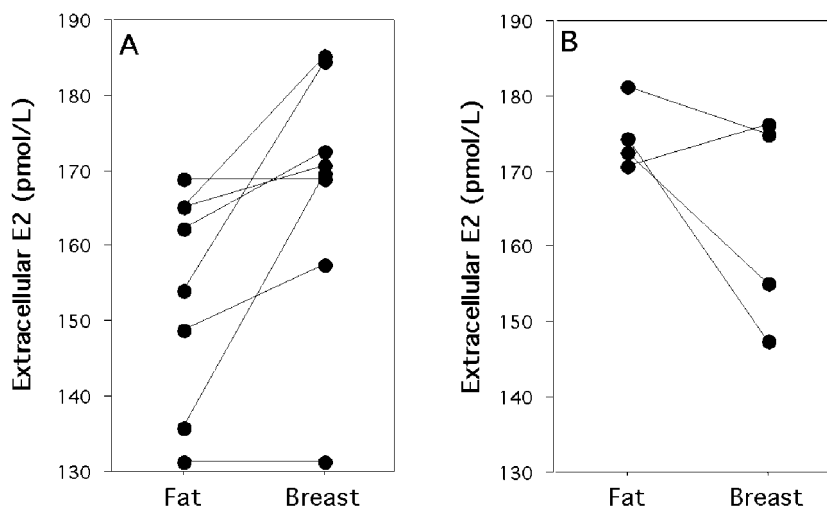


Figure 1 Extracellular estradiol in breast and fat tissue. (A) Tissue estradiol (E2) in eight women with plasma progesterone levels, representing the luteal phase of the menstrual cycle. Levels were significantly increased in breast tissue (168 ± 6 pM) compared with subcutaneous fat (154 ± 5 pM; $P < 0.05$). (B) Tissue estradiol in four women with low plasma progesterone, representing the follicular phase of the menstrual cycle. There was no significant difference between the levels; breast tissue, 163 ± 7.1 pM; fat tissue, 174 ± 2.3 pM ($P = 0.2$).

estradiol and therefore is considered as the main factor contributing to high tissue levels of estradiol in breast cancer (Vermeulen *et al.* 1986a). Several of the 17β -HSD enzymes have also been detected in normal breast tissue (Soderqvist *et al.* 1998, Miettinen *et al.* 1999) but previous investigations have not found any correlation between

enzyme expression and hormone levels (van Landeghem *et al.* 1985, Vermeulen *et al.* 1986b). This emphasizes the need for determination of the estrogen levels in the target organ before any interpretations of the physiological relevance of the expression of various enzymes can be made. As shown in this study, microdialysis can be used for *in vivo*

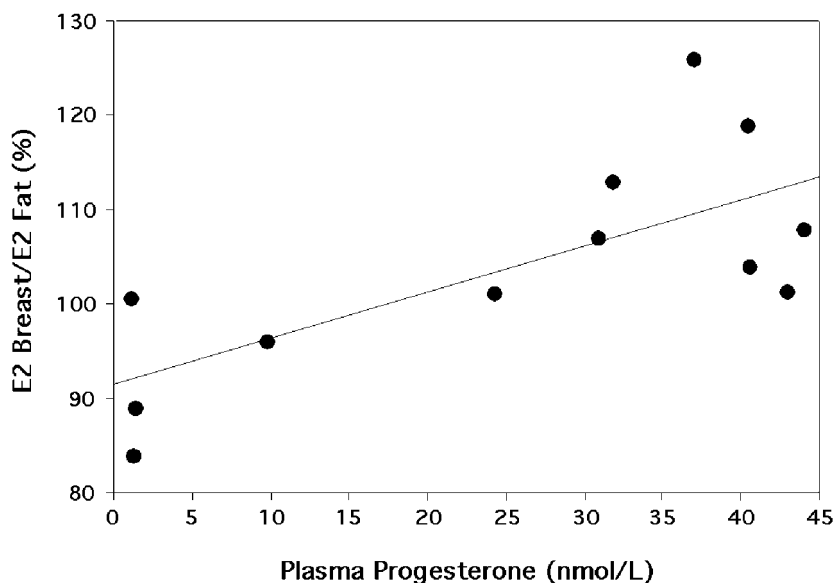


Figure 2 Correlation between plasma progesterone and the percentage of breast tissue estradiol (E2) to fat tissue estradiol. Linear regression between the percentage of breast estradiol/fat estradiol and plasma progesterone levels with progesterone as an independent variable and tissue estradiol as a dependent variable: $r = 0.709$, $t = 3.18$, $P < 0.01$.

measurements of estrogens and will be of significant value in future studies in this field of research.

In the present study the women with high progesterone levels exhibited higher breast tissue levels of estradiol compared with fat tissue. This may suggest increased enzyme activities that favour the conversion of estrone to estradiol under the influence of progesterone. This is in line with previous studies which have shown a positive correlation between serum progesterone levels and expression of 17 β -HSD type 1 in breast cancer tissue (Poutanen *et al.* 1990, 1992a, 1992b). However, there is a controversy of progestin regulation of this enzyme since other authors have demonstrated decreased reductive activity of 17 β -HSD leading to decreased formation of estradiol after exposure of progestins to breast cancer cells in culture (Chetrite & Pasqualini 2001). These studies have been performed in culture of breast cancer cells using relatively high concentrations of synthetic progestins and should be interpreted with caution regarding the regulation of these enzymes in normal breast tissue *in vivo* exposed to physiological levels of the natural occurring progesterone.

The 12 women in this study did not exhibit any difference in estrone sulphate concentrations between breast and fat tissue regardless of plasma progesterone concentrations. Although estradiol is the most potent of the endogenous estrogens and has been shown to be the most important determinant of breast cancer risk (Key 1999), estrone sulphate is the most abundant estrogen and the major precursor for local biosynthesis of estradiol. It has been shown that the estrone sulphatase activity is high in estrogen receptor positive breast cancer cell lines whereas hormone-independent cell lines express very low sulphatase activity (Chetrite & Pasqualini 2001). In normal breast tissue, adjacent to tumour tissue, estrone sulphatase activity has been shown to be elevated compared with the tumour levels (Chetrite *et al.* 2000). This suggests that normal breast tissue expresses sulphatase activity but very little is known about its regulation. The present data do not suggest any difference in the activity of estrone sulphatase in breast tissue compared with fat tissue but this has to be investigated further before any conclusions can be made. Estrone was not detectable with the 10 mm-long dialysis membrane used in this study. This could probably be overcome in future studies by using a longer microdialysis membrane and thereby increasing the recovery of estrone over the membrane.

The regulation of the enzymes involved in local biosynthesis of estrogen in peripheral tissues is complex and not fully understood. All the different cell types that exist in normal breast tissue and breast cancer including tissue macrophages and lymphocytes can regulate the enzymes involved in estrogen formation and inactivation. Several cytokines secreted by these cell types on a local level have been shown to influence the expression of 17 β -HSD, estrone sulphatase and aromatase (Turgeon *et al.* 1998, Purohit *et al.* 2002, 2003). This complex regulation

necessitates research on whole tissue, preferably *in vivo*, rather than cell culture studies. Moreover, very few studies have combined detection and quantification of enzyme activities with actual measurements of hormone levels, partly because of a lack of a suitable technique for these determinations. The microdialysis technique opens up new possibilities in such investigations.

High tissue levels of estradiol have to be considered as breast cancer-promoting for both their effects on proliferation and other non-receptor-mediated effects of estradiol, such as the carcinogenic effect of the metabolite 4-hydroxyestradiol (Liehr 2000). The high levels of estradiol in breast tissue in the luteal phase and the correlation between local breast tissue levels of estradiol and plasma progesterone shown in this study suggest that progesterone may be one regulator of local conversion of estrogen precursors into potent estradiol in normal breast tissue.

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