Mechanism of Formononetin-induced Stimulation of Adipocyte Fatty Acid Oxidation and Preadipocyte Differentiation

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Abstract Decreased adipocyte fatty acid oxidation (FAO) and impaired preadipocyte differentiation characterize hypertrophic expansion of adipose tissue (AT) from obese and insulin resistant humans and are recognized as potential mechanisms for obesity-mediated dyslipidemia. Supplementation of formononetin (FMN), one of the principal isoflavones extracted from red clover or Huangqi (Astragalus roots), has been shown to have beneficial effects on obesity-related hyperlipidemia, a well-established cardiovascular risk factor. However, a target tissue and underlying mechanism(s) through which FMN acts have been under-investigated. Thus, we investigated whether FMN promotes adipocyte FAO and preadipocyte differentiation using 3T3-L1 preadipocytes to provide potential mechanisms of FMN action. We further extended this to the culture of 10T1/2 mesenchymal stem cells (MSCs) as well as mouse AT explants to reflect in vivo effects of FMN. In fully differentiated 3T3-L1 adipocytes, FMN-treatment significantly increased the expression levels of FAO-related proteins such as pAMPK, pACC, and CPT1, all of which were consistently upregulated in AT explant cultures treated with 10 μM FMN. In addition, FMN significantly enhanced the degree of differentiation of both 3T3-L1 preadipocytes and 10T1/2 MSCs into adipocytes as evidenced by Oil Red O staining of cellular lipids. This observation correlated with increased expression levels of key adipogenic transcription factors (PPARγ and C/EBPα) and their down-stream target proteins (FABP4, Glut4 and adiponectin). Moreover, FMN failed to exert its stimulatory effects on preadipocyte differentiation in both cell types in the presence of a PPARγ antagonist, suggesting a PPARγ-dependent effect of FMN. Collectively, these data provide possible mechanisms of action of FMN on lipid metabolism and further support the favorable in vivo effects of FMN in diet and obesity-induced dyslipidemia.

Keywords: 3T3-L1 preadipocyte, 10T1/2 mesenchymal stem cell, formononetin, fatty acid oxidation, adipocyte differentiation

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1. Introduction

Adipose tissue (AT) serves as an energy reservoir and plays a critical role in the whole-body metabolic homeostasis by regulating glucose and lipid metabolism [1]. Dysregulated lipid metabolism in AT contributes to ectopic fat accumulation in peripheral tissues and hyperlipidemia (i.e., elevated levels of blood lipids and free fatty acids), which are strong risk factors for the development of metabolic and cardiovascular disease in obese individuals [2]. In humans, adipose mass expands by enlarging the size of pre-existing adipocytes (hypertrophy) and/or by increasing the number of new adipocytes (hyperplasia) through the recruitment and differentiation of precursor cells [1]. Recent evidence suggests that hypertrophic obesity is strongly associated with obesity-mediated metabolic complications such as insulin resistance and type 2 diabetes (T2D), while hyperplastic obesity is less likely linked to these metabolic consequences [3,4]. Moreover, the rates of new fat cell generation were significantly reduced in obesity and inversely associated with body mass index (BMI) and adipocyte cell size of the donors, which correlated with decreased expression of the differentiation marker genes including peroxisome proliferator-activated receptor (PPARγ), glucose transporter 4 (GLUT4) and adiponectin [5,6]. Thus, an important consequence of impaired adipocyte differentiation is the pathological hypertrophic

expansion of AT that has been linked to obesity-induced adipose dysfunction and hyperlipidemia [7,8].

Mesenchymal stem cells (MSCs) are a major source of new adipocyte generation through the commitment into preadipocytes, followed by terminal differentiation to mature adipocytes [1]. The adipogenic program is tightly regulated by a coordinated interplay of key transcription factors such as PPARγ and CCAAT/enhancer-binding protein alpha (C/EBPα) that control the downstream target genes such as fatty acid-binding protein 4 (FABP4), GLUT4 and adiponectin, thereby acquiring phenotypic functions of adipocytes [9]. In particular, PPARγ is a critical regulator of adipocyte differentiation and a nuclear receptor for anti-diabetic drugs, thiazolidinediones (TZDs) that are shown to be effective in lowering hyperlipidemia and thus reducing cardiovascular risks in diabetic patients [10]. An increasing body of evidence in animals and humans also suggests that hypertrophic obesity is closely associated with reduced mitochondrial fatty acid oxidation (FAO) with a significant decrease in AT lipid turnover [8,11-14]. A central player in the regulation of mitochondrial FAO is AMP-activated protein kinase (AMPK), a cellular energy censor, which is known to regulate carnitine palmitoyl transferase 1 (CPT1), acetyl-CoA carboxylase (ACC) and adiponectin, all of which are reduced in obesity [15,16]. Although the underlying mechanism for the development of abnormal lipid metabolism in obese AT is still unclear, it has been demonstrated that decreased adipocyte lipid oxidation and/or new fat cell formation in AT results in hyperlipidemia in humans [5,7,8,17]. Therefore, agents that promote adipocyte FAO and/or preadipocyte differentiation would not only prevent hypertrophic expansion of adipocytes, but also be a promising strategy for managing and preventing lipid abnormalities in obesity and thus cardiovascular risk.

Formononetin (FMN) is one of the principal isoflavones found in red clover and Huangqi (Astragalus roots). A recent randomized controlled trial has reported that FMN-enriched red clover extract improved hyperlipidemia in menopause women [18]. Consistently, in mice, FMN supplementation has been shown to significantly attenuate diet-induced obesity and hyperlipidemia [19]. As a potential mechanism, Gautam et. al. [19] suggested that FMN may increase energy expenditure in obese mice, but potential target tissues and underlying mechanisms have not been identified. Also, in vitro studies using 3T3-L1 cells, a well-characterized immortalized preadipocyte cell line, have generated contrasting results about the effects of FMN on preadipocyte differentiation; a stimulatory effect on adipogenesis at 5 to 50 μM [20] and an inhibitory effect at 100 pm to 1 μM [19]. Therefore, a limited and inconsistent evidence hardly explains a possible mechanism of action by which FMN attenuates obesity-mediated hyperlipidemia.

The aim of this study was to investigate the effects of FMN on adipocyte lipid oxidation and preadipocyte differentiation using 3T3-L1 preadipocytes. Potential effects on this well-established cell line were further confirmed using the culture of mouse epididymal white AT (eWAT) explants and 10T1/2 multipotent MSCs to gain insight into the mode of action of FMN at AT level. Because of the contrasting observations on the effects of

FMN on 3T3-L1 preadipocyte differentiation, we used a range of FMN concentrations (1 to 20 μM), which are within a physiologically achievable concentration range in mice [21].

2. Materials & Methods

2.1. Materials

Bisphenol A diglycidyl ether (BADGE), GW9662, insulin from bovine pancreas, 3-isobutyl-1-methylxanthine (MIX), and dexamethasone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell culture reagents including Dulbecco's modified Eagle's medium (high glucose, DMEM), DMEM/F12 medium, newborn calf serum (NCS), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Formononetin (7-hydroxy-4'-methoxyisoflavone), anti-PPARγ (Cat. sc-271392), anti-C/EBPα (Cat. sc-166258), anti-CPT-1A (Cat. sc-393072), anti-GLUT4 (Cat. sc-53566) and HRP-conjugated β-actin (Cat. sc-8432 HRP) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). anti-pAMPK (Cat. #2535), anti-pACC (Cat. #3661), anti-AMPK (Cat. #5831), anti-ACC (Cat. #3662), anti-FABP4 (Cat. #2120) and anti-adiponectin (Cat. #2789) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA).

2.2. Cell Culture

3T3-L1 preadipocytes (ATCC® CL-173™) were maintained in DMEM containing 10% NCS, 100 unit/mL penicillin, and 50 μg/mL streptomycin at 37°C under 5% CO_2 atmosphere. To induce differentiation of preadipocytes into adipocytes, post-confluent cells (designated Day 0) were cultured in a differentiation medium (DMEM media containing 10% FBS, 167 nM insulin, 0.5 mM MIX, and 1 μM dexamethasone). The cell culture medium was changed with 10% FBS-DMEM containing 167 nM insulin 2 days after and refreshed every other day until day 6. Differentiated adipocytes were subjected to Oil Red O staining and harvested for protein analysis. C3H10T1/2 cells (10T1/2 cells, ATCC® CCL-226™) were maintained in phenol red free-DMEM/F12 media. Upon reaching confluence (Day 0), cells were induced to differentiate in 5% FBS-DMEM/F12 supplemented with 167 nM insulin, 0.5 mM MIX, and 1 μM dexamethasone for 2 days. Cells were then incubated in DMEM/F12 containing 5% FBS and 167 nM insulin until day 8 to 10. Differentiated adipocytes were subjected to Oil Red O staining and harvested for protein analysis. Formononetin (FMN) was reconstituted as 100 mM stock solutions in DMSO (dimethyl sulfoxide) and added from the indicated concentrations on Day 0.

2.3. Oil-Red O Staining

To measure the degree of differentiation of preadipocytes into adipocyte, Oil Red O staining was performed. Cells were washed twice with phosphate-buffered saline (PBS, pH 7.4), fixed in 10% formalin for 20 min at room temperature (RT), and then washed with

PBS twice. Lipid droplets in the cells were stained with 0.3% Oil Red O solution at RT for 20 minutes and completely washed out with distilled water. After drying exhaustively, stained Oil Red O formazan was eluted with 100% isopropanol and quantified at 490 nm.

2.4. Explant Culture of Epididymal White Adipose Tissue (eWAT)

Epididymal white adipose tissue (eWAT) was obtained from FVB male mice aged 6 weeks (n=2) and was subject to explant culture. Briefly, 100 mg of tissues was cut in small pieces under laminar airflow and sterile conditions and placed on a 6-well plate in 10% FBS-DMEM at 37°C under 5% CO2 atmosphere for 3 hours for relaxation. Then, the media were refreshed and cultured in the presence or absence of FMN (10 μM) for 3 hours by shaking at 70 rpm and harvested for protein analysis.

2.5. Western Blotting

Cells and explants were lysed in RIPA extraction buffer [25mM Tris-HCl (pH 7.6), 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS] containing both protease and phosphatase inhibitors (Roche, Indianapolis, IN, USA). The protein concentration of each sample was determined using BCA protein assay reagent (Pierce, Rockford, IL, USA). Twenty micrograms of protein were separated on 8–12% SDS-polyacrylamide gels, followed by electrophoretic transfer to a nitrocellulose membrane. Primary antibodies were incubated overnight at 4°C at a 1:2000 dilution. After incubating with the secondary antibody at room temperature for 2 h, the proteins were detected using the ECL western blotting analysis system (Pierce). Each protein expression was normalized to β-actin.

2.6. Statistics

All data are obtained from at least three independent experiments except for explant culture of eWAT (n=2) and represented as means ± S.D. Statistical analysis was carried out by SAS version 9.1 (SAS Institute, Cary, NC). Significant differences were accepted at the significant level of 0.05 with one-way analysis of variance (ANOVA) followed by Duncan's multiple range test.

3. Results

3.1. FMN Increases CPT-1 Expression and ACC Phosphorylation through AMPK Phosphorylation in fully Differentiated 3T3-L1 Adipocytes

The activation of AMPK through the phosphorylation of AMPK on Thr172 is known to increase the expression of CPT1 and mitochondrial FAO [22]. Activated pAMPK phosphorylates and inactivates ACC, lowering malonyl-CoA levels and, thus, promoting CPT1-mediated mitochondrial

FAO while suppressing fatty acid synthesis [23]. Given the importance of adipocyte fatty acid metabolism, we first determined whether FMN increases FAO through AMPK phosphorylation in mature adipocytes. As shown in Figure 1, FMN significantly increased the phosphorylation of AMPK and ACC with their expression levels peaked at 5 μM and plateaued up to 20 μM in fully differentiated 3T3-L1 adipocytes compared with control cells (Figure 1A). However, no significant differences in total AMPK and ACC levels by FMN at all concentrations used were observed compared to the control cells (Figure 1B). FMN also markedly increased the expression of CPT1 protein with the highest expression level observed at 10 μM concentration.
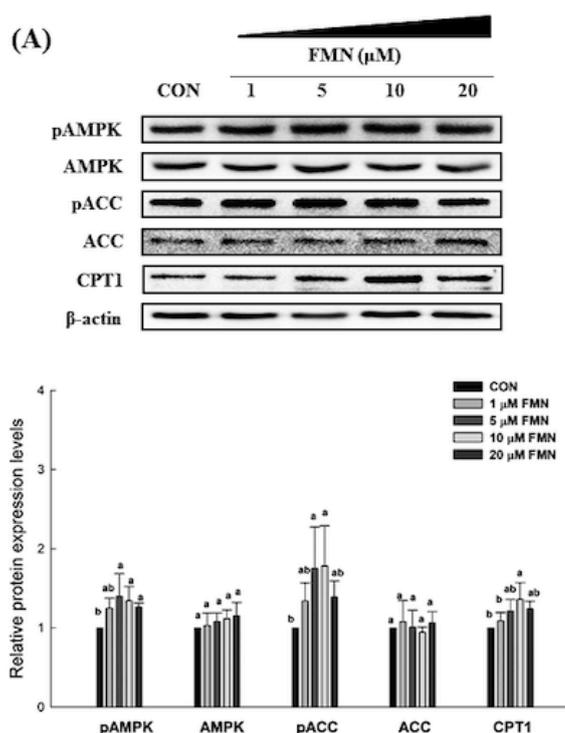

Figure 1. FMN increases AMPK-mediated lipid oxidation in in vitro 3T3-L1. (A) Representative image of protein expression in 3T3-L1 adipocytes (n=4). (B) Different letters in the bar graph indicate statistically significant difference at the level of 0.05.

3.2. FMN Consistently Increases pAMPK, pACC, and CPT-1 in Mouse eWAT Explant Culture

Next, we extended our question to determine whether the in vitro findings are replicable at the AT level. To address this, fresh mouse eWAT explants were cultured with or without FMN at 10 μM concentration that showed the highest effect on CPT1 expression in 3T3-L1 adipocytes (Figure 1). Consistent with the observations in differentiated 3T3-L1 adipocytes, FMN at 10 μM greatly increased the expression levels of pAMPK and pACC in eWAT explants compared to non-treated ones (Figure 2). We also found a concomitant increase in the expression levels of CPT1 in the tissues. Collectively, our in vitro and ex vivo findings indicate that FMN increases FAO through AMPK phosphorylation in AT.
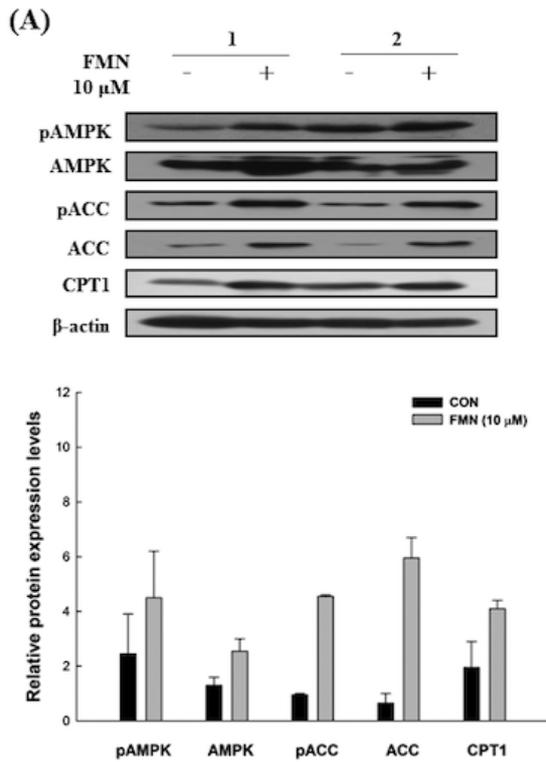
Figure 2. FMN increases phosphorylations of AMPK and ACC and CPT1 expression in mouse epididymal white adipose tissue (eWAT) explants. (A) The expression of proteins in eWAT and (B) bar graph (n=2)

3.3. FMN Stimulates Differentiation of 3T3-L1 Preadipocytes into Adipocytes via PPARγ Activation

In addition to adipocyte lipid oxidation, the effects of FMN on preadipocyte differentiation were investigated using 3T3-L1 preadipocytes. As shown in Figure 3, when compared to non-treated cells, FMN promoted lipid accumulation during 3T3-L1 preadipocyte differentiation as measured by ORO staining with the greatest abundance of lipid droplets at 20 μM. FMN treatment also significantly upregulated the protein expression levels of differentiation-related key transcription factors such as PPARγ and C/EBPα as well as adipocyte-specific genes including FABP4, GLUT4, and adiponectin (Figure 3B). The upregulation of the expression of these proteins reached the highest level at 20 μM, suggesting that FMN enhances the formation of new adipocytes through adipogenic program.

Next, we questioned whether the stimulatory effect of FMN on the differentiation of 3T3-L1 preadipocytes was dependent on PPARγ, a master regulator of preadipocyte differentiation. The co-treatment of FMN with a PPARγ antagonist, BADGE significantly reduced ORO staining of lipids compared to the cells treated with only FMN (Figure 3C). We also found that BADGE co-treatment significantly decreased the expression of PPARγ, adiponectin and FABP4 proteins, suggesting that FMN promotes adipocyte differentiation via PPARγ activation.
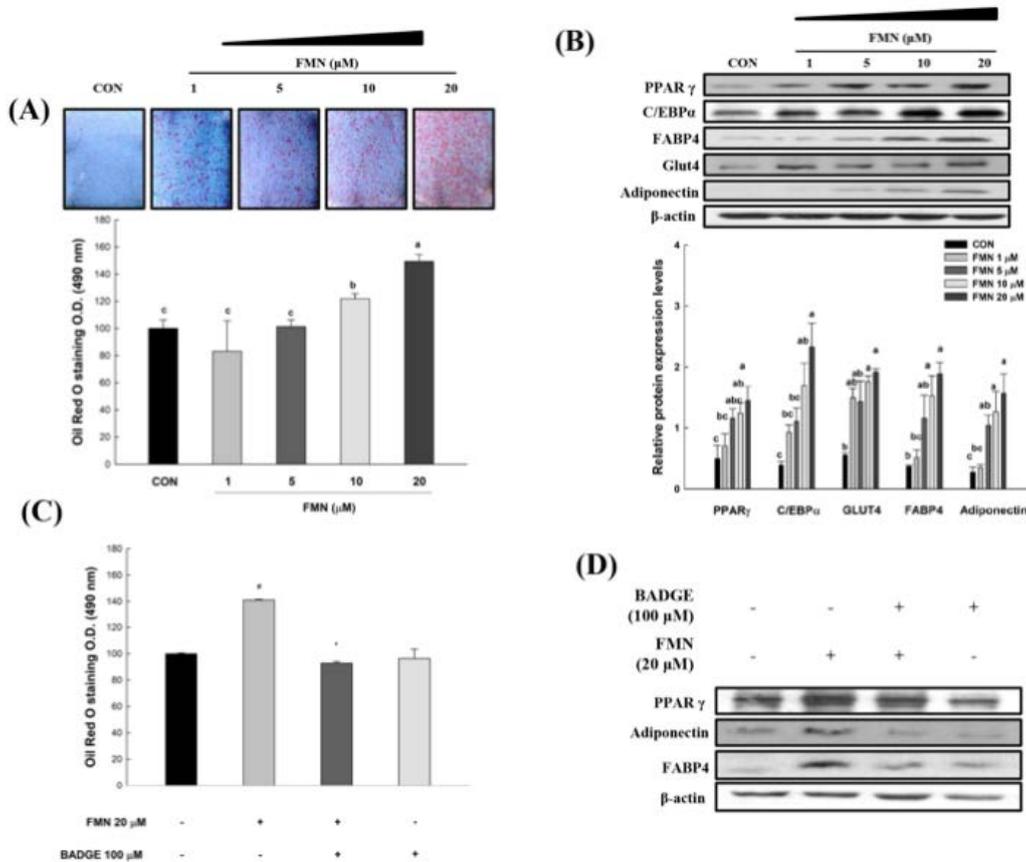

Figure 3. FMN enhances differentiation of 3T3-L1, and its stimulatory effect was blocked in the presence of a PPARγ antagonist. (A & B) 3T3-L1 preadipocytes were differentiated in the presence or absence of FMN at the indicated concentrates (n=3). (C & D) 3T3-L1 preadipocytes were co-treated with FMN and BADGE during its differentiation into adipocytes. Representative image of Oil Red O staining and protein expression in 3T3-L1 preadipocytes were presented (n=3). Different letters indicate statistically significant difference at the level of 0.05
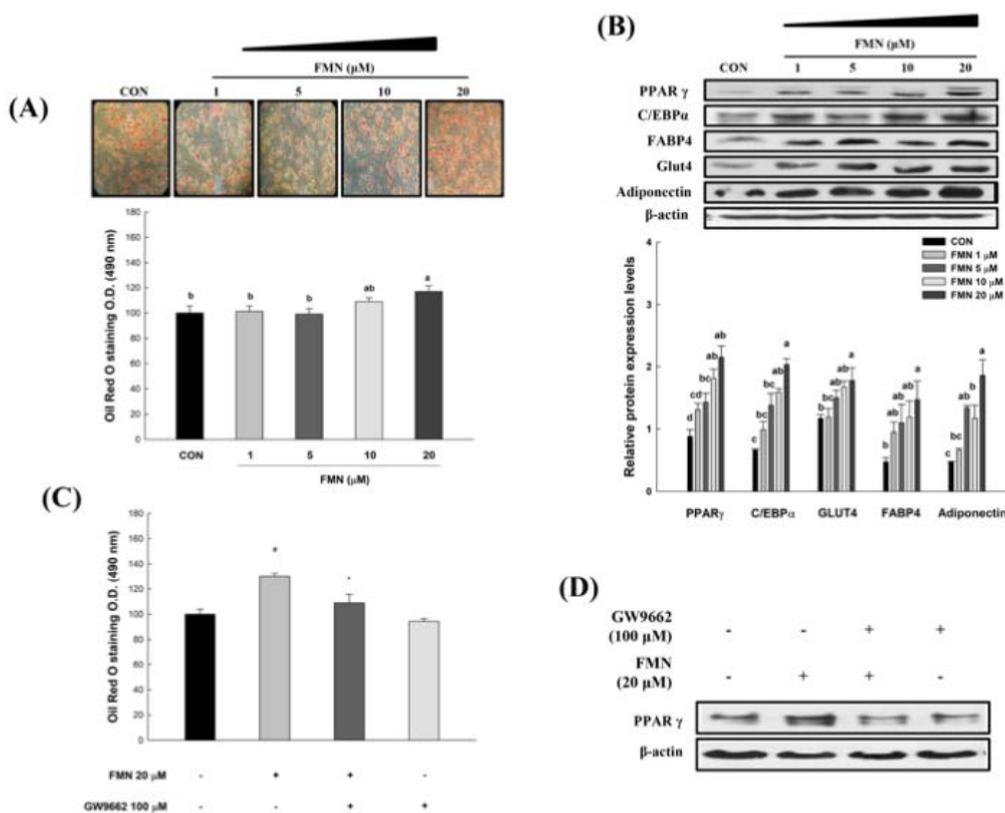
Figure 4. FMN promotes 10T1/2 MSC differentiation into adipocytes via PPARγ. (A & B) 10T1/2 MSCs were differentiated in the presence or absence of FMN at the indicated concentrates (n=3). (C & D) 10T1/2 MSCs were co-treated with FMN and GW9662 during its differentiation into adipocytes. Representative image of Oil Red O staining and protein expression in 10T1/2 MSCs were presented (n=3). Different letters indicate statistically significant difference at the level of 0.05

3.4. A PPARγ Antagonist Inhibits FMN-mediated Adipocyte Differentiation in 10T1/2 MSCs

It is well-established that a mouse embryos-derived 10T1/2 cell line is functionally similar to and pluripotent as in vivo adipose progenitor cells, MSCs [24]. Because these MSCs are important variables that influence the overall adipose cellularity, we investigated the effects of FMN on the degree of differentiation using 10T1/2 MSCs (Figure 4). Consistent with the observations in 3T3-L1 preadipocytes, FMN treatment substantially increased lipid accumulation during 10T1/2 MSC differentiation with the greatest abundance of ORO-stained lipid droplets observed at 20 μM compared to non-treated cells (Figure 4A). We also found that the protein expression levels of PPARγ, C/EBPα, FABP4, GLUT4 and adiponectin were significantly enhanced in the presence of FMN, reaching the highest levels at 20 μM (Figure 4B). Moreover, 10T1/1 MSCs co-treated with FMN and GW9662 (PPARγ antagonist) significantly reduced the abundance of ORO-stained lipid droplets and levels of PPARγ protein when compared to FMN-treated cells (Figure 4C & Figure 4D), suggesting that the stimulatory effects of FMN on 10T1/2 MSCs differentiation into adipocyte are PPARγ-dependent as observed in 3T3-L1 preadipocytes.

4. Discussion

Obesity-induced abnormalities in lipid metabolism in AT contribute to the development of hyperlipidemia which is a strong risk factor for metabolic and cardiovascular diseases [2]. A line of evidence suggests that decreased fat cell turnover through preadipocyte differentiation and/or reduced adipocyte lipid oxidation in obese humans characterize obesity-mediated metabolic abnormalities, particularly the development of dyslipidemia and ectopic fat accumulation [7,8,25]. It has been reported that FMN, a major isoflavone abundantly found in red clover and Huangqi (Astragalus roots), has blood lipid-lowering potentials in menopausal women and obese mice [18,19]. However, potential target tissues and mechanisms responsible have been still unknown. In this study, we demonstrated that FMN can promote adipocyte lipid oxidation through AMPK phosphorylation and also enhance preadipocyte differentiation via PPARγ activation using 3T3-L1 preadipocyte cell line. Our consistent findings using the culture of mouse eWAT explants and 10T1/2 MSCs further support the dual mode of biological action of FMN at the AT level. Thus, our data indicate that FMN-enriched diets or supplements may reduce the risk for hyperlipidemia and its related cardiovascular outcomes by promoting preadipocyte differentiation and adipocyte lipid oxidation in AT in obesity.

Preadipocyte differentiation involves the recruitment of MSCs that undergo commitment and differentiation into preadipocytes, followed by terminal differentiation into adipocytes [1]. There is a continuous turnover of fat cells in humans through preadipocyte differentiation with approximately 10 % per year [25]. Moreover, recent studies demonstrated that the rates of preadipocyte differentiation are reduced in obese and diabetic humans and inversely correlated to existing adipocyte size and

BMI [5,6]. The transcription factor PPARγ is well-known as a master regulator of preadipocyte differentiation and metabolism as evidenced by the demonstration that PPARγ overexpression was sufficient to trans-differentiate non-adipocytes into adipocyte-like cells [26]. In addition to the critical role of PPARγ in regulating preadipocyte differentiation, PPARγ expression in AT was reduced in obese and diabetic humans with low preadipocyte differentiation capacity, adipocyte hypertrophy, and metabolic dysregulation [5,6,27]. However, the synthetic ligands of PPARγ, TZDs, significantly improved lipid dysregulation and insulin sensitivity in obese and diabetic subjects [10,28], supporting the notion that reduced PPARγ expression and a limited ability to expand AT by differentiating precursor cells lead to increased lipid levels in the blood and non-adipose cells.

A growing body of evidence in humans and animals has indicated the potential blood lipid-lowering effects of FMN [18,19]. Shen et al [29] reported that FMN along with soy isoflavones, such as daidzein and genistein, is a potential PPAR agonist, with consistent observations that 3T3-L1 preadipocyte differentiation was significantly enhanced by FMN treatment at 5 to 50 μM [20]. However, in contrast to the previous reports, recent in vitro data by Gautam et al [19] showed that FMN suppressed 3T3-L1 preadipocyte differentiation by suppressing PPARγ expression at 100 pm to 1 μM. These conflicting results obtained from different studies could be mainly attributable to the differences in the concentrations of FMN. Considering pharmacokinetic analysis in mice showing that 4 mg/kg intravenous injection resulted in approximately 1.3 μM in 30 minutes [21], we used the range of concentrations (1 to 20 μM) that are physiologically relevant and achievable through dietary means. In contrast to Gautam et al [19], our data showed that FMN promoted 3T3-L1 preadipocyte differentiation with the comparable effects on 10T1/2 MSCs at the concentration range from 1 to 20 μM. The co-treatment of FMN with PPARγ antagonists further indicates that the stimulatory effect of FMN on 3T3-L1 and 10T1/2 cells was PPARγ-dependent, consistent with the finding by Shen et al [29]. Therefore, our results suggest that FMN enhances preadipocyte differentiation by targeting PPARγ, which could be a possible mechanism by which FMN exerts blood lipid-lowering effects in humans and animals.

An important outcome of impaired formation of new fat cells through adipocyte differentiation is the pathological expansion of hypertrophic adipocytes that is closely linked to metabolic complications such as insulin resistance and dyslipidemia [7,8]. When compared to lean adipocytes, hypertrophied adipocytes exhibit decreased lipid and glucose sequestration, reduced lipid hydrolysis and oxidation that correlated closely to hyperlipidemia in obese humans [8,11,12]. Gautam et al [19] have shown that the blood lipid-lowering effects of FMN supplementation in obese mice were attributable to increased thermogenesis based on the increased mRNA levels of uncoupling protein 1 (UCP1) in eWAT. In support of this, Nie et al [30] has demonstrated that FMN increases the mRNA expression of UCP1 and PR domain containing 16 (PRDM16, a marker of thermogenic adipocytes) in inguinal and epididymal WAT from HFD-fed mice. In addition to the pivotal role of UCP1 in the regulation of thermogenesis in brown adipocytes, white adipocyte UCP1 functions as a futile substrate cycle to control mitochondrial FAO via the activation of AMPK, which is a sensor of cellular energy status [31]. Thus, this led us to hypothesize that FMN may increase AMPK-dependent FAO in adipocytes. The present study is, to the best of our knowledge, the first work showing that FMN promotes AMPK phosphorylation in adipocytes. In addition, FMN significantly increased expression levels of key proteins involved in FAO, such as pAMPK, pACC, and CPT-1. Furthermore, we confirmed the potential effects of FMN on lipid oxidation in AT using mouse eWAT explant culture as supported by the consistent upregulation of pAMPK, pACC and CPT-1 proteins.

In conclusion, FMN is one of the widely used bioactive constituents, and both FMN research and market demands have substantially increased in the past decades. Our in vitro and ex vivo data provide additional information for a potential target tissue and molecular mechanisms by which FMN increases AT energy expenditure that may account for its blood lipid-lowering effects observed in previous in vivo studies [18,19]. We further showed that FMN increased lipid oxidation in adipocytes through AMPK-ACC-CPT1 pathway and stimulated preadipocyte differentiation via PPARγ activation. Although our data suggest that FMN supplementation may attenuate obesity-induced dyslipidemia by targeting AT lipid metabolism, further studies are needed to determine the in vivo effects of dietary FMN using pre-clinical and/or clinical models.

Acknowledgements

Conflicts of Interest Statement

The authors declare that there is no conflicting interest in this manuscript.

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