Effects of estrogen receptor α and β on the expression of visfatin and retinol-binding protein 4 in 3T3-L1 adipocytes

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Received April 3, 2013; Accepted July 8, 2013

DOI: 10.3892/ijmm.2013.1440

Abstract. The aim of this study was to investigate the effects of estrogen and estrogen receptor α (ERα) and β (ERβ) on the expression of visfatin and retinol-binding protein 4 (RBP4) by treating 3T3-L1 adipocytes with estradiol (E2), estrogen receptor agonists and antagonists. Mature adipocytes were exposed to E2, the ERα agonist, 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT), the ERβ agonist, 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN), E2 with the ERα antagonist, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP), and E2 with the ERβ antagonist, (5R, 11R)-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol [(R,R)-THC], at various concentrations. To determine the effects of ER subtypes on the expression of adipokines, quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) and western blot analyses were performed. E2 concentrations of 10^{-5} and 10^{-6} mol/l induced a statistically significant increase in the expression of RBP4 (P=0.012 and P=0.011, respectively). In the cells treated with 10^{-5} mol/l PPT, RBP4 expression significantly increased (P<0.05) in a dose-dependent manner. Treatment with the ERα antagonist, MPP (10^{-5} mol/l), and E2 suppressed the expression of RBP4 (P=0.032). However, the expression of RBP4 was not significantly altered when the cells were treated with the ERβ agonist or antagonist. The expression of visfatin was not affected by different concentrations of E2 and ERs. 17β-estradiol significantly increased the secretion of RBP4 and upregulated RBP4 expression via ERα but not ERβ in 3T3-L1 adipocytes. RBP4 expression was regulated by estrogen in the 3T3-L1 adipocytes and this effect was selectively mediated by ERα.

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Key words: adipocytes, adipokines, visfatin, retinol-binding protein 4

Introduction

Adipose tissue is not only a simple energy store, but is increasingly being recognized as an important organ in the regulation of metabolism and pathological processes. Adipokines are a series of soluble factors secreted by adipose tissue. There are several known types of adipokines, such as relatively well known leptin, adiponectin, interleukin-6 and resistin, as well as visfatin and retinol-binding protein 4 (RBP4) (1-3). Visfatin is a 52-kDa cytokine secreted by visceral fat and its expression level in plasma increases with the severity of obesity. Visfatin exerts insulin-mimetic effects and its plasma levels increase in overweight and obese patients with metabolic syndrome (2,4). RBP4 was initially thought to function only in the delivery of retinol to tissues (5). However, the transgenic overexpression of human RBP4 causes insulin resistance and glucose intolerance through a retinol-independent mechanism, whereas the normalization of serum RBP4 induces insulin sensitivity. In obese patients with type 2 diabetes mellitus, serum levels of RBP4 are increased (1,6). Substances involved in the regulation of adipokine expression in adipocytes are currently being investigated. Such substances may participate in energy elevation, sugar or lipid metabolic processes and the overall process of obesity within the body.

Estrogen deficiency during menopause causes excessive visceral adipose tissue accumulation which is known to be linked to metabolic syndrome (7). Loss of ovarian function is related to both an increase in total fat and an accumulation of central fat, which increases the risk of cardiovascular and metabolic disease (8-11). Adipose tissue metabolism is directly influenced by sex hormones, particularly estrogen, and the estrogen receptor (ER) is expressed at the mRNA and protein level in human adipose tissues (12). ERα and ERβ are both expressed in adipose tissue and bind estrogen with different affinities (13,14). The physiological role of ERβ appears to be a modulator of ERα activity *in vitro* (15). However, very little is known about the effects of the two subtypes of ERs on the expression of visfatin and RBP4.

In this study, we aimed to demonstrate the effects of estrogen via ERs on visfatin and RBP4 expression by manipulating

the concentration of estradiol (E2) and ERα- and β-selective agonists and antagonists in 3T3-L1 adipocytes.

Materials and methods

Materials. We used 17β-estradiol (1,3,5[10]-estratriene-3, 17β-diol, cell culture tested) purchased from Sigma (St. Louis, MO, USA). The ERα selective agonist, 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT), the ERβ selective agonist, 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN), the ERα selective antagonist, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP), and the ERβ pure antagonist and partial ERα agonist, (5R, 11R)-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol [(R,R)-THC], were purchased from Tocris Bioscience (Ellisville, MO, USA). Anti-mouse RBP4 and anti-mouse visfatin antibodies were obtained from R&D Systems (Minneapolis, MN, USA). Anti-GAPDH antibody was purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Cell culture. Mouse 3T3-L1 fibroblasts (American Type Culture Collection, Manassas, VA, USA) were plated at $5x10^4$ cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 25 mM glucose, 10% fetal bovine serum (FBS), 500 units/ml penicillin and 500 μg/ml streptomycin (medium A) at 37˚C in a 5% CO_2 humidified atmosphere. The cells were grown in the same medium until three days after confluence and were then differentiated into mature adipocytes by treatment with 500 μM 3-isobutyl-1-methylxanthine (IBMX), 250 nM dexamethasone and 330 nM insulin in medium A. The cells were then incubated for two days in medium A containing 330 nM insulin, followed by four days of incubation in medium A. The medium was changed every two days. After eight days of incubation, the intracytoplasmic accumulation of lipid droplets was observed in the fully differentiated 3T3-L1 adipocytes and they were stained with Oil Red O solution (0.5% Oil Red O in isopropanol). For all the experiments, mature adipocytes were serum-starved for 12 h and then incubated with or without chemical reagents at various concentrations. At first, the cells were incubated in sterile medium containing various concentrations of E2. To modulate ERα and ERβ expression in adipocytes, the ER agonists, PPT or DPN, were added to the cells at several concentrations. In subsequent experiments, the ER antagonists, MPP or (R,R)-THC, were added to cells along with a constant dose of E2 for stimulation. These cells were used for the measurement of RBP4 and visfatin expression.

Assessment of cell viability and cell number. Cultured cells were detached from the culture dishes with 0.05% trypsin-EDTA (Gibco BRL, Life Technologies, Merelbeke, Belgium) at 72 h of culture under different culture conditions. The cells were stained with trypan blue (Gibco BRL, Life Technologies) and viable cells were counted on a hemocytometer without staining.

Total RNA isolation and reverse transcription reaction. RNA was extracted and purified using an RNeasy lipid tissue mini kit as suggested by the manufacturer (Qiagen, Valencia, CA, USA). The RNA concentration was measured using a spectrophotometer (DU®530; Beckman Coulter, Fullerton, CA, USA) and RNA quality was confirmed on agarose gels. A total RNA sample (2 μg/sample) was used for cDNA synthesis in a volume of 20 μl using a SuperScript™ III First-Strand Synthesis System for RT-PCR kit (Invitrogen, Milano, Italy). RNA was reverse-transcribed under the following conditions: 25 mM $MgCl_2$, 10 mM dNTP mix, 10X RT buffer, 0.1 M DTT, 200 U of SuperScript™ III (Invitrogen), 40 U of RNaseOut and 50 μM oligo(dT) primers in a final volume of 20 μl. The reaction was incubated at 65˚C for 5 min and 50˚C for 50 min and the enzyme was then heat-inactivated at 85˚C for 5 min. Four microliters of the reaction product were used for quantitative PCR.

Quantitative PCR. Quantitative PCR was used to quantify the mRNA expression of RBP4 and visfatin. The expression was normalized using the GAPDH housekeeping gene product as an internal reference. The primers and probes were designed for mouse RBP4 and visfatin using Primer Express® Software version 2.0 (Applied Biosystems, Foster City, CA, USA). RBP4 and visfatin mRNA levels were quantified using TaqMan Real-Time PCR with an ABI 7700 system (Applied Biosystems). Gene-specific probes and primer pairs for RBP4 (Assays-on-Demand, Mm00803264_m1; Applied Biosystems) and visfatin (Assays-on-Demand, Mm00451938_m1; Applied Biosystems) were used. For each probe/primer set, a standard curve was generated, which was confirmed to increase linearly with increasing amounts of cDNA. The amplification conditions were 2 min at 50˚C, 10 min at 95˚C and a two-step cycle of 95˚C for 15 sec and 60˚C for 60 sec for a total of 45 cycles.

Western blot analysis. The cells were lysed using a buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1% Triton X-100 and a mixture of protease inhibitors [aprotinin, phenylmethyl sulfonyl fluoride (PMSF) and sodium orthovanadate]. Equal amounts of total protein were resolved on a 12% SDS-polyacrylamide gel and proteins were transferred onto a nitrocellulose membrane. After blocking (TBS, 0.1% Tween-20) at 4˚C overnight, the membranes were incubated with primary antibodies for anti-mouse RBP4 (dilution 1:1000) or anti-mouse visfatin (dilution 1:1000) for 2 h followed by incubation with secondary antibodies linked to HRP, anti-mouse GAPDH (dilution 1:2000). Immunoreactive proteins were visualized by chemiluminescence using SuperSignal® West Dura Extended Duration Substrate (Pierce Chemical Co., Rockford, IL, USA) and a Fujifilm Luminescent Image Analyzer LAS-3000 with a charge-coupled device camera (Science Imaging Scandinavia AB).

Statistical analysis. To compare the mRNA expression levels of RBP4 and visfatin in adipocytes, analysis of variance (ANOVA) with a post-hoc Dunnett's test was used. Data are presented as the means ± standard error of the mean. To evaluate the presence of a correlation, Pearson's correlation coefficient and linear regression analysis were used. Null hypotheses of no difference were rejected if $P<0.05$. All statistical analyses were performed using the SPSS statistical package version 10.0 (SPSS, Inc., Chicago, IL, USA).

Results

Effects of estradiol on expression of RBP-4 and visfatin in adipocytes. To investigate the effects of E2 on the expression

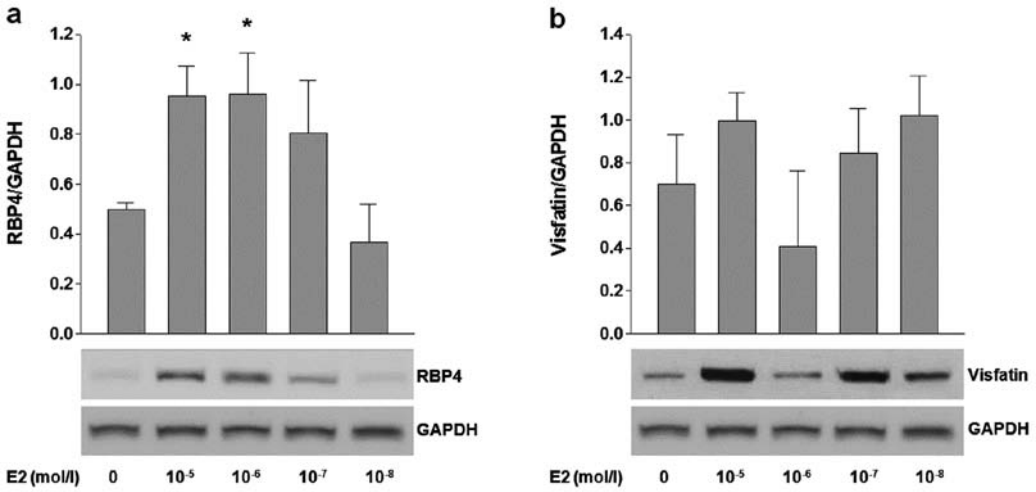

Figure 1. Effects of estradiol (E2) on the protein and mRNA expression of (a) retinol-binding protein 4 (RBP4) and (b) visfatin. 3T3-L1 adipocytes were incubated in medium containing E2 at the indicated range of concentrations for 24 h. Total RNA was extracted and (a) RBP4 and (b) visfatin mRNA levels were measured using quantitative reverse transcription PCR. Western blot analysis was used to measure the protein expression of estrogen receptor (ER) in 3T3-L1 adipocytes under the same conditions. Data are the means ± SD of three independents experiments. Results were analyzed by ANOVA and post-hoc Dunnett's test. *P<0.05 vs. control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

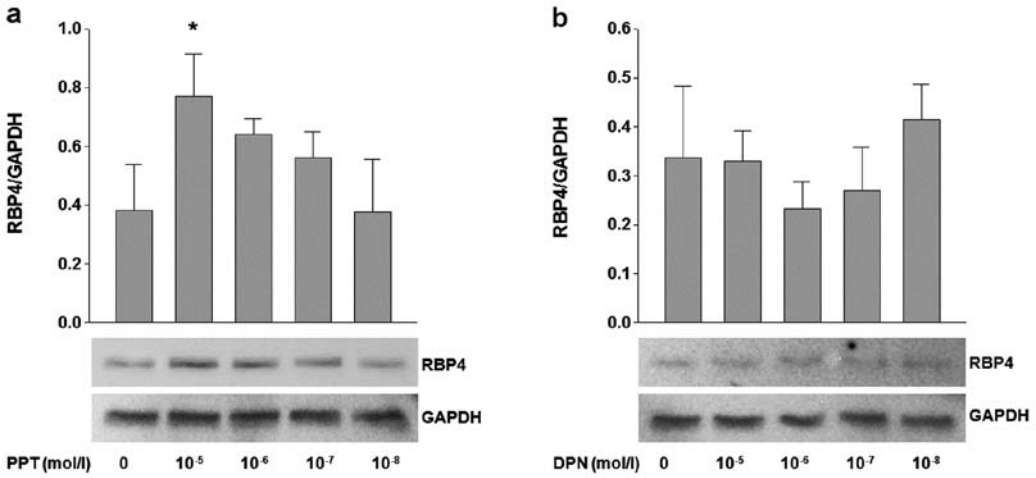

Figure 2. Effects of estrogen receptor (ER) agonists on the protein and mRNA expression of retinol-binding protein 4 (RBP4) in 3T3-L1 adipocytes. The 3T3-L1 adipocytes were incubated in medium containing (a) 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) and (b) 2,3-bis(4-hydroxyphenyl)-propionitrile at the indicated concentrations for 24 h. Total RNA was extracted and RBP4 mRNA levels were measured using reverse transcription PCR. Western blot analysis was used to measure the protein expression of RBP4 in 3T3-L1 adipocytes under the same conditions. Data are the means ± SD of three independent experiments. Results were analyzed by ANOVA and the post-hoc Dunnett's test. *P<0.05 vs. control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

of RBP4 and visfatin, 3T3-L1 adipocytes were treated with various concentrations (10^{-5}-10^{-9} mol/l) of E2. Treatment with high concentrations (10^{-5} and 10^{-6} mol/l) of E2 significantly increased the RBP4 mRNA levels (P=0.012, P=0.011, respectively), as well as RBP4 protein expression (Fig. 1a). However, the expression of visfatin was not influenced by any tested concentration of E2 (Fig. 1b).

Effects of ERα and ERβ agonists (PPT and DPN) on the expression of RBP4 and visfatin. The adipocytes were treated with various concentrations of PPT (ERα agonist) and DPN (ERβ agonist) to investigate the effects of ERα and ERβ on the expression of RBP4 and visfatin. The cells treated with 10^{-5} mol/l PPT showed a significant and dose-dependent increase in RBP4 mRNA (P<0.05) and protein expression (Fig. 2a). On the other hand, the adipocytes treated with DPN showed no difference in expression (Fig. 2b). Of note, the mRNA and protein expression of visfatin was not influenced by treatment with PPT and DPN (Fig. 3a and b).

Effects of ERα and ERβ antagonists [MPP and (R,R)-THC] on the expression of RBP4 and visfatin. In parallel with the ER agonist experiment, ER antagonists [MPP and (R,R)-THC] were used to verify the functions of ER subtypes in fat cells. The aim of this experiment was to isolate the effects of each ER subtype. The cells were treated simultaneously with a fixed concentration of E2 (10^{-7} mol/l) to provide an appropriate stimulus. Treatment with high concentrations (10^{-5} mol/l) of MPP and E2 (10^{-7} mol/l) resulted in the reduced expression of RBP4 at the mRNA and protein level (P=0.032). No significant

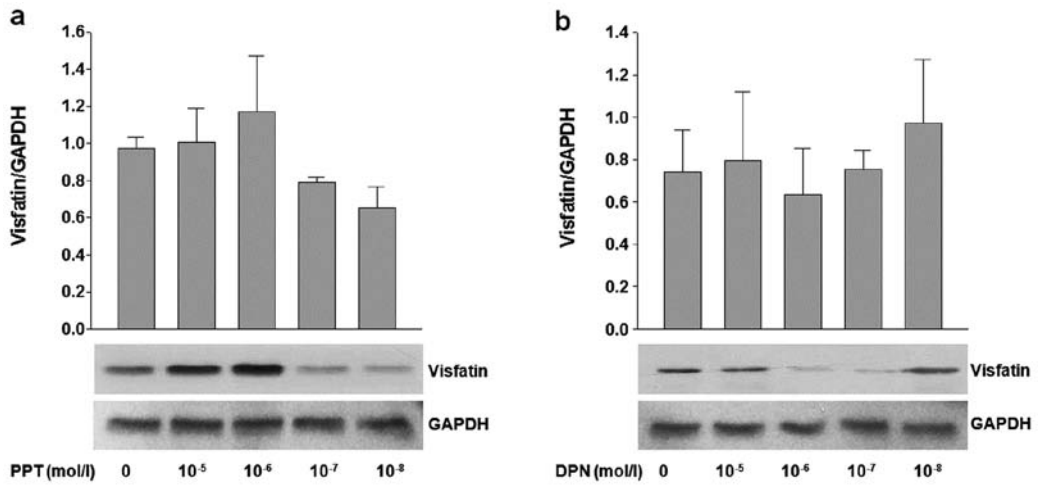

Figure 3. Effects of estrogen receptor (ER) agonists on protein and mRNA expression of visfatin in 3T3-L1 adipocytes. The 3T3-L1 adipocytes were incubated in medium containing (a) 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) and (b) 2,3-bis(4-hydroxyphenyl)-propionitrile at the indicated concentrations for 24 h. Total RNA was extracted and visfatin mRNA levels were measured using reverse transcription PCR. Western blot analysis was used to measure the protein expression of visfatin in 3T3-L1 adipocytes under the same conditions. Data are the means ± SD of three independent experiments. Results were analyzed by ANOVA and the post-hoc Dunnett's test. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

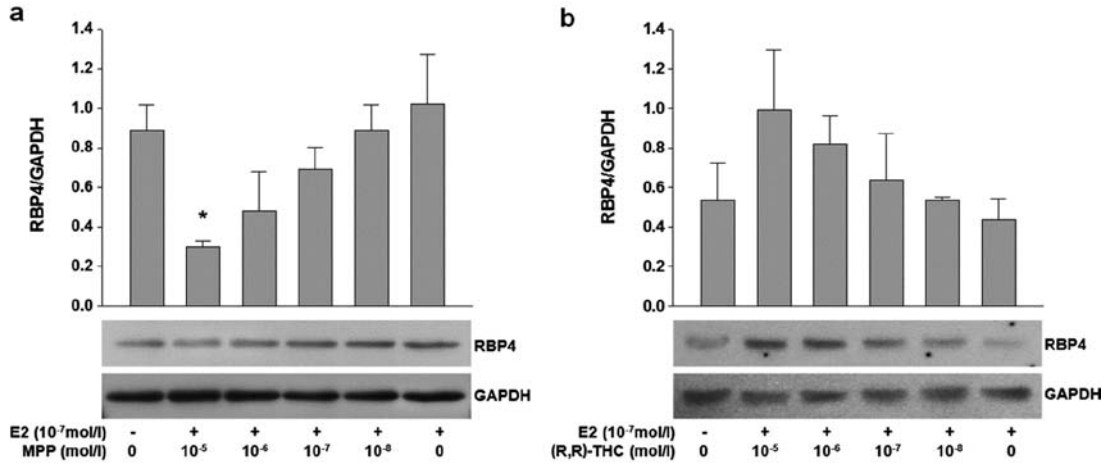

Figure 4. Effects of estrogen receptor (ER) antagonists on protein and mRNA expression of retinol-binding protein 4 (RBP4) in 3T3-L1 adipocytes.The 3T3-L1 adipocytes were incubated in medium containing (a) 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP) and (b) (5R, 11R)-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol [(R,R)-THC] in conjunction with E2 at the indicated concentrations for 24 h. Total RNA was extracted from the adipocytes and the RBP4 mRNA levels were measured using quantitative reverse transcription PCR. Western blot analysis was used to measure protein expression of RBP4 in 3T3-L1 adipocytes under the same conditions. Data are the means ± SD of three independent experiments. Results were analyzed by ANOVA and the post-hoc Dunnett's test. *P<0.05 vs. control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

change in the mRNA level of RBP4 was observed following treatment with variable concentrations of (R,R)-THC (Fig. 4a and b). There was no significant change in the expression of visfatin upon treatment with various concentrations of MPP or (R,R)-THC (Fig. 5a and b).

Discussion

In this study, we evaluated the effects of 17β-estradiol on RBP4 and visfatin expression in 3T3-L1 adipocytes. RBP4 mRNA expression and protein production was increased in a dose-dependent manner in adipocytes by estradiol. RBP4 does not reach adult plasma levels until puberty, after which plasma levels are changeable, based on the menstrual cycle (16-18). This variation in the plasma RBP level appears to correlate with peak levels of estradiol and menopausal status in females. Previous studies have reported that plasma RBP4 levels in post-menopausal women are higher than those in pre-menopausal women, which is associated with insulin resistance (19,20). After menopause, women develop increased amounts of visceral fat due to fat redistribution. RBP4 expression is highly elevated, not just in serum, but also in visceral fat, and serum RBP4 protein levels are considered a marker of intra-abdominal fat mass (21). Thus, we initially assumed that a correlation may exist between specific adipokines in visceral fat and estrogen. ERα and β densities are more dependent on the location of adipose deposition than on gender, with visceral depots showing higher mRNA densities (22). Our results demonstrated that 17β-estradiol significantly increased the secretion of RBP4 and upregulated the expession of RBP4

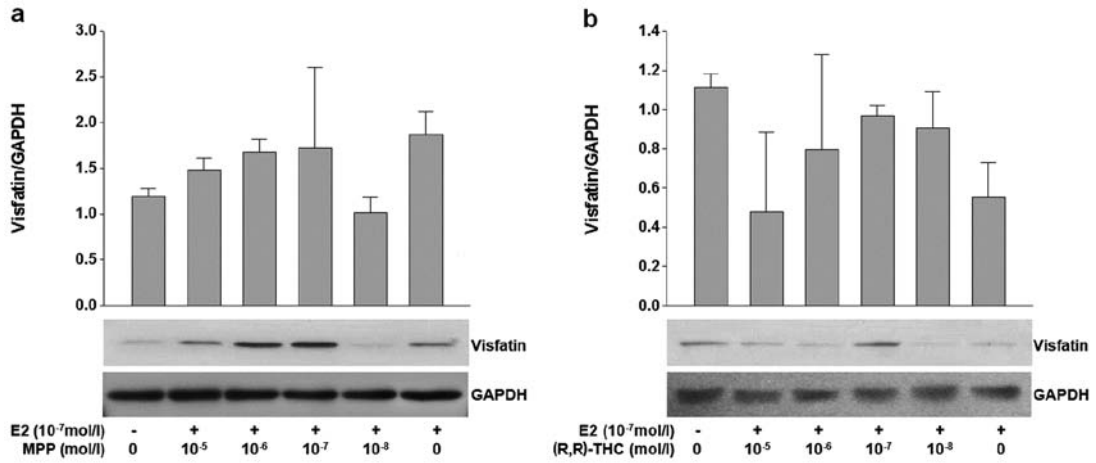

Figure 5. Effects of estrogen receptor (ER) antagonists on protein and mRNA expression of visfatin in 3T3-L1 adipocytes. The 3T3-L1 adipocytes were incubated in medium containing (a) 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP) and (b) (5R, 11R)-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol [(R,R)-THC] in conjunction with E2 at the indicated concentrations for 24 h. Total RNA was extracted from the adipocytes and visfatin mRNA levels were measured using quantitative reverse transcription PCR. Western blot analysis was used to measure the protein expression of visfatin in 3T3-L1 adipocytes under the same conditions. Data are the means ± SD of three independent experiments. Results were analyzed by ANOVA and the post-hoc Dunnett's test. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

in 3T3-L1 adipocytes, which is consistent with the results of previous studies (23,24).

In a previous study, Janke *et al* reported that RBP4 gene expression in adipose tissue was decreased in obese menopausal subjects and that there were no differences in serum RBP4 levels among lean, overweight and obese menopausal subjects (25). However, their experiment used human subcutaneous adipose tissue *in vivo* and the RBP4 level is not related to subcutaneous fat mass. The serum RBP4 level does not correlate with the subcutaneous fat diameter, but rather with the visceral fat diameter (26).

The reason estrogen significantly increases RBP4 expression in adipose tissues is unclear. Vitamin A (retinol) is taken up by peripheral tissue, such as the genital tracts in the form of free retinol by passive diffusion based on the concentration gradient between the blood and cytosol. Retinol is therefore thought to play a pivotal role in the female reproductive organs. In addition, estrogen itself appears to control retinoic acid biosynthesis. Estrogen has been shown to markedly increase the cellular RBP4 mRNA content in rat vagina and uterus tissues, which participate in the uptake and/or intracellular metabolism of retinol (27). We found that estrogen significantly upregulated the expression of RBP4 in 3T3-L1 adipocytes. Thus, the estrogen-induced upregulation of RBP4 expression in adipose tissue is expected to result in a shift in the equilibrium of retinoic acid in the reproductive organs. Our results suggest that estrogen mediates retinoic acid metabolism by the regulation of RBP4 expression in adipose tissue. The systemic deficiency of serum estrogen may activate a specific regulatory process and consequently, may induce the overexpression of RBP4 through ER in visceral adipocytes. These data, together with our finding that estrogen stimulates RBP4 expression in adipocytes, this process may be partially due to a decrease in serum estrogen levels after menopause.

Visfatin may play an important role in adipocyte metabolism in association with metabolic syndrome-related diseases. Visfatin is regulated by conditions associated with metabolic syndrome and visfatin mRNA expression is regulated by sex hormones in 3T3-L1 pre-adipocytes (28). The estriol treatment of 3T3-L1 cells has been shown to increase visfatin gene expression, but estradiol had insignificant effects on visfatin gene expression (29). These results are partly consistent with our finding that visfatin was not directly influenced by estrodiol in 3T3-L1 adipocytes.

Estradiol selectively influences adipose tissue, according to the type of adipokine. ERα and β control the expression of leptin in different ways (30). However, our results verified that adipokines can be influenced independently by ERα without being affected by ERβ. There are many existing studies on the effect of sex hormones on adipokine expression, but the results of these studies differ according to the type of adipokine or the experimental design (31-33). These differences suggest that the pathway may be dependent on adipokine type and may be established through the specific ER type. However, a comprehensive analysis of the effects of steroidal hormones on adipokine expression is warranted.

We found that estrogen significantly increased RBP4 expression via ERα in 3T3-L1 adipocytes without influencing visfatin expression, suggesting a novel role for ERα in the regulation of RBP4 expression. To the best of our knowledge, this is the first demonstration that 17β-estradiol significantly increases the secretion of RBP4 and upregulates RBP4 expression via ERα, but not ERβ, in 3T3-L1 adipocytes. RBP4 may have a specific function in visceral fat redistribution in menopausal women. Through the control of sex hormones in menopausal women, the regulation of RBP4 expression in adipose tissue may potentially inhibit visceral fat redistribution and ultimately protect post-menopausal women from obesity-related diseases. Further *in vivo* studies are required to investigate the link between RBP4 expression and estrogen, while considering the effects of estrogen status, including the menstrual cycle. In addition, the results of this study need to be clinically verified. The development of novel therapeutics for metabolic syndrome-related diseases will require further understanding of the correlation between the effects of estrogen and adipokines.

Acknowledgements

This study was supported in part by the Konyang University Myunggok Research Fund of 2009.

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