Pig testis extract augments adiponectin expression and secretion through the peroxisome proliferator-activated receptor signaling pathway in 3T3-L1 adipocytes

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Abstract Adiponectin is a key molecule whose upregulation may ameliorate symptoms of type 2 diabetes mellitus and disorders of lipid metabolism. Several plant-derived components have been shown to enhance adiponectin secretion; however, there have been no reports on the effects of animal-derived products. Therefore, in the current study, we investigated whether hot-water extracts of specific livestock by-products induce the expression of adiponectin in mouse 3T3-L1 adipocytes. Out of the 11 extracts tested, pig testis extract (PTE) was found to enhance adiponectin expression and secretion by 3T3-L1 cells. Furthermore, simultaneous treatment with PTE and daidzein, a soy phytoestrogen, synergistically enhanced adiponectin secretion. Moreover, pretreatment with an estrogen receptor β antagonist (PHTPP) diminished adiponectin secretion from daidzein-treated cells but not from PTE-treated cells. Transcriptome analyses revealed that both daidzein and PTE

regulate the peroxisome proliferator-activated receptor signaling pathway, although differences in the regulation of gene expression were observed between PTE- and daidzein-treated cells. These results suggest that PTE ameliorates lipid metabolic dysfunction by promoting adipocyte differentiation and enhancing adiponectin secretion via a mechanism different from that of daidzein.

Keywords Pig testis extract · Adiponectin · PPAR · 3T3-L1

Introduction

The excessive accumulation of body fat, called obesity and, in some cases, metabolic syndrome, has become one of the most important health issues worldwide. Obesity can lead to numerous health problems such as hyperlipidemia, type 2 diabetes mellitus, hypertension, cardiovascular disease, cancer, and dementia (Huang 2009; Giovannucci 2007; Raffaitin et al. 2009). Previous studies have shown that obesity is the most critical factor in the development of insulin resistance and type 2 diabetes mellitus (Guilherme et al. 2008). Therefore, the adipose tissue is recognized as an endocrine organ that secretes factors such as tumor necrosis factor- α and interleukin-6, with adverse physiological effects (Hotamisligil et al. 1993; Rotter et al. 2003). In addition, adipose tissue plays

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important roles in the energy metabolism and secretion of functional molecules. Adiponectin is an adipocytokine mainly secreted from adipose tissue; adiponectin induces adipocyte differentiation. Adiponectin is controlled by peroxisome proliferator-activated receptor- γ (PPAR γ), and it enhances insulin sensitivity and lipid metabolism through the adiponectin receptors AdipoR1 and AdipoR2 (Maeda et al. 2001; Kadowaki and Yamauchi 2005). Previous studies in humans have shown that serum adiponectin levels are reduced in people with type 2 diabetes mellitus and obesity (Weyer et al. 2001; Spranger et al. 2003), and that administration of adiponectin ameliorates insulin resistance in rodents (Maury and Brichard 2010).

Various components derived from natural products promote adiponectin expression. Phytoestrogens ameliorate obesity and diabetes, and daidzein, a phytoestrogen, promotes adiponectin expression (Bhathena and Velasquez 2002; Sakamoto et al. 2014). Phytoestrogens interact as ligands with estrogen receptors (ERs), especially ER β (Kuiper et al. 1998), which may be an important factor in their antiobesity and antidiabetic effects. Furthermore, natural products such as mulberry leaf extract, unripe kiwi fruit extract, ashitaba extract, resveratrol, and puerarin have adiponectin-activating effects (Naowaboot et al. 2012; Abe et al. 2010; Zhang et al. 2015; Rivera et al. 2009; Lee et al. 2010). Although several plant-derived components have been shown to enhance the production of adiponectin, there have been no reports of animal-derived products that enhance the production of adiponectin. Therefore, in the current study, we investigated the effects of livestock by-product extracts on adiponectin expression.

Most livestock by-products are rarely used; however, some have been reported to exert specific effects in living organisms. For e.g., collagen derived from pig skin, chicken legs, and cartilage suppresses rheumatoid arthritis, hypertension, and dry skin (Trentham et al. 1993; Zhang et al. 2010; Okawa et al. 2012). Placenta extract has been demonstrated to protect the skin from inflammation in mice (Jash et al. 2011). Therefore, examination of the potential effects of other animal by-products that are routinely consumed via the diet is necessary. In this study, we investigated whether livestock by-products, particularly pig testis extract, enhance adiponectin secretion from 3T3-L1 preadipocytes. To the best of our

knowledge, this is the first report describing the effects of livestock by-product extracts on adipocyte differentiation and adiponectin expression. This study demonstrates the potential therapeutic benefits of livestock by-products in the amelioration of obesity and lipid metabolism disorders.

Materials and methods

Cells and reagents

Mouse 3T3-L1 cells were purchased from DS Pharma Biomedical (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies (Gaithersburg, MD, USA). Isobutylmethylxanthine, dexamethasone, and insulin were purchased from Takara Bio (Shiga, Japan). Imida 15 (low-concentration chicken breast extract), Imida 40 (high-concentration chicken breast extract), C-mucola (chicken cartilage extract), P-mucola (pig cartilage extract), P-elastin (pig vessel extract), P-chondroitin (pig cartilage extract), CCOP (highly pure chicken leg extract), C-LAP (chicken leg extract), P-LAP (pig skin extract), P-placenta (pig placenta extract), and P-testis (pig testis extract, PTE) were manufactured by NH Foods (Osaka, Japan). Daidzein was purchased from LC Laboratories (Woburn, MA, USA) and reconstituted in dimethylsulfoxide (Wako, Osaka, Japan). PHTPP, 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol, was purchased from Abcam (Cambridge, MA, USA).

Differentiation of 3T3-L1 cells

3T3-L1 preadipocytes were cultured in DMEM supplemented with 10% FBS at 37 °C in an atmosphere of 5% CO₂. When the cells reached confluence, the medium was replaced with DMEM containing 10% FBS, 0.5 mM isobutylmethylxanthine, 2.5 μ M dexamethasone, and 10 μ g/mL insulin (day 0). After 2 days, the medium was replaced with DMEM containing 10% FBS and 10 μ g/mL insulin. The medium was changed every 2 or 3 days, and 100 μ g/mL of each extract was continually added from day 0 until the cells were collected for analysis (days 6–7).

Quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated from 3T3-L1 cells using TRIzol reagent (Life Technologies) and purified using the SV total RNA isolation system (Promega, Madison, WI, USA). cDNA was synthesized using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan), according to the manufacturer's protocol. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using the THUNDERBIRD SYBR Fast qPCR kit (Toyobo) and a CFX96 deep-well real-time system (Bio-Rad Laboratories, Hercules, CA, USA). Gene expression levels were normalized to those of β -actin. The PCR primer sequences were as follows: mouse adiponectin (forward: 5'-GTTCTACTGCAACATTCCGG-3', reverse: 5'-TACACCTGGAGCCAGACTTG-3'), mouse PPAR γ (forward: 5'-AGACC ACTCGCATTCCTTTGAC-3', reverse: 5'-TTGGG TCAGCTCTTGTGAATGG-3'), mouse adipocyte protein 2 (aP2) (forward: 5'-ATGAAATCACCGCAGACGAC-3', reverse: 5'-TCGACTTTCCATCCCCTTG-3'), and mouse β -actin (forward: 5'-AAATCGTGCGTGACATCAAA-3', reverse: 5'-AAGGAA GGCTGGAAAAGAGC-3'). The experiments were performed in triplicate.

Nile red staining and lipid droplet area quantification

Lipid droplets formed in the 3T3-L1 adipocytes were stained with Nile Red (Wako). First, after 7 days of culture, cells were fixed with 4% paraformaldehyde for 20 min at room temperature. Then, the cells were stained with Nile Red (final concentration, 5 μ g/mL) for 10 min. The cells were rinsed three times with phosphate-buffered saline (PBS) after each treatment. Images of lipid droplets were captured using a fluorescence microscope BZ-X700 (Keyence, Osaka, Japan), and fluorescence intensities were calculated.

Enzyme-linked immunosorbent assay

The concentration of adiponectin protein in the culture supernatant was determined using a mouse/rat adiponectin enzyme-linked immunosorbent assay (ELISA) kit (Otsuka Pharmaceutical, Tokyo, Japan) according to the manufacturer's protocol.

DNA microarray

Total RNA was isolated from 3T3-L1 cells as described above. DNA microarray was performed according to a previously reported method (Kadooka et al. 2015). To identify genes with altered expression, we calculated intensity-based Z-scores and ratios from the normalized signal intensities of each probe and then compared the values between control and experimental samples. We established the following criteria for significantly up-/downregulated genes: upregulated genes, Z-score ≥ 2.0 and ratio ≥ 1.5 -fold; downregulated genes: Z-score ≤ -2.0 and ratio ≤ 0.66 -fold. To determine significantly overrepresented gene ontology (GO) categories, we used the tools and data provided by the Database for Annotation, Visualization, and Integrated Discovery (DAVID; <http://david.abcc.ncifcrf.gov>).

Statistical analysis

All results are expressed as the mean $\pm$ standard error of the mean. Statistical differences were determined by a two-sided Student's *t* test, with $p < 0.05$ considered statistically significant.

Results

Effects of P-testis on adiponectin production

3T3-L1 preadipocytes were cultured with the extracts of various livestock by-products or with daidzein in the presence of the differentiation mixture, and expression of adiponectin mRNA was examined by qRT-PCR. The concentrations of the extracts were predetermined by investigating the level that does not affect cell growth (data not shown). The results showed that PTE and daidzein significantly increased the expression of adiponectin mRNA. Compared to the control, adiponectin mRNA expression in cells treated with 100 μ g/mL PTE and 25 μ M daidzein increased 3.1- and 5.9-fold, respectively (Fig. 1a). Next, we evaluated the effects of PTE and daidzein on the accumulation of lipid droplets in the cells. Nile Red staining showed that PTE as well as daidzein enhanced the accumulation of lipid droplets (Fig. 1b–d).

Fig. 1 Effect of livestock by-product extracts on adiponectin expression and lipid droplet accumulation in 3T3-L1 cells. **a** Relative levels of adiponectin in 3T3-L1 cells treated with livestock by-product extracts were determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Cells were treated with the differentiation mixture and 100 $\mu\text{g/mL}$ livestock by-product extracts or 25 μM daidzein for 2 days. The medium was then changed every 2 days, and each extract was added continually with 10 $\mu\text{g/mL}$ insulin until the cells were collected on day 7. For lipid droplet quantification, 3T3-L1 cells were cultured with the dimethylsulfoxide control (**b**), 25 μM daidzein (**c**), and 100 $\mu\text{g/mL}$ PTE (**d**). **e** On day 7 cells were analyzed by Nile Red staining. The lipid droplet area was determined using BZ-X700. Statistical differences were determined by using a two-sided Student's *t* test. $*p < 0.05$ versus the corresponding control group (white bar)

Furthermore, we examined the expression of genes involved in adipogenesis. The mRNA levels of PPAR γ , its target gene aP2, and adiponectin were significantly increased in the cells treated with PTE. PTE at concentrations of 100 and 1000 $\mu\text{g/mL}$ and 25 μM daidzein increased the expression of PPAR γ by 1.2-, 1.8-, and 3.0-fold, that of aP2 by 2.8-, 5.6-, and 4.8-fold, and that of adiponectin by 2.1-, 6.2-, and 6.3-fold, respectively (Fig. 2a–c). Adiponectin secretion was also increased by PTE in a dose-dependent manner (Fig. 2d). We used phosphate buffered saline

(PBS) and DMSO as a solvent/vehicle and as the negative control.

Synergistic effect of PTE and daidzein

Next, we treated 3T3-L1 cells with PTE and daidzein simultaneously. The results revealed that simultaneous treatment further enhanced adiponectin secretion compared with treatment with each individual agent (Fig. 3a). After treatment with 100 $\mu\text{g/mL}$ PTE treatment, 36.8 ng/mL of adiponectin was detected.

Fig. 2 Effects of pig testis extract (PTE) on the mRNA expression levels of adipocyte differentiation genes. Cells were treated with 100 and 1000 $\mu\text{g/mL}$ PTE and 25 μM daidzein. mRNA was isolated from the 3T3-L1 cells on day 7. Relative levels of adiponectin (a), PPAR γ (b), and aP2 (c) were determined by qRT-PCR. Results are expressed relative to control cells treated with PBS after normalization to β -actin. **d** Effects of PTE on protein secretion compared with PBS control. Secretion levels of adiponectin were analyzed by enzyme-linked immunosorbent assay (ELISA). These experiments were performed in triplicate. Statistical differences were determined by using a two-sided Student's *t* test. **p* < 0.05 versus the corresponding control group (white bar)

Daidzein-treatment at concentrations of 25, 50, 75, and 100 μM induced 41.0, 115.9, 102.5, and 39.6 ng/mL of adiponectin, respectively. On the other hand, the concentrations of secreted adiponectin were 80.6, 215.6, 161.2, and 62.9 ng/mL when cells were treated with 100 $\mu\text{g/mL}$ PTE plus 25, 50, 75, and 100 μM daidzein, respectively, indicating that the simultaneous treatment increased adiponectin secretion by approximately two-times compared with treatment with each agent separately. Interestingly, secretion of adiponectin by daidzein-treated cells was suppressed by the selective ER β antagonist PHTPP, while that by PTE-treated cells was not suppressed (Fig. 3b).

Gene expression profiles of 3T3-L1 adipocytes

A whole-genome microarray analysis of 39,430 genes was performed to identify genes whose

expression changed in 3T3-L1 cells after treatment with 25 μM daidzein, 100 $\mu\text{g/mL}$ PTE, 25 μM daidzein plus 100 $\mu\text{g/mL}$ PTE, and PBS (control). Compared with the control, statistically significant changes in the expression of 725 genes (476 upregulated and 249 downregulated), 446 genes (380 upregulated and 66 downregulated), and 972 genes (777 upregulated and 195 downregulated) were observed after treatment with 25 μM daidzein, 100 $\mu\text{g/mL}$ PTE, and 25 μM daidzein plus 100 $\mu\text{g/mL}$ PTE, respectively. Genes associated with the GO term “Fat cell differentiation” were analyzed in detail (Table 1). Among the Compare1 (daidzein vs. PBS), Compare2 (PTE vs. PBS), and Compare3 (daidzein + PTE vs. PBS) data, expression of these genes was most significantly enhanced in Compare3. Although some key factors involved in fat cell differentiation, such as cluster of differentiation

Fig. 3 Changes in adiponectin secretion from 3T3-L1 cells after treatments with daidzein, PTE, and PHTPP. **a** Cells were exposed to different concentrations of daidzein alone (white bars) or in combination with 100 μg/ml PTE (black bars). **b** Cells were treated with either 25 μM daidzein or 100 μg/mL

PTE alone or in combination with 0.1 nM PHTPP. Control cells were treated with DMSO. These experiments were performed in triplicate. Statistical differences were determined by using a two-sided Student's *t* test. **p* < 0.05 versus the corresponding control group

Table 1 Gene expression alterations in 3T3-L1 adipocytes exposed to daidzein, pig testis extract (PTE), and daidzein + PTE, selected using the key phrase “fat cell differentiation”

^aCompare1: comparison of gene expression profiles between PBS- and daidzein-treatments

^bCompare2: comparison of gene expression profiles between PBS- and PTE-treatments

^cCompare3: comparison of gene expression profiles between PBS- and (daidzein + PTE)-treatments. Statistical significance of the gene expression alteration was set as a ratio of ≥ 1.5 or ≤ 0.66

Gene symbol	Compare1_ratio ^a	Compare2_ratio ^b	Compare3_ratio ^c
<i>Adig</i>	5.76938	2.36314	10.8526
<i>Adipoq</i>	5.05347	2.17217	8.9254
<i>Arxes1</i>	12.45707	2.60907	23.48178
<i>Arxes2</i>	6.0181	1.81889	9.69668
<i>Cebpa</i>	2.88025	1.66037	5.48135
<i>Fabp4</i>	5.83233	2.15635	8.27744
<i>Insig1</i>	2.39386	1.40236	3.16221
<i>Itga6</i>	1.94159	1.28326	3.12645
<i>Lpin1</i>	2.81018	1.42019	4.20011
<i>Mrap</i>	6.75176	2.83762	15.52308
<i>Pex11a</i>	1.76507	1.2956	2.53142
<i>Pparg</i>	2.04734	1.33948	3.33894
<i>Rarres2</i>	2.12701	1.69052	4.43443
<i>Retn</i>	5.72421	2.55964	11.83163
<i>Retn</i>	5.90687	2.56649	12.77504
<i>Scd1</i>	4.55525	2.35615	8.95009
<i>Sh2b2</i>	3.8436	1.77239	6.10506
<i>Slc2a4</i>	6.10822	2.56673	16.44747
<i>Sort1</i>	3.51746	1.65165	5.94378
<i>Wfdc21</i>	2.40178	1.94134	5.25578

36, were not included in this analysis, the expression of these genes was confirmed to be notably augmented (data not shown).

DAVID analysis

The data for the genes with significantly altered expression in comparison with PBS-treated cells were

imported into the DAVID v. 6.7 annotation tool. The enrichment of specific pathway components in functionally regulated gene groups was characterized with reference to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (Table 2). Upon comparison of the changes induced by daidzein and PTE, “PPAR signaling pathway”, “Metabolism of xenobiotics by cytochrome P450”, and “Adipocytokine signaling pathway” were found to be commonly altered.

Heat maps and Venn diagram analysis

To verify the differences in gene expression among 3T3-L1 adipocytes after the different treatments, we performed clustering analysis (Fig. 4a). In Fig. 4a, each column shows the gene expression profiles of 3T3-L1 adipocytes treated with PBS, daidzein, PTE, and PTE plus daidzein, respectively. The heat maps demonstrate that the gene expression patterns were significantly different among the treatments. Moreover, we analyzed the differences in the number of genes with altered expression among Compare1, Compare2, and Compare3 (Fig. 4b). Analysis of the Venn diagram revealed that only 86 (7.4%) upregulated genes and 31 (9.5%) downregulated genes were

common. We also found that 118, 272, and 397 upregulated genes and 114, 19, and 56 downregulated genes were exclusively regulated by treatment with daidzein or PTE alone, as well as by simultaneous treatment with daidzein plus PTE, respectively.

Discussion

It is known that adiponectin improves insulin resistance and lipid metabolism. Here, we demonstrated that PTE increased the expression and secretion of adiponectin by 3T3-L1 adipocytes. This result indicates that PTE contains an activating component(s) that accelerates adipocyte differentiation and adiponectin secretion. PTE is comprised of approximately 80% protein, 10% ash, and > 5% carbohydrates (Table S1 and S2).

Sex hormones impair PPAR γ activity and adiponectin expression (Yoon 2009; Jeong and Yoon 2011; Sato et al. 2013). Although PTE is extracted from a reproductive organ, it is unlikely that endogenous sex hormones influence its effects; furthermore, PTE was extracted before sexual maturation. In this study, adiponectin secretion induced by daidzein, but not that induced by PTE, was inhibited by PHTPP. Thus, the

Table 2 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway-based functional classification of genes differentially expressed in 3T3-L1 adipocytes exposed to daidzein (a) or PTE (b)

Term	Count	<i>p</i> value
(a)		
PPAR signaling pathway	18	3.48E–10
Valine, leucine, and isoleucine degradation	8	6.36E–04
Alanine, aspartate, and glutamate metabolism	6	0.002558
Citrate cycle (TCA cycle)	6	0.00297
Steroid biosynthesis	4	0.016535
Metabolism of xenobiotics by cytochrome P450	7	0.019882
Adipocytokine signaling pathway	7	0.021266
Glycolysis/gluconeogenesis	7	0.022714
Starch and sucrose metabolism	5	0.028524
Butanoate metabolism	5	0.031193
Drug metabolism	7	0.034724
Insulin signaling pathway	10	0.035589
Pyruvate metabolism	5	0.043295
(b)		
PPAR signaling pathway	11	6.28E–08
Metabolism of xenobiotics by cytochrome P450	5	0.011022
Adipocytokine signaling pathway	5	0.011607
Linoleic acid metabolism	4	0.022552

Fig. 4 mRNA expression profiles of adipocyte differentiation. **a** Hierarchical clustering showing up- and downregulated mRNAs among the four treatment groups. Rows represent genes, and columns represent treated cell samples. Colors indicate the distance of each row from the median. **b** Venn

diagram visualizing the overlapping results between the differentially regulated genes found in Compare1 (daidzein vs. PBS control), Compare2 (PTE vs. PBS control), and Compare3 (daidzein + PTE vs. PBS control)

upregulation of adiponectin secretion by daidzein was controlled, at least partly, through ER β , whereas the upregulation of adiponectin secretion by PTE was not. This finding suggests that the activating component of PTE is not an ER β ligand such as a sex hormone. If a sex hormone(s) was the main activating component(s), the effect of PTE would likely be inhibited by PHTPP; furthermore, PTE would not exhibit synergistic effects with daidzein, as indicated by the present experiments.

The adiponectin-enhancing activity of PTE was not diminished by heating or treatment with proteinase K. However, it was enhanced by ammonium sulfate fractionation, which was used to concentrate PTE (data not shown). The active ammonium sulfate

fractions were extracted from a highly hydrophobic fraction, and the activity was distributed among several fractions, suggesting that the activating component(s) is able to form a multimeric complex. This hypothesis needs to be confirmed in future studies.

It is possible that the main active component of PTE is an animal-derived compound such as a peptide, proteoglycan, or mucopolysaccharide. In fact, PTE is rich in mucopolysaccharides. Mucopolysaccharides and proteoglycans have previously been reported to show moisturizing, anti-inflammatory, antioxidant, antiobesity, and antidiabetes effects; however, only a few studies have reported on their adiponectin-promoting effects (Merline et al. 2011; Teng et al.

2012; Goto et al. 2012). Our findings suggest that livestock by-products have previously unreported physiological effects, and further investigation of their therapeutic potential is necessary.

In this study, we observed that simultaneous treatment with PTE and daidzein synergistically enhanced adipocyte differentiation and adiponectin secretion. Synergistic effects have been reported previously in many cases (Hataya et al. 2001; Zeng et al. 2005; Delfosse et al. 2015), including those mediated through estrogen receptors (Sharma et al. 2004; Yang et al. 2001). However, to our knowledge, synergistic effects on adiponectin secretion have not been reported to date.

Using KEGG pathway analysis, we first found that the same pathways, “PPAR signaling pathway”, “Metabolism of xenobiotics by cytochrome P450”, and “Adipocytokine signaling pathway”, were affected by the treatments with daidzein and PTE. Daidzein and PTE apparently promoted some of the same alterations, although the overall gene expression profiles, illustrated by the heat map and Venn diagram, were notably different. These results suggest that daidzein- and PTE-treatments may affect adipocyte differentiation and adiponectin expression through different mechanisms, which may explain the synergistic effect. Detailed investigations are necessary to clarify this hypothesis.

In future studies, we plan to identify the effective component(s) in PTE and explore the mechanisms of adiponectin activation through various experiments, including in vivo studies. The results from these studies may lead to novel treatments for type 2 diabetes mellitus and disorders of lipid metabolism.

In conclusion, this study demonstrated that PTE modulates adipocyte differentiation and adiponectin secretion in 3T3-L1 cells. Our results also demonstrated that simultaneous treatment with PTE and daidzein synergistically enhanced adiponectin expression. We also revealed that the overall gene expression profiles of 3T3-L1 cells were significantly different after PTE- and daidzein-treatments. These results suggest that a component(s) present in PTE may be beneficial for the treatment of type 2 diabetes mellitus and disorders of lipid metabolism, demonstrating the health potential of livestock by-products.

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Authors' contribution KK performed the research, MS and TM analyzed the data, SK performed informatics analysis, YK wrote the paper, TF designed the research study and wrote the paper.

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