EXPERIMENTAL STUDY

Evidence for selective estrogen receptor modulator activity in a black cohosh (*Cimicifuga racemosa*) extract: comparison with estradiol-17 β

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Abstract

Objective: Some phytoestrogens are believed to have selective estrogen receptor modulator (SERM) activity with no action in the uterus but beneficial effects in the hypothalamo/pituitary unit and in the bone and are presently the focus of clinical interest. In the present experiments, the effects of the clinically used *Cimicifuga racemosa* (CR) extract BNO 1055 in the uterus, in the bone and on serum luteinizing hormone (LH) were compared with the effects of estradiol-17 β (E₂) under acute and chronic conditions in ovariectomized rats.

Methods: Ovariectomized rats were treated either acutely (6 h) or chronically (3 months) with E₂ or the CR extract. Gene expression of some estrogen-regulated genes in the metaphysis of the tibia and the uterus was determined. Furthermore, bone mineral density was measured by quantitative computer tomography.

Results: When given acutely, both E₂ and the CR extract inhibited LH secretion and slightly stimulated gene expression of IGF-I, collagen-1 α 1, osteoprotegerin and osteocalcin (all osteoblast products), and of tartrate-resistant acid phosphatase (TRAP, an osteoclast product) in the metaphysis of the femur. While E₂ stimulated uterine weight and expression of progesterone receptor (PR), the complement protein (C3) and IGF-I genes, and inhibited gene expression of the estrogen receptor β (ER β) in the uterus, no such effect was observed under acute CR treatment. After chronic application with pelleted food over 3 months E₂ had profound effects in the uterus on weight and gene expression (ER β , PR, C3 and IGF-I) which were not seen in the CR-treated animals. Within 3 months after ovariectomy, control rats had lost more than 50% of the metaphyseal bone mass of the tibia, an effect prevented by E₂ and partially by CR supplementation.

Conclusions: These data confirm the concept that the CR extract BNO 1055 contains as yet unidentified substances with SERM properties which act in the hypothalamo/pituitary unit and in the bone but not in the uterus.

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Introduction

Estrogen receptors (ER) are present in almost all organs of the body. The two cloned ERs (ER α and ER β) show characteristic distribution in the body (for review see 1) and they transmit estrogenic messages by acting as transcription factors on the estrogen response element (ERE) of a variety of estrogen-regulated genes (for review see 1). We have earlier shown that *Cimicifuga racemosa* (CR) extracts have estrogenic effects in the hypothalamo/pituitary unit to suppress pituitary luteinizing hormone (LH) secretion in ovariectomized rats but to lack an estrogenic, i.e. stimulatory effect on uterine weight (2). At this time an explanation for such organ-selective effects of the CR extract cannot

be given. It is now well established that substances exist which have estrogenic effects in some organs but no or even anti-estrogenic effects in others. These substances are called selective estrogen receptor modulators (SERMs) (for review see 3). Our increasing knowledge about such SERM activities may now offer a plausible explanation for the organ-selective effects of the CR extract.

The pituitary LH-producing cells express gonadotropin-releasing hormone (GnRH) receptors which need to be exposed to the ligand – namely GnRH – in a pulsatile fashion (4). The neuronal network that causes pulsatile GnRH release is called the GnRH pulse generator. Following ovariectomy, the GnRH pulse generator becomes hyperactive which results in

pulsatile LH secretion at a high rate with high LH pulse amplitudes. The overactivation of the GnRH pulse generator causes co-activation of other hypothalamic neurons which regulate body temperature and thereby vasodilation of skin vessels which are experienced as hot flushes in climacteric women (5). Estradiol substitution of ovariectomized rats dampens the overactivity of the GnRH pulse generator, thereby hot flushes are diminished or cease and pituitary LH secretion is reduced. Hence, this so-called negative feedback action of estrogens on pituitary LH secretion in ovariectomized rats can be used as an indirect measure of hot flushes.

The uterus is a highly sensitive target for estrogen action and this effect is primarily mediated via the ER of the α -subtype (1, 6). When administered acutely, estradiol-17 β (E_2) causes an edematous swelling (uterine ballooning). Upon chronic administration, uterine tissue proliferates and the myometrium and endometrium become hypertrophic. Both acute and chronic effects of E_2 are in part due to activation of the insulin-like growth factor-I (IGF-I) gene and protein expression (for review see 1, 7, 8). In addition, a protein belonging to the complement family (C3), the function of which is largely unknown, is dramatically stimulated by E_2 and some phytoestrogens (9).

The bone is also a target organ for estrogens. Both receptor subtypes have been demonstrated in the bones of immature rats and osteoblast cell lines (10–13). Whether the bones of adult rats express both or only the ER α subtype is controversial (14, 15). It appears that it is primarily the osteoblast cell line that expresses the ER of the α -subtype (10–12, 14). The dominant effect of E_2 in these cells is to stimulate production of osteocalcin (OC) and collagen-1 α 1, a bone-specific matrix protein (16). Also osteoprotegerin (OPG), a soluble receptor belonging to the tumor necrosis factor receptor family (17, 18), appears to be E_2 regulated (19, 20). The function of OPG is to bind and thereby to inactivate osteoclast differentiation factor. As a result, osteoclast differentiation and hence activity is inhibited (17–20). Many of these effects are mediated by a stimulation of osteoblast IGF-I production (21, 22). The question as to whether or not osteoclasts are estrogen receptive and, if so, which ER type is being expressed, is much less clear (11, 14, 15). The effects of estrogens on tartrate-resistant acid phosphatase (TRAP), an osteoclast product, have been described which, however, could be indirectly exerted via paracrine mechanisms, through factors released by osteoblasts. In each case, E_2 has osteoprotective effects in that it prevents the development of osteoporosis following ovariectomy. To estimate the bone mineral density of the proximal metaphysis of the tibia, computer-assisted tomography (CT) allowed determination of trabecular density of the metaphysis of the tibia. It is well documented that loss of estrogens results in increased fat accumulation

in animals (23). In the same CT plane in which the tibial metaphysis was scanned a paratibial fat depot was visible, the surface of which could also be quantified. This allowed not only a functional estimate of the degree of osteoporosis developing during the course of the 3-month investigation period and whether or not E_2 and the CR extract have osteoporosis-preventing effects but also an exact measurement of fat accumulation in this area.

The fact that, in earlier experiments, CR extracts had a negative feedback effect on LH secretion in the hypothalamo/pituitary unit but no effects on uterine weight (2) suggested to us that SERM activities are possibly present in the rhizome of this plant and prompted us to investigate the acute and chronic effects of the CR extract (BNO 1055) on serum LH levels and on the expression of the above-mentioned estrogen-regulated genes in the uterus and bone.

Materials and methods

Female Sprague–Dawley rats were used for the present experiments. Permission to perform these experiments was obtained from the Landesregierung Braunschweig (permission no. Az. 509.42502/01-13.00 dated 17 July 2000). Upon arrival from the supplier (Winkelmann, Borcheln, Germany) the rats were fed with pelleted chow in which soy proteins were replaced by potato proteins. One week after arrival they were ovariectomized. Food and water were available *ad libitum*. The animal quarters were illuminated from 0600 to 1800 h. Room temperature was 25 °C at a relative humidity of 55%.

The CR extract was obtained from Bionorica (Neumarkt, Germany) and was an aqueous/ethanolic extract of the rhizomes of cultivated plants. The preparation of the CR extract BNO 1055 was as follows: finely ground CR rhizomes were extracted with five times the amount of 50% (v/v) water/ethanol for 48 h with a percolation speed of 500 kg/h. After filtration, the extract was concentrated under vacuum and evaporated to dryness at 100–140 mbar according to a patent-protected process. The product temperature did not exceed 40 °C.

Acute experiments

Three to four weeks after ovariectomy the jugular vein of each animal was catheterized. After recovery for 24 h, blood samples (100 μ l) were withdrawn starting at 0700 h at 10-min intervals for 60 min. Thereafter, E_2 (3.5 μ g/animal), the CR extract BNO 1055 (62.5 mg/animal) or the solvent (1 ml 5% cremophor) was slowly infused i.v. within 1 min. Further blood samples were withdrawn at 10-min intervals for the next 2 h. Six hours after i.v. injection of test substances the animals were decapitated, blood was collected from

the trunk, the uteri were removed and weighed and the metaphysis of the femurs prepared. Both organ specimens were then immediately frozen in liquid nitrogen and stored at -80°C .

Chronic experiments

Two chronic experiments were performed in which animals remained either untreated or were treated with E_2 or the CR extract BNO 1055 immediately after ovariectomy. From experience with the animals' food intake in the last 2 weeks prior to the beginning of the experiments it was known that the mean food intake of each animal was 20 ± 2 g. On this basis, E_2 and the CR extract (BNO 1055) was added such that 20 g food contained either 1.0 mg E_2 in experiment 1, 0.5 mg E_2 in experiment 2, 200 or 1000 mg CR extract BNO 1055 in experiment 1, or 50 or 200 mg CR extract BNO 1055 in experiment 2. Each group of animals consisted of 15–18 or 12 rats in experiments 1 or 2 respectively.

Further details about experiment 1

Animals were weighed and vaginal smears taken once per week. After 3 months they were decapitated, blood was collected from the trunk, and the uteri were removed, cleaned and weighed. In addition, the metaphysis of the femur was prepared and both tissue specimens frozen immediately in liquid nitrogen and stored at -80°C .

Further details about experiment 2

Animals were weighed and vaginal smears taken twice weekly. Prior to experimentation, 1 day after ovariectomy, 2, 4, 8, and 12 weeks after the onset of E_2 or CR-supplemented food animals were anesthetized (inhalation anesthesia with isoflurane) and the density of the metaphysis of the tibia was recorded by CT (24) (XCT Research SA+STRATEC Medizintechnik GmbH, Pforzheim, Germany).

Extraction of RNA

Immediately after decapitation of the animals, the uterus and femur were dissected and snap-frozen in liquid nitrogen within 3 min to minimize degradation of RNA. Samples were stored at -70°C until RNA preparation.

Total RNA was extracted from the metaphysis of the distal part of the femur and the uterus. Tissue samples were chilled in liquid nitrogen and pulverized in a 5 ml Teflon container with a tissue homogenizer (Micro-dismembrator; Braun, Melsungen, Germany). The RNeasy Mini Kit (Qiagen, Hilden, Germany) was used for further processing of the samples following the manufacturer's instructions. Tissue powder (50 mg) was

suspended in 600 μl (uterus) or 300 μl (femur) RT-lysis buffer and further homogenized by brief ultrasound sonication (10 s). The homogenates were applied onto QIA shredder columns (Qiagen, Hilden, Germany) and the eluates were subsequently loaded onto the extraction columns. Total RNA was eluted with 50 μl diethylpyrocarbonate (DEPC)-treated water. To determine RNA concentrations, 5 μl RNA solution was diluted with 75 μl water. Absorption was measured at 260 and 280 nm with a biophotometer (Eppendorf, Hamburg, Germany). Concentrations of the RNA solutions were adjusted to 50 ng RNA/ μl (uterus) or 15 ng/ μl (femur) with DEPC-treated water and stored at -70°C until further analysis.

Reverse transcription

The RT reaction was carried out with either 500 ng total RNA/10 μl (uterus) or 150 ng total RNA/10 μl (femur). In addition, the reaction mixture contained 4 μl 5 $\times$ reaction buffer with 250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl_2 and 50 mM dithiothreitol. The concentrations of random primers and deoxy-NTPs were 100 ng and 10 mM respectively, in a volume of 1 μl each. The mixture was completed with Superscript RNase H⁻ reverse transcriptase (1 μl containing 200 U) and 1 μl ribonuclease inhibitor (1 U; Promega, Mannheim, Germany). The final volume was adjusted with DEPC-treated water to 20 μl . All reagents except the ribonuclease inhibitor were purchased from GibcoBRL (Karlsruhe, Germany). Reverse transcription was initiated by incubation of samples at 22°C for 10 min followed by the enzymatic reaction conducted at 42°C for 50 min. At the end of incubation, the samples were heated at 95°C for 10 min to inactivate the enzyme and denature RNA-cDNA hybrids.

Real-time PCR

Real-time PCR reactions were based on the 5' nuclease assay (25) which was run on an ABI Prism 7700 Sequence Detection System (TaqMan; PE Applied Biosystems, Foster City, CA, USA). The sequences of primers and probes for the genes analyzed in the present study are summarized in Table 1. Primers and probes were chosen with the assistance of the Primer Express software (PE Applied Biosystems). Oligonucleotides were purchased from Eurogentec (Seraing, Belgium).

Each PCR run included six duplicate cDNA samples of defined concentrations to generate a standard curve (derived from the *in vitro* transcription described below), a no-template control and the respective sample cDNAs. Amplification reactions (25 μl) contained 1 $\times$ TaqMan Universal PCR Master Mix (PE Applied Biosystems), 50–900 nM of each primer (for details see Table 1), 175–225 nM probe and

Table 1 Sequences of primers and probes analysed.

Gene	Primers: forward and reverse	Probe (5'-FAM-3'TAMRA)	Product size	Reference (Accession no.)
ER- α	5'-AAGCTGGCCTGACTCTGCAG-3' 5'-GCAGGTCATAGAGAGGCACGA-3'	5'-CGTCTGGCCCAGCTCCTCCTCATC-3'	144 bp	(26) (X61098)
ER- β	5'-GAGGAGATACCACTCTTCGCAATC-3' 5'-GGAGTATCTCTGTGTGAAGGCCAT-3'	5'-CAGGGCATCTGTACCGCGTTTCAG-3'	159 bp	(27) (U57439)
PR	5'-CTGGTTCCGCCACTCATCA-3' 5'-TCAGGCTCATCCAGGAGTACTGA-3'	5'-CCGACACTTCCAGCTCTTTGCTGACCA-3'	229 bp	(28) (L16922)
IGF-I	5'-TGTCGTCTTTCACATCTCTTCTACCTG-3' 5'-CCACACACGAAGTGAAGAGCGT-3'	5'-TTACCAGCTCGGCCACAGCCGGAC-3'	121 bp	(29) (X04482)
C3	5'-CTGTACGGCATAGGGATATCACG-3' 5'-ATGCTGGCCTGACCTTCAAGA-3'	5'-ATGCCATCCTCACAACACTTCCGCAG-3'	199 bp	(30) (X52477)
Type I-procollagen α chain	5'-GGCGAAGGCAACAGTCGAT-3' 5'-TCCATTCCGAATTCCTGGTCT-3'	5'-TGCACGAGTCACACCGGAAGTGG-3'	173 bp	(31) (Z78279)
Osteoprotegerin	5'-CCTCTTTCTTTCTGCCTCTGATAGTC-3' 5'-CCAAGTCTGCAACTCGAATCAAAT-3'	5'-CGTATCAGGTGCACGAGCCTTATCCCA-3'	150 bp	(17) (U94330)
Osteocalcin	5'-CAAAGCCCAGCGACTCTGA-3' 5'-AGGTAGCGCCGGAGTCTATTCT-3'	5'-CCTTCATGTCCAAGCAGGAGGGCAGT-3'	85 bp	(32) (M11777)
Tartrate-resistant acid phosphatase type 5	5'-GATCACCTTGGCAATGTCTCG-3' 5'-GGCTGACAAAGTCGTCGGAAT-3'	5'-TGCCTACTCCAAGATCTCCAAGCGCTG-3'	175 bp	(33) (M76110)

PR, progesterone receptor. FAM, 6-carboxy-fluorescein; TAMRA, 6-carboxy-tetramethyl-rhodamine.

2–4 μ l cDNA. The cycling conditions were 2 min incubation at 50 °C for eliminating carryover PCR products by uracil DNA glycosylase treatment, 10 min at 95 °C for activation of the AmpliTaq Gold DNA polymerase followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

To generate cRNA, each PCR product was cloned into the pCR II-TOPO plasmid using the TOPO TA Cloning Kit following the manufacturer's instructions (Invitrogen, Groningen, The Netherlands). After confirming the sequence and orientation of the cloned PCR product by commercial sequencing (Seqlab, Göttingen, Germany), the plasmid construct was linearized with an appropriate restriction endonuclease and purified using the Wizard DNA Clean-Up System (Promega). RNA was synthesized according to the manufacturer's instructions using the RiboMAX Large Scale RNA Production System with either SP6- or T7-polymerase respectively (Promega). After photometric determination of concentrations of the RNA stock solutions, serial dilutions (tenfold intervals) of the RNA were reverse transcribed as described above. The standard curve was generated by plotting the known cDNA concentrations versus the corresponding threshold cycle (C_t) value obtained in the real-time PCR reaction using Sequence Detection Software (PE Applied Biosystems). To determine the relative expression levels of the tissue samples, the respective C_t values were interpolated from the standard curve.

LH radioimmunoassay

The blood samples collected from animals in the acute or the chronic experiments were centrifuged (3000 *g*,

10 min) and the serum used for measurement of LH by a specific radioimmunoassay supplied by the NHPP (Dr A F Parlow, Harbor-UCLA Medical Center, Torrance, CA, USA).

CT analysis

Measurement of bone density by CT yielded metaphyseal bone densities given in mg/cm^3 (24). The means of the controls at each measured time-point were calculated and set at 100%. Each individual value of the treatment groups was % related to this 100% value and the resulting means were compared statistically. In the CT scan of the metaphysis of the femur, a paratibial fat depot was visible, the surface of which within the CT plane could easily be quantified by perimetry. The percentage of this fat depot surface in relation to the total surface of the CT plane at the height of the metaphysis of the tibia can be calculated as an exact measure of the size of this fat depot.

Statistical evaluation

In the present study relative changes of mRNA levels were analyzed by comparison between the control and treatment groups. Based on the C_t values of the PCR reactions measured in the control group, an average expression level of the analyzed target gene was calculated from the standard curve, which was set at 100%. Individual C_t values in the treatment groups were transformed in relation to this average value of the control group.

Data are expressed as means $\pm$ S.E.M. Significant differences between the control and treatment groups

were analyzed by one-way ANOVA followed by Dunnett's *post hoc* test for multiple comparisons (Graph Pad; Prism, San Diego, CA, USA). *P* values <0.05 were considered significant.

Results

Acute experiments

Figure 1 shows mean serum LH levels prior to and within the 120 min after i.v. injection of 0.5 µg E₂ (Fig. 1a), 62.5 mg CR extract (Fig. 1b) and 1 ml solvent (Fig. 1c). Both E₂ and the CR extract suppressed serum LH levels significantly. Figure 2 shows that the acute E₂ injection largely increased uterine weight within 6 h, an effect not seen in the CR-treated animals. Expression of uterine ERα, ERβ, IGF-I and C3 genes is shown in Fig. 3. While the acute E₂ treatment had a slight but significant suppressive effect on ERα (Fig. 3a), it strongly downregulated the expression of the ERβ gene (Fig. 3b). Gene expression of IGF-I and C3 was largely upregulated by E₂ (Fig. 3c,d). All parameters remained unaffected by the CR extract.

The acute effects of E₂ and CR on the expression of a variety of genes in the metaphysis of the femur are shown in Fig. 4. While the concentrations of ERα (data not shown) and OPG mRNA were not affected by E₂ or CR, expression of the IGF-I and the collagen-1α1 gene were stimulated by E₂ and by the CR extract, though these effects were statistically not significant. Gene expression of TRAP was significantly stimulated by the CR extract, an effect also seen in the E₂-treated animals.

Chronic experiments

Similarly, under chronic conditions (3-month treatment in experiment 1) uterine weights (Fig. 5a) were significantly higher in the E₂-treated animals in comparison with controls while the CR extract at two doses had no effect. Body weights (Fig. 5b) were significantly lower in the E₂-treated animals in comparison with the controls, an effect also exerted by the higher CR (1000 mg/kg) but not by the lower (200 mg/kg) dose. The stimulating effect of E₂ on uterine weight was due to stimulation of the thickness of both the endometrium and the myometrium (Fig. 6). Neither endometrial nor myometrial thickness or histological appearance (data not shown) were affected by the two concentrations of the CR extract.

Figure 7 shows that also under chronic conditions (in experiment 1), E₂ had no uterine effect on ERα but suppressed ERβ and stimulated PR (though not significantly) gene expression, an effect not seen in the animals treated either with low or high amounts of the CR extract. Expression of the IGF-I and C3 genes in the uterus (Fig. 7) was, as under acute treatment conditions, largely stimulated in the E₂-treated

Figure 1 Effects of (a) 0.5 µg E₂, (b) 62.5 mg CR extract and (c) solvent (5% cremophor = Crem) on serum LH levels before and after acute i.v. injection of the compounds. For statistical reasons mean LH levels in the hour before and for 1 and 2 h after injection of the compounds were calculated. They are shown as horizontal bars in each figure and the absolute values±S.E.M. are also given.**P* < 0.05 vs pretreatment values.

animals. Neither the low nor the high amounts of CR fed to the animals had any significant effects on these uterine parameters (Fig. 7).

The chronic effects of E₂ and the CR extract in the metaphysis of the femur were in striking contrast

Figure 2 An acute i.v. injection of E₂, the CR extract or the solvent resulted in stimulated uterine weight only by E₂ while the CR extract was totally ineffective. * $P < 0.05$ vs control.

to those seen under acute administration (Fig. 8). The expression of the marker genes for both osteoblast and osteoclast activity (collagen-1 α 1, OPG, OC and TRAP respectively) were reduced by E₂ and the higher dose of the CR extract. As under acute conditions, expression of the ER α gene remained unaffected (data not shown). In none of the metaphyses of the femur was expression of the ER β gene demonstrable (data not shown).

Figure 9 shows the CT-calculated bone densities of the metaphysis of the tibiae which were determined in

the second chronic experiment. Animals receiving unsupplemented food lost 53.7% of their metaphysal bone density. This effect was statistically significantly reduced to 38.7% by the higher dose of the CR extract. The CR-treated animals lost more metaphysal bone density in comparison with the E₂-treated animals but had significantly higher bone density than the unsupplemented controls.

Figure 10 shows the size of the paratibial fat depot in the untreated animals. The fat depot had doubled in size in comparison with the preovariectomy value. This effect was significantly reduced by E₂ and by both doses of the CR extract.

Discussion

In earlier experiments we had shown that the CR extract BNO 1055 was able to displace radiolabeled E₂ from a cytosolic ER preparation (34). The main new observation made in the present study is that this (these) yet unidentified substance(s) have estrogenic effects not only in the hypothalamo/pituitary axis to reduce serum LH levels but also in the bone where they (it) mimic(s) both acute and chronic effects of E₂. In agreement with earlier observations, the CR extract at neither dosage had any significant estrogenic effect in the uterus (2). The acute and chronic effects of E₂ on uterine weight are best explained by the stimulation of the IGF-I gene (7,

Figure 3 Effects of E₂, the CR extract and the solvent on expression of various uterine genes 6 h after i.v. injection of the compounds. (a) While E₂ significantly reduced ER α gene expression, the CR extract had no significant effect on this gene. (b) E₂ largely inhibited ER β gene expression, an effect not seen for the CR extract. Gene expression of (c) IGF-I and (d) C3 was stimulated by E₂ while no effects were observed under the CR treatment. * $P < 0.05$ vs control.

Figure 4 An acute injection of E₂ or CR extract had a significant stimulatory effect on gene expression of IGF-I in (a) the metaphysis of the femur, while expression of (c) OPG and (d) OC genes was unaffected. An acute stimulation of (b) collagen-1α1 (Col 1α1) and (e) TRAP was seen after the injection of the CR extract, an effect also seen following E₂ injection. **P* < 0.05 vs control.

8). IGF-I is a growth factor which causes proliferation of both the endometrium and the myometrium. The initial acute stimulatory effect of E₂ on uterine weights cannot, most likely, be attributed to proliferation but

to increased vascular endothelial growth factor (VEGF) action. VEGF was formerly also called the permeability factor because it causes increased capillary permeability thereby soaking the interstitial endo-

Figure 5 Chronic treatment of ovariectomized rats with E₂ or the CR extract over a period of 3 months (the compounds were applied through phytoestrogen-free pelleted food) yielded a strong stimulatory effect of E₂ on uterine weight, (a) an effect not observed under two doses of the CR extract (b) while the E₂ and the CR administration at a higher dose significantly reduced the bodyweight of the animals. **P* < 0.05 vs control.

Figure 6 The chronic application of E₂ but not of the CR extract over 3 months with the food stimulated the thickness of the endometrial epithelium significantly. * $P < 0.05$ vs control.

metrial and myometrial tissue and filling the uterine lumen with a serum ultrafiltrate (7, 35). The uteri of the chronically E₂-treated animals were solid streaks with hypertrophied endometrial and myometrial tissue which was most likely due to increased IGF-I action. The complement protein C3 has as yet unknown functions in the uterus but has been demonstrated in numerous experiments to be highly sensitively upregulated by E₂. It was therefore not surprising to see largely elevated C3 gene expression in the acutely and chronically E₂-treated animals but even this sensitive parameter did not indicate any estrogenic activity in the CR extracts as neither dose had any effect on gene expression of C3. It was interesting to observe an acute and a chronic inhibitory effect of E₂ on ER β but not on ER α gene expression. Gustafsson *et al.* (6, 27, 36) claim that ER α may mediate proliferative and ER β differentional effects (so-called Yin-Yang theory). If this is

Figure 7 A chronic treatment with E₂ had no significant effect on uterine gene expression of (a) ER α and (c) PR but inhibited gene expression of (b) ER β and stimulated gene expression of (d) IGF-I and (e) C3. None of these parameters was significantly affected by the two doses of the CR extract. * $P < 0.01$ vs control.

Figure 8 Chronic E₂ treatment suppressed gene expression of (a) collagen-1 α 1, (d) TRAP and (c) OC in the metaphysis of the femur significantly and (b) that of OPG though this effect was not statistically significant after 3 months of treatment. The high dose of CR (1000) also suppressed collagen-1 α 1 and OPG gene expression significantly. Expression of the TRAP and OC genes was also suppressed under the high CR dose; this effect, however, was not statistically significant. * $P < 0.05$ vs control.

true, the downregulation of ER β by E₂ would allow or enhance the effect of E₂ via ER α to stimulate uterine parameters involved in ballooning and growth. Indeed, ER α knock-out mice do not show uterine bal-

looning or proliferation in response to E₂ (1). This allows the conclusion that compounds in the CR extract do not address the ER of the α -subtype.

CT (24) of the metaphysis of the tibia revealed a dramatic loss of bone mass in the ovariectomized animals of more than 50% of the trabecular structures over 3

Figure 9 Trabecular density of the metaphysis of the tibia (MT) was reduced by more than 54% within 3 months after ovariectomy (ovx). This effect was significantly counteracted by E₂ and to a lesser extent (but also significantly) by CR supplementation to the pelleted food. * $P < 0.05$ vs trabecular density prior to ovariectomy. # $P < 0.05$ vs sham-fed controls (3 months after ovariectomy).

Figure 10 Size of the paratibial fat depots in the variously treated animals. Note that the size was largest in the ovariectomized (ovx) controls and significantly smaller in the E₂-treated and in the CR BNO 1055-fed rats. * $P < 0.05$ vs preovariectomy, # $P < 0.05$ vs sham-fed controls (3 months after ovariectomy).

months. This bone loss was totally prevented by E_2 and significantly reduced in the animals receiving CR-substituted food. In our experiments, the metaphysis of the femur of rats expressed only $ER\alpha$ and no $ER\beta$. This is in agreement with some, but in disagreement with the results of others (10–14, 37, 38). Most ER studies were performed in osteosarcoma cell lines, which are dedifferentiated into an embryonic state (13, 14, 36, 37) or in bones of immature rats or individuals with healing fractures or various bone diseases (11). Indeed, in developing bones our quantitative real-time RT-PCR system picks up $ER\beta$ mRNA readily (authors' unpublished data). Few publications indicate the presence of $ER\beta$ in the bone of adult rats or mice (10, 37, 38). If $ER\beta$ are present in adult bones their number is extremely low. Lim *et al.* (12) demonstrated 0.7×10^2 copies of $ER\beta$ but 2×10^5 copies of $ER\alpha$ in 1 μ g RNA.

Utilizing our RT-PCR system we have demonstrated the presence of estrogen-regulated $ER\beta$ mRNA in the uterus. Hence our method picks up $ER\beta$ in other organs but not in the metaphysis of the femur. It is interesting that $ER\beta$ protein is readily detectable in both osteoblasts and osteoclasts (13) which may point to an extremely high translational efficiency of $ER\beta$ mRNA in these bone cells.

E_2 had a clear osteoporosis-preventing effect, which appears to be mediated via the amply expressed $ER\alpha$. Since the CR extract, however, also had osteoprotective effects it is unlikely that this osteoprotective effect of CR is mediated via the $ER\alpha$ because the CR extract should then also have uterine effects which were not observed. Hence, the receptor type involved in mediating the osteoprotective effects of CR remains obscure at present. Possibly splice variants of $ER\alpha$ or $ER\beta$ which are not present in the uterus and which are not picked up by our PCR are mediating the estrogenic effects of CR. Alternatively, another, not yet cloned ER or another estrogen-binding protein which affects transcriptional efficiency of $ER\alpha$ may be involved.

It is known that many of the estrogenic effects in the bone are exerted via IGF (39–41). It was therefore not surprising to observe a slight acute stimulatory effect of E_2 on IGF-I gene expression. Estradiol appears also to activate directly (via an ERE) or indirectly (via paracrine mechanisms) collagen-1 α 1, OC and OPG gene expression. The TRAP was also rapidly activated within 6 h after E_2 treatment. Hence, not only osteoblast but also osteoclast activities are acutely upregulated by E_2 and this effect was in part shared by the higher dose of the CR extract.

The observation that in the chronically E_2 -treated rats all acutely E_2 -upregulated osteoblast parameters were downregulated below control values was not surprising because osteoclast activity was also reduced. Both bone-forming compartments appear to be highly active in estrogen-deprived animals with osteoclasts being the dominant compartment. Hence, bone turnover is increased with bone degradation being higher

than bone formation which results in osteoporosis (42, 43). In response to E_2 treatment after the above-detailed acute stimulation of both compartments, osteoblast and osteoclast activity appeared to be decreased which results in an equilibrated low bone turnover. Similar mechanisms appear to take place in the metaphysis of the femur of the CR-treated animals as the expression of the estrogen-regulated genes are qualitatively similarly affected in the CR-treated animals. Quantitatively, the effects, however, are smaller. This reflects also in the loss of bone mineral density. In the E_2 -substituted ovariectomized rats, loss of trabecular bone density of the metaphysis of the tibia as determined by quantitative CT was almost negligible in comparison with the pretreatment values. Such an anti-osteoporotic effect was also clearly present in the CR-treated animals although not as profound as in the E_2 -treated rats. However, significantly less bone was lost in comparison with the control animals.

In the same CT plane in which the metaphysis of the tibia is located, a small fat depot is visible, the size of which could be quantified. In good agreement with the generally increased bodyweight of the ovariectomized animals, this fat depot increased in size during the 3 months following ovariectomy. In the E_2 -treated animals, this effect was almost as small as prior to ovariectomy and was partially shared by the two doses of the CR extract in a dose-dependent manner. Hence, the putative phytoestrogens present in the CR extract did not only exert an estrogenic and therefore osteoprotective effect in the bone but it had also an estrogenic effect on the total bodyweight and this appears to be due to a reduced fat accumulation which had occurred in the ovariectomized control-fed animals. Hence, these substances may have anti-lipotropic or lipolytic effects.

The chemical identity of the estrogenic substance(s) in the CR extract BNO 1055 deserves some discussion. Earlier we had shown that another CR isopropanolic extract contained small amounts of formononetin (44). Later, extracts produced by the same extraction procedure were devoid of this isoflavone (45) but recently another group purified fukinolic acid out of a CR extract which had estrogenic effects (46). In the extract used for the present experiments no attempt was made to purify the estrogenic compound(s). In a recent double-blind, placebo and conjugated estrogen controlled clinical study, however, the CR extract BNO 1055 proved to have SERM activities as major climacteric complaints and bone turnover parameters as measured in the serum (crosslaps, bone-specific alkaline phosphatase, OC) were influenced as in the patients receiving conjugated estrogens (47).

In summary, we have demonstrated that the CR extract BNO 1055 contains as yet unidentified substances which have estrogenic effects in the hypothalamo/pituitary axis to reduce pituitary LH secretion and

in the bone to partially prevent osteoporosis. The CR extract BNO 1055, however, was devoid of estrogenic activities in the uterus. From these results we have concluded that in the CR extract BNO 1055 estrogenic compounds are present which have SERM activities with desirable effects in the hypothalamo/pituitary unit and in the bone but with no undesirable effects in the uterus.

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