Investigations on the Effects of Palmitate on Neuronal Cells

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Investigations on the Effects of Palmitate on Neuronal Cells
By Ryan Gelsinger

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Abstract

Many studies have demonstrated that increased levels of free fatty acids (FFAs) are associated with increased apoptosis in hepatocytes, podocytes, myocytes, and pancreatic islet cells. However, the effects of FAAs on neuronal cell function are not well characterized. In this study, mouse neuroblastoma cells from the Neuro-2a (N2a) cell line were treated with either bovine serum albumin (BSA) or different concentrations of BSA-conjugated palmitate (PA) and examined cell viability using MTT assay. Concentrations of PA at or above 200 μM in the growth media were associated with a decrease in cell viability. In order to examine whether N-acetylcysteine (NAC) or sodium phenylbutyrate (PB) affected the PA-induced decrease in cell viability, N2a cells were treated with PA in combination with either NAC or PB and cell viability was measured using MTT assay. Neither NAC in concentrations of up to 200 μM nor PB in concentrations of up to 1 mM was able to attenuate the PA-induced decrease in N2a viability. When viewed under a microscope PA treated cells exhibited increased irregularity in size and shape as compared to cells treated with BSA. In order to examine whether PA treatment affected insulin signaling in this cell line, N2a cells were treated with 200 μM PA for 24 hours followed by stimulation with 25 ng/mL insulin for 0, 5, or 10 minutes. Activation of AKT1 and GSK-3β was then analyzed by Western blot assay. Preliminary results suggested that while insulin-induced GSK-3β activation may not be affected by PA treatment, insulin-induced AKT1 activation appeared to be attenuated by PA treatment as compared to BSA-treated controls. These results remain to be confirmed and the mechanism of the PA-induced changes in viability and insulin signaling remain to be defined.
Introduction

Obesity, defined as a body mass index (BMI) of 30 or greater, is a growing public health concern in the United States. According to the 2010 data from the CDC approximately 36% of adults and 17% of children in the U.S. were categorized as obese.\textsuperscript{1} Obesity is associated with a number of comorbid medical conditions including hypertension, coronary artery disease, stroke, sleep apnea, non-alcoholic fatty liver disease, the development of several types of cancer, and type 2 diabetes.\textsuperscript{2-5}

Individuals with type 2 diabetes experience a systemic insensitivity to the effects of insulin, a decreased compensatory insulin secretion response, and a consequent increase in blood glucose levels.\textsuperscript{6} Insulin is a peptide hormone that is central to proper glucose metabolism. When blood glucose levels become elevated insulin is released from the β islet cells of the pancreas. On binding to its receptor, this hormone initiates a complex intracellular signal transduction cascade.\textsuperscript{7} One of the most important metabolic effects of this cascade is the translocation of the Glut4 transporter from intracellular stores to the plasma membrane in adipocytes and myocytes, which promotes glucose uptake by these cells. Insulin also promotes glycogen synthesis and inhibits lipolysis, both of which are important for maintaining the proper energy balance in an organism.\textsuperscript{8} In addition to its metabolic effects, this hormone also promotes protein synthesis as well as cell survival, differentiation, and proliferation.\textsuperscript{9-10}

While insulin may alter the subcellular localization of neuronal glucose transporters, unlike some other tissues neurons do not appear to experience increased glucose uptake solely in response to insulin exposure.\textsuperscript{11} Despite this fact, neurons express the insulin cell-surface receptor and studies suggest that insulin signaling plays an important role in proper neuronal function.\textsuperscript{12} Insulin signaling is impaired in the neurons of several mouse strains that are used as
models for type 2 diabetes and are prone to developing diabetic neuropathy, a condition characterized by peripheral nerve dysfunction.\textsuperscript{13-14} Attenuated insulin signaling has also been associated with decreased cognitive performance and the formation of \( \beta \)-amyloid plaques commonly found in Alzheimer’s disease.\textsuperscript{15-16}

Protein kinase B, also known as AKT, serves a crucial role in mediating many of the effects of the insulin signaling pathway. This serine/threonine kinase is activated by phosphorylation as part of the cascade which, in turn, phosphorylates a number of downstream effector molecules. Among these targets are glycogen synthase kinase 3\( \beta \) (GSK-3\( \beta \)), which is involved in regulating glycogen synthesis, and Bcl-2-associated death protein, which is involved in initiating apoptosis.\textsuperscript{17}

One of the most common clinical findings in obese individuals is an elevated level of free fatty acids (FFAs) in the blood.\textsuperscript{18} In vitro studies have been performed in a number of different tissues, including hepatocytes, podocytes, myocytes, and pancreatic islet cells to examine the effects of exposure to these elevated FFA levels. One of the common cellular effects of FFA exposure for periods greater than 12 hours is the induction of apoptosis in these cell types.\textsuperscript{19-22} Prolonged treatment with elevated FFA levels has also been associated in vitro with impaired AKT1 activation and a consequent decrease in insulin signaling in hepatocytes and adipocytes.\textsuperscript{23-24}

While the exact mechanism underlying these changes remains unclear, endoplasmic reticulum (ER) and oxidative stress caused by FFA exposure have both been proposed as causative factors in impaired insulin signaling. According to the ER stress theory prolonged exposure to FFAs causes unfolded proteins to accumulate in the ER. This buildup triggers the initiation of the cellular unfolded protein response. As a part of this response c-Jun amino-
terminal kinase (JNK) is activated and subsequently adds inhibitory serine phosphorylations to components of the insulin signaling cascade. The oxidative stress theory states that the increased intracellular FFA load leads to an increase in β-oxidation and a consequent rise in reactive oxygen species (ROS) production. These ROS, in turn, activate kinases such as JNK and p38 which phosphorylate and attenuate the action of components of the insulin signaling pathway. Both ER stress and ROS generation have also been implicated in the induction of apoptosis.

Though there have been significant studies to examine the effects of FFA exposure on cell survival and insulin signaling in liver, muscle, pancreatic islet cells, and other peripheral tissues, the impact of such treatments on neuronal tissue has not been well characterized. We first examined the effects of PA exposure on the viability of the N2a cells by MTT assay. We then treated cells with PA in combination with either NAC, an ROS scavenger, or PB, a chemical that has been shown to reduce ER stress, in an effort to determine whether oxidative or ER stresses contribute to PA-induced changes in N2a viability. We also used a microscope to visualize cells after PA treatment to study any morphological changes. Finally, we sought to examine the effects of PA treatment on insulin signaling in N2a cells through Western blotting.
Materials and Methods

Cell Culture

Neuro-2a (N2a), a murine neuroblastoma cell line, was purchased from the American Type Culture Collection (ATCC, Manassas, VA). These cells were maintained in Eagle’s Modified Essential Medium (EMEM) containing 10% fetal bovine serum (FBS) and 1% pen/strep antibiotic. Cells were cultured at 37°C in a humidified incubator with 5% CO₂.

MTT Assay

N2a cells were seeded into a 96-well cell culture plate at 2.5x10³ cells per well in EMEM containing FBS and pen/strep as described above. One day after seeding, cells were treated as follows. For the PA dose response experiment, cells were treated with 0, 25, 50, 100, 200, 400, or 600 μM PA for 24 hours. For the PA/NAC cotreatment experiment, cells were treated with either 2.4 mg/mL BSA or 200 μM PA in combination with 0, 25, 50, 100, or 200 μM NAC for 24 hours. For the PA/PB cotreatment experiment, cells were treated with either 2.4 mg/mL BSA or 200 μM PA in combination with 0, 25, 50, 100, 200, 500, or 1000 μM PB for 24 hours. Each well then received 10 μL of 5 mg/mL MTT and incubated for 4 hours. At the end of this incubation, 100 μL of solubilization reagent was added to each well of the cell culture plate. The plate was rested at room temperature for 10 minutes to allow complete dissolution of all formazan crystals and read at 560 nm using a Spectramax M5 plate reader (Molecular Dynamics, Sunnyvale, CA). Absorbance values for each treatment group were normalized to the control group to determine relative cell viability.
**Microscopy**

N2a cells were seeded into 6-well culture plate at $2.0 \times 10^5$, incubated overnight, and rinsed with PBS. The media was then replaced with serum-free EMEM and treated with 2.4 mg/mL BSA or PA at 100, 200, or 400 μM and incubated for 24 hours. At the end of the treatment period, the cells were washed with PBS to remove non-adherent cells before being imaged at 400X magnification using an Amscope MU1000 camera and the ToupView software (ToupTek Photonics).

**Insulin Time Course**

N2a cells were seeded into 6-well culture plates at $1.8 \times 10^5$ cells per well. One day after seeding the cells were rinsed with PBS. The media was then replaced with serum-free EMEM supplemented with 2.4 mg/mL BSA and incubated for 24 hours before being treated with 25 ng/mL insulin for 0, 5, or 10 minutes.

**Palmitate Treatment**

N2a cells were seeded into 6-well culture plates at $1.8 \times 10^5$ cells per well for control wells and $3.0 \times 10^5$ cells per well for PA treatment wells. One day after seeding, the cells were rinsed with PBS. The media was then replaced with serum-free EMEM and treated with 2.4 mg/mL BSA for control wells and 200 μM PA for PA treatment wells. Cells were incubated for 24 hours before being treated with 25 ng/mL insulin for 0, 5, or 10 minutes.
**Protein Extraction**

At the end of the treatment period, media was aspirated from the wells of 6-well cell culture plates. The cells were washed with Hank's balanced salt solution, then 100 μL of 1X cell lysis buffer (0.0625M Tris, 0.05 M dithiothreitol, 10% glycerol, 2% sodium dodecyl sulfate, 0.01% bromophenol blue) was added to each well. Samples were then collected and sonicated using a 550 Sonic Dismembrator (Fischer Scientific, Pittsburgh, PA) for 15 pulses while on ice.

**Western Blot Analysis**

Aliquots of lysed protein samples were taken from the protein extraction samples, heated to 95°C for 5 minutes, and then centrifuged at 10000 rpm for 5 minutes. Aliquots were then loaded into 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and resolved by electrophoresis performed at 100V. Protein was then transferred from the gels to nitrocellulose membranes electrophoretically. The membranes were blocked with Superblock (ThermoScientific, Logan, UT) for 1 hour at room temperature, then incubated with primary antibodies immunoreactive against phospho-AKT1 (Epitomics, Burlingame, CA) or phospho-GSK-3β (Santa Cruz Biotechnology, Dallas, TX) at appropriate dilutions overnight at 4°C. Membranes were washed with tris-buffered saline with 0.1% Tween-20 (TBST), then incubated in horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour at room temperature. Membranes were washed again in TBST and developed using the ECL Plus detection kit (ThermoScientific, Logan, UT). Images of the membranes were generated using a Storm 860 Molecular Imager (Molecular Dynamics) and quantified using the Image J program from the National Institutes of Health. Following this, the membranes were stripped and reprobed with primary antibodies immunoreactive against total AKT1 or total GSK-3β. Band
intensities for p-AKT1 and p-GSK-3β were then normalized to total AKT-1 or GSK-3β respectively and plotted.
Results

Effects of PA Treatment on N2a Cell Viability

To determine the effects of PA exposure on neuronal cell viability, cells were seeded into a 96-well culture plate and allowed to rest overnight. They were then treated with PA at 0, 25, 50, 100, 200, 400 or 600 μM for 24 hours before cell viability was analyzed by MTT assay.

Analysis of the data revealed an 8% increase in N2a cell viability following treatment with 25 μM PA, while the 50 μM and 100 μM treatment groups showed no significant difference in cell viability when compared to control. As the concentration of PA treatment continued to increase, cell viability decreased in a dose-dependent manner. The 200 μM, 400 μM, and 600 μM treatment groups experienced a 20%, 28%, and 34% reduction in cell viability respectively (Fig. 1).

Effects of PA/NAC Cotreatment on N2a Cell Viability

To study the effects of NAC on PA-induced changes in cell viability we seeded N2a cells into a 96-well culture plate and, following an overnight rest, treated them with either 2.4 mg/mL BSA or 200 μM PA for 24 hours. Cells in each group were cotreated with 0, 25, 50, 100, or 200 μM PA and 25 or 50 μM NAC.
μM NAC for the same duration. At the end of this incubation period an MTT assay was used to determine cell viability in each treatment group. Data were normalized to the untreated control group.

None of the concentrations of NAC used for treatment induced a significant change in cell viability in the BSA treatment groups when compared to the untreated control. Cells treated with PA and 0 μM NAC exhibited a 22% decrease in cell viability compared to BSA treated cells. Increased concentrations of NAC up to 200 μM did not have any significant effects on the reduction in cell viability induced by PA (Fig. 2).

![Fig 2 Effects of PA/NAC Cotreatment on N2a Cell Viability as Measured by MTT Assay](image)

Effects of PA/PB Cotreatment on N2a Cell Viability

To investigate the effects of PB on PA-induced changes in cell viability N2a cells were seeded into a 96-well culture plate and allowed them to rest overnight. We then treated them with either 2.4 mg/mL BSA or 200 μM PA and cotreated with 0, 25, 50, 100, 200, 500 μM, or 1
mM of PB for 24 hours. Following this incubation an MTT assay was performed to determine cell viability in each treatment group. Data were normalized to the control group.

None of the concentrations of PB used for treatment induced a significant change in cell viability in the BSA treatment groups when compared to the untreated control. Cells treated with PA and 0 μM PB displayed a 21% reduction in cell viability when compared to cells treated with BSA. Cotreatment with concentrations of PB from 25μM to 1 mM did not result in a statistically significant reversal of this decrease in cell viability (Fig. 3).

![Fig 3 Effects of PA/PB Cotreatment on N2a Cell Viability as Measured by MTT Assay](image)

*Fig 3 Effects of PA/PB Cotreatment on N2a Cell Viability as Measured by MTT Assay* N2a cells were seeded and treated with 2.4 mg/mL or 200 μM PA and cotreated with PB at the indicated concentrations for 24 hours. Untreated cells were used as control group. Cell viability was then measured via MTT assay. Data are presented as mean ± SD; n=6

**Cell Imaging**

To directly visualize the effects of PA treatment on N2a cell viability and morphology cells were plated and allowed to rest overnight. Cells were then washed with PBS before serum-free EMEM supplemented with 2.4 mg/mL BSA or 100, 200, or 400 μM PA was added. After a
24 hour incubation period the culture wells were washed to remove non-adherent cells before being imaged at 400X magnification.

Imaging of the BSA treatment group showed a fairly consistent size and morphology among all cells in the field of view. They were generally round and each extended several projections from the cell body into surrounding environment (Fig. 4A). PA treatment was associated with a dose-dependent decrease in the number of adherent cells, as well as the number of projections extending from each cell. PA treatment also appeared to induce an increased variability in cell size when compared to the BSA treatment group (Fig 4B-D).

![Fig 4 Effects of PA Treatment on Cell Confluence and Morphology](image)

**Insulin-Induced AKT1 Activation**

To determine whether insulin exposure induced AKT1 phosphorylation in our cell line, N2a cells were plated and allowed to rest overnight. Cells were then washed and incubated in serum-free media containing 2.4 mg/mL BSA for 24 hours. Cells were subsequently challenged
with 25 ng/mL insulin for 0, 5, or 10 minutes. Protein was isolated from these cells and analyzed by Western blotting to determine the levels of the phosphorylated and total forms of AKT1. The intensity of each protein band was then quantified and plotted as the relative level of phospho- to total AKT1.

Analysis of the Western blotting quantification data showed an increase in AKT1 activation in both the 5 and 10 minute treatment groups (Fig. 5).

**Insulin-Induced GSK-3β Activation**

To determine whether insulin exposure induced GSK-3β phosphorylation in the N2a cell line, aliquots were taken from the protein samples collected in the previous insulin time course experiment and analyzed by Western blotting to determine the levels of the phosphorylated and total forms of GSK-3β. The intensity of each protein band was then quantified and plotted as the relative level of phospho- to total GSK-3β.
Analysis of the Western blotting quantification data for GSK-3β phosphorylation showed that cells treated with insulin exhibited increased GSK-3β activation in at both 5 and 10 minutes following treatment. The increase in phosphorylation seen at 5 minutes did not reach statistical significance when compared to the control group, however (Fig. 6).

Impact of PA Treatment on Insulin-Induced AKT1 Activation

To investigate the effects of PA treatment on the degree of AKT1 phosphorylation in our cell line, we seeded N2a cells and incubated them in serum free media supplemented with 2.4 mg/mL BSA or 200 μM PA for 24 hours. At the end of this treatment period the cells were stimulated with 25 ng/mL insulin for 0, 5, or 10 minutes. Protein was then collected and analyzed by Western blotting to determine the levels of phospho- and total AKT1. The intensity
of each protein band was then quantified and plotted as the relative level of phospho- to total AKT1.

Preliminary results from this experiment were consistent with our previous finding. BSA-treated cells that were challenged with insulin for 0 minutes showed a relatively low level of AKT1 activation. Insulin challenge for 5 and 10 minutes in the BSA treatment group was associated with an increase in AKT1 phosphorylation. The PA treatment group that was challenged with insulin for 0 minutes also displayed a low level of baseline AKT1 phosphorylation. Insulin challenge for 5 and 10 minutes in the PA treatment group appeared to be associated with an increase in AKT1 phosphorylation, though neither of these result represented statistically significant increase when compared to the PA-treated 0 minute challenge group (Fig. 7). Insulin-induced AKT1 activation in PA treated cells appeared to be attenuated when compared to activation in BSA treated cells, but we were unable to replicate these provisional results with further experimentation due to technical complications.
Impact of PA Treatment on Insulin-Induced GSK-3β Activation

To determine whether PA treatment had an impact on GSK-3β activation in the N2a cell line, aliquots were taken from the protein samples collected in the previous PA treatment experiment and analyzed by Western blotting to determine the levels of phospho- and total GSK-3β. The intensity of each protein band was then quantified and plotted as the relative level of phospho- to total GSK-3β.

Preliminary results for this experiment suggested that BSA-treated cells that were challenged with insulin for 0 minutes showed a relatively low level of GSK-3β activation. Insulin challenge for 5 and 10 minutes in the BSA treatment group resulted in increased GSK-3β. The 0-minute insulin challenge group that had been pretreated with PA also displayed a low
level of baseline GSK-3β phosphorylation. Insulin challenge for 5 minutes also appeared to increase GSK-3β phosphorylation (Fig. 8). Insulin-induced activation of GSK-3β in PA-treated cells did not appear to differ substantially from the activation seen in control cells. We were unable to repeat these provisional results with further experimentation due to technical complications.

Fig 8 Effects of PA Treatment on Insulin-Induced GSK-3β Activation
(A) Western blot to detect p-GSK-3β and total GSK-3β performed using protein extracted from N2a cells treated with 2.4 mg/mL BSA or 200 μM PA for 24 hours and then challenged with 25 ng/mL insulin for 0, 5, or 10 minutes as indicated.
(B) Quantification of Western blot bands plotted as the level of p-GSK-3β normalized to the amount of total GSK-3β. Data are presented as mean ± SD; n=3.
**Discussion**

This study was aimed at investigating the impact of PA treatment on cell viability and insulin signaling in neuronal cells. We first sought to study the impact that exposure to elevated FFA levels had on cell viability. Cells from the mouse neuroblastoma line N2a were initially treated with varying concentrations of PA for 24 hours. At the end of this incubation period an MTT assay was used to determine the relative number of viable cells remaining. Treatment with 25 μM PA for 24 hours was associated with a slight increase in cell viability compared to the 0 μM PA control group. The relatively low level of PA in this treatment group may have caused this increase by providing an additional source of energy for the cells without negatively impacting cell health, thereby facilitating increased cell proliferation. Cells that were treated 50 μM and 100 μM did not show a significant alteration in viability when compared to controls, but treatment with 200, 400, and 600 μM PA was associated with a dose-dependent reduction in viability. These results correspond to findings in other cell types and suggest that elevated levels of FFAs can have a marked impact on the health of neuronal cells.

Next, an attempt was made to determine whether treatment with the ROS scavenger NAC or the ER stress attenuator PB was able to reverse the decrease in cell viability caused by PA treatment. Cells were treated with 2.4 mg/mL BSA or 200 μM PA as well as varying concentrations of NAC or PB for 24 hours before an MTT assay was used to determine the relative cell viability. In both the NAC and PB cotreatment experiments, PA treated cells experienced a significant reduction in cell viability when compared both to untreated controls and the BSA treatment groups. None of the concentrations of NAC or PB used for cotreatment was able to generate significant alterations in cell viability in either the BSA or PA treatment groups. These results suggest that the reduction in cell viability we observed may not be caused
by ER stress or ROS. Despite the fact that the results did not reach statistical significance, treatment with 200 μM, 500 μM, and 1 mM PB appeared to cause a small increase in cell viability in both the BSA- and PA-treated cells. Studies using a larger number of samples may help clarify the impact of this chemical on cell survival. Treatment with other ER stress attenuators, such as phenylpropionate, or ROS scavengers, such as coenzyme Q10, may also be justified to clarify the role of these processes following FFA exposure.  

In addition we performed an imaging study where cells were treated with different concentrations of PA and visualized with a microscope to support the findings of our initial MTT assay. When compared to the BSA treated control group the 100, 200, and 400 μM PA treatment groups displayed a dose-dependent decrease in cell confluence. Exposure to elevated levels of PA was also associated with alterations in cell size and morphology when compared to controls. These data taken with the results of our MTT assay suggest that PA, especially at high levels, has a profound impact on cell health and consequently on cell survival. It is possible that these effects may be mediated by PA acting on mitochondria to impair their function, thereby reducing the amount of ATP available for proper cell growth and proliferation. Exposure to PA may also cause the mitochondria to generate pro-apoptotic signals.

Next we investigated the time course of insulin signaling in N2a cells and the effects of PA on this signaling pathway. We found that insulin challenge induced significant AKT1 activation at 5 and 10 minutes and significant GSK-3β activation at 10 minutes when compared to controls. Cells were then treated with either 2.4 mg/mL BSA or 200 μM PA for 24 hours before challenging with insulin for 0, 5 or 10 minutes. Preliminary results suggest that 5 and 10 minute insulin challenge in the BSA-treated group may generate an increase in AKT1 activation. Insulin challenge in the PA-treated group for both 5 and 10 minutes, however, was not associated
with any significant alteration in AKT1 phosphorylation. Cells in the BSA treatment groups also displayed a statistically significant increase in GSK-3β phosphorylation with 5 and 10 minute insulin challenge when compared to the 0 minute group. Insulin challenge for 5 minutes in the PA treatment group appeared to generate a significant increase in GSK-3β activation when compared to the PA-treated 0 minute challenge group. No significant alteration in GSK-3β activation was noted between the PA-treated 0 and 10 minute insulin challenge groups.

These data suggest that exposure to PA may result in attenuated insulin signaling in neuronal tissue, but we were unable to replicate these results with further experimentation due to technical complications. Though initial experiments probing for phospho- and total AKT1 yielded acceptable results, subsequent Western blots performed according to the same procedure were marked by a band of unknown identity. This band, though it did not overlie the bands of interest, was of such high intensity that it obscured the signals for phospho- and total AKT1 and prevented accurate analysis of these bands. Experimentation regarding the effects of PA on GSK-3β activation was similarly hindered by technical issues. Initial experiments utilizing a different antibody set were unable to generate a reliable signal for p-GSK. Though this was eventually resolved through the use of the reagents listed above, funding and time constraints prevented the continued experimentation needed to validate our results. It is also possible that the cell death caused by PA treatment in these experiments may have confounded our results. Though the PA treatment groups were initially seeded with more cells than the BSA treatment groups there were notably more cells in the wells that had received BSA at the time protein was collected. The degree of cell death appeared to be higher than that observed during the MTT assays for cell viability and may be a result of the media used for each experiment. Experiments in other cell lines have suggested that FBS exhibits a protective effect against the effects of PA
on cell viability. Since the media used for the MTT assays contained FBS and the media used for the insulin treatment experiments was serum-free, this may account for the increased cell death noted in the insulin signaling experiments. Based on these findings future studies could be warranted to clarify the effects of PA treatment on AKT1 and GSK-3β activation, as well as to determine whether PA treatment is associated with the activation of JNK or p38 as is seen in other tissues. Should these experiments be performed, however, steps should be taken to further compensate for PA-induced cell death and ensure that approximately equal amounts of protein are collected from all treatment groups. Studies looking for the addition of inhibitory serine or threonine phosphate groups to upstream elements of the insulin signaling cascade in response to PA treatment may also be reasonable.
Literature Cited


