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Intracellular Mediators of Procyanidin-Induced Lipolysis in 3T3-L1 Adipocytes

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We have previously reported that grape seed procyanidins stimulate long-term lipolysis on 3T3-L1 fully differentiated adipocytes. To unravel the molecular mechanism by which procyanidins exert this effect, we checked the involvement of two main cellular targets in adipose cells: protein kinase A (PKA) and peroxisome proliferator-activated receptor-γ (PPAR-γ). Procyanidin treatment increased intracellular cAMP levels in 3T3-L1 adipocytes, and their lipolytic effect was inhibited by simultaneous treatment with H89, a PKA specific inhibitor. BRL49653, a very highly specific ligand of PPAR-γ, totally abolished the lipolytic effect of procyanidins. Simultaneous to this long-term lipolytic effect, the mRNA levels of some differentiation adipocyte markers decreased, although there were no changes in the triglyceride content of the cells. BRL49653 did not antagonize the decrements of differentiation markers. These results support a mediation of PPAR-γ and PKA on the lipolytic effects of procyanidins on 3T3-L1 adipocytes.

KEYWORDS: Procyanidin; lipolysis; protein kinase A; PPAR-γ2; 3T3-L1

INTRODUCTION

Procyanidins, a group of flavonoids, are oligomeric forms of catechins that are abundant in red wine, grapes, chocolate, and apples (*1*). Our group has previously shown that long-term treatment with grape seed procyanidins increases lipolytic rate in 3T3-L1 adipocytes (*2*). We also know that procyanidins decrease mRNA levels of hormone-sensitive lipase (HSL) and the activity of glycerol-3-phosphate dehydrogenase (G3PDH) (*2*), but we do not know the underlying molecular mechanisms to explain these effects. There is scarce information about the mechanism of action of procyanidins. Only Rosenkranz has shown that wine procyanidins inhibit the activation of platelet-derived growth factor β (β-PDGF) receptor on vascular smooth muscle cells (*3*). There is more information about the cellular targets of some monomeric flavonoids, which also exert a lipolytic effect. Quercetin and fisetin act synergistically with epinephrine on β-adrenergic receptor (*4*), while genistein inhibits tyrosine kinase activity (*5*) and inhibits CCAAT/enhancer binding protein-β (C/EBP) activity (*6*). To determine the mechanisms of action of procyanidins that explain their lipolytic effect on the adipose cell, we must bear in mind that this effect only works with a long-term treatment. Hormonal short-term modulation of lipolysis is mainly driven by cAMP-dependent protein kinase A (PKA), which controls both HSL lipolytic activity (*7*, *8*) and non-HSL lipolytic activity (*9*). Long-term lipolysis is modulated by several factors such as cAMP, phorbol esters, dexamethasone, and tumor necrosis factor-α ((TNFα)) (*7*, *10*). Of all of these, TNF-α is the one whose lipolytic effect very much resembles that of the procyanidins and is counteracted by peroxisome proliferator-activated receptor-γ (PPARγ) ligands such as thiazolidinediones (TZD) (*11*), both in the presence of HSL and in the absence of HSL (*9*).

The aim of this study was to find cellular targets of procyanidins that explain their long-term lipolytic effect on adipocytes. To do this, we evaluated the interaction of procyanidins with PKA-mediated signaling pathways and the procyanidin modifications of PPAR-γ mediated pathways.

MATERIALS AND METHODS

Chemicals. Grape seed procyanidins extract (PE) was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, this procyanidin extract had a mean molecular weight of 1399 and contained essentially monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%), and oligomeric (5−13 units) (31.7%) procyanidins. Thiazolidinedione BRL49653 was kindly provided by GlaxoSmithKline (UK) and Galderma R&D (France). (±)Epinephrine and DL-propanolol were purchased from Sigma Chemical Co. (St. Louis, MO). Cell culture reagents were obtained from BioWhitthaker (Verviers, Belgium). H89 was purchased from Calbiochem (Merck KGaA, Darmstadt, Germany).

Cell Culture and Differentiation. 3T3-L1 preadipocytes were cultured and induced to differentiate as previously described (*2*). Briefly, confluent preadipocytes were treated with 0.25 μmol/L dexamethasone, 0.5 mmol/L 3-isobutyl-methylxanthine, and 5 μg/mL insulin for 2 days in 10% fetal bovine serum containing DMEM. Cells were then switched to 10% FBS/DMEM media containing only insulin for 2 more days, and then switched to 10% FBS/DMEM media without insulin. Ten days after differentiation was induced, cells were treated with the grape

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seed procyanidins and various ligands (BRL49653, H89, epinephrine and propanolol), as shown in detail in the figures. We determined the lipid content by staining the cells with Oil Red O.

Measurement of Lipolytic Activity. Fully differentiated adipocytes were treated for 15 h with procyanidins, with or without H89, epinephrine, propanolol, or BRL49653, depending on the experimental design (see figures). Lipolysis was monitored as the amount of glycerol released into the media or as the amount of nonesterified fatty acid (NEFA) released into the media. Glycerol was measured by the Garland and Randle method (*12*). NEFA was measured by the NEFA-C kit (WAKO Chemicals, Germany). Glycerol and NEFA values were corrected by their protein content, which was measured by the Bradford method (*13*) using the Bio-Rad protein reagent.

Measurement of Glycerol-3-phosphate Dehydrogenase Activity. After treatment, differentiated 3T3-L1 adipocytes were rinsed twice with PBS, scraped into 750 μL of 50 mmol/L Tris-HCl, 1 mmol/L EDTA, 1 mmol/L β-mercaptoethanol, and sonicated. The resulting extract was used to measure glycerol-3-phosphate dehydrogenase activity (G3PDH), in accordance with the Wise and Green method (*14*).

Cyclic AMP Determination. Differentiated 3T3-L1 adipocytes were exposed for 8 min to procyanidin extract or epinephrine. After the treatment, the cells were scraped in PBS. Part of the cell suspension was deproteinized with 3% perchloric acid and neutralized with 1 mmol/L NaOH. We used the supernatant of this extract to measure cyclic AMP content in the cells with a cyclic AMP assay kit (Amersham). We used the rest of the cell suspension to quantify protein content by the Bradford method (*13*).

Quantitative RT-PCR. PPAR-γ2 and HSL mRNA levels were measured by real-time RT-PCR analyses in a fluorescent thermal cycler (GeneAmp 5700 Sequence Detection System, Applied Biosystems). The level of mRNA for each gene was normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA detected in each sample. 3T3-L1 adipocytes were grown in 6-well multiplates, and total RNA was isolated using the High Pure RNA Isolation Kit (Roche). 1 μg of RNA was reverse transcribed by the SuperScript II Rnase H_ Reverse Transcriptase according to the manufacturer's instructions (LifeTechnologies). 2/100 of each RT reaction was amplified according to the protocols provided by the manufacturer (Applied Biosystems, Warrington, UK). The following primers were used: GAPDH CATGGCCTTCCGTGTTCCT (forward), and CCTGCTTCACCACCTTCTTGA (reverse); PPAR-γ2 CTGTTGACCCAGAGCATGGT (forward), and AGAGGTCCACAGAGCTGATTCC (reverse); HSL GGAGCACTACAAACGCAACGA (forward), and AATCGGCCACCGGTAAAGAG (reverse).

Statistics. Results are expressed as mean ± SEM. Effects were assessed using One-way or T-test. We used Tukey's Test of honestly significant differences to make pairwise comparisons. All calculations were performed using SPSS software.

RESULTS

PKA Participates in the Lipolytic Action of Procyanidins. PKA participates in the control of both the HSL-mediated and the non-HSL-mediated lipolytic activity of adipocyte (*9*,*15*). As **Figure 1** shows, the lipolytic effect of procyanidins requires PKA involvement, because treatment of cells with the PKA inhibitor H-89 reduced procyanidin-induced lipolysis in a dose-dependent manner. In control cells, 20 μmol/L H-89 did not significantly modify the lipolysis rate (1.25 ± 0.04 vs 1.01 ± 0.01). Procyanidins also increased cAMP levels of the cells, by a similar amount as epinephrine (**Figure 2**). Because of the oligomeric structure of procyanidins, the most suitable way to activate this intracellular-signaling pathway should be procyanidin interaction with β-adrenergic receptors. To check this hypothesis, we applied a simultaneous treatment with procyanidins and a β-adrenergic agonist (epinephrine) or a β-adrenergic antagonist (propanolol). **Figure 3** shows that procyanidin-induced lipolysis remains even at high epinephrine concentrations. And propanolol, both at 10 and at 100 μM, was not able to antagonize the lipolytic effect of procyanidins (PE 140 mg/L, 2.495 ± 0.212; PE + propanolol 10 μM, 1.945 ± 0.033; PE + propanolol 100 μM, 2.536 ± 0.553).

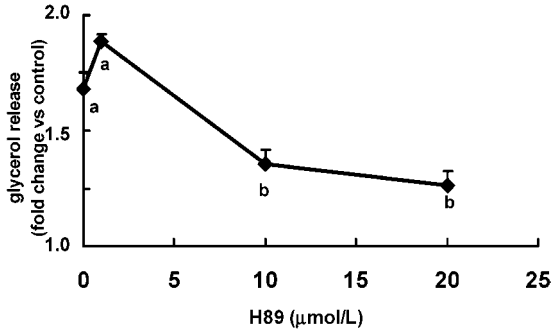

Figure 1. Effect of H89 on procyanidin-induced lipolysis. Fully differentiated 3T3-L1 adipocytes were treated with 140 mg/L grape seed procyanidin extract (PE) in the presence of various concentrations of H89 for 15 h. H89 treatment was done 10 min before PE addition. Data are expressed as glycerol release versus PE-induced lipolysis. Each treatment was previously corrected by its own vehicle control. Values represents mean ± SEM. The letters (a, b) indicate statistically significant differences between H89 concentrations.

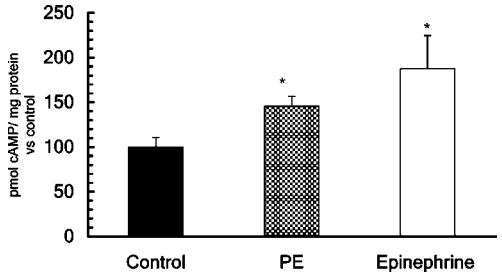

Figure 2. Effect of procyanidin and epinephrine on cyclic AMP levels. Differentiated 3T3-L1 adipocytes were exposed for 8 min to 210 mg/L grape seed procyanidin extract (PE) or 1 μmol/L epinephrine. cAMP levels (pmol/mg protein) are normalized to the control levels (100%). Each value represents mean ± SEM. *p < 0.05 as compared to control.

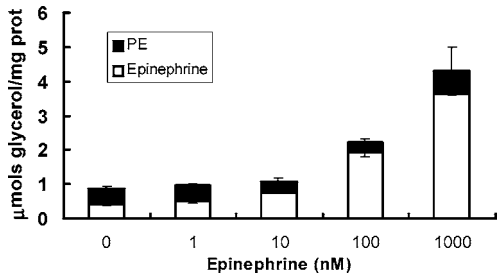

Figure 3. Effect of procyanidins on epinephrine-induced lipolysis. 3T3-L1 adipocytes were incubated for 15 h with 0–1000 nmol/L epinephrine in the absence or presence of 140 mg/L grape seed procyanidin extract (PE). Full column shows simultaneous treatment. ■ represents PE effect, and □ represents epinephrine effect. Values represent mean ± SEM.

PPARγ Participates in the Lipolytic Action of Procyanidins. Of all of the molecules so far described as having lipolytic action, the one that has effects most similar to those of the procyanidins is TNF-α. Thiazolidinedione BRL49653, a high affinity PPARγ agonist (*16*), blocks the lipolytic action of TNF-α (*17*). **Figure 4** shows that BRL49653 cancels out the lipolytic effect of procyanidins. This was a clear dose−response effect, with an IC50 ≈ 70 nmol/L. Total inhibition was achieved around 1 μmol/L BRL49653. However, this clear antagonism between BRL49653 and procyanidins was only observed in the lipolytic effect of procyanidins. Simultaneous treatment with

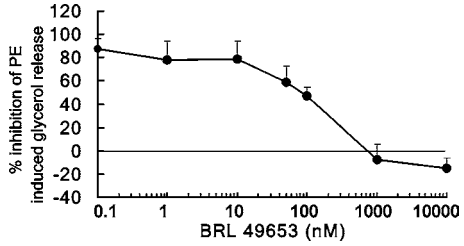

Figure 4. Effect of BRL49653 on procyanidin-induced lipolysis. Fully differentiated 3T3-L1 adipocytes were treated with 140 mg/L grape seed procyanidin extract (PE) in the presence of different concentrations of BRL49653 for 15 h. Data are expressed as % of PE-induced lipolysis. Values represents mean ± SEM.

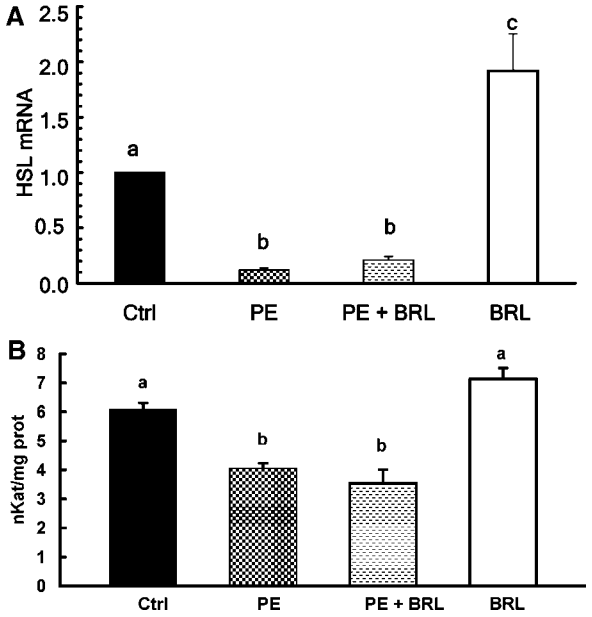

Figure 5. Effect of simultaneous treatment with BRL49653 and procyanidin on HSL mRNA levels (**A**) and G3PDH activity (**B**). Fully differentiated 3T3-L1 adipocytes were treated for 15 h with 140 mg/L grape seed procyanidin extract (PE), 0.1 μmol/L BRL49653 (BRL), and a combination of the two. (**A**) After treatment, total RNA was extracted and gene expression was quantified by real-time RT-PCR. HSL gene expression, normalized by GAPDH mRNA levels, is expressed relative to control cells. (**B**) After treatment, cells were scraped and sonicated. Glycerol-3-phosphate dehydrogenase activity (nKat/mg protein) was assayed in this homogenate. Values represent mean ± SEM. a, b, c indicate groups significantly different with $p < 0.05$.

BRL49653 and procyanidins did not antagonize the effects of procyanidins on adipocyte markers either in the previously published effects (*2*), that is, the decrease in HSL mRNA (**Figure 5A**) or the decrease in G3PDH activity (**Figure 5B**), or in the decrease in PPARγ mRNA levels shown in **Figure 6**.

Although we found no differences in lipid depots due to 24-h procyanidin treatment (**Figure 7**), there was a reduction of some adipocyte markers (mRNA HSL and PPARγ; G3PDH activity). To check whether cells dedifferentiate due to procyanidin treatment, we first exposed cells to 140 mg/L procyanidins for 24 h, and then changed the cells to a new cell culture medium without procyanidins for 3 days. The lipolytic rate, measured as glycerol or as nonesterified fatty acid (NEFA) released to the medium, decreased very strongly after 24 h of procyanidin treatment (**Figure 8A**). Three days after treatment, HSL and PPARγ mRNA levels tended to recover (**Figure 8B**).

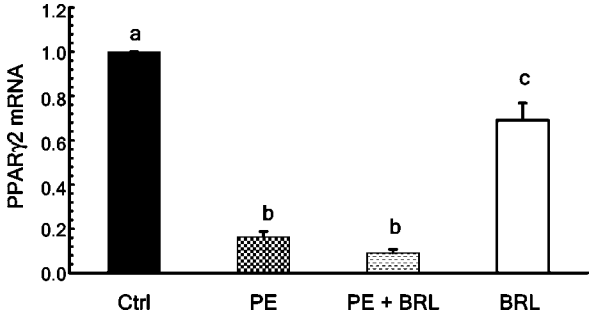

Figure 6. Procyanidin effects on PPARγ2 mRNA levels. 3T3-L1 adipocytes were incubated for 15 h with 140 mg/L grape seed procyanidin extract (PE), 0.1 μmol/L BRL49653 (BRL), and a combination of the two. After treatment, total RNA was extracted and gene expression was quantified by real-time RT-PCR. PPARγ2 gene expression, normalized by GAPDH mRNA levels, is expressed relative to control cells. Values represents mean ± SEM. a, b, c indicate groups significantly different with $p < 0.05$.

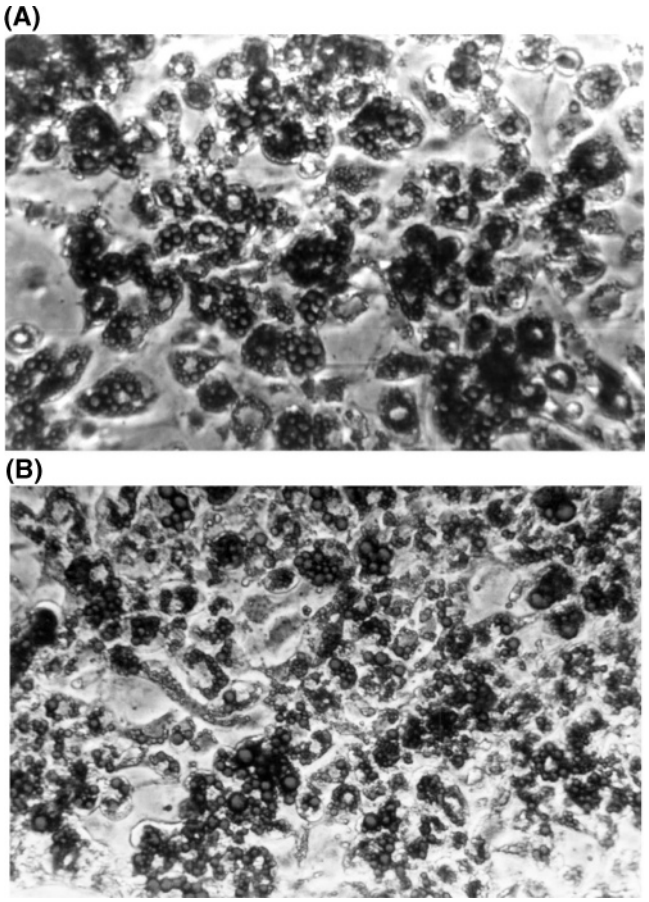

Figure 7. Oil Red O staining of differentiated cells. Fully differentiated (day 10) 3T3-L1 cells were treated for 15 h with (**B**) and without (**A**) 140 mg/L grape seed procyanidin extract (PE) and subsequently stained for lipid accumulation with Oil Red O.

DISCUSSION

We previously described the lipolytic and antilipogenic effects of grape seed-derived procyanidins on 3T3-L1 adipocytes (*2*). We now describe two intracellular mediators (PKA and PPAR-γ) that help to explain the long-term lipolytic effect of procyanidins. Two pieces of evidence support PKA participation: the inhibition of the lipolytic effect by H-89 and the increase in cAMP induced by procyanidins. PPAR-γ involvement was deduced from BRL49653 experiments. BRL49653, at concentrations very close to those at which it binds to PPARγ

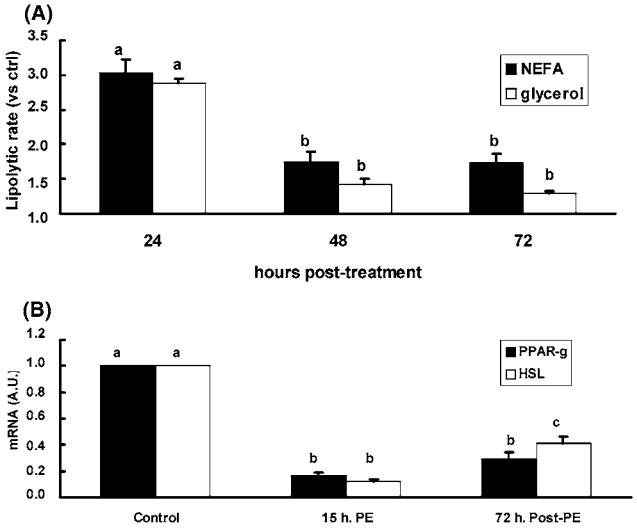

Figure 8. Lypolitic rate and mRNA levels post-procyanidin treatment. 3T3-L1 adipocytes were first incubated for 24 h with 140 mg/L grape seed procyanidin extract (PE). Cells were then changed to a new cell culture medium without PE for 3 days. (**A**) Lypolitic rate versus nontreated cells was measured as glycerol or as NEFA released to the medium. (**B**) PPARγ2 and HSL mRNA levels were quantified by real-time RT-PCR. PPARγ2 and HSL gene expression, normalized by GAPDH mRNA levels, is expressed relative to control cells. Values represents mean ± SEM. a, b, c indicate groups significantly different with $p < 0.05$.

(*16*), antagonized the procyanidin effect, and the inhibition obtained with the simultaneous treatment was total. From our experiments, we cannot say how procyanidins interact with these intracellular mediators. Liang and co-workers reported that some flavonoids (those with a monomeric structure) act as allosteric effectors, bind to PPARγ, and activate it (*18*).

Although Kupussamy (*4*) and Harmon (*19*) reported that flavonoids potentiate β-adrenergic agonist-mediated lipolysis, our results suggest that procyanidins do not interact with β-adrenergic receptors for two reasons: first, because a typical β-adrenergic stimulation of lipolysis works in a short time and the lipolytic effect of procyanidins was observed only after long-term exposure (*2*) and, second, because β-adrenergic antagonists do not remove the lipolytic effect of procyanidins and this lipolytic activity remains after simultaneous treatment with epinephrine. We believe it is much more likely that procyanidins act on some signal transduction pathway that modulates PPARγ activity through PKA and/or mitogen-activated protein kinases (MAPK) (*20*, *21*). PKA involvement has already been shown, and the previous results of our group support procyanidin modulation of MAP kinases (*22*). Williams and co-workers recently revised the role of flavonoids as signaling molecules or as antioxidants (*23*). Their revision reinforced the idea that flavonoids may exert modulatory actions in cells as signaling molecules.

Procyanidin effects show similarities to the TNF-α reported effects (*17*, *24*, *25*): long-term lipolytic activity, down-regulation of adipocyte markers, removal by PPAR-γ agonists of their lipolytic activity, and hypothetical modulation of MAPK signaling mechanism. However, PPAR-γ agonists also remove the down-regulation of adipocyte markers induced by TNF-α (*17*, *25*). In our results, this did not occur. Another difference were the effects on the triglyceride content of the cells. Doses of TNF-α that down-regulate mRNA HSL significantly decrease the amount of triglycerides in the cells (*26*). We did not find statistical differences between the triglyceride contents of procyanidin-treated cells and those of control cells. Despite all of these differences between TNF-α effects and procyanidins effects, we decided to check what post-treatment effect procyanidins had. Most effects of procyanidins after 15−24 h of treatment were reversed 24 or 72 h after treatment. At 72 h after treatment, only PPARγ showed mRNA levels close to those observed during procyanidins treatment. However, adipogenic compounds such as thiazolidinediones also led to decreases in PPARγ mRNA levels in mature adipocytes (*20*). There are many different opinions about the PPARγ mRNA levels that are required for PPARγ to play their important role in maintaining the characteristics of mature adipocyte (*20*, *26*).

In conclusion, from our present results, we define two intracellular mediators of grape-procyanidin-induced lipolysis. The main one is the peroxisome proliferator-activated receptor-γ (PPAR-γ), although protein kinase A (PKA) is also involved. More work is needed to determine the mechanisms that explain the complete sequence of signaling events and ascertain the role of procyanidins on the adipocyte differentiation program.

ABBREVIATIONS USED

HSL, hormone sensitive lipase; G3PDH, glycerol-3-phosphate dehydrogenase; β-PDGF, platelet-derived growth factor β; C/EBPβ, CCAAT/enhancer binding protein-β; PKA, cAMP-dependent protein kinase A; TNFα, tumor necrosis factor-α; PPARγ, peroxisome proliferator-activated receptor-γ; TZD, thiazolidinediones; PE, grape seed procyanidin extract; NEFA, nonesterified fatty acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinases.

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