Thesis for the Degree of Master of Science

Effect of Phosphorylated Glucosamine on Adipocyte Differentiation in 3T3-L1 Preadipocytes

by

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The Graduate School

Pukyong National University

February 2009

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Phosphorylated glucosamine이 3T3-L1 지방세포 분화에 미치는 영향

Advisor: Prof. Se-Kwon Kim

by

Jung-Ae Kim

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Abstract

Obesity is related directly or indiectly with several diseases such as diabetes, hypertension cancer, etc. For these reasons, obesity became one of the major social problems. Glucosamine was phosphorylated by phosphorous pentoxide (P_2O_5). Briefly N-phtalamide glucosamine was dissolved in methanesulfonic acid with adding phosphorous pentoxide (P_2O_5) followed by stirring at 5ºC for 4 h. In this study, effects of glucosamine-6-phosphate (Glc6-P) on adipocyte differentiation of 3T3-L1 cells were investigated by measuring triglyceride level and oil red o staining as indicators of lipid accumulation. In order to understand the mechanism by which lipid accumulation in adipocytes is decreased by phosphorylated glucosamine, the expression levels of several genes and proteins associated with adipogenesis and lipolysis were examined by using reverse transcription-polymerase chain reaction (PCR), real-time PCR and western blot analyses. Treatment with phosphorylated glucosamine significantly reduced lipid accumulation during adipocyte differentiation and induced down-regulation of PPARγ, SREBP1 and C/EBPα in a dose-dependent manner. Moreover, treatment with phosphorylated glucosamine during adipocyte differentiation induced significant up-regulation of Pref-1 mRNA as well as down-regulation of the adipocyte specific gene promoters such as aP2, FAS, LPL and leptin. As the lipolytic response, phosphorylated glucosamine up-regulated HSL mRNA expression and suppressed the expression levels of TNFα mRNA compared to fully differentiated adipose tissue. These results suggest that inhibitory effect of phosphorylated glucosamine on adipocyte differentiation might be mediated through the down-regulation of adipogenic transcription factors such as PPARγ, SREBP1c and C/EBPα, related to the downstream of adipocyte specific gene promoters.

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List of Abbreviation

ACC	Acetyl-CoA carboxylase
BAT	Brown adipose tissue
C/EBP	CCAAT/enhancer-binding protein
DEX	Dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
FAS	Fatty acid synthase
FBS	Fetal bovine serum
Glc-6-P	Glucosamine 6 Phosphate
HSL	Hormon sensitive lipase
IBMX	Methylisobutylxanthine (0.5 mM)
LPL	Lipoprotein lipase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PCR	Polymerase chane reaction
PPARγ	Peroxisome proliferator-activated receptor-γ
Pref-1	Preadipocyte factor-1
SREBP	Sterol regulatory element binding proteins
TG	Trigliceride
TNF-α	Tumor necrosis factor alpa
VLDL	Very-low-density lipoproteins
WAT	White adipose tissue

Introduction

The worldwide obese population has been increasing dramatically and obesity has become one of the serious public health problems. The obesity defined as an excessive body weight in the form of fat. Obesity causes numerous diseases such as type 2 diabetes, hypertension, cancer, and osteoarthritis (Xavier et al., 2002). As a result, obesity has been found to reduce life expectancy. Cause of Obesity is related to energy intake compared with energy expenditure such as person's basal metabolic rate and level of physical exercise. Psychiatric illness is contributing to obesity but cases are limited.

To maintain energy balance in physiological system and avoidance of obesity would be expected to have several requirements (Bruce et al., 1996). The first requirement is the level of energy stores in adipose tissue by using energy incoming protein such as Lipoprotein lipase (LPL), glucose transporter 4 (GLUT4), and fatty acid trasproter. Most of all, LPL play an important role in differentiation of adipocyte and increased gene expression level in initial stage of adipogenesis. Adipose tissue lipoprotein lipase (LPL) hydrolyzes TG in chylomicrons, and very-low-density lipoproteins (VLDL) to free fatty acids and monoacylglycerols, which are transported to fat cells. Free fatty acids and monoacylglycerols are stored as TG in fat cells via re-esterification. Thus, adipose tissue LPL is regulated TG deposition in adipocyte. (Yamaguchi et al., 2002). Adipose tissue LPL activity differs in different conditions, for example with the nutritional state and on induction of diabetes mellitus (Bergo et al., 1996; Bergo et al., 2007). Starvation causes decrease of LPL activity in adipose (Lacasa et al., 1991).

The second requirement is a mechanism whereby such a signal can be received, most likely in the central nervous system. The hypothalamus has been known to be a key

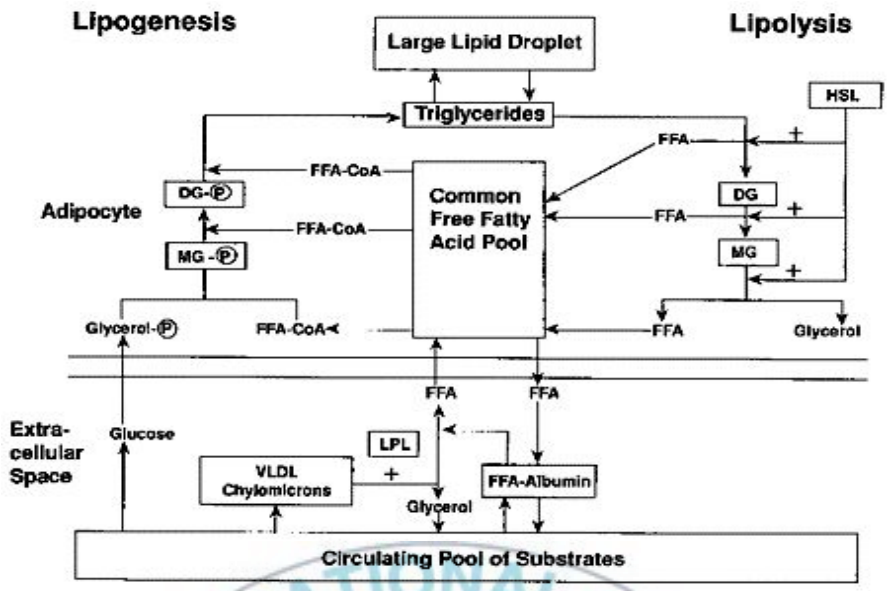


Figure 1. Diagrammatic representation of triglyceride storage (lipogenesis) and breakdown (lipolysis) in adipocytes. (Leibel et al., 1983).

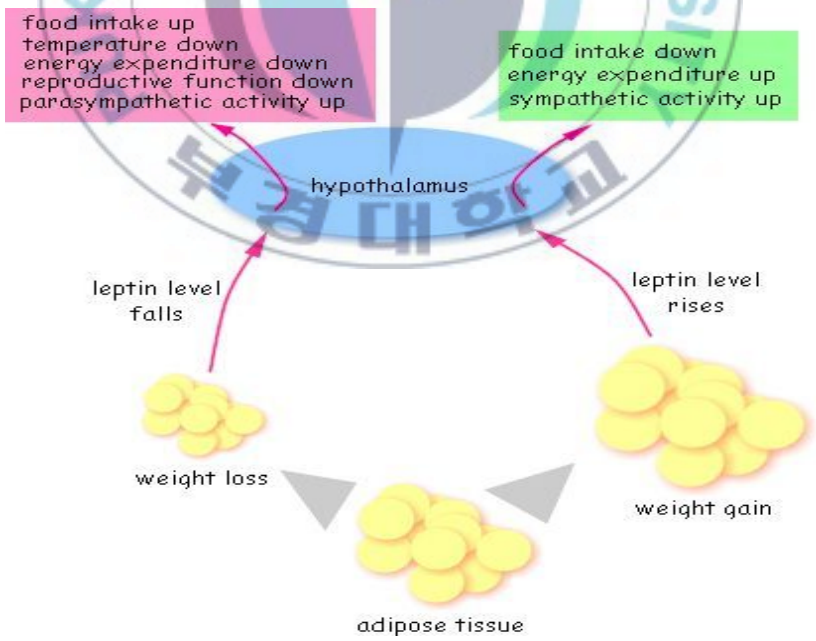


Figure 2. Control of leptin secretion and synthesis.

brain region to regulate energy balance. Identification of the leptin receptor as the product of another obesity gene and demonstration of its expression in the hypothalamus (Islam1et al., 2000), provides a key link between peripheral signals and the central pathways involved in the regulation of energy balance (Bruce et al., 1996). Leptin is secreted form adipocyte with an important function in regulating food intake and energy expenditure (Hollung et al., 2004). An important function of leptin is probably to protect tissues from overload of TG by stimulation of fatty acid oxidation (Wang et al., 1999). So deficiency of leptin or leptin receptor result obesity. However, most human obesity correlated with elevated leptin levels. An increases plasma leptin levels are directly proportional to body fat as assessed by BMI and even more tightly with fat cell size (Lee et al., 2007).

The third requirement is consuming processes that are related to the support of cellular functions for the purpose thermogenesis. In the body, there are two types of adipose tissue termed white adipose tissue (WAT) and brown adipose tissue (BAT). WAT stores excess energy as triglycerides and releases free fatty acids. BAT is involved in non-shivering and diet-induced thermogenesis through the uncoupling of oxidative phosphorylation, which is carried out by the action of a BAT-specific uncoupling protein (UCP). WAT is distributed in a number of locations throughout the body. In contrast, BAT is restricted to specific areas. WAT consists of adipocytes having a single fat droplet within the cells, whereas BAT has a multi locular disposition of fat droplets, i.e. a number of individual droplets within each adipocyte. Adipocyte differentiation is a complex process involving a cascade of expression of many transcription factors and adipocyte-specific genes (Takashi et al., 1997)

PPARγ controlled lipid homeostasis through enhance adipocyte differentiation and have been identified as a major transcription factor of adipogenesis (Rosen et al.,

2000). To regulate adipocyte differentiation, PPARγ coexpression with other transcription factors such as C/EBPs and SREBPs. These transcription factors effect the complex changes together in gene expression necessary for the development of the functional adipocyte (Robert et al., 2002). During adipocite differentiation PPARγ is highly expressed in adipose tissue. The expression of PPARγ in fibroblasts results in intracellular accumulation of lipid droplets, features that are specific for mature white adipocytes (Tontonoz et al., 1994). Recent studies reveal that mutation of PPARγ inhibited adipogenesis in cultured preadipocytes (Gurnell et al., 2000).

The other transcription factor is CCAAT/ enhancer-binding proteins (C/EBPs) in adipocyte gene expression and differentiation. The C/EBPs belong to the large family of basic leucine zipper (bZip) transcription factors. There are six members of C/EBPs have been reported and characterized: C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε, and CHOP (Takashi et al., 1997). These proteins can bind each other both homodimerize and heterodimerize to the same C/EBP consensus sequences. According to them, C/EBPα, β, and δ are expressed in both WAT and BAT and have been investigated extensively in adipogenesis (Manchado et al., 1994).

Sterol regulatory element binding proteins (SREBPs) are also important transcription factors that regulate the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids (Shimomura et al., 2000). In the liver three SREBPs (SREBP-1α, SREBP-1c, and SREBP-2) modulate the production of lipids for export as lipoproteins and as bile. The promoter of the SREBP-1c gene contains response elements for insulin, glucagon and liver X-activated receptors (LXR). Knockout mice that lack all SREBPs die at an early stage of embryonic development (Mohamed et al., 2007). SREBP-1α expressed in most tissues but its stimulator has not been identified (Zhou et al., 2001). Over expression of SREBP-1c produces a triglyceride-enriched fatty liver, while

SREBP-2 over expression resulted that increases cholesterol synthesis (Matsuda et al., 2001). Insulin treatment increases the amount of SREBP-1c. SREBP-1α expressed in most tissues. Furthermore, SREBP-1c may also contribute to the regulation of glucose uptake and glucose synthesis. Thus, SREBP-1c may play a critical role in the regulation of hepatic glucose production and triglyceride involved in lipid accumulation.

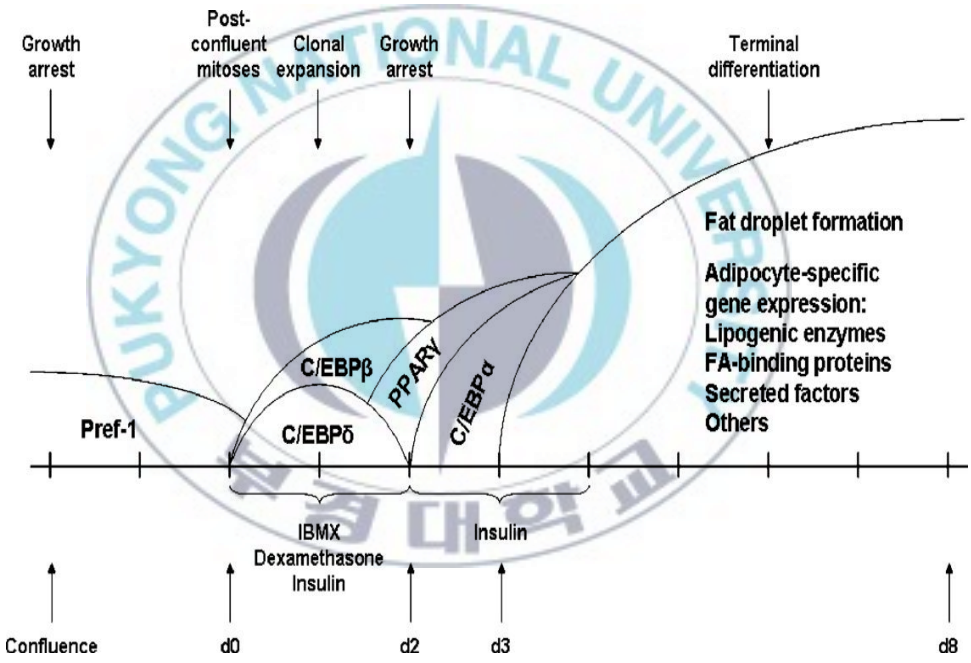


Figure 2. The transcriptional control of adipogenesis in 3T3-L1 preadipocyte maturation stages and related-gene expression. C/EBP: CCAAT/enhancer binding protein, FA: fatty acid, PPARγ : peroxisome proliferator-activated receptor (pascal et al., 2008)

Perilipins are phosphoproteins that regulate lipolytic signaling through interaction with hormone- sensitive lipase (HSL). Overexpression of perilipin A (periA) in fibroblasts increases storage of triglyceride associated with a decrease in lipolysis (Susan et al., 2005; Yanxin et al., 2003). Hormone-sensitive lipase (HSL) is an 84- kDa cytoplasmic protein of adipocytes, in which it catalyzes the hydrolysis of triacylglycerols. In adipocytes, HSL mediated lipolysis is activated via a series of events, including b-adrenergic stimulation, a complex combination of cyclic adenosine monophosphate-dependent serine phosphorylations mediated by protein kinase A (Su et al., 2001; Kelehmainen et al., 2002).

Preadipocyte factor-1 (pref-1) is an epidermal growth factor (EGF) repeat domain-containing transmembrane protein and exists as multiple discrete forms with a molecular weight range of 45–60 kDa in preadipocytes (Nicholson et al., 2007). However, pref-1 expression levels are completely abolished after adipocyte differentiation (Smas et al., 1993). The induction of adipocyte differentiation by glucocorticoids is also partially due to the suppression of pref-1 expression (Smas et al., 1999). A recent study has been reported that pref-1 inhibits expression of PPARγ and C/EBPα (Kim et al., 2007).

Tumour necrosis factor (TNF-α) is an important regulating factor of obesity (Bullo et al., 1999). TNF-α produced by the adipocyte that can help limit obesity but there are no direct evidence to support this. TNF-α inhibit LPL activity, the down regulation of the expression of the glucose transporter GLUT, the inhibition of insulin receptor activity, and the induction of adipose leptin production in cultured cell and animals (Bullo et al., 2002). In obese humans, over expression of TNF-α is proportional to the extent of the fat depot in adipose tissue. However, lower production of TNF-α is involved in the maintenance and progression of obesity (Kern et al., 1995). TNF-α inhibits the synthesis of lipoprotein lipase, ACC, and FAS (key enzymes involved in

lipogenesis) and induces lipolysis and apoptosis. Despite of these highly regulated physiological system, obesity still increasing.

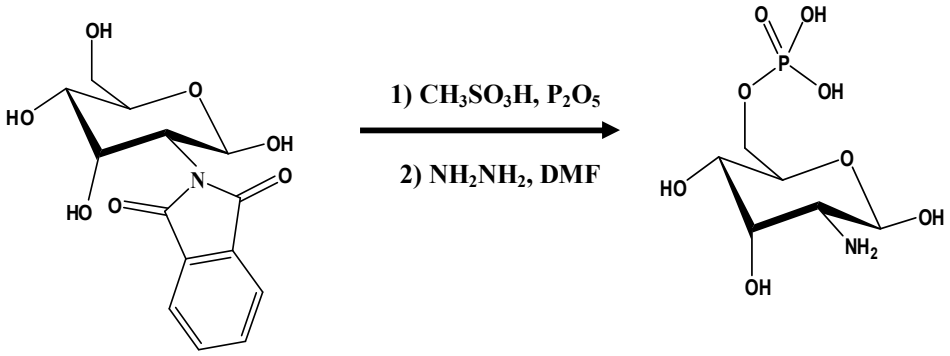
Glucosamine (Glc) is an amino sugar which has been tested widely for therapeutic effectiveness in the treatment of arthritis and well tolerance in patients. Mumerous clinical trials have tested widely for glucosamine supplements. Three kinds of glucosamine such as glucosamine hydrochloride, sulfated glucosamine, and N-acetyl-glucosamine are generally used as glucosamine nutritional supplements (Kim et al., 2007) Among them, the positive effect of sulfated glucosamine on MMP and osteoblastic differentiation has been reported (Rajapakse et al., 2007). Threse glucosamines can be derived from chitin, which is biopolymer included in exoskeleton of marine invertebrate animals. Chitosan obtained by alkaline deacetylation of the chitin has also attended considerably for its commercial applications in biomedical, food, and chemical industries. Among derivatives of chitosan, phosporylated chitosan oligosaccharide exhibited the high inhibitory activity on the formation of calcium phosphaed (Kim et al., 2005). Therefore, it could be expected that phosphorylation of glucosamine contribute to some kind of biomedical activity.

In this study, we synthesised phosphorylated glucosamine (glucosamine-6-phosphate, Glc-6-P) and investigated its effects on lipid accumulation in cultured 3T3-L1 adipocytes by measuring triglyceride (TG) contents and Oil-Red O staining as indicators of lipid accumulation. To understand the mechanism by which phosphorylated glucosamine decreases lipid accumulation in adipocytes, the expression levels of several genes and proteins associated with adipogenesis and lipolysis were examined by using reverse transcription-polymerase chain reaction (PCR), real-time PCR and western blot analyses.

Experimental Procedure

1. Synthesis Gulcosamine-6-Phosphate

Gulcosamine-6-phosphate was phosphorylated by phosphorous pentoxide (P_2O_5) (Nishi et al., 1986). Briefly, N-phtalamide glucosamine (1 mmol) was dissolved in 10 ml of methanesulfonic acid with adding phosphorous pentoxide (P_2O_5) and followed by stirring at 5ºC for 4 h. The products were precipitated with ether and centrifuged. The precipitates were air-dried and dissolved in DMF (50 ml), hydrazine monohydrate (20 ml) and water (40 ml) in order to remove the residue phtalamide. The mixture was heated to 100°C under N_2 atmosphere with stirring. After reaction for 15 hr, the suspension was filtrated, 50 ml of water was added into the filter layer and then the filtrates were dried in vacuum with a rotary evaporator. 50 ml of water was added into the dried product. The resultant solution was cooled and then dialyzed exhaustively against distilled water using a 100 Da molecular weight cut-off dialysis membrane. Small amount of non-reacted glucosamine and impurities were removed following cation-exchange chromatography using a Dowex-50 cataion exchange resin.



N-Phtalamide glucosamine **Glucosamine-6-phosphate**

Figure 4. Synthesis pathway of phosphorylated glucosamine.

2. Cell culture and adipocytes differentiation

Mouse 3T3-L1 preadipocytes were grown to confluence in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) at 37ºC in a humidified atmosphere of 5% CO_2. At 1 day postconfluence (designated "day 0"), cell differentiation was induced with a mixture of methylisobutylxanthine (0.5 mM), dexamethasone (0.25 μM), and insulin (5 μg/ml) in DMEM containing 10% FBS. After 48 h (day 2), the induction medium was removed and replaced by DMEM containing 10% FBS supplemented with insulin (5 μg/ml) alone. This medium was changed every 2 days. Samples were treated into culture medium of adipocytes at day 0 day. After the 4 days treatment of glucosamine and glucosamine-6-phosphate, the medium was removed for analysis of leptin.

3. Cell viability assay

Cytotoxicity of glucosamine and glucosamine-6-phosphate was evaluated by MTT assay, a method based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Weislow et al., 1989), after the incubation with glucosamine and glucosamine-6-phosphate for 4 days.

The medium containing 3T3L-1 cells were cultured into a 48-well plate at a density of 10^5 cells/ml. The plate was incubated overnight and treated with 100 μl of DMEM medium containing different concentrations. After 72 h of incubation, 100 μl of 500 μg/ml MTT solution was added to each well and the plate was incubated for another 4 hours at 37ºC. The blue formazan salt was dissolved in 50% DMSO and 4% triton X-100. Optical density was measured at 540 nm with a GENios microplate reader (Tecan, Austria GmbH, Austria). The optical density of formazan formed by untreated cells was taken as 100% of viability.

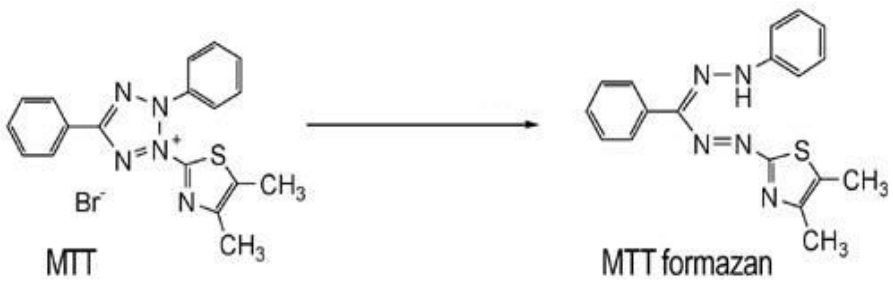


Figure 5. Molecular structure of MTT and its corresponding reaction product

4. Apoptotic assay using Hoechst 33258 staining

Cells were fixed with 3.7% buffered neutral formalin for 10 min, rinsed with phosphate-buffered saline three times and incubated in 1 μM Hoechst 33258 (Sigma) for 30 min in the dark. After another three washes with phosphate-buffered saline for 5 min each, cells were observed with a fluorescence microscope equipped with a 4', 6-amidino-2-phenylindole filter. To quantify apoptosis, the preparation was examined under × 100 magnification.

5. Oil Red O staining

For Oil-Red O staining (McNeel and Meramann, 2003, Havel PJ, 2000), cells were washed gently with phosphate buffered saline (PBS) twice, fixed with 3.7% fresh formaldehyde (Sigma) in PBS for 1 h at room temperature, and stained with filtered Oil-Red O solution (60% isopropanol and 40% water) for at least 1 h. After staining of lipid droplets with red, the Oil Red O staining solution was removed and the plates were rinsed with water and dried. Images were collected on an Olympus microscope (Tokyo, Japan). Finally, the dye retained in the cells was eluted with isopropanol and quantified by measuring the optical absorbance at 500 nm.

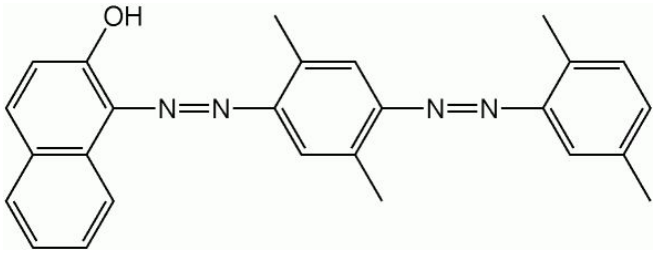


Figure 6. Chemical structure of Oil red O

6. Measurement of triglyceride content

Cellular triglyceride contents were measured using a commercial triglyceride assay kit (Triglyzyme-V**,** Eiken Chemical, Tokyo, Japan) according to the manufacturer's instructions. Cells were treated with glucosamine-6-phosphate with the concentration of 50, 100, and 200 μg/ml in 12-well plates during the adipocyte differentiation for 6 days. The cells were washed twice with PBS, scraped in 75 μl of homogenizing solution (154 mM KCl, 1 mM EDTA, 50 mM Tris, pH 7.4), and sonicated to homogenize the cell suspension. The residual cell lysate was centrifuged at 3000 x *g* for 5 min at 4ºC to remove fat layer. The supernatants were assayed for triglyceride content and protein content. Triglyceride was normalized to protein concentration determined by the BSA as standard. Results were expressed as milligrams of triglyceride per milligram of cellular protein.

7. RNA extraction and reverse transcription-polymerase chain reaction

Total RNA was isolated using a Trizol reagent (Invitrogen Co., CA, USA) following the manufacture's recommendations. Total RNA was digested with RNase-free DNase (Roche, IN, USA) for 15 min at 37ºC and repurified by the RNeasy kit according to the manufacture's protocol (Quiagen, CA, USA). cDNA was synthesized from 2 μg total

RNA. By incubation at 37ºC for 1 h with AMV reverse transcriptase (Amersham) with random hexanucleotide according to the manufacture's instruction. Primers to specifically amplify the genes interested were showed in Table 4. Amplification was performed in a master-cycler (Eppendorf, Hamburg, Germany) with cycles of denaturation at 94ºC 30 sec, annealing at 58ºC 30 sec, and extension at 72ºC for 30 sec, respectively. The amplified PCR products were run in 1.0% agarose gels and visualized by ethidium bromide (EtBr).

Table 1: Chemicals used for reverse transcription PCR

PCR Chemical	Amount used	Stock
MMLV reverse transcriptase	0.5 µl	200 U/ µl
dNTP mixture	1 µl	10 mM
DTT	1 µl	100 mM
5X reaction buffer	4 µl	
RNase inhibitor	0.5 µl	80 U/ µl

Table 2: RT-PCR conditions

Temperature	Time
42ºC	1h 30 min
95ºC	5 min
4ºC	∞

Table 3. Chemicals used for PCR reaction

PCR Chemical	Amount used	Stock
Taq polymerase	0.5 µl	5 U/ µl
dNTP	2 µl	2.5 mM
Reverse Primer	0.5 µl	50 pmole/ µl
Forward Primer	0.5 µl	50 pmole/ µl
10X reaction buffer	2 µl	
DW	10.5 µl	

Table 4. PCR conditions

Temperature	Time	Cycle
94ºC	5 min	1
94ºC 61ºC 72ºC	45 sec 45 sec 1 min	30
72ºC	10 min	1
4ºC	∞	

Table 5. Gene-Specific Primers Used for the RT-PCR and Real-Time RT-PCR

Gene	Direction	Sequence
PPARγ	Forward	5'-TTT TCA AGG GTG CCA GTT TC-3’
	Reverse	5'-AAT CCT TGG CCC TCT GAG AT-3’
SREBP1c	Forward	5'-TGT TGG CAT CCT GCT ATC TG-3'
	Reverse	5'-AGG GAA AGC TTT GGG GTC TA-3'
C/EBPα	Forward	5’-TTA CAA CAG GCC AGG TTT CC-3’
	Reverse	5’-GGC TGG CGA CAT ACA GTA CA-3’
Pref-1	Forward	5’-CTA ACC CAT GCG AGA ACG AT-3’
	Reverse	5’-GCT TGC ACA GAC ACT CGA AG-3’
aP2	Forward	5’-TCA CCT GGA AGA CAG CTC CT-3’
	Reverse	5’-AAT CCC CAT TTA CGC TGA TG-3’
Leptin	Forward	5’-GGA TCA GGT TTT GTG GTG CT-3’
	Reverse	5’-TTG TGG CCC ATA AAG TCC TC-3’
LPL	Forward	5’-TCC AAG GAA GCC TTT GAG AA-3’
	Reverse	5’-CCA TCC TCA GTC CCA GAA AA-3’
FAS	Forward	5’-TTG CTG GCA CTA CAG AAT GC-3’
	Reverse	5’-AAC AGC CTC AGA GCG ACA AT-3’
HSL	Forward	5’-GAG GGA CAC ACA CACACC TG-3’
	Reverse	5’-CCC TTT CGC AGC AAC TTT AG-3’
Perlipin	Forward	5’-AAG GAT CCT GCA CCT CAC AC-3’
	Reverse	5’-CCT CTG CTG AAG GGT TAT CG-3’
TNFα	Forward	5’-AGG CCT TGT GTT GTG TTT CCA-3’
	Reverse	5’-TGG GGG ACA GCT TCC TTC TT-3’
β-actin	Forward	5'- CCA CAG CTG AGA GGG AAA TC-3'
	Reverse	5'-AAG GAA GGC TGG AAA AGA GC-3'

8. Real-time RT-PCR analysis of mRNA expression

Gene expression was measured by real time RT-PCR in an ExiCycler (Bioneer, Daejeon, Korea). Briefly, total RNA was isolated from 3T3-L1 adipocytes using Trizol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA synthesis was performed with 1 μg of total RNA using Superscript II reverse transcriptase (BD Bioscience, Palo Alto, CA). One microliter of each RT reaction was amplified in a 20 μL PCR assay volume containing 2.0 mM $MgCl_2$, 0.5 μM each primer (Table 5), and 1× Greenstar™ PCR Master Mix (Bioneer). Samples were incubated in the ExiCycler for an initial denaturation at 94ºC for 10 min, followed by 40 PCR cycles. Each cycle proceeded at 94ºC for 40 sec, 59ºC for 30 sec, and 72ºC for 30 sec. Relative quantification was calculated using the 2-($\Delta\Delta$ C_T) method.

To confirm amplification of specific transcripts, melting curve profiles (cooling the sample to 40ºC and heating slowly to 95ºC with continuous measurement of fluorescence) were produced at the end of each PCR.

9. Western blot analysis

Western blotting was performed according to standard procedures. Briefly, cells were lysed in RIPA buffer containing 50 mM Tris–HCl (pH 8.0), 0.4% Nonidet P-40, 120 mM NaCl, 1.5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 80 μg/ml leupeptin, 3 mM NaF and 1 mM DTT at 4°C for 30 min. Cell lysates (50 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech., England, UK), blocked with 5% skim milk, and hybridized with primary antibodies(diluted 1:1000). After incubation with horseradish-peroxidase-conjugated secondary antibody at room temperature, immunoreactive proteins were detected using a chemiluminescent ECL assay kit (Amersham Pharmacia

Biosciences, England, UK) according to the manufacturer's instructions. Western blot bands were visualized using a LAS3000® Luminescent image analyzer (Fujifilm Life Science, Tokyo, Japan).

10. Statistical analysis

Data were expressed as mean ± SE and analyzed using the analysis of variance (ANOVA) procedure of Statistical Analysis System (SAS Institute, 1999-2001). Significant differences between treatment means were determined by using Duncan's multiple range tests. Significance of differences was defined at the $p<0.05$ level. Each experiment was replicated at least 3 times.

Results

1. Synthesis of phosphorylated glucosamine

Synthesized glucosamine-6-phosphate was identified by IR (Figure 7), ^{1}H NMR (Figure 8), ^{13}C NMR (Figure 9) and ^{31}P NMR (Figure 10) spectrums.

Glc-6-P: white solid; IR (KBr) ν_{max} 3412 (OH), 2864 (CH), 1578 (NH), 1309 (CN), 1200 (P=O), 1000 (POC), 900 (PO); ^{1}H NMR (D_2O, 400 MHz) δ 5.33, 4.83 (1H, H-1_α, H-1_β), 3.22, 3.0 (1H, H-2_α, H-2_β), 4.0-3.47 (1H, H-3, 1H, H-4, 1H, H-5 and 2H, H_2-6), 4.7 (D_2O); ^{13}C NMR (D_2O, 400MHz) δ 89.40, 92.98 (C-1_α, C-1_β), 54.4,56.84(C-2_α, C-2_β), 69.40 (C-3), 75.22 (C-4), 75.22,70.82 (C-5_α, C-5_β), 63.90 (C-6).

2. Effect of Phosphorylated glucosamine on viability of 3T3-L1 cells

Cytotoxicity of phosphrylated glucosamine on the viability of 3T3-L1 adipocyte cell lines were investigated using MTT assay and Hoechst staining. 3T3-L1 cells were treated with or without Glc-6-P in various concentrations. None of the Glc-6-P did exhibit any significant cytotoxicity as expected (Figure 11). Results obtained from MTT assay and Hoechst staining revealed that Glc-6-P is safe compounds for *in vitro* cell culture experiments.

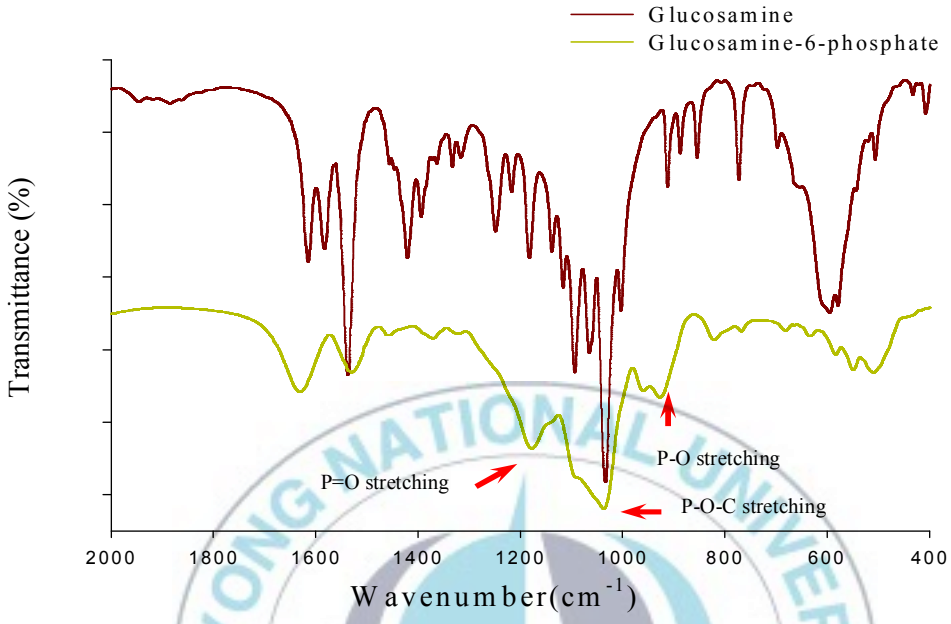


Figure 7. IR spectrum of Glc-6-P.

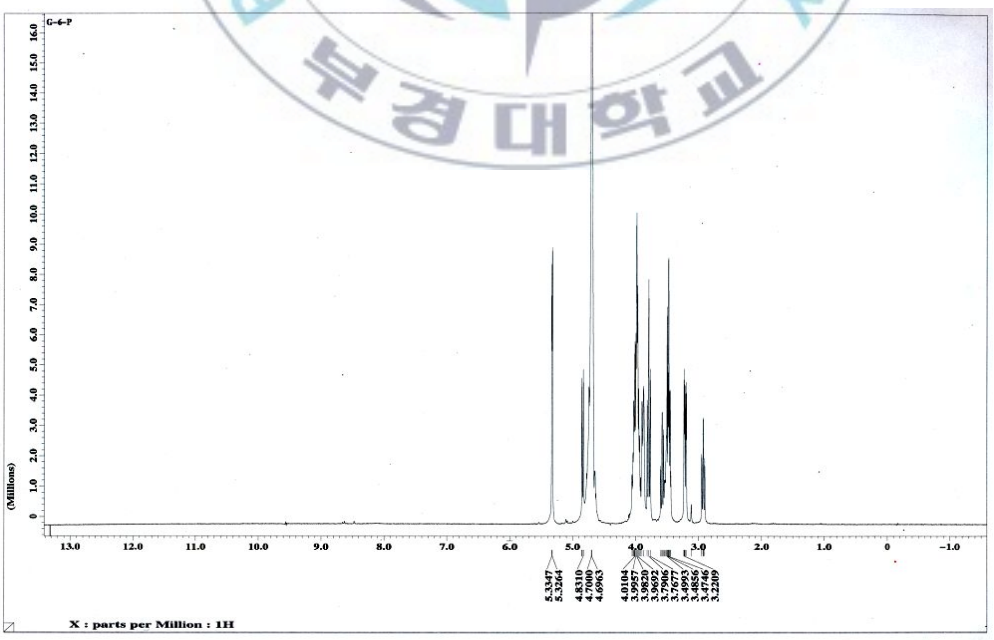


Figure 8. ^{1}H-NMR spectrum of Glc-6-P.

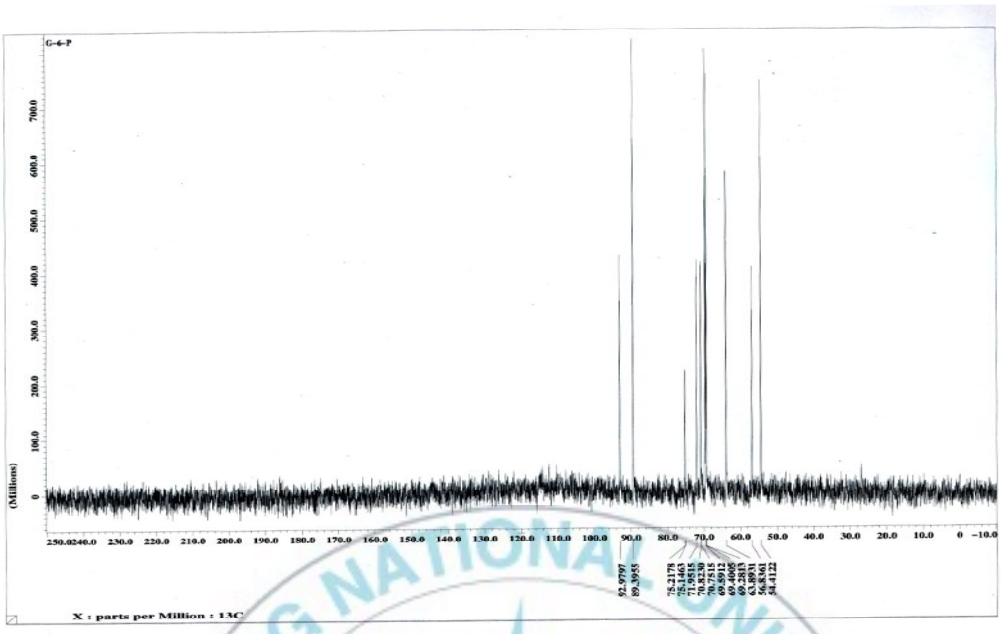


Figure 9. ^{13}C-NMR of Glc-6-P.

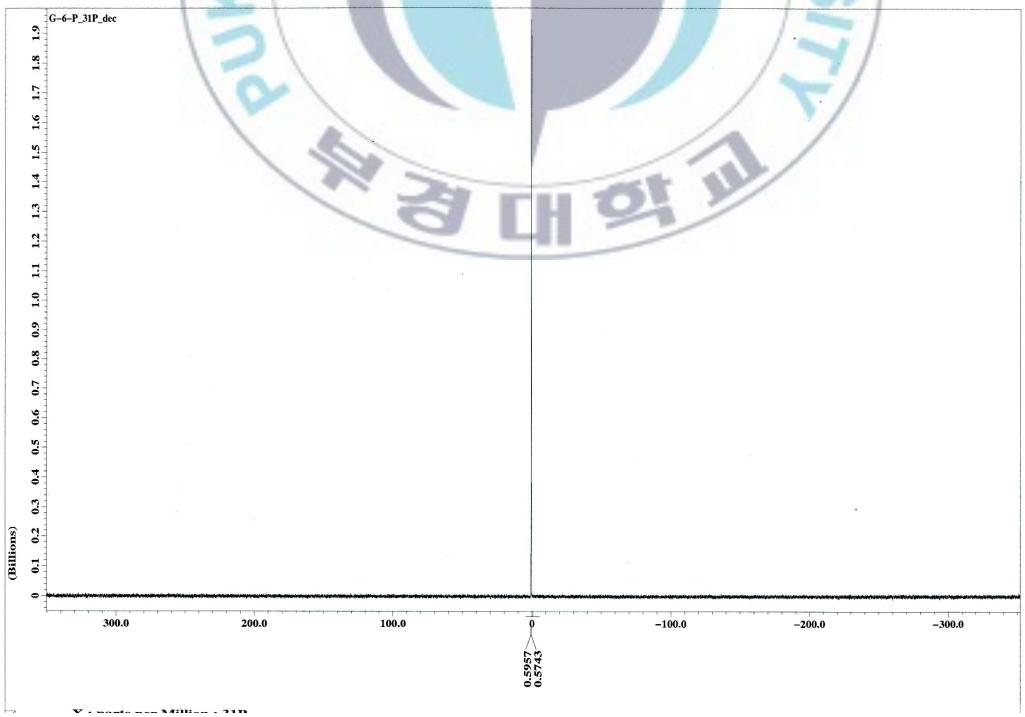
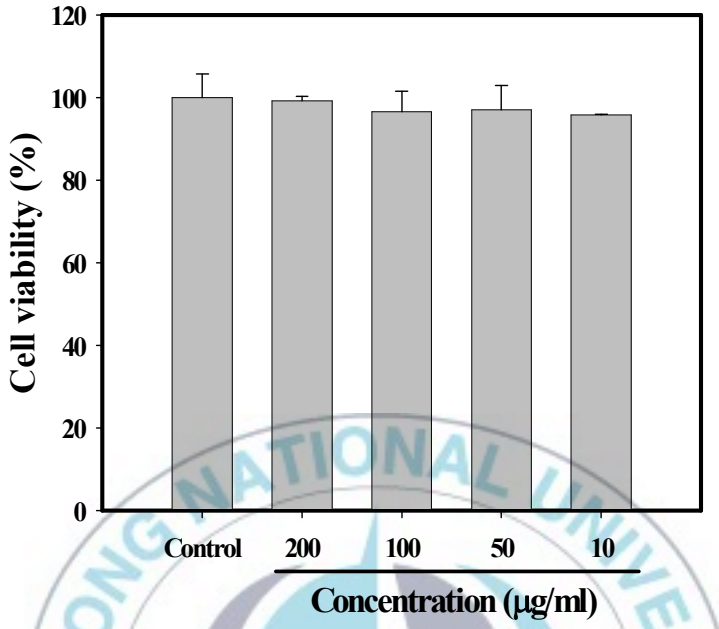


Figure 10. ^{31}P-NMR spectrum of Glc-6-P.

(A)



(B)

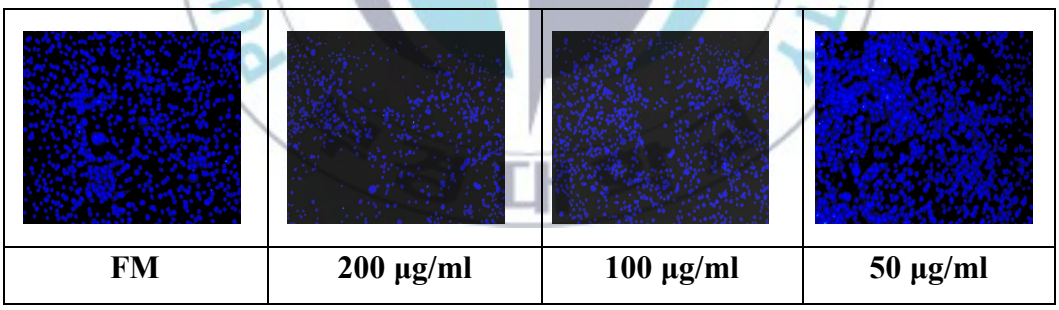


Figure 11. Cytotoxic effect of Glc-6-P in 3T3-L1 cells using MTT assay (A) and Hoechst 33258 staining (B). 3T3-L1 preadypocyte and adipocyte cells were treated with different concentration of Glc-6-P for 24 h and during differentiation for 6 days, respectively.

3. Effect of phosphorylated glucosamine on lipid accumulation in 3T3-L1 adipocytes

To explore the anti-adipogenic potential of phosphorylated glucosamine, 3T3-L1 preadipocyte were differentiated with phosphorylated glucosamine for 6 days (from day 0 to day 6). Lipid accumulation as a major marker of adipogenesis was quantified by direct triglyceride (TG) measurement and Oil Red O staining (Figures 12-14). Treatment with phosphorylated glucosamine reduced TG content of differentiated adipocyte lysate in a dose-dependent manner ($p<0.05$), but glucosamine did not. We also stained triglycerides of fully differentiated adipocytes with Oil Red O staining solution. OD value of Oil Red O eluted solution represents lipid droplet accumulation in the cytoplasm. Phosphorylated glucosamine inhibited lipid accumulation in the cytoplasm of treated cells in a dose-dependent manner ($p<0.05$). The OD value of eluted dye decreased according to the concentrations, which means that phosphorylated glucosamine inhibits adipogenesis during the adipocytes differentiation.

4. Inhibition of adipogenesis

To determine whether phosphorylation of glucosamine affects the expression of transcriptional factors, RT-PCR, and western blotting analyses were conducted (Figures 15-17). Although glucosamine did not had any effect on down-regulation of PPARγ, C/EBPα, and SREBP1c expressions, phosphorylation of glucosamine induced significant down-regulation in a dose-dependent manner compared to differentiated adipocytes without sample treatment. Treatment with Glc-6-P also induced down-regulated protein expressions of PPARγ, C/EBPα, and SREBP1c in a dose-dependent manner. The down-regulation of C/EBPα, and SREBP1c by treatment with GLC-6-P might reduce fatty acid synthesis as well as the synthesis and activity of PPARγ, resulted in inhibition of lipid accumulation by blocking adipogenesis.

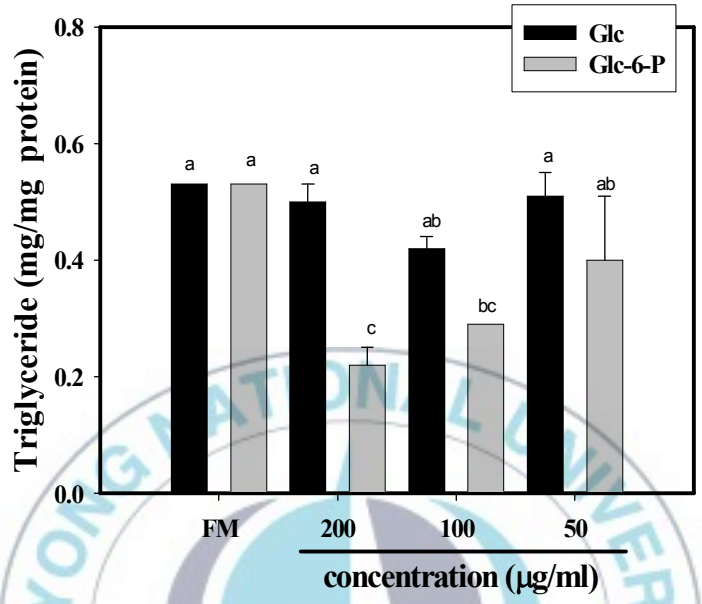


Figure 12. Effects of glucosamine and phosphorylated glucosamine on TG consumptions in 3T3-L1 cells. Cells were cultured in the medium contained with 50, 100, and 200 μg/ml from day 0 to day 6. Data are mean ± standard error values (n = 3). [a-c] Means with the different letters are significantly different (p<0.05) by Duncan's multiple range test.

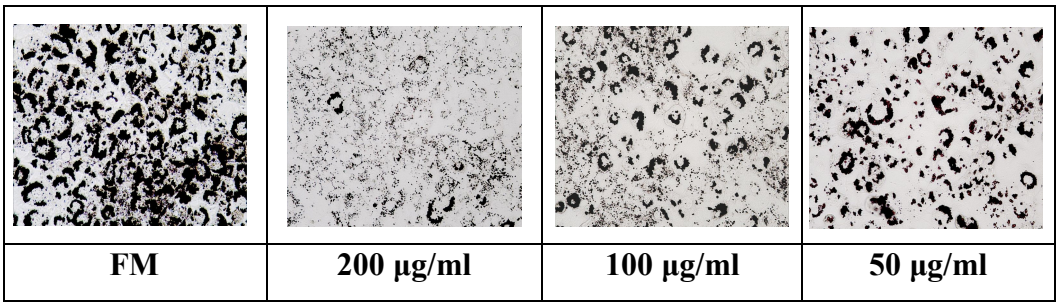


Figure 13. Microscopic image of 3T3-L1 cell morphology after Oil red O staining.

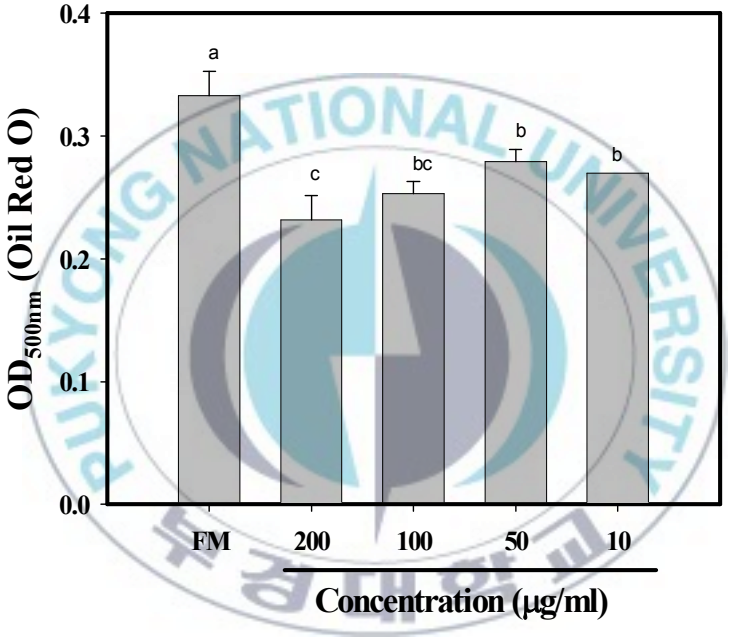


Figure 14. Effects of glucosamine and phosphorylated glucosamine on adipocyte differentiation in 3T3-L1 cells. Cells were cultured in the medium contained with 50, 100, and 200 μg/ml from day 0 to day 6. This data shows OD values of eluted dye. Data are mean ± standard error values (n = 3). $^{a-c}$ Means with the different letters are significantly different (p<0.05) by Duncan's multiple range test.

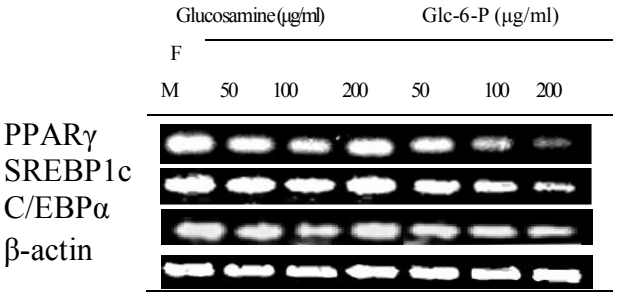


Figure 15. Effects of glucosamine and phosphorylated glucosamine on PPARγ, C/EBPα, and SREBP1c mRNA and protein expressions in 3T3-L1 cells.

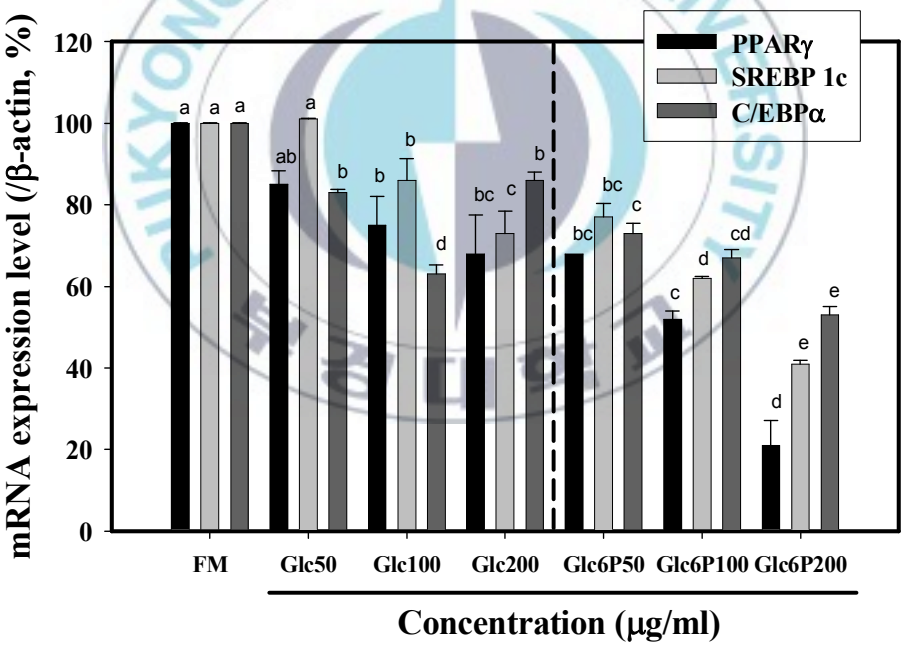


Figure 16. Effects of glucosamine and phosphorylated glucosamine on PPARγ, C/EBPα, and SREBP1c mRNA and protein expressions in 3T3-L1 cells. Glucosamine and phosphorylated glucosamine were treated into differentiated adipocyte for 6 days. [a-e] Means with the different letters are significantly different (p<0.05) by Duncan's multiple range test.

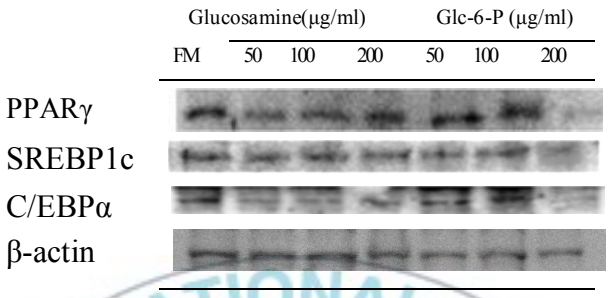


Figure 17. Effects of glucosamine and phosphorylated glucosamine on PPARγ, C/EBPα, and SREBP1c protein expressions in 3T3-L1 cells. Glucosamine and phosphorylated glucosamine were treated into differentiated adipocyte for 6 days.

5. Effect of phosphorylated glucosamine on pref-1, aP2, FAS, LPL, and leptin mRNA expression

We further studied whether the phosphorylated glucosamine regulates the expressions of adipogenic target genes such as ap2, FAS, LPL, and leptin as well as Pref-1 gene of characteristic of an undifferentiated state. Treatment with phosphorylated glucosamine during adipocyte differentiation induced significant up-regulation of Pref-1 mRNA and down-regulation of the expression level of aP2 mRNA (Figures 18, 19). Moreover, the expression levels of FAS, LPL, and leptin mRNA were also dose-dependently suppressed by treatment with phosphorylated glucosamine (Figures 20, 21).

6. Effect of phosphorylated glucosamine on lipolysis and inhibition of TNFα mRNA expression

Since phosphorylated glucosamine inhibited lipid formation during adipocytes differentiation, we explored whether the reduction effect of phosphorylated glucosamine on lipid accumulation is associated with lipolysis. As the lipolytic response during differentiation of adipocytes, the gene expression levels of perilipin, HSL, and TNFα were determined by using reverse transcription-polymerase chain reaction (Figures 22, 23) and real-time PCR (Figures 24, 25). Treatment with phosphorylated glucosamine up-regulated HSL mRNA expression, and down-regulated perlipin mRNA expression. Also, treatment with phosphorylated glucosamine induced down-regulation of TNFα mRNA expression compared to fully differentiated adipose tissue. We can assume that the inhibition of phosphorylated glucosamine on lipid accumulation is not mediated by TNFα.

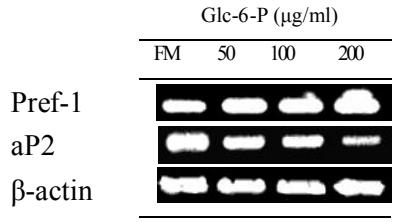


Figure 18. Effect of phosphorylated glucosamine on pref-1 and aP2 mRNA expressions in 3T3-L1 cells.

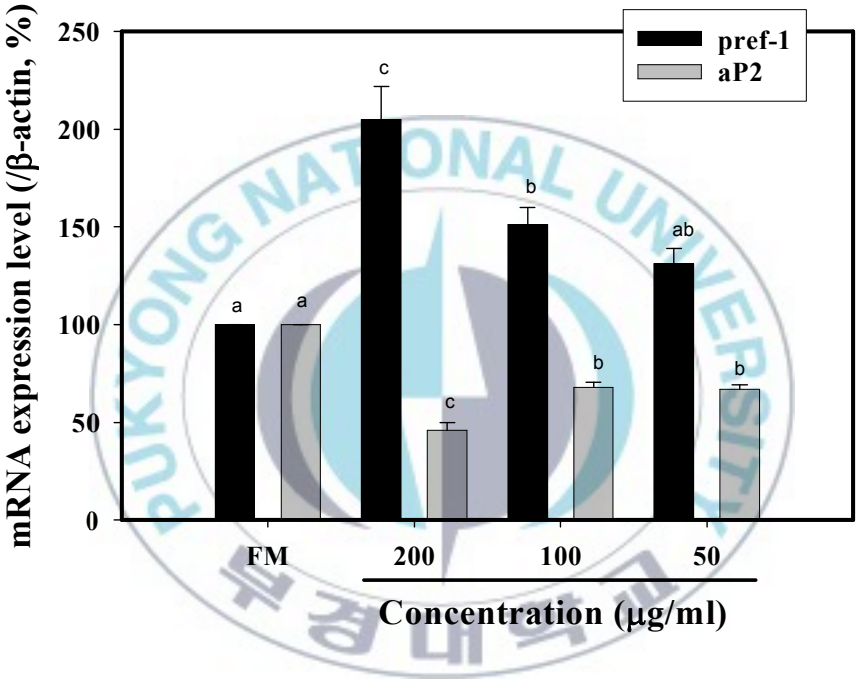


Figure 19. Effect of phosphorylated glucosamine on pref-1 and aP2 mRNA expressions in 3T3-L1 cells. Phosphorylated glucosamine was treated into differentiated adipocyte for 6 days and total RNA was isolated and RT-PCR was performed using indicated primers. The amplified PCR products were run in 1% agarose gel and visualized by EtBr staining. β-actin was used as a house-keeping control gene. [a-c] Means with the different letters are significantly different (p<0.05) by Duncan's multiple range test.

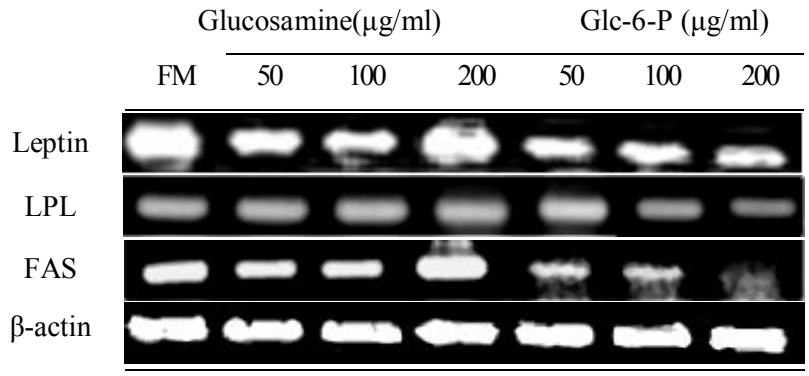


Figure 20. Effects of glucosamine and phosphorylated glucosamine on Leptin, LPL, and FAS mRNA expressions in 3T3-L1 cells.

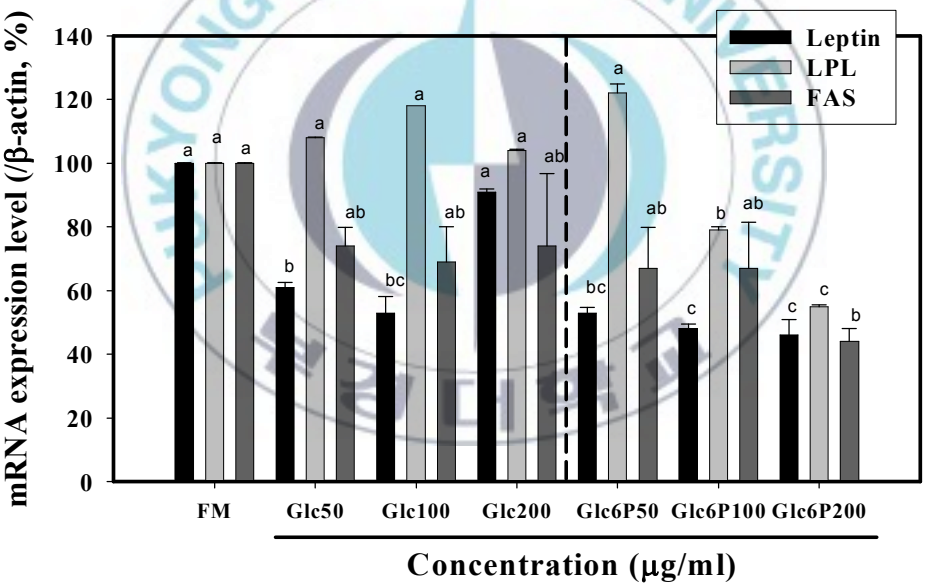


Figure 21. Effects of glucosamine and phosphorylated glucosamine on Leptin, LPL, and FAS mRNA expressions in 3T3-L1 cells. Glucosamine and phosphorylated glucosamine were treated into differentiated adipocyte for 6 days and total RNA was isolated and RT-PCR was performed using indicated primers. [a-c] Means with the different letters are significantly different (p<0.05) by Duncan's multiple range test.

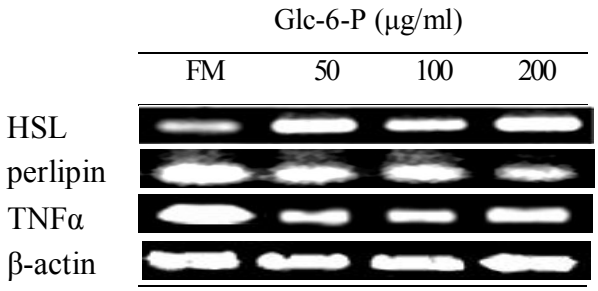


Figure 22. Effect of phosphorylated glucosamine on HSL, perlipin, and TNFα mRNA expressions in 3T3-L1 cells.

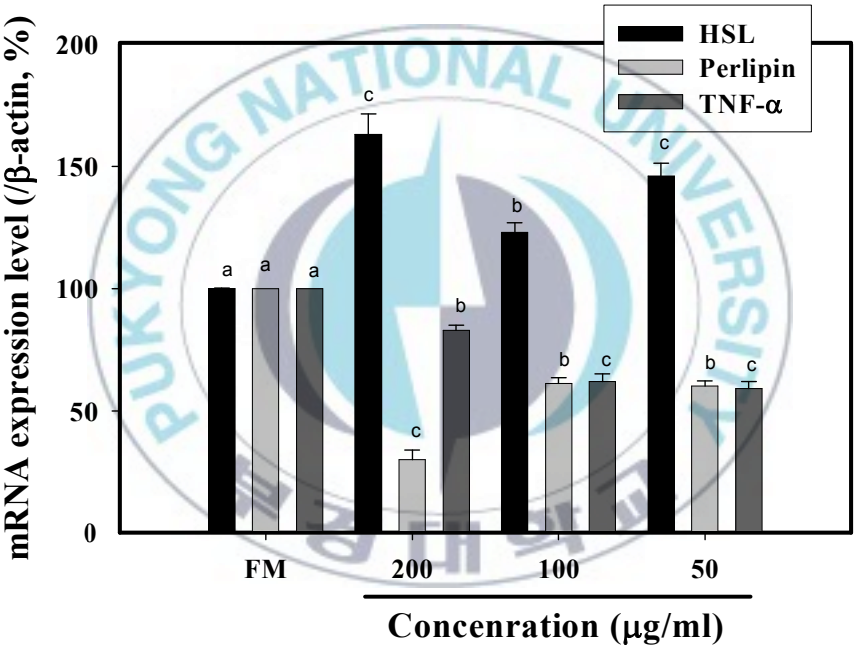


Figure 23. Effect of phosphorylated glucosamine on HSL, perlipin, and TNFα mRNA expressions in 3T3-L1 cells. Phosphorylated glucosamine was treated into differentiated adipocyte for 6 days and total RNA was isolated and RT-PCR was performed using indicated primers. The amplified PCR products were run in 1% agarose gel and visualized by EtBr staining. β-actin was used as a house-keeping control gene.

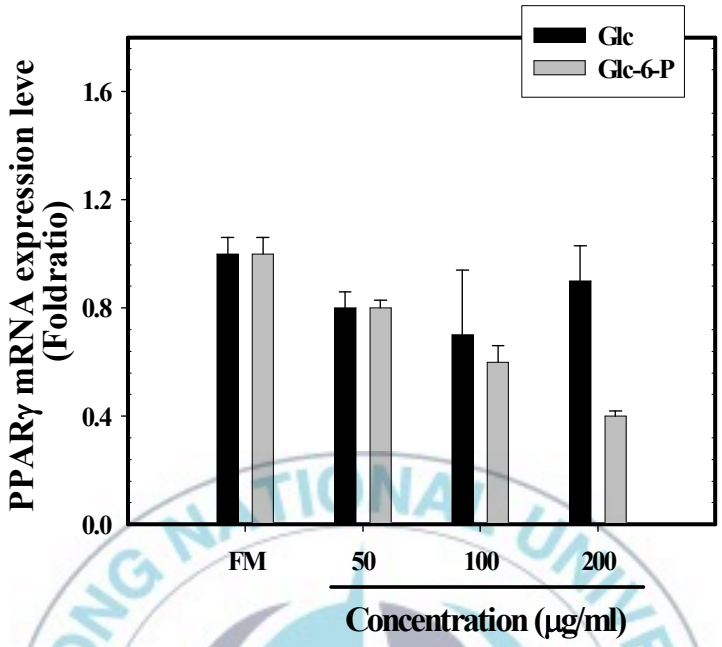


Figure 24. Effect of Glucosamine and phosphorylated glucosamine on PPARγ mRNA expression in 3T3-L1 cells determined by Real Time PCR.

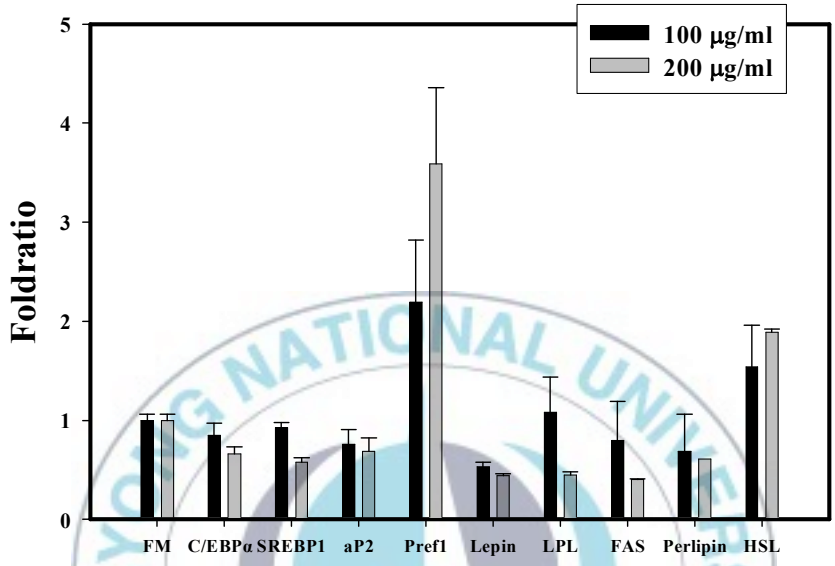


Figure 25. Effect of Phosphated glucosamine on mRNA expressions determined by Real-Time PCR.

Discussion

The obesity has been recognizing worldwide as a one of the serious socioeconomic health problems. Obesity is a heavy accumulation of fat in the body's fat cells to such a serious degree that it greatly increases the risk of obesity-associated diseases such as type-2 diabetes, hypertension, cancer, osteoarthritis, heart disease, etc (Lee et al., 2005). It is known that adipocyte differentiation and the amount of fat accumulation are associated with the occurrence and development of obesity (Jeon et al., 2004). Adipocytes play a vital role in lipid homeostasis and energy balance by relating to TG storage and free fatty acids release. For these reasons, many studies have been conducted to search for new health benefit materials for obesity or weight control. Evans et al. (2002) suggested possible mechanisms in reference to antiobesity actions as following: reducing incorporation of glucose and free fatty acids into triglyceride, increasing oxidation of glucose and/or fatty acids, or increasing lipolysis. In this study, we synthesised phosphorylated glucosamine (Glc-6-P) as a sample and investigated its effects on lipid accumulation in cultured 3T3-L1 adipocytes by measuring TG contents and Oil-Red O staining as indicators of lipid accumulation. We also examined the mRNA expression of several genes associated with adipogenesis and lipolysis in order to understand the mechanism by which phosphorylated glucosamine decreases lipid accumulation in adipocytes. 3T3-L1 preadipocyte was differentiated with phosphorylated glucosamine for 6 days and lipid accumulation was quantified by measuring TG contents and Oil Red O staining. Treatment with phosphorylated glucosamine reduced TG content of differentiated adipocyte lysate in a dose-dependent manner ($p<0.05$) compared to differentiated adipose tissue, but glucosamine did not. Treatment with phosphorylated glucosamine reduced OD value of Oil Red O eluted solution in the cytoplasm of treated

cells according as the concentrations, which means that phosphorylated glucosamine inhibits adipocytes differentiation. There are no reports how phosphorylated glucosamine works on adipogenesis or lipolysis in 3T3-L1 adipocyte. It could be interesting to study the action mechanism of phosphorylated glucosamine in 3T3-L1 adipocytes.

Adipocyte differentiation includes a series of programmed changes in specific gene expression. Adipogenesis can be induced through the action of several enzymes such as FAS, ACC, acyl-CoA synthetase (ACS), and glycerol-3-phosphate acyltransferase. The expressions of these genes are regulated by transcription factors such as peroxisome proliferator-activated receptor-γ (PPARγ), CCAAT/enhancer-binding proteins (C/EBPα), and differentiation-dependent factor 1/sterol regulatory element-binding protein (SREBP1c), which are known to be critical activators for adipogenesis and showed early changes in gene expression during adipocyte differentiation (Latasa et al., 2000; Luong et al., 2000; Ericsson et al., 1997). PPARγ and C/EBPα, two central transcriptional regulators, are induced prior to the transcriptional activation of most adipocyte specific genes. They play vital roles in adipocyte differentiation and coordinate expression of genes involved in creating or maintaining the phenotype of adipocytes (Rosen, 2005). Overexpression of these transcription factors can accelerate adipocyte differentiation. SREBP1c is known to critically cross-activate a ligand binding domain of PPARγ as well as promote the production of an endogenous PPARγ ligand (Bruce and Jeffery, 2001). SREBP1c also regulates the expression of the enzymes involved in lipogenesis and fatty acid desaturation (Rosen et al., 2000). Phosphorylation effect of glucosamine on the expression of these transcriptional factors was determined. Although glucosamine did not have any effect on down-regulation of PPARγ, C/EBPα, and SREBP1c expressions, phosphorylation of glucosamine induced significant down-regulation in a dose-dependent manner compared to differentiated adipocytes without

sample treatment. Treatment with phosphorylated glucosamine also induced down-regulated protein expressions of PPARγ, C/EBPα, and SREBP1c in a dose-dependent manner. The down-regulation of C/EBPα and SREBP1c by treatment with phosphorylated glucosamine might reduce fatty acid synthesis as well as the synthesis and activity of PPARγ, resulted in inhibition of lipid accumulation by blocking adipogenesis.

Phosphorylation of glucosamine inhibited adipogenesis in 3T3-L1 cells through down-regulating PPARγ, C/EBPα, and SREBP1c expression. PPARγ and C/EBPα synergistically activate the downstream of adipocyte specific gene promoters such as adipocyte fatty acid binding protein aP2, fatty acid synthase (FAS), lipoprotein lipase (LPL), and leptin (Gregoire et al., 1998). Thereby, we further studied whether phosphorylated glucosamine regulated the gene expressions of adipogenic target genes such as ap2, FAS, LPL, and leptin as well as Pref-1. Preadipocyte factor 1(Pref-1) is known as an adipogenesis inhibitor serves as a marker for preadipocytes. Pref-1 is of characteristic of an undifferentiated state and is down-regulated during adipocyte differentiation (Wang et al., 2006). A remarkable down-regulation of Pref-1 expression induces increase in the expression of C/EBPα and PPARγ (Rosen et al., 2002). The aP2 gene is terminal differentiation marker of adipocytes and plays central roles in the pathway that link obesity to insulin resistance and fatty acid metabolism. Treatment with phosphorylated glucosamine during adipocyte differentiation induced significant up-regulation of Pref-1 mRNA and down-regulation of aP2 mRNA.

Moreover, the expression levels of FAS, LPL, and leptin mRNA were also dose-dependently suppressed by treatment with phosphorylated glucosamine. Traditionally FAS has been seen as a terminal marker of adipocyte differentiation. Activated PPARγ and SREBP1c are involved in a metabolic cascade leading expression of FAS and clearly

able to cross-activate the FAS promoter (Palmer et al., 2002). LPL catalyzes the hydrolysis reactions of triglyceride, which plasma triglyceride is metabolized to free fatty acids for TG synthesis with adipose cells (Yamaguchi et al., 2002). Adipose tissue LPL involves the extent of the TG depot in fat cells and the high regulation of LDL activity in adipocyte is closely linked with obesity (Bullo et al., 2002). The leptin is exclusively secreted in adipose tissue in proportion to their TG stores and cell size in adipose tissue and may be important in the development and extent of obesity (Bullo et al., 2002). Leptin concentration in the serum is positively associated with adipose tissue mass (Maffei et al., 1995). Therefore, leptin is used as an indicative marker of obesity. These results suggest that phophorylation of glucosamine might suppress differentiation of adipocyte and adipogenesis through C/EBPα, SREBP1c, and PPARγ mediated adipogenesis mechanism, related to the downstream of adipocyte specific gene promotors, including ap2, FAS, LPL, and leptin.

Since phosphorylated glucosamine inhibited lipid formation during adipocytes differentiation, we also explored whether the reduction effect of phosphorylated glucosamine on lipid accumulation is associated with lipolysis. Lipolysis includes some critical processes such as phosphorylation of perilipin and Hormone-sensitive lipase (HSL) translocation into lipid droplets (Ardevol et al., 2000). HSL mediate the hydrolysis of triglyceride into fatty acids and glycerol. Moreover, the cytokine tumor necrosis factor α (TNFα) has been known to accumulate adipose tissue mass and related to inducing lipolysis and apoptosis of adipocytes (Zhang et al., 2008). But these actions are not well understood in adipose tissue and there was no mechanistic evidence to support this. In both human and experimental models, leptin and TNFα expressions in adipose tissue are related with body fat. The expression of these molecules increases during weight gain and decreases upon weight loss (Kirchgessner et al., 1997). So overexpression of TNFα in

adipose tissue is proportional to the extent of the fat depot. TNFα expression level was increased concentration dependant manner but not significant effect compared to fully differentiated group. Therefore this result indicate that not lypolitic effect but also inhibit adipocyte differentiation. The expression levels of perilipin and HSL during differentiation of adipocytes were determined as the lipolytic response. Treatment with phosphorylated glucosamine up-regulated HSL mRNA expression, but did not perlipin mRNA expression. We can assume that the inhibition of phosphorylated glucosamine on lipid accumulation is not mediated by perilipin, HSL and TNFα.

In conclusion, our results revealed that phosphorylated glucosamine inhibited adipocyte differentiation and adipogenesis in 3T3-L1 cells. Moreover, at molecular level, phosphorylated glucosamine enhanced the inhibited regulation of PPARγ, C/EBPα, and SREBP1c and adipogenic specific promoter genes. Therefore, our study suggests that GLC-6-P may also be involve in direct binding to PPARγ to increase its activity, in addition to promoting PPARγ, C/EBPα, and SREBP1c expressions through adipogenesis mechanism related to the downstream of adipocyte specific gene promotors, including ap2, FAS, LPL, and leptin gene. However, further molecular mechanism of phosphorylated glucosamine on the transcription factor in adipogenesis or apoptosis of adipocytes through signaling pathways remains to be elucidated.

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