Quercetin, a functional compound of onion peel, remodels white adipocytes to brown-like adipocytes

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Abstract

Adipocyte browning is a promising strategy for obesity prevention. Using onion peel-derived extracts and their bioactive compounds, we demonstrate that the onion peel, a by-product of the onion, can change the characteristics of white adipocytes to those of brown-like adipocytes in the white adipose tissue of mice and in 3T3-L1 cells. Expression of the following brown adipose tissue-specific genes was increased in the retroperitoneal and subcutaneous adipose tissues of 0.5% onion peel extract-fed mice: PR domain-containing 16, peroxisome proliferator-activated receptor gamma coactivator 1 α , uncoupling protein 1, and cell death-inducing DFFA-like effector. In 3T3-L1 adipocytes, onion peel extract induced the expression of brown adipose tissue-specific genes and increased carnitine palmitoyltransferase 1 α . This effect was supported by decreased lipid levels and multiple small-sized lipid droplets. The ethyl acetate fraction of the onion peel extract that contained highest portion of hydrophobic molecules showed the same browning effect in 3T3-L1 adipocytes. A high performance liquid chromatography analysis further identified quercetin as a functional compound in the browning effect by onion peel. The quercetin-associated browning effect was mediated in part by activation of AMP-activated protein kinase. In summary, our study is the first to demonstrate the browning effects of onion peel and quercetin using both animal and cell models. This result indicates that onion peel has the potential to prevent obesity by remodeling the characteristics of white adipocytes to those of brown-like adipocytes.

51

52 **1. Introduction**

53 Obesity, indicated by excess fat accumulation in white adipose tissue (WAT), mainly
54 develops when energy expenditure is less than energy intake [1]. Long-term excess fat
55 deposition in the body is associated with chronic diseases, including cardiovascular disease,
56 diabetes, and insulin resistance [2]. Increased energy expenditure is crucial to successfully
57 reducing body fat (i.e., weight loss) in obese individuals [1]. Physical activities, including
58 exercise, are interventions that increase energy expenditure and are commonly combined with
59 dietary modifications [1]. Because energy expenditure through exercise require steady and
60 long-term efforts to reduce body fat, the long-term maintenance of a healthy body is difficult
61 for obese people.

62 The discovery of brown adipose tissue (BAT) in human adults and children has
63 provided a new approach toward increasing energy expenditure [3, 4]. In contrast to WAT,
64 which stores extra energy as fat, BAT expends energy, i.e., stored fat, by generating heat
65 through non-shivering thermogenesis [5]. Uncoupling protein 1 (UCP1), a BAT-specific
66 thermogenin, promotes this heat generation by leaking protons into the mitochondrial matrix
67 to uncouple oxidative phosphorylation from ATP synthesis [6]. Notably, the induction of
68 UCP1 expression contributes to fat reduction in obese people [7]. Thus, UCP1 expression has
69 been indicated as a therapeutic target to increase energy expenditure. In response to
70 physiological stimuli, such as exposure to cold temperatures, some adipocytes in WAT are
71 differentiated into brown like-adipocytes, known as beige or brite adipocytes, that acquire
72 UCP1 expression [8]. This process requires the same major transcriptional factors and
73 indicators as those used to develop brown adipocytes, and they include PR domain-
74 containing 16 (PRDM16), peroxisome proliferator-activated receptor gamma coactivator 1 α
75 (PGC1 α), and cell death-inducing DFFA-like effector (CIDEA) [9]. Therefore, the browned

white adipocytes may be able to prevent obesity through a UCP1-induced thermogenic effect by burning fat that is directly stored in WAT [5]. Recent studies have indicated that phytochemicals, including curcumin, berberine, capsaicin, monoterpene, and resveratrol, enhance adipocyte browning in WAT [10-14]. However, phytochemicals with browning effects have not been heavily investigated.

Onion peel is a by-product of the onion. It contains high levels of quercetin derivatives, a flavonoid subgroup known as the flavonols, compared with those of the onion and other vegetables and fruits [15]. Quercetin has shown various biological functions, including anti-oxidant, anti-inflammatory, and anti-obesity effects [15-18]. Onion peel extract has been reported to inhibit lipogenesis and adipogenesis and increases fatty acid oxidation [15, 17]. However, the effects of onion peel extract on adipocyte browning in WAT remain unexplored. The aims of this study were to examine the browning effects of onion peel extract in WAT using high fat diet-induced obese C57BL/6 mice and to investigate the onion peel extract-derived compounds for adipocyte browning and its underlying mechanism using 3T3-L1 cells.

2. Materials and methods

2.1. Onion peel extraction

Onion peels were kindly provided by Boardman Foods Inc. (Boardman, Oregon, USA). Fresh onion peels were washed and air-dried overnight. One hundred grams of dried onion peel were pulverized using a homogenizer (Kinematica, New York, USA) for 3 min with 1 L of 60% aqueous ethanol as the extracting solvent. To increase the extraction rate, the homogenized sample was further extracted in an ultrasonic bath for 20 min at room temperature. The extract was filtered through Whatman No. 2 filter paper (Whatman International Limited, Kent, England) using a chilled Büchner funnel. The filtration residues

were extracted once more using the procedure described above. The solvent of the filtrate was evaporated using a rotary evaporator (Buchi RE120 Rotovapo, Flawil, Switzerland) in a water bath at 45 °C until the solvent was completely removed. The onion peel extract (OPE) was scraped from the evaporator flask and stored at -80 °C until use.

2.2. Fractionation of OPE by liquid-liquid extraction

OPE was fractionated into the ethyl acetate fraction (OPEF) and water fraction (OPWF) using the liquid-liquid extraction method. Briefly, 20 g of OPE was dissolved in 200 mL of deionized water (DW) and transferred to a separatory funnel. After adding an equal volume of ethyl acetate to the separatory funnel, the mixture was vigorously shaken and maintained overnight at room temperature. The mixture was divided into the ethyl acetate layer (OPEF) and water layer (OPWF) according to the immiscibility of solvents. All OPE fractions were separately collected and evaporated using a rotary evaporator until they were completely dried. The fractions were stored at -80 °C until use.

2.3. Identification and quantification of the main compounds in OPE, OPEF, and OPWF by reversed-phase high-performance liquid chromatography (HPLC)

HPLC was performed to identify and quantitate the major compounds in OPE, OPEF, and OPWF using a Waters HPLC system (Waters, Milford, MA, USA) equipped with a 1525 binary pump and 2748 dual wavelength absorbance detector (DAD). Separations were performed on a C₁₈ reversed-phase symmetry analytical column (5 µm x 300 mm x 4.6 mm; Waters, Milford, MA) using a gradient of mobile phases at a flow rate of 1.0 mL/min. The gradient elution was performed by changing the ratio of solvent A (HPLC-grade water with 0.5% H₃PO₄) to solvent B (methanol with 0.5% H₃PO₄) as follows: 95% A and 5% B, 0-10 min; 85% A and 15% B, 10-15 min; 70% A and 30% B, 15-20 min; 65% A and 35% B, 20-

25 min; 30% A and 70% B, 25-28 min; and 95% A and 5% B, 28-30 min. The injection volume for each sample was 10 μ L. The detection was performed at 360 nm. Quercetin and isoquercetin in OPE, OPEF, and OPWF were quantitated by comparing their retention times to those of pure quercetin and isoquercetin standards (Sigma-Aldrich, St. Louis, MO, USA). All organic solvents used in the analysis were HPLC grade (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Animal study

Fifteen 4-week-old male C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were randomly assigned to two dietary groups as follows: one was fed a high-fat diet (HFD, 60% Kcal from fat, $n = 9$) and the other was fed HFD supplemented with 0.5% OPE (w/w, HFD-OPE, $n = 6$). The diets were established in the Comparative Medicine Diet Laboratory at Wake Forest School of Medicine (Winston-Salem, NC, USA) (Supplementary Table 1). The mice were housed in a room maintained at 25 °C with a 12-h light-dark cycle and provided with free access to food and water for 8 weeks. Their body weights and dietary consumption were recorded weekly. At the end of the experimental feeding, mice were fasted for 16 h and anesthetized with isoflurane using a Somnosuit-small animal anesthesia system (Kent Scientific Cooperation, St. Torrington, CT, USA). Blood was drawn by cardiac puncture under anesthesia using a syringe that contained ethylenediaminetetra acetic acid. Plasma was separated from the whole blood by centrifugation at 12,000 $\times$ g for 10 min at 4 °C. The mice were euthanized by cervical dislocation. The epididymal WAT (EWAT), retroperitoneal WAT (RWAT) and subcutaneous WAT (SWAT) were dissected and immediately frozen in liquid nitrogen. The plasma and WATs were stored at -80 °C until use. All procedures were approved by the

Institutional Animal Care and Use Committee of the Wake Forest University and North Carolina Agricultural and Technical State University.

2.5. Culture and differentiation of 3T3-L1 cells

The 3T3-L1 cells were purchased from the American Type Culture Collection (ATCC®CL173™, Manassas, VA, USA). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% calf bovine serum (37 °C, 5% CO₂). To differentiate preadipocytes into white adipocytes, cells were seeded into 12-well plates (BD Biosciences, San Jose, CA, USA) at a density of 1.75×10^4 cells/cm². When the cells reached confluence (on day 3 after seeding), the medium was changed to DMEM supplemented with 10% fetal bovine serum (FBS), 1 µg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine and 0.25 mM dexamethasone. After two days, the differentiation medium was replaced with post-differentiation medium containing 10% FBS and 1 µg/mL insulin. The cells were cultured for 6 days with fresh post-differentiation medium replacements every other day until preadipocytes became fully differentiated into mature white adipocytes (typically by day 11 after cell seeding). For experiments, OPE, OPEF and OPWF (50, 100 and 150 µg/mL) and quercetin or isoquercetin (25, 50, and 100 µM) were added to the post-differentiation medium every other day until the cells were harvested for RNA extraction or stained with Oil Red O (day 11).

2.6. Cytotoxicity assay

The cytotoxicities of OPE, OPEF, OPWF, quercetin and isoquercetin were determined using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) cell proliferation assay kit (Cayman, Ann Arbor, MI, USA) according to the manufacturer's instructions. Preadipocytes were seeded into 96-well plates at a density of 1.75×10^4 cells/cm². On the following day, OPE, OPEF, and OPWF (0 - 40 µg/mL) and quercetin and

isoquercetin (0 - 240 μ M) were added to the cells. After 48 h, the culture medium was replaced with fresh culture medium containing 0.5 mg/mL MTT, and the cells were incubated for 3 h. After removing the MTT-containing medium, 100 μ L of dimethyl sulfoxide was added to dissolve the purple formazan crystals. The absorbance that proportionally indicated the number of viable cells was measured at 570 nm using a Synergy HT microplate reader (BioTek, Winooski, VT, USA). Cell viability (%) was calculated as 100 x (absorbance of sample-treated cells / absorbance of the control cells without samples).

2.7. Oil Red O staining

Lipid droplets in the cells were observed by Oil Red O staining (Thermo Fisher Scientific). Fully differentiated white adipocytes (day 11) were washed with phosphate buffered saline (PBS) and rinsed with 10% buffered formalin (Thermo Fisher Scientific). The cells were subsequently incubated with fresh 10% buffered formalin for 1 h at room temperature, washed with 60% isopropanol and completely dried. Cells were further incubated with an Oil Red O working solution, which was prepared by adding 2 parts of water to 3 parts of the stock solution (0.35 g Oil Red O in 100 mL isopropanol) for 30 min at room temperature. After four washes with DW, cells were visualized using an Evos XL-microscope (Thermo Fisher Scientific). To quantitate the lipids in the cells, 1 mL of isopropanol per well was added to dissolve the Oil Red O reagent. The absorbance was measured at 510 nm using a Synergy HT microplate reader (BioTek).

2.8. Quantitative real-time polymerase chain reaction (qPCR) analysis

Total RNA was extracted from the EWATs, RWATs, SWATs and fully differentiated white adipocytes using TRIzol (Thermo Fisher Scientific) according to the manufacturer's instructions. The isolated RNA was quantified using a Take3 micro-volume plate equipped with a Synergy HT microplate reader (BioTek). cDNA was synthesized from

1 µg of total RNA using the XLAScript cDNA MasterMix (Exella GmbH, Feucht, Germany) according to the manufacturer's instructions. Gene expression was quantitated using the Fast Start Essential DNA Green Light Master kit (Roche, Indianapolis, IN, USA) in a Light Cycler 90 (Roche). The PCR conditions were as follows: 10 min at 95 °C, followed by 50 cycles of denaturation for 10 sec at 95 °C, annealing for 10 sec at 60 °C, and extension for 10 sec at 72 °C. The primers were designed using Primer3, a web-based software (<http://bioinfo.ut.ee/primer3-0.4.0/>), and are listed in Table 1. Ribosomal protein L 32 (RPL32) was used as the invariant gene.

2.9. Inhibition of AMP-activated protein kinase (AMPK)

3T3-L1 cells were treated with dorsomorphin, an AMPK inhibitor, to examine the role of AMPK signaling in the browning of 3T3-L1 cells by quercetin. The cells were cultured and differentiated using the procedure described above. After differentiation, the cell culture medium was replaced with the post-differentiation medium including 5 µM dorsomorphin, 100 µM quercetin, or 5 µM dorsomorphin plus 100 µM quercetin on days 5, 7 and 9. On day 11, the cells were harvested to measure PRDM16, UCP1, PGC1α, and carnitine palmitoyltransferase (CPT) 1α gene expression.

2.10. Statistical analysis

The data are expressed as the mean ± standard error of the mean (SEM). The differences between the control and the various concentrations of OPE, OPEF, OPWF, quercetin, and isoquercetin were evaluated by one-way analysis of variance (ANOVA) with Tukey's post hoc test (Prism 5.0, GraphPad Software Inc., La Jolla, CA, USA). The data obtained from the *in vivo* study and AMPK inhibition experiments were analyzed using the unpaired Student's *t*-test (Prism 5.0, GraphPad Software Inc.). P values less than 0.05 were considered significant.

3. Results

3.1. OPE induces adipocyte browning in WAT

To investigate whether OPE can change the characteristics of white adipocytes to those of brown-like adipocytes in WAT, WAT from HFD-OPE-fed mice were evaluated by comparing them to those of HFD-fed mice. At the end of the 8-week feeding period, the HFD-OPE-fed mice had similar body weights (HFD, 35.0 ± 1.1 g, $n = 9$; HF-OPE, 35.1 ± 2.2 g, $n = 6$), EWAT weights (HFD, 2.2 ± 0.2 g, $n = 9$; HFD-OPE, 2.4 ± 0.3 g, $n = 6$) and RWAT weights (HFD, 0.70 ± 0.04 , $n = 9$; HF-OPE, 0.74 ± 0.06 , $n = 6$) to those of the HFD-fed mice. Figure 1A-H shows the expression changes of an adipogenesis-related gene (peroxisome proliferator-activated receptor gamma, PPAR γ), lipogenesis-related genes (fatty acid synthase, FAS and acetyl-CoA carboxylase, ACC), BAT-specific genes (PRDM16, UCP1, PGC1 α , and CIDEA) and a fatty acid oxidation-associated gene (CPT1 α) in EWAT, RWAT, and SWAT of HFD-OPE fed mice. OPE did not affect PPAR γ expression in the WATs (Fig. A). FAS expression was unchanged in all three WAT types when HFD-OPE-fed mice were compared to HFD-fed mice, whereas ACC was downregulated by nearly 50% in the EWATs but not in the RWATs and SWATs (Fig. 1B and C). Consistent with the expression increase of PRDM16, which encodes a coregulator for brown adipocyte development, the UCP1, PGC1 α and CIDEA genes were upregulated in the RWATs of the HFD-OPE-fed mice (Fig. 1D-G). In the SWATs, only UCP1 and CIDEA were upregulated, while the PRDM16 and PGC1 α genes remained unchanged (Fig. 1D-G). There was no change in CPT1 α gene expression (Fig. 1H).

3.2. OPE induces white adipocytes to become brown like-adipocytes

OPE and OPEF did not exhibit cytotoxicities at or below 150 $\mu\text{g/mL}$, and OPWF was not cytotoxic at or below 400 $\mu\text{g/mL}$. Quercetin and isoquercetin did not exhibit cytotoxicities at or below 100 μM and 400 μM , respectively. Thus, the concentration ranges chosen for this 3T3-L1 cell study were 50 to 150 $\mu\text{g/mL}$ for OPE, OPEF and OPWF and 25 μM to 100 μM for quercetin and isoquercetin. Figure 2 shows the browning effect of OPE on adipocytes using 3T3-L1 cells. PPAR γ gene expression gradually increased as the treatment concentration increased to 100 $\mu\text{g/mL}$ OPE compared to adipocytes without the OPE treatment, which were used as the control. However, adipocytes treated with 150 $\mu\text{g/mL}$ OPE exhibited an equal expression level to that observed in adipocytes treated with 50 $\mu\text{g/mL}$ OPE (Fig. 2A). As shown in Figure 2B and C, the FAS and ACC genes were unaltered by the 50 and 100 $\mu\text{g/mL}$ OPE treatments, but both genes were decreased by 150 $\mu\text{g/mL}$ OPE. Adipocytes that were treated with 50 and 100 $\mu\text{g/mL}$ OPE showed increased PRDM16 expression levels, but this gene was suppressed by 150 $\mu\text{g/mL}$ OPE (Fig. 2D). However, UCP1 expression was induced by increasing the OPE concentration (Fig. 2E). Treatments with either 100 or 150 $\mu\text{g/mL}$ OPE increased PGC1 α expression (Fig. 2F). The CIDEA gene was significantly downregulated by OPE in a dose-dependent manner (Fig. 2G). Consistent with the increased expression of BAT-selective genes, the 100 or 150 $\mu\text{g/mL}$ OPE treatments increased CPT1 α expression (Fig. 2H). Adipocyte images that were obtained by Oil Red O staining to visualize lipid droplets are shown in Figure 2I. Adipocytes without OPE treatment showed a single large lipid droplet, which was characteristic of white adipocytes. By increasing the OPE concentration, the lipid droplet contracted and became multiple droplets, and the overall lipid accumulation in the adipocytes was reduced (Fig. 2I and J).

3.3. OPEF, but not OPWF, induces adipocyte browning

We investigated whether OPEF and OPWF, which were fractionated from OPE, exhibited the same browning effect in 3T3-L1 adipocytes. The expression levels of the PPAR γ , FAS and ACC genes were decreased by 150 μ g/mL OPEF compared with those of the control, but these genes were unchanged in the OPWF-treated adipocytes, as shown in Figure 3A-C. PRDM16 expression was increased in adipocytes treated with 50 and 100 μ g/mL OPEF (Fig. 3D). However, its expression was dramatically suppressed by 150 μ g/mL OPEF. Adipocytes that were treated with all three OPWF concentrations showed identical inductions in their expression levels of PRDM16 compared with that of the control (Fig. 3D). As shown in Figures 3E and 3F, the UCP1 and PGC1 α genes were upregulated in a dose-dependent manner by OPEF. These genes were also moderately induced by OPWF. CIDEA expression was reduced by OPEF, but there was no change in the OPWF-treated adipocytes (Fig. 3G). Consistent with the increased expression of BAT-specific genes, CPT1 α was also increased by OPEF but remained unchanged by OPWF (Fig. 3H).

3.4. Quercetin and isoquercetin are the major flavonoids in OPE, OPEF, and OPWF

To identify and quantify the major dietary compounds in OPE, OPEF, and OPWF, three extracts were analyzed by HPLC. The chromatograms of the quercetin and isoquercetin separations from OPE, OPEF, and OPWF by HPLC are shown in Figure 4A-C. According to the standard curve-based quantification of pure quercetin and isoquercetin, the quercetin and isoquercetin concentrations in OPE and OPEF were 6.8 ± 0.1 mg and 8.1 ± 0.1 mg/100 mg dried weight (dw) and 7.8 ± 0.1 mg and 9.1 ± 0.1 mg/100 mg dw, respectively. Further calculations showed that the OPE and OPEF used in our cell study contained mixtures of up to 12.15 μ g/mL isoquercetin (26.16 μ M) plus 10.2 μ g/mL (33 μ M) quercetin and 13.65 μ g/mL isoquercetin (29.4 μ M) plus 11.69 μ g/mL (38.7 μ M) quercetin, respectively. As

shown in Figure 4C, an isoquercetin peak was observed in OPWF with a lower limit of quantification. Quercetin was not detected in OPWF (Fig. 4C).

3.5. Quercetin, but not isoquercetin, changes the characteristics of adipocytes to those of BAT-like adipocytes

We investigated the effects of the major dietary compounds found in OPE and OPEF, quercetin and isoquercetin, on adipocyte browning. The 100 μ M quercetin treatment slightly increased PPAR γ gene expression but decreased FAS and ACC gene expression (Fig. 5A-C). PRDM16 was upregulated in adipocytes that were treated with either 50 or 100 μ M quercetin. However, adipocytes that were treated with 100 μ M quercetin showed the same PRDM16 expression level as the control (Fig. 5D). When treated with 100 μ M quercetin, expression of the UCP1 and PGC1 α genes was significantly increased (Fig. 5E and F). CIDEA was reduced by increasing the concentration of quercetin (Fig. 5D). Consistent with the increased expression of BAT-specific genes, CPT1 α was also increased by quercetin (Fig. 5H). None of the genes tested in this study were affected by isoquercetin (Fig. 5A-H).

3.6. Quercetin partially regulates adipocyte browning through the AMPK signaling pathway

To determine the mechanism by which quercetin converts the characteristics of white adipocytes to those of BAT-like adipocytes, the expression of BAT-specific genes was examined using 3T3-L1 adipocytes treated with or without 100 μ M quercetin in the absence or presence of an AMPK inhibitor (dorsomorphin). As shown in Figure 6A, dorsomorphin decreased PRDM16 gene expression to the level observed in adipocytes treated with quercetin in the absence of dorsomorphin. There was no additional reduction by quercetin (Fig. 6A). Dorsomorphin increased UCP1 gene expression (Fig. 6B), and the induction was increased further with quercetin. PGC1 α gene expression was increased by quercetin (Fig.

6C), but this effect was not observed in the AMPK-inhibited adipocytes (Fig. 6C). CPT1 α was upregulated in quercetin-treated adipocytes, but this effect was not observed with AMPK inhibition (Fig. 6D).

4. Discussion

This study used HFD-induced obese mice and 3T3-L1 cells to examine whether onion peel exhibits a browning effect, where white adipocytes are converted to brown-like adipocytes. Several studies have provided evidence that onion peel has the potential to alleviate obesity by inhibiting adipogenesis and increasing fatty acid oxidation. However, the onion peel-mediated browning effect that may prevent obesity has not been investigated. In this study, OPE supplementation for 8 weeks did not lower body weight or fat mass in HFD-induced obesity, but the HFD-OPE groups showed increased expression of several BAT-specific genes (PRDM16, UCP1, PGC1 α and CIDEA) in their RWATs and SWATs.

PRDM16, a transcriptional coregulator, is essential for brown adipocyte development in BAT and beige cells in WAT [8]. Together with the increased PRDM16 expression in this study, UCP1, a BAT-specific thermogenin, and PGC1 α , a transcriptional factor that regulates UCP1-induced thermogenesis, were induced in both the RWATs and SWATs of HFD-OPE-fed mice. PGC1 α positively regulates CIDEA expression by stimulating estrogen-related receptor (ERR) α and nuclear respiratory factor (NRF)-1 [19]. CIDEA, another BAT-specific gene, plays a major role in lipid droplet formation in BAT and beige cells [19, 20]. Consistent with the increased PGC1 α expression, CIDEA was dramatically induced in the RWATs of HFD-OPE-fed mice. Beige cells develop in response to stimuli (i.e., exposure to cold temperatures) in SWAT, which is an abundantly inguinal fat, and in RWAT [21] [22]. In this study, the HFD-OPE-fed mice showed an increase in all BAT-specific genes in their RWATs, but only the UCP1 and CIDEA genes were induced in their SWATs. None of the

BAT-specific genes were increased in the EWATs by OPE supplementation. Although OPE did not reduce WAT weights during 8-week feeding, long-term OPE consumption may prevent fat accumulation by changing the characteristics of adipocytes to those of brown-like adipocytes in WAT.

To investigate functional compounds for the browning effects of OPE, we added OPE, OPEF, OPWF, quercetin, and isoquercetin to 3T3-L1 cells during their post-differentiation phase. Consistent with the increased expression of BAT-specific genes in the RWATs of the HFD-OPE-fed mice, OPE induced PRDM16, UCP1 and PGC1 α , but not CIDEA expression, in mature white adipocytes. Recently, CIDEA has been reported to promote lipid droplet enlargement by transferring triacylglycerol between lipid droplets [23]. CIDEA-deficient mice, which are resistant to obesity development, showed increased non-shivering thermogenesis in BAT after being exposed to cold temperatures [24]. In this study, OPE-treated 3T3-L1 cells exhibited reduced expression of the CIDEA gene. This reduction is typically associated with a decline in fat accumulation and with small lipid droplets. BAT-specific genes were associated with the regulation of lipolysis and lipogenesis. Together with the increase in UCP1 expression, CPT1 α was upregulated in the OPE-treated 3T3-L1 cells. This result suggests that OPE may induce lipolysis for fatty acid oxidation, further activating UCP1 expression. In contrast to the *in vivo* study, OPE increased PPAR γ at lower concentrations but reduced PPAR γ , FAS, and ACC expression at 150 μ g/mL in the 3T3-L1 cells, which suggests decreased adipogenesis and lipogenesis. The inhibition of adipogenesis by OPE may have also resulted from ACC inactivation through AMPK activation by quercetin, a bioactive compound of the onion peel [16]. OPE-mediated inhibition of adipogenesis and lipogenesis in 3T3-L1 cells has been previously reported [15, 17].

OPE was fractionated into its hydrophobic and hydrophilic fractions (OPEF and OPWF, respectively). In this study, OPEF-treated 3T3-L1 cells showed similar browning

effects to those observed in OPE-treated adipocytes. OPWF did not affect the genes involved in regulating adipogenesis, lipogenesis, and fatty acid oxidation or the BAT-specific genes. This result indicates that OPEF contained functional compounds that could convert white adipocytes to brown-like adipocytes. A previous study showed that isoquercetin and quercetin are major compounds in the onion and onion peel [25]. Our data, obtained by HPLC, revealed the presence of these two compounds in OPE and OPEF. Our data suggested that OPE and OPEF, which we used in the cell study, comprised similar concentrations of isoquercetin and quercetin. However, only quercetin induced the gene expression pattern observed in the OPE- and OPEF-treated adipocytes. This finding indicates that quercetin is a functional compound in onion peel that converts the characteristics of white adipocytes to those of brown-like adipocytes.

Several reports have shown that white adipocyte browning is mediated by the AMPK signaling cascade [10, 14, 26, 27]. AMPK is a key regulator of energy balance that stimulates catabolic ATP-generating pathways (i.e., fatty acid oxidation), induces mitochondrial biogenesis and activates BAT [28, 29]. Wang et al. showed that resveratrol increased BAT-specific gene expression in the inguinal WATs of mice by activating the phosphorylation of AMPK α 1 [14]. The browning effect of curcumin in 3T3-L1 cells was also achieved by inducing AMPK [10]. Additionally, quercetin is a known activator of the AMPK cascade [16]. Therefore, we investigated whether the observed quercetin-induced browning effect was mediated by AMPK activation. When AMPK was inhibited, the basal mRNA levels of PRDM16, PGC1 α and CPT1 α were reduced, and this decline was not reversible with quercetin. However, the dorsomorphin-treated cells showed an increase in UCP1 gene expression compared with that of cells without dorsomorphin. Quercetin still increased UCP1 gene expression when AMPK was inhibited, indicating that the quercetin-induced browning

effect was, in part, AMPK-dependent. However, there may be alternative mechanisms by which quercetin promotes adipocyte browning to brown-like adipocytes.

Overall, OPE promoted the remodeling of white adipocytes to brown-like adipocytes by inducing BAT-like genes in the WATs of mice and in 3T3-L1 cells *in vitro*. The OPE-induced browning effect was mediated by quercetin, a functional compound in onion peel whose effect was partially dependent on the AMPK signaling cascade. Although further animal studies are necessary to investigate the long-term effects of exposure to OPE or quercetin and to uncover the mechanisms behind quercetin's regulation of the browning effect, our findings are the first to report the browning effects of onion peel and quercetin *in vivo* and *in vitro*. This study demonstrates the anti-obesity effect of onion peel through adipocyte browning in WAT. The onion peel, a by-product that contains a high concentration of quercetin, may be a potential candidate for development into food products that economically prevent obesity.

Acknowledgements

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Figure Legends

Figure 1. The OPE effect on gene expression in WATs. Expression changes were measured in the EWATs, RWATs and SWATs of mice that were fed HFD (n = 9) and HFD-OPE (n = 6) for 8 weeks. Expression of selected genes related to adipogenesis, (A) PPAR γ , and lipogenesis, (B) FAS and (C) ACC. Other genes included the BAT-specific genes—(D) PRDM16, (E) UCP1, (F) PGC1 α , and (G) CIDEA—and one fatty acid oxidation-associated gene, (H) CPT1 α . * $P < 0.05$, HFD vs. HFD-OPE.

Figure 2. The OPE effect on gene expression in 3T3-L1 adipocytes. Gene expression was determined in 3T3-L1 adipocytes that were treated with or without OPE (0, 50, 100 and 150 $\mu\text{g/mL}$) every other day during differentiation (days 5, 7, and 9). The selected genes related to adipogenesis, (A) PPAR γ , and lipogenesis, (B) FAS and (C) ACC. Other genes included the BAT-specific genes—(D) PRDM16, (E) UCP1, (F) PGC1 α , and (G) CIDEA—and one fatty acid oxidation-associated gene, (H) CPT1 α . (I) Lipid droplets were visualized after Oil Red O staining in fully differentiated 3T3-L1 adipocytes that were treated with OPE (0, 50, 100 and 150 $\mu\text{g/mL}$). (J) Lipid accumulation in fully differentiated 3T3-L1 adipocytes was quantified after extracting the Oil Red O stain. A different letter indicates a statistically significant difference ($P < 0.05$).

Figure 3. The effect of OPEF and OPWF on gene expression in 3T3-L1 adipocytes. Gene expression was determined in 3T3-L1 adipocytes that were treated with or without OPEF and OPWF (0, 50, 100 and 150 $\mu\text{g/mL}$) every other day during differentiation (days 5, 7, and 9). The selected genes related to adipogenesis, (A) PPAR γ , and lipogenesis, (B) FAS and (C) ACC. Other genes included the BAT-specific genes—(D) PRDM16, (E) UCP1, (F) PGC1 α ,

and (G) CIDEA—and one fatty acid oxidation-associated gene, (H) CPT1 α . A different letter indicates a statistically significant difference ($P < 0.05$).

Figure 4. Identification of isoquercetin and quercetin in OPE, OPEF and OPWF. Isoquercetin and quercetin were detected in OPE, OPEF and OPWF by HPLC.

Figure 5. The effect of OPEF and OPWF on gene expression in 3T3-L1 adipocytes. Gene expression was determined in 3T3-L1 adipocytes that were treated with or without quercetin and isoquercetin (0, 25, 50 and 100 μ M) every other day during differentiation (days 5, 7, and 9). The selected genes related to adipogenesis, (A) PPAR γ , and lipogenesis, (B) FAS and (C) ACC. Other genes included the BAT-specific genes—(D) PRDM16, (E) UCP1, (F) PGC1 α , and (G) CIDEA—and one fatty acid oxidation-associated gene, (H) CPT1 α . A different letter indicates a statistically significant difference ($P < 0.05$). QCT, quercetin; IsoQCT, isoquercetin

Figure 6. The effect of quercetin on the expression of BAT-specific genes under AMPK inhibition. Gene expression of (A) PRDM16, (B) UCP1, (C) PGC1 α , and (D) CPT1 α was determined in 3T3-L1 adipocytes that were treated with or without 100 μ M quercetin (on days 5, 7, and 9) in the absence or presence of an AMPK inhibitor (5 μ M dorsomorphin). * $P < 0.05$, without quercetin vs. with quercetin; ** $P < 0.01$, without quercetin vs. with quercetin.

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Gene	Forward primer	Reverse primer
ACC	TGCATTCTGACCTTCACGAC	ACATCCACTTCCACACACGA
CIDEA	ATCACAACTGGCCTGGTTACG	TACTACCCGGTGTCCATTTCT
CPT1 α	TTTGACTTTGAGAAATACCCTGATA	TGGATGAAATTCTCTCCCACAATAA
FAS	TGGGTTCTAGCCAGCAGAGT	ACCACCAGAGACCGTTATGC
PGC1 α	TGCCCAGATCTTCCTGAACT	TCTGTGAGAACCGCTAGCAA
PPAR γ	TTTGACTTTGAGAAATACCC	TGGATGAAATTCTCTCCAC
PRDM16	CAGCACGGTGAAGCCATTC	GCGTGCATCCGCTTGTG
RPL32	CACCAGTCAGACCGATAT	TTCTCCGCACCCTGTTG
UCP1	TCAGGATTGGCCTCTACGAC	TGCATTCTGACCTTCACGAC

Table 1. Quantitative real-time PCR primers

Abbreviations: ACC, acetyl-CoA carboxylase; CIDEA, cell death-inducing DFFA-like effector; CPT1 α , carnitine palmitoyltransferase 1 alpha; FAS, fatty acid synthase; PGC1 α , peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PPAR γ , peroxisome proliferator-activated receptor gamma; PRDM16, PR domain-containing 16; RPL32, ribosomal protein L 32; UCP1, uncoupling protein 1

Figure 1

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