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Gene expression profiling of 3T3-L1 adipocytes exposed to phloretin☆

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Abstract

Adipocyte dysfunction plays a major role in the outcome of obesity, insulin resistance and related cardiovascular complications. Thus, considerable efforts are underway in the pharmaceutical industry to find molecules that target the now well-documented pleiotropic functions of adipocyte. We previously reported that the dietary flavonoid phloretin enhances 3T3-L1 adipocyte differentiation and adiponectin expression at least in part through PPARγ activation. The present study was designed to further characterize the molecular mechanisms underlying the phloretin-mediated effects on 3T3-L1 adipocytes using microarray technology. We show that phloretin positively regulates the expression of numerous genes involved in lipogenesis and triglyceride storage, including GLUT4, ACSL1, PEPCK1, lipin-1 and perilipin (more than twofold). The expression of several genes encoding adipokines, in addition to adiponectin and its receptor, is positively or negatively regulated in a way that suggests a possible reduction in systemic insulin resistance and obesity-associated inflammation. Improvement of insulin sensitivity is also suggested by the overexpression of genes associated with insulin signal transduction, such as CAP, PDK1 and Akt2. Many of these genes are PPARγ targets, confirming the involvement of PPARγ pathway in the phloretin effects on adipocytes. In light of these microarray data, it is reasonable to assume that phloretin may be beneficial for reducing insulin resistance, in a similar way to the thiazolidinedione class of antidiabetic drugs.

Keywords: Phloretin; Adipocyte; Microarray; Nutrigenomics; Lipogenesis; Insulin signaling

1. Introduction

Adipocytes are highly specialized cells that play a central role in energy homeostasis [1]. The primary role of fat cells is to store energy as triglycerides during periods of calorie excess and to release it during periods of calorie deficit. Adipocytes also express and secrete numerous bioactive substances, called adipokines, which act at both local (autocrine/paracrine) and systemic (endocrine) levels to regulate whole-body energy metabolism [2]. Adipocytes possess the full complement of enzymes and regulatory proteins required to execute both lipogenesis and lipolysis. Under normal conditions, these processes are tightly controlled by hormonal and biochemical signals in response to changes in nutritional state. Adipocyte dysfunction is known to play a crucial role in the development of obesity, type 2 diabetes and cardiovascular disease. In addition, it is well established that excess adipose tissue in obesity and markedly reduced adipose tissue in lipodystrophy are both associated with insulin resistance and related metabolic complications [3,4]. Therefore, proper regulation of adipogenesis is required not only for appropriate lipid storage, but also for systemic energy and lipid homeostasis. Adipogenesis is a complex process accompanied by coordinated changes in morphology, hormone sensitivity and gene expression [5]. Several transcription factors act cooperatively and sequentially to drive the adipogenic program, including CCAAT/enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptor γ (PPARγ) [6]. Convincing evidence supports that PPARγ is the central regulator of adipogenesis and is also an important modulator of lipogenesis, glucose homeostasis and insulin sensitivity [6,7]. Thiazolidinediones (TZDs), as synthetic PPARγ ligands and antidiabetic agents, are able to improve insulin sensitivity by promoting adipocyte differentiation and lipid storage [8,9].

More recently, much attention has been focused on flavonoids, a group of phenolic compounds that have been linked to a reduced risk of major chronic diseases [10]. Phloretin belongs to the chalcone class of flavonoids and is present as glucosides in apples [11] and

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strawberries [12]. Phloretin was shown to display numerous biological properties: antioxidant capacity [11], competitive inhibition of sodium D-glucose cotransporter 1 [13], prevention of cytokine-induced expression of endothelial adhesion molecules and reduction of human platelets activation [14]. In addition, in our previous study, we have shown that phloretin significantly enhances 3T3-L1 adipocyte differentiation by increasing C/EBPα and PPARγ mRNA levels and by inducing PPARγ transcriptional activity [15]. We also provided evidence that phloretin stimulates the expression and secretion of adiponectin, an insulin-sensitizing adipokine. These data suggested for the first time that phloretin may be beneficial for reducing insulin resistance through its potency to regulate adipocyte differentiation and function. The present study was designed to extend our understanding of the molecular mechanisms underlying the phloretin-mediated effects on adipocytes. We used DNA microarray technology to identify large-scale gene expression changes in mature 3T3-L1 adipocytes treated with phloretin during differentiation.

2. Materials and methods

2.1. Cell culture reagents

Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Invitrogen (Carlsbad, CA, USA) and charcoal-stripped fetal bovine serum (FBS) was obtained from Biowest (Nuaille, France). Phloretin, isobutylmethylxanthine, dexamethasone, insulin and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture and stimulation

3T3-L1 preadipocytes (ATCC, Manassas, VA, USA) were seeded in 6-cm-diameter dishes at a density of 15×10^4 cells/well. Cells were grown in phenol red-free DMEM supplemented with 10% charcoal-stripped FBS at 37°C in a 5% CO_2 humidified atmosphere. To induce differentiation, 2-day postconfluent 3T3-L1 preadipocytes (Day 0) were stimulated for 48 h with 0.5 mM isobutylmethylxanthine, 0.25 μM dexamethasone and 1 μg/ml insulin in phenol red-free DMEM supplemented with 10% charcoal-stripped FBS. Then preadipocytes were maintained in and refed every 2 days with phenol red-free DMEM supplemented with 10% charcoal-stripped FBS and 1 μg/ml insulin. To examine the effect of phloretin on adipocyte differentiation, 2-day postconfluent 3T3-L1 preadipocytes received 50 μM phloretin every 2 days until the end of the experiment at Day 12 (time of full differentiation). Phloretin was reconstituted as 50 mM stock solutions in DMSO and stored at −20°C. Three independent experiments, each in triplicate, were performed.

2.3. RNA Extraction

Total RNA was extracted from 3T3-L1 cells at Day 12 after adipogenic induction using TRIzol reagent (Invitrogen) according to the manufacturer's instructions.

2.4. Microarray

Quantity and quality of the extracted RNA were checked with 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using the Agilent RNA 6000 Nanokit (Part Number 5067-1511). RNA samples with an RNA integrity number (RIN) greater than 9 were used for further analysis in a two-color microarray experiment using Agilent 4×44K Mouse Whole-Genome 60-mer oligo-chips (ref G4122F, Agilent Technologies). Aliquots representing equal amounts of total RNA were pooled from the nine samples (triplicates of three experiments) of each condition at Day 12. Each pool (control and treated) was then run in quadruplicate on four individual arrays, two of which being dye swapped. Treated and control RNA samples were labeled using the Agilent low RNA Input Fluorescent Linear Amplification Kit (p/n 5184-3523) and with cyanine-5 and cyanine-3 cytidine triphosphate dyes, according to the manufacturer's instructions The dye incorporation rate was assessed with a Nanodrop ND-1000 spectrophotometer and was between 1.2 and 1.4 pmol/μl. Hybridization was carried out using the Agilent Gene Expression Hybridization Kit (p/n 5188-5242), following the manufacturer's instructions. Briefly, 825 ng of treated sample cRNA was mixed with 825 ng of control sample cRNA and this solution was subjected to fragmentation (30 min at 60°C) and then hybridized to the arrays in a rotary oven (65°C, 17 h, 10 rpm). Slides were disassembled and washed in Solutions I and II according to the manufacturer's wash buffer kits instructions and scanned with the Agilent DNA Microarray Scanner model G2505B (Agilent Technologies).

2.5. Microarray data analysis

Signal intensity per spot was generated from the scanned image with Feature Extraction Software 7.5 (Agilent Technologies) using default setting. Spots that do not pass quality control procedures are automatically flagged in this procedure. The Lowess (locally weighted linear regression curve fit) and dye-swap normalization methods were applied to the ratio (Cy5/Cy3) of the signal intensities generated in each microarray with GeneSpring GX 7.3 (Agilent Technologies). Genes corresponding to spots that were flagged in more than one of the four replicates were removed from further analysis. Results were further filtered on confidence using Student's *t* test with a *P* value cut-off set at .05. From these selected genes, lists of statistically significant differentially regulated genes between treated and control cells (1.5-fold up or down) were generated. Gene changes common to at least three of the quadruplicates were considered significant. To further characterize sets of functionally related genes, the following software applications were used: MetaCore (GeneGo), Pathway Miner (Bio Resource for Array Genes at http://www.biorag.org) and Pathway Express [16] (available at http://vortex.cs.wayne.edu/projects.htm). Briefly, each software extracted relevant biological processes from the widely used biological databases (such as KEGG, BIOCARTA, GENMAPP, *etc*.) and ordered them on the basis of the most significant representation of genes within each biological process as compared to the expected distribution due to chance alone.

2.6. Real-time quantitative RT-PCR

Total RNA (1 μg) was reverse transcribed to cDNA in a final 20 μl using random primers (Invitrogen) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Real-time quantitative RT-PCR (qRT-PCR) analysis was performed using the Mx3005P Real-Time PCR System (Stratagene, La Jolla, CA, USA). Reactions were performed in a final 20 μl containing 10 ng of cDNA, optimized specific primers and probes (TaqMan Gene Expression Assays, Applied Biosystems, Tokyo, Japan) and TaqMan Gene Expression Master Mix (Applied Biosystems) according to the manufacturer's instructions. The assay identification numbers of the TaqMan Gene Expression Assays used are available on the Applied Biosystem website (http://www.appliedbiosystems.com). For each condition, expression was quantified in each of the triplicate of the three independent experiments, and 18S rRNA was used as the endogenous control in the comparative cycle threshold (C_T) method [17]. The data are expressed as means±S.D. Significant differences between the control and the treated groups, set at $P<.05$, were determined by Student's *t* test using Statview Software (Abacus Concepts, Cary, NC, USA).

3. Results

3.1. Analysis of phloretin-induced changes in adipocyte gene expression

Phloretin effects on 3T3-L1 differentiation-related gene expression (PPARγ, C/EBPα, LPL, FABP4, adiponectin) had been preliminary tested for phloretin concentrations ranging from 1 to 50 μM. A tendency towards up-regulation was observed starting at 20 μM but reached statistical significance only at 50 μM (data not shown). Gene expression profiling of mature 3T3-L1 adipocytes using DNA microarrays was then conducted on cells treated with 50 μM phloretin, a concentration that can be reached in plasma of phloretin-supplemented rats [18]. The screening process led to the identification of 1641 genes that were differentially expressed with a fold change higher than 1.5-fold. Of these, 1093 had an increased expression in adipocytes treated with phloretin, and 548 had a decreased expression level. Since RNAs were pooled from biological replicates and results filtered on confidence using technical replicates, it can be assumed that these data represent truly differentially regulated genes at levels above technical and biological background variability. In order to find the most relevant biological processes involving these regulated genes, the two sets of up- and down-regulated genes were further analyzed using the software applications described in Materials and Methods. This article is focused on regulatory and metabolic pathways that exhibited the highest level of significance concerning the representation of functionally annotated genes from our lists when compiling the results obtained in the three software applications.

3.2. Phloretin increases expression of genes associated with carbohydrate metabolism

Expression of several genes involved in carbohydrate metabolism was up-regulated by phloretin (Table 1). These included the gene

Table 1
Phloretin-induced mRNA changes in genes associated with carbohydrate metabolism

Gene symbol	Gene description	Genbank ID	Fold change
Glucose uptake			
SLC2A4	Glucose transporter 4; GLUT4	NM_009204	2.5
Glycolysis and gluconeogenesis			
HK2	Hexokinase 2	NM_013820	2.0
ALDOA	Aldolase 1, isoform A	NM_007438	1.7
BPGM	2,3-Bisphosphoglycerate mutase	NM_007563	1.7
PKM2	Pyruvate kinase, muscle	NM_011099	1.5
LDH2	Lactate dehydrogenase 2, B chain	NM_008492	1.5
PC	Pyruvate carboxylase	NM_008797	2.1
MDH1	Malate dehydrogenase 1, NAD (soluble)	NM_008618	1.7
PEPCK1	Phosphoenolpyruvate carboxykinase 1 (soluble)	NM_011044	3.0
Pyruvate dehydrogenase complex			
PDHB	Pyruvate dehydrogenase (lipoamide) beta	NM_024221	1.6
PDHX	Pyruvate dehydrogenase complex, component X	NM_175094	1.5
DLAT	Dihydrolipoamide S-acetyltransferase	NM_145614	1.7
DLD	Dihydrolipoamide dehydrogenase	NM_007861	3.4
TCA Cycle			
CS	Citrate synthase	NM_026444	1.8
ACO2	Aconitase 2	NM_080633	1.5
IDH3A	Isocitrate dehydrogenase 3 (NAD+) alpha	NM_029573	2.6
OGDH	Oxoglutarate dehydrogenase (lipoamide)	NM_010956	1.9
SDHA	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	NM_023281	2.0
MDH2	Malate dehydrogenase 2, NAD (mitochondrial)	NM_008617	1.7
Citrate transport			
SLC25A1	Citrate transport protein (CTP)	NM_153150	2.2
Pentose phosphate pathway			
G6PDX	Glucose-6-phosphate dehydrogenase X-linked	NM_008062	1.5
PGD	Phosphogluconate dehydrogenase	BC011329	2.1
RPE	Ribulose-5-phosphate-3-epimerase	NM_025683	2.2
TKT	Transketolase	NM_009388	1.8
TALDO1	Transaldolase 1	NM_011528	1.9

encoding glucose transporter 4 (GLUT4, 2.5-fold) as well as genes encoding enzymes of glycolysis, such as hexokinase 2 (HK2, 2.0-fold) and muscle-type pyruvate kinase (PKM2, 1.5-fold). The expression of both pyruvate carboxylase (PC, 2.1-fold) and phosphoenolpyruvate carboxykinase 1 (PEPCK1, 3.0-fold), two rate-limiting enzymes of the gluconeogenic pathway, was also up-regulated by phloretin. In addition, genes encoding enzymes of the pyruvate dehydrogenase complex and of the tricarboxylic acid (TCA) cycle, such as dihydrolipoamide dehydrogenase (DLD, 3.4-fold) and citrate synthase (CS, 1.8-fold), respectively, were up-regulated. Phloretin also increased the mRNA levels of genes involved in the pentose phosphate pathway, including phosphogluconate dehydrogenase (PGD, 2.1-fold), transketolase (TKT, 1.8-fold) and transaldolase (TALDO1, 1.9-fold).

3.3. Phloretin increases the expression of genes associated with lipid metabolism

Several genes involved in adipocyte differentiation and lipogenesis were up-regulated by phloretin (Table 2). These included genes responsible for fatty acid uptake and transport, such as lipoprotein lipase (LPL, 2.2-fold), fatty acid translocase (CD36, 2.2-fold), fatty acid transport protein 4 (FATP4, 1.8-fold) and fatty acid binding protein 4 (FABP4, 3.4-fold). Genes encoding enzymes of the fatty acid and triglyceride synthesis were also significantly induced, including fatty acid synthase (FASN, 1.9-fold), stearoyl-CoA desaturase 1 (SCD1, 2.4-fold) as the rate-limiting enzyme in the synthesis of monounsaturated fatty acids, and diacylglycerol acyltransferases (DGAT1, 2.4-fold; DGAT2, 2.8-fold), which catalyze the last step in triglyceride synthesis. In addition, phloretin increased the mRNA levels of acyl-CoA synthetase long-chain family member 1 (ACSL1, 3.8-fold) and glycerol-3-phosphate dehydrogenase 1 (GPD1, 2.6-fold) respectively involved in fatty acid activation and glycerol phosphorylation. Consistent with a rise in triglyceride synthesis, genes encoding lipid droplet-associated proteins, including adipose differentiation-related protein (ADFP, 2.0-fold), fat-specific protein 27 (FSP27, 3.2-fold) and perilipin (PLIN, 3.3-fold), were up-regulated.

3.4. Phloretin modulates the expression of genes encoding adipokines

Transcript levels of adiponectin (ADIPOQ, 2.3-fold), adiponectin receptor 2 (ADIPOR2, 1.8-fold) and visfatin (PBEF1, 2.1-fold) were significantly increased by phloretin (Table 3). Adiponectin and visfatin play significant roles in energy metabolism mainly by increasing whole-body insulin sensitivity [2]. Phloretin also enhanced the expression of genes related to vascular function, including angiotensinogen (AGT, 2.4-fold), the precursor of angiotensin II involved in blood pressure regulation, vascular endothelial growth factor A (VEGFA, 2.0-fold) and angiopoietin-like 1 (ANGPTL1,

Table 2
Phloretin-induced mRNA changes in genes associated with lipid metabolism

Gene symbol	Gene description	Genbank ID	Fold change
Fatty acid uptake and transport			
LPL	Lipoprotein lipase	NM_008509	2.2
CD36	CD36 antigen/fatty acid translocase	NM_007643	2.2
SLC27A4	Fatty acid transport protein 4; FATP4	NM_011989	1.8
FABP4	Fatty acid binding protein 4, adipocyte; aP2	NM_024406	3.4
FABP5	Fatty acid binding protein 5, epidermal	NM_010634	2.5
Fatty acid synthesis			
FASN	Fatty acid synthase	NM_007988	1.9
SCD1	Stearoyl-CoA desaturase 1	NM_009127	2.4
ACSL1	Acyl-CoA synthetase long-chain family member 1	NM_007981	3.8
FADS1	Fatty acid desaturase 1	NM_146094	1.6
FADS2	Fatty acid desaturase 2	NM_019699	1.9
Triglyceride synthesis			
GPD1	Glycerol-3-phosphate dehydrogenase 1 (soluble)	NM_010271	2.6
AGPAT2	1-Acylglycerol-3-phosphate *O*-acyltransferase 2	NM_026212	1.9
AGPAT3	1-Acylglycerol-3-phosphate *O*-acyltransferase 3	NM_053014	1.9
DGAT1	Diacylglycerol *O*-acyltransferase 1	NM_010046	2.4
DGAT2	Diacylglycerol *O*-acyltransferase 2	NM_026384	2.8
Lipid droplet-associated proteins			
ADFP	Adipose differentiation-related protein	NM_007408	2.0
FSP27	Fat-specific protein 27	NM_178373	3.2
PLIN	Perilipin	NM_175640	3.3
CAV1	Caveolin 1	NM_007616	2.8

Table 3
Phloretin-induced mRNA changes in genes encoding adipokines

Gene symbol	Gene description	Genbank ID	Fold change
Insulin sensitivity			
ADIPOQ	Adiponectin	NM_009605	2.3
ADIPOR2	Adiponectin receptor 2	NM_197985	1.8
PBEF1	Pre-B-cell colony-enhancing factor 1; visfatin	NM_021524	2.1
Vascular function			
AGT	Angiotensinogen	NM_007428	2.4
VEGFA	Vascular endothelial growth factor A	NM_009505	2.0
ANGPTL1	Angiopoietin-like 1	NM_028333	2.3
Inflammation			
IL6	Interleukin 6	NM_031168	0.64
IL1RL1	Interleukin 1 receptor-like 1	NM_010743	0.47
PTX3	Pentraxin-related gene	NM_008987	0.66
CCL7	Chemokine (C-C motif) ligand 7	NM_013654	0.66
CCL8	Chemokine (C-C motif) ligand 8	NM_021443	0.54
CCL27	Chemokine (C-C motif) ligand 27	NM_011336	0.66
CXCL4	Chemokine (C-X-C motif) ligand 4	NM_019932	0.6
CXCL10	Chemokine (C-X-C motif) ligand 10	NM_021274	0.66
Extracellular matrix			
FN1	Fibronectin 1	NM_010233	0.56
COL1A1	Procollagen, type I, alpha 1	NM_007742	0.45
COL3A1	Procollagen, type III, alpha 1	NM_009930	0.56
THBS1	Thrombospondin 1	NM_011580	0.65
LOX	Lysyl oxidase	NM_010728	0.56
MMP3	Matrix metalloproteinase 3	NM_010809	0.59
MMP13	Matrix metalloproteinase 13	NM_008607	0.53
TIMP1	Tissue inhibitor of metalloproteinase 1	NM_011593	0.6
TIMP2	Tissue inhibitor of metalloproteinase 2	NM_011594	0.6

2.3-fold), both involved in angiogenesis. In contrast, expression of genes encoding inflammatory factors was down-regulated by phloretin. These included the pro-inflammatory cytokine interleukin 6 (IL6, 0.64-fold), the acute-phase protein pentraxin 3 (PTX3, 0.66-fold) and several chemokines that control the recruitment and migration of immune cells to a site of infection. In addition, phloretin decreased the transcript levels of proteins involved in extracellular matrix (ECM) remodeling. These included matrix metalloproteinases (MMP3, 0.59-fold; MMP13, 0.53-fold) and their tissue inhibitors (TIMP1, 0.60-fold; TIMP2, 0.60-fold). There was also a marked down-regulation of genes encoding ECM components, such as fibronectin (FN1, 0.56-fold) and collagens (COL1A1, 0.45-fold; COL3A1, 0.56-fold), the expressions of which are negatively correlated with adipocyte differentiation [19,20].

3.5. Phloretin increases the expression of transcriptional regulators associated with adipocyte phenotype

Key transcription factors regulating adipocyte differentiation and phenotype maintenance had higher gene expression levels under phloretin treatment (Table 4). These included C/EBPα (2.0-fold), PPARγ (1.9-fold), retinoid x receptor γ (RXRγ, 2.1-fold) and liver X receptor α and β (LXRα, 2.5-fold; LXRβ, 1.5-fold). Phloretin also increased the mRNA levels of Krüppel-like factor 15 (KLF15, 1.9-fold) and decreased those of GATA binding protein 2 (GATA2, 0.65-fold), respectively, shown to promote and inhibit adipocyte differentiation [21,22]. Interestingly, lipin 1 gene (LPIN1) described as a candidate gene for lipodystrophy was significantly up-regulated by phloretin (2.6-fold) [23]. In contrast, the gene encoding aryl hydrocarbon receptor (AHR) was significantly down-regulated (0.53-fold). AHR is mainly involved in the control of xenobiotic metabolism but it has been also reported to negatively regulate adipocyte differentiation by inhibiting both PPARγ activation and clonal expansion, a prerequisite event for adipocyte differentiation [24].

3.6. Phloretin increases the expression of genes associated with insulin signal transduction

Insulin action is mediated through a complex network of signaling pathways that trigger rapid changes in protein phosphorylation and function, and changes in gene expression (for review, see Ref. [25]). As represented in Fig. 1, based upon the insulin signaling pathway available in the KEGG database [26], several genes encoding insulin signal transduction molecules were up-regulated by phloretin. These included genes encoding pivotal effectors of the phosphatidylinositol 3-kinase (PI3K) pathway, such as the catalytic β-subunit of PI3K (PIK3CB, 1.8-fold), phosphoinositide-dependent kinase 1 (PDK1, 2.2-fold) and the serine/threonine kinase Akt2 (1.6-fold). Akt2 activates or inhibits, by phosphorylation, a number of substrates primarily involved in the metabolic actions of insulin. Among them, phosphodiesterase 3B (PDE3B) is responsible for the antilipolytic action of insulin and its gene expression was shown to be significantly increased by phloretin (2.0-fold). A separate insulin signaling pathway localized in lipid raft microdomains involves the tyrosine phosphorylation of Cbl proto-oncogene by insulin receptor. This phosphorylation step requires the recruitment of Cbl to the adaptor protein APS, while the Cbl-associated protein, CAP, targets Cbl to the lipid raft protein flotillin. Both APS and CAP genes, as well as the gene encoding the adaptor protein CrkII, were significantly up-regulated by phloretin (1.9-, 2.7- and 1.6-fold, respectively). Insulin also activates the mitogen-activated protein kinase (MAPK) signaling pathway responsible for its mitogenic actions. In this pathway, phloretin increased the gene expression of the serine/threonine kinases Raf1 (1.8-fold) and MNK2 (1.8-fold).

3.7. Confirmation of microarray data by qRT-PCR

The microarray expression data for a subset of genes differentially regulated by phloretin were validated using qRT-PCR. In addition, the mRNA levels of sterol regulatory element binding protein 1c (SREBP1c), which displayed no change in its expression using microarray, were examined. Overall, qRT-PCR confirmed that the data from the microarray analysis were robust and that in many cases the intensity of fold changes was on the same order of magnitude (Fig. 2). Of all genes quantified, only qRT-PCR data for PBEF1 differed

Table 4
Phloretin-induced mRNA changes in genes associated with transcriptional regulation of adipocyte differentiation and function

Gene symbol	Gene description	Genbank ID	Fold change
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha	NM_007678	2.0
PPARG	Peroxisome proliferator activated receptor, gamma	NM_011146	1.9
PGC1B	Peroxisome proliferative activated receptor, gamma, coactivator 1 beta	NM_133249	1.9
RXRG	Retinoid X receptor, gamma	NM_009107	2.1
LXRA	Liver X receptor, alpha	NM_013839	2.5
LXRB	Liver X receptor, beta	NM_009473	1.5
KLF15	Kruppel-like factor 15	NM_023184	1.9
GATA2	GATA binding protein 2	NM_008090	0.65
NRIP1	Nuclear receptor interacting protein 1	NM_173440	1.7
LPIN1	Lipin 1	NM_015763	2.6
RB1	Retinoblastoma 1	NM_009029	1.5
RORG	RAR-related orphan receptor, gamma	NM_011281	2.2
TSHR	Thyroid stimulating hormone receptor	NM_011648	3.0
AHR	Aryl-hydrocarbon receptor	NM_013464	0.53

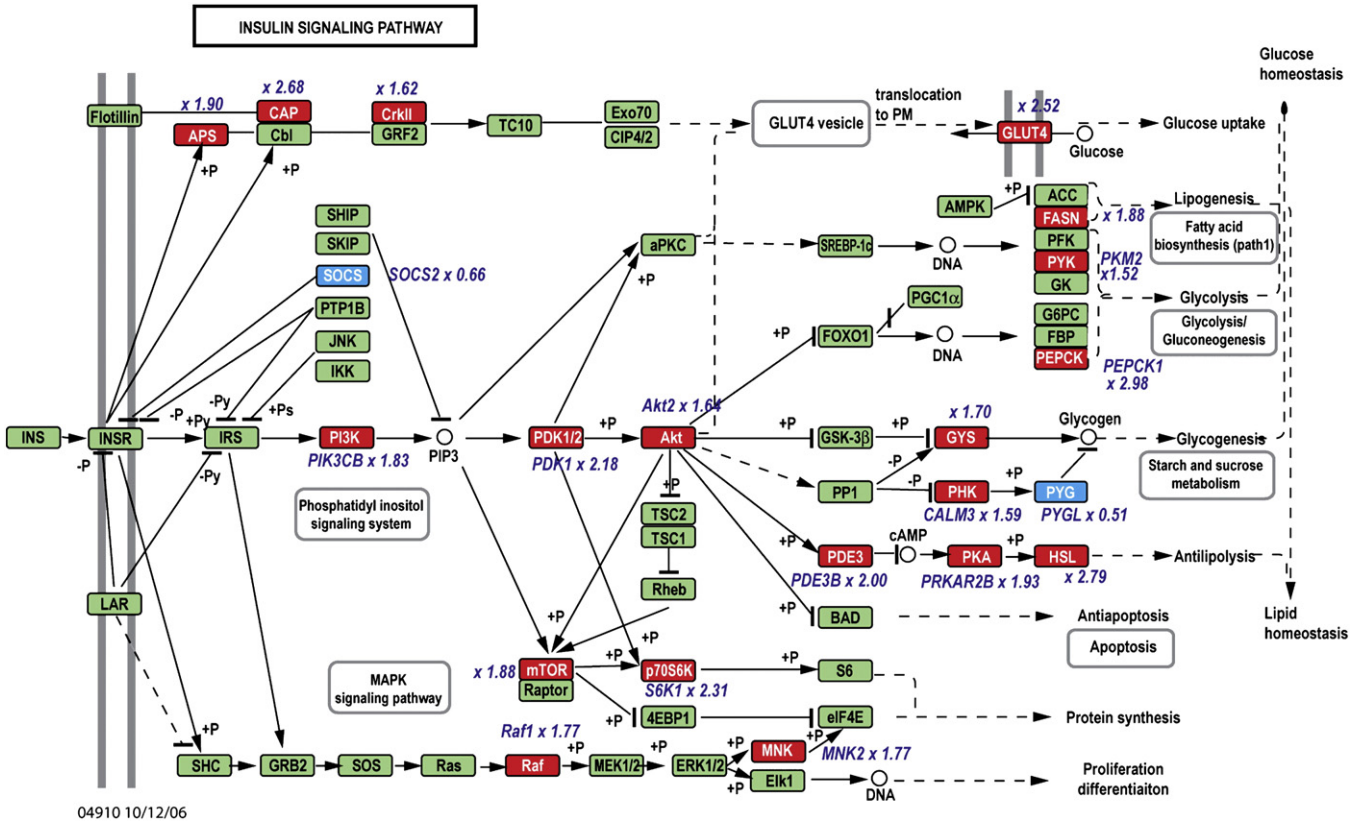

Fig. 1. KEGG Insulin signaling pathway. Genes significantly up- or down-regulated by phloretin are represented respectively in red or blue with their corresponding fold change from the microarray data. PIK3CB: Phosphatidylinositol 3-kinase, catalytic, beta; PDK1: 3-phosphoinositide-dependent kinase 1; Akt2: thymoma viral proto-oncogene 2; APS: adaptor protein containing PH and SH2 domains; CAP: Cbl-associated protein; CrkII: v-crk avian sarcoma virus CT10 oncogene homolog; GLUT4 : glucose transporter 4; FASN: fatty acid synthase; PKM2: pyruvate kinase 2, muscle; PEPCK1: phosphoenolpyruvate carboxykinase 1; GYS: glycogen synthase 1; CALM3: calmodulin 3; PYGL: glycogen phosphorylase, liver; PDE3B: phosphodiesterase 3B, cGMP-inhibited; PRKAR2B: protein kinase, cAMP-dependent, regulatory, type II beta; HSL: hormone-sensitive lipase; mTOR: mammalian target of rapamycin; S6K1: ribosomal protein S6 kinase 1; Raf1: v-raf-1 murine leukemia viral oncogene homolog 1; MNK2: MAPK-interacting kinase 2; SOCS2: suppressor of cytokine signaling 2.

from microarray data. Although PBEF1 mRNA levels showed no significant change in the qRT-PCR analysis, they showed significant up-regulation in the microarray analysis.

4. Discussion

In this study, we show that phloretin positively regulates the expression of genes involved in all the metabolic pathways leading to the synthesis of triglycerides and their subsequent storage in 3T3-L1 adipocytes. This is consistent with the increased triglyceride accumulation that we have previously observed in adipocytes treated with phloretin [15]. The up-regulation of metabolic genes also further characterizes the enhancement of adipocyte differentiation under phloretin treatment [15]. Additionally, pathway-oriented analysis of the microarray data shows that, in 3T3-L1 adipocytes, several key regulatory functions for the control of energy balance and glucose homeostasis are modulated by phloretin at the transcriptional level. These transcriptional effects share common features with those exerted by synthetic TZDs.

The two major sources of metabolites used for triglyceride synthesis are glucose, imported via the insulin-sensitive glucose transport system, and fatty acids, available from the hydrolysis of extracellular lipoprotein-bound triglycerides catalyzed by LPL [27]. Here we show that phloretin significantly increases transcripts for the gene encoding the insulin-sensitive glucose transporter GLUT4, and for numerous key genes involved in fatty acid uptake and transport such as CD36, FATP4 and FABP4. For triglyceride synthesis, the glycerol backbone and fatty acids are transformed into glycerol-3P and fatty acyl-CoA esters. Since glycerol kinase activity is negligible in adipocytes under physiological conditions [28], glycerol-3P is produced from a metabolic intermediate of glycolysis via GPD1, and from glyceroneogenesis, an abbreviated version of gluconeogenesis involving PC and PEPCK1 [28]. Fatty acyl-CoA esters are synthesized by

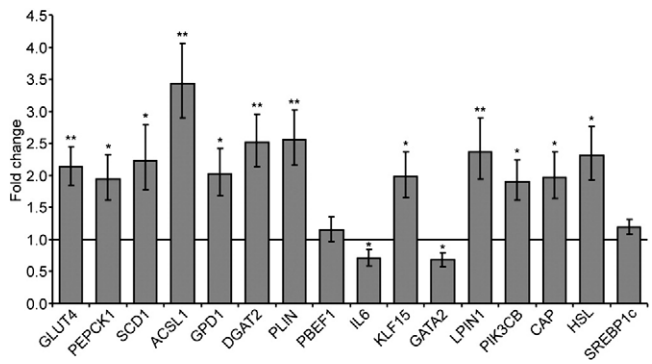

Fig. 2. Expression levels of selected genes quantified by qRT-PCR. Two-day postconfluent 3T3-L1 preadipocytes (Day 0) were treated with 50 μM phloretin every 2 days for 12 days. Cells treated with 0.1% DMSO were used as controls. Total RNA was extracted from cells at Day 12, and mRNA levels of the indicated genes were measured by qRT-PCR using specific primers and probes. Results were expressed relative to control cells (not shown) after normalization to 18S rRNA. The bars represent mean values±S.D. *P<.05; **P<.01.

acyl-CoA synthetase (ACS) enzymes, including ACSL1. Remarkably, the murine fatty acid transport protein FATP4 has been reported to exhibit acyl-CoA synthetase activities [29]. A small part of fatty acids used for triglyceride synthesis comes from *de novo* fatty acid synthesis from glucose. In keeping with the increased expression of genes involved in glycolysis and in the pyruvate dehydrogenase reaction, we can expect an increase in acetyl-CoA that could lead to promotion of fatty acid synthesis under phloretin treatment. Additionally, CS and CTP (citrate transport protein) responsible for the transport of acetyl-CoA from the mitochondria into the cytosol where fatty acid synthesis occurs have their gene expression significantly increased by phloretin. Consistent with a rise in fatty acid synthesis, several genes of the pentose phosphate pathway providing NADPH as reducing power for this synthesis are up-regulated.

In addition to the central role of lipid storage, adipocytes actively contribute to endocrine signaling. Among adipokines, adiponectin has recently attracted much attention because a decrease in its plasma levels appears to play an important causal role in insulin resistance, type 2 diabetes and the metabolic syndrome [30]. Adiponectin has been described to modulate both glucose and lipid metabolism in skeletal muscle and liver, and to act as an insulin sensitizer [30]. Recent evidence also suggests that adiponectin operates as a key autocrine regulator of adipocyte differentiation and secretory function [31,32]. We have previously shown that phloretin markedly enhances adiponectin gene expression and secretion [15]. Complementing our previous results, we report here a significant increase in ADIPOR2 mRNA levels, raising the possibility that phloretin promotes the autocrine action of adiponectin. Therefore, we could hypothesize that the reduced gene expression of IL6, TIMP1 and TIMP2 observed under phloretin treatment might account for their reduced secretion previously reported in response to adiponectin [32]. Interestingly, it has been suggested that, by decreasing the secretion of TIMPs, adiponectin could directly contribute to adipose tissue remodeling by increasing the number of small adipocytes more sensitive to insulin than large adipocytes [32]. Phloretin may also influence adipocyte differentiation by increasing AGT gene expression and possibly secretion. Adipose tissue possesses a complete functional renin–angiotensin system (RAS) that is regulated independently of the systemic RAS [33], and for which any possible impact on blood pressure regulation remains unclear [33,34]. There are several lines of evidence supporting that local formation of AGT (via angiotensin II) promotes adipocyte growth and differentiation, both directly by promoting lipogenesis and indirectly by stimulating prostaglandin synthesis [33].

Insulin, the most potent anabolic hormone known, promotes adipocyte triglyceride storage by a number of mechanisms, including fostering the differentiation of preadipocytes to adipocytes and, in mature adipocytes, stimulating glucose uptake and triglyceride synthesis, as well as inhibiting lipolysis [25]. As evidenced in this study, phloretin induces gene expression of several insulin signal transduction molecules. We can hypothesize that its effects on adipocyte differentiation and function may be related to an improvement of adipocyte insulin sensitivity. Insulin increases glucose uptake by stimulating the translocation of GLUT4 from intracellular storage sites to the plasma membrane. This complex process is well known to be mediated through the activation of the PI3K pathway [35]. For the full insulin-stimulated glucose uptake, emerging evidence suggests, however, the requirement of a second insulin signaling pathway originating from lipid rafts and that links actin dynamics to GLUT4 translocation [35,36]. In this line and of particular interest, phloretin significantly increases the transcript levels of the adaptor protein CAP, the expression of which well correlates with insulin sensitivity. The protein is found predominantly in insulin-sensitive tissues, and expression is up-regulated in animal and human adipocytes by TZDs [37].

Insulin counteracts lipolysis mainly through the activation of PDE3B in adipocytes [38]. The significant increase in PDE3B mRNA levels induced by phloretin could lead to a decrease in intracellular cAMP levels and thus to inhibition of lipolysis through dephosphorylation of hormone-sensitive lipase (HSL) [39]. Although phloretin significantly increases HSL mRNA levels, its enzyme activity essentially depends on its phosphorylation by cAMP-dependent protein kinase (PKA). The increase in perilipin mRNA levels could also play a part in the regulation of lipolysis under phloretin treatment. Perilipin is the major protein found in association with adipocyte lipid droplets and has been suggested to function as a barrier restricting the access of lipases, thereby maintaining a low rate of basal lipolysis [40]. In response to increase in intracellular cAMP levels, perilipin, however, undergoes phosphorylation by PKA and this phosphorylation may serve as a docking for HSL on the lipid droplets, facilitating the ensuing of hydrolysis. In addition, phloretin increases the transcript levels of FSP27, a novel lipid droplet protein that shares many features characteristic of perilipin, including the ability to enhance triglyceride accumulation when it is ectopically expressed in 3T3-L1 preadipocytes [41]. Conversely, FSP27 depletion in mature adipocytes has been shown to significantly stimulate lipolysis and to reduce the size of lipid droplets [41]. On the whole, it appears that phloretin treatment substantially affects various mechanisms of insulin signaling in a way that improves insulin sensitivity.

The microarray data presented here agree with our previous data and extend them, suggesting that phloretin-mediated effects on adipocyte differentiation and function are directed by a combination of increased PPARγ and C/EBPα mRNA levels and induced PPARγ transcriptional activity [15]. Thus, a number of genes up-regulated more than twofold by phloretin are target genes of PPARγ, including those encoding GLUT4, LPL, CD36, FATP4, FABP4, ACSL1, PEPCK1, CAP, perilipin and adiponectin [42–44]. Moreover, we show that phoretin significantly increases the gene expression of RXRγ as the obligate heterodimeric partner for PPARγ [45]. This may contribute to induce PPARγ transcriptional activity through promotion of PPARγ-RXR heterodimer binding to PPAR responsive elements in the promoter of target genes. Phloretin also significantly increases the expression of LXRα and to a lesser extent the expression of LXRβ, both receptors being able to heterodimerize with RXR. Both LXRs are highly expressed in adipose tissue, and LXRα expression increases during adipocyte differentiation [46] and is regulated by PPARγ [47]. LXRs are involved in the regulation of lipid metabolism in mature adipocytes, but their role in adipocyte differentiation is controversial [46,48,49]. The transcription factor SREBP1c mediates some of the metabolic actions of insulin by inducing genes involved in lipogenesis [50,51]. It is noteworthy that we did not find any significant induction in the expression of SREBP1c using both microarray and qRT-PCR analysis, suggesting that this factor does not contribute to the phloretin-mediated effects on lipogenesis. Supportively, a recent study reported that, in adipocytes, unlike in hepatocytes, the up-regulation of SREBP1c gene has negligible effects on the expression of lipogenic genes, despite a concomitant increase in nuclear active SREBP1c; the authors proposed that lipogenic genes are controlled almost independently of SREBP1c in adipocytes [52].

Interestingly and unexpectedly, we show that phloretin significantly increases lipin-1 mRNA levels. Lipin-1 deficiency has been identified as the cause of generalized lipodystrophy characterized by impaired adipose tissue development and insulin resistance in the fatty liver dystrophy (*fld*) mouse [23]. Studies on lipin-1-deficient mice and cells have demonstrated that lipin-1 expression is required upstream of PPARγ for normal adipocyte differentiation [53]. Nevertheless, transgenic mice overexpressing lipin-1 specifically in adipose tissue exhibit increased fat cell triglyceride content and lipogenic gene expression [54]. Despite their higher adiposity, these transgenic mice have improved insulin sensitivity, likely due to more efficient fatty

acid trapping in adipose tissue as protection against lipid deposition in skeletal muscle and liver. Collectively, these studies suggest important functions for lipin-1 in adipocyte differentiation, lipogenesis and insulin sensitivity that might underlie, at least in part, the phloretin-mediated effects on adipocytes. More recently, lipin-1 has been identified as a phosphatidate phosphatase type 1 (PAP1) enzyme, which catalyzes the conversion of phosphatidate to diacylglycerol, the immediate precursor of triacylglycerol [55]. This new biochemical role of lipin-1 may indirectly account for gene expression changes previously considered to be a direct regulatory effect on transcription [56].

The inability to store fat in adipose tissue leading to chronic elevations in plasma free fatty acid levels combined with ectopic accumulation of triglycerides in nonadipose tissues is strongly associated with the pathogenesis of insulin resistance and type 2 diabetes [3,8]. TZDs, as antidiabetic drugs and activators of PPARγ, promote adipocyte differentiation along with the up-regulation of key genes involved in lipogenesis and triglyceride storage [8,9]. Increased storage capacity of adipose tissue upon TZD treatment results in a repartitioning of triglycerides toward adipose tissue and consequently reverses lipotoxicity-induced insulin resistance and lowers plasma glucose levels in patients with type 2 diabetes. In addition, TZDs intensify the signal transduction associated with insulin response by inducing expression of adiponectin and simultaneously reducing adipocyte expression of several insulin-resistant factors, like IL6 [8,9]. TZDs may also directly affect the insulin signaling pathway in adipose tissue, as suggested by the induction of CAP. More recently, lipin-1 expression in human adipose tissue and in cultured adipocytes has been shown to be up-regulated by TZDs, raising the possibility that lipin-1 is a mediator of TZD action [57]. Taken together, our microarray data highlighted that the transcriptional effects of phloretin are reminiscent of those of TZDs on adipocyte lipid metabolism and secretory function. Consequently, it is reasonable to assume that phloretin may be beneficial for reducing insulin resistance. Besides the "TZD-like" effects described above, we observed that phloretin positively regulates the expression of genes encoding Phase II enzymes, including glutathione S-transferases and epoxide hydrolases (data not shown). Such activation of detoxification pathways was often reported for many flavonoids in various biological models [58] but never for TZD antidiabetic drugs. Thus, in addition to TZD-like effects and as expected, phloretin is able to modulate biological pathways that are typical targets of flavonoids.

In conclusion, we provide evidence that the naturally occurring flavonoid phloretin, in line with the enhancement of adipocyte differentiation, profoundly influences lipogenesis and triglyceride storage in 3T3-L1 cells. This may be due in part to improved insulin sensitivity as indicated by the up-regulation of several key insulin signaling effectors. In addition, this microarray-based study has largely confirmed the involvement of PPARγ in the phloretin-mediated effects on adipocyte differentiation and function. Further functional and physiological studies are required to evaluate whether phloretin has potentially beneficial effects on insulin resistance.

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