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Oleate Induces Apoptosis in 3T3-L1 Adipocytes

A. Rohana, A. M., Fadzilah Adibah and M. S. Muhammad Roji

***Abstract*—**Oleic acid (C18:1) play an important role in proliferation of fat cells. In this study, the effect of oleate on cells viability in 3T3-L1 cells (fat cells) was investigated. The 3T3-L1 cells were treated with various concentrations of oleate in the presence of 23 mM glucose. Oleate was added to adipogenic media (day 0) to investigate the influence of oleate on proliferation of postconfluent preadipocytes after 24 h induction. 0.1 mM oleate promoted cell division by increasing 33.9% number of cells from basal control in postconfluent preadipocytes. However, there were no significantly different in cells viability with control cells when oleate concentrations were increased up to 0.5 mM. When added to differentiated adipocytes (day 12) for 48 h, the number of cells decreased as oleate concentrations increased. 92.7% of cells lost demonstrated apoptosis and necrosis after 48 h with 0.5 mM oleate. The fluorochrome staining was examined under fluorescence microscopy using acridine orange and ethidium bromide double staining. Furthermore, the presence of high lactate (60.6% increased from basal control) released into plasma has shown the direct cytotoxicity of 0.5 mM oleate on adipocytes.

***Keywords*—**adipocytes, apoptosis, oleate, postconfluent preadipocytes

I. Introduction

FATTY acids play an important role in cellular energy metabolism. Fatty acids are also an integral part of cells membrane components, which can influence the cells' fluidity and cells' receptor. Moreover, specific fatty acids are needed for hormonal action (eicosanoids) and can also act as signaling molecules involved in regulating gene expressions in fatty acid transport and metabolism. However, the cells response depending on the concentration of fatty acids presence in the plasma which relative to their different fatty acid chain lengths and the degree of unsaturations [1].

Growing evidence indicates that fatty acids are responsible for the loss of insulin sensitivity causing insulin resistance, leading to type 2 diabetes mellitus and obesity [2]-[4]. The deleterious effect is shown in insulin sensitive tissues including hepatocytes, myocytes and adipocytes [5]-[7]. It is becoming a serious debate concerning the effect as human consume fatty acids for dietary lipid. Adipocytes play a central role in the development of obesity as characterized by increases in number or size of adipocytes, or a combination of both. The proliferation and differentiation of adipocytes and fat accumulation are the direct cause of obesity. Thus, in this work, the effect of oleate on proliferation of 3T3-L1 postconfluent preadipocytes and adipocytes was investigated. Besides, the effect of oleate on lactate release by the cells and apoptosis were also investigated.

A. Rohana is with Faculty of Chemical & Natural Resources Engineering, Universiti Malaysia Pahang, Lebuhraya Tun Razak, 26300 Gambang Kuantan, Malaysia. (phone: 609-5492866; fax: 609-5492889; e-mail: rohanaa@ump.edu.my).

A. M. Fadzilah Adibah is with Faculty of Chemical Engineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia. (e-mail: adibah@cheme.utm.my).

M. S. Muhammad Roji is with Institute of Bioproduct Development, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia. (e-mail: author@ibd.utm.my).

II. Material And Methods

A. Material

Dulbecco's Modified Eagle's Medium (DMEM), foetal bovine Serum (FBS) and Penicillin-Streptomycin (PS) were purchased from Gibco BRL (Grand Island, NY). Foetal calf serum (FCS) was obtained from Biowest (France). Sodium salt of oleic acid (C18:1) and fatty acid free-bovine Serum Albumin (FAF-BSA) were obtained from Sigma-Aldrich (USA).

B. Cell Culture and Treatment

The mouse fibroblast 3T3-L1 cells (European Collection of Cell Cultures, UK) were cultured and differentiated into adipocytes. Briefly, cells were cultured in DMEM supplemented with 10%FCS and 1%PS at 37^{o}C in 5%CO_2. Once the cells were fully confluent, the cells were incubated with additional 48 h before initiating differentiation. Subsequently, the cells were induced (day 0) to differentiate with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.25uM dexamethasone (DEX) and 1ug/ml insulin in DMEM with 10%FBS for two days. Cells were then maintained with insulin and 10%FBS in DMEM for another 2 days. Thereafter, the media were changed in DMEM with 10%FBS for every second day. Between days 12, the cells became mature. Cells were used 12 days after post-confluent.

C. Fatty Acid Treatment

The hydrophobic properties of oleate required albumin as a physiologic carrier to enter the cells. Oleic acid bound with albumin solution was prepared according to [8]. Sodium salt of FFA was added in phosphate buffer saline (PBS) and gently warmed to facilitate solubility. The warmed fatty acid salt solution was complexed to 5%FAF-BSA in PBS at a 6:1 fatty acid to BSA molar ratio (FFA/BSA). The sterile filtered, complexed fatty acid solution was added to 10%FBS in DMEM to obtain indicated final FFA concentration. The final concentration in the medium was obtained using enzymatic colorimetric assay (NEFA HR II, Wako Japan) by measuring the absorbance of blue purple colour [10].

For postconfluent cells, oleate was added to MDI differentiation medium containing 23 mM glucose and 100 nM insulin at day 0 and cultured for 24 h. During this phase, the cell reentered the cell cycle and underwent a postconfluent

mitosis where the cell experienced at least one round of DNA replication and cell division. For, the differentiated adipocytes, the cells were serum-starved overnight incubation in DMEM and supplemented with 0.1%FAF-BSA [11]. Then, the cells treated with oleate and cultured in a high glucose level (23 mM) and in the presence of 100 nM insulin. Control cells were treated as treatment except FAF-BSA was added to the medium instead of FA bound with BSA.

D. Quantification of Cell Viability via MTT assay

MTT assay is a standard colorimetric assay for measuring cellular proliferation (cell growth). Yellow 3-(4,5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) is reduced to purple formazan in the mitochondria of living cells. The number of surviving cells is directly proportional to the level of the formazan product created. The amount of colour produced is directly proportional to the number of viable cells and can be read on a multi-well scanning spectrophotometer [12]. Briefly, MTT of 5 mg/ml was dissolved in PBS. The solution was filtered through a 0.2 μM filter and stored at 4°C for frequent use or frozen for extended period. Routinely, 3T3-L1 adipocytes are washed three times with PBS. 10 μl of MTT stock solution was added in each well and incubated for 3-4 hr at 37°C. 200 μl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the insoluble purple formazan product into colour solution. The absorbance of this coloured solution was quantified by measuring at wavelength of 570 nm and reference wavelength of 630 nm with ELISA plate reader.

E. Quantification of Lactate in Spent Media

The presence of lactic acid in the spent medium after treatment of the cells was measured [13], [14] using Biochemistry Analyzer (YSI 2700 SELECT, USA). YSI 2700 SELECT uses the immobilized oxidase enzymes principle for chemistry measurements. 100 μl of spent medium was assayed using YSI 2700 SELECT to determine the L-lactate concentration. The measurements were unaffected by colour, turbidity, density, pH or the presence of reducing substances. Data are presented as the percentage of lactate released into medium relative to control.

F. Staining Cells for Apoptosis

Acridine orange (AO) and ethidium bromide (EB) are intercalating, nucleic acid specific, fluorochromes which emit a green and orange fluorescence respectively when they are bound to DNA. Viewed by fluorescence microscopy viable cells appear to have a bright green nucleus with intact structure while apoptotic cells exhibit a bright green nucleus showing condensation of chromatin as dense green areas. Late apoptotic cells and necrotic cells will stain both AO and EB. However, EB produces the highest intensity emissions and cells appear orange. Late apoptotic cells have an orange nucleus showing condensation of chromatin and necrotic cells display an orange nucleus with intact structure. Briefly, 4 μg/ml of AO/EB solution in PBS was prepared. 2 μl of AO/EB was added to the cells and incubated for 3 minutes and then observed under fluorescent microscope [15].

G. Statistical Analysis

Statistical analyses were performed using Sigma Plot 10.0. Values are expressed as means ± SE with three independent experiments. Statistical significance of treatments was determined using the paired Student's *t* test.

III. Results

A. Effect of Oleate on Postconfluent Preadipocytes and Adipocytes Viability

MDI differentiation medium in the presence of 0.1 mM oleate increased 33.9% of cell viability from control following 48 h exposure. This showed that 0.1 mM oleate stimulated proliferation of postconfluent preadipocytes. This proliferation enhances adipogenesis or differentiation of preadipocytes to form more adipocytes. However, there were no significant different in viable cells compared to control cells when increased oleate concentration up to 0.5 mM as shown in Fig. 1. This can be concluded that high concentration of oleate did not change the number of viable postconfluent preadipocytes to form more adipocytes.

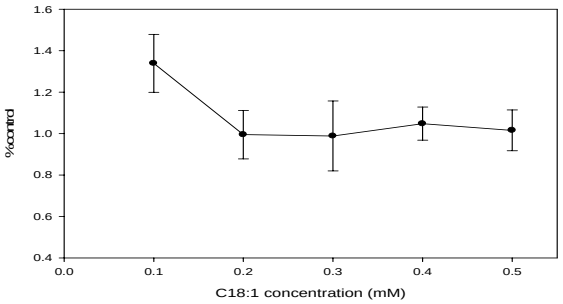

Fig. 1 Effect of oleate on cell viability during postconfluent preadipocytes. Oleate was added with MDI differentiation medium at day 0. After 48 h of treatment, the viability was determined by MTT assay. Assays were performed in 3 replicates from 3 independent experiments. Values are means ± SEM (p<0.01)

Different observations were obtained in oleate treatment of mature adipocytes. When oleate was added to differentiated adipocytes (day 12) for 48 h, the number of cells decreased as oleate concentrations increased. The significant effect was observed when treated the adipocytes with high oleate concentration (0.5 mM). As illustrated in Fig. 2, 92.7% of adipocytes were lost in the treatment.

B. Effect of Oleate on Apoptosis

Fluorochrome staining was carried out to examine whether the cells death are due to excess oleate in culture and toxic to cells, leading to apoptosis and necrosis. Fig. 3a shows the viable cells after 48 h treatment with 0.5 mM oleate which the cells appeared to have a bright green nucleus with intact structure. Whereas Fig. 3b demonstrates the cells death caused by apoptosis and necrosis. Late apoptotic cells have an orange

nucleus showing condensation of chromatin and necrotic cells displayed an orange nucleus with intact structure.

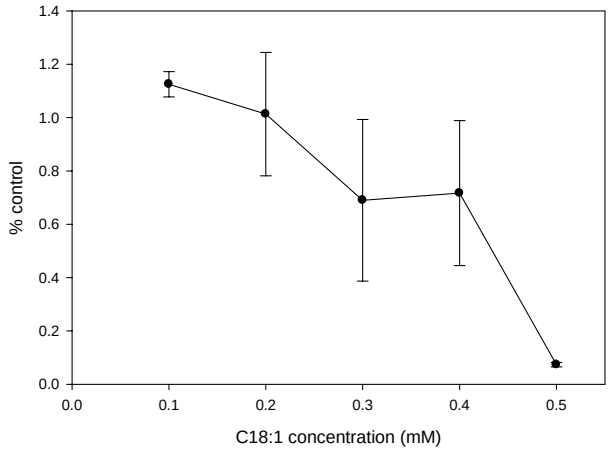

Fig. 2 Effect of oleate on cell viability. 3T3-L1 adipocytes were treated with various concentrations of fatty acids for 48 h. Following the treatment, cells viability was determined by MTT assay. Control cells were treated with vehicle treatment. Assays were performed in 3 replicates from 3 independent experiments. Values are means ± SEM (p<0.01)

C. Effect of Oleate on Lactate Released into Media

This study determined the presence of lactate dehydrogenase activity in culture medium after oleate treatment. Lactate released into culture medium was proportional to the number of dead cells [14]. As shown in **Fig. 4**, lactate released into culture medium increased 60.6% when compared to control after treatment with 0.5 mM oleate for 48 h. This suggested that high oleate concentration able to induce cytotoxicity to cells and the presence of high lactate in spent medium may explain the effect too.

IV. Discussions

Oleic acid (C18:1) play an important role in proliferation of fat cells. However, the abundance of oleate in plasma may influence the proliferation of postconfluent preadipocytes to form more adipocytes and mature adipocytes. Therefore, in this study, the effect of oleate on cells viability in 3T3-L1 cells (fat cells) was investigated. At low concentration of oleate in treatment (0.1 mM) increased the proliferation of postconfluent preadipocytes thus promoting adipogenesis to form more adipocytes. However, oleate did not affect the proliferation of the cells in high availability of oleate.

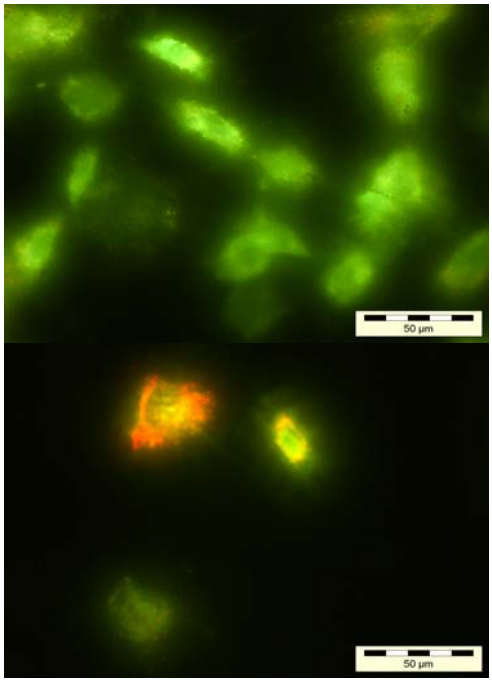

Fig. 3 (a) Viable cells to have a bright green nucleus with intact structure. (b) Apoptotic cells have an orange nucleus showing condensation of chromatin and necrotic cells displayed an orange nucleus with intact structure. The pictures were taken under fluorescent microscope after 48 h treatment with 0.5 mM oleate in adipocytes using acridine orange (AO) and ethidium bromide (EB) staining

Decosahexaenoic acid (DHA, C22:6), a (n-3) fatty acids in fish oil, caused apoptosis after 24 and 48 h of DHA treatment in postconfluent preadipocytes [16]. This induction of apoptosis during postconfluent preadipocytes showed a potential mechanism to suppress adipogenesis, leading to fewer adipocytes. DHA could mediate a reduction of body fat. In other study, conjugated linoleic acid (CLA, C18:2) induced inhibition of both proliferation and differentiation of preadipocytes, thus reducing fat formation, a mechanism to lose weight [17]. However, our findings have shown that oleate did not contribute to the formation of adipocytes nor reduced the formation of adipocytes.

Increasing oleate concentrations in mature adipocytes treatment have presented a decline trend in viable cells (Fig. 2). In one of study, 48 h incubation with 0.5 mM, 0.25 mM and 0.125 mM of palmitic acid complexed to 0.5 mM BSA and in the presence of high glucose (10 mM) caused highly toxic for the cardiomyocytes [18]. Morphological analysis showed a marker of cell death when nuclei cells become smaller and hyperchromatic. Moreover, they also observed that the normal cytoplasmic structure was completely lost. In this study, the quantification of cell viability via MTT assay indicated about 92.7% cells lost in treatment with 0.5 mM oleate and showed DNA fragmentation (data not shown).

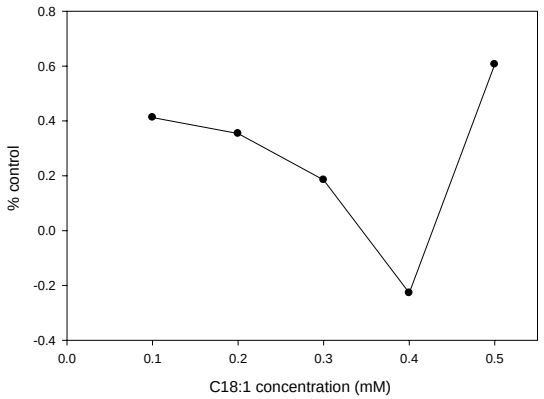

Fig. 4 Effect of oleate on lactate released in media following 48 h treatment in adipocytes. Assays were performed in 3 replicates from 3 independent experiments. Values are means ± SEM (p<0.01)

DNA fragmentation caused lost in membrane integrity and both were the marker of cell death caused by apoptosis [14]. It is not clear whether the result in cell death was due to apoptosis or necrosis. Therefore study on staining cells for apoptosis was required. Results showed that 0.5 mM oleate in culture was confirmed cause apoptosis and necrosis as demonstrated in Fig. 3b. Late apoptotic cells have an orange nucleus showing condensation of chromatin and necrotic cells displayed an orange nucleus with intact structure.

Adipocytes use glucose to produce lactate and pyruvate in addition to carbon dioxide and triglycerides. Some of the pryuvate decarboxylate via the pyruvate dehydrogenase enzyme system and then enter the tricarboxylic acid cycle as acetyl-CoA for complete oxidation. The remainder will be reduced to lactate and released into the plasma [19]. Furthermore, the high production of lactate indicates a high activity of anaerobic glycolysis process in the treatment. Lately, the presence of lactate in the spent media relative to its activity in adherent cells has been used as an index of direct cytotoxicity of exogenous material [13] and lactate released into culture medium was proportional to the number of dead cells [14]. The observation was similar with the results of the current study. 0.5 mM oleate in treatment was responsible to an increase in lactate released into culture medium (60.6% from control) and an increase in cell death (92.7% cells lost). This suggested that high fatty acids were able to induce cytotoxicity to the cells therefore causing cell to die. The presence in high lactate in spent medium may explain the effect too.

Different fatty acids may show different minimum inhibiting concentrations. Oleate concentration at some extent toxic to the cells as the number of cells declined and the presence of high lactate in medium may explain the effect as its activity was proportional to the number of dead cells. This suggested that high concentration of fatty acids were able to induce cytotoxicity to the cells therefore causing cell to die. However, the high concentration of oleate did not affect during postconfluent proliferation stage.

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