Neuroprotective effects of polyunsaturated fatty acids in an *in vitro* hypothalamic model

TA BUTTERICK-PETERSON[2], M LITTLE [2], JP NIXON [1,2], CJ BILLINGTON [1,2,3], CM KOTZ [1,2,3], CF WANG[1,2,3]

[1]VA Health Care System, Minneapolis, MN, USA, [2]University of Minnesota, Twin Cities, MN, USA, [3]Minnesota Obesity Center, St. Paul, MN, USA

Introduction

Obesity and its comorbidities are thought to be due in part to high fat diets (HFD), which are rich in saturated fatty acids (SFAs) such as palmitic acid (PA)[1-4]. In rodent models increased PA can induce hypothalamic insulin resistance, inflammation and neurodegeneration (NDG) [2, 5, 6]. Ceramides, metabolites of PA, are thought to elicit the onset of apoptotic NDG, yet these events are not fully understood in the development of obesity. Additionally, ceramides adversely alter metabolism, increase inflammation that contribute to NDG-associated disorders [7-10].

Conversely, diets high in polyunsaturated fatty acids PUFAs improve insulin sensitivity and lipid metabolism in several tissues [11]. Diets rich in ω-3 and ω-6 PUFAs such as alpha-linolenic (ALA), stearidonic (SDA), eicosapentaenoic (EPA), docosahexaenoic (DHA), and lenoleic acids (LA) have several health benefits such as cardiovascular improvement and potentially the reduction of NDG related to obesity [8, 9, 11, 12].

The specific role of dietary SFAs-induced pathological ceramide synthesis has not been fully evaluated in the brain, but increased dietary PUFAs have been linked to the prevention of NDG disorders such as Alzheimer's disease [13]. In rodent NDG models, ω-3 and ω-6 PUFAs can decrease oxidative damage (such as lipid peroxidation), but their contribution to hypothalamic integrity, an important site for regulating energy balance, has yet to be defined [4, 14, 15].

Previously, we demonstrated that HFD up to 11-weeks in multiple SD rat models can either increase 1) hypothalamic apoptotic markers of NDG in PVN neurons and ARC-POMC neurons as measured by IHC caspase-3 and cell death [16] or 2) increase both total and long chain (C18 and C20) ceramides [17]. These data agree with literature showing that HFD increases ceramides in several tissues, including hypothalamus [10, 18, 19].

Further studies are critical for determining the potential neuroprotective effect of PUFAs and their underlying mechanisms. We have begun to profile the effect of SFAs and PUFAs on hypothalamic integrity and NDG using an *in vitro* model. These studies are ongoing and we hypothesize that PUFAs protect against PA-induced NDG.

Methods

Cell line and Assays: Differentiated non-tumor derived, immortalized adult mouse hypothalamic (A12) cells (CELLutions-Cedarlane, North Carolina) were maintained in DMEM medium supplemented with 10% FBS at 37°C 5% CO_2[20]. Cells were plated overnight in a 96 well plate (3,500/well), pretreated with either: soy oil (SO), DHA, EPA, LA and then challenged with PA (Sigma, St. Louis, MO), Fig 1. Cell viability (PrestoBlue, Invitrogen) and caspase-3 (Caspase-Glo 3/7, Promega) were determined by either changes in relative fluorescent (RFU) or luminance units (RLU), which were analyzed using a microplate spectrometer reader (Spectomax-M5, Molecular Devices).

Statistical Methods: Significant differences were determined by unpaired, two-tailed test using GraphPad Prism 5 (GraphPad, San Diego, CA) for either raw RFU of RLU values. Data represented was normalized against control values and graphed as percent change relative to control.

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Results

Experimental outline

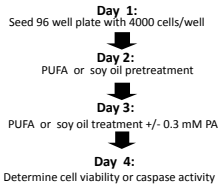

Figure 1. Schematic representation of experimental outline for determining cell viability or caspase-3/7 activity.

Standard soy oil is a complex mixture that contains both PUFAs and SFAs

Saturated Fatty Acid	Lauric	Myristic	Palmitic	Stearic	Arachidic
% Soy Oil	0.2	0.1	9.8	2.4	0.9
mM	0.09	0.04	3.50	0.77	0.26
Unsaturated Fatty Acid	**Palmitoleic**	**Oleic**	**Linoleic**	**Linolenic Acid**	
% Soy Oil	0.4	28.9	50.7	6.5	
mM	0.14	9.38	16.58	2.14	

Table 1: Composition and molar concentrations per 100 g standard soy oil (Sigma, St Louis MO).

Pretreatment with standard soy oil is neuroprotective

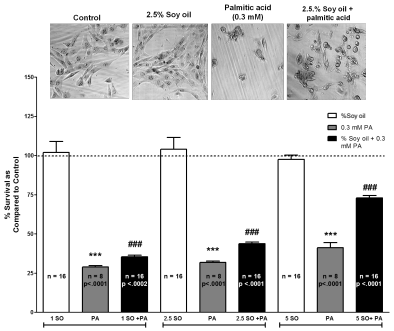

Figure 2. Standard soy oil is neuroprotective. Cells were pretreated (24 h) with increasing concentrations of SO and then cells were re-dosed and challenged with PA for 24h. Brackets on the bottom of the graph represent each treatment group. Insert is a light phase microphotograph of representative cells.

PUFAs are neuroprotective

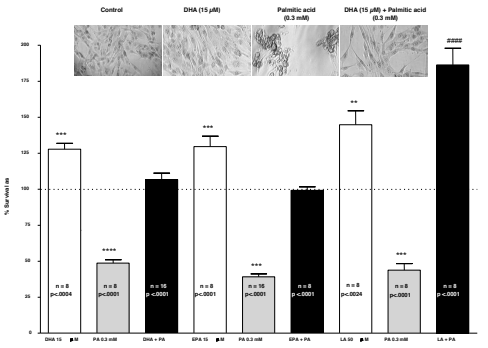

Figure 3. PUFAs are neuroprotective. Cells were pretreated (24 h) with either ω3 PUFAs (DHA or EPA) or ω6 (LA), cells were then re-dosed and then challenged with PA for 24h. Inset is a light phase microphotograph of representative cells.

PUFAs decrease caspase 3/7 induced apoptosis

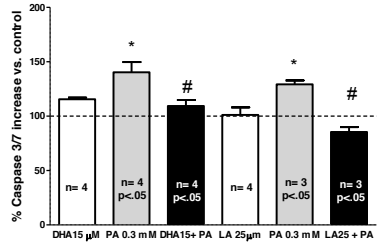

Figure 4 . PUFAs decrease caspase 3/7 induced apoptosis. Cells were pretreated (24 h) with either ω3 PUFAs (DHA) or ω6 (LA), cells were then re-dosed and then challenged with PA for 24h. Significant differences were determined, * P < .05 as compared to control and # P < .05 as compared to PA-only treated group.

Conclusions

1. Standard soy oil, a complex mixture of both SFA and PUFAs is neuroprotective in a dose dependent manner.

2. Both ω3 (DHA or EPA) and ω6 (LA) PUFAs were neuroprotective. Surprisingly, LA had the largest effect on cell proliferation and neuroprotection.

3. Both ω3 (DHA) and ω6 (LA) PUFAs decrease caspase 3 (a marker of apoptosis) activity following PA challenges.

Discussion

It is well known that HFDs can promote obesity through purely energetic effects. New evidence shows that HFDs are associated with hypothalamic inflammation and NDG, which implies effects of HFD on brain function. Ceramides, a class of lipid molecules containing sphingoid chains generated from PA provide a potential mechanistic link between HFD consumption and outcomes such as insulin resistance, diabetes mellitus, and NDG. The development of normal brain structure and function is critical for the central regulation of energy metabolism, and thus hypothalamic NDG may result in disordered energy metabolism, including obesity (Fig 5).

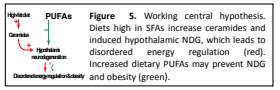

Figure 5. Working central hypothesis. Diets high in SFAs increase ceramides and induced hypothalamic NDG, which leads to disordered energy regulation (red). Increased dietary PUFAs may prevent NDG and obesity (green).

Our preliminary data and ongoing studies are supported by a recent publication demonstrating that PUFA's can directly alter hypothalamic function [12]. There have been many health benefits attributed to PUFAs, yet Americans do not consume the amounts of 0.05-1.0g per day recommended by both the American Heart Association (AHA) and the American Dietetic Association (ADA) [21].

There is virtually little data regarding the benefit of PUFAs to hypothalamic health and hypothalamic integrity [12, 21]. We are in the process of evaluating the mediators of PUFA induced neuroprotection in both *in vitro* models and *in vivo* (rodent) feeding studies. The contributions of Bcl-2 (an anti-apoptotic protein), mitochondrial integrity and changes in ceramide synthesis are ongoing. It is clear that in addition to pharmacological and physical activity, use of PUFAs should be explored as a potential strategy for mitigating obesity.

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