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Effects of black cohosh on estrogen biosynthesis in normal breast tissue *in vitro*

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Abstract

Objectives: To investigate the effect of black cohosh on the estrogen biosynthesis in the breast *in vitro*.
Methods: Steroid sulfatase (STS) activity was studied in normal breast tissue obtained from pre- and postmenopausal women undergoing reduction mammoplasty. STS protein expression was studied by immunohistochemistry and western blotting. Breast tissue was incubated *in vitro* without or with black cohosh (iCR) at concentrations ranging from 0.1 mg/ml to 1 ng/ml. STS activity was evaluated by incubating homogenized breast tissue with [^{3}H]-estrone sulfate, separating the formed products, estrone (E_1) and estradiol (E_2), by thin layer chromatography and measuring the amounts of E_1 and E_2 by scintillation counting.
Results: STS protein expression and enzymatic activity were detected in all specimens investigated. In all groups, significantly more E_1 than E_2 was produced. Local estrogen formation was decreased in premenopausal breast tissue by treatment with iCR at 0.1 mg/ml ($p \leq 0.05$).
Conclusions: iCR decreases local estrogen formation in normal human breast tissue *in vitro*. This may contribute to the lack of hormonal effects of black cohosh in breast tissue observed in previous studies.

Keywords: Breast; Black cohosh; *Actaea racemosa*; *Cimicifuga racemosa*; Sulfatase activity

1. Introduction

Postmenopausal hormone therapy (HT) has been seen as a specific treatment for climacteric symptoms in the short term and as a prevention of chronic diseases in the long term [1–3]. However, several clinical trials have indicated an increased risk of breast cancer (BC) associated with HT [4–8]. In recent years, extracts of the rhizome of black cohosh (*Actaea*, syn. *Cimicifuga racemosa*) (CR) have been recognized as a rational choice for treatment and prevention of menopausal disorder [9,10]. So far, *in vitro* and *in vivo* studies

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suggest CR to be safe for the breast [11–16]. However, the precise mode of action of CR is not yet fully understood. According to the concept of intracrinology [17] suggesting that inactive hormones released by hormone-producing organs are transported via blood vessels to their target tissues where enzymes secure their conversion into active hormones, the amount of steroids locally available is more important than their serum concentration. The most biologically active estrogen in breast tissue is 17β-estradiol (E_2). Breast tissue and mammary cancer cells have been shown to possess the enzymatic systems necessary for the intratumoral biosynthesis of estrogens from precursor molecules circulating in plasma. Three main enzymes are important in this process: aromatase, which converts androgens to estrogens [18–20], E_1S-sulfatase (STS) which hydrolizes E_1S to E_1 [20–28], and 17β-hydroxysteroid dehydrogenase type 1 (17βHSD-1) which reduces E_1 to E_2 [29–32]. The activity of STS in breast tumors has been shown to be 10–500-fold higher than aromatase activity [33–35]. In BC, STS mRNA has been shown to be an independent prognostic indicator in predicting shorter relapse free survival [36] and to correlate positively with tumor size and lymph node metastasis [37]. Thus, STS seems to play a crucial role in local biosynthesis of estrogens in breast (cancer) tissue. Estradiol and various progestogens have been shown to influence STS in BC cells *in vitro* [38–44]. *In vitro*, the effect of steroids on local estrogen formation in normal and malignant breast cells is rather due to changes in STS activity than in its mRNA and protein levels [45]. We recently demonstrated that long-term HT alters local estrogen formation in breast tissue of postmenopausal cynomolgus monkeys (*Macaca fascicularis*) and women depending on breast tissue composition [46,47].

The aim of this study was to investigate the effect of an isopropanolic-aqueous (40%, v/v) extract of CR (iCR) on estrogen biosynthesis in breast tissue *in vitro*.

2. Methods

2.1. Chemicals, reagents, and treatment

The iCR (B. Nr. 010720) was provided by Schaper & Brümmer GmbH & Co.KG. Salzgitter, Germany. The concentration of the extract was 100 mg/ml in relation to the dry residue. Alcohol concentration was 40% (v/v). The final concentration of isopropanol in tissue homogenates during iCR treatment did not exceed 0.5% (v/v). The same concentration of isopropanol was present in the control samples. Breast tissue homogenates were incubated without and with iCR at a concentration range from 10 mg/ml to 0.1 μg/ml leading to a final concentration range in the homogenate from 0.1 mg/ml to 1 ng/ml, respectively. The radioactive labeled steroid [^{3}H]-E_1S (specific activity 57.3 Ci/mmol), was purchased from Perkin Elmer Life and Analytical Sciences (549 Albany Street, Boston, MA, USA). Unlabeled E_2 and E_1 were obtained from Sigma. Unless stated otherwise, all chemicals were from Sigma–Aldrich (Deisenhofen, Germany).

2.2. Subjects

E_1S metabolism in normal breast tissue was studied in five post- and five premenopausal women undergoing reduction mammoplasty (Department of Plastic Surgery, Clinic Centrum Hohenzollernring, Muenster, Germany, Department of Gynecology and Obstetrics, St. Barbara Clinic, Hamm, Germany, and Department of Obstetrics and Gynecology, University Clinic of Muenster, Germany). The postmenopausal women's age was in the range from 48 to 70 years (mean 56 ± 7.7 years), and the premenopausal women's age in the range from 31 to 57 years (mean 43 ± 9 years; $p = 0.05$). None of the women received hormonal treatment prior to breast surgery. Immediately following surgical removal, the tissue samples were shock frozen in liquid nitrogen and stored at $-70\,^{\circ}$C until thin layer chromatography for enzyme activity and western blotting for protein quantification were performed. Since one postmenopausal woman was extremely overweight (body mass index (BMI) 50) she was excluded from the subset of postmenopausal women when STS activity was evaluated. In addition, normal breast tissue obtained from another subset of women ($n = 5$) undergoing breast reduction mammoplasty (Department of Plastic Surgery, Fachklinik Hornheide, Germany) was immediately fixed in 10% neutral phosphate-buffered formalin for H&E staining and immunohistochemistry. Histological evaluation was performed by a board-specified pathologist (G. Edel, M.D., Ph.D.,

Institute of Pathology at the Franziskus Hospital in Muenster, Germany, Institute of Pathology Diebold, Niemann & Schönfeld, Hamm, Germany). The breast tissue examined did not contain any malignant lesions.

Women gave written informed consent to their breast tissue being analyzed in addition to established pathological analysis.

2.3. Immunohistochemistry

Normal breast tissue obtained from a reduction mammoplasty of a 49-year-old healthy woman was formalin-fixed and paraffin-embedded using standard techniques. Consecutive sections of 2–3 μm were cut from the paraffin blocks, dewaxed and re-hydrated. Antigen retrieval was performed by hot water steaming in citrate buffer (pH 6; 30 min). Following a blocking step with Aurion BSAc solution (DAKO, Glostrup, Denmark) for 30 min, sections were incubated with a monospecific rabbit-anti human STS-antiserum [48], diluted 1:1000 in phosphate-buffered saline (PBS) containing 1% bovine serum albumine (BSA), or a control rabbit serum overnight at 4 °C. Endogenous peroxidase activity was quenched with methanol/0.6% H_2O_2, followed by three washes with PBS. STS was detected using the DAKO cytomation rabbit-EnVision plus-horseradish peroxidase (HRP) system and the AEC substrate (DAKO), followed by counterstaining with Mayer's Hemalum (Merck, Darmstadt, Germany). Sections were observed and documented using a Zeiss Axiovert 100 microscope equipped with an Axiophot Mrc camera.

2.4. Western blotting

For Western blotting, breast tissue extracts of five pre- and five postmenopausal women were diluted with SDS-sample buffer to a protein concentration of 1 μg/μl. Twenty microliters of sample/lane were subjected to electrophoresis on 12% SDS-polyacrylamide gels and electro-transferred to nitrocellulose membranes as previously described [50]. Subsequently, the membranes were blocked with 5% non-fat dry milk in TBS containing 1% (v/v) Tween 20 for 1 h at room temperature, washed 3× 5 min with TBS and incubated with anti-STS-antiserum [48] diluted 1:1500 in TBS containing 5% BSA and 1% (v/v) Tween at 4 °C for 16 h. The membrane was washed 3× 5 min with TBS (1% Tween) and incubated with a peroxidase-conjugated goat-anti rabbit IgG antibody (Calbiochem, Nottingham, UK) diluted 1:2000 in TBS containing 5% BSA and 1% (v/v) Tween at room temperature for 1 h. The blot was washed 3× 5 min with TBS (1% Tween), followed by an enhanced chemoluminescence reaction (Super Signal, Pierce, Rockford, MA) and exposition to ECL–hyperfilm (Amersham, Braunschweig, Germany). To normalize for a loading control, blot membranes were stripped with 0.2 M glycine, pH 2.5 [50] and reprobed as described above, using a monoclonal mouse-anti tubulin primary antibody (Sigma–Aldrich, dilution 1:4000) and HRP-conjugated goat-anti-mouse IgG secondary antibodies (Calbiochem, dilution 1:10,000), respectively. Scanned images of the exposed films were analyzed densiometrically using the NIH Image J software (NIH, Bethesda, USA), normalizing STS expression to tubulin content.

2.5. E_1S-sulfatase (STS) activity assay

STS activity was measured as described previously [45,46]. In brief, breast tissue (150 mg) was finely minced with scissors and homogenized on ice, in 2 ml 0.06 M Tris–HCl-buffer pH 7.0 (assay buffer throughout the entire procedure) for approximately 1 min at maximum speed using a Polytron homogenizer. All incubations were carried out in duplicate. The incubation mixtures consisted of 200 μl homogenate, 19.39 pmole [^{3}H]-E_1S in 100 μl of buffer, and 100 μg of NADH and NADPH in 100 μl of buffer (total incubation volume 400 μl). Simultaneous background incubations using buffer instead of tissue homogenate were performed in order to correct for non-enzymatic transformation of the substrate. After 15 and 45 min of incubation in air at 37 °C in a water bath, 200 μg of non-radioactive E_1 and 17β-E_2 were added in 100 μl of ethanol together with 0.5 ml of 0.5 M sodium phosphate buffer, pH 7.0. The unconjugated steroids were extracted with 2.0 ml toluene. After inverting the tubes 100 times they were centrifuged at 1500 × *g* for 10 min. The lower aqueous phase was frozen in liquid nitrogen. Hundred microliters of the upper toluene phase was removed and added to 100 μl of buffer and 3.0 ml of scintillation fluid to determine total hydrolysis. The remaining toluene phase was evaporated to dryness in nitrogen atmosphere and dissolved in 100 μl

Folch-Solution (2:1 chloroform:methanol). Thin layer chromatography (TLC) on Alugram Sil G/UV 254 (Roth; Macherey-Nagel Düren, Germany) was performed using 13% ethanol in toluene as solvent. After visualization in 254 nm UV light, the zones in the plate corresponding to the estrogens as well as the non-fluorescent zones were cut apart and separately eluted with 3 ml ethanol and 6 ml scintillation fluid. Aliquots were taken from each fraction from the TLC plate for counting of the [^{3}H] activity. A Wallac 1409 (β-Counter) scintillation spectrometer was used for the radioactivity measurements and Riafluor (New England Nuclear Corporation, Boston, MA, USA) was used as scintillation fluid. The enzyme activity was expressed as the amount of unconjugated estrogen ($E_1 + E_2$) formed per minute and per milligram of protein. The total protein was determined by the method of Bradford [49].

2.6. Statistical methods

Results were expressed as the mean ± standard error of the mean (S.E.M.). Statistical comparisons were made using the student's two-tailed test. A p-value < 0.05 was considered to be significant. All treatment experiments were repeated at least four times. Negative STS activity values were set to zero and repeated measures were aggregated by calculating the mean.

3. Results

3.1. Subjects' characteristics

There were no significant group differences regarding age, BMI, number of pregnancies, deliveries, and

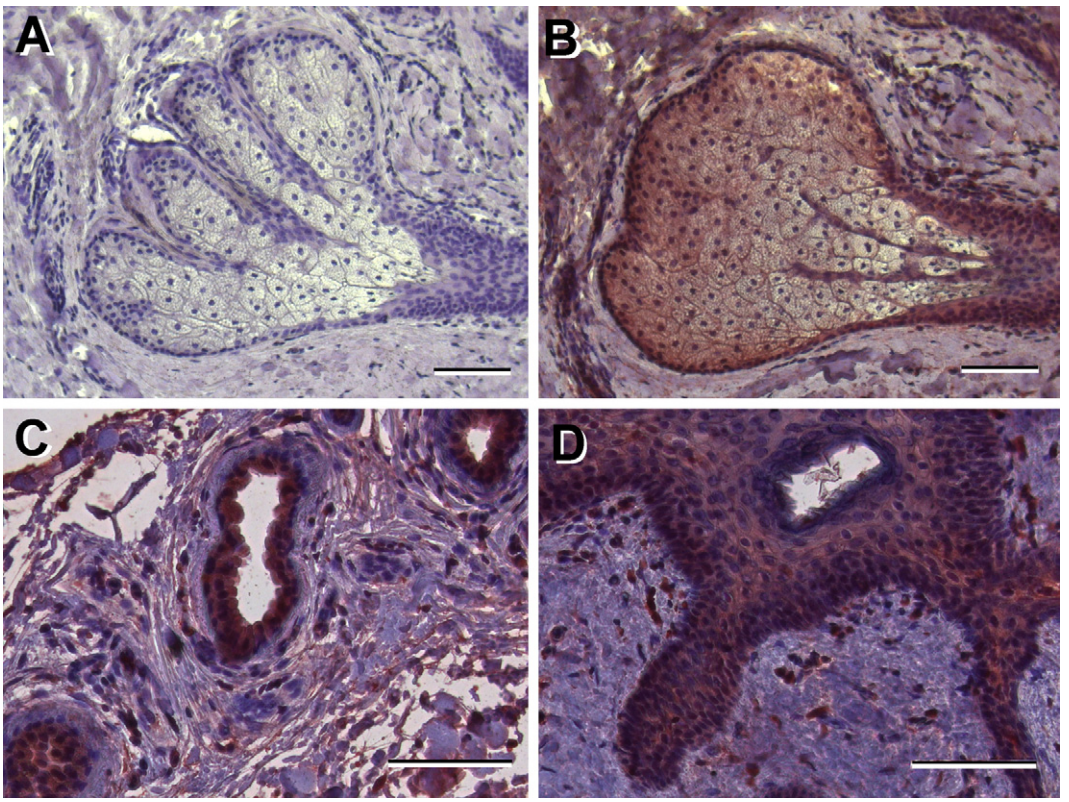


Fig. 1. Immunolocalization of STS in normal human breast tissue. Paraffin sections of normal breast tissue were immunostained with a monospecific antiserum against STS [48] (B–D), or a preimmune rabbit control antiserum (A). STS staining was predominantly found in epithelial cells. Bar = 50 μm.

miscarriages. Mean BMI was within the overweight range (postmenopausal women: $30 \pm 11\,kg/m^2$, and premenopausal women: $26 \pm 4\,kg/m^2$, respectively; $p = 0.53$). Hysterectomy without ovarectomy had been performed in two of the five post-, and one of the five premenopausal women, respectively. None of the postmenopausal but two of the premenopausal women were current smoker. The family history (first and second degree) referring to breast cancer was negative for both groups. Breast cancer had occurred earlier in the mammary gland which was now subject of reduction mammoplasty in three of the five postmenopausal women. In the premenopausal subset one in five women had been treated for contralateral breast cancer prior to reduction mammoplasty. Reduction mammoplasty for cosmetic reason only was performed in one in five post- and four in five premenopausal women. Prior to breast surgery a mammogram was performed to prove lack of suspicious lesions.

3.2. E_1S-sulfatase (STS) protein expression and localization

To confirm the expression of STS in normal breast tissue, we performed immunohistochemistry on formalin-fixed, paraffin-embedded specimens using a well-characterized monospecific anti-STS-antiserum [48]. STS protein was predominantly localized in epithelial cells of lobuloalveolar and ductal tissue. However, STS protein staining was also occasionally present in stromal cells (Fig. 1). To quantify STS protein in the breast tissue samples studied, we performed western blots of tissue extracts. A specific band corresponding to the Mr of dimeric STS [51] was detected in all breast tissues investigated (Fig. 2). Interestingly, STS protein expression was significantly increased by approximately 30% in breast tissue of premenopausal women, resulting in a signal overload (Fig. 2, lanes 6–10).

3.3. Local E_1 and E_2 formation as indicative for E_1S-sulfatase (STS) activity in vitro

The activity of STS in kryoconserved normal breast tissue could be demonstrated. The enzyme effectively metabolized the radioactive substrate E_1S. E_1 was the most abundant, labeled estrogen found in breast tissue after incubation with labeled E_1S. On an average, the concentration of locally formed E_1 was about 18 (untreated postmenopausal women; $p = 0.08$) and 53 (untreated premenopausal women; $p \leq 0.05$) times higher than that of E_2. In untreated postmenopausal women, mean E_1 and E_2 formation was 9.4 ± 2.9 and 0.5 ± 0.2 fmol/(mg(protein) min), respectively. In contrast, in untreated premenopausal women, mean E_1 and E_2 formation was 55.5 ± 16.3 and 1.0 ± 0.5 fmol/(mg(protein) min), respectively. Neither E_1 ($p = 0.06$) nor E_2 ($p = 0.4$) formation were significantly different comparing pre- and postmenopausal women.

Interestingly, total estrogen formation was decreased after 45 min of incubation when compared to 15 min of incubation (n.s.).

3.4. Influence of iCR on E_1S-sulfatase (STS) activity in vitro

Both, E_1 and E_2 formation after 15 min of incubation were dose-dependently decreased by iCR treatment compared to controls regardless of menopausal status. However, the reduction of total estrogen formation ($E_1 + E_2$) was only significant for iCR treatment at 0.1 mg/ml in premenopausal women (Figs. 3 and 4). Similarly, after 45 min of incubation total estrogen formation ($E_1 + E_2$) was also significantly decreased by treatment with iCR at 0.1 mg/ml ($p \leq 0.05$) in comparison to controls (Fig. 5). E_1, E_2, and total estrogen formation ($E_1 + E_2$) after treatment with iCR at 0.1 mg/ml to 1 ng/ml did not differ significantly between pre- and postmenopausal women.

Interestingly, the local estrogen formation of one postmenopausal woman was measured in the premenopausal range. This woman had a BMI of 50 suggesting that the higher amount of body and breast fat tissue might have an impact on the local estrogen formation the mammary gland.

4. Discussion

To our knowledge we are the first to investigate the effect of iCR on STS activity in normal breast tissue *in vitro*. Although the amount of epithelial tissue in the mammary gland changes due to age and reproductive stage [52] STS activity has been shown to be present regardless of menopausal stage [53]. We

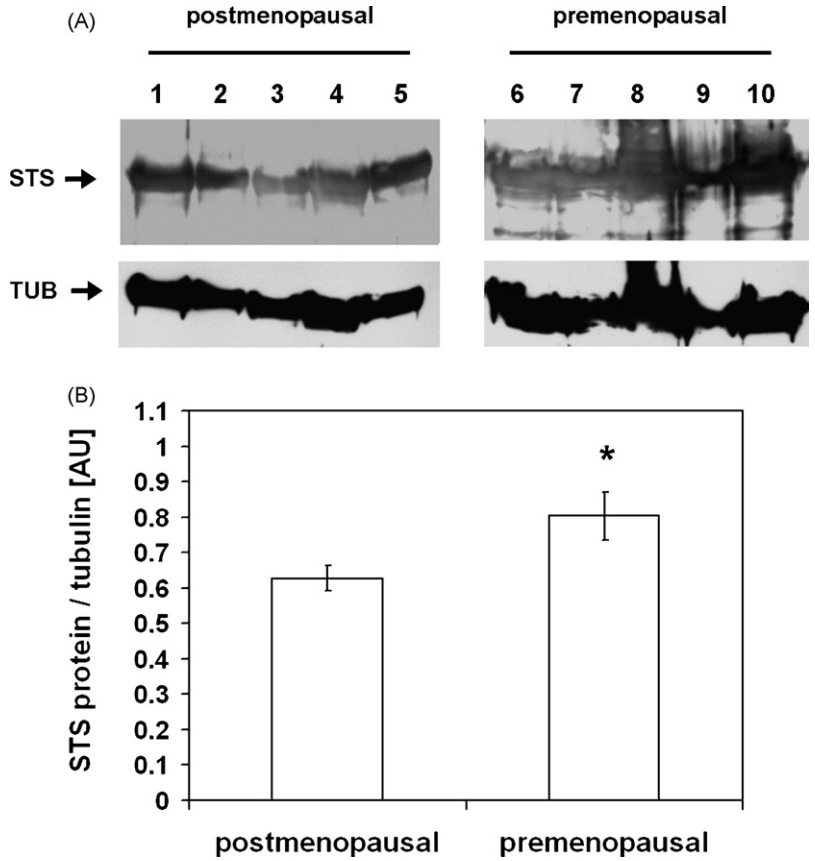
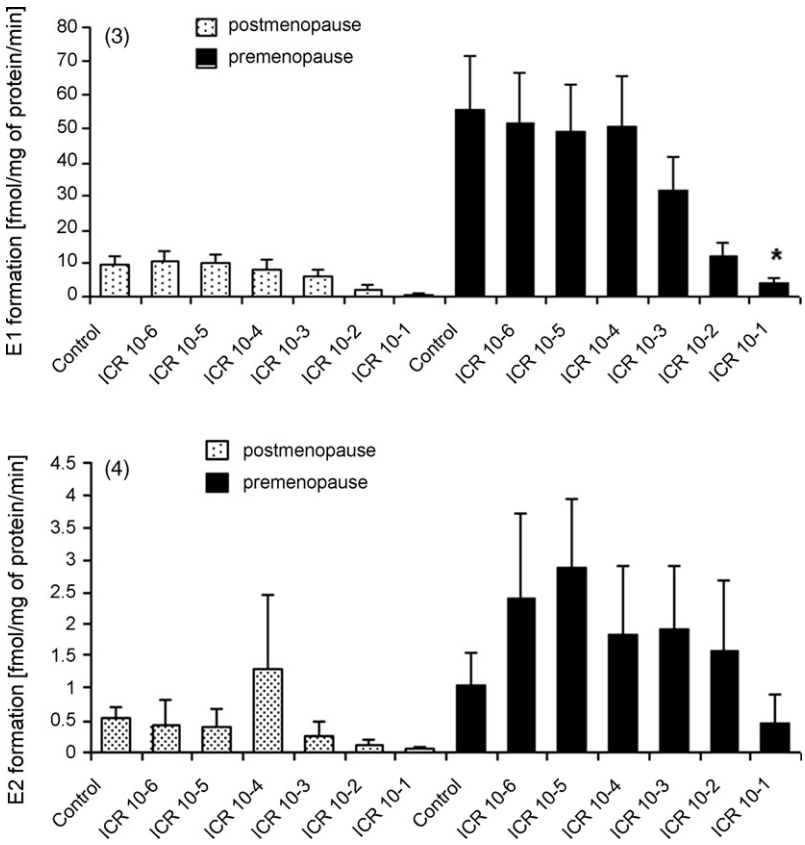


Fig. 2. Western blot analysis of STS protein expression in normal breast tissue from postmenopausal (1–5) and premenopausal (6–10) women. Twenty micrograms protein/lane were subjected to SDS-PAGE and Western blotting using a monospecific STS-antiserum [48]. (A) STS protein expression was detected in all breast tissue samples. High STS protein expression levels in specimens obtained from premenopausal women resulted in a chemoluminescence signal overload (lanes 9–10). (B) Densitometric analysis of STS protein expression normalized for tubulin expression. The signal intensities of the STS and tubulin bands shown in panel A were quantified using NIH image J software. AU = arbitrary units, $*p > 0.05$ ($n = 5$).

demonstrated local estrogen formation being higher in untreated pre- than in postmenopausal breast tissue supporting previous results [53]. Treatment with iCR induced a dose-dependent decrease of both, E_1 and E_2 formation in pre- and postmenopausal breast tissue which was significant at 0.1 mg/ml in premenopausal breast tissue.

Regarding our enzyme activity assay, we focused on rapid changes in desulfation. After 45 min of incubation STS activity was not significantly decreased in comparison to 15 min of incubation. Various Km for STS activity have been reported by others ranging from 4 to 27 μM in human BC tissue [34,35,54–56], 5.8 μM in dysplastic human breast tissue [52], and from 4.1 to 18.2 μM in normal human breast tissue homogenates [57]. V_{max} for STS activity has been reported to range between 0.8 and 125 μmol E_1/(g(protein) h) in human BC tissue [35], and between 18.2 and 75.9 μmol E_1/g in normal breast tissue homogenates [57]. Since the substrate concentration in our assay was in the physiological range, no further increase in STS activity was to be expected after 45 min of incubation, as a steady state of biosynthesis of estrogens had obviously been reached.

Supposing a 100% bioavailability, the human daily recommended dose, i.e. 50 μl iCR, would result in a serum concentration of 1.7 μg/ml [58]. Therefore, the active doses of iCR as present in the tissue homogenate



Figs. 3 and 4. E_1S-sulfatase (STS) activity in breast tissue after 15 min of incubation. Formation of E_1 (Fig. 3), and E_2 (Fig. 4) is reported. STS activity was evaluated as indicated in the text. Values (in fmol estrogen/(mg(protein) min)) are expressed as means and standard error of the mean (S.E.M.). Changes from control are significant for treatment with iCR at 0.1 mg/ml in premenopausal women. *Significantly different at $p \leq 0.05$. *Abbreviations*: E_1 = estrone; E_2 = estradiol; iCR = isopropanolic extract of *Cimicifuga racemosa*; 10^{-1} to 10^{-6} = concentration at 0.1 mg/ml to 1 ng/ml.

incubation steps, i.e. 0.1 and 0.01 mg/ml, are well within the range that might be expected locally in certain target tissues.

The decrease of local estrogen formation by iCR could be either a change in the enzyme's amount or its activity. Previous studies in BC cell lines have demonstrated that treatment with estradiol, and various progestins, respectively, rather affect enzyme activity than its protein levels and mRNA expression [40,59]. We previously reported similar effects in non-malignant human breast cells *in vitro* [45]. Thus, the enzymatic effects leading to a decreased E_1 and E_2 formation could be mediated by a decreased STS, elevated sulfotransferase, or by a shift in the balance of 17β-HSD type 1 and 2 activity. Progestins have been shown to inhibit the reductive 17β-HSD activity as well as to stimulate the oxidative 17β-HSD activity, respectively in BC cell lines. In contrast, sulfotransferase activity was shown to be increased in MCF-7 and T-47D cell lines when progestins were added [32,41,60,61]. A recent study in BC cells demonstrated different effects on various enzymes depending on what type of progestin and estradiol was combined with [44]. However, which mechanism is responsible for the effects observed in our study remains to be resolved.

However, in *in vivo* studies conventional HT, especially estrogen plus progestogen (EPT), has been shown to increase the amount of locally formed estrogens which might at least partly explain the adverse effects on the mammary gland observed by

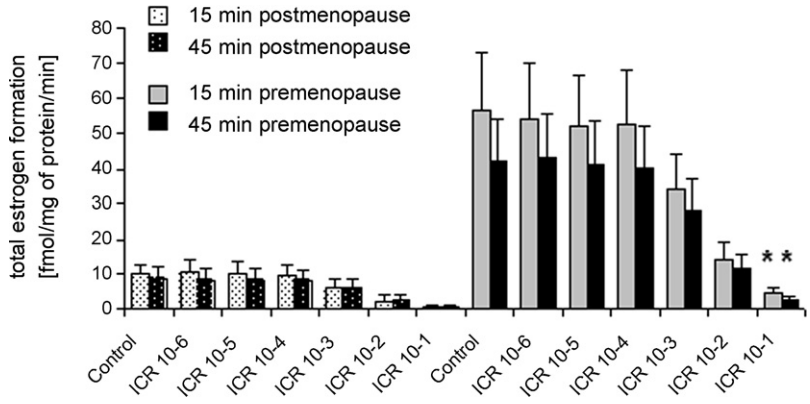


Fig. 5. E_1S-sulfatase (STS) activity in breast tissue after 15 and 45 min of incubation. Formation of total estrogen formation ($E_1 + E_2$) is reported. STS activity was evaluated as indicated in the text. Values (in fmol estrogen/(mg(protein) min)) are expressed as means and standard error of the mean (S.E.M.). Changes from control are significant for treatment with iCR at 0.1 mg/ml in premenopausal women. *Significantly different at $p \leq 0.05$. *Abbreviations*: iCR = isopropanolic extract of *C. racemosa*; 10^{-1} to 10^{-6} = concentration at 0.1 mg/ml to 1 ng/ml.

long-term EPT [46,47]. On the other hand, tibolone, a unique tissue-selective agent for postmenopausal women, has been shown to inhibit tumor growth in the therapeutic rat DMBA induced tumor model and to almost abolish tumor development in the preventive model [43]. One explanation for the breast protective effect is the inhibition of STS activity which was demonstrated for tibolone and its metabolites [42]. Likewise, iCR did not stimulate cancerous growth in the therapeutic rat DMBA induced tumor model either [15]. Furthermore the lack of estrogenity of (isopropanolic and ethanolic extracts of) CR was confirmed by its antiproliferative activity in both, hormone-dependent, and -independent BC cell lines, due to activation of caspases and induction of apoptosis [11,12]. Similarly, CR has been shown to induce cell cycle arrest in G1 in MCF-7 and MDA-MB-453 cells [13]. These findings are supported by Lupu et al. reporting no estrogenic activity in RNAse protection assays, estrogen-responsive-element-luciferase assay, the Ishikawa cell system, and in colony formation of ER-expressing breast cancer cells [14]. In healthy postmenopausal women, 6 months of treatment with black cohosh did neither increase mammographic density nor breast cell proliferation [16]. In BC survivors, black cohosh did not lead to an increased but moreover to a slightly decreased risk of BC recurrence [62]. We suggest, that the lack of estrogenicity and thus the breast protective effects observed might be due to a decrease of locally available active estrogens in breast tissue.

However, future studies investigating long-term effects of CR on breast tissue should consider menopausal and reproductive stage.

5. Conclusions

We conclude that iCR alters local estrogen formation in normal human breast tissue leading to a decrease of local E_1 and E_2 biosynthesis *in vitro*. This may explain the lack of estrogenic effects of black cohosh in breast tissue observed in previous studies. Further *in vivo* studies in normal breast tissue are warranted.

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