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Abstract

Adipose tissue is responsible for insulating internal organs, storing energy for times of negative energy balance, and secreting adipokines into circulation. The major cell types found in adipose tissue include undifferentiated adipocytes (pre-adipocytes), adipocytes, and adipose tissue macrophages (ATMs). Adipocytes and ATMs are the major sources of adipokines, adipose tissue cytokines with functions ranging from inflammatory mediators to metabolic regulators. Polyunsaturated fatty acids (PUFAs) have been shown to affect differentiation and lipid droplet formation in 3T3-L1 adipocytes, but few studies have examined their effects on inflammatory adipokine production from these cells. Adipocyte differentiation, which is marked by lipid droplet (triacylglycerol; TAG) accumulation, is regulated by various transcription activators, including a splice variant of the nuclear receptor PPARγ. PUFAs are major regulators of PPARγ. The objectives of this experiment were to determine the effects of n-3 and -6 PUFAs on TAG metabolism, as measured by free fatty acid (FFA) release and lipid droplet formation, and inflammatory cytokine production, as measured by interleukin (IL)-6 and macrophage inflammatory protein (MIP)-1α production. In order to examine these effects, the 3T3-L1 cells were differentiated in media with 100μM of PUFA treatment (α-linoleic acid [LA], arachidonic acid [ARA], and eicosapentaenoic acid [EPA]), followed by a 6 h, 1μg/mL lipopolysaccharide (LPS) challenge. Fully differentiated 3T3-L1 adipocytes under these treatments did not release FFA in quantifiable amounts. The ARA+LPS treatment produced a significantly higher concentration of MIP-1α in conditioned media compared to control, LPS, and LA+LPS treatments. There was no significant difference in the amount of IL-6 produced between treatments. These data suggest that differentiating 3T3-L1 adipocytes in the presence of

the n-6 PUFA ARA results in increased release of LPS-stimulated MIP-1α and indicate a role of this n-6 fatty acid on adipocyte derived inflammation.

Introduction

Adipose tissue is responsible for insulating internal organs, storing energy for times of negative energy balance, and secreting adipokines into circulation (Surmi and Hasty, 2008). The major cell types found in adipose tissue include undifferentiated adipocytes (pre-adipocytes), adipocytes, and adipose tissue macrophages (ATMs). Adipocytes and ATMs are the major sources of adipokines. Adipokines are signaling proteins with functions ranging from inflammatory mediators to metabolic regulators (Bai and Sun 2015). An example of an adipokine is macrophage inflammatory protein 1α (MIP-1α), which also acts as a chemoattractant, and when measured can be an indicator of the presence of other proinflammatory cytokines (Schall 1994). Interleukin 6 (IL-6) is another adipokine that is secreted by adipocytes and macrophages and known to induce a proinflammatory response (Fain 2010)

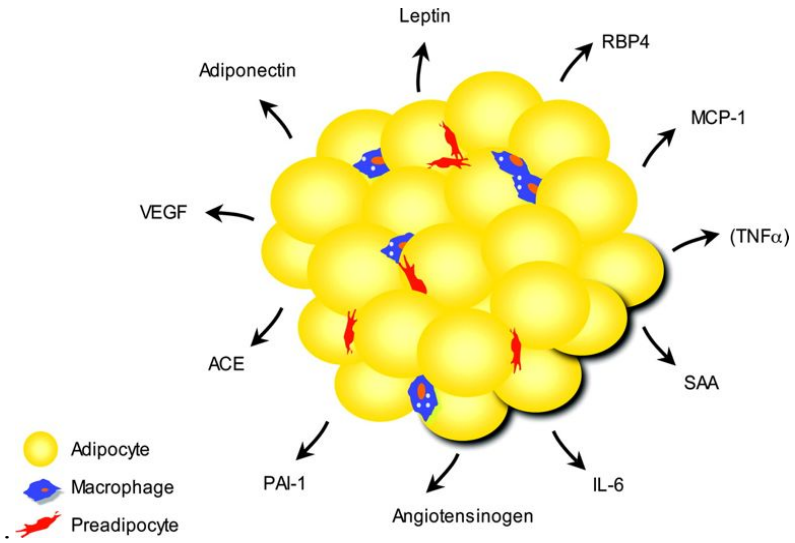

Figure 1 Major adipokines released by adipose tissue. Credit: Gustafson et al Inflamed Adipose Tissue: A Culprit Underlying the Metabolic Syndrome and Atherosclerosis. Arterioscler Thromb VascBiol. 27:2276.

The 3T3-L1 cell line of fibroblasts can be differentiated into adipocytes for *in vitro* adipocyte studies. During differentiation, these fibroblasts undergo a series of biochemical and morphological changes resulting in the accumulation of lipid droplets (Tong and Hotamisligil 2001). The formation of lipid droplets is due to the synthesis of triacylglycerol (TAG) which provides a concentrated form of metabolic energy (Gurr et al. 2002). During times of negative energy balance, or stimulation by other factors, TAGs are catabolized releasing long-chain FAs that are imported to the mitochondria for degradation through FA oxidation, which yields ATP (Gurr et al. 2002). If the long chain FAs are not catabolized they can be released by the adipocytes as free fatty acids (FFAs). Polyunsaturated fatty acids (PUFAs) such as omega-3 and omega-6 fatty acids can be released during lipolysis, leading to an increase in local FA concentration and subsequent uptake by surrounding adipocytes during differentiation and lipid droplet development (Teusink et al. 2003). Adipocyte differentiation, which is marked by lipid droplet accumulation, is regulated by various transcription activators, including a splice variant of the nuclear receptor PPARγ, as well as by transcription repressors (MacDougald and Lane 1995). Throughout the differentiation process, genes whose function is to inhibit adipogenesis are suppressed through transcriptional regulation (Jessen, Stevens 2002; Gregoire et al. 1998). During the differentiation process, the adipocyte also undergoes a change in shape to a more spherical form rather than the stringy fibroblast precursor (Kuri-Harcuchet al., 1978). This shape change is independent of the formation of the lipid droplet which is an indicator of the presence of lipid binding proteins and the terminal stage of differentiation (Gregoire et al. 1998).

The size of the adipocyte is an important marker of what adipokines will be secreted (Manickam et al. 2010). Increase in TAG storage and total adipose tissue mass can occur through

adipocyte hypertrophy and/or adipocyte hyperplasia. Adipocyte hyperplasia is an increase in the number of adipocytes whereas hypertrophy is characterized by an increase in the size of adipocytes (Jo et al. 2009). Typically, larger adipocytes release proinflammatory adipokines whereas smaller adipocytes secrete anti-inflammatory adipokines (Manickam et al. 2010).

Fatty acids (FAs) are carboxylic acids and are named based on the number of double bonds the FA contains. Furthermore, there are two main types of polyunsaturated fatty acids (PUFAs), omega-3 and omega-6 . Omega-3 PUFAs have their first double bond located on the 3rd carbon atom while omega-6 PUFAs have a double bond on the 6th carbon from the methyl end of the molecule (Bayly, 2014). Fatty acids are derived from adipose tissue triglycerides through the action of hormone-sensitive lipase which is activated by hormones like glucagon and epinephrine (Bayly, 2014). Recent research has shown that the length and degree of unsaturation of fatty acids present in media used to culture adipocytes influence PPARγ regulated gene expression which in turn dictates lipid droplet formation during differentiation (Manickam et al. 2010).

One of the major FA regulators of PPARγ expression is eicosapentaenoic acid (EPA) which is a long chain omega-3 polyunsaturated fatty acid (Manickam et al. 2010). Eicosapentaenoic acid has been shown to have an effect on lipid droplet size through effects on lipid catabolism (Manickam et al. 2010), as well as mediate reductions in pro-inflammatory adipokine secretion in favor of anti-inflammatory secretions (Flachs et al 2009). Reports on the effects of omega-3 and omega-6 PUFAs on adipocyte differentiation and TAG accumulation (as measured by lipid droplet size) are conflicting. Madsen et al. (2005) reported that the omega-3 PUFAs EPA and docosahexaenoic acid (DHA), as well as the omega-6 PUFAs α-linoleic acid

(LA) and arachidonic acid (ARA) stimulated the differentiation and increased TAG accumulation in 3T3-L1 cells at concentrations of 100 μM. In contrast, Kim et al. (2006) found that 50 and 200 μM doses of DHA inhibited differentiation and lipid droplet accumulation in 3T3-L1 cells. Lastly, treatment of 3T3-L1 cells with 100 μM of EPA markedly reduced lipid droplet size and total lipid accumulation (Manickam et al. 2010). In addition to PUFAs, monounsaturated fatty acids (MUFAs) have also been shown to have proinflammatory effects on 3T3-L1 cells. Previous research has found 3T3-L1 preadipocytes that were acutely exposed to MUFAs produced higher levels of MIP-1α and IL-6 compared to mature 3T3-L1 cells (Dordvic et al. 2014).

It is important to clarify the effects of PUFAs on adipocyte differentiation and to determine if these fatty acids have effects on inflammatory molecule production. As common dietary fats, PUFAs have the possibility of improving metabolic status by decreasing fat mass size, as well as decreasing inflammation coming from adipose tissue. The objectives of these experiments were to determine the effects of the PUFAs EPA (omega-3), α-linoleic acid (LA, omega-6) and arachidonic acid (ARA, omega-6) on 3T3-L1 differentiation, as measured by lipid droplet size, and inflammatory cytokine production as measured by MIP-1α and IL-6 production.

Methods and Materials

Reagents

Murine pre-adipocytes (3T3-L1) were purchased from ATCC (Manassass, VA). Dulbecco's modified Eagle's medium (DMEM), phosphate buffered saline (PBS), fetal bovine serum (FBS), penicillin-streptomycin, dexamethasone, biotin, insulin, and 3-Isobutyl-1-methylxanthine (IBMX) were purchased from Fisher Scientific (Pittsburg, PA). Bovine serum albumins (BSA) was purchased from Sigma Aldrich (St. Louis, MO). Sodium salts of fatty acids were from NU-CHEK Prep, Inc. (Waterville, MN). The interleukin (IL)-6 and macrophage inflammatory protein-1α (MIP1-α) enzyme-linked immunosorbent assays (ELISA) were purchased from R&D systems (Minneapolis, MN), while the Griess assay for nitric oxide (NO) quantification was obtained from Promega (Madison, Wisconsin).

Cell Culture

3T3-L1 cells were grown in 10cm plates with DMEM containing 10% FBS, 1% Pen-Strep, and 4µg Biotin (complete growth medium; CGM). Cells were incubated in a 95% O_2 and 5% CO_2 humidified atmosphere. The medium was changed every two-three days, until cells reached 75% confluency. Three days after reaching 75% confluence and when cells looked clumped together losing fibroblast morphology, differentiation was initiated by aspirating growth medium and replacing it with the differentiation cocktail (CGM+ 2.5% IBMX, 0.25% DEX, 0.1 %insulin). This medium was left on the cells for 48hrs. Following this period, they were fed with the post-differentiation cocktail (CGM + 0.1 % insulin). These post- differentiation feedings

occur three times, withmedium being left on the cells for 48hrs between feedings. Cell differentiation was confirmed by staining differentiated 3T3-L1 cells with Oil Red-O.

Oil Red-O staining

Fully differentiated 3T3-L1 cells were washed with 1X PBS. Cells were fixed with the addition of 10% formalin and 60% isopropanol followed with a 30 min incubation (after addition of each solution).The Oil Red-O solution was added to the cells and left for 20 mins to be discarded and washed 3-5 times with ddH_2O. The Oil Red-O solution stains the lipid droplets in the adipocytes. Hematoxylin was added to the plate for 1 minute and then discarded and washed with ddH_2O. The hematoxylin stains the nuclei of the cells. When viewed under a microscope the lipid droplets appeared red and nuclei appeared blue.

3T3-L1 differentiation with PUFAs

PUFA stock solutions were complexed to BSA in serum free media (SFM) at a 2:1 molar ratio for a period of 2 hours prior to plating each day of feeding. Following this period, each treatment was mixed with the corresponding differentiation media along with 200μM α-tocopherol, resulting in 100 μM PUFA treatments. The α-tocopherol solution was added to prevent oxidation of PUFAs. Treatments were added to duplicate wells using a 0.2 μm syringe filter. The cells incubated for forty eight hours at a time. Forty eight hours following the last post-differentiation feeding, the adipocytes underwent an LPS challenge. The fully differentiated 3T3-L1 cells were washed 2x with 1X PBS. SFM was added to each well and followed by the addition of 0.1μg/ml of LPS. The cells were then incubated for 6 hours at 37^{o}C. Following the

challenge, the supernatant was collected and stored for use in FFA assays, MIP-1α and IL-6 ELISAs.

Enzyme-linked immunosorbent assay

Analyses of samples for determination of cytokine levels were done using IL-6 and MIP-1α ELISAs according to manufacturer's instructions. These ELISAs use the sandwich technique where the cytokines produced by the samples are bound to the antibody of the cytokine in the wells of the plate. From here a polyclonal antibody specific to the cytokine being examined is added. Finally a substrate solution is added, causing the wells to change colors as well as a stop solution before reading the plate. The intensity of the color is proportional to the amount of cytokine present. Samples were tested in triplicate, and a standard curve was produced and used to extrapolate the cytokine concentrations in the samples.

Free fatty acid quantification

Analysis of samples for determination of free fatty acid levels were done using a Fatty acid assay from BioAssay Systems according to manufacturer's instructions. The free fatty acids are converted into acyl-CoA and then H_2O_2. The H_2O_2 reacts with dye and produces a pinkish color that is directly related to the concentration of free fatty acids present. Samples were tested in triplicate, and a standard curve was produced and used to extrapolate the cytokine concentrations in the samples.

Statistical analyses

Concentrations of IL-6 and MIP-1α in cell culture medium and were analyzed using two-way ANOVAs (JMP Pro 13). Interleukin-6 and MIP-1α data were normalized using a log transformation. The sources of variation included experiment, treatment, and experiment x treatment interaction, with the experiment and treatment x experiment interaction considered as random variables. When treatment effects were detected ($P < 0.05$), means were separated using Tukey's HSD.

Results

Oil Red-O staining

3T3-L1 cells were stained following the differentiation protocol in order to confirm successful differentiation (Figure 2). The Oil Red-O stains the lipid droplets which develop following the differentiation process. From these images, one can see the morphological differences between them. The right image shows fibroblasts that are elongated. This shape is lost during the differentiation process as the cells become larger, rounded, and take up triglycerides which are stored in the visible lipid droplets shown in the left image.

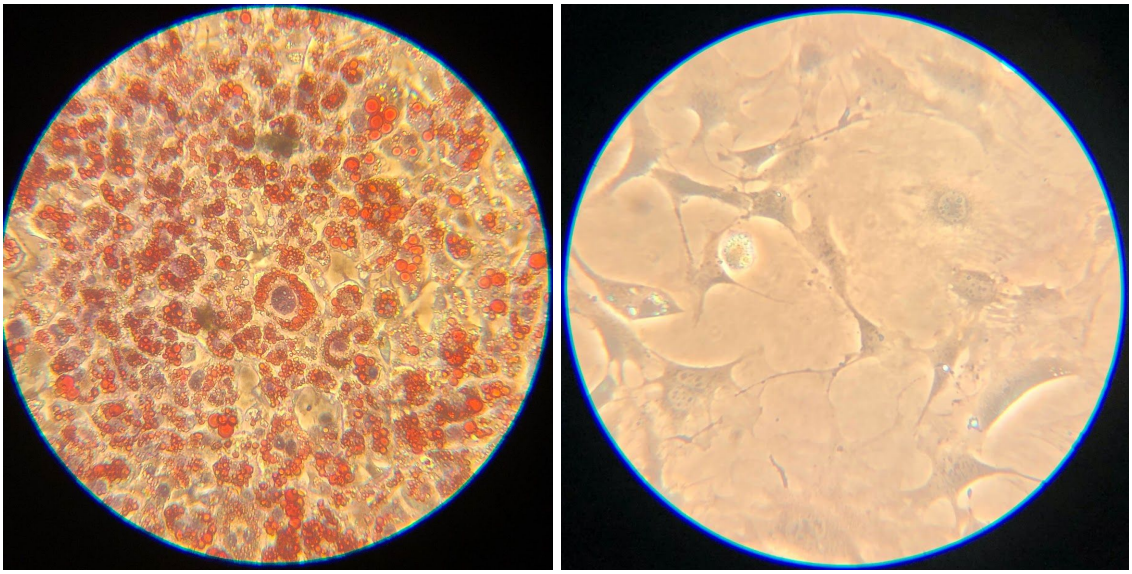

Figure 2 Left image: Fully Differentiated 3T3-L1 cells (passage 5) stained with Oil Red-O. Right image: 3T3-L1 (passage 5) at 70% confluency before initiation of the differentiation process.

MIP-1α concentrations in conditioned media

3T3-L1 cells differentiated with arachidonic acid and then stimulated with LPS (1 μg/ml) showed the highest concentration of MIP- 1α in conditioned media (26.315 ± 2.54 pg/mL) whereas cells treated with only LPS produced the lowest concentration of MIP- 1α (0.06 ± 2.54

pg/mL) (Tukey-Kramer; $p<0.05$) (Figure 3). These results are consistent with previous research from Cranmer-Byng et al. 2015, which showed that 3T3-L1 cells produced higher concentration of MIP- 1**α** when ARA and LPS were present in culture conditions. Although differentiating the adipocytes with linoleic acid resulted in numerically higher MIP-1**α** concentrations following LPS stimulation (7.482 ± 2.54 pg/mL) compared to control (0.55 ± 2.54 pg/mL) and LPS-challenged cells, there was no statistical difference in MIP-1**α** production among these three treatments. Additionally, MIP-1**α** production by LA-differentiated adipocytes was not significantly different than MIP-1**α** production by ARA or EPA-differentiated cells (14.93 ± 2.54 pg/mL) following LPS challenge.

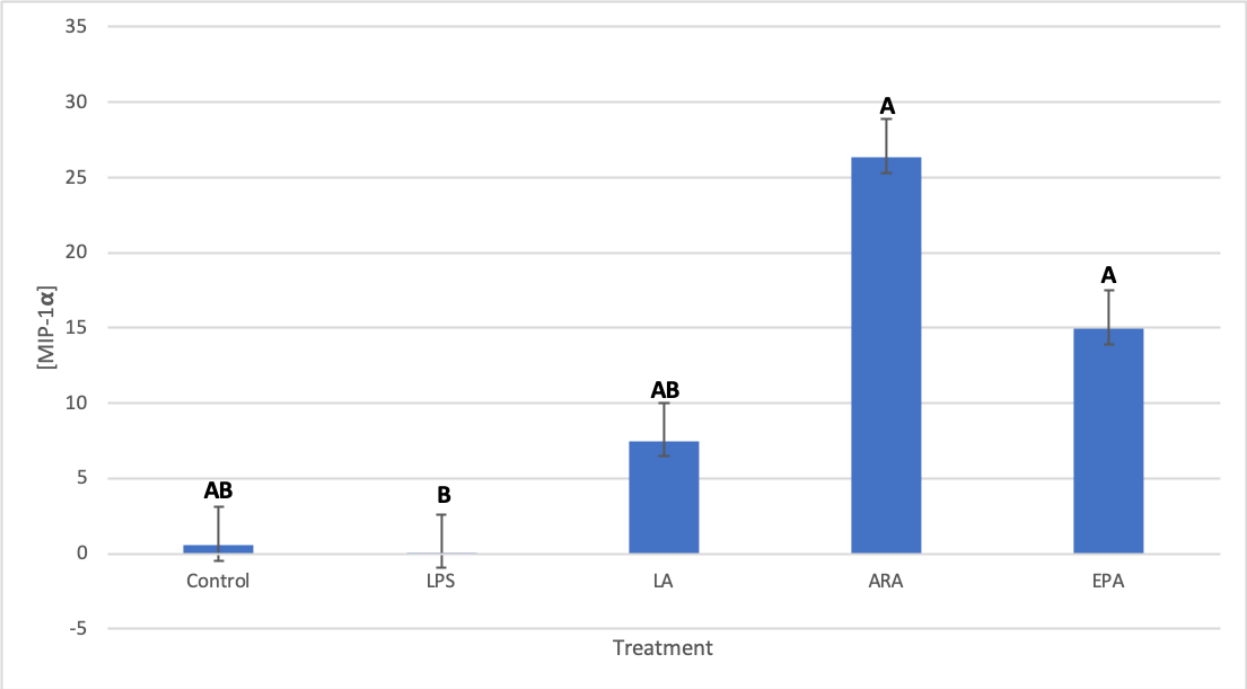

*Figure 3 Mean MIP-1**α** (pg/ml) production of 3T3-L1 cells challenged with LPS and preincubated with various PUFA treatments. ARA treatment had the highest MIP- 1**α** production compared to other treatments ($p=0.027$). Bars are mean; error bars are SEM. Groups were compared by two-way ANOVA, followed by post-hoc Tukey- Kramer HSD; significant group differences are represented with letters ($p<0.05$). Treatments were performed in duplicate. Values are the means of four experiments.*

IL-6 concentrations in conditioned media

There was no significant difference in the production of IL-6 between treatments (two-way ANOVA; p=0.95). The average concentration of IL-6 produced by the differentiated adipocytes was 115.36 ± 68.70pg/ml.

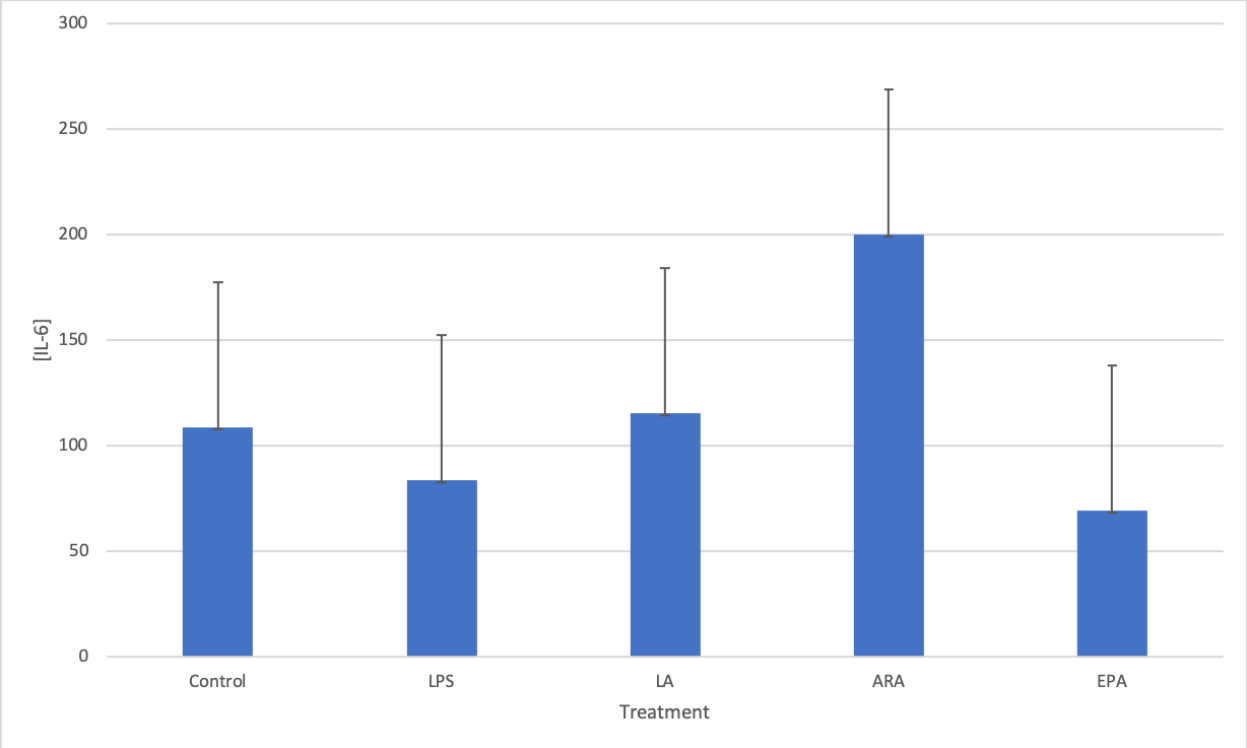

Figure 4 Mean IL-6 (pg/ml) production of 3T3-L1 cells challenged with LPS and preincubated with various PUFA treatments. There was no differences in IL-6 production among treatments (p=0.95). Bars are mean; error bars are SEM. Groups were compared by two-way ANOVA. Treatments were performed in duplicate. Values are the means for four experiments.

Lipid droplet appearance

Figure 5 contains images of all six treatments after the cells were fully differentiated. This comparison allowed us to both confirm that differentiation with the PUFA treatments was

successful as well as visually compare the differences in lipid droplet development, a hallmark of differentiation. While one can see visual differences in size of lipid droplets across the treatments, we were unable to statistically quantify any differences in size.

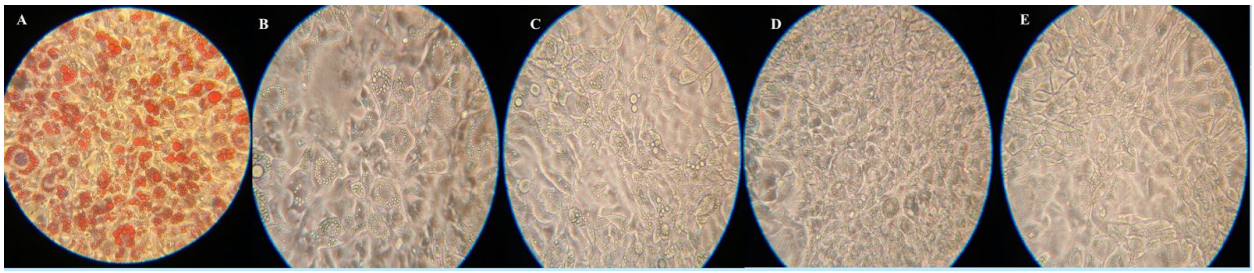

Figure 5 Images of differentiated 3T3-L1 cells treated with LPS and PUFAs. Image A shows control cells stained with Oil Red-O, which stains the lipid droplets. Images B-E were not stained with Oil Red-O. B=LPS treatment; C=LPS+LA treatment; D=LPS+ARA treatment; E=LPS+EPA treatment.

Free Fatty Acid concentrations in conditioned media

The amount of free fatty acid produced by the treatments was less than the lower standard of the assay (<7 micromoles/liter) and therefore below the level of detection of this assay. Data is not shown.

Discussion

Fully differentiated 3T3-L1 adipocytes did not release FFA in quantifiable amounts (data not shown), but did produce measurable amounts of both IL-6 and MIP-1α. The values we quantified were much lower than those reported by Chirumbolo et al. (2014) despite the use of the same LPS concentration (1 μg/ml) and similar LPS-incubation length (6 hours vs 8 hours). Chirumbolo et al. reported that incubation of differentiated 3T3-L1 cells with 1 μg/ml of LPS for 8 hours resulted in production of 800 pg/ml of IL-6 and 50 pg/ml of MIP-1α. We quantified 115.36±68.7 pg/ml of IL-6 and 9.86±2.54 pg/ml MIP-1α produced by differentiated adipocytes treated with 1 μg/ml LPS for 6 hours. There was no difference in MIP-1α cytokine production between control and LPS stimulated cells (0.55 ±2.54 pg/ml and 0.06±2.54 pg/ml, respectively). This could be attributed to the adipocytes inherently producing MIP-1α and other cytokines regardless of the presence of LPS. These results suggest that our current methodology of differentiating 3T3-L1 cells with fatty acids and LPS may cloud our ability to examine the amount of cytokines produced by the cells in the presence of FAs. Additional experiments need to be conducted with 3T3-L1 cells differentiated with FAs but without the LPS challenge in order to further examine this.

Our results suggest that 3T3-L1 adipocytes differentiated with 100 μM of ARA produced significantly more MIP-1α after 6 hours of LPS-stimulation compared to the other treatments. However, there was not a significant difference in production of IL-6 between the LPS and LPS+fatty acid treatments following 6 hours of LPS-stimulation. This differs from results obtained by Cranmer-Byng et al. (2015) who reported that a 24 hour treatment of fully differentiated 3T3-L1 adipocytes with ARA and LPS increased pro- inflammatory monocyte

chemoattractant protein-1 (MCP)-1 and interleukin IL-6 secretion and gene expression, but did not observe an increase in production of proinflammatory proteins in adipocytes treated with other omega-6 and omega-3 PUFAs. Monocyte chemoattractant protein-1 (MCP-1) is also referred to as CCL2 and functions similar to MIP-1α, by recruiting immune cells to sites of inflammation. Despite the differences in methodology, the difference in results obtained by our lab and Cranmer-Byng's results (2015) suggests that treating 3T3-L1 cells with ARA can induce proinflammatory adipokine release independent of if the treatment occurs pre- or post-differentiation. Additional investigation is needed to determine whether the adipocytes produce these cytokines as a response to LPS or if it is an inherent occurrence. We hypothesize that the increase in proinflammatory adipokines is related to an increased production of prostaglandin E2 (PGE2), an inflammatory prostanoid, which subsequently results in increased production of other proinflammatory adipokines. Prostanoids are made from ARA, and research has shown that ARA inhibits lipogenic gene expression in 3T3-L1 adipocytes through a prostanoid pathway (Mater et al., 1998). If ARA is inhibiting lipogenesis the result should be smaller adipocytes and less inflammatory cytokines being produced. Our results seem to contradict this sentiment since ARA produced the highest concentration of MIP-1α. Further investigation is needed to determine if ARA-differentiated adipocytes produce higher concentrations of PGE2 and if there is a difference in LD development.

Unfortunately, we were not able to develop a methodology to quantify adipocyte hypertrophy, hyperplasia, or lipid droplet size. We are currently seeking assistance in developing a methodology to measure these. Since Oil Red-O stains lipid droplets of differentiated 3T3-L1 adipocytes, perhaps using an image software such as ImageJ to measure the intensity of the stain

will allow us to measure LD development. Another possibility is running a colorimetric assay, similar to some ELISAs and GREISS assays to measure differences in intensity of color produced (in this case Oil Red-O stain). This method is similar to Manickam et al. (2010), who reported quantifying LD development using Oil Red-O stain and spectrophotometric analysis.

Future experiments should examine if there is an effect on cytokine production when the 3T3-L1 cells are differentiated with FAs but without the LPS challenge. Additionally, analysis of our samples using a PGE2 ELISA to explore the proposed hypothesis above regarding the effects of ARA on PGE2 production should be performed. The PPARγ antagonist GW9662 can also be used to determine if ARA is exerting its proinflammatory effects through this nuclear receptor. In an effort to further explore the relationship between adipocytes and other adipose tissue cells the 3T3-L1 cells can be differentiated in the presence of these other cells using transwells plates. This method would allow for the further study of cross communication between the adipocytes and other adipose tissue cells.

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