Available online at www.sciencedirect.com
SCIENCE DIRECT
European Journal of Pharmacology 538 (2006) 1–4
www.elsevier.com/locate/ejphar

Raloxifene promotes adipocyte differentiation of 3T3-L1 cells

Yuko Murase [a], Junji Kobayashi [a,*], Atsushi Nohara [a], Akimichi Asano [a], Naoto Yamaaki [a], Kaoru Suzuki, Hiroshi Sato [b], Hiroshi Mabuchi [a]

[a] *Department of Molecular Genetics of Cardiovascular Disorders, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa, 920-8640, Japan*
[b] *Department of Molecular Virology and Oncology and Center for the Development of Molecular Target Drugs, Cancer Research Institute, Kanazawa University, Japan*

Received 12 January 2006; received in revised form 13 February 2006; accepted 13 March 2006
Available online 27 April 2006

Abstract

To explore the possibility that raloxifene might influence an adipocyte differentiation and lipogenesis, we studied the effects of raloxifene on the expression of adiponectin and other peroxisome proliferator-activated receptor γ targeting genes using the 3T3-L1 adipocytes. With standard adipogenic inducers, we added raloxifene at various doses for the adipocyte differentiation. Higher doses of raloxifene facilitated lipid accumulation of the 3T3-L1 cells. We next examined the differentiating and differentiated adipocytes and found that raloxifene augmented mRNA levels of adiponectin, adipocyte-specific fatty acid binding protein, and lipoprotein lipase dose-dependently in both. These effects were opposite those of 17β-estradiol treatment. These findings suggest that raloxifene promotes adipocyte differentiation, providing a novel insight into the treatment of postmenopausal metabolic syndrome with hypoadiponectinemia.

Keywords: Raloxifene; 3T3-L1 adipocyte; Adiponectin; Lipoprotein lipase

1. Introduction

Recent studies indicate that estrogen is inversely associated with antiatherogenic and insulin-sensitizing adipokine adiponectin (Tanko et al., 2004; Gavrila et al., 2003), which is known to be a marker of adipocyte differentiation (Combs et al., 2002). Estrogen itself has direct effects on adipocytes to inhibit lipogenesis (Homma et al., 2000; Palin et al., 2003). Recently, raloxifene, a selective estrogen receptor modulator, has been increasingly used for the treatment and prevention of osteoporosis. Raloxifen is a nonsteroidal benzothiophene compound (Fig. 1), and several modifications to the 2-arylbenzothiophene core have shown that the 6-hydroxy and 4-hydroxy substitutions of raloxifene are important for estrogen receptor binding and for mimicking the corresponding 3- and 17β-hydroxyl groups of 17β-estradiol (Grese et al., 1997).

We recently found that raloxifene raised circulating adiponectin in non-diabetic postmenopausal women (unpublished data). To explore the possibility that raloxifene might influence adipocyte differentiation and lipogenesis in vitro, this study researched the effects of raloxifene on the expression of adiponectin and other peroxisome proliferator-activated receptor γ (PPARγ) targeting genes, including adipocyte-specific fatty acid binding protein (aP2) and lipoprotein lipase (LPL) using 3T3-L1 adipocytes.

2. Materials and methods

NIH 3T3-L1 cells (Green and Kehinde, 1975, 1976) (Health Science Research Resource Bank, Osaka, Japan) were grown in a phenol red-free Dulbecco's modified Eagle's medium (DMEM/F-12) (Gibco) with 10% calf serum. Two days after cell confluence (designated as day 0), differentiation was initiated with 10% fetal bovine serum (FBS) containing 0.5 mM isobutylmethylxanthine (IBMX) and 1 μM dexamethasone for 2 days. The cells were then incubated in a DMEM/F-12 supplemented with 10% FBS and 1 μg/ml insulin for 2 days and

* Corresponding author. Tel.: +81 76 265 2268; fax: +81 76 234 4271.
E-mail address: junji@med.kanazawa-u.ac.jp (J. Kobayashi).

0014-2999/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2006.03.033

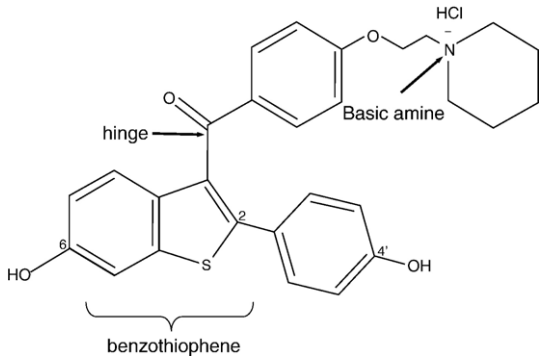

Fig. 1. Chemical structures of raloxifene hydrochloride.

maintained thereafter with 10% FBS/DMEM/F-12 to day 8. Raloxifene 0.1 to 10 μM (Sigma), 17β-estradiol 0.1 to 10 μM (Sigma), pioglitazone 1 μM (Takeda Chemical), and vehicle dimethyl sulfoxide (DMSO) were added to the medium over the full course of differentiation within 0.1% of the volume. Medium was changed every other day. At day 8, the cells were washed with phosphate-buffered saline (PBS) twice, and then stained with 0.6% Oil Red O solution for 2 h at room temperature. Stained Oil Red O was0 eluted with isopropanol after the cells were washed, and the optical density (OD) of the solution at 520 nm was measured.

To determine adiponectin, aP2, and lipoprotein lipase (LPL) mRNA expression during the course of adipocyte differentiation, all compounds were added to the medium on day 2 (differentiating state) or on day 8 (mature adipocyte) and then incubated for another 24 h. Media without insulin were used at day 2 in the case of extracting RNA on day 3. Total RNA of

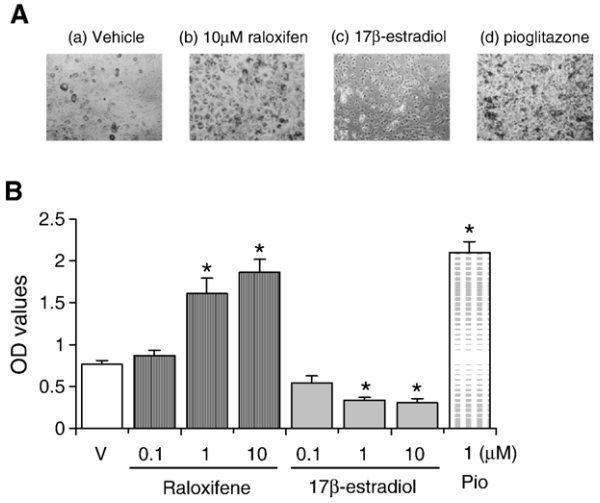

Fig. 2. Effects of raloxifene on adipose differentiation of 3T3-L1 cells. Vehicle (DMSO), raloxifene, E_2, and pioglitazone were added to the induction medium from day 0 to 8. (A) Oil Red O staining of 3T3-L1 cells at day 8 in vehicle medium (a) or in medium supplemented with 10 μM raloxifene (b), 10 μM 17β-estradiol (c), or 1 μM pioglitazone (d). Original magnification ×100. (B) Quantification of the lipid accumulation was based on the OD values in destained Oil Red O. Data are expressed as means±S.E.M. from triplicate experiments. *$P<0.05$, versus vehicle (*V*). Pio=pioglitazone.

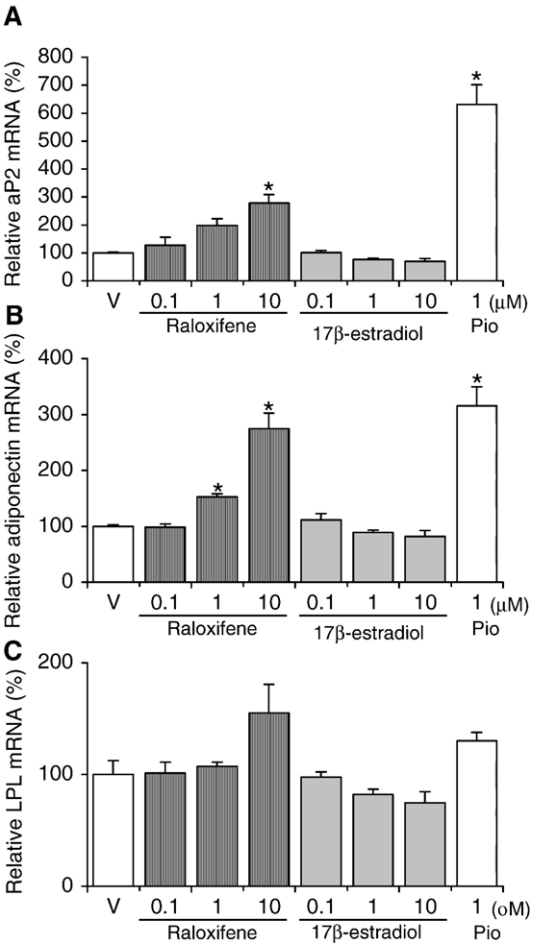

Fig. 3. Effects of raloxifene on adipogenesis in differentiating 3T3-L1 cells (day 3). Cells were treated with compounds on day 3 for 24 h. Total RNA was prepared and subjected to quantitative analysis of aP2 (A), adiponectin (B), and lipoprotein lipase (LPL) (C) by real-time PCR. Results were normalized to 18S mRNA. Data are expressed as means±S.E.M. from triplicate experiments. *$P<0.05$, versus vehicle (V). Pio=pioglitazone.

3T3-L1 cells was extracted using an ISOGEN (Nippon gene, Japan), followed by the synthesizing cDNA by a reverse transcription polymerase chain reaction (RT-PCR) kit (TaKaRa Bio Inc., Japan). A quantitative real-time RT-PCR was performed using the ABI Prism 7000 sequence detection system (Applied Biosystems). Reactions were performed in a 50 μl volume containing 25 μl of 2×SYBR Premix Ex Taq (TaKaRa Bio Inc.), 4 μl of cDNA (corresponding to 1 ng total RNA input), and 0.2 μM primers. The primers were designed and synthesized by the TaKaRa Bio Inc. (sequences and PCR conditions available upon request). For each condition, expression was quantified in duplicate, and 18S mRNA was used as the endogenous control in the comparative cycle threshold (C_T) method (Livak and Schmittgen, 2001). Prostacyclin concentrations in the culture media for 3T3-L1 preadipocytes were measured using an immunoassay system (R and D system Inc.). The data are presented as means±S.E.M. Student's *t* test was used to compare the data between the vehicle and the treated groups, with $P<0.05$ considered significant.

3. Results

3.1. Effect of raloxifene and 17β-estradiol on lipogenesis in 3T3-L1 preadipocytes

As shown in Fig. 2, higher doses of raloxifene exhibited more extensive deposits of lipid droplets compared with non-treated cells. In contrast, 17β-estradiol suppressed lipogenesis in a dose-dependent manner, which is consistent with previous reports (Homma et al., 2002; Palin et al., 2003).

3.2. Regulation of PPARγ targeting genes by raloxifene and 17β-estradiol on differentiating state of 3T3-L1 cells (day 2 to day 3)

We next examined the regulation of PPARγ targeting genes by raloxifene and E_2 on differentiating state (from day 2 to day 3) or differentiated state (from day 8 to day 9) in the 3T3-L1 cells. Fig. 3A and B show that 10 μM raloxifene at the differentiating state increased aP2 and adiponectin mRNA expression by 2.8±0.3-fold (P<0.05) and 2.7±0.3-fold (P<0.05), respectively, compared to the vehicle treatment. Also, such increases were observed dose-dependently. The levels of mRNA for LPL tended to increase in the presence of raloxifene or pioglitazone (Fig. 3C). In contrast, 17β-estradiol tended to decrease the mRNA expression of aP2, adiponectin and LPL in a dose-dependent manner (Fig. 3A, B, and C).

3.3. Regulation of PPARγ targeting genes by raloxifene and 17β-estradiol on differentiated state of 3T3-L1 cells (day 8 to day 9)

Similarly, as shown in Fig. 4, after cells were completely differentiated into adipocytes, aP2, adiponectin, and LPL mRNA levels were elevated in the presence of 10 μM raloxifene (2.0±0.2-fold ($P<0.05$), 1.7±0.1-fold ($P<0.05$), and 1.9±0.1-fold ($P<0.01$), respectively), whereas those markers were suppressed in the presence of 10 μM 17β-estradiol.

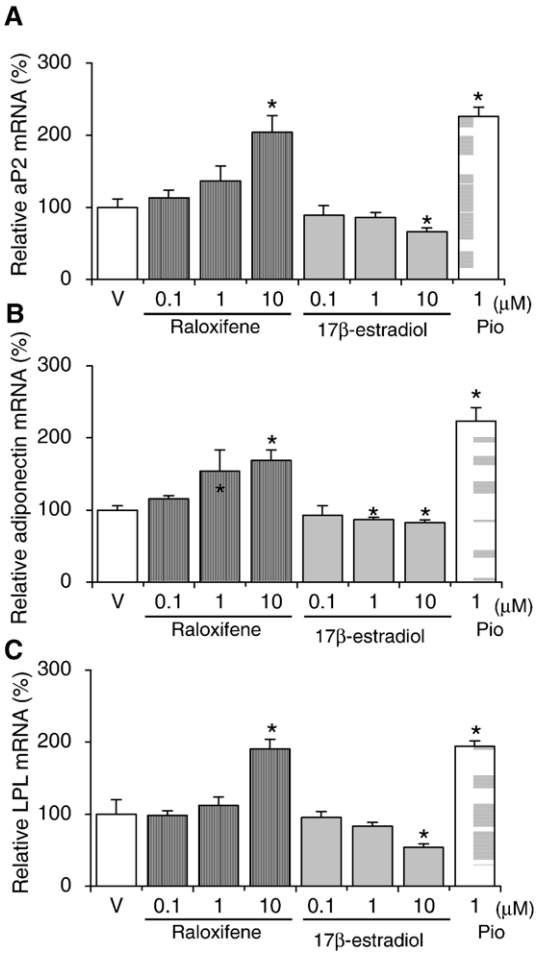

Fig. 4. Effects of raloxifene on adipogenesis in differentiated 3T3-L1 cells (day 9). Cells were treated with compounds on day 8 for 24 h. Total RNA was prepared and subjected to quantitative analysis of aP2 (A), adiponectin (B), and LPL (C) by real-time PCR. Results were normalized to 18S mRNA. Data are expressed as means±S.E.M. from triplicate experiments. *$P<0.05$, versus vehicle (V). Pio=pioglitazone.

3.4. Effect of raloxifene or pioglitazone on prostacyclin concentrations in 3T3-L1 preadipocytes

To determine if the observed effect of raloxifene in the present study might be due to its promoting prostacyclin release in the 3T3-L1 preadipocytes, we investigated the effect of raloxifene and pioglitazone on prostacyclin concentrations in the 3T3-L1 preadipocytes. The prostacyclin concentrations in the culture media in the presence of 0, 0.1, 1 and 10 μM of raloxifene were 796, 355, 399 and 251 pg/ml, respectively, whereas that in the presence of 1 μM of pioglitazone was 502 pg/ml, suggesting that raloxifene did not promote the production of prostacyclin in the 3T3-L1 preadipocytes.

4. Discussion

In this study, we demonstrated that raloxifene augmented dose-dependent lipogenesis and PPARγ downstream gene expression, including adiponectin in the 3T3-L1 adipocytes. It should be noted that these effects were opposite to those observed with E_2 treatment. Our data also showed that in both differentiating and differentiated 3T3-L1 adipocytes raloxifene enhanced the adipogenesis. These findings suggest that raloxifene might possess another pathway to activate PPARγ other than the signal interacted transduction pathway via estrogen receptor (Wang and Kilgore, 2002). This may in part be due to the fact that the expression of estrogen receptor-α , the dominant subtype in adipocytes, is extremely low in undifferentiated preadipocytes (Homma et al., 2000; Somjen et al., 1997; Dieudonne et al., 2004). Our findings that raloxifene did not promote the release of prostacyclin in the culture media of 3T3-L1 cells suggested that it was unlikely that the adipogenesis by raloxifene was due to the action of prostacyclins on adipogenesis.

Further studies will be needed to fully elucidate the underlying signaling mechanisms by which raloxifene promotes adipogenesis.

A better understanding of this kind of compound may provide hints for treatment of postmenopausal women with metabolic syndrome, which is associated with hypoadiponectinemia (Ryo et al., 2004; Salmenniemi et al., 2004).

References

Combs, T.P., Wagner, J.A., Berger, J., Doebber, T., Wang, W.J., Zhang, B.B., Tanen, M., Berg, A.H., O'Rahilly, S., Savage, D.B., Chatterjee, K., Weiss, S., Larson, P.J., Gottesdiener, K.M., Gertz, B.J., Charron, M.J., Scherer, P.E., Moller, D.E., 2002. Induction of adipocyte complement-related protein of 30 kilodaltons by PPARgamma agonists: a potential mechanism of insulin sensitization. Endocrinology 143, 998–1007.

Dieudonne, M.N., Leneveu, M.C., Giudicelli, Y., Pecquery, R., 2004. Evidence for functional estrogen receptors alpha and beta in human adipose cells: regional specificities and regulation by estrogens. Am. J. Physiol., Cell Physiol. 286, C655–C661.

Gavrila, A., Chan, J.L., Yiannakouris, N., Kontogianni, M., Miller, L.C., Orlova, C., Mantzoros, C.S., 2003. Serum adiponectin levels are inversely associated with overall and central fat distribution but are not directly regulated by acute fasting or leptin administration in humans: cross-sectional and interventional studies. J. Clin. Endocrinol. Metab. 88, 4823–4831.

Green, H., Kehinde, O., 1975. An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. Cell 5, 19–27.

Green, H., Kehinde, O., 1976. Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. Cell 7, 105–113.

Grese, T.A., Cho, S., Finley, D.R., Godfrey, A.G., Jones, C.D., Lugar III, C.W., Martin, M.J., Matsumoto, K., Pennington, L.D., Winter, M.A., Adrian, M.D., Cole, H.W., Magee, D.E., Phillips, D.L., Rowley, E.R., Short, L.L., Glasebrook, A.L., Bryant, H.U., 1997. Structure-activity relationships of selective estrogen receptor modulators: modifications to the 2-arylbenzothiophene core of raloxifene. J. Med. Chem. 40, 146–167.

Homma, H., Kurachi, H., Nishio, Y., Takeda, T., Yamamoto, T., Adachi, K., Morishige, K., Ohmichi, M., Matsuzawa, Y., Murata, Y., 2000. Estrogen suppresses transcription of lipoprotein lipase gene. Existence of a unique estrogen response element on the lipoprotein lipase promoter. J. Biol. Chem. 275, 11404–11411.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25, 402–408.

Palin, S.L., McTernan, P.G., Anderson, L.A., Sturdee, D.W., Barnett, A.H., Kumar, S., 2003. 17Beta-estradiol and anti-estrogen ICI: compound 182,780 regulate expression of lipoprotein lipase and hormone-sensitive lipase in isolated subcutaneous abdominal adipocytes. Metabolism 52, 383–388.

Ryo, M., Nakamura, T., Kihara, S., Kumada, M., Shibazaki, S., Takahashi, M., Nagai, M., Matsuzawa, Y., Funahashi, T., 2004. Adiponectin as a biomarker of the metabolic syndrome. Circ. J. 68, 975–981.

Salmenniemi, U., Ruotsalainen, E., Pihlajamaki, J., Vauhkonen, I., Kainulainen, S., Punnonen, K., Vanninen, E., Laakso, M., 2004. Multiple abnormalities in glucose and energy metabolism and coordinated changes in levels of adiponectin, cytokines, and adhesion molecules in subjects with metabolic syndrome. Circulation 110, 3842–3848.

Somjen, D., Tordjman, K., Waisman, A., Mor, G., Amir-Zaltsman, Y., Kohen, F., Kaye, A.M., 1997. Estrogen stimulation of creatine kinase B specific activity in 3T3L1 adipocytes after their differentiation in culture: dependence on estrogen receptor. J. Steroid Biochem. Mol. Biol. 62, 401–408.

Tanko, L.B., Bruun, J.M., Alexandersen, P., Bagger, Y.Z., Richelsen, B., Christiansen, C., Larsen, P.J., 2004. Novel associations between bioavailable estradiol and adipokines in elderly women with different phenotypes of obesity: implications for atherogenesis. Circulation 110, 2246–2252.

Wang, X., Kilgore, M.W., 2002. Signal cross-talk between estrogen receptor alpha and beta and the peroxisome proliferator-activated receptor gamma1 in MDA-MB-231 and MCF-7 breast cancer cells. Mol. Cell. Endocrinol. 194, 123–133.