Antiobesity activity of a sphingosine 1-phosphate analogue FTY720 observed in adipocytes and obese mouse model

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Abbreviations: ATGL, adipose TG lipase; C/EBP, CCAAT/enhancer binding proteins; cAMP, cyclic AMP; DG, diglycerides; GSK3, glycogen synthase kinase 3; HSL, hormone-sensitive lipase; PPAR, peroxisome proliferator-activated receptor γ; S1P, sphingosine-1-phosphate; TGs, triglycerides

Abstract

Higher levels of body fat are associated with an increased risk for development numerous adverse health conditions. FTY720 is an immune modulator and a synthetic analogue of sphingosine 1-phosphate (S1P), activated S1P receptors and is effective in experimental models of transplantation and autoimmunity. Whereas immune modulation by FTY720 has been extensively studied, other actions of FTY720 are not well understood. Here we describe a novel role of FTY720 in the prevention of obesity, involving the regulation of adipogenesis and lipolysis in vivo and in vitro. Male C57B/6J mice were fed a standard diet or a high fat diet (HFD) without or with FTY720 (0.04 mg/kg, twice a week) for 6 weeks. The HFD induced an accumulation of large adipocytes, down-regulation of phosphorylated AMP-activated protein kinase α (p-AMPKα) and Akt (p-Akt); down-regulation of hormone-sensitive lipase (HSL), adipose triglyceride lipase (ATGL) and perilipin mRNA as well as up-regulation of phosphorylated HSL (p-HSL, Ser563) and glycogen synthase kinase 3 α/β (p-GSK3α/β). All these effects were blunted by FTY720 treatment, which inhibited adipogenesis and promoted lipolysis. Also, FTY720 significantly decreased lipid accumulation in maturing preadipocytes. FTY720 down-regulated the transcriptional levels of the PPARγ, C/EBPα and adiponectin, which are markers of adipogenic differentiation. FTY720 significantly increased the release of glycerol and the expression of the HSL, ATGL and perilipin, which are regulators of lipolysis. These results show that FTY720 prevented obesity by modulating adipogenesis and lipolysis, and suggest that FTY720 is used for the treatment of obesity.

Keywords: adipocyte; adipogenesis; antiobesity; FTY720; lipolysis

Introduction

An imbalance between energy intake and expenditure can result in excess triacylglycerol accumulation in adipose tissue, resulting in obesity (Duncan et al., 2007). In morbid obesity an increased adipocyte number (hyperplasia) may occur through adipocyte differentiation of precursor cells present in adipose tissue (Ntambi and Kim, 2000; Rosen and MacDougald, 2006). However, obesity is largely attributed to adipocyte hypertrophy that occurs when triacylglycerol synthesis exceeds breakdown (lipolysis), resulting in elevated triacylglycerol storage (Duncan et al., 2007; Jaworski et al., 2007). Indeed, unlike triacylglycerol synthesis (Dircks and Sul, 1999; Jaworski et al., 2009) that occurs at high levels in other tissues, lipolysis for the liberation of fatty acids that can then be used as an energy source by other tissues is unique to adipocytes (Duncan et al., 2007). Thus, lipolysis and hyperplasia of adipocytes might be important factors in the development of obesity.

Lipolysis is one of the most important metabolic pathways regulating energy homeostasis and the pathogenesis of obesity (Zimmermann et al., 2004). Lipolysis is governed by two major enzymes that are hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) (Bézaire et al., 2009). ATGL initiates lipolysis by cleaving the first FA from
Triglyceride (TG) and then HSL act on diacylglycerol (DAG), releasing two additional FAs and one glycerol molecule (Gaidhu et al., 2010). Whereas HSL is present in the cytosol, ATGL is localized in the cytosol and also on lipid droplets (Zimmermann et al., 2004; Lasa et al., 2012).

Adipocytes are derived from mesenchymal stem cells, which have the potential to differentiate into myoblasts, chondroblasts, osteoblasts or adipocytes (Rayalam et al., 2008). Adipogenesis has been investigated extensively using 3T3-L1 cells. Adipocyte differentiation involves an elaborate network of transcription factors that regulate the expression of numerous genes responsible for the phenotype of mature adipocytes (Lee et al., 2009). Among the various transcription factors that promote preadipocyte differentiation and influence adipogenesis, peroxisome proliferator-activated receptor gamma (PPARγ) has been termed the “master regulator of adipogenesis” (Tontonoz and Spiegelman, 2008; Wakabayashi et al., 2009). Other adipogenic transcription factors include the CCAAT/enhancer binding proteins (C/EBP) (Nerurkar et al., 2010). They are necessary for the expression of adipocyte-specific genes, such as adiponectin (Xing et al., 2010).

Akt (protein kinase B, PKB), a PH domain containing serine/threonine kinase, regulates growth factor signaling to stimulate glucose uptake, glycogen synthesis (Cross et al., 1995) and protein synthesis by influencing the glycogen synthase kinase 3 (GSK3)α/β and tuberous sclerosis complex 2 (TSC2)-mTOR signaling pathways. Increased protein translation following Akt activation elicits skeletal muscle hypertrophy (Rommel et al., 2001) and augments hepatic fatty acid oxidation with reduced fat accumulation (Izumiya et al., 2008). GSK3β, which influences insulin resistance, is phosphorylated and inhibited by Akt (Cross et al., 1995). Akt and GSK3β activity are reciprocally regulated in insulin resistant and obesity. Akt/mTOR activity is decreased (Shao et al., 2000; Funai et al., 2006) and GSK3β increased (Kaidanovich and Eldar-Finkelman, 2002) in insulin-resistant tissues of aging and obese mice (Chakraborty et al., 2010).

AMP-activated protein kinase (AMPK) activation leads to increased fat oxidation by the upregulation of the transcription factor NRF-1, which stimulates mitochondrial biogenesis and inhibits both lipolysis and lipogenesis, affecting directly the enzymes engaged in lipid metabolism and down-regulating PPARγ expression (Moreno-Navarrete et al., 2009). FTY720 is a prodrug phosphorylated by type 2 sphingosine kinase to form FTY720-phosphate (FTY720-P) (Kihara and Igarashi, 2008). The S1P1 receptor regulates the egress of lymphocytes from secondary lymphoid organs. By inducing the internalization and degradation of the S1P1 receptor, FTY720-P reduces circulating lymphocyte counts, and is therefore considered a promising therapy for immune disorders (Takabe et al., 2008). However, other functions of FTY720 excluding immune modulation remain largely unknown. Recently, FTY720 was found to be able to prevent high-fat diet (HFD-induced weight gain, insulin resistance and adipose tissue inflammation in C57BL/6 mice (Kendall and Hupfeld, 2008). However, this previous study mainly focused on the effect of FTY720 on lymphocytes sequestration. The effect of FTY720 on adipocytes has not been studied, especially on adipogenesis and lipolysis, which are important on obesity development.

Investigation of the direct effect of FTY720 on adipocytes will provide some novel insights for additional inhibitory mechanism of FTY720 on obesity. We report that administration of FTY720 prevents obesity from high fat feeding by not only regulating lipolysis through HSL activation but also inhibiting adipogenesis via phosphorylation of Akt.

Results

**FTY720 administration mice are resistant to obesity**

In this study, obesity was induced in normal mice by feeding a HFD for 10 weeks. After 3 weeks, mice fed the SD weighted $25.7 \pm 0.6 \, g \, (n = 5)$, while control mice fed the HFD (HFD-CON) weighted $31.4 \pm 2 \, g \,(n = 6)$. The respective weights at 8 weeks were $31.3 \pm 1.5 \, g \,(n = 6)$ and $42.4 \pm 1.7 \, g \,(n = 6)$. Compared with mice fed the SD, mice fed the HFD increased their body weight by 24.6% after 3 weeks of feeding and became 51.1% heavier after 8 weeks of feeding (Figures 1A and 1B). Parallel to the body weight change, the weights of white adipose tissue (WAT) were significantly higher in obese mice than in normal mice at the end of 16 weeks (data not shown). FTY720 administration to mice fed the HFD (FTY720) effectively suppressed the increase in body weight compared with HFD-CON mice. After one week of injection, HFD-FTY720 mice weighted $33.8 \pm 1.3 \, g \,(n = 6)$, while HFD-CON mice weighted $37.4 \pm 1.9 \, g \,(n = 6)$. Compared with HFD mice,
Figure 1. FTY720 administration mice are resistant to obesity. (A) Gross appearance of standard diet (n = 5, SD) and high fat-diet (n = 6, HFD) fed mice. (B) Time course of body weights over 15 weeks in mice fed a SD and HFD. (C) Gross appearance of HFD controls (n = 6, CON), PBS-treated (n = 6, PBS) and FTY720-treated (n = 6, FTY) obese mice. (D) Time course of body weights over 15 weeks in mice fed with HFD and intraperitoneally injected with none (n = 6, CON), PBS-treated (n = 6, PBS) and FTY720-treated (n = 6, FTY) obese mice. (E, F) Average body weights (E), epididymal fat pad weights (F) and organ weights (G) of mice at 15 weeks. (H, I) Representative images (H) of hematoxylin and eosin-stained sections of gonadal WAT and adipocyte cell size (I) in gonadal WAT. (J, K) Body fat composition of HFD controls (n = 6, CON), PBS-treated (n = 6, PBS) and FTY720-treated (n = 6, FTY) obese mice was calculated percent of CON. Data are expressed as the means ± SD for 6 mice. *P < 0.05; and **P < 0.01, significant differences between HFD control and each treatment group; ##P < 0.01, significant differences between PBS treatment group and FTY720 treatment group.
Figure 2. FTY720 phosphate (FTY-p) decreases lipid droplets during the differentiation of 3T3-L1 preadipocytes. (A) Preadipocytes were induced to differentiate with FTY-p of increasing concentrations for 6 days. The AdipoRed assays were performed on day 6 and were photographed with a light microscope (× 200). (B) Fluorescence was measured with an excitation wavelength of 485 nm and an emission wavelength of 572 nm. Bar graph indicates the mean ± S. E. M. (n = 3). The experiments were repeatedly performed to confirm the results. The data were analyzed using analysis of variance (ANOVA) and Duncan multiple range test (P < 0.05). Means sharing a common alphabetical symbol do not differ significantly. (C-E) Preadipocytes were induced to differentiate with FTY-p (1 μM) and harvested at day 7 during the differentiation period. The mRNA expression of PPARγ (C), C/EBPα (D) and adiponectin (E) was analyzed by real-time RT-PCR. Values are mean ± S. E. M. of data from three separate experiments (n = 3); each experiment was performed in triplicate. The data were analyzed using analysis of variance (ANOVA) and Duncan multiple range test (P < 0.05). Means sharing a common alphabetical symbol do not differ significantly. (F) Preadipocytes were induced to differentiate with or without FTY-p 0.1 and 1 μM for 2 days. At day 2, the protein levels of p-Akt were analyzed by Western blot. The experiments were repeatedly performed to confirm the results.

HFD-FTY720 mice displayed an 11% reduced body weight 1 week after the administration of FTY720. This ratio was maintained until the end of the experiment (Figures 1C and 1D). The lower body weight of the mice administered FTY720 was largely accounted for by a reduction in WAT weight (Figures 1F and 1G). At 16-weeks-of-age, HFD-FTY mice had effectively smaller WAT depots compared to HFD mice, with a combined WAT depot weight that was 20% lower (Figure 1F). Body composition analysis indicated that FTY treatment decreased triacylglycerol content (Figure 1H). Histological analysis showed that the gonadal WAT from HFD-CON contained significantly large adipocytes (Figures 1I-1K, P < 0.01) compared with SD, but FTY720 mice displayed considerably smaller adipocytes compared with those of HFD-CON mice (Figures 1I-1K).

FTY720 inhibits adipocyte differentiation of 3T3-L1 cells

A decrease in adipose tissue mass can be a result of a decrease in adipocyte size and/or number due to reduction of adipocyte differentiation (Gregoire et al., 1998). Also, FTY720 is phosphorylated in vivo by sphingosine kinase 2 to become the active drug metabolite (S)-FTY720-phosphate (FTY-p) and only the (S)-phosphorylated form of FTY720 is capable of activating S1P receptors in vitro (Brinkmann et al., 2004; Valentine et al., 2010). Therefore, we studied the influence of FTY-p on adipocyte differentiation in the 3T3-L1 cells. When 3T3-L1 cells were allowed to differentiate over 6 days in the presence of increasing concentrations of FTY-p in the adipogenic medium, a reduction in TG accumulation was observed (Figures 2A and 2B). The effect of FTY-p was significantly detected at 0.1 μM and was maximal at 1 μM.

To confirm the anti-adipogenic differentiation of FTY-p, the mRNA levels of biochemical markers of differentiation (PPARγ, C/EBPα and adiponectin) were determined (Figures 2C-2E). When the 3T3-L1 preadipocytes were differentiated with MDI treatment, the mRNA levels of the adipogenic markers of differentiation increased compared to the control. However, FTY-p treatment led to a significant reduction in the mRNA level of PPARγ, C/EBPα and adiponectin (Figures 2C-2E). The evidence was consistent with an influence of FTY720 on adipocyte differentiation.

Besides altering insulin sensitivity, Akt and its downstream effectors can reduce fat accumulation by reducing adipogenesis via GSK3β (Ross et al.,...
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Figure 3. FTY720 induces Akt phosphorylation and inhibits the activation of GSK-3α/β. (A) Western blot analysis for p-Akt (Ser 473) (Upper panel) and p-GSK3α/β (Tyr279/216) (Middle panel) protein in WAT of standard diet (n = 3, SD), high fat-diet (n = 5, HFD CON) and FTY720-treated obese mice (n = 5, HFD FTY720). (B) Levels of p-Akt (Ser 473) and p-GSK3α/β (Tyr279/216) were normalized to α-actin. The experiments were repeatedly performed to confirm the results. The data were analyzed using student's t-test. **P < 0.01, significant differences between SD and each treatment group; ##P < 0.01, significant differences between HFD-CON and HFD-FTY720 group.

Figure 4. FTY720 induces AMPK phosphorylation in vivo as well as in vitro. (A) Western blot analysis for p-AMPKα (Thr172) protein in WAT of standard diet (n = 4, SD), high fat-diet (n = 5, HFD CON) and FTY720-treated obese mice (n = 5, HFD FTY720). (B) Levels of p-AMPKα (Thr172) were normalized to α-actin. The experiments were repeatedly performed to confirm the results. The data were analyzed using student's t-test. **P < 0.01, significant differences between SD and each treatment group.

As mentioned above, phosphorylation of Akt is important in vivo for improving the insulin sensitivity and exerting an anti-obesity effect through a reduction in adipogenesis via GSK3β. Akt-mediated inhibition of GSK3β inhibits adipogenesis (Ross et al., 2000; Tang et al., 2005). Therefore, we detected that the levels of p-Akt (Ser 473) and p-GSK3α/β (Tyr279/216) in HFD-FTY compared with SD and HFD-CON (Figure 3). In mice fed the SD, the level of phosphorylation of Akt (Ser 473) was high, but obesity was declined. The administration of FTY720 showed a dramatic rise of the activation of Akt. Reciprocally, the phosphorylation of GSK3α/β (Tyr279/216) was decreased in SD and HFD induced an incremental increase, but FTY720 tended to decrease it (Figure 3).

Also, phosphorylation of AMPK is pivotal for increasing energy expenditures such as fatty acid oxidation and for inhibiting energy accumulation such as lipogenesis and adipogenic differentiation.
The role of AMPK in adipogenesis is not entirely clear, but several AMPK-activating molecules have anti-adipogenic effects via MCE inhibition and down-regulation of adipogenic transcriptional pathways (Vingtdeux et al., 2011). Therefore, we measured the expression of p-AMPKα (Thr 172) in HFD-FTY compared with SD and HFD-CON (Figures 4A and 4B). In mice fed with SD, the level of p-AMPKα (Thr 172) was high, but obesity attenuated it. FTY720 showed a significant rise of the phosphorylation of AMPK. In 3T3-L1 cells, FTY720-p increased the level of phosphorylation of AMPK in a dose-dependent manner as well (Figures 4C and 4D).

These observations supported the view that reduced fat accumulation in FTY720 treated mice is a result of reduced adipogenesis through AMPK and Akt- GSK3α/β pathway.

**FTY720 enhances fat breakdown through up-regulation of HSL, ATGL, perilipin and phosphorylation of HSL**

Lipolysis in the WAT of humans and rodents is regulated by ATGL, HSL and perilipin (Gaidhu et al., 2010; Sawada et al., 2010). To investigate whether the markedly decreased adiposity observed in HFD-FTY mice was the result of increased lipolysis, we measured the expression of HSL, ATGL and perilipin in gonadal WAT of SD, HFD-CON and HFD-FTY mice. When compared with the WAT of SD, HFD feeding caused significant down-regulations of HSL, ATGL and perilipin mRNA levels (Figures 5A-5C), but FTY720 treatment increased the mRNA levels of HSL, ATGL and perilipin (Figures 5A-5C). The activity of HSL is regulated by both phosphorylation and translocation to the lipid droplet. PKA phosphorylates HSL at Ser 563 (Yin et al., 2003; Zhou et al., 2011). In accordance with these studies, we detected the HSL phosphorylation at Ser 563 in WAT of SD and HFD-FTY mice (Figures 5D and 5E). In SD mice, HSL phosphorylation at Ser 563 in WAT was expressed at high levels, but HFD feeding markedly dropped the phosphorylation of HSL (Ser 563). The HSL phosphorylation at Ser 563 was considerably increased by the administration of FTY720 (Figures 5D and 5E). These results indicated that decreased adiposity in FTY720 treated mice is a result of enhanced lipolysis through the up-regulation of HSL, ATGL and perilipin, as well as phosphorylation of HSL.

**FTY720-p increases adipocyte lipolysis of 3T3-L1 cells**

To study the effect of FTY720-p on lipolysis in vitro, the differentiated adipocytes were incubated with various concentrations of FTY720-p for 24 h or 1 μM of FTY720-p for various times, and the glycerol level was determined in the medium. FTY720-p treatment led to a significantly higher release of glycerol after the two treatment periods (12 h and 24 h) analyzed (Figure 6A). Also, FTY720-p substantially increased lipolysis in a dose-dependent fashion (Figure 6B).

To assess the effects of FTY720-p on major molecular mechanisms that regulate lipolysis in vitro, we examined the phosphorylation of HSL on key serine residues as well as the mRNA content of ATGL, HSL and perilipin. The differentiated adipocytes were incubated with 1 μM of FTY720-p for 12 h and 24 h, and the ATGL, HSL and perilipin mRNA levels were significantly increased (Figure 6C). After 24 h of treatment with 0.1 and 1 μM of FTY720-p, ATGL, HSL and perilipin expression were enhanced in
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HSL induces lipolysis after phosphorylation of serine residues 563, 659 and 660 (Anthonisen et al., 1998; Shen et al., 1998; Arner, 2005), leading to translocation of HSL to lipid droplets and to a marked enhancement of lipolysis. Phosphorylation of perilipin A, a protein associated with the lipid droplet, by protein kinase A (PKA) has also been demonstrated to be necessary for activation of HSL and for catecholamine-induced lipolysis to occur (Miyoshi et al., 2006). Conversely, the cellular energy sensor AMPK has been demonstrated to phosphorylate serine-565 of HSL, which prevents PKA-mediated phosphorylation of this enzyme and impairs catecholamine-stimulated lipolysis (Garton et al., 1989). While phosphorylation of HSL at Ser 563 was increased, phosphorylation of HSL at Ser 565 was dose-dependently decreased by FTY720-p (Figures 6E and 6F). The effect of FTY720-p was significantly detected at 1 μM from 4 h and was prolonged to 48 h (Figures 6E and 6F). These results revealed the pro-lipolytic activity of FTY720-p in vitro.

Discussion

The data presented here show for the first time that FTY720 acts as an anti-obesity drug, which induces resistance to HFD-induced obesity and that the action of FTY720 is mediated by both anti-adipogenic differentiation and pro-lipolytic action. Administration of FTY720 to HFD-induced obese mice effectively reduced body weights as well as body fat mass by increasing anti-adipogenic action via the regulation of Akt-GSK 3α/β signaling and diminished lipolysis.
via regulation of HSL. in vitro, treatment with exogenous FTY720-p effectively inhibited adipogenesis by down-regulating the expression of adipocyte specific differentiation markers. Moreover, treatment with exogenous FTY720-p significantly enhanced lipolysis by up-regulating the expression and phosphorylation of HSL, ATGL and perilipin.

Excessive adipose tissue accumulation is a result of an increase in both adipocyte number (hyperplasia) that occurs through adipocyte differentiation and adipocyte size (hypertrophy) that occurs when triacylglycerol synthesis exceeds breakdown (lipolysis). From this perspective, FTY720 might behave as a novel anti-obesity drug, because FTY720 effectively prevented HFD-induced obesity, both by blocking the adipocyte number and adipocyte size that through anti-adipogenic action and pro-lipolytic action, respectively.

FTY720 was originally derived as an immunomodulatory compound exerting beneficial effects in several animal models of chronic inflammation (Brinkmann et al., 2004; Chiba, 2005). A large number of preclinical studies have demonstrated the efficacy of FTY720 in models of organ transplantation, autoimmune disease and cancer (Brinkmann et al., 2004). FTY720 has focused over only immune suppression or proliferation, so we investigated other action of FTY720. Recently, consistent with our results (Figure 1), a new study found that FTY720 administration prevents HFD-induced weight gain, insulin resistance and adipose tissue inflammation (Kendall and Hupfeld, 2008). Moreover, this report revealed that resistance to HFD-induced obesity was caused by reduction of adipose tissue lymphocytes and macrophages, as well as the favoring of a CD11c-negative, anti-inflammatory macrophage phenotype, because adipose tissue activated macrophages and lymphocytes contribute to insulin resistance through the secretion of factors that impair insulin signaling (Shoelson et al., 2006; Kendall and Hupfeld, 2008). However, the study only clarified that FTY720 reduced adipose tissue lymphocytes and macrophages, as well as favoring a CD11c-negative, anti-inflammatory macrophage phenotype and focused on only immune modulating function of FTY720 (Kendall and Hupfeld, 2008). Furthermore, the administration dose of FTY720 (0.04 mg/kg twice per week) we used has been shown not to alter the leukocyte profile or the B-cell counts (Nofer et al., 2007). Our results presented here show for the first time previously unknown actions of FTY720, as a negative regulator of adipocyte differentiation (Figure 2) via the up-regulation of p-Akt (Figures 2F and 3) and a positive regulator of lipolysis (Figures 5 and 6) in vivo as well as in vitro.

Akt is related to lipogenesis. HFD-induced hepatic steatosis is correlated with decreased Akt phosphorylation upon insulin treatment (Pinto Lde et al., 2010). Skeletal muscle-specific overexpression of Akt 1 reduces fat accumulation while increasing fatty acid oxidation in the liver with less steatosis (Izumiya et al., 2008). Akt/mTOR-mediated skeletal muscle hypertrophy (Rommel et al., 2001) leads to increased insulin sensitivity (Izumiya et al., 2008), which may be physiologically associated with the alterations in insulin sensitivity of FTY720-treated mice. Moreover, GSK3β is adipogenic (Ross et al., 2000) so its inhibition in FTY720 administration mice may contribute to their leanness. Thus, the role of Akt in lipogenesis is complex and may reflect isoform- and tissue-specific effects.

The molecular machinery involved in triacylglycerol breakdown and fatty acid release works in an orderly and regulated fashion, conferring to WAT the capacity to respond to various feeding conditions and to the energy demands of the body (Gaidhu et al., 2010). Importantly, conditions that lead to overeating and obesity disrupt normal regulation of WAT lipolysis. In fact, basal lipolysis has repeatedly been reported as elevated, whereas catecholamine-induced lipolysis is suppressed in obese humans and rodents (Zimmermann et al., 2004; Jocken and Blaak, 2008). The classical mechanism to explain this condition is centered on the fact that the largely expanded WAT of obese subjects becomes resistant to insulin, impairing the major lipogenic and antilipolytic effects of this hormone (Rodén, 2004; Zimmermann et al., 2004; Arner, 2005; Stumvoll et al., 2005; Gaidhu et al., 2010). In HFD-induced obese mice, HSL and perilipin content are severely reduced (Gaidhu et al., 2010). In line with this report, presently HSL and perilipin mRNA levels were seriously decreased and phosphorylation of HSL at Ser 563 was also markedly reduced in HFD-CON, but FTY720 reversed the content of HSL and perilipin and phosphorylation of HSL at Ser 563 in WAT (Figure 5). Also, FTY720 had effect on inducing lipolysis in vitro. FTY720-p treatment time- and dose-dependently increased glycerol release, lipolytic related gene expression and phosphorylation of HSL at Ser 563. Although increased lipolysis in HFD feeding led to the release of FFA to serum, resulting in insulin resistance terminally, other mouse models displaying increased lipolysis and decreased adipose tissue mass also reported unchanged or reduced serum non-esterified fatty acid (Martinez-Botas et al., 2000; Tansey et al., 2001; Lucas et al., 2003; Hertzel et al., 2006; Jaworski et al., 2009). Despite increased lipolysis resulted in ectopic triacylglycerol accumulation in the liver and skeletal muscle, oxygen consumption is increased and Ucp1, Dio2...
and Ppard mRNA expression in WAT is substantially increased, suggesting higher oxidation and thermogenesis in WAT (Jaworski et al., 2009). Moreover, AMPK has been shown to increase fatty acid oxidation in WAT. AMPK stimulates pathways, which increase fatty acid oxidation (Daval et al., 2006). Presently, HFD decreased the phosphorylation of AMPK, but FTY720 increased the phosphorylation of AMPK in WAT (Figure 4). Therefore, we propose that FTY720 might increase energy production (glucose transport, fatty acid oxidation).

In conclusion, the results of this study show that exposure of HFD-induced obese mice to FTY720 inhibits the development of obesity. There are two suggested mechanisms of FTY720 to resistance to obesity. First, FTY720 inhibits adipogenesis in vitro as well as in vivo, as confirmed by a reduction in triglyceride accumulation, a reduction in the expression of adipocyte specific genes and regulating Akt pathways. Secondly, FTY720 enhances lipolysis in vitro as well as in vivo, as confirmed by an increase in glycerol release and lipolysis-associated gene expressions.

This is the first study to identify the anti-obesity action of FTY720 by the modulation of adipogenesis and lipolysis. These results suggest that FTY720 might be therapeutic for obesity.

Methods

Animals
All studies received approval from the Chonbuk University laboratory animal research center. We used mice on a pure C57BL/6J background. In total, 20 male and 5-week-old C57BL/6J mice initially weighing 20-22 g obtained from Samtako biokorea (Osan, Korea). The animal room was maintained at 23 ± 1°C with alternating 12-h light and dark cycles. After a 1-week acclimatization period, 20 mice were divided randomly into two groups. The normal group was fed with a standard diet (SD), whereas the model group was fed with a HFD. The SD was composed of 10 kcal% fat, 70 kcal% carbohydrates and 20 kcal% protein, whereas the HFD was composed of 45 kcal% fat, 35 kcal% carbohydrate, and 20 kcal% protein (%kcal of total energy). After 3 weeks of feeding with the HFD, the resulting obese mice were subdivided randomly into three groups consisting of five mice each. One was an obese control model group. In the second group, mice were injected intraperitoneally (i.p.) with FTY720 (0.04 mg/kg in phosphate buffered saline (PBS) twice a week for 6 weeks). In both groups, body weight was measured weekly. The third group was administered the same volume (0.1 ml) of only the PBS vehicle. Whole body fat composition of live animals was determined after the treatment period using a Minispec nuclear magnetic resonance analyzer (Bruker Optics, Billerica, MA). The epidymal fat-pad samples were stored at -70°C until they were analyzed. All animal procedures were performed in accordance with the institutional guidelines for the Chonbuk University laboratory animal research center.

Reagents
FTY720 was purchased from Cayman Chemical (Ann Arbor, MI). FTY720 phosphate (FTY720-p) was purchased from Echelon Biosciences (Salt Lake City, UT). FTY720 was prepared as a 1 mg/ml solution in PBS. FTY720-p was prepared as a 2 mM solution in methanol and then further diluted in cell culture medium.

Cell culture and differentiation
The 3T3-L1 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% calf serum and antibiotics (100 μg/ml gentamicin and 100 μg/ml penicillin-streptomycin). To induce differentiation, 2-day post-confluent 3T3-L1 cells were incubated in MDI induction media (DMEM containing 10% fetal bovine serum, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 μM dexamethasone and 1 μg/ml of insulin) for 2 days. In some experiments, FTY720-p (1 μM) was added at the time of the induction of differentiation. Two days after MDI (DAY 2) the media was changed to insulin media.

Quantification of lipid content
Lipid content was quantified using AdipoRed Assay Reagent (Lonza, Verviers, Belgium) according to the manufacturer’s instructions. In brief, adipocytes grown in 24-well plates were incubated with MDI medium alone or medium + test compounds during the adipogenic phase and, on day 7. The cells were then filled with 300 μl PBS and 30 μl of Adipored reagent was added and incubated for 10 min at 37°C. Fluorescence was measured with an excitation and emission wavelength of 485 nm and 572 nm, respectively.

Adipolysis assay
Glycerol release was measured using the Adipolysis Assay Kit (Cayman Chemical) according to the manufacturer’s instructions. Briefly, differentiated adipocytes in a 96-well plate were stimulated with MDI-p for 24 h. After stimulation, the cell culture supernatants were collected from each well and stored until use at -20°C. One hundred microliters of free glycerol assay reagent was added to 25 μl of each supernatant. After incubation for 15 min at room temperature, the absorbance was measured at 540 nm.

Quantitative real-time polymerase chain reaction (qRT-PCR)
Total RNA was extracted from 3T3-L1 cells treated with S1P using the Easy-spin™ total RNA extraction kit (iNtRON Biotechnology, Seoul, Korea). cDNA synthesis was carried out following the instructions of the Prime Script™ 1st strand cDNA synthesis kit (TaKaRa Bio, Tokyo, Japan). For qRT-PCR, 1 μl of gene primers with SYBR Green (Bio-Rad Laboratories, Hercules, CA) in 20 μl of reaction volume was applied. The sequences of the primers used for the
Real-time PCR were as follows: \( \text{PPAR} \) (forward 5′CCGGAAACGCCAAACATGC3′, reverse 5′TGGTGTGAAAGTGCAGAGC3′), C/EBP-\( \alpha \) (forward 5′CCGGAAACGCCAAACATGC3′, reverse 5′TGGTGTGAAAGTGCAGAGC3′), Adiponectin (forward 5′TGGTGTGAAAGTGCAGAGC3′, reverse 5′TGGATCTGTGTCGTGGAA3′), Perilipin (forward 5′CAGGATCCTCAGGGATTG3′, reverse 5′GAAGCTGCCAATTATTGTCAGCTTCTTCAAGG3′), HSL (forward 5′AGACCACATCGCCCACAA3′, reverse 5′CCCTCACAAC3′), p-GSK3\( \beta \) (forward 5′CCCTCACAAC3′, reverse 5′CACATCTCTCGGAGGACCA3′), C/EBP-\( \alpha \) (forward 5′CCGGAAACGCCAAACATGC3′, reverse 5′TGGTGTGAAAGTGCAGAGC3′), Adiponectin (forward 5′TGGTGTGAAAGTGCAGAGC3′, reverse 5′TGGATCTGTGTCGTGGAA3′), Perilipin (forward 5′CAGGATCCTCAGGGATTG3′, reverse 5′GAAGCTGCCAATTATTGTCAGCTTCTTCAAGG3′), and \( \beta \)-actin (forward 5′TGGAGGAAATCGTGGATGC3′, reverse 5′GCTCGTTGCCAATAGTGATGA3′).

All reactions with iTaq SYBR Green Supermix (Bio-Rad Laboratories) were performed on the CFX96 real-time PCR detection system (Bio-Rad Laboratories).

**Western blot**

The 3T3-L1 cells were lysed in a lysis buffer (25 mM HEPES: pH 7.4, 100 mM NaCl, 1 mM EDTA, 5 mM MgCl\( _2 \), 0.1 mM dithiothreitol and protease inhibitor mixture). Proteins were electrophoretically resolved by 8-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting was performed as previously described (Moon et al., 2011). Images were captured using the Fusion FX7 acquisition system (Vilbert Lourmat, Eberhardzell, Germany) (Seo et al., 2012). The antibodies used for immunoblotting were p-Akt (Ser 473) (Epitomics, Burlingame, CA), p-GSK3\( \beta \) (Tyr279/216) (Santa Cruz Biotechnology, Santa Cruz, CA), p-HSL (Ser 565) (Cell Signaling Technology, Beverly, MA), p-HSL (Ser 565) (Cell Signaling Technology) and \( \beta \)-actin (Sigma-Aldrich, St. Louis, MO).

**Statistical evaluation**

All data are expressed as the mean ± SEM, and the data were compared using the Student’s t-test and the ANOVA Duncan test with the SAS statistical package. The results were considered significant for values of \( *P < 0.05 \) or \( **P < 0.01 \).

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