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Effects of High Sugar Consumption on Transcriptome of Adipose Tissues and Metabolome of Blood in Mice

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Abstract

Effects of High Sugar Consumption on Transcriptome of Adipose Tissues and Metabolome of Blood in Mice

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It is well known that sustained sugar consumption leads to the increase in prevalence of metabolic syndrome. Previously, animal model experiments were conducted for demonstrating the effects of sugar intake on metabolic syndrome-related markers in two different levels of dietary fat. Mice fed sugar solution with normal fat diet were compared with those fed plain water with normal fat diet, plain water with high fat, or sugar solution with high fat diet. Surprisingly, heightened level of serum triglyceride (TG) was found only in normal fat with sugar treated group, not in high fat and high fat with sugar fed groups, compared to control group. Another observation that there was dramatic increase in lipid accumulation in adipose tissue of high fat treated groups, but no increase in normal fat with sugar treated group, led us to hypothesize there might be an impairment in TG clearance from blood to adipocytes in this model.

Therefore, it was aimed to investigate the changes in transcriptional networks in adipose tissues of normal fat and sugar treated group compared to those of control, high fat and high fat with sugar treated groups. Firstly, RNA sequencing was performed in epididymal adipose tissues of three individual mice in each group. 6,849 differentially expressed genes in one group from the others were identified by ANOVA test. Functional enrichment analysis of the significant genes demonstrated that sugar ingestion with normal fat diet upregulated expression of genes involved in energy metabolism, especially in mitochondrial function. Moreover, the changes in mitochondrial functions affected the expression of transcription factor CREB and downstream molecules implicated in TG accumulation. The transcripts expression of CREB downstream molecules were reduced in normal fat diet and sugar drink treated mice, and this showed us the possibilities that the size of adipocytes did not expand in normal fat diet and sugar treated mice due to decrease of genes involved in TG accumulation in adipocytes. Also the expressions of fatty acid transporters were reduced by normal fat diet and sugar consumption, and this was accompanied by elevation of the level of fatty acids as well as TG in serum lipid metabolomics profile derived by LC-MS analysis. The concentration of majority of TG species in serum lipid profiles was increased by normal fat and sugar drink intake, whereas the concentration of TG species which were including double bond more than six was not elevated. These findings demonstrate the mechanism by which high sugar diet develop impairment of serum TG clearance into adipose tissues and provide new candidate for markers to target for hypertriglyceridemia prevention.

Keywords: Sugar, High fat diet, Mouse, Adipose Tissue, Transcriptomics, Metabolomics

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List of Abbreviations

ACSL1	Acyl-CoA Synthetase 1
ATP5	ATP Synthase
CAV	Caveolin
CBP	CREB-binding protein
CE	Cholesteryl ester
COX	Cytochrome C Oxidase Subunit
CYTB	Ubiquinol-Cytochrome-C Reductase Complex Cytochrome B Subunit
ETC	Electron transport chain
FA	Fatty acid
FABP	Fatty acid binding protein
FATP	Fatty acid transport protein
GPIHBP1	Glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1
GPR119	G protein-coupled receptor 119
LC-MS	Liquid chromatography-Mass spectrometry
LMF1	Lipase maturation factor 1
LPL	Lipoprotein lipase
LysoPC	Lysophosphatidylcholine
LysoPE	Lysophosphatidylethanolamine
ND	NADH Dehydrogenase Subunit
NDUF	NADH:Ubiquinone Oxidoreductase Subunit
PA	Phosphatidic acid
PC	Phosphatidylcholine
PCA	Principal component analysis
pCREB	Phosphorylated CREB
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
RNAseq	RNA sequencing
RPKM	Reads Per Kilobase of transcript per Million mapped reads
SDH	Succinate Dehydrogenase Complex

SM	Sphingomyelin
TG	Triglyceride
UQCR	Ubiquinol-Cytochrome C Reductase
VLDL	Very low-density lipoprotein
WAT	White adipose tissue

I. Introduction

Sugar is disaccharides composed of fructose and glucose, and contributes to energy density of diets (Gaby, 2005). It confers sweet taste to various food, so many people consumes sugar as sweeteners or processed food such as sugar-sweetened beverages (Edwards *et al.*, 2016). The availability of processed foods, the primary sources of sugar, has been increased, thus the consumptions of sugar has exceeded the recommendations (BBates, 2014). In the previous study, sugar was reported to enhance the mood temporarily, and it possesses addictive effect (Avena *et al.*, 2009). The excessive ingestion of sugar has been a worldwide phenomenon, therefore many countries exert the strong restraints regarding sugar intake, also World Health Organization suggested that adults and children decrease their consumption of free sugar to less than 10% of total energy intake (Edwards *et al.*, 2016) (WHO, 2015).

It is also worth noting that sustained sugar supplement results in increased prevalence of metabolic syndrome (Brownell and Frieden, 2009). The previous studies suggested that sugar ingestion was associated with development of cardiovascular diseases (Basu *et al.*, 2013), diabetes mellitus and obesity (MacGregor and Hashem, 2014). The most important cause of prevalence of metabolic diseases by sugar intake is that sugar consumption elevates the level of serum triglyceride (TG) (Mittendorfer and Sidossis, 2001). Increased amount of TG in serum is implicated in occurrence of hypertriglyceridemia (Yi *et al.*, 2013), also the elevated level of TG rich lipoproteins in serum are related with appearance of coronary artery diseases such as atherosis in

coronary artery (Colhoun *et al.*, 2002) and artery hardening (Van Eck *et al.*, 2000).

White adipose tissues are a major organ associated with regulating the level of TG in serum by uptake TG in serum and accumulating it as lipid droplets in adipocytes (Kershaw *et al.*, 2006). WAT is composed of nucleus, cytoplasm, mitochondria and lipid droplet (Wilson-Fritch *et al.*, 2004), and it stores energy as TG in lipid droplet when the amount of energy intake exceeds energy requirements in the body (Kershaw *et al.*, 2006). Also, adipose tissue generates energy in the starvation by hydrolyzing the lipid droplets and promoting fatty acid oxidation (Zimmermann *et al.*, 2004). However, although adipose tissues have a crucial role in regulation of amount of serum TG, it also plays main roles in developing overweight and obesity (Ibrahim, 2010).

Previously, the experiments were conducted for identifying the effect of sugar intake in normal fat diet and high fat diet fed mice (Lee, 2015). Diets were supplied into four groups: normal-fat diet; normal-fat diet with sugar drink; high-fat diet; high-fat diet with sugar drink. The results of experiments showed that the analysis of the quantity of total energy intake revealed no significant differences in normal fat diet with sugar drink treated group and high fat diet provided group, however, the level of serum TG was significantly elevated in normal fat diet and sugar drink treated group. The concentration of TG in blood is regulated by uptake of TG into myocytes or adipocytes (Kershaw *et al.*, 2006), and adipocytes store surplus TG as lipid droplet. The size of epididymal adipocytes was measured through H&E staining, and the results showed that the adipocytes did not expand in normal fat diet with sugar drink

treated group despite the high level of TG in serum. Furthermore, the weight of epididymal adipose tissues did not increase in normal fat with sugar treated group. These findings suggested that there might be defects in TG clearance by adipocytes.

Although the relationship between high sugar intake and the outbreak of metabolic diseases is obvious, the causes of failure to regulate TG homeostasis in serum by sugar ingestion remain poorly understood. To investigate the mechanism of failure in TG clearance caused by sugar consumption, RNA sequencing in epididymal adipose tissue was performed, and lipidome profile in serum of C57BL/6 mice fed experimental diets was analyzed.

II. Materials and Methods

1. Animal care and experimental design

Animal was raised according to the method of previous study (Lee, 2015). Five-week-old male C57BL/6 mice were purchased from Central Lab. Animal Inc. (Seoul, South Korea), maintained and accommodated under standard laboratory conditions during one week. Mice were assigned to four groups (C, HS, HF, HFHS) after accommodation and raised for 10 weeks. C was given normal fat diet and plain water; HS received normal fat diet and sugar drink, sugar drink was 23% (w/v) sugar solution; HF was treated with high fat diet and plain water; HFHS received high fat diet and sugar drink. Normal fat diet, 10% Kcal from fat (D12450J); high fat diet, 60% Kcal from fat(D124592) were acquired from Research Diets Inc. (New Brunswick, New Jersey, United States). All food and drink was supplied ad libitum. All experimental procedures were approved by the institutional animal care and use committee (IACUC) in Seoul National University (Approval number: SNU-131029-1-1; Seoul, South Korea)

2. Sample Preparation

Mice were sacrificed on the 70th day of experiment. Animals were fasted overnight and all food and drink was withdrawn except plain water. 16 hours after fasting, mice were anesthetized by intraperitoneal injection of 20% urethane solution (1.0-1.5 mg/g bw). Whole blood samples were obtained by cardiac puncture and coagulated at room

temperature. Whole blood were centrifuged at 3,000 × g for 10 min in 4℃ and serum was separated. The epididymal white adipose tissue was harvested and stored at -80℃ until further analysis.

3. Isolation of RNA from Adipose Tissues

Frozen white adipose tissues stored at -80°C were homogenized with TissueLyser II (Qiagen, Velenica, California, United States). Total RNA was isolated from epididymal adipose tissue using RNA isolation kit (RNAqueous-4PCR Kits, Invitrogen, Carlsbad, California, United States) according to the manufacturer's protocols. The integrity of the RNA was checked with a Nanodrop 2000 UV-Vis spectrophotometer (Thermo Scientific, Waltham, Massachusetts, United States).

4. mRNA Isolation from Total RNA

Polyadenylated mRNA was isolated from total RNA of adipose tissues using Dynabeads® mRNA DIRECT™ Micro Purification Kit (Invitrogen, Carlsbad, California, United States). Superparamagnetic beads adhered with oligo(dT) sequences was delivered into samples of total RNA to develop complementary combination of poly(A) chain at 3' terminus and oligo$(dT)_{25}$ residue, and substances except mRNA was washed and removed.

5. RNA Library Construction

mRNA was fragmented with RNase III and fragmented RNA was purified using Magnetic Bead Purification Module (Ion Torrent, Gilford, New Hampshire, United States). The quality of fragmented mRNA was assayed with Qubit® RNA Assay Kit and Qubit® 3.0 Fluorometer (Invitrogen, Carlsbad, California, United States). The size distribution of fragmented mRNA was identified with Agilent 2100 Bioanalyzer using Agilent RNA 6000 Pico Kit (Agilent Technologies, Santa Clara, California, United States).

Fragmented RNA was hybridized, ligated with Ion Total RNA-Seq Kit v2 (Ion Torrent, Gilford, New Hampshire, United States). Complementary DNA (cDNA) was synthesized through reverse transcription of fragmented RNA. cDNA was purified with Magnetic Bead Purification Module (Ion Torrent, Gilford, New Hampshire, United States) and barcode was tagged at the terminus of cDNA with Ion Xpress™ RNA-Seq Barcode and Ion Xpress™ RNA 3' Barcode Primer (Ion Torrent, Gilford, New Hampshire, United States). Barcode tagged cDNA was amplified through Polymerase Chain Reaction (PCR), and purified with Magnetic Bead Purification Module (Ion Torrent, Gilford, New Hampshire, United States). The concentration and the size distribution of amplified cDNA were verified with Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, United States) using Agilent DNA 1000 Kit (Agilent Technologies, Santa Clara, California, United States).

6. RNA Sequencing

Ion PI™ Hi-Q™ OT2 200 Kit and Ion OneTouch™ 2 Instrument (Ion Torrent, Gilford, New Hampshire, United States) was utilized to generate template-positive Ion PI™ Ion Sphere™ Particle (ISPs). Template-positive ISPs was produced through emulsion PCR, and the quality of ISPs pellet was validated with Qubit® 3.0 Fluorometer (Invitrogen, Carlsbad, California, United States). The defective template-positive ISPs was eliminated using Ion One Touch™ ES (Ion Torrent, Gilford, New Hampshire, United States).

The template-positive ISPs and control ISPs were compounded and sequencing primer was annealed to enriched ISPs to analyze the sequences of amplified libraries using Ion PI™ Hi-Q™ Sequencing 200 Kit (Ion Torrent, Gilford, New Hampshire, United States). The template-positive ISPs was loaded into Ion PI™ Chip Kit v3 (Ion Torrent, Gilford, New Hampshire, United States), and Ion PI™ Hi-Q™ Sequencing Polymerase (Ion Torrent, Gilford, New Hampshire, United States) was delivered to the chip. The analysis of sequences in cDNA was conducted through Ion Proton™ System (Ion Torrent, Gilford, New Hampshire, United States).

7. RNA-Seq Data Analysis

Reads alignment and annotation were performed with Partek Genomics Suite v6.6.

The RNA sequences were aligned to the UCSC mouse reference genome (version mm10) and annotated to the 'GENCODE Genes-release M7'. The data was normalized into RPKM (Reads Per Kilobase of transcript per Million mapped reads) values. Differential expression of genes was determined after conduct ANOVA with cutoff significance level of unadjusted p-value<0.05 in Partek Genomics Suite v6.6. Functional enrichment and pathway analysis were performed with Ingenuity Pathway Analysis software.

8. Protein Extraction from Adipose Tissues

Adipose tissue was homogenized using TissueLyser II (Qiagen, Velenica, California, United States). Nuclear protein was extracted from adipose tissues with NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Waltham, Massachusetts, United States) and protease and phosphatase in extracted protein was restrained using Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Waltham, Massachusetts, United States). The concentration of protein in nuclear and cytoplasmic fraction was measured with Micro BCA™ Protein Assay Kit (Thermo Scientific, Waltham, Massachusetts, United States).

9. Blotting Analysis

Nuclear protein samples of adipose tissues were loaded in Bolt™ 4-12% Bis-Tris Plus

Gels (Invitrogen, Carlsbad, California, United States) for SDS-PAGE, and separated protein was transferred to PVDF membranes (Invitrogen, Carlsbad, California, United States). Membranes were incubated for detection of phosphorylated CREB on ser133 (pCREB), and nuclear loading protein TATA-binding protein (TBP). Antibodies of pCREB and TBP were purchased from Cell Signaling Technology (Danvers, Massachusetts, United States) and diluted to 1:2000. Membranes were incubated with primary antibodies for 1 hour, then with anti-rabbit IgG (Cell Signaling Technology, Danvers, Massachusetts, United States) at a 1:2000 dilution for 1 hour. The blots were washed after incubation and visualized with ECL solution (Thermo Scientific, Waltham, Massachusetts, United States). Protein bands were quantified with Quantity One (Bio Rad, Hercules, California, United States) and visualized using GraphPad Prism 5 (San Diego, California, United States).

10. Serum Lipidome Preparation

Serum lipid extraction was performed with a chloroform:methanol mixture (2:1, v/v) as modified method of Folch et al. (Folch *et al.*, 1957). Briefly, 50 µl of serum was placed in a 2-ml vial. Next, 450 µl of a chloroform:methanol mixture (2:1, v/v) and the vial was thoroughly vortexed. Next, 100 µl of water was added and the vial was thoroughly vortexed. After centrifuging for 10 min at 13,000 rpm and 4°C, the lower phase was transferred to a new vial and evaporated under a stream of nitrogen. And this process were repeated a total of three times in order to increase the efficiency of

lipid extraction. Serum lipid extracts were diluted with 200 ul of an isopropanol:acetonitrile:water mixture (2:1:1, v/v/v), and a 10 µl injection was used for UPLC/Q-TOF MS.

11. Serum Lipidome Profiling Analysis

LC-MS was performed on a triple TOF™ 5600 MS/MS system (AB SCIEX, Concord, Canada) combined with a UPLC system (Waters, Milford, MA). Separations were performed on an Acquity UPLC BEH C18 column (2.1 × 100 mm with 1.7-µm particles; Waters). The binary gradient system comprised 10 mM ammonium acetate in an acetonitrile:water mixture (4:6, v/v) (solvent A) and 10 mM ammonium acetate in an acetonitrile:isopropanol mixture (1:9, v/v) (solvent B). The flow rate was maintained at 0.35 ml/min for 18 min.

The mass spectrometer was operated in the electrospray ionization-positive and -negative modes, and the mass range was set at m/z 100–1500. The following parameter settings were used: ion spray voltage of 5500 V, temperature of 500°C, curtain gas of 30 psi, declustering potential of 90 V, and collision energy of 10 V. In addition, information-dependent acquisition was used to trigger the acquisition of MS/MS spectra for ions matching the information-dependent acquisition criteria. MS/MS experiments were run with collision energy of 40 V and collision energy spread of 15 V. Mass accuracy was maintained with automated Calibrant Delivery System (AB Sciex, Concord, Canada) interfaced to the second inlet of the DuoSpray

source. Experiments were performed in duplicate, and the percent relative standard deviation (% RSD) for repeated measures of quality control was used to measure precision.

All MS data, including retention times, m/z, and ion intensities, were extracted by the Markerview software (AB Sciex) incorporated within the instrument, and the resulting data were assembled into a matrix. Metabolites were identified using the LipidMap (www.lipidmaps.org) and Human Metabolome Databases (www.hmdb.ca) and confirmed by standard samples samples (Avanti Polar Lipids, Alabaster, AL) based on both retention times and mass spectra.

III. Results

1. Transcriptome Profiles of Adipose Tissue

The experimental diet was supplied to mice into four groups: C, normal-fat diet; HS, normal-fat diet with sugar drink; HF, high-fat diet; HFHS, high-fat diet with sugar drink to demonstrate the effect of sugar consumption in normal fat and high fat diet fed mice. In the study, the level of serum TG was significantly increased in HS, whereas, the size of epididymal adipocytes which are involved in regulating serum TG did not expand. To identify the mechanism of defect in TG clearance resulted in normal fat diet with sugar intake, RNA sequencing was performed in epididymal adipose tissues acquired from mice of experimental groups because adipose tissues perform significant functions in regulating the level of serum TG by fetching immoderate TG and accumulating it as a form of lipid droplets (Kershaw *et al.*, 2006).

The libraries of mRNA in adipose tissues were constructed, and libraries were loaded on ISP beads and amplified, then ISP beads were placed into three Ion PI™ Chips. The loading density of ISP is approvable when the value of loading density is more than 50%, and the results of our loading density of Ion PI™ Chips were 87-89% (Figure 1). The sequencing of cDNA libraries was conducted, and the results of RNA-seq are represented in Table 1. The proportion of aligned reads in each sample from RNA-seq results was composed of coding region, ribosomal region, UTR, intronic and intergenic regions (Figure 2). The expression of sequences of coding regions was analyzed to acquire the knowledge about gene expression regulated by

normal fat diet and sugar treatment in mice. The expression values of coding sequences were converted and normalized into RPKM(Reads Per Kilobase of transcript per Million mapped reads) value, and the RPKM value was used in further analysis.

Principal component analysis (PCA) confirmed that the transcriptome profiles of normal fat diet and sugar treated mice were definitely distinguishable from other experimental groups (Figure 3). Differentially expressed genes in normal fat diet with sugar drink fed mice was established by ANOVA with the significant level of p-value < 0.05 restriction. The hierarchical clustering of gene expression in experimental diet treated mice represent that gene expression is divided into two sections subject to provision of high fat diets (Figure 4). The effects of sugar intake on alteration of gene expression have more influence in normal fat diet fed group than high fat diet fed group.

To gain a further understanding of the biological processes affected by normal fat diet with sugar drink consumption, functional enrichment analysis of the differentially expressed genes was performed based on IPA knowledge (Table 2). Consequently, the significantly enriched pathways by normal fat and high sugar consumption are revealed including oxidative phosphorylation, mitochondrial dysfunction, as well as TCA cycle. These pathways are involved in energy metabolism (Zheng, 2012); (Short *et al.*, 2005); (Akram, 2014) and take an important part in generating ATP. Therefore, it was found that high sugar consumption significantly changes the expression of genes involved in energy metabolism related to mitochondrial processes.

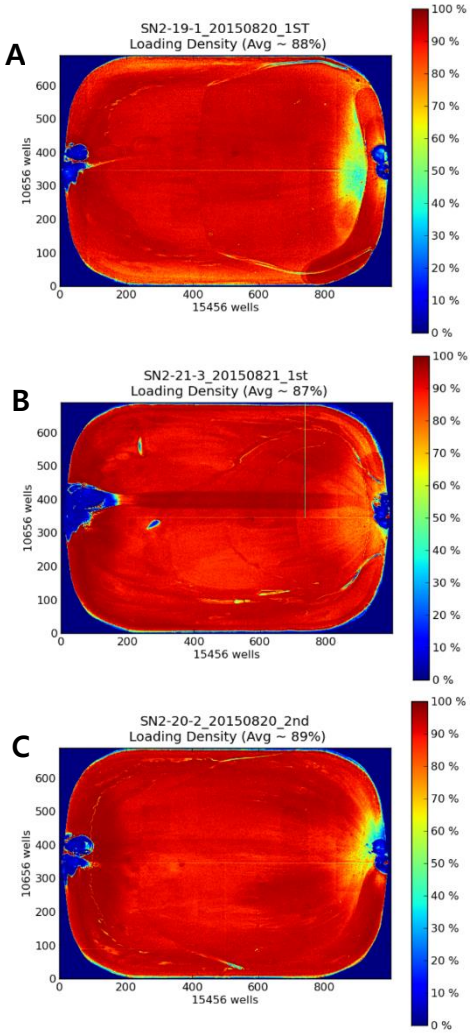

Figure 1. Loading density of ISP into Ion PI™ Chips

A, loading density of IPS beads of cDNA libraries from C1A, HS2A, HF2B, HFHS3A; B, loading density of IPS beads of cDNA libraries from C4B, HS4C, HF5A, HFHS1D; C, loading density of IPS beads of cDNA libraries from C2A, HS5B, HF1A, HFHS1C. Colors represent the degree of loading density of ISP into wells on Ion PI™ Chips.

Table 1. Summary of results from RNA sequencing

Group	Sample ID	Total Reads	Aligned Reads	Percent Aligned	Mean Read Length (bp)	Genes Detected
C	C1A	19994155	19723365	0.9865	109.2	16514
	C2A	17179457	16919557	0.9849	123.9	18553
	C4B	17130117	16900845	0.9866	104.7	16621
HS	HS2A	17052823	16896502	0.9908	106.2	16459
	HS4C	18917522	18496442	0.9777	109.2	16496
	HS5B	15531912	15338044	0.9875	120.4	15648
HF	HF1A	20393905	19854679	0.9736	97.7	15884
	HF2B	19049950	18681503	0.9807	113	15466
	HF5A	21700874	21130798	0.9737	108.1	15885
HFHS	HFHS1C	26510664	26031990	0.9819	115.5	17729
	HFHS1D	19756499	19310147	0.9774	112.5	15529
	HFHS3A	26327979	26006137	0.9878	107.5	16327

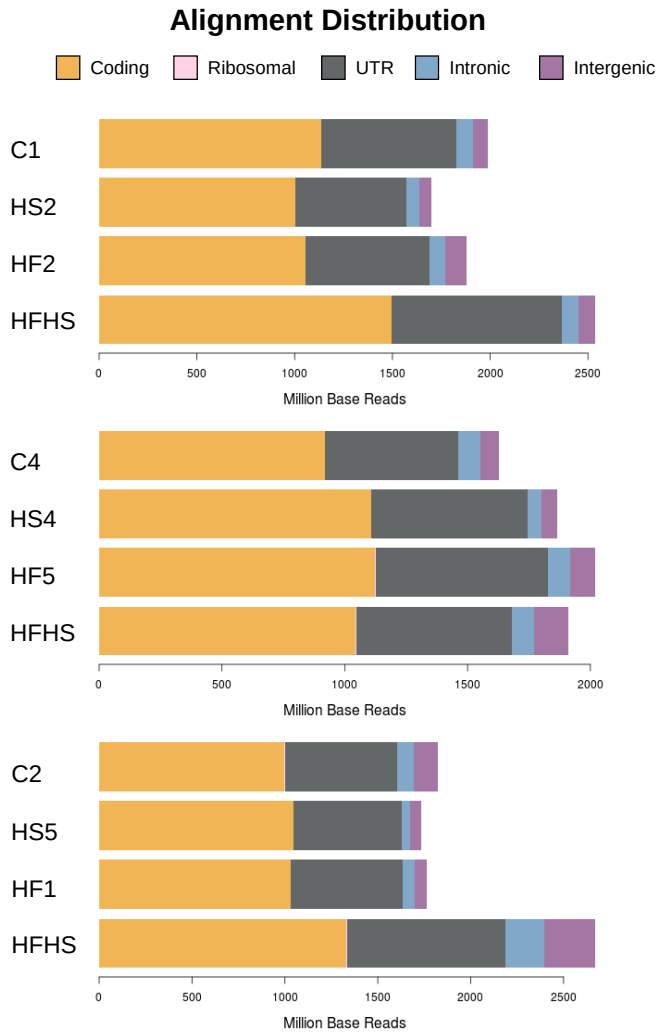

Figure 2. Alignment distribution of results from RNA sequencing

Summary of alignment distribution of library sequences of experimental groups. Coding, the proportion of coding sequences of a gene in entire reads; Ribosomal, the proportion of ribosomal RNA sequences; UTR, sequences of 3' and 5' untranslated region in RNA; Intronic, the sequences of intron in RNA; Intergenic, the proportion of sequences in intergenic region.

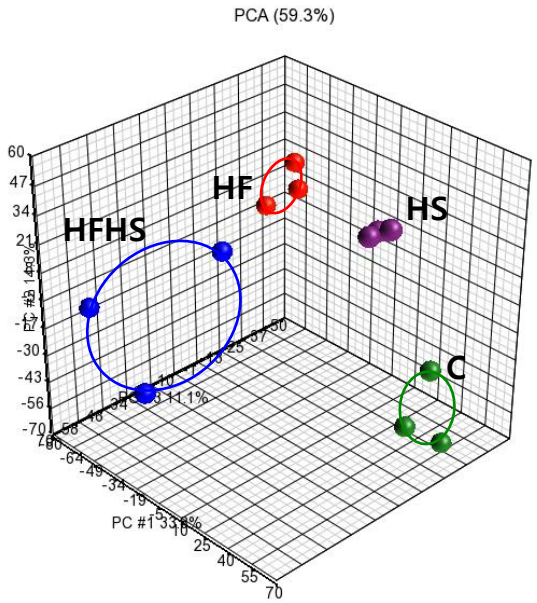

Figure 3. Principal component analysis of transcriptome in adipose tissue

Principal component analysis of transcripts from the RNA-seq dataset. C, control diet fed group; HS, control diet with 23% sugar drink treated group; HF, high fat diet fed group, HFHS, high fat diet with 23% sugar drink fed group. Each ball represents individual mouse.

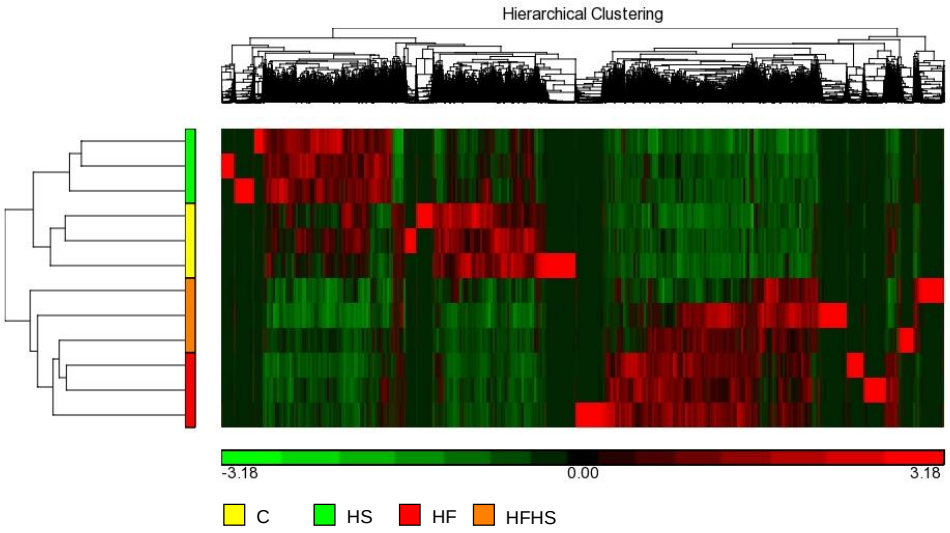

Figure 4. Hierarchical clustering of transcriptome in adipose tissue

Hierarchical clustering of transcripts from the RNA-seq dataset. C, control diet fed group; HS, control diet with 23% sugar drink treated group; HF, high fat diet fed group, HFHS, high fat diet with 23% sugar drink fed group.

Table 2. Functional enrichment analysis of transcriptome in adipose tissue induced by sugar treatment

Canonical Pathways	-log (p-value)	Genes in Term	Target Genes in Term	Molecules
Oxidative Phosphorylation	13.3	99	23	ATP5J,SDHA,NDUFV1,SDHB,COX7B,NDUFA9,ATP5A1,COX10,NDUFB5,UQCR11,NDUFB8,NDUFA2,NDUFB10,Atp5e,UQCRB,ATP5C1,NDUFB9,ATP5B,UQCRC2,NDUFS6,NDUFS2,NDUFA10,NDUFA3
Mitochondrial Dysfunction	12.8	159	28	HSD17B10,SDHB,COX7B,NDUFA9,COX10,NDUFB5,NDUFB8,NDUFA2,Atp5e,NDUFB10,PDHA1,NDUFB9,PARK7,NDUFS6,NDUFS2,NDUFA10,SDHA,ATP5J,NDUFV1,ATP5A1,UQCR11,UQCRB,ATP5C1,FIS1,ATP5B,UQCRC2,NDUFA3,PINK1
Protein Ubiquitination Pathway	4.36	245	21	FZR1,UBE4B,USP5,HLA-A,PSMD13,UBE3B,USP19,HSPD1,DNAJB2,PSMC5,PSMD8,ANAPC4,PSMD11,HSP90AB1,BAG1,UBE2V1,DNAJB6,PSMD4,DNAJB1,DNAJC7,UBE2J2
TCA Cycle II (Eukaryotic)	4.29	22	6	SDHA,SDHB,IDH3G,FH,MDH2,IDH3B
Isoleucine Degradation I	4.28	14	5	HSD17B10,ECHS1,AUH,ACADSB,EHHADH

2. Gene Expression in Mitochondrial Dysfunction and beta oxidation

The observation that 'mitochondrial dysfunction' was the most significant normal fat diet with sugar intake-induced pathway (Table 2) prompted us to examine further the expression of genes involved in mitochondrial function. There is electron transport chain(ETC) in mitochondrial inner membrane, and mitochondria generates ATP through ETC which is composed of complex I-V (Zhao *et al.*, 2014). Electrons are transported from NADH to ATP synthases in ETC, then ATP is synthesized in complex V. Many enzymes are associated in regulation of generation of ATP in mitochondrial electron transport chain complex.

Complex I is multisubunit of ubiquinone oxidoreductase (Adam-Vizi, 2005), and electron is transferred from NADH to ubiquinone in complex I. The expression of NADH:ubiquinone oxidoreductase subunit genes was significantly upregulated in normal fat and sugar drink treated group (Figure 5), whereas downregulated in high fat fed group (Figure 6) and high fat with sugar drink treated group (Figure 7). In ETC complex I, *Nd4l*, *Ndufa2*, *Ndufa3*, *Ndufa6*, *Ndufa9*, *Ndufa10*, *Ndufb2*, *Udufb5*, *Ndufb8*, *Ndufb9*, *Ndufb10*, *Ndufs2*, *Ndufs6*, *Ndufs7*, *Ndufv1* are upregulated by sugar treatment in normal fat condition (Figure 8), while downregulated in high fat and high fat with sugar treated groups. Complex II is composed of succinate dehydrogenase complex, it conveys electron from succinate to ubiquinone (Hao *et al.*, 2009). The

transcripts of succinate dehydrogenase complex, *Sdha*, *Sdhb*, *Sdhc* are highly expressed in HS, whereas down expressed in high fat and high fat with sugar treated groups (Figure 8). In complex III, electron is transferred from reduced ubiquinone, ubiquinol, to cytochrome c (Solmaz and Hunte, 2008). The transcripts of complex III such as *Cytb*, *Uqcr10*, *Uqcrb*, *Uqcrc1*, *Uqcr2* are decreased by high fat and high fat with sugar consumption, however, increased by normal fat diet and high sugar intake (Figure 8). Complex IV is consist of cytochrome c oxidase, and removes four electrons from cytochrome C and transfers it to dioxygen to generates two molecules of H_2O (Balsa *et al.*, 2012). In complex IV, *Cox1*, *Cox2*, *Cox4i1*, *Cox7B*, *Cox10* are upregulated in normal fat and sugar drink treated group. The eight protons transferred from cytochrome C are eliminated from mitochondrial matrix and gradation of proton concentration is constructed, and ATP is synthesized by ATP synthase in complex V (Hubal *et al.*, 2011). While the transcripts of ATP synthases such as *Atp5a1*, *Atp5b*, *Atp5c1*, *Atp5d*, *Atp5e*, *Atp5g3*, *Atp5j* are downregulated by high fat and high fat with sugar intake, the high sugar consumption with normal fat diet upregulated the mRNA expression of ATP synthases.

In results of functional enrichment analysis, 'beta oxidation' was the one of the top enriched pathway, thus the expressions of transcripts related in beta oxidation in adipose tissue was identified (figure 9). The transcripts level of *Hsd17b10, Echs1, Auh, Ehhadh, Edi1* was higher in sugar treated group than in high fat treated group.

Taken together, the transcript expression of enzymes of electron transport

chain I-V shows the tendency of upregulation by normal fat with high sugar consumption. In contrast, high fat and high fat with sugar treated group represent decreased mRNA expression of enzymes of ETC. Moreover, the expressions of majority genes involved in beta oxidation in adipose tissues was upregulated in normal fat and sugar treated group.

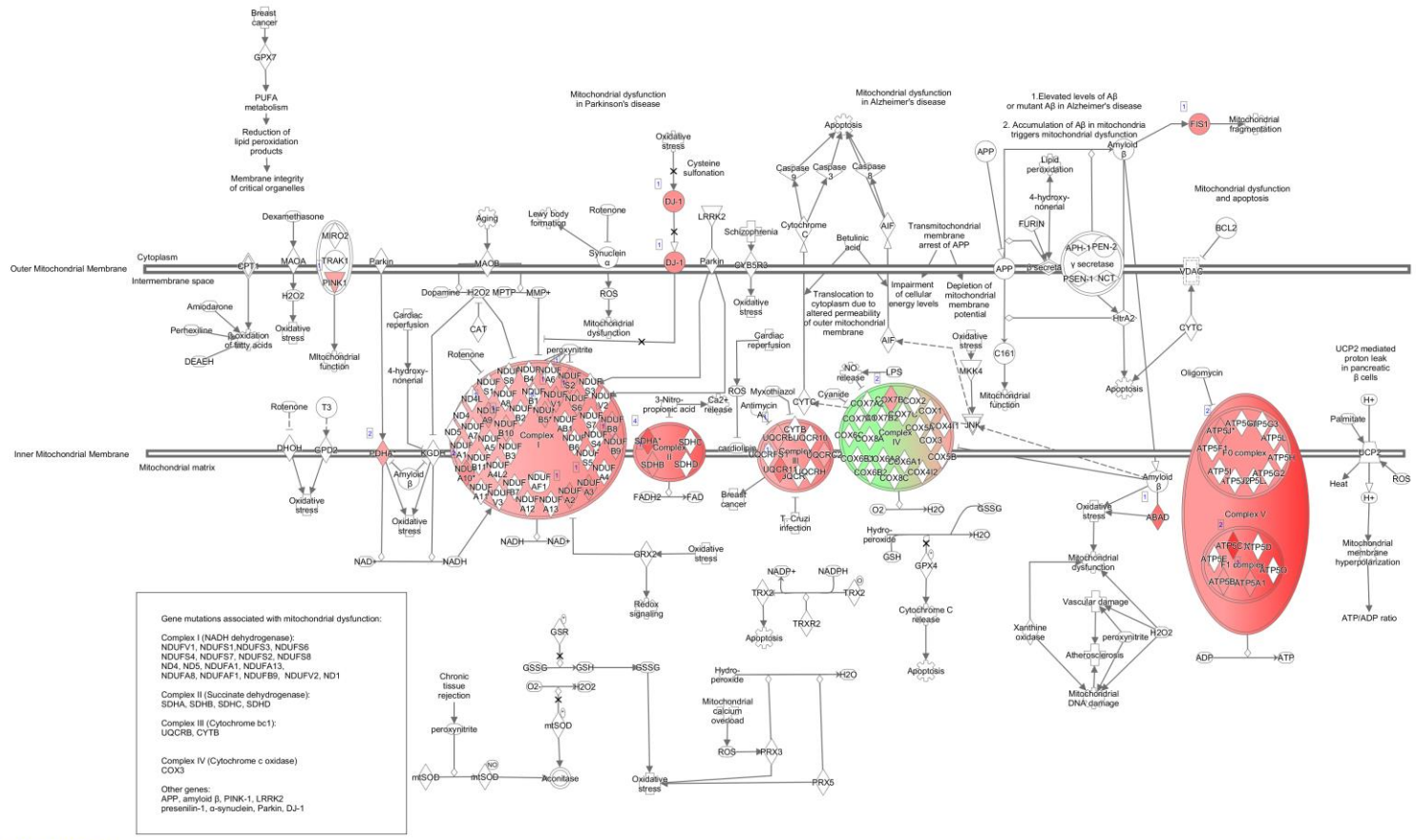

Figure 5. Genes involved in mitochondrial dysfunction in adipose tissue of normal fat with sugar drink treated mice

Diagram of genes implicated in mitochondrial function. The expression value of genes in diagram is fold change of HS toward C (control). Red colored complex is upregulated in HS toward C (control). Cutoff value of genes is RPKM >2, p-value<0.05. Pathways are based on IPA knowledge.

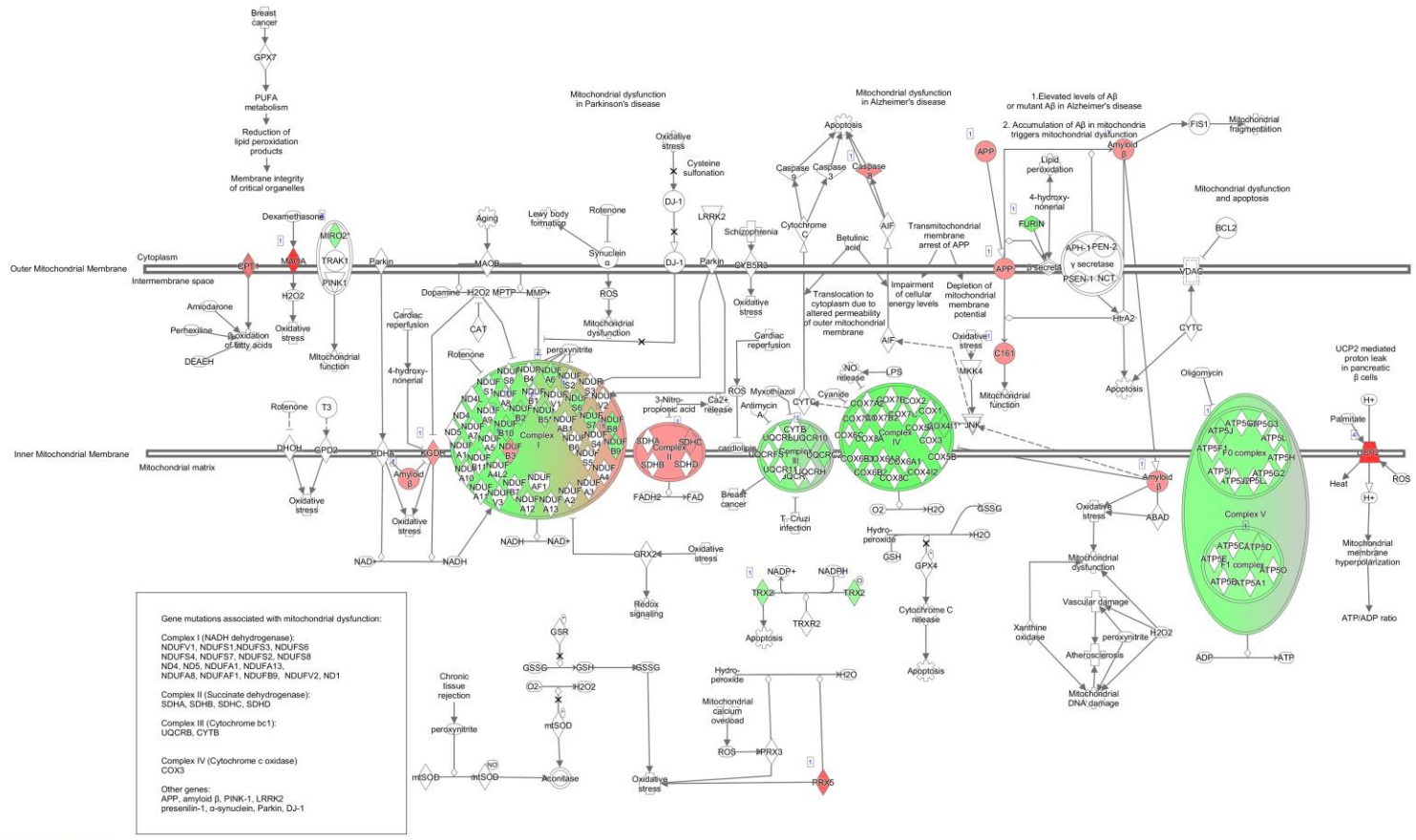

Figure 6. Genes involved in mitochondrial dysfunction in adipose tissue of high fat fed mice

Diagram of genes implicated in mitochondrial function. The expression value of genes in diagram is fold change of HF toward C (control). Red colored complex is upregulated in HF toward C (control). Cutoff value of genes is RPKM >2, p-value<0.05. Pathways are based on IPA knowledge.

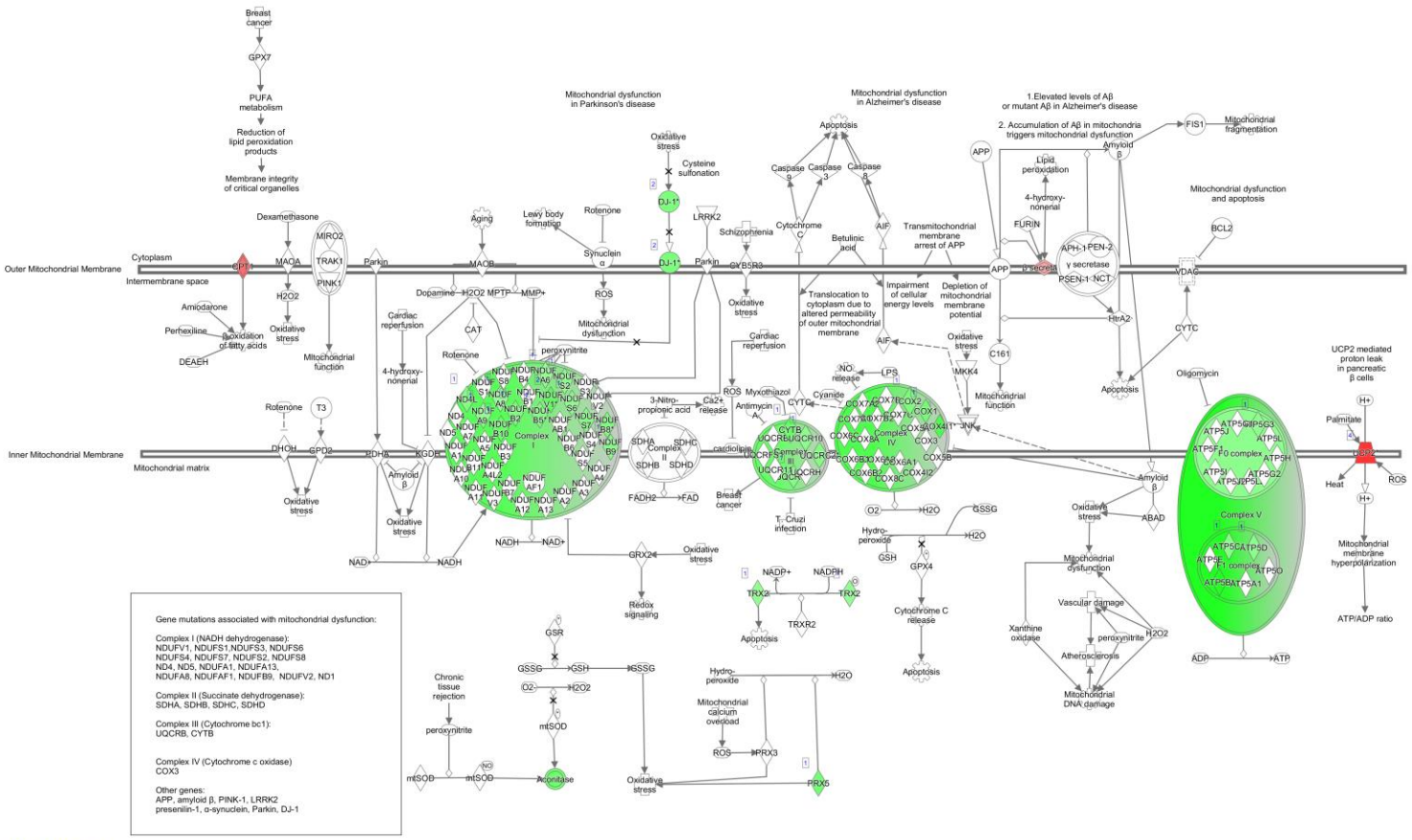
Mitochondrial dysfunction in Parkinson's disease
Mitochondrial dysfunction in Alzheimer's disease
1.Elevated levels of Aβ or mutant Aβ in Alzheimer's disease
2. Accumulation of Aβ in mitochondria triggers mitochondrial dysfunction
Mitochondrial fragmentation
Mitochondrial dysfunction and apoptosis
Breast cancer
GPX7
PUFA metabolism
Reduction of lipid peroxidation products
Membrane integrity of critical organelles
Dexamethasone
MIRO2
TRAK1
PINK1
MAOA
CPT1A
Parkin
Cytoplasm
Intermembrane space
Outer Mitochondrial Membrane
Inner Mitochondrial Membrane
Mitochondrial matrix
Amiodarone
Perhexiline
Oxidation of fatty acids
DEAEH
H2O2
Oxidative stress
Mitochondrial function
Aging
Lewy body formation
Rotenone
Synuclein α
MAOB
Dopamine
H2O2
MPTP
MPP+
ROS
Mitochondrial dysfunction
Oxidative stress
Cardiac reperfusion
CAT
peroxynitrite
4-hydroxy-nonenal
Rotenone
T3
DHOH
GPD2
Oxidative stress
Amyloid β
Oxidative stress
NAD+
NADH
KGDH
PDHA
Complex I
NADH
NAD+
Oxidative stress
GRX2
Redox signaling
Oxidative stress
Oxidative stress
DJ-1*
Cysteine sulfonation
DJ-1*
LRRK2
Parkin
Schizophrenia
GYB5R3
Oxidative stress
Cardiac reperfusion
ROS
Ca2+ release
3-Nitro-propionic acid
SDHA
SDHB
Complex II
SDHC
SDHD
FADH2
FAD
Myxothiazol
Antimycin A
CYTC
Cyanide
Complex III
UQCRB
UQCRC1
UQCRC2
UQCRH
UQCRFS1
UQCR10
UQCR11
Breast cancer
T. Cruzi infection
Apoptosis
Caspase 9
Caspase 3
Caspase 8
Cytochrome C
AIF
Betulinic acid
Transmitochondrial membrane arrest of APP
Translocation to cytoplasm due to altered permeability of outer mitochondrial membrane
Impairment of cellular energy levels
Depletion of mitochondrial membrane potential
AIF
Oxidative stress
MKK4
JNK
Mitochondrial function
C161
NO release
LPS
Complex IV
COX7A1
COX7A2
COX2
COX1
COX7B
COX7B2
COX7C
COX3
COX4I1
COX4I2
COX5A
COX5B
COX6A1
COX6A2
COX6B1
COX6B2
COX6C
COX8A
COX8C
O2
H2O
GSSG
Hydro-peroxide
H2O
GSH
GPX4
Cytochrome C release
Apoptosis
NADP+
NADPH
TRX2
TRXR2
TRX2
Apoptosis
APP
Lipid peroxidation
4-hydroxy-nonenal
FURIN
APP
β-secretase
APH-1
PEN-2
γ secretase
PSEN-1
NCT
HtrA2
APP
Amyloid β
FIS1
Mitochondrial fragmentation
BCL2
VDAC
CYTC
Apoptosis
Amyloid β
ABAD
Oxidative stress
Mitochondrial dysfunction
Vascular damage
Xanthine oxidase
peroxynitrite
H2O2
Atherosclerosis
Mitochondrial DNA damage
Oligomycin
Complex V
F1 complex
F0 complex
ATP5A1
ATP5B
ATP5C
ATP5D
ATP5E
ATP5O
ATP5J
ATP5J2
ATP5L
ATP5H
ATP5I
ATP5G2
ATP5G3
ATP5F1
ATP5L
ADP
ATP
UCP2 mediated proton leak in pancreatic β cells
Palmitate
UCP2
Heat
ROS
Mitochondrial membrane hyperpolarization
ATP/ADP ratio
GSR
GSSG
GSH
GSSG
O2
H2O2
mtSOD
Chronic tissue rejection
peroxynitrite
mtSOD
NO
mtSOD
Aconitase
Hydro-peroxide
H2O
Mitochondrial calcium overload
ROS
PRX3
Oxidative stress
PRX5
Gene mutations associated with mitochondrial dysfunction:
Complex I (NADH dehydrogenase):
NDUFV1, NDUFS1,NDUFS3, NDUFS6
NDUFS4, NDUFS7, NDUFS2, NDUFS8
ND4, ND5, NDUFA1, NDUFA13,
NDUFA8, NDUFAF1, NDUFB9, NDUFV2, ND1
Complex II (Succinate dehydrogenase):
SDHA, SDHB, SDHC, SDHD
Complex III (Cytochrome bc1):
UQCRB, CYTB
Complex IV (Cytochrome c oxidase)
COX3
Other genes:
APP, amyloid β, PINK-1, LRRK2
presenilin-1, α-synuclein, Parkin, DJ-1
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Figure 7. Genes involved in mitochondrial dysfunction in adipose tissue of high fat with sugar drink treated mice

Diagram of genes implicated in mitochondrial function. The expression value of genes in diagram is fold change of HFHS toward C (control). Red colored complex is upregulated in HFHS toward C (control). Cutoff value of genes is RPKM >2, p-value<0.05. Pathways are based on IPA knowledge.

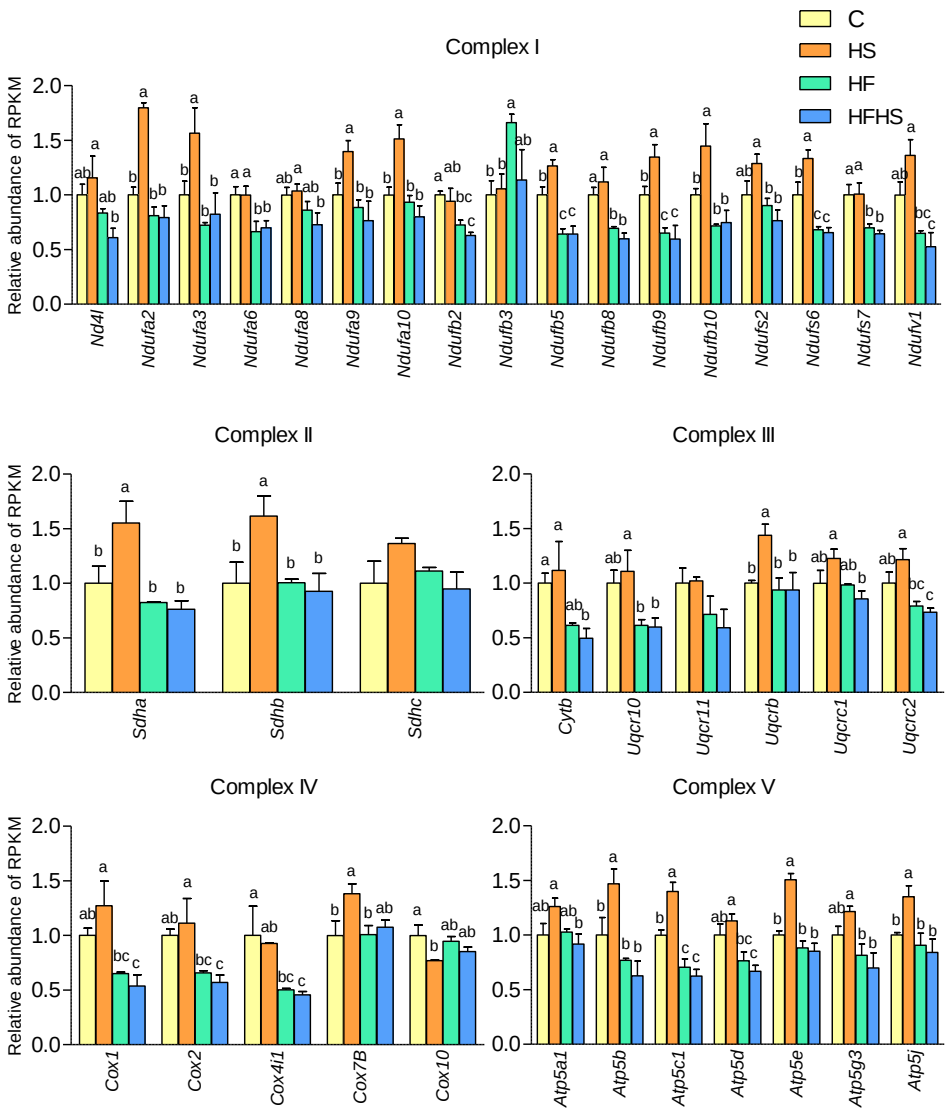

Figure 8. The expression of genes related in mitochondrial electron transport chain

The relative expression values of genes involved in mitochondrial electron transport chain complex I-V. Values are relative abundance of relative expression of RPKM

toward control group in adipose tissues. * p-value <0.05, One-way ANOVA followed by Duncan's MRT post hoc comparison. ND, NADH Dehydrogenase Subunit; NDUF, NADH:Ubiquinone Oxidoreductase Subunit; SDH, Succinate Dehydrogenase Complex; CYTB, Ubiquinol-Cytochrome-C Reductase Complex Cytochrome B Subunit; UQCR, Ubiquinol-Cytochrome C Reductase, Complex III Subunit; COX, Cytochrome C Oxidase Subunit; ATP5, ATP Synthase.

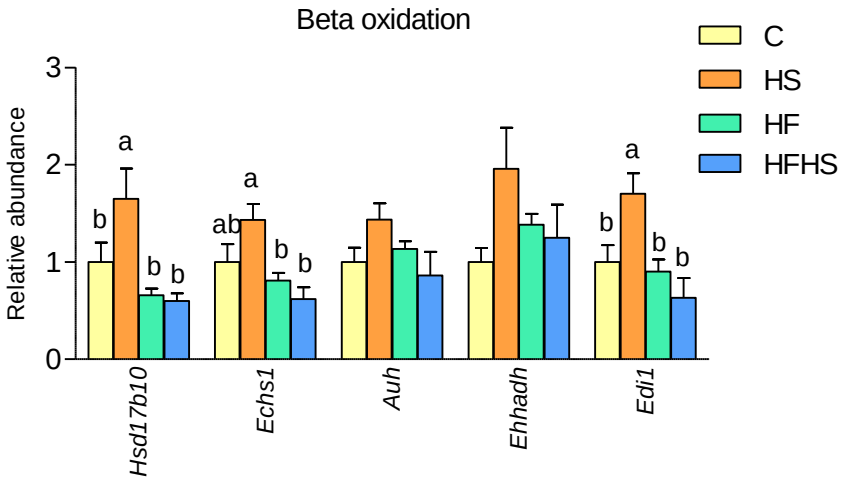

Figure 9. Transcripts expression of molecules related in beta oxidation

Relative transcripts expression of molecules involved in beta oxidation. The expression values are relative expression of RPKM toward control group. * p-value <0.05, One-way ANOVA followed by Duncan's MRT post hoc comparison.

3. Inhibition of CREB Pathway by High Sugar Intake

Previous studies showed that mitochondrial dysfunction induced by a mitochondrial electron transport chain complex inhibitor leaded to phosphorylation of transcription factor CREB to Ser133. (Vankoningsloo *et al.*, 2006); (De Pauw *et al.*, 2009). The phosphorylated CREB recruits co-activator CREB-binding protein (CBP) (Mayr and Montminy, 2001) and initiates the transcription of several genes in adipose tissues. CREB arouses the transcription of genes associated in differentiation of adipose tissues, (Vankoningsloo *et al.*, 2006) and regulates adipogenesis through TG accumulation in adipocytes. (Reusch *et al.*, 2000). The CREB pathway was downregulated in HS (Figure 10), whereas upregulated in HF (Figure 11) and HFHS (Figure 12).

Next, the expression of phosphorylated CREB (pCREB) protein was examined to estimate the degree of activation of CREB (Figure 13). Nuclear protein was extracted from adipose tissues of experimental diet treated mice, then TATA-binding protein(TBP) was examined as a nuclear loading control and the relative expression of pCREB protein was measured. The quantification of this assay showed us that the level of pCREB protein was lower in HS than in HF and HFHS.

The downstream transcripts of CREB such as *Cebpb*, *Ppargc1a*, *Insig1*, *Insig2*, *Srebf1* are known as a regulator of accumulation of TG in adipocytes (Park *et al.*, 2010); (Spiegelman *et al.*, 2000); (Engelking *et al.*, 2005); (Ka *et al.*, 2009); (Carobbio *et al.*, 2013); (Wang *et al.*, 2015). The relative mRNA expressions of *Cebpb*, *Insig1* in HS toward C was lower than the relative transcripts level in HF, HFHS

toward C (Figure 14), whereas the gene expression of *Ppargc1A*, *Insig2*, *Srebf1* is higher in HSvsC than in HFvsC, HFHSvsC.

In summary, high sugar consumption with normal fat diet upregulates the expression of mitochondrial genes, then induces the downregulation of CREB transcript and protein expression. Also the expression of CREB transcripts has an effect of modulation in CREB downstream molecules associated with TG accumulation in adipocytes.

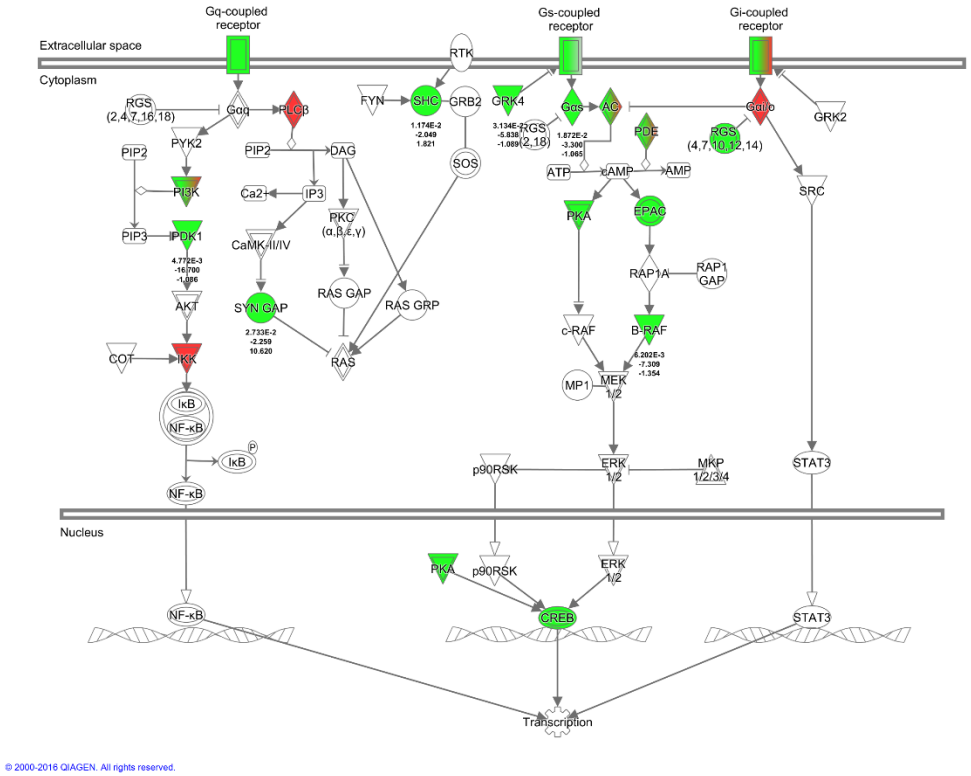

Figure 10. CREB pathway induced by sugar drink treatment in adipose tissue of normal fat diet with sugar drink treated mice

Diagram of G-protein coupled signaling in HS. The expression value of genes in diagram is transcripts level in adipose tissue. The color represents fold change in HS toward C (control). Green colored genes are downregulated, red colored genes are upregulated. Cutoff value is p-value < 0.05, Fold change $\geq |2|$. Pathways are based on IPA knowledge.

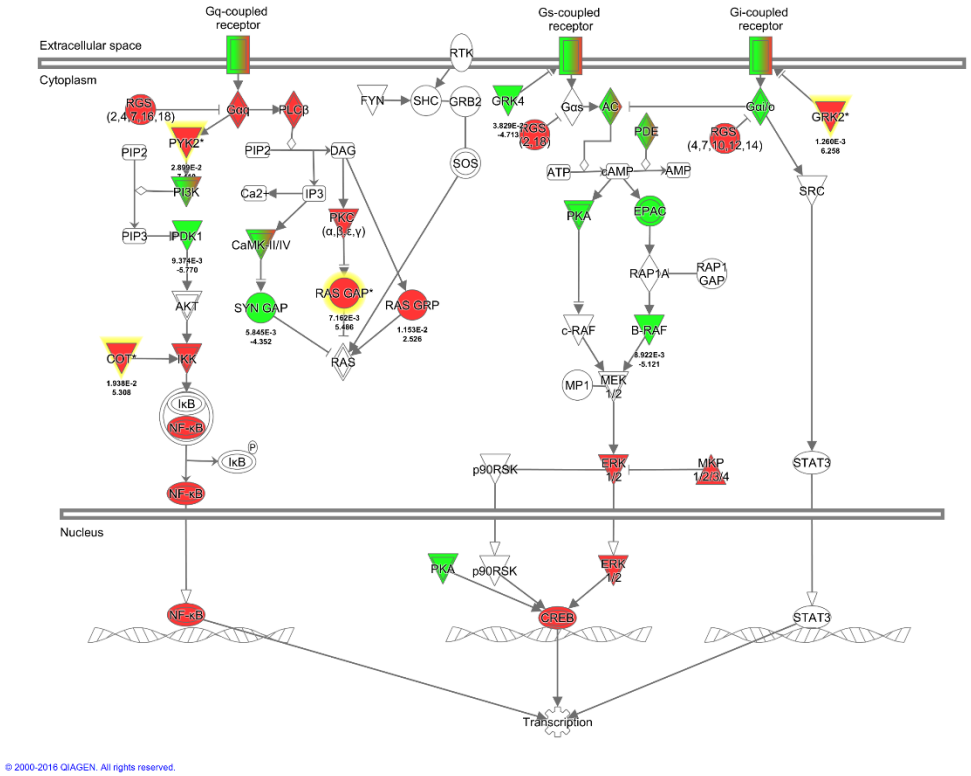

Figure 11. CREB pathway induced by sugar drink treatment in in adipose tissue of high fat fed mice

Diagram of G-protein coupled signaling in HF. The expression value of genes in diagram is transcripts level in adipose tissue. The color represents fold change in HF toward C (control). Green colored genes are downregulated, red colored genes are upregulated. Cutoff value is p-value < 0.05, Fold change $\geq |2|$. Pathways are based on IPA knowledge.

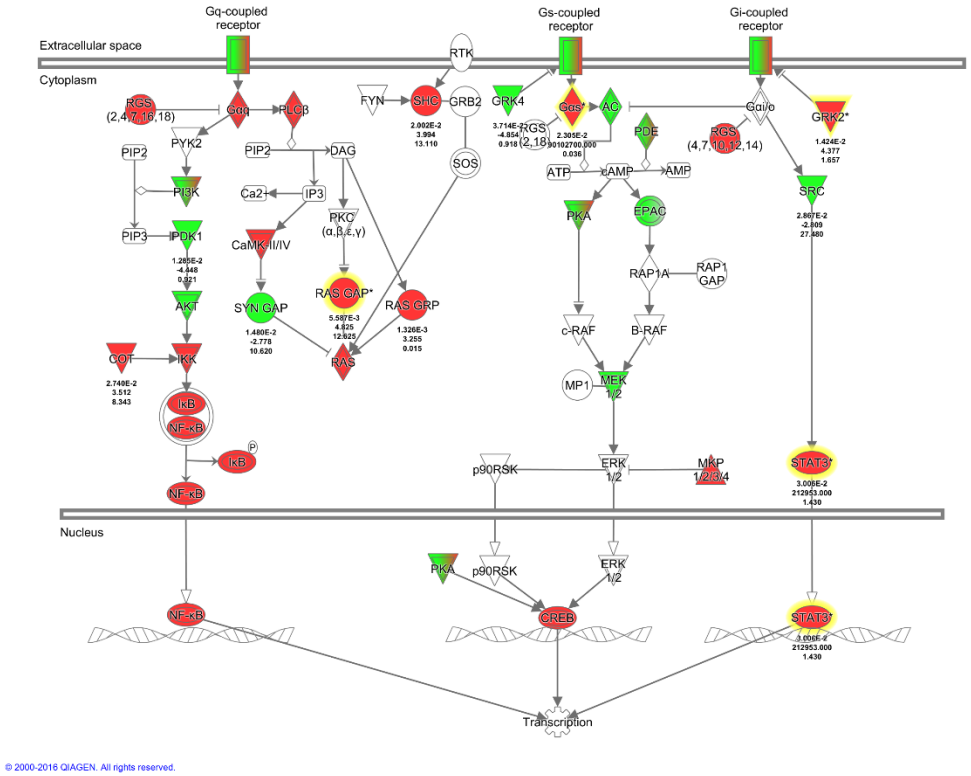

Figure 12. CREB pathway induced by sugar drink treatment in in adipose tissue of high fat with sugar drink treated mice

Diagram of G-protein coupled signaling in HFHS. The expression value of genes in diagram is transcripts level in adipose tissue. The color represents fold change in HFHS toward C (control). Green colored genes are downregulated, red colored genes are upregulated. Cutoff value is p-value < 0.05, Fold change $\geq |2|$. Pathways are based on IPA knowledge.

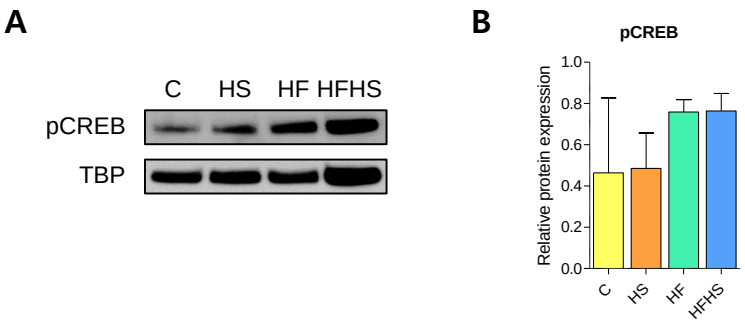

Figure 13. Western blot assay of phosphorylated CREB.

A, Protein expression of phosphorylated CREB and TATA-binding protein assayed by Western blot. Representative samples in each group are shown above. B, Phosphorylated CREB protein expression quantified from western blot assay.

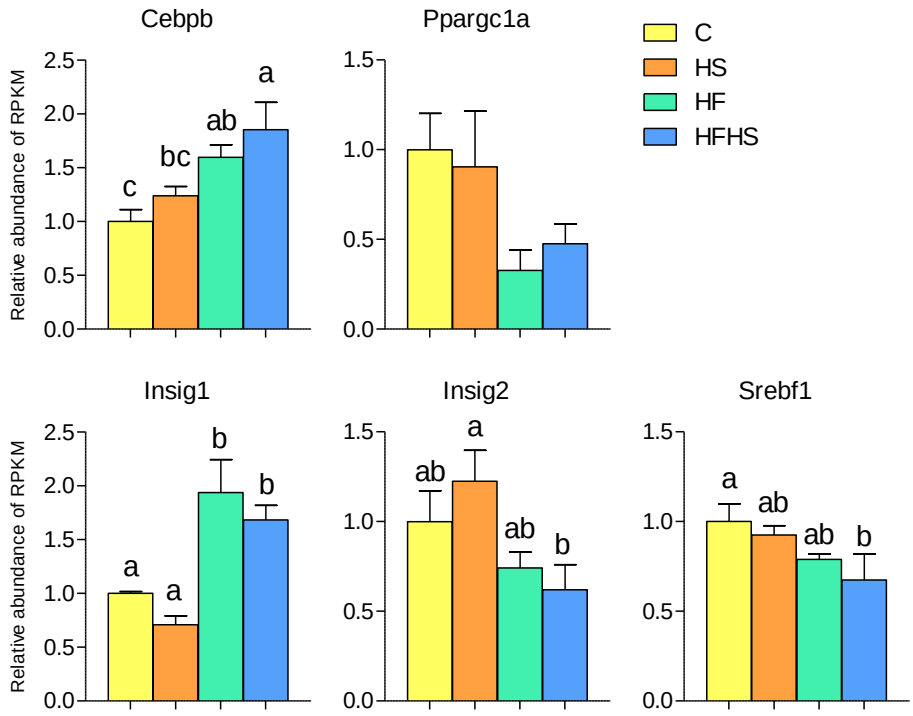

Figure 14. Downstream molecules of CREB

Relative expression of downstream molecules of CREB which are involved in accumulation of TG in adipocytes. The expression values are relative expression of RPKM toward control group. * p-value <0.05, One-way ANOVA followed by Duncan's MRT post hoc comparison.

4. Transcripts Levels of Fatty Acid Transporters in Adipocytes

Next, the gene expressions of fatty acid (FA) transporters in adipose tissue was investigated to see any defect in TG clearance to the adipose tissues in HS group. Adipose tissue has an important role in regulating the concentration of TG in serum. (Kershaw *et al.*, 2006). TG is transported in blood stream as a form of very low-density lipoprotein (VLDL). Free fatty acid which is generated from hydrolysis of TG is transported to adipocyte, then reesterified and accumulated as lipid droplets (Lobo *et al.*, 2007). Several molecules have been involved in uptake of long chain fatty acid from blood stream to adipocytes. (Figure 15).

Lipoprotein lipase(LPL) hydrolyzes TG of chylomicron or VLDL and generates free glycerol and fatty acid (Attie, 2007) which is transported to adipocyte. Several factors are implicated in appropriate operation of LPL. LMF1 has a role in maturation of LPL in ER of adipocytes (Péterfy *et al.*, 2007), and matured LPL migrates to capillary endothelium through transcytosis, then anchored to the luminal surface by GPIHBP1. (Fisher, 2010); (Williams, 2009). Immovable LPL hydrolyzes TG in VLDL and generates free fatty acids. (Attie, 2007). The expression of genes involved in activation of LPL was not decreased by normal fat diet and sugar intake (Figure 16A) compared to high fat and high fat with sugar fed groups. The level of transcripts of maturation factor *Lmf1* was higher in HS group than in HF, HFHS groups, and the expression of *Gpihbp1*, *Lpl* was not lower in group HS compared to HF.

Free fatty acid generated from hydrolysis of TG is transported to adipocytes by long chain fatty acid transporters. Fatty acid is facilitated diffused by CD36 (Coburn *et al.*, 2000) or transported by FATP (SLC27A) into adipocytes. (Glatz *et al.*, 2010). FABP binds to fatty acid at the surface of endothelial cell and translocate it to site of action of CD36. (Brinkmann *et al.*, 2002). CAV1 has a role in migrating CD36 to membrane for uptake long chain fatty acids. (Ring *et al.*, 2006). The transcripts level of fatty acid transporters such as *Fabp5, Fatp5, Fatp6* was lower in HS than in HF, HFHS, and the expressions of *Cav1, Cd36* were lower in HS in comparison with HF group (Figure 16B). These results suggest that the hydrolysis of TG is not impeded by with sugar consumption with normal fat diet, however, TG is less transported to adipocyte in normal fat diet with high sugar treated group than in high fat and high fat with sugar fed groups.

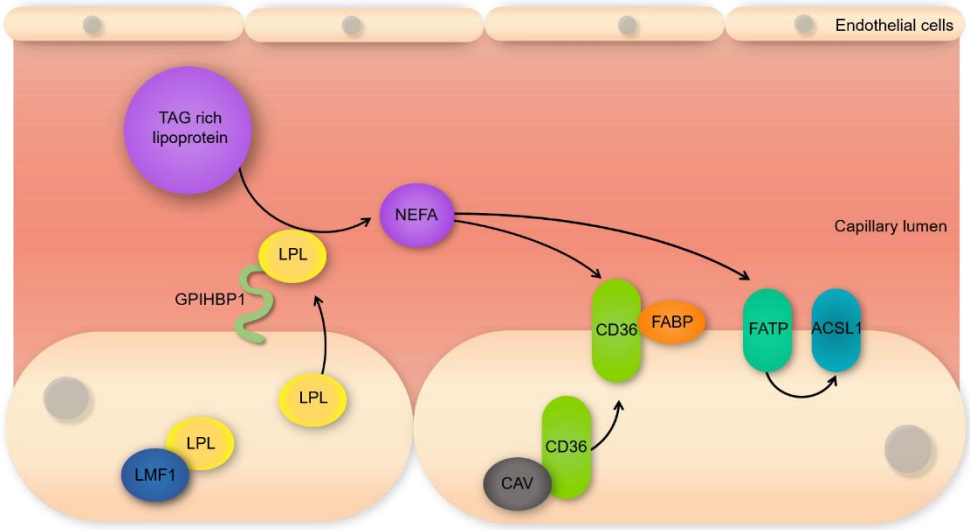

Figure 15. Molecules involved in fatty acid uptake from blood stream to adipocyte

Diagram of mechanism associated with fatty acid uptake to adipocyte. NEFA, non-esterified fatty acid; LMF1, lipase maturation factor 1; LPL, lipoprotein lipase; GPIHBP1, glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1; CAV, caveolin; FABP, fatty acid binding protein; FATP, fatty acid transport protein; ACSL1, acyl-CoA Synthetase 1.

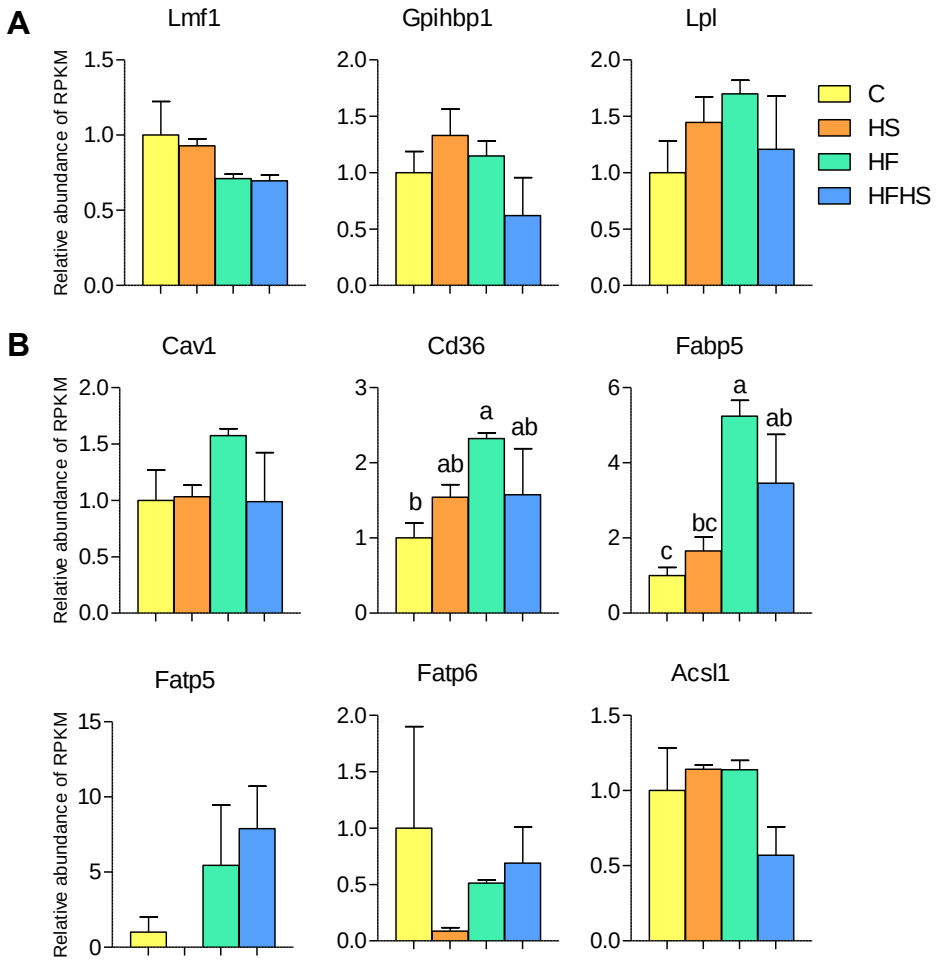

Figure 16. The expression of genes involved in fatty acid uptake from blood stream to adipocyte

A. Molecules involved in TG hydrolysis; B, Molecules encoding fatty acid transporter proteins. The expression values are relative expression of RPKM toward C (control). * p-value <0.05, One-way ANOVA followed by Duncan's MRT post hoc comparison.

5. Global Profiling of Serum Lipid Metabolomics

The analysis of serum lipid metabolomics was conducted through liquid chromatography-mass spectrometry. The concentrations of TG containing fatty acid chains are represented in Figure 17. The concentration of global TG species was elevated in sugar treated group, whereas reduced in high fat treated group. TG species are divided into four parts subject to number of double bond contained in three fatty acids. TG containing fatty acids including double bonds less than five, TG48:1, TG50:1, TG46:2, TG48:2, TG50:2, TG48:3, TG50:3, TG56:3 were significantly increased in normal fat and sugar treated group, decreased in high fat treated group.

In the global lipid profiling of each group, several lipid species were detected (Figure 18), and the level of respective lipids was different in each group. Lysophosphatidylethanolamine (LysoPE) is generated from hydrolysis of phosphatidylethanolamine and acts as a ligand of GPR119. (Soga *et al.*, 2005). The level of serum LysoPE is increased in HS, whereas decreased in high fat fed mice. Lysophosphatidylcholine (LysoPC) is created from phosphatidylcholine by hydrolysis and directly activates the G2A receptor and release of two Gα subunits. (Khan *et al.*, 2010). LysoPC represents the tendency of decrease in high fat consumption group, high fat and high fat with sugar treated group.

Triglyceride (TG) participates in various biological function such as accumulation of fat-soluble toxic material, storage of energy, or providing fatty acids. (Shi and Cheng, 2009). TG concentration in serum differs in HS and HF, HFHS, and this

is in line with the elevated amount of serum TG in normal fat diet with normal fat with sugar treated group of previous study (Lee, 2015). The concentration of TG in serum was increased in NF, whereas decreased in HF, HFHS (Figure 17). Fatty acid (FA) has a role as a ligand of various transcription factor, (Jump *et al.*, 2013), as a component of diverse phospholipids or triglycerides (Simopoulos, 2013), or as a regulator of fluidity of cell membrane. (Yao and Rock, 2015). The level of fatty acids was elevated in normal fat with high sugar consumption group (Figure 17).

In the global profile of lipid metabolomics, it was found that the level of TG in metabolomics was higher in HS than in HF, HFHS. In addition, the expression level of lysoPE, lysoPC, FA was different between normal fat diet with high sugar consumption group and high fat fed groups, also the composition of TG including three fatty acids containing double bonds represents noticeable distinctions in HS, HF and HFHS.

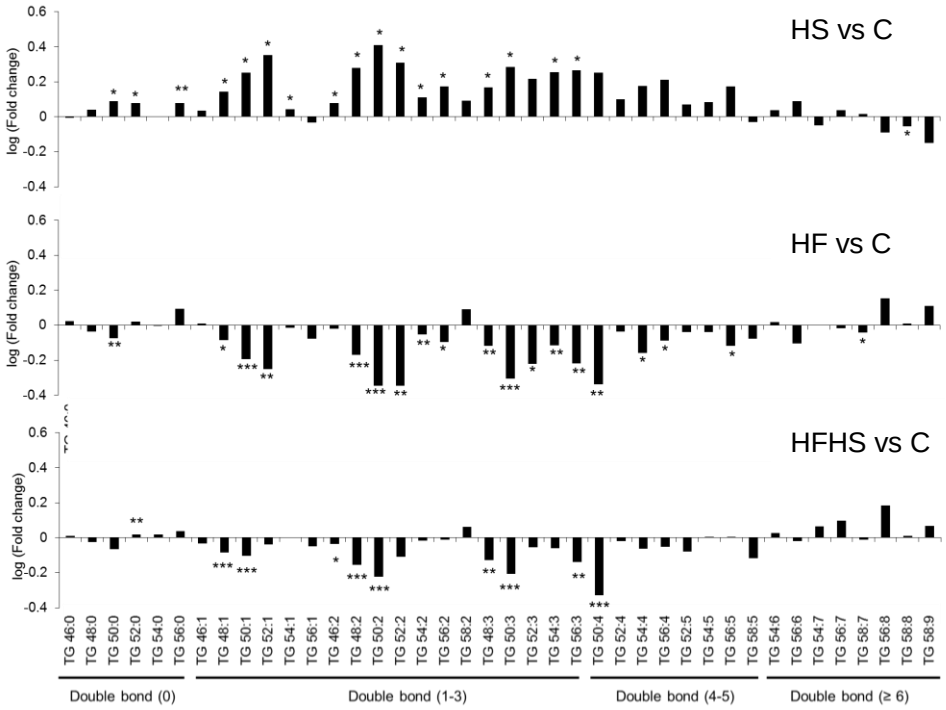

Figure 17. The concentration of TG species

The concentration of serum TG species containing fatty acids in normal fat diet with sugar drink treated (HS), high fat (HF) and high fat diet with sugar drink (HFHS) treated mice. The relative value of concentration is log(fold change) toward C (control). TG species are grouped into four clustering according to the number of double bonds in three fatty acid chains. Student's t-test; * p-value <0.05, ** p-value <0.01, *** p-value <0.001

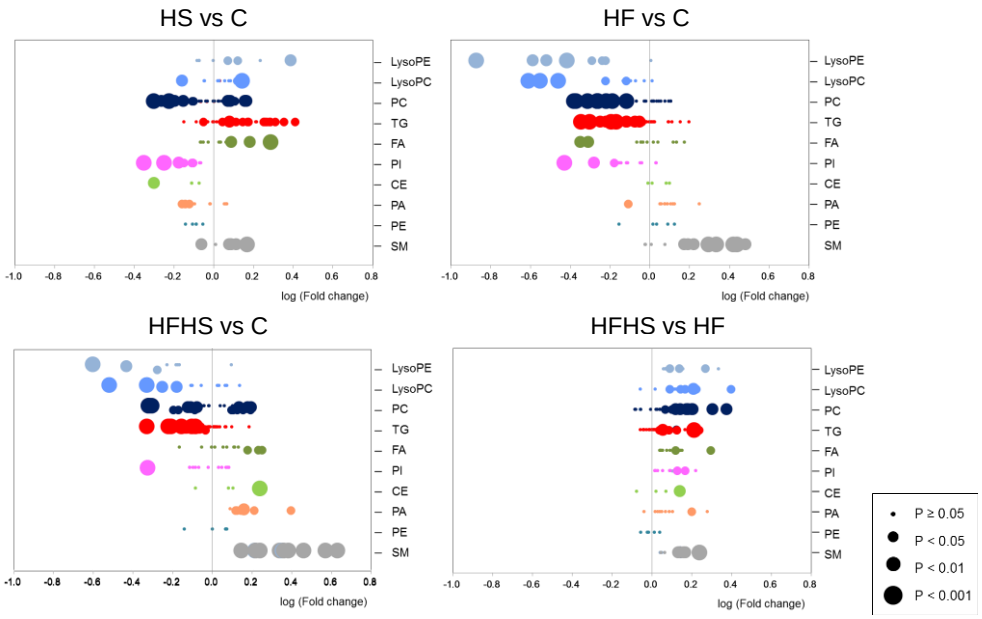

Figure 18. Serum lipid metabolites profiles of experimental diet treated mice

Total serum lipids profiles in normal fat diet with sugar drink treated (HS), high fat fed (HF) and high fat with sugar drink treated (HFHS) mice. The analysis of serum lipid metabolomics was identified through LC-MS. The relative expression value is log(fold change) toward C (control). Each dot indicates a lipid species, and the dot size represents statistical significance. LysoPE, lysophosphatidylethanolamine; LysoPC, lysophosphatidylcholine; PC, phosphatidylcholine; TG, triglyceride; FA, fatty acid; PI, phosphatidylinositol; CE, cholesteryl ester; PA, Phosphatidic acid; PE, phosphatidylethanolamine; SM, Sphingomyelin.

IV. Discussion

The identification of effect of high sugar intake on failure of TG clearance required multistep analysis of transcriptome, protein expression and lipidome. In this study, the mechanism of failure in TG clearance affected by high sugar intake with normal fat diet was attempted to establish through RNA sequencing in adipose tissues and LC-MS based lipidomics in serum. The transcriptome profile of adipose tissue was in normal fat diet, normal fat diet with sugar, high-fat, high-fat with sugar treated groups was integrated to demonstrate the function of genes which are influenced by sugar intake. The results of our study indicate that the ingestion of sugar with normal fat diet induces the gene programs related to energy metabolism especially implicated in electron transport chain in mitochondria. Furthermore, high sugar intake diminished the transcripts level of fatty acid transporters in adipose tissues. In the same vein, the concentration of TG and fatty acid of global serum lipid profile was raised in sugar drink treated group.

Previously, the study was conducted to demonstrate the effect of sugar intake in normal fat or high fat diet fed mice, so sugar was supplied as drink, and the level of serum TG was increased in sugar drink with normal fat diet treated group at the end of the provision of experimental diets. In the preceding studies, excessive intake of carbohydrate promoted the elevation of plasma TG by affecting the synthesis of TG in the liver (Mittendorfer and Sidossis, 2001). The elevation of serum TG is accompanied the risk of prevalence of cardiovascular disease (Nakagawa *et al.*,

2006), thus the level of TG in serum maintains homeostasis in normal state (Franssen *et al.*, 2011). The adipocyte and myocyte have a role in regulation of the level of serum TG by uptake the TG in blood stream to store or utilize it as energy sources (Kershaw *et al.*, 2006). Adipose tissue switches surplus energy to TG and conserve it as lipid droplet (Kershaw *et al.*, 2006). Thus, in the previous study, the histological analysis of adipose tissue was performed through H&E staining to examine the interaction between serum lipid and adipose tissues, and the result was that the size of adipocyte was not enlarged in normal fat diet with sugar drink treated group, whereas high fat and high fat with sugar fed groups showed expanded adipocytes. Therefore, it was concluded that the TG clearance was not performed appropriately in normal fat diet with sugar drink fed group.

Therefore, RNA-seq was conducted in adipose tissues to identify the cause of malfunction in TG clearance to adipocytes, and implemented functional enrichment analysis to demonstrate the functions of genes which are significantly affected by normal fat diet with sugar intakes. One of the several interesting results of our study was that the mRNA level of genes involved in function of electron transport chain of mitochondria, which was related in genesis of energy was increased by normal fat with sugar drink ingestions. These genes such as ubiquinone oxidoreductase, succinate dehydrogenase, ubiquinol-cytochrome c reductase, cytochrome c oxidase and ATP synthase encode enzymes which are associated with production of ATP through oxidative phosphorylation (Adam-Vizi, 2005); (Hao *et al.*, 2009); (Solmaz and Hunte, 2008); (Balsa *et al.*, 2012); (Hubal *et al.*, 2011). Previous studies reported

that the upregulation of these genes is implicated in reduction of body fat (Boden *et al.*, 2005).

The gene expression of mitochondrial electron transport chain is reduced in high fat fed groups, and it has been reported earlier that high fat diet induces down-regulation of mitochondrial oxidative phosphorylation (Sparks *et al.*, 2005). Coupled with this, the expression of genes associated in 'oxidative phosphorylation' was decreased in the results of functional enrichment analysis in high fat fed groups in this study. Interestingly, our study indicates the upregulation of transcripts involved in beta oxidation in sugar consumption group. Lipid oxidation in mitochondria promotes the oxidation of fatty acids, and induces the reduction of reesterification of fatty acids, consequently causes decreased accumulation of lipid droplet (Serra *et al.*, 2013) in adipocytes. Therefore, the increase of beta oxidation and the expression of genes associated with function of electron transport chain may facilitates the generation of ATP, and decreases collection of extra energy as lipid droplet. Also it demonstrates the possibility that the size of adipocytes has not been expanded in normal fat diet with sugar drink treated group due to decrease of accumulation of TG.

In this study, the expression of transcripts encoding CREB proteins was decreased in normal fat diet and sugar drink ingested group. It has been previously reported that the expression of transcription factor CREB was increased by mitochondrial dysfunction inducing agent, and it caused the adipogenesis and accumulation of lipid droplet in adipocytes (Vankoningsloo *et al.*, 2006). In this study, the mitochondrial function is improved and the transcripts level of some CREB transcripts was

reduced in normal fat diet with sugar drink fed group. Moreover, the protein expression assay of our study showed the lower level of CREB protein in sugar treated group than in high fat fed group. The downstream molecules of CREB are associated in lipid accumulation and adipogenesis. *Cebpb* and *Insig1* are known as regulator of TG accumulation in adipocytes (Park *et al.*, 2010); (Carobbio *et al.*, 2013), and the level of transcripts of these genes was lower in normal fat diet and sugar drink treated group, higher in high fat and high fat with sugar drink treated groups. Whereas, in the previous study, *Insig2* was demonstrated to suppress the synthesis and deposit of TG, and the transcripts level of *Insig2* was higher in HS, lower in HF. In line with this, the size of adipocytes in normal fat with sugar treated group in earlier study conducted in this laboratory was not widened despite high level of serum TG. Interestingly, according to preceding studies, *Ppargc1a* was identified to act as a coactivator *Pparg* and induce an adipogenic differentiation (Spiegelman *et al.*, 2000). However, the transcripts level of *Ppargc1a* was higher in HS and lower in HF. Previous study showed that *Ppargc1a* encoded the mitochondrial protein, therefore, the lower mRNA level of *Ppargc1a* in HF, HFHS might induce the lower transcripts level of mitochondrial ETC genes.

Our data indicated that the exposures to sugar drink with normal fat diet leaded to the downregulation of fatty acid transporters in adipose tissues. The liver synthesizes TG from dietary carbohydrates, then the TG is stored in liver or transported through the blood stream as a form of very low density lipoprotein (VLDL) (Adiels *et al.*, 2006). There are lipoprotein lipases (Lpl) on endothelial cells of blood vessels of several tissues, and lpl-mediated hydrolysis generates free fatty acid from

TG in VLDL (Attie, 2007). The long chain fatty acid transporters have a role in uptake of fatty acid to the adipocyte for esterifying and storing it as lipid droplet (Glatz *et al.*, 2010); (Xu *et al.*, 2013). In the previous study, the malfunction of fatty acid transporters caused the hypertriglyceridemia (Cai *et al.*, 2014), and the reduction of transcripts level of fatty acid transporters caused the elevation in the level of TG in plasma by impeding the TG clearance (Jun *et al.*, 2012). In our data, mRNA level of most fatty acid transporters was lower in normal fat diet with sugar drink fed mice than in high fat and high fat with sugar treated groups, therefore it might have affected the occurrence of defect in TG clearance to adipocytes, then elevated level of serum TG and not expanded size of adipocytes.

The alteration of global serum lipid profile in response to experimental diets treatment was observed. The sugar-induced changes in the concentration of serum lipid species was demonstrated using LC-MS. The concentration of several lipid species showed different level between HS, HF and HFHS. The increase in TG of global lipidome analysis in sugar drink treated group was similar with the elevated level of serum TG in normal fat diet with sugar drink treated group of previous study performed in this laboratory. The increased level of FA in serum indicates that LPL was activated and hydrolyzed TG in VLDL to fatty acids, however, the activity of fatty acid transporters was not enough to transport the large amount of fatty acids into adipocytes.

Moreover, the drastic change of serum LysoPE and LysoPC between HS and

HF, HFHS was observed. Previous study demonstrated that lysophosphatidylethanolamine and lysophosphatidylcholine is generated by hydrolysis of phosphatidylethanolamine and phosphatidylcholine by the action of phospholipase A2 (Pla2) (Gonçalves *et al.*, 2012). In our study, the level of Pla2 transcripts was highest in HS and lower in HF, HFHS. This results suggest that the elevated mRNA levels of Pla2 might have induced the enhanced amount of LysoPE and LysoPC through the facilitated hydrolysis of PE and PC.

In summary, the sugar-induced alterations in normal fat diet treated mice was demonstrated using RNA-seq based transcriptome and LC-MS based metabolome. This study suggests us that the changes caused by normal fat diet with sugar intake mainly leaded to upregulation of gene expression involved in energy metabolism, especially implicated in the mitochondrial function. Furthermore, sugar consumption of normal fat treated mice reduced the transcripts level of long chain fatty acid transporters, thus decreased the flux of fatty acid from blood stream to adipocytes. Consequently, the key finding of this study is that the increase of gene expression associated with energy generation had prevented the expansion of adipocytes, also the downregulated transcripts level of fatty acid transporters caused the elevated level of serum TG in normal fat diet with sugar drink treated group. These findings propose the effect of sugar ingestion on lipid metabolism, and establish new markers for treatment of hypertriglyceridemia. Further studies regarding the alteration of serum lipid metabolomics profiles are required to obtain more detailed perception into the relationship between transcriptome and serum lipid contents.

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국문초록

설탕 섭취에 의한 지방조직에서의 전사체 및 혈중 대사체 변화 연구

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박 미 연

설탕 섭취에 의해 비만, 당뇨와 같은 대사성 질환의 발생 위험이 높아진다는 사실은 널리 알려져 있다. 본 연구실에서는 각기 다른 식이 지방 조건에서의 설탕 섭취가 대사성 질환 지표 변화에 미치는 영향을 규명하고자 선행연구를 진행하였다. 실험식이는 '대조군 식이; 대조군 식이와 설탕물; 고지방 식이; 고지방 식이와 설탕물' 총 네 군으로 나누어 제공하였다. 실험결과, 고지방식이 및 고지방식이와 설탕물 병행섭취 그룹과는 다르게 정상지방식이 조건에서 설탕을 섭취한 그룹에서 혈중 중성지방 수치가 유의적으로 증가하였다. 또한 정상지방식이 조건에서 설탕 섭취시 혈중 중성지방 조절을 담당하는 지방세포의 크기는 증가하지 않은 것을 알 수 있었고, 연구 결과들을

바탕으로 하여 혈액에서 지방세포로의 TG clearance가 정상적으로 작동하지 않은 것을 확인하였다. 따라서 본 연구에서는, 지방대사에서 주요한 역할을 담당하는 지방조직에서 차세대 염기서열 분석을 시행함으로써 정상지방식이 조건에서 설탕을 섭취하였을 경우 혈액에서 지방세포로의 TG clearance 작용이 저하되는 원인을 규명하고자 하였다. 지방조직에서의 전사체 분석 결과를 바탕으로 정상지방식이와 설탕 병행섭취에 의해 발현이 유의하게 변한 유전자들에 대해 Functional enrichment analysis를 진행한 결과, 에너지 대사 및 미토콘드리아 기능에 관여하는 전사체들의 발현이 증가한 것을 알 수 있었다. 또한 설탕 및 정상지방식이 병행섭취 그룹에서 미토콘드리아의 기능 증가는 CREB과 중성지방 축적에 관련된 하위 유전자들의 발현 감소를 유발하였고, 이는 결국 지방세포 내의 중성지방 축적 감소로 이어져 지방세포의 크기가 증가하지 않았다. 혈중 중성지방의 지방세포 내 유입 감소에 대한 이해를 위해 지방조직에서 지방산 운반 단백질의 전사체 발현을 관찰한 결과, 중성지방 및 설탕 병행섭취군에서 지방산 운반 단백질의 유전자 발현이 감소하였다. 혈청에서의 액체크로마토그래피-질량분석법에 의한 지방대사체 성분 분석 결과, 혈중 중성지방과 지방산의 농도 증가를 확인하였다. 위 결과를 종합하면, 정상지방식이 조건에서 고설탕을 섭취함으로써 미토콘드리아 기능에 관련된 전사체 발현이 증가하였고, 전사인자 CREB의 발현이 변화하여 지방세포 내의 중성지방 축적을

저해하였다. 또한, 지방산 운반 단백질의 전사체 발현 저하로 혈중 중성지방 수치가 상승하였다. 본 연구는 고설탕 식이에 의해 지방조직으로의 혈중 TG clearance 의 저하 메커니즘을 규명하였으며, 고중성지방혈증의 치료에 적응할 수 있는 새로운 지표를 제시하였다는 점에서 그 의의가 있다.

주요어 : 설탕, 고지방식이, 마우스, 지방조직, 전사체, 대사체

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