Theaflavins Improve Insulin Sensitivity through Regulating Mitochondrial Biosynthesis in Palmitic Acid-Induced HepG2 Cells

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Abstract: Theaflavins, the characteristic and bioactive polyphenols in black tea, possess the potential improvement effects on insulin resistance-associated metabolic abnormalities including obesity and type 2 diabetes. However, the molecular mechanisms of theaflavins improving insulin sensitivity are still not clear. In this study, we investigated the protective effects and mechanisms of theaflavins on palmitic acid-induced insulin resistance in HepG2 cells. Theaflavins could significantly increase glucose uptake of insulin-resistant cells at noncytotoxic doses. This activity was mediated by upregulating the glucose transporter 4 protein expression, increasing the phosphorylation of IRS-1 at Ser307, and reduced the phosphor-Akt (Ser473) level. Moreover, theaflavins were found to enhance mitochondrial DNA copy number through down-regulate the PGC-1β mRNA level and up-regulate PRC mRNA expression in insulin-resistant HepG2 cells. These results indicated that theaflavins could improve free fatty acid-induced hepatic insulin resistance by promoting mitochondrial biogenesis, and were promising functional food and medicines for insulin resistance-related disorders.

Keywords: Theaflavins; Hepatocyte; Insulin resistance; Insulin signaling pathway; Mitochondrial biogenesis; Peroxisome proliferator-activated receptor coactivator-1 (PGC-1).

1. Introduction

Insulin resistance (IR) is a pathological condition in which cells fail to respond to the normal physiological dose of insulin, and plays important roles in the pathogenesis of metabolic syndrome such as obesity and type 2 diabetes mellitus (T2DM)[1]. Accumulating studies have demonstrated that consumption of high-energy diets lead to the glycometabolic disorder and impairment of insulin sensitivity, which increased the risk of the development of metabolic abnormalities[2]. The current clinical drugs for treatment of diabetes and insulin resistance, including sulfonylureas, metformin and thiazolidinediones, always have some side effects such as weight gain and hypoglycemia [3-5]. Search for new functional foods and medicines for IR and T2DM from natural resources with fewer adverse effects has become an urgent need.

Black tea, the most popular tea in the world, has been found to effectively improve high-energy diet induced metabolic syndromes such as obesity, hyperlipidemia and diabetes in animal models and adults [6,7]. Theaflavins, the characteristic polyphenols generating from enzymatic oxidation of appropriate pairs of catechins during the black tea production, were reported to contribute importantly to these health benefits of black tea. The major theaflavins are theaflavin (TF), theaflavin-3-gallate (TF-3-G), theaflavin-3′-gallate (TF-3′-G) and theaflavin-3, 3′-digallate (TFDG) [8]. A randomized pilot study showed that oral administration of theaflavins had a beneficial effect on body fat and muscle in healthy individuals[9]. Black tea polyphenols containing theaflavins were demonstrated to promote insulin-sensitive glucose transporter 4 (GLUT4) translocation through
both PI3K and AMPK-dependent pathways in L6 skeletal muscle cells[10]. Jin et al reported that administration of black tea extracts, TFs and TF1 all significantly lowered the serum insulin levels, improved the insulin sensitivity and suppressed fat accumulation in high-fat diet induced obese rats, and no obvious toxicity were observed[11]. Liver, skeletal muscle and adipose are major organs involved in the glucose metabolism and insulin resistance[12]. Among the three organs, liver is the most possible target of theaflavins based on their bioavailability and tissue concentrations[13]. However, the effect and molecular mechanisms of theaflavins on improving liver insulin sensitivity are still not clear.

Mitochondria are primarily responsible for providing cells with energy in the form of adenosine triphosphate (ATP), and play an important role in many cellular processes. The association between mitochondria dysfunction and insulin resistance have been observed in insulin resistant or diabetic patients and animal models[14]. Reduced mitochondrial capacity will contribute to the accumulation of reactive oxygen species or lipid intermediates, desensitizing insulin signaling and leading to insulin resistance[15]. The lower mitochondrial content is usually associated with reduced mitochondrial function. Improving mitochondrial function and biogenesis may lead to new therapeutic or preventive options for IR and T2DM [16]. The peroxisome proliferator-activated receptor coactivator-1 (PGC-1) family, composed of PGC-1α, PGC-1β and PGC-1-related coactivator (PRC), play a vital role in a regulatory network governing mitochondrial biogenesis and respiratory function. Several studies in humans and rodents have described the associations between the PGC-1 family and IR [3].

Redox-active compounds such as resveratrol, pyrroloquinoline, quinone and hydroxytyrosol have been reported to improve mitochondrial function and biogenesis through counteracting reactive oxygen species[17]. Theaflavins were found to be potent inhibitors of the membrane-bound complex I and ATP synthase, and could eliminate superoxide produced from the respiratory chain of Escherichia coli [8]. Considered together, the present study was designed to examine whether theaflavins was able to promote liver mitochondrial biogenesis and alleviate insulin resistance using an insulin-resistant HepG2 cell model. The possible molecular mechanisms were also elucidated.

2. Results
2.1. Chemical compositions of TFs

HPLC analysis showed that TFs used in this work contained 12.04% TF, 18.10% TF-3-G, 24.14% TF-3’-G and 38.49% TFDG. The total content of the four theaflavin monomers in TFs was 92.77% (Fig. 1).

![Figure 1. HPLC chromatogram of theaflavins (TFs). 1, Theaflavin (TF): R1=R2=H; 2, Theaflavin-3-gallate (TF-3-G): R1=H, R2=galloyl; 3, Theaflavin-3’-gallate (TF-3’-G): R1=galloyl, R2=H; 4, Theaflavins-3, 3’-digallate (TFDG): R1= R2=galloyl.](image-url)

2.2. Effect of TFs on HepG2 cell viability

The cytotoxicity of TFs on HepG2 cells was evaluated using the MTT assay after 24 h incubation. There was no significant difference (p>0.05) among the cell viabilities of the negative control and TFs-treated groups (10-40 μg/ml), indicating TFs had no cytotoxic effects on HepG2 cells
within the selected concentrations (Fig. 2). The TFs treatment used in the next experiments were between 0 - 10 μg/ml, in order to explore whether TFs could influence cell insulin sensitivity at lower and safer doses.

Figure 2. Effect of theaflavins (TFs) on HepG2 cell growth at 24 h. Cell viability was determined by MTT assay. Data represent means ± SD from five replicates. Significant differences among different treatments are indicated by different letters (p < 0.05).

2.3. Establishment of IR HepG2 cell model induced by PA

In order to determine the most optimal concentration of PA for inducing IR HepG2 cells, the effects of PA on cell viability and glucose uptake were tested. The MTT assay showed that PA (150-450 μM) could inhibit the proliferation of HepG2 cells in a dose-dependent manner after 24 h treatment (p<0.05), and the cell viability varied from 105.09 ± 6.75% to 21.12 ± 1.69% (Fig. 3A). Then the cell 2-NBDG uptake was determined at lower concentrations of PA (150-350 μM), and Fig. 3B showed that 2-NBDG uptake was significantly decreased from 62.23±4.89% to 27.65±5.81% in HepG2 cells (p<0.05). These results suggested that PA could stimulate IR in HepG2 cells at 150- 250 μM without obvious cytotoxicity, and 250 μM was chosen for establishing IR HepG2 cell model because of its higher efficiency.
Figure 3. Palmitic acid (PA) induces insulin resistance in HepG2 cells. (A) Effect of PA on HepG2 cell growth at 24 h. Cell viability was determined by MTT assay. (B) PA reduces 2-NBDG uptake of HepG2 cells. Data represent means ± SD from five replicates. Significant differences among different treatments are indicated by different letters (p < 0.05).

2.4. Effects of TFs on glucose uptake of IR HepG2 cells

In order to determine whether TFs could ameliorate IR of hepatocytes, glucose uptake assay was performed in IR HepG2 cells induced by PA. As shown in Fig. 4, PA (250 μM) significantly decreased the 2-NBDG uptake of HepG2 cells, while TFs (2.5-10 μg/ml) and metformin (5 μg/ml, positive control) obviously reversed the reduction of 2-NBDG uptake at 24 h treatment (p<0.05). This result indicated that TFs could improve the insulin sensibility of HepG2 cells treated by PA.

Figure 4. Effects of theaflavins (TFs) on 2-NBDG uptake of insulin-resistant HepG2 cells induced by palmitic acid (PA). Metformin is used as a positive control. Data represent means ± SD from five replicates. Significant differences between groups are indicated by different letters (p < 0.05).

2.5. Effects of TFs on insulin signaling pathway

To confirm the improvement of TFs on PA-induced insulin resistance, the expression of insulin signaling pathway-associated proteins in HepG2 cells were determined. As shown in Fig. 5 A-C, PA significantly reduced the phosphor-Akt (Ser473) protein level (p<0.05), increased the protein expression of phosphor-IRS-1 (Ser307), and had no significant effects on the protein expressions of...
GLUT4, Akt and IRS-1 (p>0.05). TFs could enhance the GLUT4 protein level in a dose-dependent manner, and remarkably reverse the phosphorylation of Akt and IRS-1 induced by PA (p<0.05). TFs’ activities on phospho-IRS-1 (Ser307) and GLUT4 could be comparable to that of metformin. These results indicated that TFs might improve the glucose uptake and insulin sensitivity of PA-induced HepG2 cells through IRS-1/Akt/GLUT4 pathway.

![GLUT4 protein expression](image1)

![Akt and p-Akt(S473) protein expression](image2)

![IRS-1 and p-IRS-1(S307) protein expression](image3)
2.6. **TFs improve mitochondrial biogenesis in PA-induced HepG2 cells**

The mtDNA copy number was determined to evaluate the mitochondrial mass. As shown in Fig. 6, the relative mtDNA content in PA-treated HepG2 cells was reduced by around 20% compared to that of normal cells. TFs significantly increased the mtDNA copy number in a dose-dependent manner, and their activity was stronger than that of metformin (p<0.05). These data suggested that the TFs could improve mitochondrial biogenesis in IR HepG2 cells.

**Figure 6.** Effect of theaflavins (TFs) on the mtDNA copy number of insulin-resistant HepG2 cells at 24 h. Data represent means ± SD from five replicates. Significant differences among different treatments are indicated by different letters (p < 0.05).

2.7. **Effects of TFs on mRNA expression of PGC-1 family in PA induced HepG2 cells**

Two PGC-1 family members associated with mitochondrial biogenesis under energy stimulus, PGC-1β and PRC genes were determined by RT-PCR. In the PA-induced HepG2 cells, the mRNA
expression was up-regulated, and the PRC mRNA level was decreased in comparison to the control cells. TFs obviously reversed the mRNA expressions of PGC-1β and PRC (p<0.05), and their activities were stronger or similar compared with that of metformin at the same dose (Fig. 7).

**Figure 7.** Effects of theaflavins (TFs) on the PGC-1β (A) and PRC (B) mRNA expressions of insulin-resistant HepG2 cells at 24 h. The relative mRNA level was determined by quantitative real-time PCR, and calculated by the mean value with the comparative Ct method (ΔΔCt). Data represent means ± SD from five replicates. Significant differences among different treatments are indicated by different letters (p < 0.05).

3. Discussion

Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible. Future research directions may also be highlighted.

High calorie dietary habits coupled with low levels of physical activity are prominent cause of metabolic syndrome in modern world. IR is a key risk factor in the pathogenesis of various chronic diseases including T2DM, cardiovascular disease, chronic kidney disease and cognitive disorders [18-20]. Natural products are thought to be the important sources for anti-T2DM drug discovery[21]. Many natural compounds such as flavanols[22] and anthocyanins [23] have been reported to ameliorate insulin resistance through different signaling pathways. Theaflavins, as one
of the major flavor and bioactive ingredient of black tea, has been demonstrated to protect against cancer, inflammation, hyperlipidemia, hypertension and obesity etc. in vivo and in vitro [7,24-26]. Although theaflavins showed potential preventive and therapeutic effects on IR-associated metabolic abnormalities as candidate nutritional supplements and medicines, the researches on the direct correlation between theaflavins and IR are still limited.

Notwithstanding the bioavailability of tea polyphenols in vivo was thought to be low, theaflavins were accumulated in the small and large intestine, liver and prostate of mice primarily in free forms [13]. The liver is crucial for the maintenance of normal glucose homeostasis [27]. Impairment in hepatic insulin signaling resulted in glucose intolerance, lipid synthesis and chronic IR [28]. Although PA-induced impaired insulin signaling cascade in human or mouse hepatocytes, is a well-documented model of experimental IR, the PA dosage varied in different studies [29,30]. In the present work, an IR HepG2 cell model was established with 250 μM of PA (Fig. 3). The fluorescent glucose analog, 2-NBDG, was used as a marker to detect glucose transport. Tfs significantly enhanced the 2-NBDG uptake of PA-induced HepG2 cells, indicating that these compounds could improve the insulin sensitivity of IR hepatocytes (Fig. 4).

The GLUT4, a member of glucose transporters family, is identified as the most important type of glucose transporters in mediating insulin-dependent glucose uptake and maintaining glucose homeostasis. Overexpression of GLUT4 is a good strategy for treatment of IR[31]. Several natural compounds and medicines such as leanolic acid and liraglutide have been reported to attenuate IR at least partly through increasing GLUT4 expression in liver [32,33]. Previous study showed that black tea polyphenols could promotes GLUT4 translocation in skeletal muscle cells[10]. Our data indicates that Tfs increased the expression of GLUT4 in PA-induced HepG2 cells (Fig. 5 A), which explained the enhanced glucose uptake of IR hepatocytes treated with Tfs.

The IRS-1/PI3K/Akt signaling pathway plays an important role in the regulation of insulin signaling transduction and glucose metabolism in liver[34]. Akt as the downstream effector of phosphatidylinositol 3-kinase (PI3K), could mediate effects of insulin on glucose uptake, glycolysis, gluconeogenesis and glycogen synthesis in hepatocytes[35-37]. The PI3K/Akt signaling pathway was shown to increase GLUT4 expression and promote translocation of GLUT4 vesicles to the plasma membrane[38]. Insulin receptor substrate-1 (IRS-1) is essential for recruiting and activating downstream PI3K/Akt pathway[39]. The serine phosphorylation of IRS-1 (particularly on Ser636/639 and Ser307) could inhibit tyrosine phosphorylation of IRS-1 and then block the downstream effector pathways and impair insulin signaling[40]. In this work, PA reduced the phosphorylated Akt (Ser473) protein level and increased the phosphorylation of IRS-1(Ser307) in HepG2 cells, which were in accordance with the previous study [41]. Tfs significantly reversed phosphorylation of Akt and IRS-1 induced by PA (Fig. 5B and 5C), indicating that Tfs could modulate insulin signaling transduction and increase glucose uptake via the IRS-1/Akt/GLUT4 pathway in liver cells.

Defects in mitochondrial biogenesis leads to excess reactive oxygen species and the subsequent decrease in energy expenditure, which are the main disruptors of insulin signaling in obesity. Accumulation of FFAs in the liver may be connected with mitochondrial dysfunction including mitochondrial DNA (mtDNA) depletion, decreased activity of respiratory chain complexes and impaired mitochondrial β-oxidation[42]. Some natural products such as resveratrol and the extract of Parkinsonia aculeata have been proved to improve high-fat diet-induced insulin resistance through stimulating mitochondrial biogenesis[43,44]. Our data showed that theaflavins increased the relative mtDNA copy numbers of IR HepG2 cells (Fig. 6), indicating that they could improve mitochondrial biogenesis of high-fat induced hepatocytes.

The PGC-1 family modulate mitochondrial biogenesis and energy metabolism in a specific and subtle manner that depends on the tissue and physiological context[45]. In liver, PGC-1α regulates gluconeogenesis in response to fasting, while PGC-1β governs lipid metabolism in response to specific nutritional stimuli such as fructose and fatty acids. PGC-1β is a transcriptional coactivator for SREBP-1, the master regulator of hepatic lipogenesis. High-fat feeding stimulates the expression of both PGC-1α and SREBP1c and 1a in liver[46]. Nagai et al. proved that knockdown of PGC-1α in liver protected rats from fructose induced hepatic insulin resistance, which mostly be attributed to
reduction in hepatic lipogenesis resulting in reduced hepatic DAG content and decreased PKC3 activation[47]. Additionally, PGC-1β knockdown decreased mitochondrial copy numbers, and reduced genes involved with mitochondrial fatty acid oxidation, biogenesis and function. PGC-1β inhibition may be a therapeutic target for treatment of NAFLD, hypertriglyceridemia, and insulin resistance associated with increased denovo lipogenesis[48][48][52]. PRC, the least characterized member of PGC-1 family, appears to be restricted to the regulation of mitochondrial biogenesis in proliferating cells. Knockdown of PRC in vitro resulted in the generation of abnormal mitochondria that exhibited disorganized cristae, severe membrane abnormalities, reduced ATP production, and down-expressed respiratory protein subunits from complexes I, II, III, IV and ATPase [45].

Previous study showed that oral administration of a purified theaflavin mixture (10 mg/kg BW) increased energy expenditure via induction of uncoupling proteins (UCP-1 and UCP-3) and PGC-1α in fasting mice[49]. However, it is not clear whether PGC-1αs are involved in TFs-regulated mitochondrial function with fat stimuli. Considering the importance of PGC-1β and PRC for regulating lipid metabolism and mitochondrial biogenesis, their RNA expressions were determined in this work. Compared with IR HepG2 cells induced by PA, TFs could significantly decreased the PGC-1β RNA level, and enhanced the PRC RNA expression (Fig.7). These results were similar with the previous report that H2S regulated liver mitochondrial biogenesis associated with downregulating the mRNA and protein level of PGC-1β and upregulating the mRNA and protein levels of PRC in mouse hepatocytes[50]. Based on our results and the literature reports, TFs might promote mitochondrial biogenesis via modulation of PGC-1β and PRC in insulin resistant hepatocytes induced by free fatty acids.

4. Materials and Methods

4.1. Preparation and analysis of theaflavins

A highly purified theaflavins mixture (TFs) were prepared according to the method developed in our lab [51]. The sample was analyzed by Shimadzu LC-2010A HPLC system equipped with a Shimadzu SPD10A UV detector (Shimadzu, Kyoto, Japan). Chromatographic separation of TFs was carried out on an Intertil ODS-SP C18 reversed-phase column (5 μm, 250x4.6 nm, Shimadzu, Kyoto, Japan). The mobile phase A was acetic acid/acetonitrile/water (0.5: 3: 96.5, v/v/v), and B was acetic acid/acetonitrile/water (0.5: 30: 69.5, v/v/v). Gradient elution was performed with 40–85% B from 0 to 20 min and 85% B until 70 min at a flow rate of 1 mL/min. The column was kept at 25 °C throughout the analysis and the wavelength was set at 280 nm.

4.2. Cell culture and regent

The human liver cancer cell line HepG2 was purchased from the cell bank of Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Genom, Hangzhou, China) containing 10% fetal bovine serum (Hyclone, NSW, Australia). 100 units/ml penicillin (Biological Industries, CT, USA) and 0.1 mg/ml streptomycin (Biological Industries, CT, USA). Cells were maintained at 37 °C with humidified air and 5% CO2. Palmitic acid (PA) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The primary antibodies against Akt, phosphor-Akt (Ser473), IRS-1, phosphor-IRS-1 (Ser307), GLUT4 and GAPDH were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

4.3. Cell viability

Cell viability was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphe-nyltetrazolium bromide (MTT) assay. HepG2 cells were seeded into 96-well plates at 1.5x104 per well, cultured for 48 h, and then treated with different concentrations of PA (0 - 450 μM) or TFs (0 - 40 μg/mL) for 24h. Subsequently, 100 μl MTT (0.5 mg/mL) was added to each well and incubated for 4 h at 37 °C in the dark. After removing the supernatant, 150 μL DMSO was added to dissolve the formazan crystals. The absorbance was measured at 492 nm with a microplate reader (Bio-Technie, MN, USA).
4.4. Induction of insulin-resistant HepG2 cells

Insulin-resistant HepG2 cells were induced by PA. Briefly, a 100 mM PA stock solution was prepared in 0.1 M NaOH at 70 °C, and then diluted in 10% (w/v) BSA solution to produce various concentrations of PA[52]. HepG2 cells were treated with PA (0, 150, 250, 350 μM) for 24 h in black 96-well plates, and cell glucose uptake was measured as aforementioned to determine the optimum PA concentration used for building the insulin-resistant HepG2 cell model.

4.5. Glucose uptake assay

Glucose uptake in HepG2 cells was measured by using 2-(N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG, Sigma-Aldrich, USA) according to the previous report[53] with a few modifications. In brief, cells cultured on black 96-well plates were treated with PA in the presence of TFs (0 - 10 μg/ml) or Metformin (5 μg/ml, positive control) for 24 h. Then cells were kept in glucose-free DMEM for 4h, and stimulated by insulin (500 nM) for another 10 min. Following incubation with 50 nM 2-NBDG in glucose-free DMEM for 10 min, the cells were quickly washed twice with ice-cold PBS buffer to terminate reaction. The fluorescence was monitored at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

4.6. Total RNA preparation and Real-time PCR analysis

Total RNA was extracted from HepG2 cells using a Eastep Super Total RNA Extraction Kit (Promega, USA). RNA was quantified by a K5500 Micro-Spectrophotometer (Kaiao, Beijing, China), and 500 ng RNA was reverse transcribed into cDNA using HiScript Reverse Transciptase Kit (Vazyme biotech, USA). Real-time PCR was performed on a 7500 Real-Time PCR System according to the procedure of ChamQTM SYBR qPCR Master Mix Kit (Vazyme biotech, USA).

GAPDH was used as the reference, and relative mRNA expression was calculated by the mean value with the comparative Ct method (ΔΔCt). The primer pairs for PGC-1β, PRC and GAPDH were chosen from the Primer Bank website (http://www.rtprimerdb.org/). The sequences of the primers for each gene are shown in Table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5’-3’)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGC-1β</td>
<td>Forward: TGA CTC CGA GCT CTT CCA G</td>
<td>54.7</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGA AGC TGA GGT GCA TGA TA</td>
<td>54.8</td>
</tr>
<tr>
<td>PRC</td>
<td>Forward: AGT GGT TGG GGA AGT CGA AG</td>
<td>54.8</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCT GCC GAG AGA GAC TGA C</td>
<td>56.9</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: GAA GGT GAA GGT CCG AGT C</td>
<td>54.8</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAA GAT GGT GAT GGG ATT TC</td>
<td>55.0</td>
</tr>
</tbody>
</table>

4.7. Determination of mitochondrial DNA copy number

The relative mitochondrial DNA (mtDNA) copy number was indicated by the ratio of mtDNA to nuclear DNA (nDNA) as previously described[54] NADH dehydrogenase subunit 1 (ND1) gene was used to represent mtDNA, and the nuclear-encoded 18S rRNA gene was used to represent nDNA. Total DNA was extracted from HepG2 cells using a DNA Extraction Kit (BioVision, Shanghai, China). Relative amounts of mtDNA and nDNA were determined by real-time quantitative PCR. The primer sequences were: mtDNA fwd, 5’-ATGGCCAACCTCCTACTCCT-3’; mtDNA rev, 5’-GGCGTTAGTGAGAAGGTAGT-3’; nDNA fwd, 5’-ACGGACCAAGCGAAAGCA-3’; nDNA rev, 5’-GACATCTAAGGCATCAGCAGAC-3’.
4.8. Western blot

Cells were harvested with protein extraction reagent (Tiangen, Beijing, China), and total protein levels were determined by the BCA protein kit (Tiangen, Beijing, China). Cell lysates were separated by SDS-PAGE, and blotted onto polyvinylidene difluoride (PVDF) filters membrane with a Mini-Protean 3 System (Bio-Rad). The membrane was blocked with 5% skim milk for 1 h, and then incubated with specific primary polyclonal/monoclonal antibodies overnight at 4 °C. After incubation with horseradish peroxidase conjugated secondary antibody for 1 h, immunoreactive proteins were visualized with the ECL Plus Western Blotting Detection Reagents (Fude-bio, Hangzhou, China) and exposed using a Mini-Protein System (Bio-Rad, GA, USA). Protein bands were quantitated with NIH ImageJ software and normalized by GAPDH bands for analysis.

4.9. Statistical analysis

Data were presented as the mean ± standard deviation (SD). Multiple comparisons were performed by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls (SNK) test., and p<0.05 was considered statistically significant. Statistical analysis was performed using Statistical Analysis System (SAS) for windows V8.

5. Conclusions

Taken together, theaflavins at noncytotoxic doses could protect HepG2 cells against PA-induced insulin resistance by increasing glucose uptake and modulating the IRS-1/Akt/GLUT4 pathway. These effects were mediated through improving mitochondrial biogenesis and regulating PGC-1 family member PGC-1β and PRC. These findings extend the understanding of the physiological function played by theaflavins in obesity, insulin resistance and diabetes. Theaflavins may exert a therapeutic effect on hepatic insulin resistance with few side effects, and could be promising functional food and medicines for IR-related disorders in the future.

Author Contributions: T.-T.T. and N.R. designed the experiments and wrote the paper; and B.L. conceived and organized the study; J.-F.W. and N.G. performed the experiments for cell culture and glucose uptake assay of theaflavins; H.K. and E.K. contributed the isolation of theaflavins from the leaves of black tea; Y.-Y.T., W.-Y.W. and P.-M.H. revised the manuscript; and all authors approved the final version manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.
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Sample Availability: Not available.