The Effect of Eicosapentaenoic Acid on Hepatic Metabolism and Intracellular Lipid Droplet Dynamics in An in Vitro Model of Hepatic Steatosis

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Abstract

Background Intra-hepatocyte triglyceride accumulation is the hallmark of non-alcoholic fatty liver disease (NAFLD). Little is known of the role of lipid droplets in the pathogenesis of this increasingly common condition. Eicosapentaenoic acid (EPA), an omega-3 fatty acid, has been suggested as a potential treatment for NAFLD. Within this study we explore different energy substrates with and without EPA and evaluate biochemical markers alongside lipid droplet characteristics.

Methods EPA was added to C3A cells incubated in standard media, oleate (which mimics simple steatosis) or lactate, pyruvate, octanoate and ammonia (LPON), mimicking pathophysiology of NAFLD. At 72 hours, glucose, ketone bodies and various markers of liver function were assessed and triglyceride content was determined using confocal images of cells stained with BODIPY 493/503 and DAPI. Z stacks of confocal images were analysed using Volocity 3D image analysis and Visual Basic software to estimate the physical characteristics of lipid droplets. Liver function was assessed by measuring AST, albumin and LDH.

Results Albumin levels increased in control and oleate and LDH showed EPA did not affect viability in concentrations up to 250µm. There was no effect to AST with addition of EPA. Glucose and ketone bodies were reduced in LPON treated cells in a dose dependent manner. EPA reduced triglyceride concentrations and lipid droplets per nucleus in untreated cells compared with control, however, there was no significant difference to total triglyceride concentration of cells treated with oleate + EPA or LPON + EPA. Lipid droplet volume increased in both fat loaded models with the addition of EPA, but untreated control remained the same. Surface area of lipid droplets was higher in oleate model compared to LPON.

Conclusion The LPON + EPA model shows reduction of glucose and ketone bodies in a dose dependent manner indicating EPA may be useful as an adjunct to treatment of type 2 diabetes. Accumulation of lipid droplets at different rates and physical characteristics compared to oleate may reflect differences in FFA metabolism or their incorporation into the cell and lipid droplet membrane.

1. Introduction

The global epidemic of obesity has triggered an inexorable rise in the prevalence of non-alcoholic fatty liver disease (NAFLD). As a result NAFLD is now the third commonest reason for receiving a liver transplant in the United States[1]. Intrahepatic triglyceride accumulation is the hallmark of NAFLD. Stored within the lipid droplets in the hepatocyte cytoplasm, triglyceride is an efficient fatty acid storage molecule, not only crucial for energy homeostasis but also vital for protecting cells against the deleterious effects of free fatty acids. Lipid droplets are highly dynamic, which in response to the appropriate signals, can be esterified, hydrolyzed or reesterified to meet cellular demand[2]. Altered liver metabolism with an accumulation of lipid droplets is an early pathophysiological marker of insulin resistance and steatosis leading to cirrhosis and possibly hepatocellular carcinoma [3,4,5]. The size of

lipid droplets is maintained by a tightly regulated mechanism and they are known to take part in various biological processes such as neutral lipid storage and the storage and degradation of proteins [4,5]. Despite the abundance of lipid droplets, surprisingly little is known about their role in the pathogenesis of NAFLD.

Omega-3 polyunsaturated fatty acids, of which eicosapentaenoic acid (EPA) is among the most physiologically active and has been suggested as a potential treatment for NAFLD [6,7]. Cell and animal studies have suggested that omega-3 fatty acids may act as regulators of hepatic gene expression [8, 9]. The mechanisms whereby omega-3 fatty acids affect gene expression are complex but may involve interplay between the activation of peroxisome proliferator-activated receptor (PPAR) α , and the suppression of sterol regulatory element binding protein (SREBP) [10,11]. Such influence on lipid homeostasis can potentially alter the characteristics of lipid droplets. For instance, PPAR activation has been shown to modulate lipid droplet proteins-perilipin, adipophilin, and TIP47 (PAT proteins) [12]. However, the effect of omega-3 fatty acids on physical characteristics and biological properties of lipid droplets in NAFLD is yet to be established.

We have previously shown that C3A cells treated with energy substrates; lactate (L), pyruvate (P), octanoate (O) and ammonia (N) *in vitro* recapitulate the events that have been proposed to occur in human NAFLD [15]. While oleate mimics simple steatosis *in vitro*, octanoate has been shown to mimic the pathophysiology of NAFLD through increasing β oxidation and upregulating genes causing fibrosis and synthesis of cholesterol [15,16]. Indeed, LPON-induced cellular steatosis manifests many of the key features associated with steatohepatitis such as impaired mitochondrial function, enhanced oxidative stress with production of reactive oxygen species (ROS), increased ketogenesis and altered glucose metabolism [15, 16]. However, when cells are pre-treated with oleate, we have shown no mitochondrial dysfunction despite triglyceride accumulation [15].

The main mechanism that underpins the triglyceride accumulation is different between these models. The medium chain free fatty acid, in LPON, octanoate, cannot be directly esterified thus the triglyceride accumulation with LPON relies on *de novo* lipogenesis [13]. By comparison, triglyceride accumulation with oleate is the result of direct esterification in proportion to its concentration [13, 14]. Whether such a contrast can influence the characteristics of lipid droplets remains unknown.

In this study we also explore the effects of both oleate and LPON with and without EPA on human hepatic C3A cells and evaluate several markers on liver function alongside the dynamics of lipid droplets for each model. Liver function was assessed by measuring lactate dehydrogenase (LDH), albumin and aspartate transaminase (AST). Further assessment of the effect of EPA on glucose, ketone bodies and reactive oxygen species donor was performed to assess how EPA may effect basic functionality of the cell and be of importance in the study of type 2 diabetes which is often associated with NAFLD and metabolic disease. Finally, we defined the dynamics of lipid droplets within our models to see if this could be a useful marker in the context of histopathological characterization of NAFLD examining

triglyceride concentrations, lipid droplet sphericity and volume to size ratio which has been implicated in lipid homeostasis and insulin sensitivity[17].

2. Materials And Methods

2.1 Cell culture

Human hepatoblastoma C3A cells were cultured in 75 cm² tissue culture flasks in minimal essential medium Eagle (MEME) with 10% fetal calf serum (FCS) and antibiotics (100IU/ml penicillin, 100mg/ml streptomycin) at 37°C and 5% CO₂ until 70% confluent before supplementation with combinations of oleic acid (Ol)(0.25mM)complexed with 4 grams bovine serum albumin (BSA) in 20ml media, lactate (L) (10mM), pyruvate (P)(1mM), octanoate(O)(2mM) and ammonia (N)(4mM) with or without EPA (50 or 250mM). Unless stated otherwise, cells were cultured in triplicates [9].

Optimal concentration of EPA in LPON model using LDH measurement

C3A cells were grown in 6 well plates and incubated with LPON plus either 10, 50, 100, 250 and 500µM EPA. extracellular LDH concentration was used to assess cell viability. This assay is based on the procedure of Gay et al.,[18]Here the LDH concentration in the cell lysates and supernatant were determined by following the rate at which NAD is reduced to NADH measured as an increase in absorbance at 340nm in the presence of lactate using a LDH kit method (Sentinel Diagnostics, Italy) modified for use on the Cobas-Fara centrifugal analyser (Roche Diagnostics, Welwyn Garden City, UK). The rate of decrease in absorbance at 340nm, measured at 37C, is directly proportional to LDH activity in the sample. Results were expressed as % LDH released calculated as follows – intracellular LDH/ (extracellular LDH + Intracellular LDH) x 100.The data is presented as % of LDH in the supernatant/ total LDH. The results are also presented in Figure 3.1 as means and standard error of the mean (SEM).

Quantitative analysis of glucose

Principle: (Hexokinase (HK)) $\text{Glucose} + \text{ATP} \leftrightarrow \text{Glucose-6-phosphate} + \text{NAD}^+$ (G6PdH) $\text{Glucose-6-phosphate} + \text{NAD}^+ \leftrightarrow \text{Gluconolactone 6-phosphate} + \text{NADH}$ The assay detects the concentration of NADH produced by the two reactions above. This corresponds to the concentration of glucose in the cuvette. The glucose buffer is prepared by mixing NAD (17mg), ATP (125mL), G6PdH (12.5mL) HK (5mL) and diluting in 50mls of PBS+.200mL of each sample was pipetted into a cuvette and 1ml of glucose buffer added. This was then homogenised by gently inverting the cuvette 3-5 times. A blank well was prepared with 200mL of 95 H₂O and 1ml of buffer to act as a control. The samples were then left at room temperature for 60 minutes. Following this absorbance was read at 340nm wavelength for each cuvette and blank on Unicam UV1 spectrophotometer (Unicam Ltd, U.K). Glucose flux was then calculated according to the methods of Bergmeyer[19].

Albumin assay

Albumin working solution was made up from powdered albumin (Albumin blue 580. This was made into solution with isopropanolol (3mg/100mls isopropanolol) to give concentration 30mg/L. The absorbance of the solution was read at 580nm and the solution was diluted such that the OD was 1.00. The solution was diluted with a buffer comprising 0.6g N-morpholino-propanesulfonic acid (Mops free acid), 1.8g Mops sodium salt, 2.4g sodium chloride, 0.2g ethylene-diaminetetraacetic acid and disodium salt (EDTA disodium), 200 mL distilled water, and 20 mL isopropanol. The pH of the resulting solution is 7.4. This solution was then diluted with buffer to create standards with albumin concentration 2.5, 5.0, 10, 20, 40, 50, 75, 100, 150, 200 mg/mL. 80mL of each standard or sample was added in duplicate to wells in a microtitre place. 160mL of dye was added to each well. The plate was then shaken for 30seconds before the fluorescence was read (excitation 590nm, emission 645nm) on Cytofluor Series 4000 (PerSeptiveBiosystems). A standard curve was created by inputting the data to Microsoft excel. Test values were then calculated from the standard curve. Concentration of BSA complexed with oleate was subtracted from results.

AST assay

AST was determined by a commercial kit (Randox Laboratories, UK) adapted for use on the Cobas-Fara centrifugal analyser (Roche Diagnostic Ltd, Welwyn Garden City, UK). α -oxoglutarate reacts with L-aspartate in the presence of AST to form Lglutamate plus oxaloacetate. The indicator reaction utilises the oxaloacetate for a kinetic determination of NADH consumption. Within run precision was CV<5%.

Ketone body production

Betahydroxybutyrate + NAD $\leftrightarrow$ acetoacetate + NADH

The assay detected the concentration of NADH produced by the reaction which corresponds to the concentration of betahydroxybutyrate in the sample. Two readings were taken as the changes in absorbance were small. Method A betahydroxybutyrate buffer was made by mixing NAD (50mg), glycin (3g), hydrate hydrazine (2ml). This was diluted in 100mls sterile water. For the reaction 200mL of sample was added to each cuvette. 200mL of sterile water was added to an additional cuvette to act as control. 1ml of betahydroxybutyrate buffer was then added to each sample and this was homogenised by gentle inversion. A reading was then made at 340nm wavelength on Unicam UV1 spectrophotometer (Unicam Ltd, U.K). A solution of betahydroxybutyrate dehydrogenase was then prepared by diluting 200mL of betahydroxybutyrate dehydrogenase in 1ml of sterile water. 10mL 96 of this solution was added to each cuvette and the sample homogenised. Samples were left for 1 hour at room temperature before a repeat reading was made.

Reactive oxygen species donor

Cells were grown in 6 well plates in standard MEME until confluent. Media was then replaced with either standard MEME or MEME + oleate, with or without reactive oxygen species donor (100 μ M tBOOH) and

incubated for 72 hours at 37°C. Supernatant and cells were harvested and triglyceride levels were determined as per previous methods.

2.2 BODIPY 493/503 staining

Cells were treated on chamber slides for 72 hours and fixed with 4% paraformaldehyde for 30 minutes before staining with 200ml of BODIPY 493/503 (Invitrogen, NY, USA) for 10 minutes. Cells were mounted in ProLong gold antifade reagent and were left for 24 hours at 4°C before imaging with a confocal laser microscope (Leica SP5, Mannheim, Germany). Fluorescent intensity in at least 50 cells per image was analysed using ImageJ software (National Institutes of Health, Bethesda, MD). To determine the characteristics of lipid droplets, the 3D confocal image stacks were analysed using Volocity 3D image analysis software (Perkin Elmer, Waltham, MA). Using the z stacks of BODIPY stained lipid droplets, objects smaller than 3µm³ were identified and excluded and to determine individual nuclei within the DAPI image stacks the limit was set at 50µm³.

2.3 Triglyceride concentration

To determine the triglyceride concentrations, cells were treated with the specified combination of energy substrates in 6-well plates (in triplicates) for 3 and 7 days. Intracellular triglyceride concentrations were measured using a commercial kit adapted for use on the Cobas-Fara centrifugal analyzer (Roche Diagnostics Ltd., Welwyn Garden City, UK). Triglyceride concentration was normalized to the total protein and expressed as mg per gram of total protein.

2.4 Visual Basic

Visual Basic for Applications was used in MS Excel to analyse lipid droplets from Bodipy imaging. Data was taken from Volocity imaging software and processed uniformly throughout. The dataset was filtered and sorted by volume in cubic microns. Larger objects identified as multiple droplets or nuclei were excluded. For images containing lipid droplets, objects with measured volumes of 400µm³ and higher were removed. Total, average, minimum and maximum object volume (µm³) and surface area (µm²) was assessed and average surface area to volume ratio was calculated for the data set. Objects were measured and grouped according to their volumetric size.

2.5 Statistical Analysis

All data were analysed on GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) using one way ANOVA followed by Tukey post-test. P<0.05 was deemed statistically significant.

3 Results

3.1 Dose dependent response of glucose and ketone bodies to EPA in LPON model

Endogenous glucose production was selected as the primary outcome to assess efficacy as this can be rapidly assessed by spectrophotometry. We measured glucose in micro mole of glucose per hour per gram of total protein ($\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{gTP}^{-1}$). The endogenous glucose production of LPON control is significantly higher than untreated cells ($p<0.0001$). Likewise, we see a concentration dependent trend showing a decrease in ketone bodies with increasing EPA.

3.2 Liver function tests with addition of EPA

There was no significant change in AST following incubation with EPA under any condition though the MEME control showed a trend between decreasing AST and increase of EPA ($p=0.06$) which just missed significance. Incubation with EPA significantly increased albumin in MEME control vs MEME + 250 μM EPA ($p<0.05$). Some slight significance was seen in oleate, but likely do to outlier seen in oleate control. No change was seen between other samples. At 500 μM EPA had significantly elevated LDH leakage when compared with LPON control ($p<0.001$) and was also elevated compared to other concentrations of EPA.

3.3 Ketone body production across all conditions

No significant difference in production of ketone bodies could be seen in control (MEME media only) + EPA or oleate + EPA, however, there was significant reduction of ketone bodies between LPON control and LPON + EPA 50 μM ($p<0.05$) and LPON + EPA 250 μM ($p<0.01$)

3.4 Reactive oxygen species donor

Our LPON model is associated with increased ROS while control and oleate models are not. This experiment was designed to measure total triglyceride concentration in control and oleate models in the presence of a ROS donor to rule out ROS in the LPON model preventing the lowering of triglycerides. The presence of a reactive oxygen species donor did not prevent the reduction in hepatocyte triglycerides in the Control (MEME media only) model: both with and without a ROS donor there was a significant reduction in hepatocyte triglyceride content ($p<0.0001$)

3.5 Triglyceride concentrations

We have previously shown that treatment with either oleate or LPON for 24 and 72 hours resulted in significant triglyceride accumulation which resulted in two models of NAFLD[15]. Oleate shows a model whereby lipid accumulation is the result of direct esterification[15]. Whereas, LPON shows a model characterized by de novo lipogenesis [15]. Here, we examined the impact of EPA on triglyceride concentration in these models of cellular steatosis. Cells were treated with the specified combinations of energy substrates, with or without EPA for 72 hours. Triglyceride concentrations were measured at the end of the treatment period. As shown in Figure 5, there was a significant reduction in triglyceride content when untreated cells were incubated with 250 μM EPA at both 3 ($p<0.05$) and 7 days ($p<0.05$). These equate to 21.9% (95%CI 9-35%) and 23.1% (95%CI 5-41%) reduction in triglycerides respectively. Incubation with 50 μM had no effect on hepatocyte triglyceride content. A linear trend between increasing EPA concentration and reduced hepatocyte triglyceride content was confirmed on post test analysis in the

standard media model for both day 3 ($p=0.005$) and day 7 ($p=0.006$). In contrast, EPA did not alter triglyceride concentrations in either oleate or LPON treated cells.

3.6 Lipid droplets number per nucleus

Next, we examined the effect of EPA on the quantity of lipid droplets per cell (quantified as ratio of lipid droplets to each nucleus) induced by the specified treatment. Cells were treated with LPON or oleate in chamber slides for 24 and 72-hours. The effect of adding EPA was assessed: here cells were treated for 72 hours. Using the Volocity 3D image analysis software, the number and characteristics of the lipid droplets captured by confocal microscopy were analyzed. There was no difference in the number of lipid droplets per cell in the varying treatment groups at either time point (Figure 6). The addition of EPA resulted in an increase in the number of lipid droplets per nucleus in the LPON group [$p<0.05$].

3.7 Surface Area to Volume Ratio and sphericity of lipid droplets

We then sought to determine the surface area to volume ratio (surface area (mm^2)/volume (mm^3)). The surface area to volume ratio of lipid droplets has been shown to influence crucial processes involved in lipid homeostasis including triglyceride hydrolysis and insulin sensitivity. Table 1 shows the effect of each treatment on the surface area to volume ratio. Oleate and LPON-treated cells had lower surface area to volume ratio when compared to the untreated cells after 24 hours suggesting that the initial response to lipid loading in these cells was predominantly volume expansion. After 72 hours, surface area to volume ratio of droplets induced by oleate increased. This is consistent with higher reduction in volume rather than surface area in these cells. Although a similar pattern was also seen with LPON, the ratio remained lower than that seen in oleate-treated cells. There was no difference in surface area to volume ratio on EPA treated cells.

The surface area to volume ratio influences the sphericity of lipid droplets. Here, we calculated the sphericity of the lipid droplets as: $p^{1/3}(6(\text{volume}))^{2/3}/\text{surface area}$. The sphericity of lipid droplets induced after a 24-hour treatment with oleate was lower than that seen in LPON-treated cells though it did not vary much from untreated (Table 1). After 72 hours, the rise in surface area to volume with oleate was paralleled by increased sphericity. Similarly, the reduction in the surface area to volume in oleate + EPA-treated cells was associated with diminished sphericity. In contrast, the impact of LPON treatment on sphericity was diametrical to its effect on surface area to volume ratio; the modest rise in surface area to volume ratio was mirrored by a reduction in sphericity. The sphericity of lipid droplets resulted from LPON treatment was lower than that seen in oleate and significantly lower than untreated cells with EPA ($p<0.0001$). Though the addition of EPA did not alter the sphericity of LPON treated cells overall.

3.8 Overall comparison of characteristics

Tables 1 and 2 are a summation of characteristics of hepatic lipid droplets developed under different conditions. Relative lipid volume per cell in oleate decreased by day 3 but increased with addition of

EPA. Within the LPON model, relative lipid volume per cell did not change with addition of EPA. This demonstrates that EPA has no significant effect within our in vitro model of steatosis with ROS (LPON).

Table 1: Relative physical characteristics of hepatic lipid droplets developed in different treatment conditions. Comparison of untreated cells to cells pre-treated with oleate or LPON or oleate + EPA, LPON + EPA on day 3. where day 1 is always set as 1 under all experimental conditions and results of day 3 and day 3 + EPA are shown respectively. Heatmap shows increase (red) or decrease (yellow) from day 1.

Treatment	Fold change of number of lipid droplets per cell			Fold change of total lipid volume per cell (μm^3)			Fold change of average volume of lipid droplet (μm^3)			Fold change of average Surface area: Volume		
	Day 1	Day3	Day3 + EPA	Day 1	Day 3	Day3 + EPA	Day 1	Day 3	Day3 + EPA	Day 1	Day 3	Day3 + EPA
Untreated	1	0.6	0.7	1	1	0.6	1	1.5	0.8	1	0.9	1
Oleate	1	1.2	1.4	1	0.6	0.9	1	0.4	0.6	1	1.3	1.1
LPON	1	1.4	1.9	1	1.3	1.8	1	0.9	0.9	1	1.1	1.1

Table 2: Averaged physical characteristics of hepatic lipid droplets developed in different treatment conditions. Results derived through Excel VBA. Number of droplets, total lipid volume and average volume of lipid droplet quantify the development of lipid droplets from day 1 to day 3 in size and frequency of occurrence. Surface area to volume ratio is used as a measure of lipid droplet shape and as an indicator for esterification.

Treatment	Average number of lipid droplets per cell		Average total lipid volume per cell (μm^3)		Average volume of lipid droplet (μm^3)		Average Surface area: Volume	
	Day 1	Day3	Day 1	Day 3	Day 1	Day 3	Day 1	Day 3
Untreated	11	7	301	291	30	44	1.9	1.6
Oleate	5	7	300	176	64	27	1.6	2.1
LPON	9	13	347	455	38	35	1.7	1.8
Untreated + EPA	-	7	-	194	-	24	-	1.9
Oleate + EPA	-	7	-	281	-	38	-	1.9
LPON + EPA	-	18	-	641	-	34	-	1.9

4. Discussion

Effects of EPA on ketone bodies, glucose and liver function

Our first experiments were designed to establish the optimal concentration of eicosapentaenoic acid (EPA) for use in this model. Two markers were evaluated: LDH leakage was used to assess toxicity and membrane leakage leading to cell death, and glucose production to reflect efficacy. The results showed

that EPA was effective at altering endogenous glucose production in the LPON model but that large, i.e. 500 μ M, doses of EPA resulted in a significant increase in cell death and/or membrane leakage shown by high levels of LDH (Figure 2). With these results 50 μ M and 250 μ M doses were chosen as they represented a balance of desirable and undesirable effects as well as allowing a dose-response relationship to be demonstrated.

Increase in albumin synthesis could be seen in control model supporting the suggestion that EPA has beneficial effects on the cell beyond a sole reduction of intracellular triglyceride content. There was a reduction of albumin in the oleate model compared to control, but albumin production remains higher than media only control and LPON control suggesting that overall albumin is higher in oleate loaded cells than control or LPON under all conditions. (Figure 2. B Meme control 40 μ g/mL, oleate control ~ 63 μ g/mL and LPON control 30 μ g/mL) No change in albumin synthesis was observed in the LPON + EPA model. Results showed AST was not significantly altered under any condition, although a trend of reduced AST level with increasing EPA concentration in the standard model missed statistical significance by 0.01 ($p=0.06$).

Ketone bodies are a byproduct of the breakdown of fatty acids. They have been shown to lower ROS [20], stimulate insulin release and cause lipid peroxidation which may play a role in vascular disease in diabetes [21]. However, high levels of ketone bodies can lead to life threatening ketoacidosis. As such ketone bodies were measured in our model to determine if fat loading increased production of ketones. Within controls ketone bodies were measured at 3.8 μ mol.hr MEME only control, 6 μ mol.hr oleate and 21 μ mol.hr LPON (Figure 3). This shows the LPON model to have a significantly higher level of ketone bodies at baseline than oleate or control. Addition of EPA did not change levels of ketone bodies in the standard model and oleate but significantly reduced ketones in LPON model in a dose dependent manner (Figure 1). This alongside the reduction of glucose, also in a dose dependent manner, suggest EPA may have a qualitative benefit beyond mere reduction of triglycerides (Figure 1) We have previously shown that by adding lactate, pyruvate and insulin to C3A cells that you get a dose dependent drop of insulin (Anns thesis). This is likely due to the increase of lactate and pyruvate fueling the TCA cycle and diverting pyruvate towards the gluconeogenic pathway. The presence of oxidative stress in this model augments this theory as mitochondrial dysfunction in the presence of oxidative stress can accelerate acetyl-co-A production. (Anns thesis) While more investigation is needed into the mechanisms behind this decrease of glucose, other studies have proven the effects of EPA on insulin resistance and glycemic control in *in vivo* models. [22, 23, 24]

It was considered whether the lack of triglyceride reduction in the LPON group could be a result of the increased reactive oxygen species seen in this model. Cells in the control and oleate groups were therefore co-incubated with a non-lethal dose of a reactive oxygen species donor (tBOOH). EPA continued to have an effect in cells in the standard media containing ROS donor, but there was still no significant change in the oleate group when incubated with EPA and tBOOH. Co-incubation with a reactive oxygen species (ROS) donor did not mitigate the triglyceride lowering effect seen in untreated hepatocytes when incubated with EPA. The beneficial effect on the control in terms of triglyceride reduction with ROS

suggests that the ineffectiveness of EPA in the LPON model to lower triglyceride content is not purely as a result of the increased ROS in this model. Since ROS does not affect the concentration of triglycerides in fat loaded models, the reduction seen in control must be through a different mechanism.

Lipid droplet characteristics

Characterisation of lipid droplet formation, stability and breakdown is important in understanding how fragmentation of droplets contribute to free fatty acids and ROS released within the cell. Previous work highlighted regulation of proteins involved in lipid droplet stability within the LPON and oleate models [25]. Notably, we show an upregulation of perilipin-2 (PLIN2), a gene related to stability of lipid droplets, is upregulated in our LPON model. PLIN2 is known to be involved reduction of lipolysis and decrease triglyceride turnover, which we see in Figure 5. PLIN2 is also involved in the regulation of lipid droplet stability [25]. This suggests a protective mechanism whereby stability of lipid droplet is prioritized to prevent the release of FFA and ROS which cause cytotoxicity [25]. This led us to the current study which highlights two important points.

Firstly, LPON induced steatosis, as previously shown, is associated with oxidative stress which appears to have an effect on the characteristics of lipid droplets [15]. (Figure 8) This is evidenced by the differences in lipid droplet accumulation, surface area and volume. In the oleate model there was immediate lipid volume expansion while the number of lipid droplets remained the same. By day 3 lipid volume decreased with a slight increase in the number of lipid droplets. In contrast, addition of EPA to oleate treated cells resulted in a rise of total lipid volume. This suggests that addition of EPA increases lipid accumulation to a level higher than control (Table 1). This suggests that cells pre-treated with oleate have an initial increase in fat which is metabolized by day 3 resulting in lower total volume, though addition of EPA increases total lipid volume to a level higher than control. While in contrast, LPON treatment was associated with a steady rise in number of lipid droplets and lipid accumulation but had no change in volume at day 3, which was unaffected by EPA treatment. We postulate the difference in triglyceride acquisition between LPON and oleate may be responsible for the above. Octanoate (the chosen FFA in LPON) cannot be directly esterified thus the triglyceride accumulation is largely mediated by increased de novo lipogenesis. This may explain the time dependent increase in volume and quantity of lipid droplets with LPON.

As fatty acids can become incorporated in the cell and lipid droplet membrane, another potential mechanism to explain our results is that the different fatty acids may have resulted in different phospholipids and membrane proteins. Diminished total and lipid droplet volume with oleate after 72 hours may have been attributable to enhanced PAT protein expression (an essential surfactant positioned on the surface of lipids) which promotes the packaging of lipids in smaller units thus increasing the surface area to volume ratio [26]. Such an increase in the surface area to volume ratio allows access to lipases for triglyceride hydrolysis and influences insulin sensitivity. Conversely, current hypothesis stipulates that a reduction in the surface area to volume ratio can lead to an incomplete triglyceride hydrolysis leading to DAG (diacylglycerol) formation [27]. DAG has been shown to impair insulin

signaling via the PKC isoform ϵ which phosphorylates the insulin receptor effecting all downstream effects of insulin signaling, upregulation of de novo lipogenic genes and glycogen synthesis [27,28,29]. Oleate induced cellular steatosis has been shown previously to have preserved insulin sensitivity whereas LPON (with a higher surface area to volume ratio than oleate) was associated with increased gluconeogenesis [15, 30].

The second observation is that the effects of EPA on lipid droplets differ between oleate and LPON. In untreated cells, EPA significantly reduced triglyceride concentration and the volume of lipid droplets resulting in a rise in the surface area to volume ratio. This finding of reduced hepatocyte triglyceride with EPA is consistent with several previous studies [31,32,33]. However, similar effects have not been observed in either oleate or LPON groups. Contrary to the findings in the untreated cells, EPA increased the volume of lipid droplets hence reducing the surface area to volume ratio in LPON and oleate treated cells. How might these results be accounted for? It is likely that the differences lie in the mechanisms of EPA in modulating fatty acid metabolism. EPA is thought to promote free fatty acid oxidation by activating the PPAR- α oxidative pathways [11,34,35]. FFA oxidation results in the formation of acetyl coA, which can either enter the TCA cycle, or in the presence of substrate excess, can be swiftly diverted to non-oxidative pathways including de novo lipogenesis. Therefore, it is possible that in the presence of oleate and LPON, formation of acetyl-coA was accelerated from enhanced fatty acid oxidation with EPA and resulted in de novo lipogenesis. Furthermore, decreased surface area to volume ratio of lipid droplets in these cells can hinder lipases from hydrolyzing the stored triglyceride. It is also plausible that the impact of EPA in LPON and oleate treated cells may have been mediated by the modulation of the lipid membrane composition by EPA. Addition of EPA in the context of lipid saturated cellular models may further enhance dysregulation of lipid metabolism rather than improve it.

In our previous study, we examined the effects of oleate and LPON on Cell death-inducing DFF45-like effector B (CIDEB), a member of CIDE cell death-inducing proteins, to determine if there was an association between CIDEB expression and intracellular lipid accumulation, lipid droplet size, mitochondrial β -oxidation or additional reactive oxygen species (ROS) formation [36]. CIDEB is highly expressed in the liver and modulates very low density lipoprotein (VLDL) and cholesterol homeostasis and also mediates lipid density fusion and packaging of lipids [36,37,38]

The beneficial effect of EPA in adult NAFLD remains unproven. The effect of EPA on PPAR oxidative pathways encompasses the activation of both ω -oxidation and peroxisomal β -oxidation. Unlike mitochondrial β -oxidation, these pathways can generate a significant amount of ROS [39,40]. In support of this, a metabolomic analysis has shown that an omega-3 fatty acid, DHA, increases lipid peroxidation resulting in increased isoprostanes formation mirrored by a decline in hepatic α -tocopherol and ascorbate[41]. Nevertheless, the by-product of n-3 lipid peroxidation such as 4-hydroxyhexenal from DHA but not EPA is thought to confer cardioprotective effect by enhancing antioxidative pathways mediated by NRF-2 [42]. Furthermore, DHA has been shown to suppress hepatic markers of inflammation without a reduction in hepatic steatosis[43]. It is therefore possible that any beneficial effects of omega-3 fatty acids are independent of their abilities to reduce hepatic steatosis.

The question remains whether EPA would confer benefit in vivo in the presence of mitochondrial dysfunction with high ROS burden. Mitochondrial beta-oxidation requires intact mitochondrial respiration. As such, the FFA oxidative effect of EPA may not be achieved in the presence of mitochondrial dysfunction. Our data is in agreement with the study of Du et al., where dietary supplementation with EPA in carnitine-deficient mice with impaired mitochondrial b-oxidation only served to exacerbate triglyceride accumulation [44]. Moreover, the rise in ROS formation from the overwhelming FFA oxidation with EPA can potentially exacerbate mitochondrial dysfunction further. This would limit the utility of EPA as a therapy for NAFLD.

Conclusions

NAFLD is characterized by hepatic steatosis and oxidative stress. We have shown that within our models EPA prophylactically lowers triglycerides, with and without a ROS donor and increases albumin production in control. However, there is no reduction of triglycerides in either fat loaded models with the addition of EPA though it may improve insulin sensitivity and lower glucose levels and ketone bodies associated with type 2 diabetes in patients with NAFLD.

Abbreviations

LPON – lactate, pyruvate, octanoate, ammonia

NAFLD – non alcoholic fatty liver disease

EPA – eicosapentaenoic acid

DHA – Docosahexaenoic acid

ROS – reactive oxygen species

VLDL – very low density lipoprotein

CIDEB - Cell death-inducing DFF45-like effector B

TCA cycle – tricarboxylic acid cycle

FFA – free fatty acid

LDH – lactate dehydrogenase

Declarations

Ethics – Not applicable

Consent for publication – All authors have consented for publication

Availability of data and materials – Data and materials available by request

Author Contributions:

Katie Morgan: writing the paper, analysis of data

Samantha Suppiah: experiments / experimental design / analysis of data results

Gail Masterton: experiments/ experimental design, analysis of results

VasileiosKoutsos: methodology experimental design, analysis of data, results

Khalida A. Lockman: analysis of data

Shona Johnston: microscopy, supervision, experimental design

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Figures

Figure 1

Dose dependent effect of EPA on endogenous glucose and ketone body production in the LPON model: A) Endogenous glucose production in LPON model. Statistical significance($p<0.0001$). B) Concentration dependent decrease of ketone bodies in LPON model: ketone body production in LPON model. Statistical significance ($p<0.0001$). Results expressed as mean and standard error of mean.

A

B

C

Figure 2

Liver function tests: A. Effect of EPA on AST levels: Meme control, oleate treated cells and LPON treated cells. AST measured in international unit of measure per litre. B. The effect of EPA on albumin levels: A) There was no difference between control and control + incubation with 50µm EPA, but a statistically significant increase of albumin was seen between control and incubation 250µm EPA. C. The effect of

increasing concentrations of EPA on LDH leakage in the LPON model. Results presented as % of LDH supernatant/ total LDH (i.e. LDH leakage) and expressed as mean and standard error of the mean (SEM).

Figure 3

The effect of EPA on ketone body (acetoacetate + betahydroxybutyrate) production: A) MEME control B) Oleate C) LPON Showing mean with standard error of mean. Significance LPON control vs LPON + EPA 50μM (p<0.05), LPON control vs LPON + EPA 250 μM (p<0.01)

Figure 4

Addition of ROS donor to control and oleate groups: A) Total triglyceride concentration in MEME control with and without ROS donor. B) Total triglyceride concentration in oleate model with and without ROS donor ($p<0.0001$).

Figure 5

Intra-hepatocyte triglyceride concentration at 3 and 7 days. **A)** untreated cells (MEME media only) with 50 μ M and 250 μ M. $p<0.05$ **B)** Oleate treated cells with 50 μ M and 250 μ M EPA. **C)** LPON treated cells with 50 μ M and 250 μ M EPA. are expressed as mean and standard error of the mean (SEM). Significance $p<0.05$

Figure 6

The number of lipid droplets per nucleus in different treatment groups

72 hours there is a significant rise in lipid droplet accumulation in LPON+EPA ($p<0.05$) compared to control (untreated - meme media only) and oleate. Results are expressed as mean and standard error of the mean (SEM).

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Figure 7

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Effects of eicosapentaenoic acid (EPA) on simple steatosis and NAFLD

Figure 8

Graphical representation of changes in EPA model Media only control shows lower triglycerides with no change to AST or ketone body production and an increase of albumin in the presence of EPA. The oleate model, representing simple steatosis, shows an increase in size of lipid droplets which decrease over time leading to higher lipid volume, but no increase in ROS. This model shows no change in the level of triglycerides even with an addition of a ROS donor. There was also no change to AST, albumin or levels of

ketone bodies. The LPON, NAFLD, model shows a higher number of lipid droplets with increased sphericity and area compared to control. There was no change to LDH, AST or albumin, though a decrease in glucose and ketone bodies was seen in the presence of EPA.