Influence of hyperglycemia on the apoptosis of adipocyte induced by nucleoside reverse transcriptase inhibitors for the treatment of HIV-1 infection

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The Doctoral Dissertation
Submitted to the Department of Medicine
The Graduate School of Yonsei University
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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December 2009

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December 2009

ACKNOWLEDGEMENTS

From 2004 to 2007, I worked as public health doctor in Division of AIDS in Korea National Institute of Health. During this period, I had the chance to experience basic research on HIV and it was very fresh to me. This experience led me to this work and I realized again how hardly scientific accomplishments have been obtained during the performance of my meager work. I daringly felt that I could understand a bit more about scientific accomplishment than before.

I owe every piece of my work to my mentor professor June Myung Kim, and this work couldn't be possible without his guidance. Professor Hyun Chul Lee, Young Soo Ahn, Jae Myun Lee, and Dr. Joo Shil Lee have served as committee member and given me important advices to make this article better.

And I appreciate Professor Jae-woo Kim for his advices and suggestions throughout my works.

Finally, I would like to express my gratitude to my families for their love and encouragement.

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ABSTRACT

Influence of hyperglycemia on the apoptosis of adipocyte induced by nucleoside reverse transcriptase inhibitors for the treatment of HIV-1 infection

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Nucleoside reverse transcriptase inhibitors (NRTIs) are considered responsible agents for lipoatrophy in patients receiving highly active antiretroviral therapy. Human immunodeficiency virus type 1 (HIV-1) infected patients revealing lipoatrophy usually manifest profiles of hyperinsulinemia and occasionally overt hyperglycemia. This study evaluated the effect of hyperglycemia on the apoptosis of adipocyte induced by NRTI.

Murine preadipocyte 3T3-L1 was differentiated in different glucose concentrations (1.0g/L, 4.5g/L) and 5 NRTIs (zidovudine, stavudine, didanosine, lamivudine, abacavir) were treated for 11 days with near-Cmax concentration.

The mean number of triglyceride accumulated adipocytes was higher in cells which were differentiated in a high glucose medium (80.8 vs. 134.0, $P<0.001$). However, the differentiation marker expressions such as C/EBP-α/β and FAS were not influenced by NRTI treatment or glucose concentration. In addition, mitochondria membrane potential integrity was not influenced by NRTI treatment or glucose concentration. However, apoptosis increased in zidovudine, stavudine, didanosine, and lamivudine treated adipocytes in a high glucose

medium while only zidovudine treatment induced apoptosis in a low glucose medium. Regarding the insulin induced anabolic signaling of Akt phosphorylation, both zidovudine and stavudine treatment attenuated insulin induced Akt phosphorylation in cells differentiated in a high glucose environment while only stavudine attenuated Akt phosphorylation by insulin when the adipocytes were differentiated and treated in low glucose medium.

In conclusion, glucose concentration in medium and treatment of NRTI did not alter differentiation of preadipocytes. However, NRTI induced attenuation of insulin anabolic signaling and apoptosis were augmented when preadipocytes were differentiated in a high glucose concentration medium. These findings suggest the possibility that the high glucose environment might aggravate the NRTI induced adipocyte toxicity.

Key words: Human immunodeficiency virus-1, Nucleoside reverse transcriptase inhibitor, adipocyte, apoptosis

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Ⅰ. INTRODUCTION

Metabolic complications, including dyslipidemia, insulin resistance, and altered fat distribution are common in adults infected with the Human immunodeficiency virus type 1 (HIV-1) who are receiving highly active antiretroviral therapy (HAART)[1]. With regards to altered fat distribution, subcutaneous lipoatrophy and relative or absolute accumulation of central fat may occur in HIV-infected patients. This syndrome, which includes body fat redistribution and metabolic abnormalities, was described shortly after the introduction of protease inhibitors, but peripheral fat loss was also observed in patients who had only been treated with nucleoside reverse transcriptase inhibitors (NRTI)[2].

Prospective studies investigating body composition in patients starting HAART have demonstrated initial increases in limb fat during the first few months of therapy followed by a progressive decline during the ensuing three years[3, 4]. Lipoatrophy associated with nucleoside analogues has been known to be due in part to mitochondrial injury resulting from inhibition of mitochondrial DNA polymerase-γ within adipocytes[5] and depletion of mitochondrial DNA[6]. Through these cascades, nucleoside analogues can inhibit adipogenesis and adipocyte differentiation[6] while promoting lipolysis[7] and apoptosis[8].

Hyperinsulinemia, a surrogate measure of insulin resistance, is commonly seen in association with excess truncal fat, loss of fat in the limbs, an increased waist-to-hip ratio, and buffalo humps. Among HIV-infected adults with lipoatrophy or fat accumulation, diabetes mellitus was seen in 7.0 percent, as compared with 0.5 percent of otherwise healthy control subjects matched for age

and body mass index[9]. In a longitudinal cohort study, diabetes mellitus was 4.1 times as likely to develop in HIV-infected men receiving HAART as it was in control subjects over a three-year period of observation[10]. By virtue of the advent of HAART, HIV is now a manageable chronic disease and more patients are likely to present with complications related to treatment including coronary artery disease, dyslipidemia, and diabetes.

Hyperglycemia resulting from uncontrolled glucose regulation is widely recognized as the cause of diabetic complications. Three major hypotheses about how hyperglycemia causes diabetic complications are activation of protein kinase C isoforms[11], increased formation of glucose-derived advanced glycation end products[12], and increased glucose flux through the aldose reductase pathway[13] and three of them have been recently shown to be the consequences of common mechanism, hyperglycemia-induced mitochondrial superoxide overproduction[14] .

High glucose-induced apoptosis has received much attention in recent years and the data clearly demonstrate that multiple mechanisms regulate the complicated signaling pathways that mediate high glucose-induced apoptosis[15]. One of the earliest detectable responses of a cell to a high glucose challenge is the generation of superoxide[16] and mitochondria play a central role in mediating glucose-induced apoptosis. High glucose interrupts mitochondrial electron transport which results in increased oxidation of oxygen by coenzyme Q and the subsequent generation of superoxide. Superoxide then reacts with nitric oxide to form peroxynitrite, a highly toxic molecule. Peroxynitrite causes protein dysfunction, oxidizes lipids, and modifies DNA, which often results in both apoptotic and necrotic cell death[17].

The NRTIs related lipoatrohy is inevitable in some patients who are taking specific essential drugs and the number of HIV infected patients with hyperglycemia is expected to increase in the future. The NRTIs related lipoatrophy and hyperglycemia related complications share a substantial part in the pathogenesis such as mitochondrial toxicity and apoptosis. This study aimed to evaluate the effect of hyperglycemia on the NRTI related adipocyte toxicities.

Ⅱ. MATERIALS AND METHODS

1. **Cell culture and induction of differentiation**

3T3-L1 preadipocytes were maintained in DMEM containing 10% calf serum and differentiated according to the protocol described before[18] with the exception that medium contained either 1.0 g/L or 4.5 g/L glucose since the induction of differentiation (designated as D 0) throughout the experiment. Two-day postconfluent, 3T3-L1 cells were incubated in DMEM with different glucose concentration containing 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 μM dexamethasone, and 1 μg/ml insulin for 2 days. Cells were then fed DMEM containing 10% FBS and insulin for another 2 days, after which they were fed DMEM containing 10% FBS. Adipocyte differentiation was evaluated at day 10 by the percentage of cells with lipid droplets and the protein expression of the transcription factors CCAAT/enhancer binding protein (C/EBP)-β (D 2), FAS and C/EBP-α (D7). Oil red O staining was performed on D 10. Cells were washed three times with phosphate-buffered saline (PBS) and fixed on dishes with 3% formaldehyde in PBS for 15 min. After rinsed with PBS three times, cells were incubated with oil red O solution (0.3% oil red O, 60% isopropanol, and 40% water) for 20 min. Cells were extensively washed with water to remove unbound dye. Stained cells were air dried overnight and then dissolved with 100% isopropanol for OD detection at 520 nm[19].

2. **Treatment of nucleoside analogue**

Cells were exposed to nucleoside analogue at near-Cmax concentrations for 10 days after the induction of differentiation (D10). All drugs were dissolved in DMSO and the highest concentration of solvent used in the experiment (DMSO about 0.1%) did not affect cellular viability and preadipocyte differentiation. The drugs were used at concentrations close to their maximum concentration (Cmax) values: zidovudine (AZT) 1 μM, stavudine (d4T) 10 μM, didanosine (ddI) 10 μM, lamivudine (3TC) 10 μM, and abacavir (ABC) 4 μM as described before[20]. An equal amount of solvent was applied to control the experiment.

3. **Evaluation of mitochondrial function**

Mitochondrial activity was estimated by the lysis of the tetrazolium salt, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT). NRTI treated or untreated cells cultured in 12 well plate were incubated with MTT (250 μg/ml) for 4 h at 22°C. The medium was then replaced with DMSO and MTT staining was quantified at 540 nm after 10 min of gentle shaking.

To evaluate the integrity of mitochondrial functions, JC-1 (5,59,6,69-tetrachloro-1,19,3,39-tetraethyl-benzimidazolo carbocyanine iodide, T-4069 Sigma, St. Louis, MO, USA) staining was performed. Cells in a 6 well plate were cultured and treated as described before. After trypsinization on D10, cells were suspended in PBS at approximately 1×10^6 cell/ml. 10 μL of 200 mM JC-1 was added to a final concentration of 2 μM and cells were incubated for 30 min in a 5% CO_2 incubator. After washing and resuspension in PBS, an evaluation of the mitochondrial membrane integrity was performed by FACS scan as JC-1 green (uncoupled mitochondria, detector FL-1) or red (intact mitochondria, detector FL-2) fluorescence (BD Biosciences, San Diego, CA, USA)[21]. Data were acquired and analyzed using Cell Quest software.

4. **Measurement of reactive oxygen species (ROS)**

ROS was detected with the peroxide-sensitive fluorophore 2,7-dichlorodihydrofluorescein diacetate (DCF-DA, Molecular Probes, Eugene, Oregon, USA). DCF-DA dissolved in DMSO was used at 10 μM (final concentration). 3T3-L1 cells were cultured and treated in 96-well plate. After washing and incubation with DCF-DA for 1 h at 37°C under an atmosphere including 5% CO_2, the fluorescence of DCF was analyzed in an HTS 7000 Bio Assay Fluorescent Plate Reader (PerkinElmer Life Sciences, Waltham, MA, USA) at an excitation wavelength of 485 nm and emission at 530 nm. ROS production was determined from an H_2O_2 standard curve (10–200 nmol/ml)[22].

5. Measurement of apoptosis

Apoptosis was evaluated by flow cytometry as well as the cleavage of the

caspase-3 and the Poly ADP-ribose polymerase (PARP)[23]. For flow cytometry, cells were harvested on D10 using trypsin/EDTA, washed with PBS, resuspended in 1 ml binding buffer, and stained with Annexin V-FITC and propidium iodide (PI) at room temperature for 5 min (Biovision, CA, USA). The fluorescence of Annexin V-FITC and PI was analyzed using flow cytometry[24, 25]. Active caspase-3 and PARP cleavage were estimated by western blot.

6. Measurement of insulin induced anabolic signaling

Cell responsiveness to insulin was evaluated by measuring the insulin-induced phosphorylation of Akt events in adipocyte. Differentiated cells in a 6 well plate were washed by PBS two times and stimulated by various concentrations of insulin (control, 0.1nM, 1nM, 10nM) for 20 min at 37°C. Proteins isolated from the cells were subjected to SDS-PAGE and then western blot analysis was performed[22]. To determine the influence of NRTI on the insulin signaling, Cmax concentration of AZT and d4T were added during the differentiation, which have been known to be mostly related with insulin resistance.

7. Western blotting

Cells were washed once with cold PBS and then scraped into a lysis buffer containing 1% SDS and 60 mM Tris-HCl, pH 6.8. Lysates were boiled for 10 min and verified by centrifugation, and then equal amounts of protein were subjected to SDS-PAGE and immunoblotted with antibodies to C/EBP-α/β, FAS, (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Akt, phospho-Akt, caspase-3 (Cell Signaling Technology, Inc., Boston, MA, USA) or PARP (Roche Applied Science, Mannheim, Germany).

Ⅲ. RESULTS

1. Influence of glucose concentration and NRTI treatment on preadipocyte differentiation

3T3-L1 preadipocytes were differentiated and exposed to NRTI from D0 to D10 in a different glucose concentration medium. The number of differentiated

adipocytes containing lipid droplets was counted in light microscope field of X400 magnification after oil red O staining. The mean number of cells containing lipids was significantly higher in cells differentiated in high glucose medium except for ddI and 3TC treated cells. However, the NRTI treatment did not influence the number of triglyceride accumulated adipocytes. Spectrophotometric quantification of extracted oil red O staining by isopropanol also revealed significantly higher values in cells differentiated in high glucose medium irrespective of NRTI treatment (Table 1). In the evaluation of differentiation marker proteins by western blot, C/EBP-α/β and FAS expressions were not influenced by NRTI treatment or medium glucose concentration (Fig. 1).

Table 1. Mean number of lipid droplet containing cells stained by oil red O and the spectrophotometric measurement of eluted oil red O after NRTI treatment in different glucose concentration

NRTIs*	Number of cells containing lipid droplet			Spectrophotometric measurement of eluted oil red O		
	Low glucose	High glucose	*P*-value	Low glucose	High glucose	*P*-value
Control	79.0±7.9	124.3±10.7	0.004	0.238±0.041	0.389±0.035	0.008
AZT	85.0±12.5	133.0±12.5	0.009	0.215±0.035	0.384±0.037	0.005
d4T	79.3±25.3	148.7±18.0	0.018	0.184±0.011	0.411±0.020	<0.001
ddI	99.0±21.7	119.7±24.0	0.330	0.228±0.023	0.351±0.034	0.007
3TC	77.3±17.1	130.7±58.2	0.203	0.195±0.016	0.415±0.017	<0.001
ABC	65.3±10.1	147.7±18.4	0.002	0.196±0.012	0.359±0.029	0.001
Total	80.8±17.6	134.0±26.5	<0.001	0.209±0.029	0.385±0.035	<0.001

*NRTIs treatment did not influence number cells containing lipid droplet or spectrophotometric measurement of eluted oil red O.

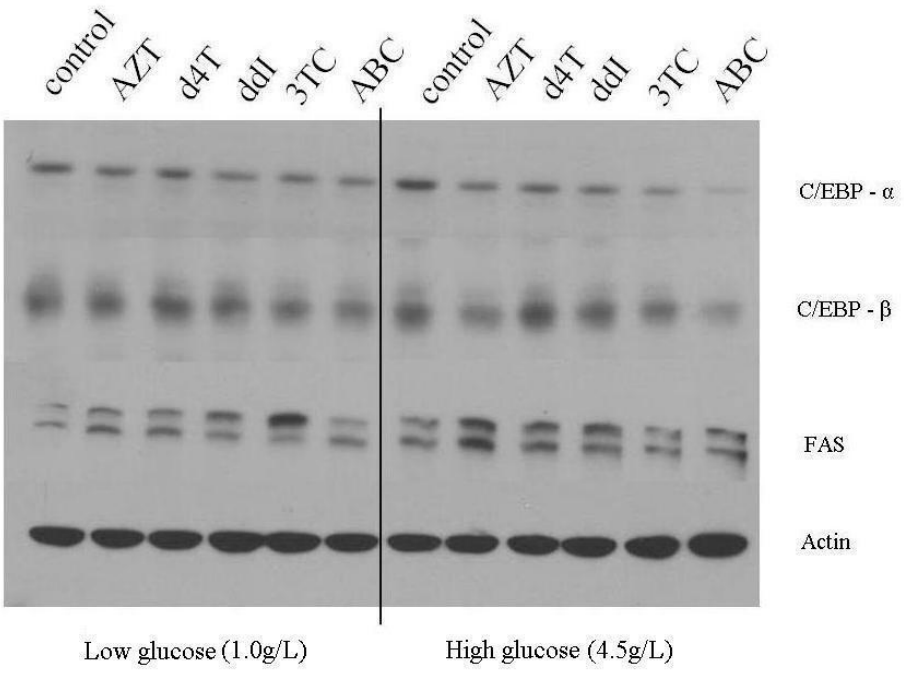

Fig. 1. Evaluation of 3T3-L1 preadipocyte differentiation after NRTI treatment in different glucose concentrations. The NRTI treatment or medium glucose concentration did not influence 3T3-L1 differentiation marker expressions in western blot such as C/EBP-α/β and FAS.

2. Evaluation of mitochondrial function

MTT assay revealed higher mitochondrial activity in cells which were differentiated in a high glucose medium except for AZT treatment (Table 2). However, when the optical density of MTT was divided by the mean number of cells observed in differentiation assay performed in identical conditions, the values were not influenced by the medium glucose concentration except for the ddI. The NRTI treatment also did not affect mitochondrial activity regardless of medium glucose concentration.

Table 2. Evaluation of mitochondrial function after NRTI treatment in different glucose concentration

NRTIs*	Optical density of MTT staining			Corrected optical density of MTT divided by the number of cells (× 100)§		
	Low glucose	High glucose	*P*-value	Low glucose	High glucose	*P*-value
Control	0.386±0.040	0.543±0.056	0.017	0.495±0.099	0.441±0.081	0.510
AZT	0.388±0.097	0.550±0.053	0.064	0.451±0.051	0.418±0.072	0.546
d4T	0.246±0.070	0.586±0.080	0.005	0.327±0.100	0.393±0.006	0.313
ddI	0.320±0.063	0.552±0.058	0.009	0.325±0.038	0.467±0.046	0.015
3TC	0.332±0.084	0.586±0.054	0.012	0.455±0.188	0.600±0.366	0.693
ABC	0.342±0.084	0.572±0.066	0.020	0.540±0.188	0.393±0.080	0.281

*NRTIs treatment did not influence MTT staining.

§The values were multiplied by 100 for better presentation.

Evaluation of mitochondrial membrane integrity was performed by FACS analysis of JC-1 green monomer to red aggregate ratio, for green monomer represents disrupted mitochondrial membrane potential. There was increasing tendency of proportion of green monomer in cells differentiated in a high glucose medium, but they were not statistically significant (Fig. 2). There was no evidence that NRTI treatment aggravated the mitochondrial membrane disruptions.

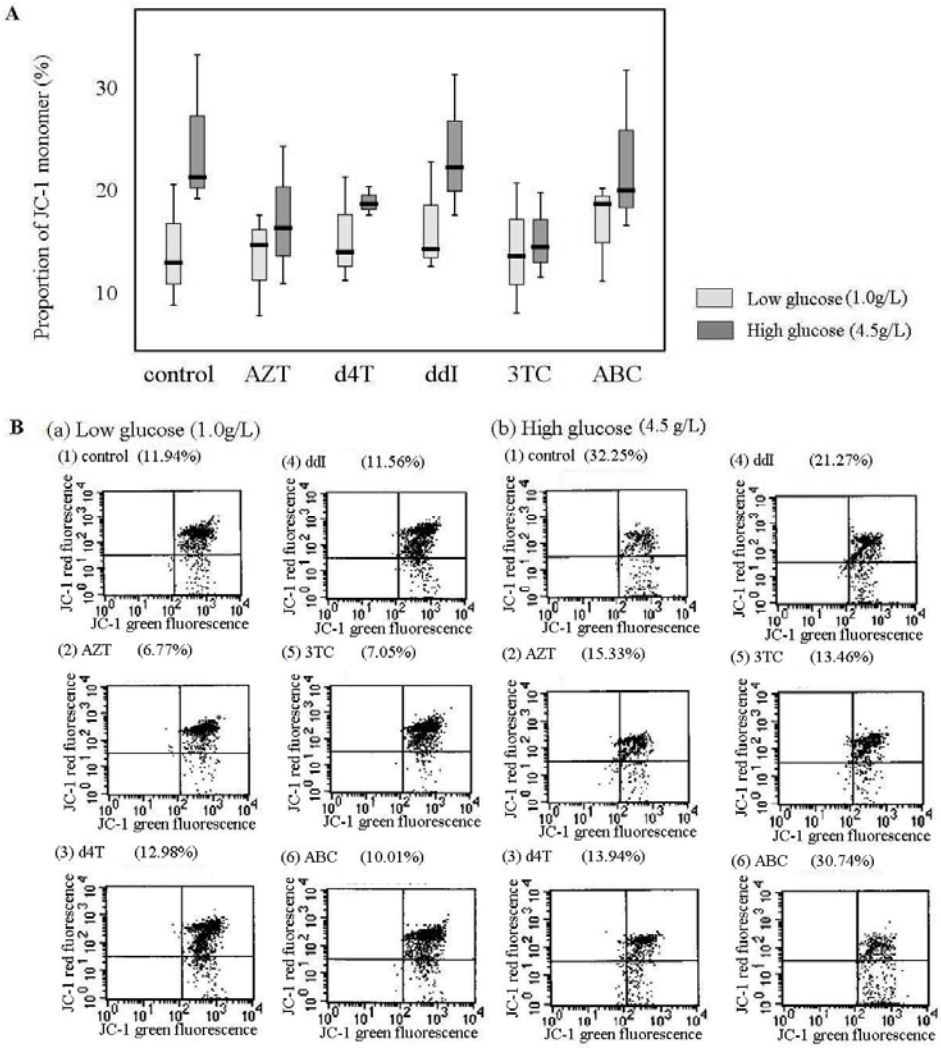

Fig. 2. Measurement of mitochondrial membrane potential integrity by JC-1 staining. Proportion of cells with membrane potential disruption (green monomer) was not influenced by the medium glucose concentration and NRTI treatment (A). Cells revealing green fluorescence in FACS were considered as green monomer dominant mitochondria with disrupted membrane potential (lower right quadrant) contrary to red aggregates (upper right quadrant) (B). Assay was performed in triplicate.

3. Measurement of reactive oxygen species

ROS production was measured by fluorescent probe DCF-DA staining. However, ROS production was not influenced by the treatment of NRTI or glucose concentration (Fig. 3).

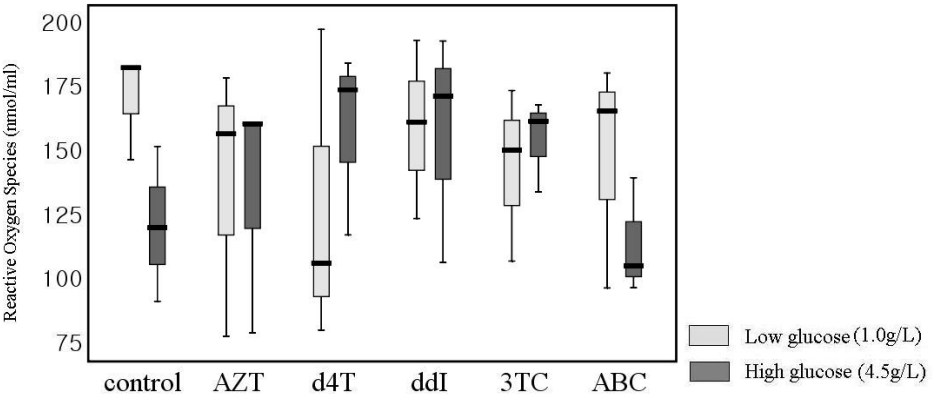

Fig. 3. Effect of glucose concentration and NRTI treatment on intracellular reactive oxygen species generation in adipocyte. Reactive oxygen species production was not influenced by the treatment of NRTI or glucose concentration.

4. Measurement of apoptosis

In western blot analysis of PARP and active form of caspase-3, increased 84.7 kDa PARP cleavage protein representing apoptosis was observed in AZT treated cells which were differentiated in a low glucose medium, while AZT, d4T, ddI, and 3TC increased PARP cleavage in the case of a high glucose medium. However, increased active-caspase 3 signal was observed during AZT and ddI treatment in cells differentiated in a low glucose medium, while ddI and 3TC augmented active-caspase 3 expression in a high glucose medium (Fig. 4).

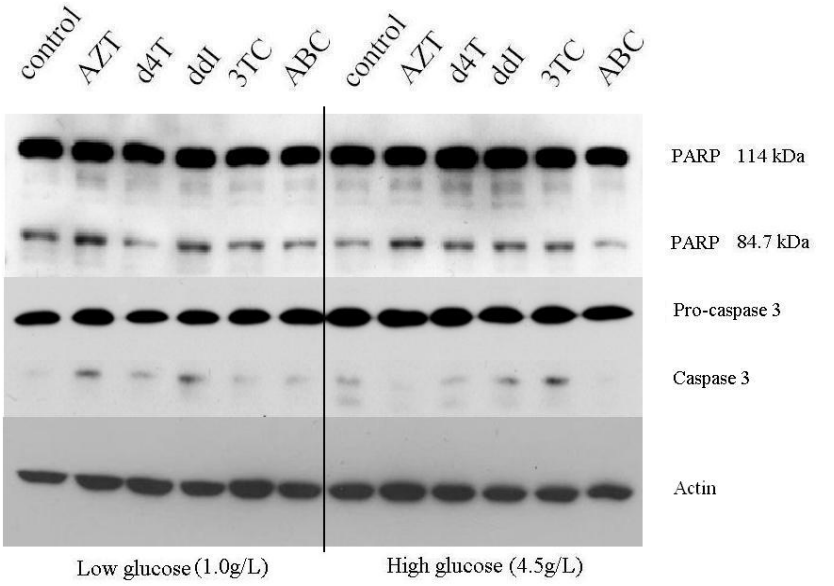

Fig. 4. Measurement of apoptosis by PARP and caspase 3 western blot. 84.7 kDa cleaved form PARP protein expression representing apoptosis was increased in AZT, d4T, ddI, and 3TC treated adipocytes in a high glucose medium while only AZT treatment induced apoptosis in a low glucose medium.

The proportion of apoptotic cells was measured by FACS analysis after Annexin V-FITC/PI staining to evaluate the influence of NRTI treatment and glucose concentration. The proportions of apoptotic cells counted by the sum of late apoptotic cells (upper right quadrant) and early apoptotic cells (lower right quadrant) were not affected by treatment of NRTI or glucose concentration (Fig. 5).

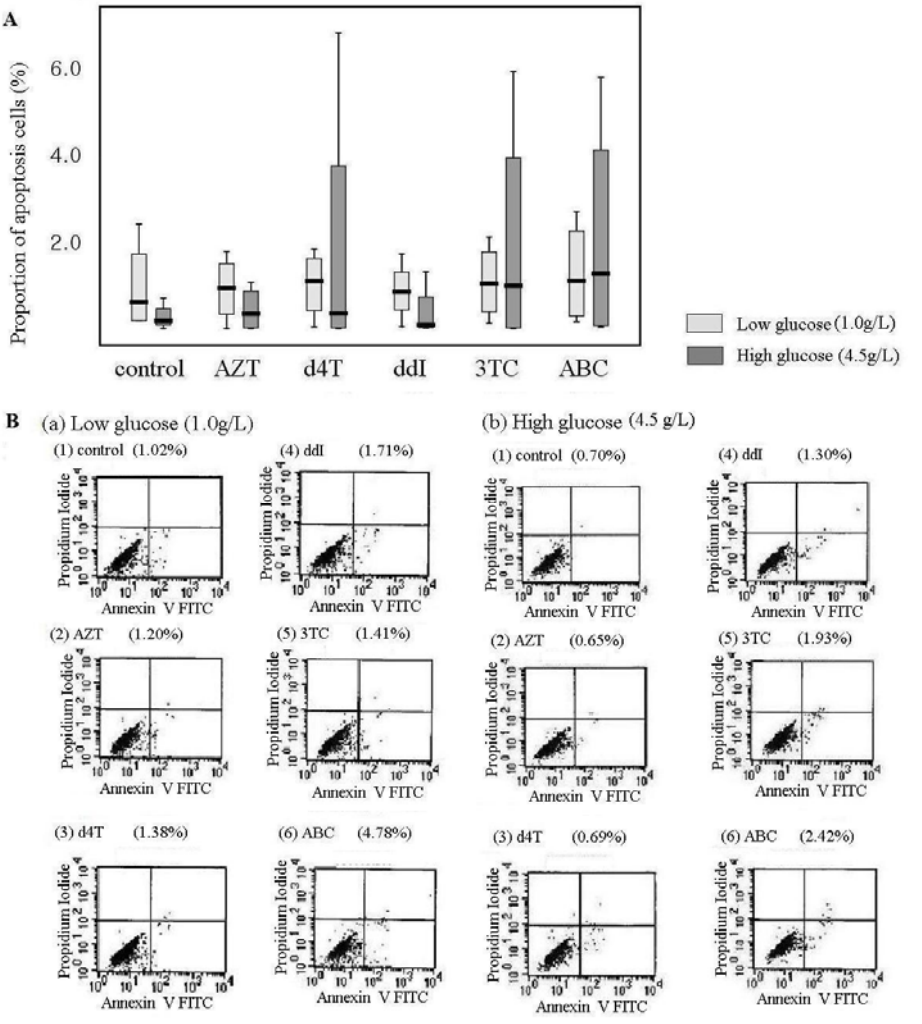

Fig. 5. Measurement of apoptosis by Annexin Ⅴ/Propidium iodide staining. Medium glucose concentration and NRTI treatment did not induce significant apoptosis during preadipocyte differentiation (A). Cells in upper right and lower right quadrants were considered as apoptotic cells in FACS analysis (B). Assay was performed in quadriplicate.

5. Measurement of insulin induced anabolic signaling

To determine whether there are functional differences when adipocytes were exposed to high glucose conditions, we evaluated the degree of insulin induced

anabolic signaling of Akt phosphorylation with differentiation protocol of different glucose medium concentrations. A dose response to insulin was performed, and the degree of Akt phosphorylation was assessed. Insulin-induced Akt phosphorylation was enhanced in cells differentiated in low glucose medium as judged by an anti-phospho-Akt antibody western blot of cell extracts (Fig. 6 A), while the total Akt levels were comparable in all instances. The enhancement of insulin induced anabolic signaling in cells differentiated in low glucose medium was reproduced in AZT and d4T treated cells. When Akt phosphorylation was compared regarding to the NRTI treatment itself, only d4T treatment attenuated Akt phosphorylation in the case of a low glucose medium (Fig. 6 B). However, in cells differentiated in high glucose medium, both AZT and d4T attenuated Akt phosphorylation.

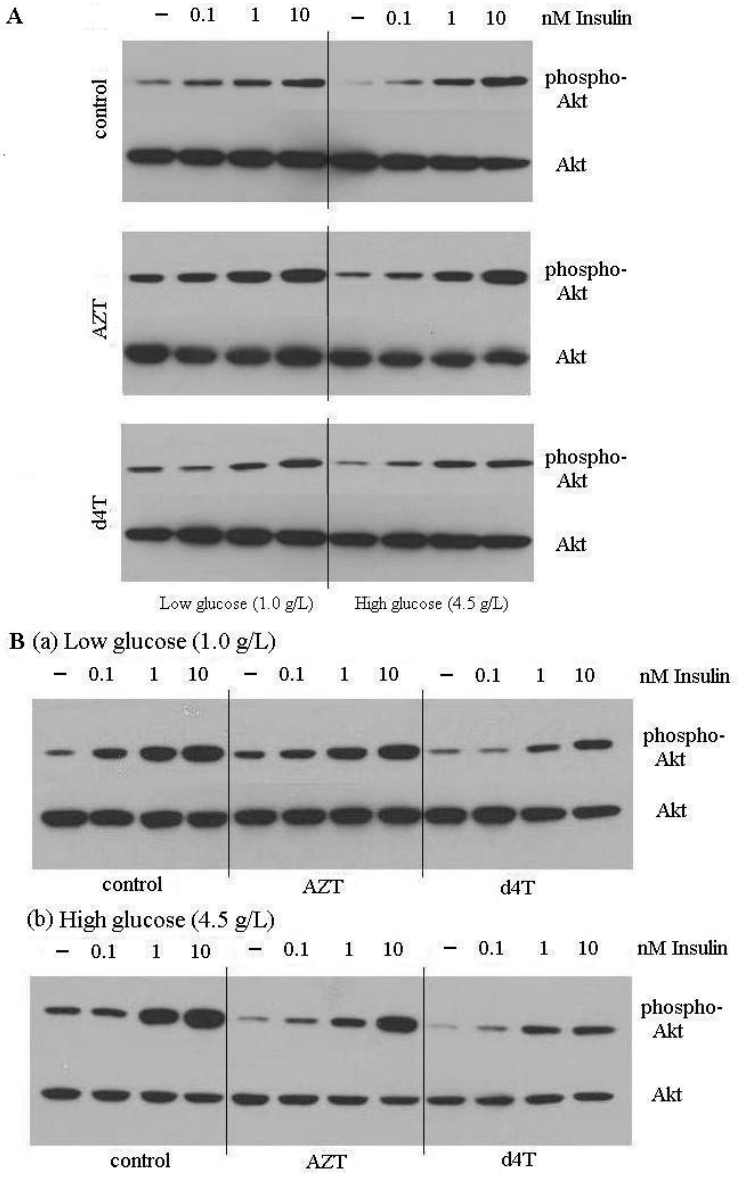

Fig. 6. Measurement of insulin induced anabolic signaling by Akt phosphorylation western blot. High glucose medium attenuated Akt phosphorylation and this phenomenon was reproducible in AZT and d4T treated cells (A). Both AZT and d4T treatment attenuated Akt phosphorylation in cases of a high glucose medium while only d4T attenuated Akt phosphorylation in a low glucose medium (B).

Ⅳ. DISCUSSION

Lipoatrophy associated with nucleoside analogues may be due in part to mitochondrial injury resulting from inhibition of mitochondrial DNA polymerase -γ within adipocytes[5] and depletion of mitochondrial DNA[6], although the exact extent and specificity of this effect remain unclear[1]. Caron reported that maximal therapeutic concentration of thymidine analogues, stavudine (10 μM) and zidovudine (1 μM) impaired lipid accumulation during preadipocyte differentiation as well as a decrease in expression of proliferator-activated receptor (PPAR)-γ and C/EBP-α in 3T3-F442A cell line[20]. In this study, impairment in preadipocyte differentiation was explained by mitochondrial dysfunction and apoptosis.

Lipodystrophy related with HAART is known to be accompanied with other metabolic complications such as diabetes mellitus and dyslipidemia. In cases of diabetes mellitus, hyperglycemia resulting from uncontrolled glucose regulation is widely recognized as the cause of diabetic complications. In addition to conventional mechanisms, hyperglycemia associated with mitochondrial function has received much attention. The study of the relationship between mitochondrial DNA and type 2 diabetes has revealed that the damage in mitochondrial DNA affects nuclear-encoded glucose transporters, glucose-stimulated insulin secretion, and nuclear encoded uncoupling proteins (UCPs) in β-cell glucose toxicity[26]. Recent data on high glucose-induced apoptosis clearly demonstrate that multiple mechanisms regulate the complicated signaling pathways that mediate high glucose-induced apoptosis[15]. Mitochondria has also been known to play a central role in mediating glucose-induced apoptosis. Lin *et al.* observed significant induction of ROS and insulin resistance in adipocytes which were exposed to high concentration glucose medium[22]. It has been known that NRTI administration contributes to development of insulin resistance within 3 months of starting HAART in treatment naïve patients irrespective of body fat change[27], and *in vitro* study also supports these findings[28].

In this study, I focused on the influence of a high glucose environment on the apoptosis and insulin induced anabolic signaling as well as adipocyte

differentiation related with NRTI treatment. Although, the lipid accumulation was enhanced in high glucose condition, the adipocytes differentiation was not influenced by glucose concentration of medium. In addition, NRTI treatment did not impair either adipocyte lipid accumulation nor differentiation markers expression such as C/EBP-α/β or FAS even in cases of AZT and d4T, which have been known to be closely related with lipoatrophy.

One of important mechanisms of lipoatrophy by NRTI is mitochondrial toxicity. This phenomenon has been observed in various NRTI related toxicities such as lactic acidosis, fatty liver, neuropathy, cardiomyopathy, and pancreatitis[29]. Caron *et al.* showed that thymidine analogues treatment induced mitochondrial dysfunction and oxidative stress in human fibroblast[30]. ROS is the byproduct of cell metabolism and mediate various intracellular signal transductions. However, overproduction of ROS or derangement of antioxidant defense mechanisms result in oxidative stress condition and this is related with mitochondrial dysfunction. Hyperglycemia results in the increased enzymatic conversion of glucose to the polyalcohol sorbitol with decreases in NADPH/glutathione, and the loss of these antioxidant results in enhanced sensitivity to oxidative stress associated with intracellular ROS[31]. NRTI treatment related toxicity and diabetes share the pathway of ROS overproduction in their pathogenesis. In this study, MTT assay and JC-1 staining were performed to evaluate mitochondrial activity and mitochondrial membrane depolarization, respectively. Enhanced mitochondrial activity was observed in cells differentiated in a high glucose medium. However, corrected values by the number of adipocytes did not reveal any differences between low and high glucose medium, and NRTI treatment also did not affect mitochondrial activity. In the JC-1 staining, an increasing tendency of green monomer proportion was observed in cells differentiated in a high glucose medium. Considering that the green monomers represent the depolarized mitochondria with membrane disruption, these results imply that a high glucose environment induced subtle mitochondrial depolarization without derangement in mitochondrial functions.

Apoptosis related with lipoatrophy during NRTI treatment has been reported in

clinical[32, 33] and *in vitro*[20, 28] studies. Subcutaneous adipose tissue of lipodystrophic HIV-1 infected patients revealed an increased percentage of apoptotic cells[6]. It has been known that AZT and d4T increase the TNF-α production independently of the adipocyte differentiation status. TNF-α plays a key role in cell apoptosis, thus increased cell death could result from NRTI-induced TNF-α overproduction. The mechanism of this phenomenon has been suggested by the TNF-α property which induces mitochondrial toxicity by releasing ROS[34]. As a whole, altered adipokine secretion resulting from patients' exposure to NRTI leads to altered adipocyte differentiation, insulin resistance and apoptosis, ultimately resulting in lipoatrophy. Treatment of some NRTIs induced apoptosis in adipocytes especially in thymidine analogues such as AZT and d4T which have been known to be closely related with lipoatrophy[20, 28]. However, different experimental cell lines or NRTI exposure protocols led to discordant results[35, 36] as well as the combination of NRTIs[8]. In this study, the treatment of AZT, d4T, ddI and 3TC revealed increased cleaved PARP expression during preadipocyte differentiation in cases of cells differentiated in a high glucose medium while only AZT treatment revealed enhanced cleaved PARP expression in case of low glucose medium. This is supposed to be related with the enhanced oxidative stress and ROS production in a high glucose medium, which might have resulted in triggering NRTI induced apoptosis as previously reported in the diabetic models[37]. However, measurements of the proportion of overt apoptotic cells by Annexin V/PI FACS analysis were not affected by NRTI treatment or medium glucose concentrations. Further investigation regarding to the significance of this finding is required.

Previous clinical studies[9, 10] proved that the duration of NRTI exposure is independently associated with insulin resistance and indirect effect of NRTI leading to the disturbance of body fat change, especially lipoatrophy, has been suggested as a plausible mechanism for insulin resistance. However, evidence from a randomized trial conducted in previously HAART-naive subjects demonstrated the early and sustained increases in fasting serum insulin levels as short as 4 weeks after treatment initiation with HAART containing a thymidine analogue (d4T plus ddI), but not with a thymidine analogue-sparing combination

(ABC plus 3TC)[38]. Furthermore, treatment for 3 months with a NRTI-containing regimen resulted in a decrease in insulin-mediated glucose disposal and an increase in fasting lipolysis in the absence of discernable changes in body composition in treatment naïve HIV-1 infected patients. Insulin resistance without overt body fat changes implies that NRTIs have directly contributed to the development of reduced insulin sensitivity through the disturbance in production and secretion of adipocytokines, which in turn could affect glucose metabolism. In an experiment performed with 3T3-L1 adipocytes, d4T and AZT decreased adiponectin production, whereas the production of TNF-α and interleukins 1/6 were enhanced[28]. There might be an important role for mitochondria in fat and muscle in the development of insulin insensitivity and NRTIs have been associated with mitochondrial toxicity. A decline in oxidative phosphorylation, resulting from mitochondrial dysfunction, could result in accumulation of free fatty acid, which, in turn, could have a negative effect on the signaling cascade of insulin and may reinforce mitochondrial dysfunction[39, 40]. NRTI-induced mitochondrial dysfunction as a cause for insulin insensitivity has been suggested by a recent study in healthy volunteers[41].

It has been known that insulin sensitivity is attenuated in adipocytes differentiated in a high glucose medium[22]. In this study, cell responsiveness to insulin was indirectly evaluated by measuring the insulin induced anabolic signaling of Akt phosphorylation events in adipocyte. Akt has been known as to play a key role in the insulin signaling pathway of anabolic metabolism[42]. Circulating insulin binds to and activates its cell surface receptor to elicit its biological actions. The insulin-receptor signaling involves the phosphatidylinositol-3-kinase (PI3-K) pathway and this mainly accounts for the metabolic responses of insulin. The critical player in the PI3-K pathway is the Ser/Thr kinase Akt, which serves as a multifaceted intermediary propagating insulin receptor signaling to the diversified downstream biological effects such as glucose uptake, glycogen synthesis, protein synthesis, lipid metabolism, and anti-apoptosis.

Consistent with previous studies[22], impairment of insulin induced Akt

phosphorylation was observed in cells differentiated in a high glucose medium and this phenomenon was reproducible in AZT and d4T treated cells, respectively. In addition, Akt phosphorylation was attenuated by AZT and d4T treatment in cells differentiated in a high glucose medium while only d4T attenuated insulin sensitivity in cells differentiated in a low glucose medium. This implies that a high glucose environment may have aggravated insulin signaling attenuation induced by NRTI.

In summary, the differentiation of 3T3-L1 preadipocyte was not influenced by glucose concentration or NRTI treatment while lipid accumulation was enhanced in cells differentiated in high glucose environment. The apoptosis induced by NRTI treatment was observed in AZT, d4T, ddI and 3TC when the preadipocytes were differentiated in a high glucose environment while only AZT induced apoptosis in cases of a low glucose concentration. Both AZT and d4T treatment attenuated insulin induced Akt phosphorylation in cells differentiated in a high glucose environment. However, only d4T attenuated insulin induced anabolic signaling when the adipocytes were differentiated and treated in a low glucose medium. Although the increasing tendency of mitochondrial membrane disruption was observed in the adipocytes which were differentiated in a high glucose medium, it was not statistically significant and differences among NRTI treatments were not observed. Further research is required with regards to the detailed mechanisms of the deleterious effect of a high glucose concentration such as mitochondrial toxicity or oxidative stresses. While AZT and d4T revealed a relationship with the apoptosis and attenuation of insulin signaling, ABC was revealed as a unique NRTI that did not induce apoptosis irrespective of glucose concentration. This is consistent with previous studies that conclude that ABC does not have substantial effects on metabolic factors[43, 44]. However, recent studies are reporting increased cardiovascular risk related with ABC use[45]. Pro-inflammatory property of ABC has been suggested as a plausible mechanism[46] and further evaluation is required related with this topic.

The major research limitation of this study was that the NRTI treatment failed to reveal overt mitochondrial toxicity or gross apoptotic features which have been

described before in many studies[6, 20, 47, 48]. However, Kosmiski *et al.* observed that only 3TC significantly altered lipid accumulation[8] and high doses of AZT (180 μM) or d4T (90 μM) treatment was required to affect preadipocyte differentiation or mitochondrial DNA depletion in another study[49]. These discrepancies between studies imply that influence of NRTI on adipocytes is affected by various factors such as *in vitro* experimental conditions. In addition, gene expression of adipose tissue has been known to be influenced by HIV infection itself irrespective of HAART[50]. In this study, subcutaneous adipose tissue from treatment naïve HIV-1 infected patients contained lower concentrations of mitochondrial DNA-encoded cytochrome c oxidase subunit II than that of healthy controls, and the expression of nuclear genes coding for mitochondrial proteins, PPAR- γ, and mRNA expression of adiponectin as well as leptin were down regulated while TNF- α was up-regulated in HIV-1 infected patients irrespective of HAART. Considering these findings and other clinical studies[51, 52], disturbance in adipose tissue is supposed to be substantially influenced by HIV-1 infection itself. However, widely used *in vitro* experimental models using adipocyte can not reflect the effect of HIV-1 infection itself and this might be related to failed reproduction of the clinical phenomenon in this experiment.

Ⅴ. CONCLUSIONS

This study aimed to evaluate the influence of hyperglycemia on the NRTI related toxicity during the differentiation of 3T3-L1 preadipocyte.

1. The mean number of triglyceride accumulated adipocytes was higher in cells which were differentiated in a high glucose medium. However, the differentiation marker expressions such as C/EBP-α/β and FAS were not influenced by NRTI treatment or medium glucose concentration.
2. Mitochondiral function and membrane integrity evaluated by MTT assay and JC-1 staining were not influenced by NRTI treatment or medium glucose concentration.
3. 84.7 kDa cleaved form PARP protein expression representing apoptosis

was increased in zidovudine, stavudine, didanosine, and lamivudine treated adipocytes which were differentiated in a high glucose medium while only zidovudine treatment induced apoptosis in a low glucose medium.

4. Insulin induced anabolic signaling of Akt phosphorylation was attenuated in cells which were treated with zidovudine and stavudine in a high glucose environment while only stavudine attenuated Akt phosphorylation in a low glucose environment.

The above findings imply that adipocyte toxicity induced by NRTI treatment might be aggravated in a high glucose concentration environment.

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ABSTRACT(IN KOREAN)

고혈당이 제 1형 인면역결핍바이러스 치료를 위한 뉴클레오사이드 역전사효소억제제에 의해 유발된 지방세포의 세포사멸에 미치는 영향

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진 범 식

뉴클레오사이드 역전사효소억제제 (Nucleoside reverse transcriptase inhibitors, NRTI)는 고강도항레트로바이러스치료 (Highly active antiretroviral therapy, HAART)를 시행받는 인면역결핍바이러스 감염환자에서 지방위축을 유발하는 것으로 알려져 있다. 지방위축을 보이는 인면역결핍바이러스 감염자들은 대개 고인슐린혈증 양상을 보이며 종종 현증 당뇨병을 동반한다. 본 연구에서는 뉴클레오사이드 역전사효소억제제에 의해서 유발되는 지방세포의 세포사멸에 고혈당이 미치는 영향을 확인하고자 하였다.

쥣과 (murine)의 지방전구세포 3T3-L1을 서로 다른 농도의 포도당 용액 배지 (1.0 g/L, 4.5 g/L) 에서 분화시키고 다섯 가지의 뉴클레오사이드 역전사효소억제제 (zidovudine, stavudine, didanosine, lamivudine, abacavir)를 치료용량의 혈중최고농도로 11일간 각각 처치하였다. 지방전구세포의 분화는 (C/EBP)-α/β, FAS의 웨스턴 블럿 및 oil red O 염색을 통해 평가하였다. 세포사멸은 PARP/caspase-3 웨스턴 블럿 및 Annexin Ⅴ/PI 염색을 통해 평가하였으며 MTT assay 및 JC-1 염색을 통해 미토콘드리아의 기능과 막전위를 각각 평가하였다. 인슐린 저항성에 대한 평가를 위해 지방위축을 빈번하게 유발하는 것으로 알려진

zidovudine과 stavudine을 처치한 세포에서 인슐린 유도 동화작용을 나타내는 Akt 단백질 인산화 정도를 웨스턴 블럿을 통해 측정하였다.

지방세포로의 분화를 나타내는 중성지방을 함유한 지방세포의 평균 수는 고농도 포도당에서 분화된 군에서 더 큰 것으로 나타났다 (80.8 vs. 134.0, $P<0.001$). 그렇지만 C/EBP-α/β, FAS 등 분화표지 물질에 대한 웨스턴 블럿에서는 뉴클레오사이드 역전사효소억제제의 처치나 배지의 포도당 농도가 영향을 미치지 않는 것으로 나타났다. 또한 MTT assay 및 JC-1 염색을 통해 평가한 미토콘드리아 기능이나 막전위도 뉴클레오사이드 역전사효소억제제의 처치나 배지의 포도당 농도에 의해 변화되지 않았다. 그렇지만 고농도 포도당 배지에서 분화시킨 지방세포에서는 zidovudine, stavudine, didanosine, lamivudine 등을 처치한 경우에 세포사멸을 나타내는 84.7 kDa 크기의 분리된 PARP 단백질의 발현이 증가되는 것이 관찰되어 zidovudine 에서만 세포사멸이 증가된 저농도 포도당 배지 분화지방세포와 대조를 보였다. 또한 저농도 포도당 배지에서 분화된 지방세포는 stavudine 에 대해서만 인슐린 유도 동화작용을 나타내는 Akt 인산화가 저하된 소견을 보이는 반면에 고농도 포도당 배지에서 배양한 경우에는 zidovudine과 stavudine을 처치한 경우 모두에서 인슐린 유발 Akt 인산화가 저하되는 소견이 관찰되었다.

결론적으로 포도당의 농도 및 뉴클레오사이드 역전사효소억제제의 처치는 지방전구세포의 분화에 영향을 미치지 않았다. 그러나 뉴클레오사이드 역전사효소억제제에 의한 세포사멸 및 인슐린 유발 동화작용 저하는 고농도포도당 배지에서 분화된 지방세포에서 악화되는 경향을 보였다. 이는 고농도 포도당 환경에서 뉴클레오사이드 역전사효소억제제에 의한 지방세포독성이 악화될 수 있다는 것을 시사한다.

핵심되는 말 : 인면역결핍바이러스, 뉴클레오사이드 역전사효소억제제, 지방세포, 세포사멸