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Ascofuranone stimulates expression of adiponectin and peroxisome proliferator activated receptor through the modulation of mitogen activated protein kinase family members in 3T3-L1, murine pre-adipocyte cell line

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ABSTRACT

Ascofuranone, an isoprenoid antibiotic, was originally isolated as a hypolipidemic substance from a culture broth of the phytopathogenic fungus, *Ascochyta visiae*. Adiponectin is mainly synthesized by adipocytes. It relieves insulin resistance by decreasing the plasma triglycerides and improving glucose uptake, and has anti-atherogenic properties. Here, we found that ascofuranone increases expression of adiponectin and PPARγ, a major transcription factor for adiponectin, in 3T3-L1, murine pre-adipocytes cell line, without promoting accumulation of lipid droplets. Ascofuranone induced expression of adiponectin, and increases the promoter activity of adiponectin and PPRE, PPAR response element, as comparably as a PPARγ agonist, rosiglitazone, that stimulates lipid accumulation in the preadipocyte cell line. Moreover, inhibitors for MEK and JNK, like ascofuranone, considerably increased the expression of adiponectin and PPARγ, while a p38 inhibitor significantly suppressed. Ascofuranone significantly suppressed ERK phosphorylation, while increasing p38 phosphorylation, during adipocyte differentiation program. These results suggest that ascofuranone regulates the expression of adiponectin and PPARγ through the modulation of MAP kinase family members.

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1. Introduction

Recent studies revealed that adipocytes are not only storage receptacles of extra fat but are also responsible for communicating with the other organs to maintain metabolic balance by secreting hormones and adipokines, such as leptin, TNF (tumor necrosis factor)-α, resistin, and adiponectin [1,2]. Adiponcetin is known as Acrp30, AdipoQ, apM1 and GBP28 and is abundant protein in plasma with a molecular mass 30 kDa [3]. Adiponectin is mainly synthesized by adipocytes and is later assembled into various complex forms through cystine-mediated disulfide linkages yielding low-, middle- and high-molecular-weight isoforms. Reduced adiponectin level is associated with obesity, type II diabetes and insulin-resistance in animal models and human patients. Adiponectin also displays anti-atherogenic properties that suppress monocyte adhesion to the endothelial cells by reducing the NF-κB signaling and mRNA expression of the adhesion molecules in the endothelial cells [4,5].

PPARγ is a member of the nuclear receptor superfamily of transcription factors [6], and plays a crucial role in transcriptional regulation for factors involved in energy metabolism and adipocyte differentiation including adiponectin, the promoter region of which has a binding site for PPARγ/retinoid X receptor (RXR) heterodimer. PPARγ can be activated by synthetic compounds, including thiazolidinediones (TZDs) [7], which are used clinically as insulin-sensitizing drugs and anti-diabetic agents. The treatment with PPARγ agonists ameliorates insulin sensitivity along with the increase in the plasma adiponectin levels in rodents and human subjects [7,8], which at least in part contributes to its ameliorative effect on insulin-resistance [9].

Ascofuranone and ascochlorin are prenylphenol anti-fungal antibiotics isolated from an incomplete fungus, *Ascochyta visiae*, through the screening system utilizing anti-viral activity (Fig. 1A) [10,11]. They and their related compounds exhibit a variety of physiological effects, including hypolipidemic activity [11,12], amelioration of type I and II diabetes [13], immunomodulation and anti-tumor activity [14,15]. Ascofuranone and ascochlorin suppresses cellular activator protein-1 (AP-1) activity and its downstream targets including plasminogen activator inhibitor (PAI)-1 and matrix metalloproteinase (MMP)-9 through the suppression of extracellular signal regulated protein kinase (ERK)-1/2 signaling pathway [16,17]. As a result, these antibiotics suppress invasion of renal carcinoma and expression of extracellular matrix proteins

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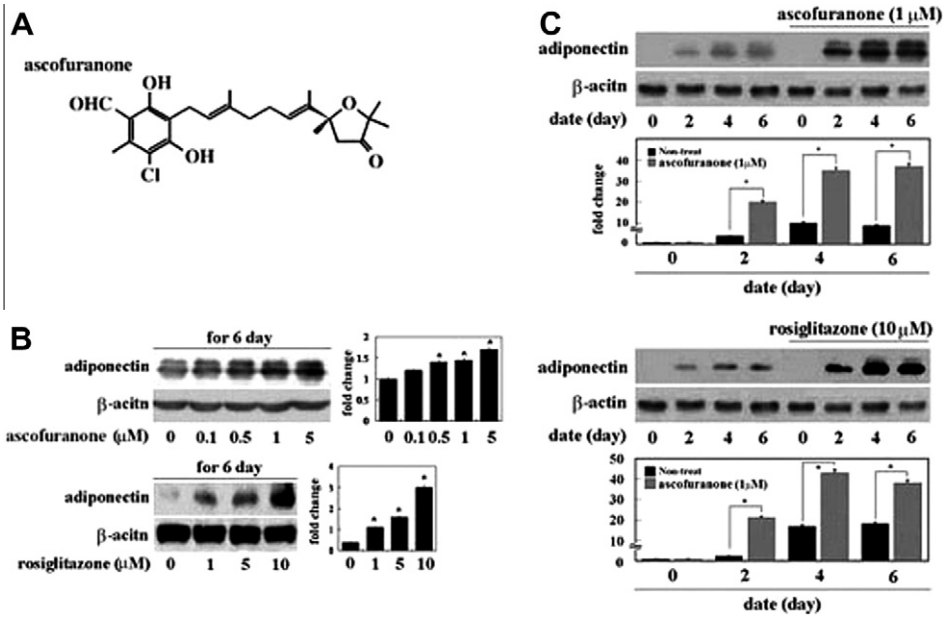

Fig. 1. Effect of ascofuranone on adiponectin expression in 3T3-L1 adipocytes. (A) Chemical structure of ascofuranone. (B and C) Post-confluent 3T3-L1 pre-adipocytes cultured in the presence of insulin, IBMX and dexamethasone for 2 days were treated with 1 μM ascofuranone, 10 μM rosiglitazone and both for 6 days in the normal culture medium containing insulin. The adiponectin expression was assessed via Western blot analysis. β-Actin expression was used as a control. A representative of three independent experiments is shown. Protein expression was quantified by densitometry using Quantity One 1-D analysis software program.

induced by growth factors such as epidermal growth factor (EGF) and transforming growth factor (TGF)-β in fibroblasts [16–18], or by oxidized low density lipoprotein (LDL) in human macrophage cell lines [19], suggesting the therapeutic potential for fibrosis and atherosclerosis. They suppress oxidative phosphorylation by inhibiting ubiquinone-dependent electron transport in isolated mitochondria [20–23], which may involve in their activity on p53 and c-Myc because respiratory inhibitors have these cellular effects in a manner similar to ascofuranone and ascochlorin.

Several ascochlorin derivatives activate nuclear receptor family members including PPARγ [24], which is relevant to some physiological activities including the induction of adipocyte differentiation, and suppression of TNF-α production in macrophages [25]. However, 4-O-methylascochlorin (MAC), a methylated derivative of ascochlorin that has no activation activity on PPARγ, still ameliorates type II diabetes in a mouse model [26], implicating additional mechanism(s) for amelioration of type II diabetes other than direct activation of PPARγ. We recently found that MAC activates hypoxia inducing factor (HIF)-1α through the activation of AMPK [27], a key regulator for energy metabolism involved in development of various metabolic diseases. In this study, we focused on adiponectin, a crucial factor for development of type II diabetes and insulin resistance, and found that ascofuranone increased the expression of adiponectin and PPARγ in 3T3-L1, murine pre-adipocyte cell lines through the modulation of MAPK family members. These results suggest that adiponectin and PPARγ plays crucial role in ameliorative effect of ascochlorin derivatives on type II diabetes.

2. Materials and methods

2.1. Chemicals and antibodies

Ascofuranone was purified from a culture broth of *A. visiae* as described previously [9]. Antibodies for total p38, total ERK1/2, phospho-Tyr204-ERK1/2, total JNK, PPARγ and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Those for phospho-Tyr182-p38 and phospho-Thr183/Tyr185-JNK antibodies were purchased from Cell Signaling Technology (Cell Signaling Technology, Inc. Beverly, MA, US). Kinase inhibitors such as SB98059, SP600125 and PD098059 were obtained from Calbiochem (Cambridge, MA, USA).

2.2. Cell culture

3T3-L1 pre-adipocytes were purchased from American Type Culture Collection and maintained in DMEM (Gibco, Grand Island, NY, US), containing 10% fetal bovine serum and 10% bovine calf serum (Gibco) and 1% antibiotic-antimycotic (Invitrogen, San Diego, CA).

2.3. 3T3-L1 adipocytes differentiation

3T3-L1 pre-adipocytes (2×10^5/ml) were cultured with a hormone cocktail containing 2 μg/ml insulin (Sigma, St. Louis, MO, US), 2 μM dexamethasone (Sigma) and 111 μg/ml isobutylmethylxanthine (IBMX, Sigma) in DMEM containing 10% fetal bovine serum and 1% antibiotic-antimycotic. After 2 days, the medium was changed to normal culture medium containing insulin 2 μg/ml, and cells were further cultured for 6 days.

2.4. Western blot analysis

Total cell lysates was prepared by lysing the cells in lysis buffer [50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.5% nonidet P-40, 100 ml phenylmethylsulfonyl fluoride, 20 mM aprotinin, and 20 mM leupeptin, adjusted to pH 8.0] at 4 °C for 30 min, followed by centrifugation at 13,000 rpm for 5 min. The cell extract was subjected on SDS-PAGE, electrotransferred to immobilon-P membranes (Millipore, Cork, Ireland), using enhanced chemiluminescence (Amersham, Piscataway, NJ, U.S.A.), as described previously [16]. Signal intensity was quantified by Quantity One 1-D software (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.).

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen, Grand Island, NY, U.S.A.). cDNA was synthesized from 1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, U.S.A.). The PCR primers used were followings; adiponectin, 5′-tgttggaatgacaggagctg-3′ (sense) and 5′-tgctgccgtcataatgattc-3′ (anti-sense); PPARγ, 5′-cgagtctgtggggataaagca-3′ (sense) and 5′-ggtcattcaagtcaaggttaa-3′ (anti-sense); β-actin used as an internal standard for RNA normalization, 5′-GCCATCGTCACCAACTGGGAC-3′ (sense) and 5′-CGATTTCCCGCTCGGCCGTGG-3′ (anti-sense). PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. Signal intensity was quantified by Quantity One 1-D software (Bio-Rad Laboratories, Inc.).

2.6. Plasmid transfection and luciferase gene assays

Adiponectin-luc (p(-908)-luc) [28] and PPAR response element, which contained 3 PPARγ, ((PPRE)x3-luc) [25] were used in transient transfection assays. Cells were plated onto 6-well dishes at a density of 1×10^4 cell/ml and allowed to grow overnight. The cell was contransfected with 2 μg of various plasmid constructs and 1 μg of the pCMA-β-galactosidase plasmid for 24 h with mirus transfection (Mirusbio, Madison, WI, U.S.A.) or lipofectamine (Invitrogen) according to the manufacturer's instructions. After 24 h incubation in fresh medium, the enzyme activities of luciferase and β-galactosidase were determined using commercial kits (Promega), according to the manufacturer's protocol. Luciferase activity was calculated as luciferase activity normalized with β-galactosidase activity in each cell lysate.

2.7. Statistical analysis

All *in vitro* results are representative of at least three independent experiments performed in triplicate. Statistically significance ($p < 0.05$) between experimental and control was calculated using analysis of variance with Newman-Kels multi-comparison test.

3. Results

3.1. Increase in adiponectin expression by Ascofuranone in a pre-adipocyte cell line, 3T3-Ll

Adiponectin is known to be exclusively expressed and secreted in adipose tissues [3] and to be gradually enhanced during the course of adipocyte differentiation of a pre-adipocyte cell line, 3T3-L1 (Fig. 1C). Ascofuranone, when included to the later phase differentiation program in the presence of insulin following to the incubation of 3T3-L1 cells in the presence of dexamethasone and IBMX and insulin for 2 days, significantly enhanced adiponectin protein expression in a time-dependent manner (Fig. 1B and C, upper panel). Rosiglitazone, a potent agonist of PPARγ, increased adiponectin expression with an extent similar to ascofuranone. (Fig. 1B and C, lower panel). Rosiglitazone stimulated differentiation of adipocytes as determined by accumulation of intracellular lipid droplets, while ascofuranone did not affect this phenotype of differentiation (data not shown), suggesting that ascofuranone mimics only the part of differentiation signals provided by rosiglitazone, through a mechanism different from direct activation of PPARγ, which is consistent with our previous observation that ascofuranone has no detectable agonistic activity for PPARγ [24].

3.2. Ascofuranone on differentiated 3T3-L1 adipocytes induces the promoter activities of adiponectin and PPARγ

The promoter activity for adiponectin in ascofuranone treated 3T3-L1 cells was quantified by a transiently transfected luciferase reporter plasmid containing adiponectin promoter sequences. As shown in Fig. 2, ascofuranone significantly increased the activity of the adiponectin promoter as comparably as rosiglitazone. Because PPARγ is a major transcription factor regulating gene expression of adiponectin [6], we also quantified effect of ascofuranone on PPRE promoter activity using luciferase promoter plasmid and found ascofuranone as well as rosiglitazone increased the promoter activity for PPRE itself. In fact, ascofuranoe significantly enhanced protein expression of PPARγ during adipocyte differentiation as comparably as rosiglitazone (Fig. 3A).

3.3. Ascofuranone regulates the expression of adiponectin via the activation/blockade of MAP kinase signalings

To decipher the signaling pathway involved in the expression of ascofuranone-induced adiponectin and PPARγ, we utilized specific MAPK inhibitors, PD98053 as a MEK inhibitor, SB203580 as a p38 MAP kinase inhibitor, and SP600125 as a c-Jun-N-terminal kinase (JNK) inhibitor, because we previously observed that ascofuranone suppressed AP-1 activity via inhibition of ERK1/2 signaling pathway [17]. As expected, inhibitors for MEK and JNK, like ascofuranone, considerably increased the expression of adiponectin and PPARγ, whereas a p38 MAP kinase inhibitor did not (Fig. 3A), suggesting that blockade of MEK and JNK signaling pathway promotes expression of adiponectin and PPARγ. Moreover these inhibitors for MEK and JNK enhanced ascofuranone-induced expression of adiponectin and PPARγ while a p38 inhibitor rather suppressed. This result was confirmed in the mRNA expression quantified by RT-PCR (Fig. 3B). These results suggest that ascofuranone stimulated expression of adiponectin and PPARγ through the inhibition of ERK/JNK signaling pathways, which is susceptible to p38 MAP kinase inhibition.

3.4. Ascofuranone regulates the activation of MAP kinase signaling pathway for induction of adiponection expression

To confirm the contribution of each MAPK to ascofuranone-mediated expression of adiponectin and PPARγ, the effect of ascofuranone on the phosphorylation status of p38 MAPK, ERK1/2 and JNK was analyzed by Western blotting using antibodies specific for each phosphorylated form. Incubation with insulin for 6 days, after the incubation for 2 days with differentiation medium containing insulin, dexamethasone, and IBMX, significantly stimulated phosphorylation of ERK1/2 and JNK, but not that of p38. Ascofuranone markedly suppressed ERK1/2 phosphorylation at the dose as low as 0.1 μM, as well as JNK phosphorylation at 1 μM (Fig. 4A). In contrast, ascofuranone stimulated p38 phosphorylation at the doses above 0.5 μM. These results suggest that ascofuranone regulates the adiponectin expression by promoting the phosphorylation of p38 MAP kinase and by inhibiting the phosphorylation of ERK and JNK.

4. Discussion

We recently found that ascofuranone that has no detectable agonistic activity on PPARγ [24] activates AMPK and improves insulin sensitivity in mouse myocyte treated with ER-stress inducing agents [29]. In this study, we focused on adiponectin for another candidate involved in anti-diabetic mechanism for ascochlorin derivatives. We found that ascofuranone increased

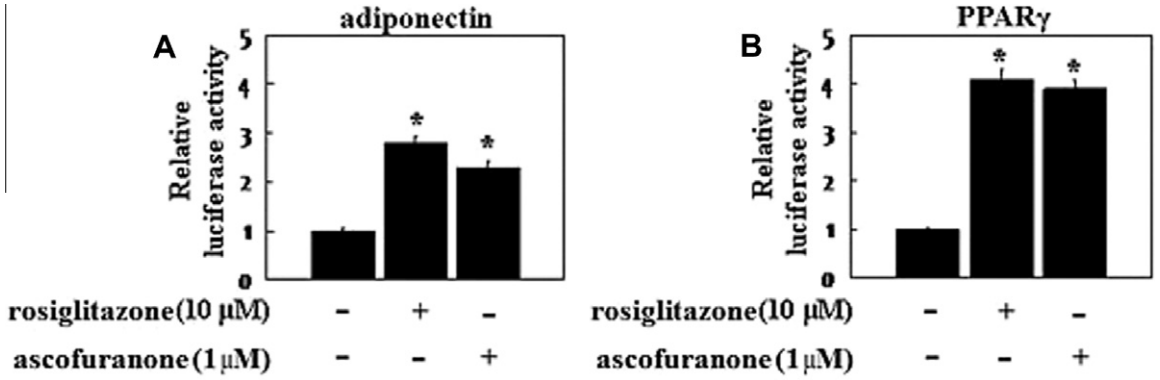

Fig. 2. Effect of ascofuranone on the promoter activities of adiponectin and PPRE in 3T3-L1 adipocytes. (A and B) 3T3-L1 adipocytes were transfected with a reporter plasmid containing adiponectin promoter or and PPRE promoter for 24 h, and were incubated with rosiglitazone or ascofuranone. The luciferase activity was measured 24 h after the drug treatment. The mean and standard error of the three independent experiments are shown. Statistically significant (*$p < 0.05$ vs. controls).

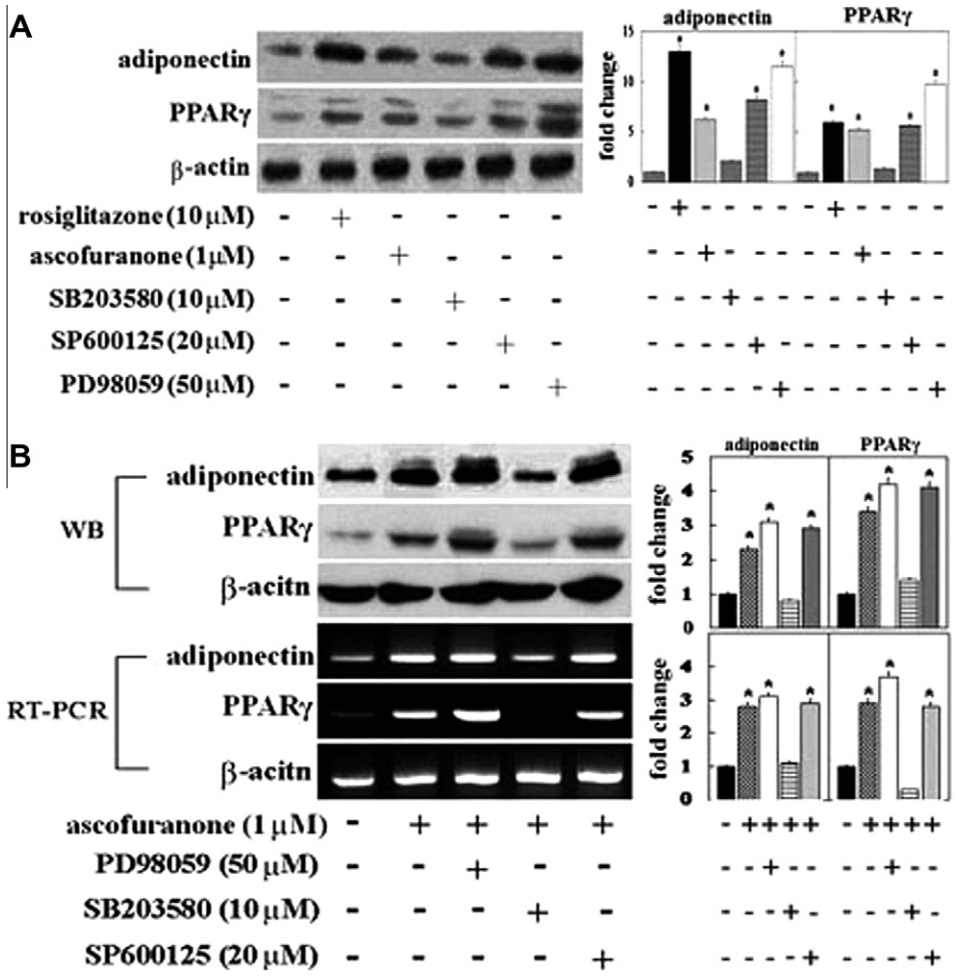

Fig. 3. Effect of MAPK inhibitors on expression of adiponectin and PPAR-γ. (A and B) Post confluent 3T3-L1 cells cultured in the presence of insulin, IBMX and dexamethasone for 2 days, were cultured with ascofuranone or kinase inhibitors in the normal culture medium containing insulin. The protein expression 6 days (adiponectin), or 2 days (PPARγ) after the addition of drugs was analyzed by Western blot, mRNA expression by RT-PCR. β-Actin expression was used as a control. A representative of three independent experiments is shown. Protein expression was quantified by densitometry using Quantity One 1-D analysis software program.

expression of adiponectin and PPARγ in 3T3-L1 preadipocytes during the later phase of differentiation program, and stimulated their promoter activity. These results suggest that enhanced expression of adiponectin and PPAR-γ could be the mechanism for anti-diabetic effect of ascochlorin derivatives.

Ascofuranone-induced expression of adiponectin and PPARγ was completely inhibited by the treatment with a p38 MAPK inhibitor SB203580, suggesting that this kinase is a positive regulator in the ascofuranone-promoted gene expression (Fig. 3A). On the other hand, inhibitors of ERK and JNK, like ascofuranone, stimulated their expression, and enhanced ascofuranone-induced one. Ascofuranone suppressed insulin-induced phosphorylation of ERK1/2 and JNK, while stimulated p38 phosphorylation. These results suggest that stimulation of p38 signaling and suppression of ERK/JNK signaling

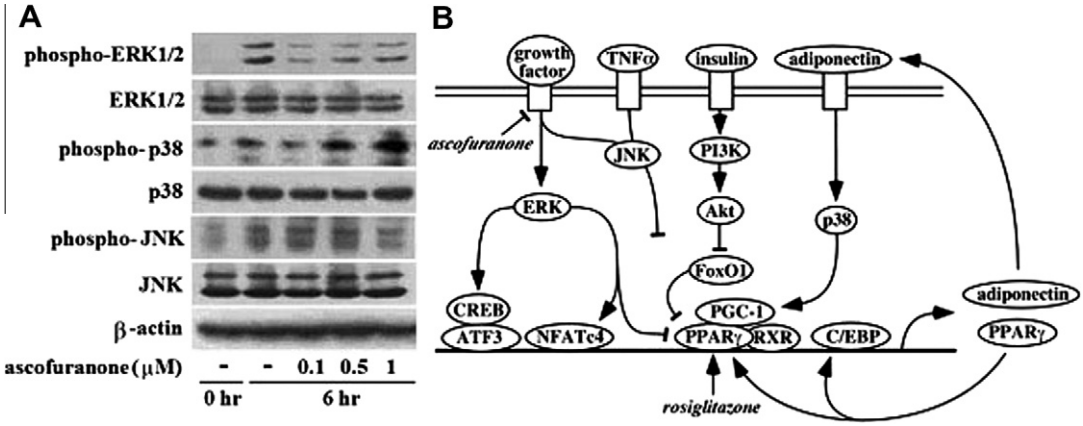

Fig. 4. Regulation of MAPK phosphorylation by ascofuranone. (A) Confluent 3T3-L1 cells cultured in the presence of insulin, IBMX and dexamethasone for 2 days were incubated with ascofuranone for 6 days in the normal culture medium containing insulin. Cellular protein was analyzed by Western blot. β-Actin expression was used as a control. A representative of three independent experiments is shown. (B) Schematic model for increase of expression of adiponectin and PPARγ by ascofuranone. Inhibition of ERK/JNK signaling pathway by ascofuranone stimulates a positive feedback loop composed of PPARγ and C/EBP, leading to stimulation of promoter activity of adiponectin. Adiponectin further activates p38 that activates PGC-1, serving another positive feedback loop. See text for detail.

promote adiponectin expression in ascofuranone-treated pre-adipocytes. The differentiation of pre-adipocytes can be modulated by MAPK, sometimes yielding opposite effects [30]. ERK is required for mitotic clonal expansion in the early phase of differentiation, while it inhibits the later phase of differentiation, at least in part, through the phosphorylation and the resultant suppression of PPARγ. The p38 MAPK pathway enhances the differentiation of adipocytes. The p38 MAPK inhibitor decreases adipocyte differentiation induced by growth hormone [31]. JNK is involved in TNF-α-mediated suppression of adiponectin expression [32], and in insulin resistance and obesity through modulation of insulin signaling [32,33].

Besides PPARγ, adiponectin expression is regulated by many transcription factors including CAAT/enhancer binding proteins (C/EBPs), cAMP-responsive element binding factor (CREB), activating transcription factor (ATF)-3, and nuclear factor of activated T cells (NFAT)c4 [6]. During adipocyte differentiation, C/EBPβ and C/EBPδ are first induced through the elevation of intracellular cAMP and dexamethasone, which is followed by direct induction of PPARγ through C/EBP binding site in its promoter. PPARγ subsequently induces C/EBPα expression that further enhances PPARγ expression, making a positive feedback loop. C/EBPζ involved in negative feedback regulation of differentiation program is expressed at the later stage when differentiation has progressed almost to completion [10,11]. ERK interferes this positive feedback loop through the phosphorylation of PPARγ at Ser82 [30]. Adiponectin activates p38 MAPK [2] that activates PPARγ coactivator-1 [34], which might serve another positive feedback loop. On the other hand, ATF3 and NFATc4 serve negative regulators for adiponectin promoter. CREB that is activated through the phosphorylation at Ser133 by kinases including ERK [35] stimulates transcription of and interaction with ATF3, resulting in repression of adiponectin gene expression [36], though mouse adiponectin promoter that has cAMP responsive element (CRE) is positively regulated by CREB and ERK [37]. Treatment of 3T3-L1 cells with IL-18 selectively suppresses expression level of adiponectin, through the ERK phosphorylation and subsequent activation of NFATc4 at Ser676 [38]. Ascofuranone may indirectly activate promoters of adiponectin and/or PPARγ through the suppression of these phosphorylation events mediated by MAPK.

Major difference between ascofuranone and a full-agonist for PPARγ, rosiglitazone, is that the former has no stimulating effect on 3T3-L1 adipocyte differentiation as judged by intracellular lipid accumulation, another prominent feature of rosiglitazone [5,7], while ascofuranone increased the expression of adiponectin and PPARγ as comparably as rosiglitazone. Ascofuranone-mediated modulation of MAPK activity, unlike rosiglitazone, may insufficiently activate this transcription cascade during differentiation, and/or suppress signal(s) required for full differentiation. In fact, high glucose-induced lipid accumulation in bone-marrow-derived mesenchymal stem cells is inhibited by PD98059 through the blockade of phosphoinositide-3-kinase (PI3K) activation and subsequent Akt phosphorylation [39], which subsequently phosphorylates FoxO1, leading to inactivation of this transcription factor that represses PPARγ expression, by preventing its nuclear import, though a number of studies demonstrate suggest that FoxO1 positively regulates adiponectin transcription [6]. Precise investigation on temporal expression of transcription factors relevant to each phenotype of adipocyte differentiation is of interest to elucidate property of ascofuranone treated pre-adipocytes.

In conclusion, our findings in this study suggest that ascofuranone augments expression of adiponectin and PPARγ in 3T3-L1 pre-adipocytes not directly through activation of PPARγ as its ligand, but through promotion of p38 MAP kinase activity and inhibition of ERK/JNK activity (Fig. 4B). From a clinical perspective, TZD is not ideal therapeutic agent because a substantial number of patients experience side effects including fluid retention, weight gain, congestive heart failure, and loss of bone mineral density. Non-agonistic PPARγ ligands blocking cdk5-mediated phophorylation at Ser273 that induce no adiopocyte differentiation, ameliorate insulin resistance in mice without these side effects, and, like ascofuranone, increase adiponectin expression [40]. Our study indicates that ascofuranone could a therapeutic agent for human metabolic diseases involving adiponectin and PPARγ.

Acknowledgments

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