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The regulation of oestrone sulphate formation in breast cancer cells

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Abstract

The formation of oestrone sulphate has been examined in MCF-7 (oestrogen receptor positive, ER+) and MDA-MB-231 (ER negative, ER−) breast cancer cells. Using intact cell monolayers and a physiological substrate concentration, progesterone (1 μM) and dexamethasone (1 μM) both increased oestrone sulphate formation in MCF-7 cells. In MDA-MB-231 cells, dexamethasone, but not progesterone, increased conjugate formation. A number of growth factors, cytokines and human serum albumin (HSA), which have previously been found to regulate oestrogen synthesis, were also examined for their ability to regulate oestrone sulphate formation. In MCF-7 cells epidermal growth factor, acidic and basic fibroblast growth factors, insulin-like growth factor-type I and insulin all stimulated oestrone sulphate formation. The cytokines, tumour necrosis factor α (TNFα) and interleukin-1β, also increased conjugate formation in the ER+ cells, as did HSA. In contrast, in MDA-MB-231 cells TNFα was without effect and HSA inhibited oestrone sulphate formation. The ability to modulate oestrone sulphate formation in ER+ cells may be an important mechanism to limit the availability of oestrogen to interact with the ER.

1. Introduction

The formation and hydrolysis of oestrogen sulphates within breast tumours may be an important mechanism to regulate the availability of oestrogen to interact with the oestrogen receptor (ER). Steroid sulphates are formed by the transfer of a sulphonate group from 3-phosphoadenosine-5′phosphosulphate to a suitable hydroxysteroid substrate [1]. It was originally thought that the formation of steroid sulphates represented the end products of metabolic inactivation. There is now evidence, however, that they may be an important storage form for steroids from which the unconjugated steroid can be liberated by the action of oestrone sulphatase (E1-STS) [2,3]. Concentrations of oestrogen sulphates in blood [4] and in normal and malignant breast tissues [5] are much higher than those of unconjugated oestrogens, and furthermore their half-life in blood (10–12 h) is considerably longer than for unconjugated oestrogens (20–30 min) [6].

The enzymes which are involved in oestrogen synthesis in breast tumours, i.e. the aromatase, oestradiol 17β-hydroxysteroid dehydrogenase (E2DH) and E1-STS, are now known to be regulated by growth factors, cytokines and prostaglandins [7,8]. These factors appear to act in a co-ordinated manner to increase the activities of all three enzymes and such a mechanism probably accounts for the high concentrations of oestradiol which are present in breast tumours [9,10].

In contrast to the information which has been gained about the regulation of the enzymes that synthesise oestrogens, little is known about the control of the sulphotransferases (ST) which convert steroids to their sulphated forms. Cytosols from breast cancer cells have previously been found to be able to convert oestrogens to their water-soluble sulphate forms [11] and oestradiol was reported to stimulate sulphate formation in MCF-7 breast cancer cells [12].

Several different ST are present in body tissues and

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include a specific ST for oestrogens (EST), hydroxysteroids (HST), such as dehydroepiandrosterone (DHA), and two forms of phenosulphotransferases (PST), the phenol sulphating P-PST and the monoamine sulphating form M-PST [13]. While the latest molecular biological techniques have helped to resolve the complex nature of the ST superfamily, there is still some debate as to which ST are present in breast cancer cells. At micromolar concentrations P-PST has been reported to be the main enzyme responsible for oestrogen sulphate formation in MCF-7 cells with no EST or HST being detected in cytosols from these cells [14,15].

Some years ago Dao and Libby originally concluded that the ability of breast tumour homogenates to form sulphates of DHA and oestrogens was associated with a favourable response to endocrine or ablative therapy [16–18]. This finding suggested that the activity of the enzyme might reflect the ER status of the tumour. The results from subsequent investigations relating the presence of ER to ST activity in breast cancer cells have generally shown higher activity in ER+ cells compared with that in ER− breast cancer cells. The ER− MDA-MB-468 cell line, however, has been reported to possess high ST activity [19].

As little is known about the regulation of oestrogen sulphate formation in oestrogen receptor positive (ER+) or negative (ER−) breast cancer cells, the ability of steroids, growth factors and cytokines to modulate the level of oestrogen sulphate formation in these cells was examined. For these investigations oestrone sulphate formation was measured in intact cell monolayers using a physiological substrate concentration.

2. Materials and methods

2.1. Cell culture

MCF-7 (ER+) and MDA-MB-231 (ER−) breast cancer cells, obtained from the American Type Culture Collection (Rockeville, MD), were routinely cultured in 25 cm^2 tissue culture flasks in Eagle's modified MEM with Hepes buffer (20 mM). This medium was supplemented with L-glutamine (2 mM), sodium hydrogen carbonate (10 mM), 1% non-essential amino acids and 5% (v/v) fetal calf serum (FCS). Cells were grown in this medium until approximately 40% confluent. Before adding test compounds cells were washed twice in phosphate-buffered saline and cultured in phenol red-free medium (MEM-AUTO POW: ICN Flow Biomedicals, Thame, Oxon, UK) supplemented as previously described with the exception that stripped FCS (SFCS) was used in place of FCS.

2.2. Treatments

Steroids were obtained from Sigma (Poole, Dorset, UK) and added to the SFCS medium in ethanol to give a final ethanol concentration of less than 0.1% (v/v). Cytokines and growth factors were obtained from Bachem (Cambridge, UK) and human serum albumin (HSA) from Calbiochem (Nottingham, UK). Cells were exposed to treatments for either 24 ± 2 or 48 ± 2 h before assaying for oestrone sulphate formation.

2.3. Measurement of oestrone sulphate formation in intact cell monolayers

The method used to measure oestrone sulphate formation in intact cell monolayers was an adaptation of a method previously developed to assay E1-STS in these cells [20]. For this, treatments were removed and cells washed with phosphate-buffered saline before the addition of [6,7-^{3}H]oestrone (1×10^5 dpm, 1–2 nM, Amersham, Aylesbury, Berks, UK) to each flask of cells. Intact cells were incubated with this substrate at 37°C for 20 h in the phenol red-free medium which lacked SFCS. At the end of the incubation period, medium (1 ml) was removed from each flask and extracted with toluene (4 ml). After removal of the toluene, aliquots (0.5 ml) of the aqueous medium were counted using a scintillation spectrometer. The remaining medium in the cell culture flasks was discarded and cell numbers determined using a Coulter counter. Results are expressed as the amount of product formed/million cells/20 h. Experiments were carried out at least twice with representative results being shown. All treatments were carried out in triplicate with results expressed as means $\pm$ SDs.

2.4. Statistics

The significance of differences in oestrone sulphate formation in cells resulting from the factors tested to that of untreated controls was assessed using Student's *t*-test.

3. Results

3.1. Assay validation

To confirm that the toluene used to remove unconjugated oestrogens from the reaction mixture was effective, ^{3}H oestrone sulphate and ^{14}C oestrone were added to the medium (1 ml) used for the assay incubation. Extraction with toluene (4 ml) revealed that 98.2 ± 5.6% of the ^{14}C oestrone was recovered in the toluene but that no ^{3}H oestrone sulphate was detected in the organic phase.

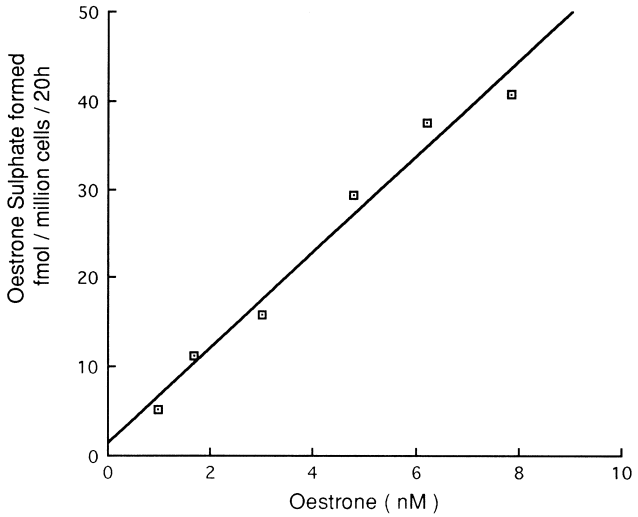

Fig. 1. Formation of oestrone sulphate in MDA-MB-231 cells was linear with respect to substrate concentration during a 20 h assay.

As it is possible that breast cancer cells possess other enzymes that are capable of rendering oestrogens water soluble (e.g. glucuronyltransferase), an experiment was carried out to confirm the sulphate nature of the product formed in the present investigations. After incubating ^{3}H oestrone with cells for a 20 h period the medium was removed and extracted with toluene to remove untransformed substrate. Aliquots of the remaining aqueous medium were then incubated for a further 2 h with a placental microsome preparation (1 mg), a rich source of E1-STS, in the absence or presence of a specific oestrone sulphatase inhibitor, oestrone-3-*O*-sulphamate (10 μM) [3]. Liberated steroids were extracted with diethyl ether and, after evaporation of the solvent, the residues were subjected to thin-layer chromatography (tlc) (dichloromethane:ethyl acetate 80:20 v/v) using oestrone as a reference standard. For the aqueous medium incubated with placental microsomes over 90% of the radioactivity eluted from the tlc plate had an identical mobility to that of oestrone. In contrast, for the medium incubated with placental microsomes in the presence of oestrone-3-*O*-sulphamate, only a background level of radioactivity was detected in the area of the tlc plate corresponding to oestrone. These results indicate that the polar product in the aqueous phase after incubation with ^{3}H oestrone is oestrone sulphate.

Using [6,7-^{3}H]oestrone as a substrate, the formation of oestrone sulphate by MDA-MB-231 cells was shown to be linear over the 1–8 nM range (Fig. 1). A similar result was also obtained using MCF-7 cells (data not shown).

3.2. Effect of steroids on oestrone sulphate formation

Oestradiol (1 nM) had only a minor effect on oestrone sulphate formation in MCF-7 cells (Fig. 2). In contrast, progesterone (1 μM) increased oestrone sulphate formation after 24 h (+96%) and 48 h (+80%) treatment. The combination of oestradiol plus progesterone resulted in a further increase in oestrone sulphate formation (+26% after 24 h, $p < 0.01$, and +61% after 48 h, $p < 0.001$) compared with the effect of progesterone. Dexamethasone (1 μM) also significantly increased oestrone sulphate formation in these cells with a greater stimulatory effect being detected after 48 h.

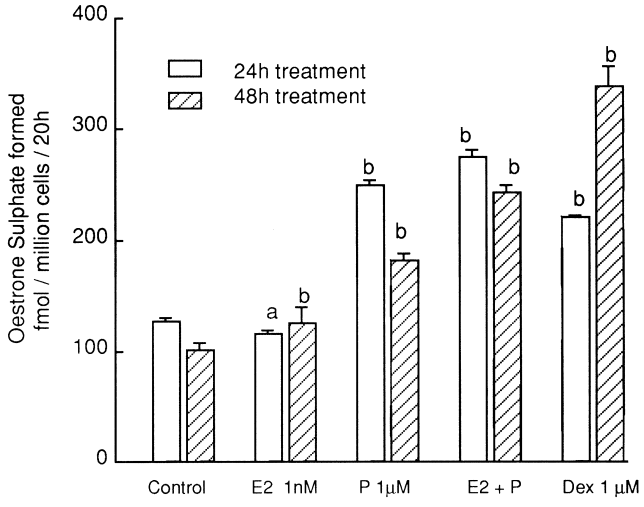

Fig. 2. Effect of treatment for 24 h or 48 h with oestradiol (E2), progesterone (P), oestradiol plus progesterone (E2 + P) or dexamethasone (Dex) on oestrone sulphate formation in MCF-7 breast cancer cells incubated with [6,7-^{3}H] oestrone (1–2 nM). (Means ± SD, $n = 3$; a, $p < 0.01$; b, $p < 0.001$ versus appropriate controls.)

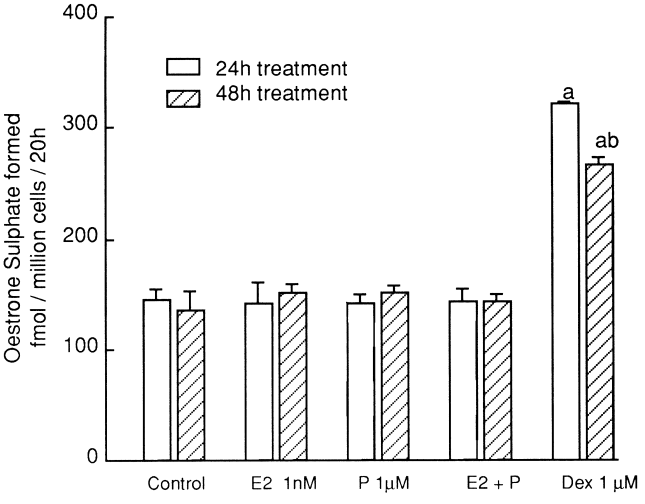

Fig. 3. Effect of treatment for 24 h or 48 h with oestradiol (E2), progesterone (P), oestradiol plus progesterone (E2 + P) or dexamethasone (Dex) on oestrone sulphate formation in MDA-MB-231 cells incubated with [6,7-^{3}H] oestrone (1–2 nM). (Means ± SD, $n = 3$; a, $p < 0.001$ versus controls; b, $p < 0.001$ versus 24 h treatment.)

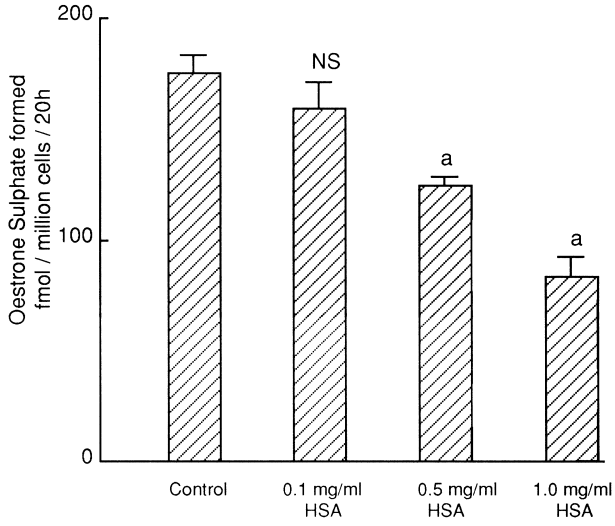

Fig. 4. Effect of treatment for 48 h with human serum albumin (HSA) on oestrone sulphate formation in MDA-MB-231 cells incubated with [6,7-^{3}H] oestrone (1–2 nM). (Means ± SD, $n = 3$; NS, not significant; a, $p < 0.001$ versus controls.)

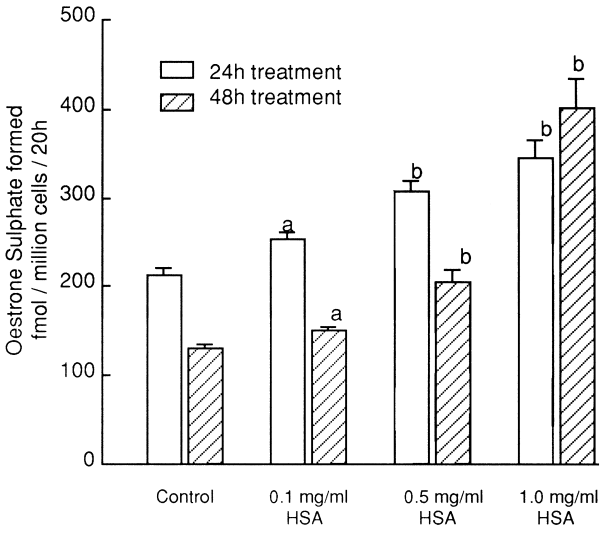

Fig. 6. Effect of treatment for 24 h or 48 h with human serum albumin (HSA) on oestrone sulphate formation in MCF-7 breast cancer cells incubated with [6,7-^{3}H] oestrone (1–2 nM). (Means ± SD, $n = 3$; a, $p < 0.01$ versus controls; b, $p < 0.001$ versus controls.)

As anticipated neither oestradiol nor progesterone were able to stimulate oestrone sulphate formation in the ER−/PR− MDA-MB-231 cells, although dexamethasone did increase its formation (Fig. 3). Basal sulphotransferase activity tended to be higher in the ER− than ER+ cells, as illustrated for the untreated cells in Figs. 2 and 3, but some variation in basal activity was detected in the different experiments.

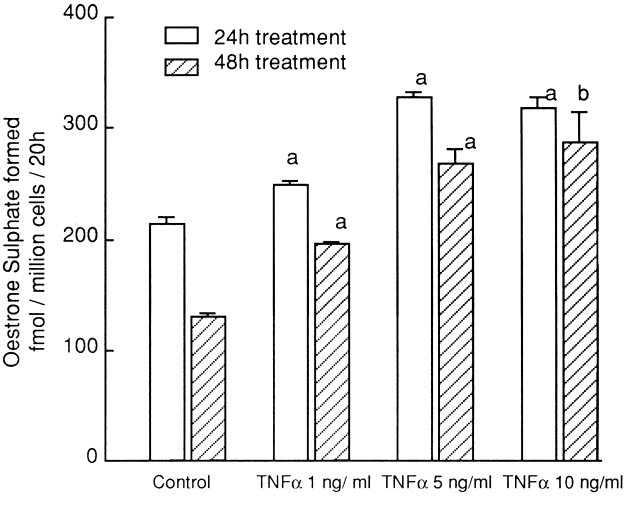

Fig. 5. Effect of treatment for 24 h or 48 h with tumour necrosis factor α (TNFα) on oestrone sulphate formation in MCF-7 breast cancer cells incubated with [6,7-^{3}H] oestrone (1–2 nM). (Means ± SD, $n = 3$; a, $p < 0.001$ versus controls; b, $p < 0.01$ versus controls.)

3.3. Effect of growth factors, cytokines and HSA on oestrone sulphate formation

In MDA-MB-231 cells, TNFα (1–10 ng/ml) failed to alter oestrone sulphate formation (data not shown) whereas HSA inhibited formation in a dose-dependent manner (Fig. 4), causing a 52% reduction in activity at the highest concentration tested.

Most of the investigations to examine the ability of growth factors and cytokines to modulate oestrone sulphate formation were carried out using MCF-7 cells. In these cells TNFα stimulated oestrone sulphate formation in a dose- and time-dependent manner with maximal stimulation being achieved at a concentration of 5 ng/ml after a 48 h treatment period (Fig. 5). Co-treatment of cells with TNFα and the protein synthesis inhibitor cycloheximide (2 μM) abolished the ability of this cytokine to increase oestrone sulphate formation (data not shown). The addition of HSA to MCF-7 cells also resulted in a dose-dependent increase in oestrone sulphate formation with maximal stimulation being produced with 1 mg/ml of 62% at 24 h and 208% at 48 h (Fig. 6). As previously found for TNFα, cycloheximide was able to block the HSA stimulation of oestrone sulphate formation in these cells.

The ability of a range of growth factors and cytokines to stimulate oestrone sulphate formation was also examined (Table 1). EGF, aFGF, bFGF, IGF-I and insulin all stimulated its formation (38–149%), whereas TGFα and IGF-II were without effect at the concentration tested. IL-1β also increased conjugate formation whereas IL-2 and IL-6 had no effect. As it has previously been shown that bFGF abolishes the ability of HSA to stimulate E1-STS activity [21], the

Table 1
Effect of growth factors, cytokines or human serum albumin (HSA) on oestrone sulphate formation in MCF-7 breast cancer cells incubated with [6,7-^{3}H] oestrone (1–2 nM) (means ± SD, $n = 3$)

Factor	Concentration	Oestrone sulphate formed (fmol/million cells/20 h)	P
Control	–	174 ± 9	
EGF	100 ng/ml	240 ± 23	< 0.01
TGFα	100 ng/ml	172 ± 8	N.S.
IGF-I	100 ng/ml	434 ± 61	< 0.01
IGF-II	100 ng/ml	187 ± 1	N.S.
Insulin	1 μg/ml	310 ± 20	< 0.001
aFGF	100 ng/ml	281 ± 23	< 0.01
bFGF	100 ng/ml	336 ± 27	< 0.01
HSA	1 mg/ml	528 ± 24	< 0.001
bFGF + HSA	100 ng/ml + 1 mg/ml	437 ± 37	< 0.001
IL-1	100 ng/ml	389 ± 20	< 0.001
IL-2	100 ng/ml	197 ± 23	N.S.
IL-6	100 ng/ml	183 ± 1	N.S.

effect of this growth factor on the HSA stimulation of oestrone sulphate formation was examined in the present study. While bFGF did reduce the ability of HSA to stimulate oestrone sulphate formation, by 17%, its stimulatory effect was by no means abolished.

3.4. Effect of steroids, growth factors and cytokines on cell growth

Cell numbers were routinely measured in order to normalise the results. However, over the short 24 or 48 h treatment periods, no significant effects on cell growth were detected.

4. Discussion

In the present investigation an intact cell monolayer system was used to examine the control of oestrone sulphate formation by breast cancer cells. This technique has been successfully used to investigate the regulation of aromatase, E2DH and E1-STS activities in breast cancer cells [20–23]. Previous investigations into the regulation of oestrone sulphate formation in breast cancer cells have used cell cytosol preparations and/or unphysiological oestrogen substrate concentrations.

The control of oestrogen sulphate formation by oestradiol and progesterone in breast cancer cells has previously been examined [12]. Changes in oestrogen sulphate formation in the human uterus, which reaches a peak during the secretory phase of the menstrual cycle, initially suggested a role for progesterone in regulating EST activity [24,25]. Using a cytosol preparation from MCF-7 cells, oestradiol increased oestrogen sulphate formation, whereas progesterone was found to be ineffective [12]. In the present investigation, while oestradiol had little effect on oestrogen sulphate formation in MCF-7 cells, progesterone was found to markedly increase its formation in these cells. While sulphotransferase activity tended to be higher in the MDA-MB-231 cells, the difference between the two cell types was relatively small. Consistent with the lack of an ER/PR in MDA-MB-231 cells, no increase in oestrone sulphate formation was detected after treatment with either oestradiol or progesterone. Dexamethasone, however, increased oestrone sulphate formation in both ER+ and ER− cells in keeping with the ubiquitous expression of glucocorticoid receptors. The endogenous glucocorticoid, cortisol, has been shown to increase the expression of P-PST mRNA in bovine tracheobronchial cells [26].

Growth factors, such as EGF and IGF-I, are important regulators of aromatase, E2DH and E1-STS activities in breast tumour-derived fibroblasts or breast cancer cells [22,27,28]. In the present study, EGF, insulin, IGF-I, aFGF and bFGF were all found to significantly enhance oestrone sulphate formation by 38% to 152% in MCF-7 cells, whereas TGFα and IGF-II had no significant effect. However, the most potent stimulator of oestrone sulphate formation was HSA (203%). This protein has previously been found to stimulate steroidogenesis in breast cancer, ovarian and testicular cells [29–31]. For E1-STS, bFGF blocks the stimulatory effect of HSA in this enzyme, but bFGF did not have a similar role in blocking the HSA stimulation of oestrone sulphate formation.

In contrast to the stimulatory effect of HSA on oestrone sulphate formation in MCF-7 cells, it inhibited formation in MDA-MB-231 cells. This is the first report, as far as we are aware, of HSA having an inhibitory effect on a steroid metabolising pathway. It still remains to be resolved as to whether albumin itself, or a molecule bound to the protein, is responsible for the effects of this protein on steroid synthesis.

In addition to growth factors, a number of cytokines

are emerging as having important roles in regulating aromatase, E2DH and E1-STS activities in breast cancer cells [7]. In the present study, IL-6, which can markedly stimulate aromatase [22] and E2DH [32,33] activities, had little effect on oestrone sulphate formation in MCF-7 cells. In contrast, IL-1β and TNFα, which both stimulate aromatase [22] and E2DH [33] activities, also stimulated oestrone sulphate formation in MCF-7 cells. In contrast, TNFα had no effect on oestrone sulphate formation in MDA-MB-231 cells, reflecting the resistance of these cells to the effects of TNFα [34]. In MCF-7 cells, treatment with cycloheximide, while having no effect on basal activity, completely abolished the ability of TNFα or HSA to stimulate oestrone sulphate formation. This indicates that both TNFα and HSA are increasing oestrone sulphate formation by inducing the synthesis of new protein.

In summary, a number of factors which have previously been found to stimulate the synthesis of oestrogens in breast cancer cells also appear to regulate the formation of oestrone sulphate in these cells. It is possible, however, that rather than simply inactivating oestrogens, such a mechanism provides an abundant supply of oestrogen sulphate within breast cancer cells where it can be acted on by E1-STS to form unconjugated oestrogens which can interact with the ER. There is some evidence, however, that oestrogen sulphate formation may be lower in malignant than in normal breast cells [35,36] and it is possible that ST activity could be lost during the malignant transformation [36]. It will therefore be important to compare the regulation of oestrone sulphate formation in normal and malignant breast epithelial cells as this may provide important new clues about the role of oestrogen sulphate formation in breast tumour development.

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