Inhibition of leptin gene expression and secretion by silibinin: possible role of estrogen receptors

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Abstract Leptin plays the role of mitogenic factor in the breast carcinogenesis. Therefore, it could be considered as a target for breast cancer therapy. Leptin gene expression could be modulated by activation of estrogen receptors. Silibinin is an herbal compound with anti-cancer activity on prostate and colorectal cancers. Based on the fact that targeting of leptin can be considered as a novel strategy for breast cancer therapy, the aim of this study was the investigation of potentiality of silibinin for inhibition of leptin gene expression and secretion, and its link with expression of estrogen receptors. Cytotoxic effect of silibinin on T47D breast cancer cells was investigated

by MTT assay test after 24, 48 and 72 h treatments with different concentrations of silibinin. The levels of leptin, estrogen receptor α and estrogen receptor β genes expression was measured by reverse-transcription real-time PCR. The amount of secreted leptin in the culture medium was determined by ELISA. Data were statistically analyzed by one-way ANOVA test. Silibinin inhibits growth of T47D cells in a time and dose dependent manner. There was significant difference between control and treated cells in the levels of leptin, estrogen receptor β expression levels and the quantity of secreted leptin was decreased in the treated cells in comparison to control cells. In conclusion, silibinin inhibits the expression and the secretion of leptin and in the future it might probably be a drug candidate for breast cancer therapy through leptin targeting.

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Introduction

Breast cancer is the most leading cancer with a high mortality rate in the world (Jemal et al. 2010). Many factors are involved in breast carcinogenesis, including adipocytokines, like leptin (Vona-Davis and Rose 2007). Leptin, a 167 amino acids hormone with a molecular mass of 16 kDa, is mainly secreted from

adipose tissue (Zhang et al. 1994). It has a central role in the control of satiety, energy expenditure, food intake and many reproductive processes (Collins et al. 1996). It also affects the metabolic and hematopoietic systems (Fantuzzi and Faggioni 2000). Adipose tissue is the main source of leptin (Löffler et al. 2001), however, other sources of leptin have also been identified in the body including testicles (Soyupek et al. 2005), ovaries (Löffler et al. 2001), the placenta (Masuzaki et al. 1997), the cartilage and bone cells (Morroni et al. 2004), the skeletal muscle (Solberg et al. 2005), and the stomach (Mix et al. 2000). Furthermore, the mitogenic, transforming or migration-inducing properties of leptin have been revealed in many different cell types such as smooth muscle cells (Oda et al. 2001), normal and neoplastic colon cells (Hardwick et al. 2001; Liu et al. 2001) and also normal and malignant mammary epithelial cells (Dieudonne et al. 2002; Laud et al. 2002). It has been shown that leptin induces growth and transformation in T47D breast cancer cells (Hu et al. 2002). Leptin acts through binding to its receptor called leptin receptor (ObR) on the target cell membrane (Tartaglia 1997). Significantly higher levels of both leptin and ObR expression have been found in cancer tissue relative to non-cancer epithelium (Ishikawa et al. 2004). In addition, numerous breast cancer cell lines such as MCF-7 and T47D could express leptin and ObR (Laud et al. 2002). All these observations confirm that leptin can not only act by endocrine and/or paracrine pathway on mammary tumor cells, but also it could act via an autocrine pathway. Additionally, a significantly positive correlation has been obtained between leptin and ObR expressions in breast cancer tissue (Koda et al. 2007). Therefore, this paracrine-autocrine leptin axis could become a target for leptin inhibiting drugs in cancer treatment and prevention. Results of a study showed that the ratio of $ER\alpha/ER\beta$ in human adipose tissue was significantly correlated with the level of serum leptin in vivo (Shin et al. 2007). Thus, $ER\alpha$ and $ER\beta$ might have different roles in the regulation of leptin expression.

Silibinin, a flavonolignan prepared from milk thistle, is involved in cancer chemoprevention in preclinical models of prostate and colorectal cancers. Interestingly, results of a study demonstrated a specific binding of silibinin to recombinant $ER\beta$, but not to $ER\alpha$ (Seidlová-Wuttke et al. 2003). Therefore, silibinin can be considered as a pure $ER\beta$ specific ligand.

Considering the important roles of leptin in the breast cancer biology, in this study, we investigated the possible variations in the leptin secretion and expression as well as expression of ERs in the T47D breast cancer cell line after its treatment with pure silibinin for different times and doses.

Materials and methods

Chemicals and reagents

Silibinin [2,3-dihydro-3-[4-hydroxy-3-methoxyphenyl]-2-[hydroxymethyl]-6-[3,5,7-trihydroxy-4-oxobenzopyran-2-yl]benzodioxin], MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], glycine and NaCl were obtained from Sigma (Deisenhofen, Germany); leptin ELISA kit was purchased from Labor Diagnostika Nord GmbH and Co, KG (Nordhorn, Germany); fetal bovine serum and phenol-red free RPMI 1640 with L-glutamine were purchased from Gibco BRL (Life Technologies, Grand Island, NY, USA); the T47D cell line was obtained from the National Cell Bank of Iran (Tehran, Iran); Sodium bicarbonate, Streptomycin and Amphotericin B were purchased from Merck (Darmstadt, Germany); Penicillin G was purchased from SERVA (Heidelberg, Germany); TRIZOL reagent was purchased from Invitrogen (Eugene, OR, USA); First-Strand cDNA Synthesis kit was purchased from Fermentas (Hanover, MD, USA); and Syber Green-I reagent was purchased from Takara Bio (Otsu, Japan).

Cell culture

T47D cells were cultured in RPMI 1640 (+L-glutamine) supplemented with 10 % FBS, penicillin G (100 IU/ml), streptomycin (100 mg/l), and amphotericin B (2.5 µg/ml) and incubated at 37 °C in a humidified atmosphere containing 5 % CO₂.

MTT assay and cell treatment

The cytotoxic effect of silibinin on T47D cells was studied by MTT assay after 24, 48 and 72 h treatments. Briefly, 2,000 cells/well were cultivated in a 96 well culture plate. After 24 h incubation at 37 °C, cells were treated with different concentrations of silibinin (0–120 µM) for 24, 48 and 72 h in quadruplicate.

Then, the medium of all wells was removed carefully and 50 μ l MTT (2 mg/ml) was added to each well and incubated for 4.5 h in the dark, followed by the addition of 200 μ l DMSO. Thereafter, Sorensen's glycine buffer (0.1 M glycine, 0.1 M NaCl, pH = 10.5) was added and absorbance of each well was read at 570 nm in 15–30 min. For data analysis, mean OD of each well was calculated. Finally, a graph was plotted using SPSS software version 18.0 (SPSS Inc., Chicago, IL, USA) and IC₅₀ of silibinin on T47D was determined (Pourhassan et al. 2010).

For studying the inhibitory effect of silibinin on leptin, ER α and ER β expression as well as leptin secretion, 1×10^5 cells/well were treated in a 6-well plate with different concentrations of silibinin (0, 20, 40, 60 and 100 μ M) for 24 h. A group containing 0.1 % DMSO without silibinin served as vehicle control.

Total RNA extraction and cDNA synthesis

Total RNA was extracted by the use of the TRIZOL Reagent according to the manufacturer's instructions. The concentration of prepared RNA was measured using a NanoDrop spectrophotometer (Termoscientific, Wilmington, DE, USA) and its integrity was confirmed by electrophoresis on a 1.2 % agarose gel containing 1 % formaldehyde.

After RNA preparation, cDNA was synthesized using the First-Strand Synthesis kit according to the manufacturer's instructions. The synthesized cDNA was immediately used in realtime PCR or stored at -70°C for later use.

Real-time PCR

The real-time PCR method was used for the analysis of leptin, ER α , and ER β gene expression levels in the

control and treated T47D cells. The β -actin gene expression was used as the internal control for normalization.

The real-time PCR reaction was done in triplicate using the syber green-I reagent in the Rotor GeneTM 6000 system (Corbett Research, Sydney, Australia) according to the manufacturer's instructions. The amplification conditions were as follows: leptin (2 min at 95°C and a two-step cycle of 95°C for 15 s and 60°C for 40 s for 40 cycles), ER α (5 min at 95°C and a two-step cycle of 95°C for 30 s and 57°C for 40 s for 40 cycles), and ER β (5 min at 95°C and a two-step cycle of 95°C for 15 s and 64°C for 40 s for 40 cycles). Sequences of primers are shown in Table 1.

Changes in leptin, ER α , and ER β gene expression levels between the control and treated T47D cells were calculated by the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen 2001).

Measurement of the secreted leptin

For the analysis of possible effect of silibinin on the amount of secreted leptin in the treated cells compared with the control cells, leptin concentrations were measured in the supernatant of cells using a human leptin ELISA kit according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed with SPSS software version 18.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean $\pm$ SD. The differences in expression levels of leptin, ER α and ER β genes as well as quantity of secreted leptin between the control and treated cells were analyzed by one-way ANOVA,

Table 1 Primers used for real-time PCR amplifications

Primer	Primer length	Sequence (5' $\rightarrow$ 3')	Product size (bp)
Leptin forward	22	CACCAAAACCCTCATCAAGACA	80
Leptin reverse	24	CTTTCTGTTTGAGGAGACTGACT	
ER α forward	20	GCCAGCAGGTGCCCTACTAC	132
ER α reverse	23	TGGTACTGGCCAATCTTTCTCTG	
ER β forward	19	AAGAGCTGCCAGGCCTGCC	268
ER β reverse	21	GCGCACTGGGGCGGCTGATCA	
β -actin forward	20	TGGACTTCGAGCAAGAGATG	137
β -actin reverse	20	GAAGGAAGGCTGGAAGAGTG	

followed by Dunett's multiple comparison tests. p value <0.05 was considered as significant.

Results

MTT assay

Data analysis of cytotoxicity assay showed that the IC_{50} s of silibinin of the T47D breast cancer cell line were 109, 75 and 31 μ M for 24, 48 and 72 h MTT assays, respectively (Fig. 1). The obtained IC_{50} s were time-dependent (p value <0.05).

Quantitative real-time PCR

Real-time PCR results showed a significant decrease in the leptin gene expression in treated cells compared to control cells (p value <0.05) (Fig. 2). We, also, measured the expression levels of $ER\alpha$, and $ER\beta$ genes in the treated and control cells. Although, no significant difference was detected in $ER\alpha$ expression levels between the treated and control cells, a significant increase was observed in the $ER\beta$ mRNA level (p value <0.05) (Fig. 3). Therefore, the $ER\alpha/ER\beta$ expression ratio has been decreased in the treated cells compared to the control cells. There was no significant difference between the DMSO control and the DMSO-free control (the 0 μ M concentration of silibinin).

Data analysis revealed a significantly negative correlation between leptin gene expression and $ER\beta$ gene expression level and no correlation between leptin and $ER\alpha$ gene expression levels in the treated cells compared to the control cells (p value <0.05). In addition, a significantly positive correlation was found between mRNA level of leptin and $ER\alpha/ER\beta$ gene expression ratio (p value <0.05).

Measurement of secreted leptin

Amounts of secreted leptin were assessed using ELISA. A significant difference was found between the control and treated cells in terms of secreted leptin (p value <0.05) (Fig. 4). This finding was in accordance with the inhibition of leptin gene expression by silibinin. Positive correlations were found between the secreted leptin, gene expression levels of leptin and $ER\beta$ but not with $ER\alpha$.

Discussion

MTT assay showed a rigorous cytotoxicity of silibinin on T47D cells which confirms a previous report on cytotoxic effects on T47D cells (Tiwari et al. 2011). The cytotoxic effects on T47D cells suggest that it may have therapeutic significance for breast cancer treatment. Our study demonstrated that silibinin can inhibit leptin gene expression and secretion in T47D breast cancer cells and this decrease in leptin gene expression and secretion was negatively associated with $ER\beta$ gene expression. These results indicate that silibinin has a strong potential to interact with the expression of leptin gene, which has significant roles in the carcinogenesis and proliferation of breast cancer cells (Vona-Davis and Rose 2007). Regarding to the critical role of leptin in breast carcinogenesis (Garofalo and Surmacz 2006), there were many attempts to inhibit leptin function and secretion. Gonzalez et al. (2009) inhibited the growth of murine mammary cancer cell and of a xenograft tumor model of human breast cancer cell lines by a leptin peptide antagonist (Gonzalez et al. 2009). In addition, leptin analog that mimics its action (Peters et al. 2007) and anti-leptin receptor monoclonal antibody (Fazeli et al. 2006) were further approaches for inhibiting the leptin function. In another study, administrations of some compounds including the β 3-adrenoreceptor agonist, conjugated linoleic acid, isoflavone, resveratrol and bitter melon led to a decreased secretion and serum levels of leptin (Ray and Cleary 2010). The current work, however, aimed to direct inhibition of leptin gene expression and secretion in human T47D breast cancer cell line by using silibinin. Due to the significant anti-cancer effects of silibinin on various types of cancers such as prostate, skin, colon, bladder and breast (Kaur and Agarwal 2007), it might be used as a chemotherapeutic agent in breast cancer therapy.

Lin et al. (2009) found that silibinin blocks the mammalian target of rapamycin signaling pathway with a concomitant reduction in the translation initiation, thus inhibiting growth of transformed cells. Singh et al. (2009) reported that silibinin/silymarin also inhibited the secretion of proangiogenic factors from tumor cells, and caused growth inhibition of endothelial cells. Our results, regarding the inhibition of leptin gene expression, confirm these findings and demonstrate another role for silibinin that it probably acts through estrogen receptors.

Fig. 1 The cytotoxic effect of silibinin on T47D cells during treatment for 24 h (a); 48 h (b) and 72 h (c) assessed by MTT assays. The IC_{50} s of silibinin on T47D cells after 24, 48 and 72 h exposure are indicated

Fig. 2 Effect of silibinin on leptin expression levels in T47D cells. Increased silibinin concentration caused significant decrease in the gene expression of leptin (p value <0.05)

It has been demonstrated that expression of leptin has a positive correlation with expression of $ER\alpha$ and a negative correlation with expression of $ER\beta$ (Yi et al. 2008). In addition, the leptin promoter has response elements for both $ER\alpha$ and $ER\beta$ (O’Neil et al. 2001); probably by which activated $ER\beta$ (bound to the specific ligand) causes the reduction of leptin gene transcription by a reduced activity of the leptin promoter. Seidlová-Wuttke et al. (2003) reported that silymarin—whose major component is silibinin—specifically bound to recombinant $ER\beta$ but not to $ER\alpha$. In the current study, we showed that changing leptin gene expression by silibinin negatively correlated with the expression of $ER\beta$. Therefore, it seems that one of the possible mechanisms of the inhibitory effects of silibinin on leptin expression may be through the activation of $ER\beta$. However, this potential pathway must be confirmed by additional studies in the future. In addition, many further studies are needed to determine the exact molecular mechanism(s) involved in the reduction of leptin gene expression and secretion by silibinin.

Conclusion

In summary, the results of this study demonstrated that silibinin could inhibit gene expression and secretion of

Fig. 3 Effect of silibinin on expression levels of $ER\alpha$ (a) and $ER\beta$ (b) genes in T47D cells. There is a significant increase in $ER\beta$ gene expression levels with an increase in the silibinin concentration (p value <0.05), unlike for the $ER\alpha$ gene expression levels

leptin in T47D breast cancer cells. In addition, the probable role of $ER\beta$ in the inhibition of leptin expression and secretion must be confirmed by future additional studies. Regarding the significant roles of leptin in breast carcinogenesis, the inhibition of its expression and secretion could be considered as a novel strategy for the treatment of breast cancer in the future.

Fig. 4 Effect of silibinin on leptin secretion in T47D cells (p value <0.05)

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Conflict of interest The authors have no competing interests.

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