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Silymarin is a selective estrogen receptor β (ERβ) agonist and has estrogenic effects in the metaphysis of the femur but no or antiestrogenic effects in the uterus of ovariectomized (ovx) rats

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Abstract

Silymarin is a widely used standardized mixture of flavonolignans and its major component Silybinin binds to cytosolic estrogen receptors. Here, we demonstrate that this binding is exclusive to the estrogen receptor β (ERβ). Treatment of ovariectomized (ovx) rats with silymarin or estradiol (E_2) may allow differentiation of biological effects mediated by the ERα or ERβ. E_2 inhibited serum LH, cholesterol, LDL and HDL concentrations in the blood and increased gene expression of IGF1, HbEGF and C3 in the uterus, while silymarin was totally ineffective or antagonistic in altering these parameters. Both, E_2 and silymarin inhibited expression of uterine ERβ gene. Hence, in the pituitary, liver (where the lipoproteins are synthesized) and uterus E_2 acts primarily via the ERα. Exclusive estrogenic effects of silymarin were observed in the metaphysis of the femur (MF), on osteoblast parameters (gene expression of IGF1, TGFβ1, osteoprotegerin, collagen-1α1, osteocalcin (OC)) and on the osteoclast activity marker tartrate resistant acid phosphatase (TRAP) gene expression of adult ovx rats. Our RT-PCR method detects ERβ gene expression in all organs including developing bones but not in the MF of adult ovx rats. We conclude therefore, that the effects of silymarin in this part of the bone cannot be exerted via the ERα because it does not bind to this receptor subtype. Despite the failure to detect ERβ mRNA in the MF of our animals the possibility exists that ERβ protein is present and may mediate the effects of silymarin. Another possibility may be that the effect of silymarin and therefore possibly also of E_2 in the MF may be mediated via other possibly not yet identified receptors or via an ERβ splice variant which is not detected by our PCR-method.

Keywords: Silymarin; Rat; Estrogen receptor; LH; Lipids; Uterus; Metaphysis of femur

1. Introduction

Silymarin was initially purified out of St. Mary's thistle (*Silybum marianum*) and is a standardized mixture of four flavonolignans (silybinin, isosilybinin, silydianin and silychristin) and the isoflavonoid taxifolin [1,2]. This purified extract is widely used to treat toxic effects in the liver [3]. Further purification into individual compounds is difficult, expensive and therefore not commercially available. There is evidence that silybinin, a hybrid molecule between an isoflavone and a lignan binds to cytosolic estrogen receptors [1]. Since the pioneering work of Gustafsson and co-workers [4] it is known that two estrogen receptors exist. The classical estrogen receptor is now called ERα, the newly discovered is the estrogen receptor β (ERβ). Little is known about the biological functions of the ERβ. According to Gustafsson, both ER receptor subtypes have "YIN-YANG" activities, i.e. they often antagonize each others function [5,6].

It is known, that estradiol (E_2) has profound and essential effects in the uterus to stimulate endo- and myometrial growth to prepare the uterus for a possible pregnancy [5]. Hence, the uterus is loaded with estrogen receptors. The dominant receptor subtype is the ERα, but ERβ is also expressed [7–9]. In ERα knock-out mice E_2 has no uterotrophic effects [10] and it is therefore believed, that the effects on uterine growth promoting factors, such as hemoglobin binding epidermal growth factor (HbEGF), insulin like growth factor 1 (IGF1) and vascular endothelial growth factor (VEGF) [11,12] are exclusively exerted via the ERα [10]. The ERβ gene in the uterus is also regulated by E_2, an effect of which the physiological significance is not understood [13]. Similarly, complement protein C3 in

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the uterus is estrogen-regulated, its upregulation by E_2 is the most sensitive estrogenic parameter available in the whole rat organism [14]. The function of C3, however, is largely undefined.

It is well accepted that E_2 has antiosteoporotic effects in the bone which appear to be mediated primarily by ERα or another yet unidentified estrogen receptor [15]. ERα knock-out mice develop osteoporosis [6] while the ERβ KO mice have little bone defects and if any, they occur primarily in the cortical bone [17]. Many of the antiosteoporotic effects of E_2 are mediated by estrogen regulated IGF1, transforming growth factor-β-1 [18–21] and osteoprotegerin [22]. Osteocalcin (OC) is an osteoblast protein with unknown function in the bone and also the bone-specific alkaline phosphatase (BAP) is of osteoblastic origin [22]. Osteoclasts are also estrogen receptive [23,24]. The tartrate resistant acid phosphatase is an osteoclast product, of which the function is to acidify the osteoclasts surrounding to enable bone demineralisation [25,26]. Hence gene expression of this enzyme is a reliable osteoclast activity marker. Osteoprotegerin is an osteoclast regulatory protein which is produced by osteoblasts [22]. It competes with osteoclasts differentiation factor (ODF) at osteoclasts for the ODF receptor [27]. Hence high osteoprotegerin concentrations in the bone will prevent osteoclast differentiation. It is highly likely that the effects of E_2 in osteoblasts and osteoclasts are mediated via the ERα or a yet nor identified ER, since ERβ KO mice have little bone defects [17].

Serum lipids, such as cholesterol, high density and low density lipoproteins (HDL and LDL) and triglycerides are also estrogen-regulated and in ovariectomized (ovx) rats total cholesterol, HDL and LDL are reduced following E_2 treatment while serum triglycerides are increased [28].

Little is known about the ER subtype to which silymarin binds. The availability of recombinant ERα and ERβ protein allows now to study binding characteristics of putatively estrogenic substances. Therefore, we tested the binding properties of commercially available silymarin to these two receptor types. It is also not known whether silymarin is able to mimic some of the earlier described actions of E_2 in the uterus, in the bone and on lipid metabolism. Hence, we performed studies in which ovx rats were subcutaneously injected with E_2 or silymarin or the solvent over a period of 7 days and measured several estrogen-regulated parameters in the uterus, in the metaphysis of the femur (MF) and in the serum.

2. Material and methods

Silymarin is a purified and standardized mixture of four flavonolignans and a flavonoid prepared of an extract of St. Mary's thistle. The preparation used for the present experiments was provided by Bionorica AG (Neumarkt, Germany) and contained the following substances: 56% silybinin and isosilybinin, 17% silychristin, 15% silydianin, 12% taxifolin.

2.1. Estrogen receptor ligand binding assay (ER-LB)

The tracer estradiol 17β-J^{125} was purchased from NEN (Dreieich, Germany). Recombinant human ERα and ERβ were obtained from PanVera (Madison, USA). All other chemicals were purchased from Sigma (Deisenhofen, Germany). Subtype specific ERα and ERβ LBs were performed according to the recently described method of Kuiper et al. [29] with the exception that bound and free tracer was separated by adsorption on dextran coated charcoal. In brief, the reaction mixture consisted of 5 μl test sample in 1% EtOH, 5 μl recombinant ER protein (0.5 pmol), 5 μl tracer (final concentration 3 nM, diluted in assay buffer) and 100 μl assay buffer (50 mM Tris, pH 7.5; 150 mM KCl, 1 mM EDTA, 10% (v/v) glycerol). Properties to displace the tracer (iodinated estradiol-17β) was tested with increasing amounts of E_2 and silymarin. Samples were incubated for 18–20 h at 6 °C in triplicates. Bound and free ligands were separated by addition of 200 μl dextran-coated charcoal buffer (0.5% charcoal, 0.05% dextran in assay buffer). After an incubation at 6 °C for 5 min, samples were centrifuged for 5 min at 1000 × *g*. The supernatant was decanted and counted in a γ-counter and the counts per min (cpm) used to construct standard errors for E_2 and silymarin.

A total of 36 Sprague Dawley rats were ovx and kept under standardized conditions (lights on 6.00 a.m. to 6.00 p.m., relative humidity 55%, phyto-estrogen-free but potatoe-protein-enriched food ad libitum) for 3 weeks. They were then divided into three subgroups consisting of each 12 animals. For the 7 following days each animal was either s.c. injected with E_2 (3.5 μg) or silymarin (3.5 mg per day) or with 1 ml 5% cremophor which was the solvent for the other compounds. Injection occurred regularly at 6.00 a.m.

Animals were sacrificed 5–6 h following the last injection, exsanguinated, the uteri and the distal metaphyses of the femures removed and immediately stored under liquid nitrogen. Total RNA was extracted from the metaphyses and the uteri from liquid nitrogen chilled pulverized samples. RT-PCR was performed as previously described utilizing the real-time PCR Taqman system (PE Applied Biosystems, Foster City, CA, USA). Details concerning primer sequences and the respective references are given in Table 1.

Serum LH levels were measured by specific radioimmunoassays supplied by the NHPP (Dr. F. Parlow, Harbour General Hospital, UCLA Medical Center, Torrance, CA, USA). Serum cholesterol, HDL, LDL, triglycerides and alkaline phosphatase concentrations were determined with commercially available kits (Roche Diagnostics, Mannheim, Germany).

For all laboratory methods standard operating procedures were available. Statistical analysis was done by Students' *t*-test. A *P*-value <0.05 was considered statistically significant.

Table 1
Primers and probes, product sizes and references and/or GenBank accession numbers for Taqman-PCR

Gene	Fwd and Rev primer	Taqman probe	Size (bp)	Reference
IGF-1	5′-TGTCGTCTTCACATCTCTTCTACCTG-3′ 5′-CCACACACGAACTGAAGAGCGT-3′	5′FAM-TTACCAGCTCGGCCACAGCCGGAC-TAMRA3′	121	[30] M15480
TGFβ	5′-GGGCTTTCGCTTCAGTGCT-3′ 5′-TCGGTTCATGTCATGGATGGT-3′	5′FAM-TCAGTCCCAAACGTCGAGGTGACCTG-TAMRA3′	121	X52498
OPG	5′-CCTCTTTCTTTCTGCCTCTGATAGTC-3′ 5′-CCAAGTCTGCAACTCGAATCAAAT-3′	5′FAM-CGTATCAGGTGCACGAGCCTTATCCCA-TAMRA3′	150	[31] U94330
Collagen-1α1	5′-GGCGAAGGCAACAGTCGAT-3′ 5′-TCCATTCCGAATTCCTGGTCT-3′	5′FAM-TGCACGAGTCACACCGGAACTTGG-TAMRA3′	173	Z78279
Osteocalcin	5′-CAAAGCCCAGCGACTCTGA-3′ 5′-AGGTAGCGCCGGAGTCTATTC-3′	5′FAM-CCTTCATGTCCAAGCAGGAGGGCAGT-TAMRA3′	85	[32] M11777
TRAP	5′-GATCACCTTGGCAATGTCTCG-3′ 5′-GGCTGACAAAGTCGTCGGAAT-3′	5′FAM-TGCCTACTCCAAGATCTCCAAGCGCTG-TAMRA3′	175	[33] M76110
VEGF	5′-GGGAGCAGAAAGCCCATGA-3′ 5′-GCTTGAAGATATACTCTATCTCATCGGG-3′	5′FAM-TACCAGCGCAGCTATTGCCGTCCAATTG-TAMRA3′	125	[29,34] M32167
HbEGF	5′-GGACTACTGCATCCACGGAGA-3′ 5′-GGTAGGGTCAGCCCATGACA-3′	5′FAM-CCACTGCCTCCCTGGTTACCATGGA-TAMRA3′	108	L05489/ L05389
C3	5′-CTGTACGGCATAGGGATATCACG-3′ 5′-ATGCTGGCCTGACCTTCAAGA-3′	5′FAM-TGCCATCCTCACAACACTTCCGCAG-TAMRA3′	199	X52477
ERβ	5′-GAGGAGATACCACTCTTCGCAATC-3′ 5′-GGAGTATCTCTGTGTGAAGGCCAT-3′	5′FAM-CAGGGCATCTGTCACCGCGTTCAG-TAMRA3′	159	[4] U57439

3. Results

Fig. 1a and b details results of a radio receptor assay utilizing recombinant ERα (Fig. 1a) or ERβ (Fig. 1b). Non-labelled E_2 was effective to displace the radioactively labelled E_2 from the ERα preparation with an EC of 2.9 nM and with an EC of 3.4 nM from the ERβ protein. Silymarin was totally ineffective in displacing radioactively labelled E_2 from the ERα protein while it displaced the tracer from the ERβ protein with an EC of approximately 5.1 μM (molecular weight of the major component of silymarin, i.e. silybinin is 500). While E_2 significantly reduced serum LH levels, silymarin had no such effect (Fig. 2). Similarly, the cholesterol, HDL and LDL lowering effects of E_2 were not shared by the flavonolignan (Fig. 3). Silymarin, however, reduced serum triglyceride levels significantly.

Fig. 4 demonstrates a stimulatory effect of E_2 on uterine growth (Fig. 4a), IGF1 (Fig. 4b) as well as on VEGF, HbEGF (Fig. 4c and d) and *C3* gene (Fig. 4e) expression but an inhibitory effect on the expression of the ERβ gene (Fig. 4f). Silymarin did not share any of the stimulatory effects of E_2 on uterine parameters. In fact, expression of C3 and of HbEGF genes were significantly reduced by the flavonolignan. As E_2, silymarin inhibited ERβ gene expression. The effects of E_2 and silymarin in the metaphysis of the femur are shown in Fig. 5. The injection of E_2 and of silymarin had identical stimulatory effects on the expression of IGF1 (Fig. 5a), TGFβ1 (Fig. 5b), osteoprotegerin (Fig. 5c), collagen-1α1 (Fig. 5d), osteocalcin (OC) (5e) and on TRAP (Fig. 5f) gene expression. ERβ gene expression was undetectable in the metaphysis of the femur of any animal, while gene expression of ERα was readily detectable but neither regulated by E_2 nor by silymarin (data not shown).

While the growth factor gene expression is increased under E_2 and silymarin treatment conditions, gene expression of osteoprotegerin is decreased (Fig. 5c) by both E_2 and silymarin. The gene expression of tartrate resistant alkaline phosphatase, an osteoclast activity marker is significantly increased by E_2, an effect which is mimicked by silymarin (Fig. 5f).

4. Discussion

Silymarin is a mixture of three flavonolignans of which silybinin is the dominant compound in the preparation used for the present experiment. Utilizing recombinant ERα and ERβ protein, we demonstrate for the first time that the flavonolignans present in silymarin bind exclusively to the ERβ but not to the ERα receptor protein. Hence, they—including silybinin—can be considered pure ERβ-specific ligands. This opens the question whether silymarin acts in an estrogen-agonistic or antagonistic way or possibly as a selective estrogen receptor modulator (SERM) on the β-subtype of the estrogen receptor. Therefore, we studied a number of biological effects of estradiol-17β in the uterus, the bone,

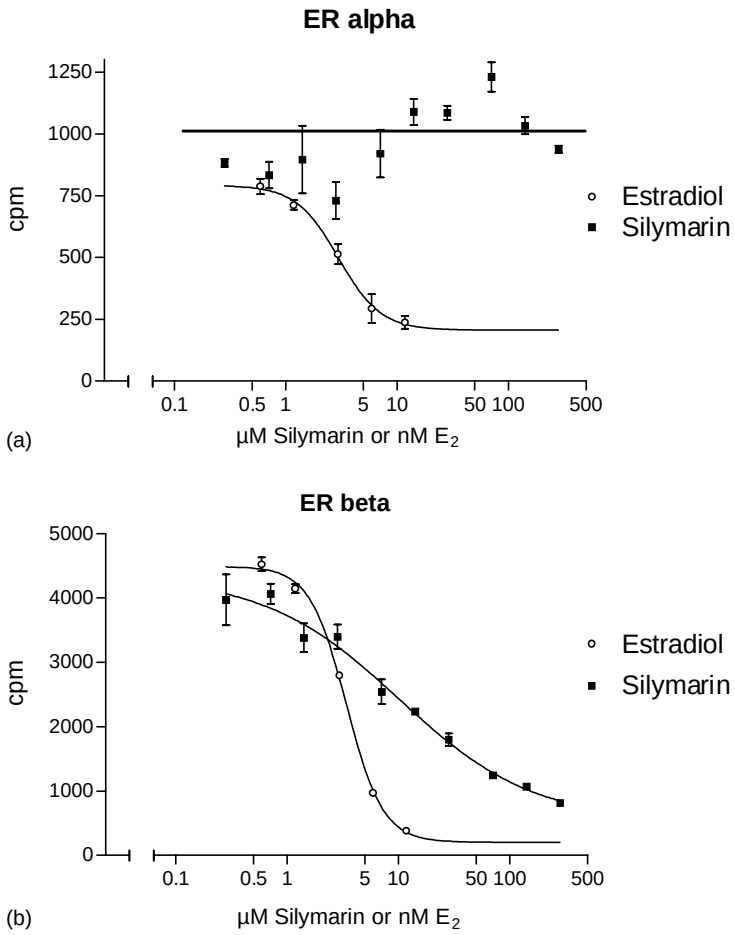

Fig. 1. Radio receptor assay curves utilizing recombinant ERα (a) and ERβ (b) as receptors for radioactively labelled E_2. Non-labelled E_2 readily displaced the radioactivity from the receptor preparations, while silymarin was totally ineffective in the ERα preparation, but competed with E_2 for the ERβ. Hence this assay indicates an exclusive ERβ selectivety of all components present in silymarin.

and on serum LH and lipids. All parameters are known to be regulated by E_2 [35].

E_2 decreased serum LH levels and this effect was not seen in the silymarin-treated animals. From gene-targeted mice it is known that the negative feedback effect of E_2 is exclusively exerted via the ERα subtype as serum LH levels in ERα knock-out mice are elevated and cannot be reduced by E_2 [16]. Hence, the β-selective action of silymarin would not be expected to exert an estrogenic negative feedback action on LH secretion.

It is known that the two major compartments of cholesterol are the high and low density lipoproteins (HDL and LDL), which are synthesized in the liver. The liver is primarily ERα-receptive and therefore the cholesterol, LDL and HDL lowering effects of E_2 can be best explained by an effect of E_2 on the ERα receptors in the liver. Therefore, the lacking effects of the ERβ-selective compound silymarin are not surprising. No effect of the 1 week lasting E_2 treatment on serum triglyceride was seen in the E_2 treated animal while these lipids were significantly reduced by silymarin; hence this effect appears to be ERβ mediated.

IGF1, HbGF and VEGF are known to be involved in the E_2 induced proliferation and vascularization of endo- and myometrial tissue [11,12]. Such effects of E_2 are not seen in ERα knock-out mice indicating that proliferation and thereby uterine growth is exclusively mediated via the ERα [13]. Hence, one would not expect to see an effect of ERβ selective compounds, such as silymarin. We did observe, however, a reduction of mRNA concentrations of HbGEF and C3 in the uterus. This may indicate, that the β-receptors in the endo- and myometrium have antiproliferative and thereby ERα antagonistic effects on the uterus. This assumption would fit with the view of Gustafsson and co-workers [5,35] that ERα and ERβ may have each other antagonizing effects (so-called “YIN-YANG” theory). It was also interesting to note, that uterine ERβ gene expression was down-regulated by both E_2 and silymarin, which may be another E2 mediated effect exerted via the ERβ.

In earlier and in the present experiments we demonstrated that the metaphysis of the femur of ovariectomized rats do not express the ERβ gene. Hence, we did not expect to observe any silymarin effects in the bone and were surprised

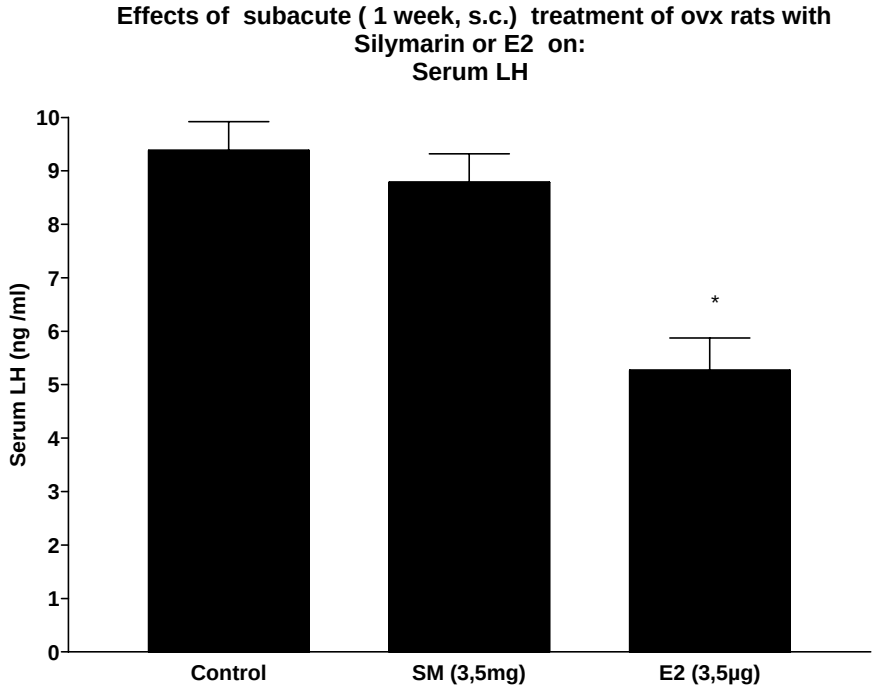

Fig. 2. Serum LH levels in ovx rats were significantly reduced 6 h after the last injection of 3.5 μg E_2, while silymarin was ineffective ($^*P < 0.05$).

to observe clear estrogenic antiosteoporotic effects in this bone structure. It is known that many of the effects of E_2 in the bone are mediated via IGF1 and TGFβ1 [18–21]. In the present experiments we show that E_2 given for 1 week stimulated IGF1 and TGFβ1 gene expression and this effect was clearly shared by silymarin. Similarly, synthesis of the bone-specific collagen-1α1 and of osteocalcin was stimulated by both, estradiol and silymarin as the mRNA of this protein was also significantly increased by both compounds. TGFβ1 is also a proliferative factor involved in proliferation of osteoblasts and therefore an antiosteoporotic growth factor [18,22]. Osteocalcin is an osteoblast protein with yet unknown functions of which the serum levels are reportedly down-regulated by E_2 [36]. Both, TGFβ1 and osteocalcin gene expression are up-regulated by E_2, an effect shared by silymarin although its effect was much less profound. It was surprising to observe increased gene expression of osteocalcin. In acutely E_2 treated animals we observed a stimulation of serum osteocalcin protein (unpublished). It is therefore, possible that E_2 acutely stimulates osteocalcin gene and protein expression, an effect which may fade off after longer lasting E_2 influence. Gene expression of osteoprotegerin was significantly inhibited by both E_2 and silymarin which may indicate a higher availability of ODF which would allow osteoclast differentiation into a mature state. On the other hand, if such osteoclast maturation had occurred it appears that their activity is decreased by E_2 and silymarin as tartrate-resistant acid phosphatase gene expression was down-regulated under both treatments. There is indeed some evidence that these two observations do not contradict each other as increased osteoclast maturation is essential as a first step for bone remodelling [37]. At a later time point these mature osteoclasts may become apopototic, which would explain their decreased total activity in the metaphysis [37].

These results indicate that the regulation of pituitary LH secretion, hepatic LDL and HDL production as well as uterine growth mediated by E_2 are exclusively ERα-regulated as the specific ERβ ligand silybinin did not have any effects on these parameters. Uterine ERβ gene expression was down-regulated by both, E_2 and silybinin indicating a direct effect on the ERβ-subtype. Surprisingly, silybinin had estrogenic effects in the metaphysis of the femur, which with our highly sensitive and selective ERβ-PCR, is devoid of ERβ mRNA. Similarly, Lim et al. [38] reported the presence of less than 100 ERβ copies per μg mRNA of bone marrow cells whereas more than 2×10^6 ERα copies per μg mRNA were found. The presence of ERβ protein however was reported in mature bone [23]. This rises the possibility that the ERβ protein stems from a splice variant which is not detected by commonly used RT-PCR and that another subtype of receptor which is not ERα and not ERβ, may transmit E_2 and silymarin estrogenic activity into osteoblasts. A third estrogen receptor subtype (ERγ) has recently been cloned in fish [39] and the possibility that such estrogen receptor is also involved in the regulation of mammalian bone metabolism has been discussed [40].

How do these results compare to the impact on the human? A lack of effect of silymarin on the hypothalamic GnRH pulse generator indicates no effect on climacteric hot flushes.

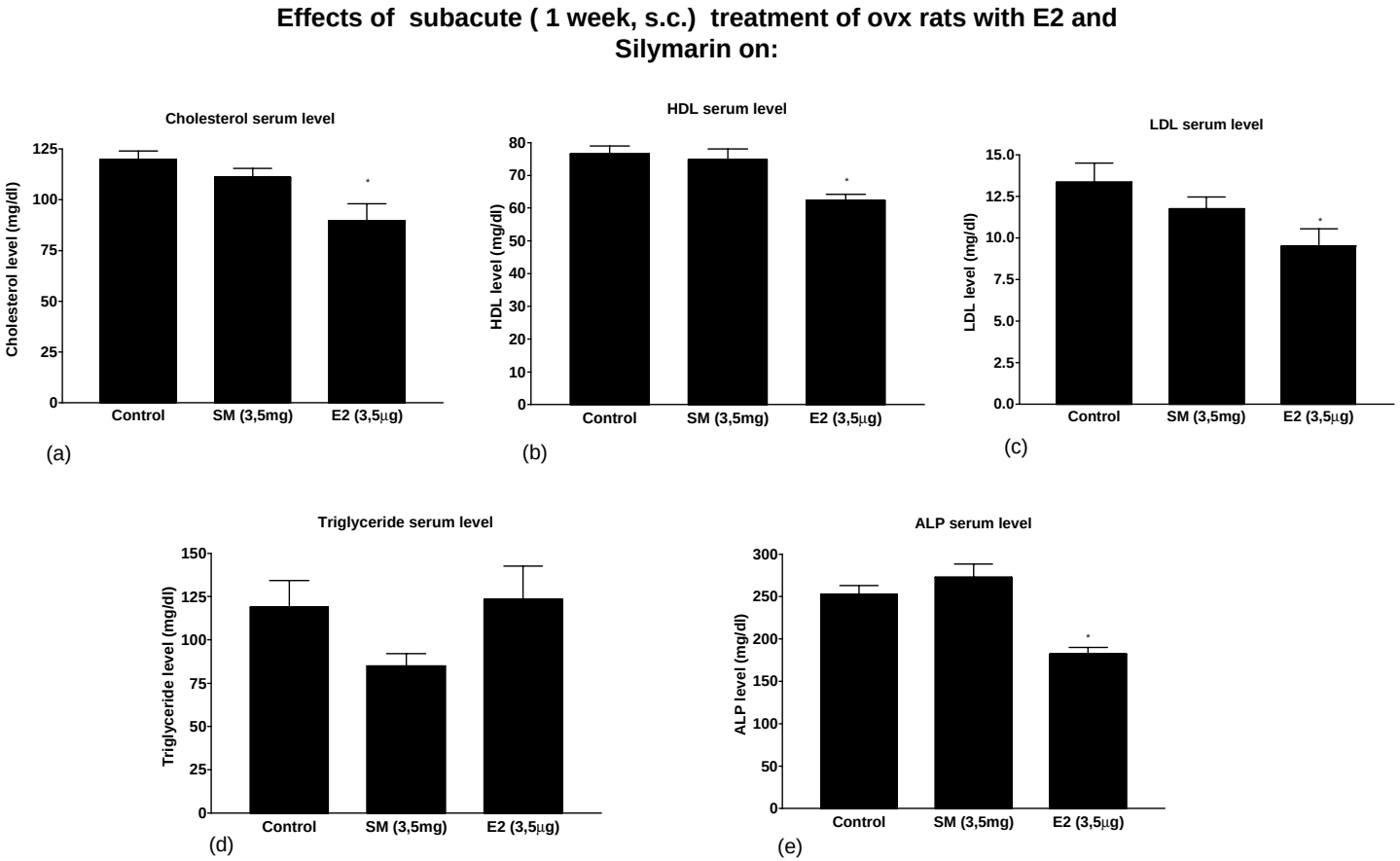

Fig. 3. Effects of E_2 and silymarin treatment on serum cholesterol (a), high density (b), low density lipoproteins (c), (HDL, LDL), on serum triglycerides (d) and alkaline phosphatase (e). Note that silymarin had no estrogenic effects on cholesterol, HDL, LDL and ALP but inhibited serum triglyceride concentrations ($^*P < 0.05$).

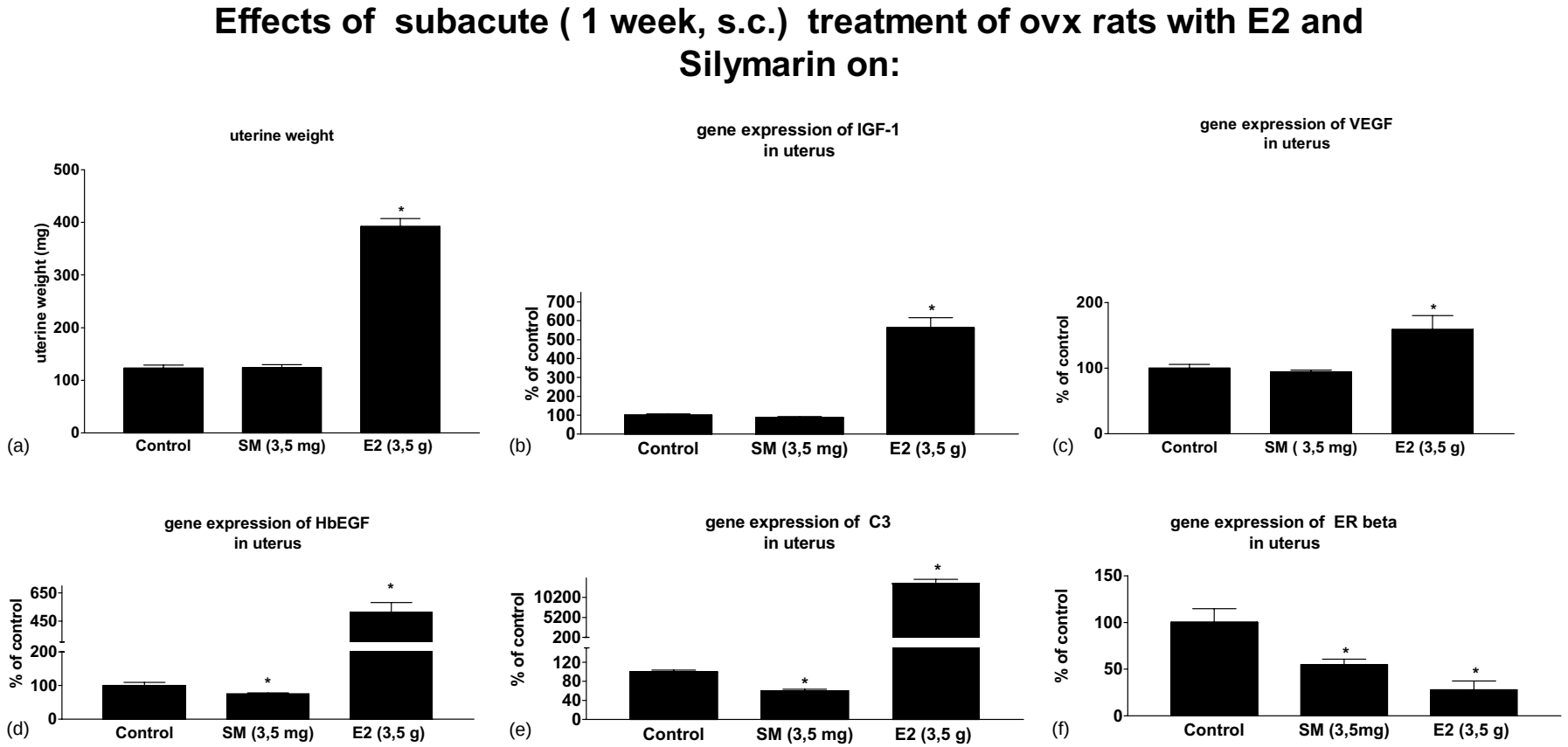

Fig. 4. Effects of E_2 and SM treatment on uterine growth (a), gene expression of IGF1 (b), VEGF (c), HbEGF (d), complement C3 (e) and ERβ (f). While E_2 stimulated gene expression of the growth factors and C3, SM had no effects. Both, E_2 and silymarin inhibited ERβ gene expression ($^{*}P < 0.05$).

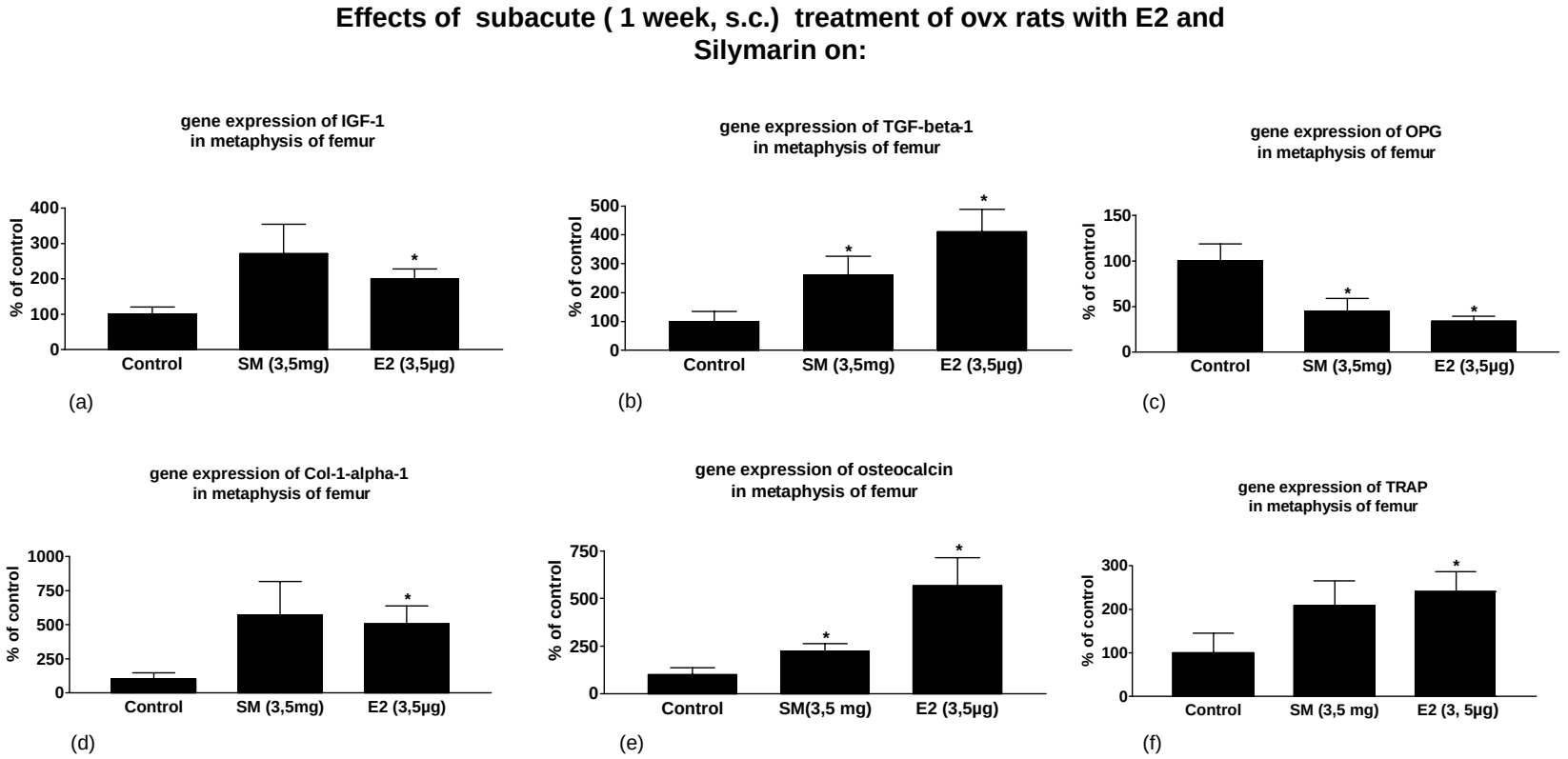

Fig. 5. Effect of E_2 and SM on gene expression of IGF1 (a), TGFβ1 (b), OPG (c), collagen-1α1 (d), osteocalcin (e) and TRAP (f) in the metaphysis of the femur. Note that silybinin mimicks all E_2 effects on osteoblast as well as on osteoclast markers, regardless whether they are stimulated or inhibited by E_2.

No effect of the flavonolignanes on serum cholesterol, HDL and LDL may be a disadvantage because estrogenic effects on HDL and LDL may be of relevance for the development of artheriosclerosis [28]. If silymarin is considered for the use in postmenopausal women it may be for the treatment of osteoporosis. Hence, silymarin fulfils the criteria of a selective estrogen receptor modulator (SERM) with similar properties as raloxifen.

Acknowledgements

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