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High-Glucose and Free Fatty Acid-Induced Adipocytes Generate Increasing of HMGB1 and Reduced GLUT4 Expression

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Abstract

BACKGROUND: High-mobility group box 1 protein (HMGB1) is released from necrotic adipocytes into the extracellular milieu as an inflammatory alarmin in obesity. Although the impact of excess nutrient on adipocytes is well known, it is not clear how specific its component drive cell size and damaged of adipocytes, and how this relates to the risk of insulin resistance.

AIM: The aim of this study was to determine HMGB1 level in adipocytes cultures after high-glucose and/or FFA exposures and to assess GLUT4 expression. We determined cellular features of adipocytes that correlate to HMGB1 released and insulin resistance.

METHODS: Differentiated adipocytes were exposed to high glucose and/or FFAs for 7 days. ELISA was performed on supernatant to assess the HMGB1 level. Total GLUT4 expression was quantified by immunofluorescence.

RESULTS: High-glucose and FFA-exposed cells have significant increase of HMGB1 level and necrotic adipocytes features. The total GLUT4 was reduced in high-glucose exposed cells ($p < 0.045$), but not in FFA-exposed cells. Hypertrophic adipocytes ($p < 0.05$) and slight decrease of GLUT4 expression were showed on HG + FFA exposures with no increase of HMGB1 level. There was a significant correlation between cell size and HMGB1 level ($r = -0.637$, $p < 0.026$)

CONCLUSION: The expression level studies between high glucose, FFA, and a combination of both on adipocytes results strongly suggest that high glucose is more induce adipocytes to release HMGB1, and reduced GLUT4 expression in adipocytes compared to FFA, and combination of both. Nevertheless, hypertrophic adipocytes which are one of characteristic signs of adipocytes dysfunction occur in high-glucose + FFA-exposed cells.

Introduction

Development of insulin resistance in adipose tissue is closely associated with adipocyte hypertrophy and pro-inflammatory responses. However, it is not yet known whether hypertrophy *per se* as triggers or through an inflammatory cascade might be sufficient to provoke insulin resistance in adipose tissue. Adipocytes hypertrophy is the main cause of energy metabolic system dysfunction, obesity, and its afflictions such as T2D [1]. Chronic excess nutrient-induced adipose tissue expansion leads to lipid droplets (LDs) hypertrophy [2], [3], and when its fully lipid-engorged, stress signals will be raised. High-mobility group box 1 (HMGB1), a non-histone chromatin-binding protein, released from predominate necrosis adipocytes on stressful insults [4]. Extracellular HMGB1 acts as an innate alarmin to stimulate the activation of resident immune cells in the adipose tissue and exhibits inflammatory responses in T2D [5].

Adipocyte death in obesity is likely to be the dominant necrosis form [6] indicated by increased level of HMGB1 [7] and cellular features of necrosis, including ruptured plasma membranes, dilated endoplasmic reticulum, cell debris in the extracellular space, and the appearance of small LDs in the cytoplasm [8], [9]. In an experimental animal model, high-level HMGB1 is associated with hyperglycemia [10], and high-fat diet-induced obesity [11]. *In vitro* studies on adipocytes have shown that distribution of HMGB1 in lean and obese normoglycemic individuals has predominantly nuclear while in obese patients with type 2 diabetes have localized to the cytosol [12]. HMGB1 release from adipocytes has studied with LPS as inflammation triggered [13]. However, according to our knowledge, no studies have been undertaken to investigate HMGB1 release with nutrient as trigger in relation to adipose tissue function in insulin-resistant states.

Glucose and FAs are transported into adipocytes through specific transporter molecules that insert into the cell membrane in response to insulin. GLUT4 is the main insulin-responsive glucose transporter in adipocytes [14], but there are also insulin-independent mechanisms for cellular GLUT4 translocation and glucose uptake [15]. Cluster of differentiation 36 is used as the fatty acid translocase

responsible for transportation of FAs across the adipocyte plasma membrane to be utilized or as energy storage [16]. The previous studies have determined that increasing fatty acid uptake of adipocytes requires greater synthesis triglycerides (TGs) in LDs, and this can lead to ER stress activating the JNK pathway, thus further increasing insulin resistance [17]. High glucose also induces adipose inflammation and insulin resistance through JNK activation [18]. Therefore, it would be very interesting to know the HMGB1 extracellular level and cellular appearance of adipocytes after affected by elevated glucose and FFA levels.

To develop successful strategies to treat obesity and T2D, studies using hypertrophic adipocytes models that adequately mimic the aspects of obesity are needed. Regarding to this, we exposed differentiated adipocytes to long-chain FFA and/or high-glucose concentration. The aim of this study was to compare the effect of high-glucose, high-fat, or a combination of both on primary rat adipocytes, and determine the correlation of adipocytes size with cellular GLUT4 and necrotic adipocytes death. We hypothesized that there is different HMGB1 level and adipose tissue function in insulin-resistant states of each treatment group. We instead demonstrate decreased cellularity of adipocytes in high-glucose exposed cells, along with breakdown LDs into small multilocular LDs in FFA-exposed cells. Our findings support a hypertrophic model of primary rat adipocytes cultures in which metabolically impaired from HG + FFA-exposed cells.

Material and Methods

Cells cultures of stromal vascular fraction

Healthy non-obese Wistar rats (4–5 weeks old) were sacrificed and submerged in 70% ethanol for 2 min. Adipose tissue was collected from peritoneal and retroperitoneal regions [19]. Fat tissue was removed and digested enzymatically by type 1 collagenase (Worthington), 1 mg in 7 ml Dulbecco's Modified Eagle Medium (DMEM) for 20 min at 37°C in a shaking bath. The cell suspension was filtered followed by centrifugation (1500 rpm for 7 min). The obtained cells were maintained in a culture flask and nourished using DMEM containing sodium bicarbonate, L-glutamine, antibiotics (100 U/ml) penicillin, and 100 mg/ml streptomycin (MP Biomedical, LCC), and supplemented with 10% of heat-inactivated fetal bovine serum (Gibco©). Adipocyte culture was kept at 37°C, 5% CO_2. Media were changed every 2 days.

Fatty acids and high-glucose supplementation

Confluent cultures were maturated by 0.1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, and 0.1 μM insulin [19] and subcultured at a density of 0.05×10^6 cells/ml/well in 24-well culture plates in growth. Cells cultures were exposed to 5 or 25 mmol/l glucose with or without 250 μmol/l palmitic acids (16:0) with daily replenishment for 7 days. At the time of harvesting cells (day 7 or 9), oil red O staining was performed according to Krauss, 2016, as described [20]. The degree of adipogenic differentiation was determined by extracting the dye from stained cells with 750 μl of isopropanol and then measured the absorbance at 500 nm [21].

Cell size quantification

Cell size was determined by microscopic measurement of cell diameter (Nikon Eclipse TE 2000-U and Q-Capture Suite 2.66.4.0, Quantitative Imaging Corp). Cell diameter was measured using ImageJ version 1.47 (National Institutes of Health, USA). A total number of 100 adipocytes were measured from each treatment group of cells. Cells <10 μm were excluded from analysis [22].

GLUT4 immunofluorescence staining

The immunofluorescence staining was performed for analysis of GLUT4 in adipocytes. Coverslips containing cells were fixed for 5 min in 75% acetone 25% ethanol. Subsequently, sections were washed 3 times for 5 min in phosphate-buffered saline (PBS, 137 mmol/L sodium chloride, 3 mmol/L potassium chloride, 8 mmol/L sodium phosphate dibasic, and 3 mmol/L potassium phosphate monobasic). GLUT4 antibody (rat IgG, Santa Cruz) was applied to the sections at a dilution of 1:200 in 5% normal serum (NGS, Invitrogen) for 2 h at room temperature. The antibody used binds the cytosolic C terminal of GLUT4 to visualize intracellular GLUT4. Following primary antibody, incubation sections were washed 3 times for 5 min in PBS. DAPI (Sigma-Aldrich) staining for cell nuclei was added to the secondary antibody at a 0.5 $\mu g \cdot mL^{-1}$ concentration. After secondary antibody, incubation sections were washed 3 times for 5 min in PBS and coverslips were mounted with 20 μL mowiol; 6 g glycerol (Sigma-Aldrich), 2.4 g mowiol 4-88 (Sigma-Aldrich), and 0.026 g 1,4-diazobicyclo-[2,2,2]-octane (Sigma-Aldrich) dissolved in 18 mL 0.2 mol/L Tris-buffer (pH 8.5) (Sigma-Aldrich). Quantitation of GLUT4 using image processing and analysis was carried out in Fiji-win 32 and was kept consistent between images. GLUT4 fluorescence intensity was quantified by measuring the signal intensity within the intracellular. Intracellular spot number and staining area were quantified by setting uniform threshold intensity and size values to identify spots within intracellular regions [23].

Measurement of HMGB1 extracellular level

The HMGB1 supernatant concentrations were determined with a recently established available ELISA

kit (BT-Lab E1635Hu). Briefly, in the wells coated with anti HMGB1 monoclonal antibody, samples to be measured and standards were incubated for 24 h at 37°C. After washing the wells for 5 times, a peroxidase-conjugated anti-HMGB1 monoclonal antibody was added into the microwells and incubated for 120 min at room temperature. After washing the wells for 5 times, 100 uL of substrate solution was added and incubated for 30 min at room temperature. A stop solution was added, and the absorbance was measured at 450 nm using microplate reader. The concentrations of the samples were then calculated from the standard curve [24].

Statistical analyses

Results are presented as mean ± SEM. Statistical significance was determined using Manova and *post hoc* Bonferroni tests. Differences were considered significant with $p < 0.05$ using SPSS 20.0 software. A Q-Q plot with observed picture the Mahalanobis distances used to evaluate (subjectively) data may have a multivariate normal distribution (qi = 1).

Results

Characterization of adipocyte differentiation

Wistar rat primary pre-adipocytes were differentiated into adipocytes and the pure adipocyte fractions were assessed by monitoring morphologically and oil red O staining (Figure 1a and b). The efficacy of differentiation reached approximately 90–95%, indicating that adipocytes could be considered as a specific homogenous cell type. As depicted in Figure 1a, no LD was observed in pre-adipocytes. Conversely, adipocytes were occupied by either medium-sized or single large LDs (Figure 1b).

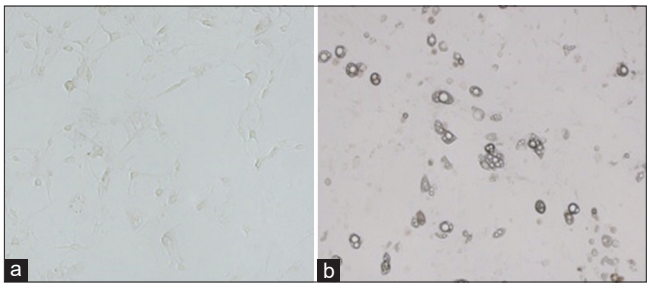

Figure 1: Differentiation of visceral pre-adipocytes to adipocytes. (a) Confluence pre-adipocytes (no lipid droplets [LDs]) were reached on day 3 of perfusion culture with the DMEM and (b) adipocytes (with medium-sized or single large LDs) following differential factor contained insulin perfusion, the cells exhibited morphological changes characteristic of adipogenic differentiation, assuming a rounded shape and forming lipid inclusion bodies visible as microscopic droplets

Combination high-glucose and FFA exposure generate adipocytes hypertrophy

To mimic a condition of chronic energy excess, we exposed adipocytes to high-glucose concentration and FFA. In this study, adipocytes in control group were occupied by either medium-sized to large-sized unilocular LDs, however, with addition of FFA daily replenishment of medium, there was a slight decrease in size, but have small multiple LDs (Figures 1 and 2). The results that are not surprising are cells with combination HG + FFA exposures that have a significant increase in cell size (hypertrophy) with large unilocular droplet (Figure 2a and 3). Comparing the two previous results, the FFA studied has divergent effects on adipogenesis.

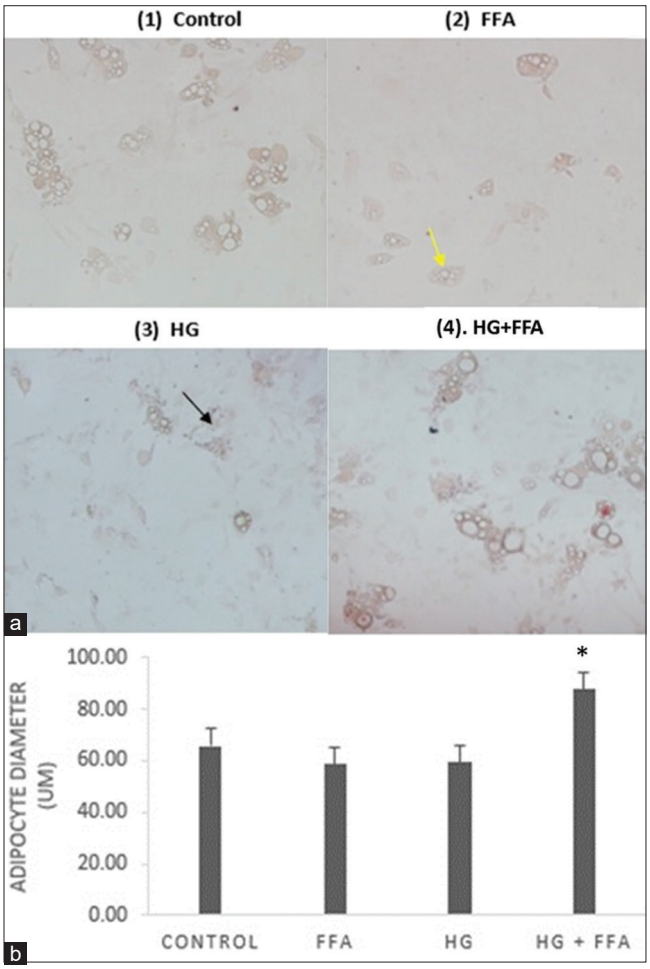

Figure 2: (a) Adipocytes appearance after treatment was visualized with oil red O staining (magnitude ×10). The treatment gives a different effect on cell size, the greatest is combination HG + FFA exposures (A4). Cells with FFA and HG exposures almost have the same size, but different cellular appearance. Cells density of HG exposures (A3) is low and comes into view cell shedding (black arrow). FFA cells (A2) appear to be predominantly multilocular lipid droplets (yellow arrow). (b) Adipocytes diameter. Cells with combination HG + FFA have greatest size compared the other cells groups ($p < 0.05$)

The most striking observation to emerge from the cell appearance comparison was high-glucose exposed adipocytes appear to have a small size with unclear cell boundaries, indicate the possibilities of necrotic cell

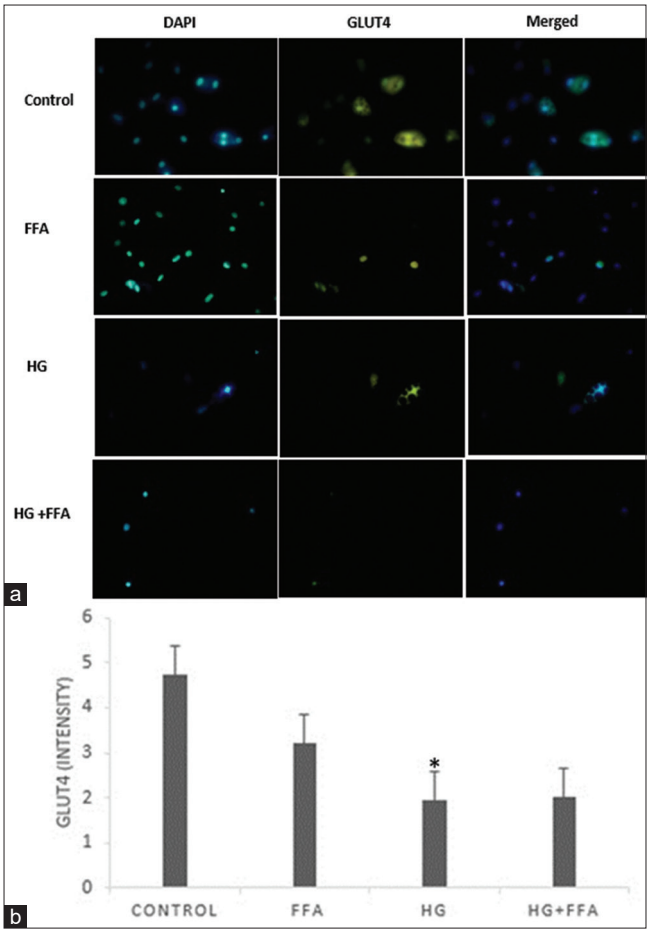

Figure 3: (a and b) High glucose suppresses total GLUT4 expression ($p < 0.05$). FFA and combination of HG + FFA decrease total GLUT4 expression but no significance differences ($p > 0.05$). Total Glut4 was analyzed using anti-GLUT4 followed by fluorescein isothiocyanate-labeled secondary antibody (magnitude ×400) stained in combination with DAPI (blue) to mark the cell nuclei

death. There were cells with very small droplet lipids, or even none at all just like pre-adipocytes, exhibit that high-glucose disrupted adipocytes differentiation process or adipogenesis (Figures 2a and 4).

High-glucose and FFA induce increasing of HMGB1 level on adipocytes

Since the release of the chromatin protein high-mobility group B1 (HMGB1) is a biomarker of necrotic cell death (Yang et al., 2014), to evaluate the effect of high glucose and FFA exposure on necrotic cell death, we used MANOVA test to analyze the different levels of extracellular HMGB1 in each group. Levels of HMGB1 in high-glucose and FFA exposure group increased significantly compared to controls ($p < 0.05$). From the graphic in Figure 4, a quite surprising result was the level of HMGB1 on combination HG + FFA exposures which was almost similar as the control ($p > 0.05$). Further statistical tests revealed that there was a significant negative correlation between cell size and HMGB1 level (Pearson's correlation coefficient, $r = -0.637$, $p = 0.026$).

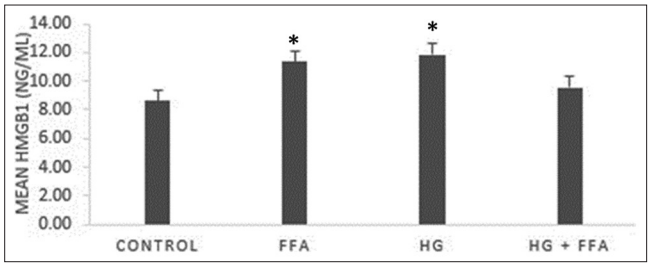

Figure 4: High-mobility group box 1 protein (HMGB1) release into the media from treated cells group. HG and FFA exposure cells increase HMGB1 level significantly than control ($p < 0.05$), rather than cells with combination HG + FFA exposures that increase slightly than control ($p > 0.05$)

High-glucose-induced insulin resistance on adipocytes

After 7 h exposure of rat adipocytes to high glucose, we observed a significant reduction in total GLUT4 relative compare to control ($p < 0.05$) (Figure 3) with impairment of adipogenesis, marked by the small size of the adipocytes and damaged cells (Figure 2). However, there was no significant difference between control cells and FFA (palmitic-exposed cells) or HG + FFA with respect to insulin-induced glucose transport.

Discussion

The present study was designed to determine the effect of overnutrition that mimic of metabolic mileu that promotes obesity and insulin resistance on visceral adipose tissue. Adipocyte enlargement is a key feature of obesity and associated with insulin resistance and metabolic disease, characterized by large and unilocular LDs [25]. High-glucose concentration and/or FFA were exposed to primary differentiated adipocytes for 7 days. Our findings indicate that high-glucose-induced adipocytes necrotic cell death, showed by increased levels of extracellular HMGB1, released by damaged cells [26]. This includes the alteration of morphologic cells, there were unclear plasma membrane border, cell shedding, and blebbing. Obesity-associated adipocyte death represents necrosis trait, such as ruptured plasma membranes, dilated endoplasmic reticulum, cell debris in the extracellular space, and the appearance of small LDs in the cytoplasm and release HMGB1 into the extracellular space [4]. Hyperglycemia is defining metabolic disruption to adipocytes that can be considered as a nutrient stress condition [27]. ER stress may be perturbed by high-glucose conditions to contribute to adipocyte dysfunction such as insulin resistance, caused by increasing advanced glycation end-products formation leading to limit insulin signaling [28]. In this study, high glucose was found to supress GLUT4 expression to the lowest level compared to others. These results are consistent with those of other studies

and suggest that GLUT4 defects in adipose tissue are early signs of metabolic alterations [29].

We found that high-glucose exposed caused cells damage whereas in the previous studies its revealed hypertrophy due to burst of the anabolic metabolism under chronic positive energy balance [30]. There are several possible explanations for this result, differentiated adipocytes are no need high-glucose supply after differentiation, proceed normally at 4 mM. They are only need high-glucose pulse only 3 days in early differentiation phase [31]. Prolonged high-glucose exposure (7 days after differentiation), cells undergo nutrient stress and impair endoplasmic reticulum (ER) function, and activate the unfolded protein response [32]. Cells type differences can also caused this discrepancy. The previous study used 3T3-L1 adipocyte that potentially modulates its cell survival genes during differentiation contributing to resistance to cell death [33].

Comparison of FFA and high glucose, combination high-glucose and FFA-exposed cells yield some surprising findings. For example, while FFA (palmitic acids) and high glucose both increased HMGB1 extracellular level and showed morphologic characteristic of damaged cells, however, the combination of high glucose and FFA in the similar dose becomes hypertrophy with low HMGB1 level and GLUT4 expression. The reason for this discrepancy is still unclear but could represent that palmitate have divergent effects on adipogenesis. The size increase of adipocytes is dependent on diet. On the other hand, the diet-induced number increase of fat cells is dependent on strain, suggesting a synergy between genetics and diet [34]. Hypertrophy occurs to meet the need for additional fat storage capacity in the progression of obesity. High-fat high-glucose intake indeed exhibits increased LD hypertrophy, these result from the excessive storage of energy in the form of TGs in LDs which link to obesity and to insulin resistance [1]. Therefore, high-fat high-glucose intakes can be developed into a hypertrophic cell model to study the effects and treatment of obesity.

We demonstrate that high FFA (palmitate 25 μmol)-exposed adipocytes for 7 days lead to multilocular LDs and release of nuclear HMGB1 that frequently used as a marker of necrosis in sites of sterile inflammation. Multilocular adipocytes could derived from proliferation of precursor cells that already existed whether new mitochondria contained in them were typical brown adipocyte or unilocular adipocytes underwent conversion to multilocular mitochondria-rich adipocytes [35] or breakdown caused by cell damage. There are small proportion multilocular adipocytes (8%) in WAT with positive UCP1 that typical for BAT [35]. Fatty acid particularly palmitic acid could activate brown adipose tissue mitochondria uncoupling protein [36]. This has to be proven by further cell morphology analysis with electron microscopy or biochemical analyses. LD morphology is closely related to the functions and characteristics of adipose tissues. Small multilocular LDs formation is ideal for the subsequent facilitation of FFA transport to mitochondria that are adjacent to LDs for β-oxidation leading to efficient intracellular lipolysis in BAT [37]. The browning of adipose tissue leads to enhanced energy expenditure and the prevention of obesity, clarification of the mechanism how administration of fatty acids in low-glucose conditions can convert WAT into BAT is very meaningful. However, this is confused with the elevation of HMGB1 level in the FFA-exposed cells. HMGB1 passively released from necrotic cells, enhancement of extracellular HMGB1 decreased autophagy that is vital to maintain cellular homeostasis and protect against inflammation of adipocyte injury after 4 days treatment [38]. LDs appear as regulator of cellular metabolism and play roles in the cellular stress response such as lipid overload [39]. Accumulation of reactive oxygen species due to functional impaired of mitochondria suspected as the cause of FFA-mediated necrosis [40] and the LDs tightly associate with mitochondria both morphologically and biochemically [41].

One unanticipated finding was that total GLUT4 in FFA-exposed cells did not decrease significantly, even had the lowest TNFα levels compared to other groups (data not shown). Another possible explanation for this is palmitate-induced lipophagy [42]. LDs are broken down by two major mechanisms: Lipolysis and lipophagy [27]. The present small multilocular LDs in FFA-exposed adipocytes indicate lipophagy, whereby parts or whole LDs are engulfed within autophagosomal membranes and fused with lysosomes for degradation by hydrolytic enzymes [43], suggesting how LD structure and dynamics define the multitude of their functions. Referring to the evidence that extracellular HMGB1 suppress autophagy, it can be assumed that the induction lipophagy by palmitate can reduce interference autophagy by HMGB1. Transcriptional factor that plays a role in lipophagy is peroxisome proliferator-activated receptor (PPAR)-γ coactivator 1α (PGC-1α) and PPAR-α target genes involved in fat and glucose metabolism, and cellular proliferation and differentiation [44]. In liver, palmitate appears to upregulate PPARγ through PGC1α [43]. However, more research on this topic needs to be undertaken before the association between FFA intake and HMGB1 release is more clearly understood.

Conclusion

The expression level studies between high glucose, FFA, and a combination of both on adipocytes results strongly suggest that high glucose is more

induce adipocytes to release HMGB1, and reduced GLUT4 expression in adipocytes compared to FFA, and combination of both. Nevertheless, hypertrophic adipocytes which are one of characteristic signs of adipocytes dysfunction occur in high-glucose + FFA-exposed cells.

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