Effects of Lipopolysaccharide and Abscisic Acid on Insulin Signaling in SH-SY5Y Cell Line

Final Master Project

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ABSTRACT

Insulin resistance is a pathophysiological mechanism closely related to neuroinflammation and present in many neurodegenerative diseases like Alzheimer's Disease. Under pathological conditions, abnormal increased phosphorylation of insulin receptor substrate 1 (IRS1) serine residues occurs, leading the disruption of the phosphoinositol 3-kinase/protein kinase B (PI3K/Akt) pathway and resulting in neuronal impairment. Lipopolysaccharide (LPS) is a pro-inflammatory molecule present in the outer cell wall of Gram-negative bacteria, which is recognized by microglial and neuronal tall-like receptors, thus leading neuroinflammation. Abscisic acid (ABA) is a sesquiterpene that has shown protective effects against peripheral insulin resistance, neuroinflammation and cognitive impairment. Besides, ABA has shown differentiation properties in glioblastoma cell line. In this study, we explored the effects of *in vitro* LPS and ABA administration on insulin signaling in neurons, acutely and chronically. In addition, we tested ABA as a neuronal differentiating stimulus on a neuroblastoma cell line. For that, functional assays were performed on the SH-SY5Y human neuroblastoma cell line. On the one hand, we pre-treated the cells with ABA (20 μM) and 20 hours later we administrated LPS (1μg/mL) acutely (24h). On the other hand, we treated cells chronically with LPS (1μg/mL) and ABA (20 μM) simultaneously for 5 days. Then, cells were treated with insulin (1 or 10 mU/mL) for 5 minutes and cell lysis was performed. Finally, we analyzed the phosphorylation of insulin signaling key proteins such as IRS1pTyr, IRS1pSer$_{616}$, pAkt and pERK$_{1/2}$ by western blot. Moreover, we analyzed qualitatively ABA and retinoic acid (RA, positive control) as neuronal differentiation stimuli in SH-SY5Y cell line by inverted optical microscope images. As a result, the comparison of groups (mean ± SEM) treated with LPS, ABA, ABA+LPS and negative control showed no significant differences in the phosphorylation of IRS1pTyr, IRS1pSer616, pAkt and pERk1/2 after insulin stimulation. On the other side, RA-treated neuroblastoma cells showed a differentiated phenotype, while ABA did not stimulate differentiation. Therefore, our data suggested that LPS did not affect insulin signaling, both acutely and chronically. Since there were no differences in terms of LPS administration, the neuroprotective effects of ABA against LPS-induced neuroinflammation could not be ascertained. Moreover, ABA did not display a neuronal differentiating stimulus on the human neuroblastoma cell line SH-SY5.

Key words: Neuroinflammation; Insulin resistance; Lipopolysaccharide; Abscisic acid; SH-SY5Y.

ABBREVIATIONS

6-OHDA: 6-Hydroxydopamine

ABA: Abscisic Acid

AD: Alzheimer's Disease

Akt: Protein Kinase B

AMPK: AMP-Activated Protein Kinsase

Aβ: β-amyloid

BBB: Blood-Brain Barrier

BCA: Bicinchoninic Acid Method

BDNF: Brain-Derived Neurotrophic Factor

BSA: Bovine Serum Albumin

CNS: Central Nervous System

DAMPs: Damage Associated Molecular Patterns

DMEM: Eagle's Minimal Essential Medium

DMSO: Dimethyl Sulfoxide

eNOS: endothelial Nitric Oxide Synthase

ERK$_{1/2}$: Extracellular signal-Regulated Kinase 1 and 2

FBS: Fetal Bovine Serum

FoxO: Forkhead box O

GABA: γ-aminobutyric acid

GPx: Glutathione Peroxidase

GSK-3β: Glycogen Synthase 3β

IGF1: Insulin-like Growth Factor-1

IKK: IkB Kinase

IL: Interleukin

IRS1: Insulin Receptor Substrate 1

JNK: c-Jun N-terminal Kinase

LanCL2: LanC Like 2

LPS: Lipopolysaccharide

mTORC1: mammalian Target Of Rapamycin Complex 1

NF-kB: Nuclear Factor kappa-light-chain-enhancer of activated B cells

NGF: Nerve Growth Factor

nNOS: neuronal Nitric Oxide Synthase

NO: Nitric Oxide

P/S: Penicillin/Streptavidin

PAMPs: Pathogen-Associated Molecular Patterns

PI3K: Phosphoinositol 3 Kinase

PKR: double-stranded RNA-dependent protein kinase

PPAR-γ: Peroxisome Proliferator-Activated Receptor γ

RA: Retinoic Acid

ROS: Reactive Oxygen Species

RXR-γ: Retinoic X Receptor γ

SOD: Superoxide Dismutase

STAT3: Signal Transducer and Activator of Transcription 3

TLR: Tall-Like Receptor

TNFα: Tumor Necrosis Factor α

INTRODUCTION

Insulin resistance is a pathophysiological mechanism closely related to neuroinflammation and present in many neurodegenerative diseases like Alzheimer's Disease (AD) (1–3). Since the brain insulin receptor was discovered in the 1970s (4), accumulated evidence has shown that insulin and insulin-like growth factor-1 (IGF1) signaling plays a key role in central nervous system (CNS) functions including memory, learning, neuronal plasticity, synapsis and survival, mood, and weight homeostasis (5,6). When insulin or IGF1 binding to their receptor, two pathways are stimulated: the Ras / Extracellular signal-regulated kinase 1 and 2 ($ERK_{1/2}$) and the phosphoinositol 3 kinase (PI3K) / protein kinase B (Akt) pathways (Fig. 1).
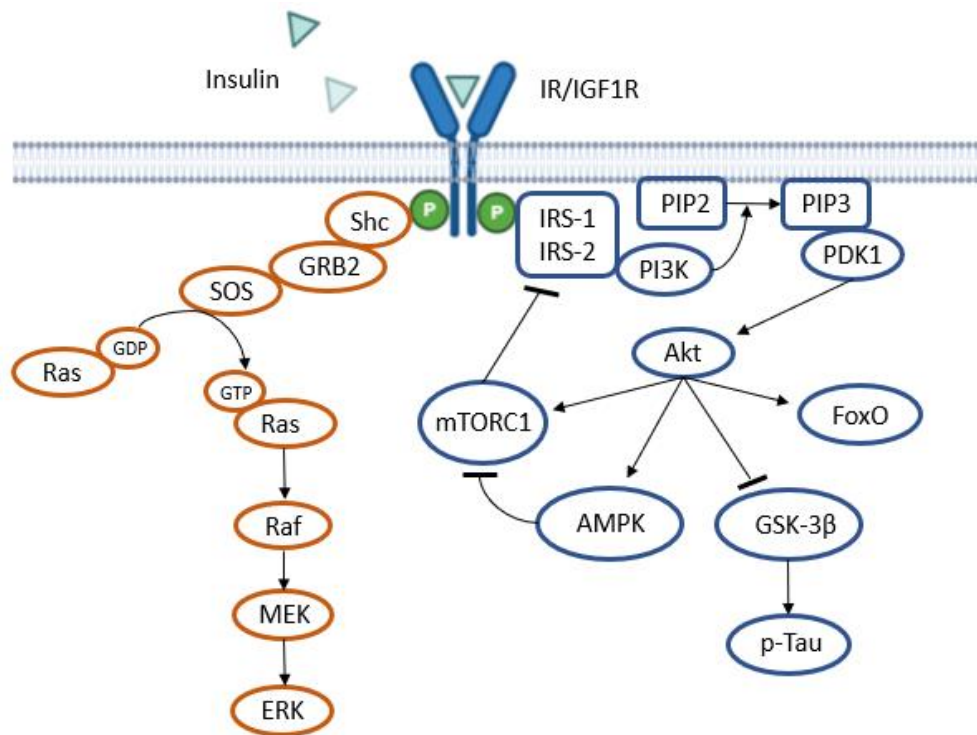

Figure 1. Insulin/IGF-1 signaling. On the left side, the Ras/$ERK_{1/2}$ pathway is represented, leading to $ERK_{1/2}$ phosphorylation and activation. On the right side is shown the PI3K/Akt pathway. Akt activation inhibits glycogen synthase 3β (GSK-3β), and Forkhead box O (FoxO), and activates AMP-Activated Protein Kinsase (AMPK) and mammalian Target Of Rapamycin Complex 1 (mTORC1) pathways.

Several data suggests that the disruption of the PI3K/Akt signaling pathway is involved in brain insulin resistance (7,8). Specifically, the insulin receptor substrate 1 (IRS1) dysfunction is the main candidate underling insulin resistance in AD (9). IRS1 is a scaffold protein that is substrate of the insulin/IGF1 receptors kinase activity. When the receptors are stimulated by their ligand the phosphorylation of multiple tyrosine residues of IRS takes place, activating the downstream

pathways. However, the phosphorylations at serine residues (mainly through serine kinases stimulated by proinflammatory cascades) act as a negative feedback mechanism turning off the signal. Under pathological conditions like AD, abnormal increased phosphorylation of IRS1's serine residues occurs, which results in a silencing of the PI3K/Akt pathway. Therefore, IRS1 dysfunction (excessive serine phosphorylation) impairs correct functioning and survival of neurons, especially in cerebral cortex and hippocampus (10). Moreover, IRS1 phosphorylation at serine 312 ($pSer_{312}$) and serine 616 ($pSer_{616}$) have been found increased in post-mortem brain tissues of AD patients, compared to other pathologies and normal aging (11). Besides, $IRS1pSer_{616}$ in the hippocampus of AD patients was correlated with episodic and working memory impairment (12), and also the specific IRS1 phosphorylation at serine 312 and 616 residues, together with the biomarker phosphorylated tau protein, have been associated as potential predictive biomarkers of AD (12,13).

Neuroinflammation is another common feature in neurodegenerative disorders. In pathological brain situations (i.e. AD brain) several toxic stimuli contribute to the microglial transition to the proinflammatory M1 phenotype (14). Amongst those important deleterious factors include: the β-amyloid (Aβ) forming extracellular deposits in plaques and misfolded proteins like hyperphosphorylated Tau, which forms intracellular neurofibrillary tangles; pathogen-associated molecular patterns (PAMPs, i.e. the components of the bacterial cell wall like lipopolysaccharide (LPS)) or damage associated molecular patterns (DAMPs), like the soluble Aβ and other cellular toxic debris. When these factors become to elevated, there is an increase in the release of reactive oxygen species (ROS) and pro-inflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin-6 (IL-6) and interleukin-1β (IL-1β). The presence of these inflammatory stimuli leads to the activation of IkB kinase (IKK), c-Jun N-terminal kinases (JNK) and double-stranded RNA-dependent protein kinase (PKR). A crucial target of these kinases is the IRS1. As mentioned above, IRS1 phosphorylation in serine residues lead to IRS1 inactivation, resulting in neuronal insulin resistance (8,10). Impaired IRS1/PI3K/Akt pathway derives in an increased glycogen synthase kinase 3β (GSK-3β) activity, which phosphorylates tau protein and favors the neurofibrillary tangles formation. In addition, there is a decrease endothelial nitric oxide synthase (eNOS) activity, which reduce nitric oxide (NO) production, generating an increase in vasoconstriction and neuroinflammation state (10). Overall, insulin resistance and neuroinflammation induce neuronal damage and aggravates AD symptoms, strongly contributing to the pathology's onset.

LPS is a pro-inflammatory molecule present in the outer cell wall of Gram-negative bacteria. It is important to note that gut microbiota dysbiosis is becoming increasingly recognized in the etiology of neurodegenerative diseases, and the onset of Alzheimer's disease has been associated with the dysbiosis of the gut microbiota. The gut microbiota is closely related to the brain through

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the gut-brain axis. Different metabolites and neurotransmitters such as short-chain fatty acids, catecholamines, norepinephrine, serotonin, γ-aminobutyric acid (GABA), acetylcholine, histamine, dynorphin and cytokines are secreted by the gut microbiota and can regulate CNS activity (15,16). In turn, many authors refer gut microbiota like "the second brain". Microbiota dysbiosis may be due to inappropriate eating habits, obesity and unhealthy lifestyle. This dysbiosis generates an increased gram-negative bacterium such as *Escherichia coli, Salmonella enterica, Salmonella typhimurium, Bacillus subtilis, Mycobacterium tuberculosis* and *Staphylococcus aureus*, which express LPS in their bacterial wall and secrete amyloid peptides in the gut. Hence, these PAMPs are recognized by the tall-like receptor (TLRs) of the intestinal immune system and the inflammatory state is induced (17). Inflammatory conditions and aging have a synergistic effect on both increased intestinal barrier and blood-brain barrier (BBB) permeability. Therefore, pro-inflammatory cytokines, LPS and amyloid peptides can cross the endothelial barrier into the bloodstream reaching the CNS, where are recognized by the TLRs (e.g. TLR2 and TLR4) of microglia, astrocytes and neurons thus contributing to neuroinflammation state (17,18). Notably, LPS levels have been found in the brain of AD patients (19), including hippocampus and neocortex (20,21).

In many models of neuroinflammation, LPS is commonly used as pro-inflammatory stimuli. Mostly, the effects of LPS have been reported on cells from the immune system (that express high levels of TLR4). Macrophages and microglia are strongly stimulated by LPS, in turn these cells secrete high levels of pro-inflammatory cytokines, like TNFα, severely affecting insulin/IGF1 signaling (as mentioned above) and consequently neuronal health (22,23). Thus, much attention has been focused on attenuating the macrophage response to inflammatory stimulus, however, the direct effect of LPS on neuronal cell line has not been studied in detail. Given the fact that anti-inflammatory therapies targeting microglia have not provided benefits in patients with AD (18), we hypothesize that in addition to LPS effect on microglial cells, LPS can damage neurons directly.

The SH-SY5Y cell line is derived from human neuroblastoma and is commonly used as an *in vitro* neuronal model. The administration of LPS directly on these cells can increase the secretion of pro-inflammatory cytokines, the ROS formation and can reduce trophic factors expression, such as the brain-derived neurotrophic factor (BDNF) and the nerve growth factor (NGF) (24,25). Hence, we used this model to test the hypothesis that LPS can impair insulin signaling in these cells, in the absence of microglia or astrocytes cells.

On the other side, several plant molecules have been used in human for their anti-inflammatory effects. This is the case of abscisic acid (ABA), a sesquiterpene essential for the correct development and functioning of plants, especially against environmental stress. However, since

Le Page-Degivry *et al.* first described ABA´s presence in mammalian brain (26), accumulated evidence suggests that mammals also synthesize ABA by different cells such as stem cells, macrophages, microglia, granulocytes and keratinocytes, and thus it could play important roles in immunity and metabolic control (27). Even though ABA's inherent function remains unknown, pharmacological administration of ABA has been reported to be protective against type 2 diabetes mellitus and prediabetes in rodents and humans (28-33). Besides, although Bruzzone *et al.* reported that ABA could act as a pro-inflammatory cytokine in human granulocytes (33), ABA has generally shown anti-inflammatory properties in several inflammatory diseases such as inflammatory bowel disease, glioma, atherosclerosis, systemic sclerosis, depression or hepatitis C, and infections like mycobacteria, influenza and malaria (27). In the CNS, ABA also exerts anti-inflammatory effects (34–36). Notably, ABA has protective effects in rodent models of AD by reducing neuroinflammation and cognitive impairment (35,36). Moreover, Ribes-Navarro *et al.* reported that ABA restored neuroinflammation-induced hippocampal alterations, including IRS1 and IRS2 expression (37). Furthermore, ABA increased neuronal survival after the administration of a neurotoxin 6-hydroxydopamine (6-OHDA) in SH-SY5Y cell line *in vitro* (38). Nevertheless, since the mechanism by which ABA exerts these effects remains elusive, it is relevant to elucidate whether ABA can provide protective effects against the failure of insulin signaling in neurons under a pro-inflammatory environment.

In addition, Zhou *et al.* reported that ABA can induce differentiation in a glioblastoma cell line via the retinoid acid (RA) signaling pathway (39). Given that RA is commonly used to differentiate SH-SY5Y neuroblastoma cells to mature neurons (40), an interesting possibility is that ABA could exert the same differentiating and anti-tumoral effect as RA on the SH-SY5Y cell line. This hypothesis has in fact not been explored.

Overall, our working hypothesis postulates that, on the one hand, LPS treatment of the SH-SY5Y cell line can reduce insulin signaling in a similar way that $A\beta$ or $TNF\alpha$ by increasing phosphorylation at IRS1 serines 616 and reducing it at tyrosine residues after insulin stimulus. Furthermore, we argue that if this model of insulin resistance by LPS is generated, treatment with ABA may be able to reduce LPS stimulus-induced insulin resistance. Moreover, just as ABA differentiates human glioma cells, ABA could differentiate SH-SY5Y cells in a similar way as RA does. Therefore, the objectives of the study are: [1] to establish a model of inflammation based on LPS administration that can engage insulin signaling in the SH-SY5Y cell line, [2] to evaluate the role of ABA in insulin signaling under LPS-induced pro-inflammatory conditions, and [3] to test the role of ABA as a differentiating stimulus for the SH-SY5Y cell line.

MATERIALS AND METHODS

Evaluation of the effects of LPS and ABA on insulin signaling in the SH-SY5Y cell line. Experimental design.

To evaluate the effects of LPS as a pro-inflammatory condition and ABA on insulin signaling, two parallel assays were performed. On the one hand, a study was carried out with acute administration of LPS. For this purpose, undifferentiated SH-SY5Y neuroblastoma cells were pretreated for 20 hours with ABA (Duchefa biochemie) at a final concentration of 20 μM (based on prior studies (39)), or with dimethyl sulfoxide (DMSO) as a control condition. Then, it was administered LPS (Sigma Aldrich) at a concentration of 1 μg/mL (based on prior studies (24,25)), removing the fetal bovine serum (FBS) from the medium to avoid interfering with the insulin signal. After 24 hours, insulin (Actrapid) was applied at a final concentration of 10 mU/mL for 5 minutes (Fig. 2A). Finally, the cells were lysed for subsequent protein analysis by Western blot. On the other hand, a parallel study was carried out with chronic administration of LPS. In this case, cells were treated with LPS (1 μg/mL) and ABA (20 μM) for 5 days (Fig. 2B). Subsequently, the cells were treated with insulin for 5 minutes, but this time at final concentration of 1 mU/mL. 12 hours before the insulin treatment, FBS was removed from the medium. Cell lysis was then performed for subsequent protein analysis by Western blot.
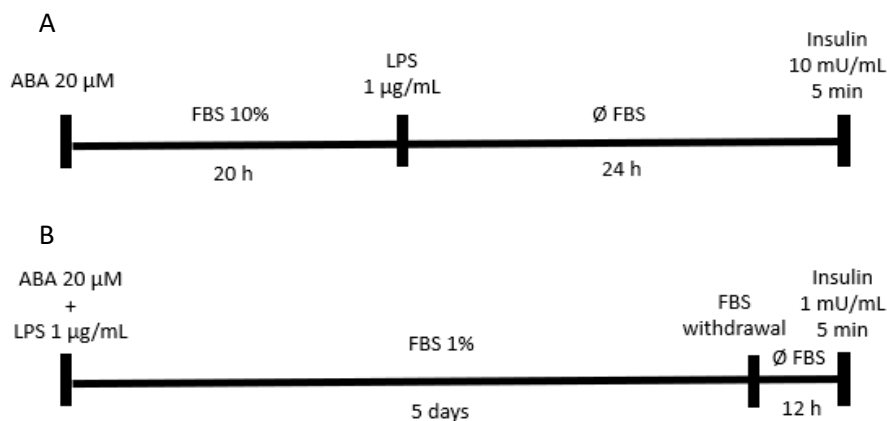

Figure 2. Experimental design of the valuation of the effects of LPS and ABA on insulin signaling in the SH-SY5Y cell line. It is shown the timeline of acute (A) and chronic (B) experiments.

Evaluation of ABA as a differentiating stimulus for the SH-SY5Y cell line. Experimental design.

To test the ability of ABA as a differentiating stimulus to mature neurons of the SH-SY5Y cell line, the study was carried out by applying three different conditions: negative control (DMSO), positive control (RA, 10 μM), and test condition (ABA, 20 μM). Cells received the treatment for 5 days and subsequently images were taken by inverted optic microscope (Leica Microsystems) and Leica Application Suite v4.11.0 software to perform qualitative morphological analysis (Fig. 3).
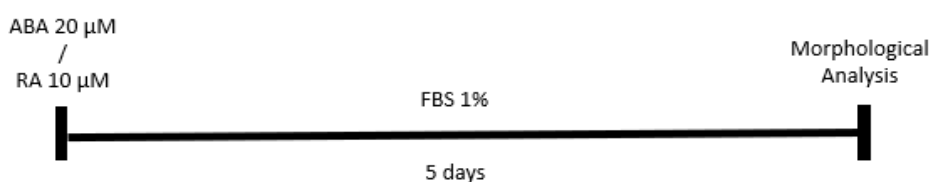

Figure 3. Experimental design of the evaluation of ABA as a differentiating stimulus for the SH-SY5Y cell line. It is shown the timeline of the experiments.

Cell cultures.

SH-SY5Y neuroblastoma cells were adherently grown in 6-well cell culture plaques (SARSTEDT) at 0.3×10^6 cells per surface area. Cells were grown with Eagle's minimal essential medium (DMEM, Glibco) supplemented with FBS (Sigma Aldrich), L-glutamine (Glibco) and penicillin/streptavidin (P/S, Sigma Aldrich) as antibiotics with a final concentration of 10%, 1% and 1% respectively. In all assays, the culture conditions were 95% humidity, 5% CO_2 and 37°C temperature. The culture medium was replaced every 2 days and cell passage was performed when the cells reached 80% confluence by releasing the cells from the surface with trypsin-EDTA (Capricorn). It should be noted that for the treatments performed chronically and for the cell differentiation assay described in the previous section, the percentage of FBS was reduced to 1%.

Cell lysis and protein quantification.

Cell lysis was performed in the same way for all assays. Keeping the temperature below 4°C, cells were scraped from the surface in the presence of cell lysis buffer (RIPA lysis buffer) together with protease and phosphatase inhibitor (Labclinics). Subsequently, they were subjected to the physical method of sonication to complete lysis. Protein quantification was performed by the bicinchoninic acid method (BCA protein assay, Thermo Fisher Scientific).

Protein analysis by Western blot.

Among 20-30 μg of the proteins resulting from cell lysis were loaded onto an 8% acrylamide SDS-PAGE gel for separation by electrophoresis and subsequent transfer to PVDF membranes

(Millipore) by electrotransfer method using the Trans-Blot Turbo Transfer System (Bio-Rad). The membranes were then blocked with bovine serum albumin (BSA, nzytech) diluted at 5% in TBS-Tween buffer (0.3% Tween 20, PanReac) for 2 hours and were then incubated with the relevant primary antibodies at 4°C until the next day. Next, after 5 washes of 7 minutes with TBS-Tween (0.3% Tween 20), the secondary antibody was incubated for 1 hour at room temperature. Afterwards, 5 washes of 7 minutes with TBS-tween (0.3%) were carried out. Finally, the membranes were developed by chemiluminescent using the Clarity Western ECL substrate kit (Bio-Rad).

Primary antibodies used: anti-IRS1pTyr (Thermo Fisher Scientific, 1:1000), anti-IRS1pSer$_{616}$ (Thermo Fisher Scientific, 1:1000), anti-pAkt (Cell-Signaling, 1:1000), anti-pERK (Santa Cruz, 1:500), anti-IRS1 (Millipore, 1:500) and anti-β-actin primary antibodies (Sigma Aldrich, 1:2000). Secondary antibodies used: peroxidase-conjugated goat anti-rabbit and peroxidase-conjugated goat anti-mouse secondary antibodies (Jackson ImmunoResearch, 1:5000).

Statistical analysis.

Western blot results were quantified using ImageJ software. The resulting data were analyzed using GraphPad Prism 8.0.1 software. The results obtained were averaged and the standard error of the mean was calculated. The data were subjected to the Anderson-Darling test to check the normal distribution. After the nonparametric result of the data, the means were compared using the Mann-Whitney U test. We considered a type I error of 0.05 ($p<0.05$) to reach statistical significance.

RESULTS

Effects of acute administration of LPS and ABA on insulin signaling in the SH-SY5Y cell line.

To study the effect of acute treatment with LPS and ABA on SH-SY5Y cells, we created four experimental conditions: a control condition, without the administration of ABA or LPS (CTRL); a control with ABA, but without LPS (ABA); a control with LPS without ABA (LPS); and the condition of ABA together with LPS (ABA+LPS). Furthermore, the four experimental conditions were subdivided into two groups, untreated (wo INS) and insulin-treated (INS) cells. The results were shown visually by the images of the Western blot, and graphically as a result of quantification, showing the mean and standard error of the mean (mean ± SEM). IRS1pTyr and IRS1pSer$_{616}$ levels were normalized to total IRS1 expression, whereas pAkt and pERK levels were normalized to expression of the constitutive protein β-actin. The results of all conditions were normalized to the control condition without insulin (e.g., LPS INS / CTRL wo INS, Fig. 4).

We first focused on the phosphorylation of tyrosine residues, and thus on IRS1 activation (Fig. 4 A, B). We found statistically significant differences (p<0.05) between the conditions with and without insulin (wo INS vs. INS) within the control group (1,00 ± 0,00 vs. 2,52 ± 0,26; n=3), the ABA group (0,99 ± 0,05 vs. 2,89 ± 0,18; n=3), the LPS group (0,88 ± 0,25 vs. 2,46 ± 0,09; n=3) and within the ABA+LPS group (0,79 ± 0,24 vs. 2,46 ± 0,18; n=3). These results indicate that insulin treatment activates its signaling cascade. However, no statistically significant inter-group differences were observed if we compare the means between the four experimental groups when we administered insulin.

Next, we show the results of IRS1 phosphorylation at Ser_{616} residues (Fig. 1 A, C). In this case, no statistically significant differences were observed within groups if we compared insulin administration or non-administration, since phosphorylation of this residue is not so dependent on hormonal stimulus, and neither when comparing the four experimental groups: CTRL wo INS (1,00 ± 0,00; n=3), CTRL INS (0,96 ± 0,10; n=3), ABA wo INS (1,06 ± 0,14; n=3), ABA INS (1,04 ± 0,04; n=3,), LPS wo INS (0,96 ± 0,19; n=3), LPS INS (0,94 ± 0,14; n=3), ABA+LPS wo INS (1,03 ± 0,22; n=3) and ABA+LPS (0,97 ± 0,13; n=3). We expected to find an increase in this marker in the cells treated with the pro-inflammatory molecule LPS, but we also did not see differences between the experimental groups.

As for the phosphorylation and activation of the Akt protein (Fig. 1 A, D), we can see differences in terms of the insulin signal within groups, but in this case, we have the limitation of n=2, which did not allow us to reach statistical significance. We also did not see significant changes when comparing the four experimental groups stimulated with insulin: CTRL wo INS (1,00 ± 0,00; n=3), CTRL INS (7,12± 1,69; n=2), ABA wo INS (1,30 ± 0,48; n=2), ABA INS (6,81 ± 1,05 n=2,), LPS wo INS (1,42 ± 0,54; n=2), LPS INS (6,52 ± 1,70; n=2), ABA+LPS wo INS (1,63 ± 0,19; n=2) and ABA+LPS (9,41 ± 2,53; n=2).

Finally, following ERK activation (Fig. 1 A, E), we highlighted a statistically significant difference in activation between CTRL wo INS (1,00 ± 0,00; n=3) and CTRL INS (10,72 ± 4,77; n=3), and between ABA wo INS (2,02 ± 0,81; n=3) and ABA INS (12,63 ± 6,40; n=3). The differences in activation between LPS wo INS (4,69 ± 1,69; n=2) and LPS INS (14,50 ± 7,406; n=2), and between ABA+LPS wo INS (3,76 ± 1,45; n=2) and ABA+LPS INS (16,10 ± 8,94; n=2), did not give statistical significance due to the low sample size available and the dispersion of the data, however, the differences were clearly visible. No statistically significant differences were observed between the four groups after insulin administration. Nonetheless, LPS-treated cells without insulin seemed to exhibit higher levels of pERK compared to control group.
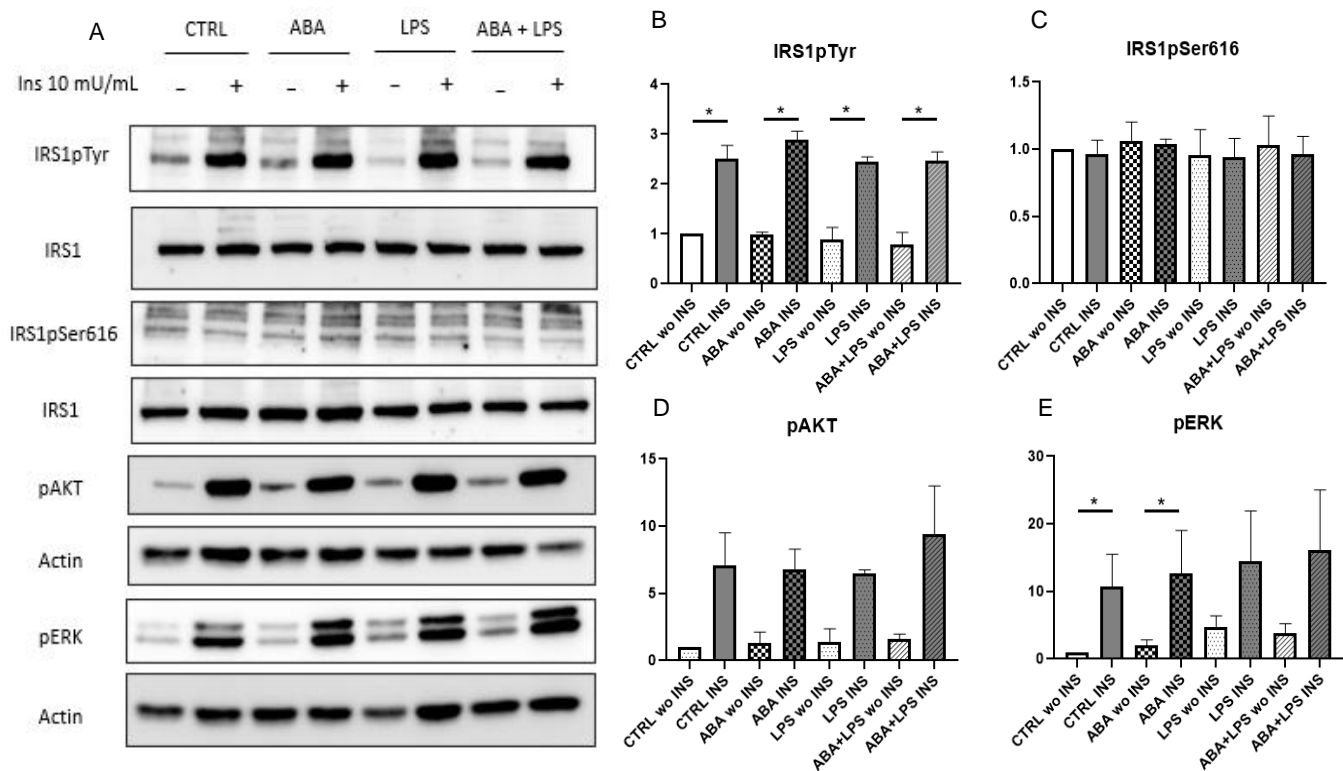
Figure 4. Western blot analysis showing the effect of acute administration of DMSO (control), LPS, ABA and ABA+LPS on insulin signaling proteins in SH-SY5Y cells. Panel A shows the images obtained by western blot, panels B-E show the quantification and graphical representation (mean ± SEM) of the activation of proteins involved in insulin signaling, such as IRS1pTyr (B), IRS1pSer (C), pAkt (D) and pERK (E). Data were analyzed by Mann-Whitney U test (n = 2-3). *P < 0,05.

Effect of chronic administration of LPS and ABA on insulin signaling in the SH-SY5Y cell line.

To evaluate the effect of chronic administration for 5 days of LPS and ABA on insulin signaling, we formed the same 4 experimental groups, with insulin and without insulin, as in the acute condition. In this case, it is important to note that we could only perform n=2, a limitation that did not allow us to reach statistical significance in the results of this condition. As in the previous section, the results were shown visually by means of the images of the Western blot development, and graphically as a result of quantification, showing the mean and standard error of the mean (mean ± SEM). The results of all conditions were normalized to the control condition without insulin (e.g., LPS INS / CTRL wo INS, Fig. 5).

We first focused on the phosphorylation of tyrosine residues, and thus on the activation of IRS1. Although statistical significance was not reached, we could appreciate that insulin enhanced IRS1 tyrosine phosphorylation for all experimental groups: CTRL wo INS (1,00 ± 0,00; n=2) and CTRL INS (1,87 ± 0,17; n=2), ABA wo INS (0,81 ± 0,09; n=2) and ABA INS (2,58 ± 0,93; n=2),

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LPS wo INS ($0,64 \pm 0,22$; n=2) and LPS INS ($2,60 \pm 0,15$; n=2), ABA+LPS wo INS ($1,19 \pm 0,03$; n=2) and ABA+LPS INS ($2,77 \pm 0,29$; n=2). However, similar to the acute treatment conditions, no differences were observed between groups after insulin administration (Fig. 5 A, B).

Regarding phosphorylation at Ser_{616} residues (Fig. 5 A, C), no differences were observed between CTRL wo INS ($1,00 \pm 0,00$; n=2) and CTRL INS (0.82 ± 0.32; n=2) as expected. Between ABA wo INS ($1,41 \pm 0,17$; n=2) and ABA INS ($0,72 \pm 0,12$; n=2) there was a more marked difference, a result that was not expected and that will have to be verified by increasing the sample size. No differences were perceived between LPS wo INS ($0,62 \pm 0,06$; n=2) and LPS INS ($0,52 \pm 0,04$; n=2), and neither between ABA+LPS wo INS ($0,50 \pm 0,02$; n=2) and ABA+LPS INS ($0,66 \pm 0,02$; n=2). In this case, we expected to find an increase of this marker ($IRS1pSer_{616}$) in cells treated with the pro-inflammatory molecule LPS, but we also saw no difference between the experimental groups treated with insulin.

Regarding Akt, no statistically significant differences were observed between CTRL wo INS ($1,00 \pm 0,00$; n=2) and CTRL INS ($4,68 \pm 3,17$; n=2); nor between ABA wo INS ($0,72 \pm 0,62$; n=2) and ABA INS ($4,16 \pm 2,58$; n=2); nor between LPS wo INS ($0,83 \pm 0,13$; n=2) and LPS INS ($3,89 \pm 2,37$; n=2); and nor between ABA+LPS wo INS ($1,04 \pm 0,38$; n=2) and ABA+LPS INS ($2,92 \pm 1,13$; n=2). If we look at the insulin-treated experimental groups, we could appreciate a tendency to downregulation of Akt activation, but we could not remark a statistical significance (Fig. 2 A, D).

Finally, no statistically significant differences in ERK activation were observed between CTRL wo INS ($1,00 \pm 0,00$; n=2) and CTRN INS ($1,26 \pm 30,04$; n=2); nor between ABA wo INS ($0,82 \pm 0,07$; n=2) and ABA INS ($1,23 \pm 0,02$; n=2); nor between LPS wo INS ($0,54 \pm 0,24$; n=2) and LPS INS ($0,91 \pm 0,28$; n=2); and nor between ABA+LPS wo INS ($0,39 \pm 0,04$; n=2) and ABA+LPS INS ($0,89 \pm 0,05$; n=2). In that case, we could appreciate a trend toward a decrease in ERK activation with and without insulin stimuli in LPS-treated cells, but we could not note a statistical significance (Fig. 2 A, E).

Abscisic acid does not act as a differentiating stimulus for the neuroblastoma cell line SH-SY5Y.

Analyzing qualitatively the appearance and morphology of cells treated with either DMSO (negative control), RA (positive control) or ABA (test condition), we could see that cells treated with DMSO showed an undifferentiated epithelial phenotype, whereas cells treated with RA showed a clearly differentiated neuron phenotype. Mature neurons are more elongated, larger and with larger projections and neurites than undifferentiated. However, ABA-treated cells expressed an undifferentiated phenotype of SH-SY5Y neuroblastoma cells, similarly to negative control (Fig. 6).
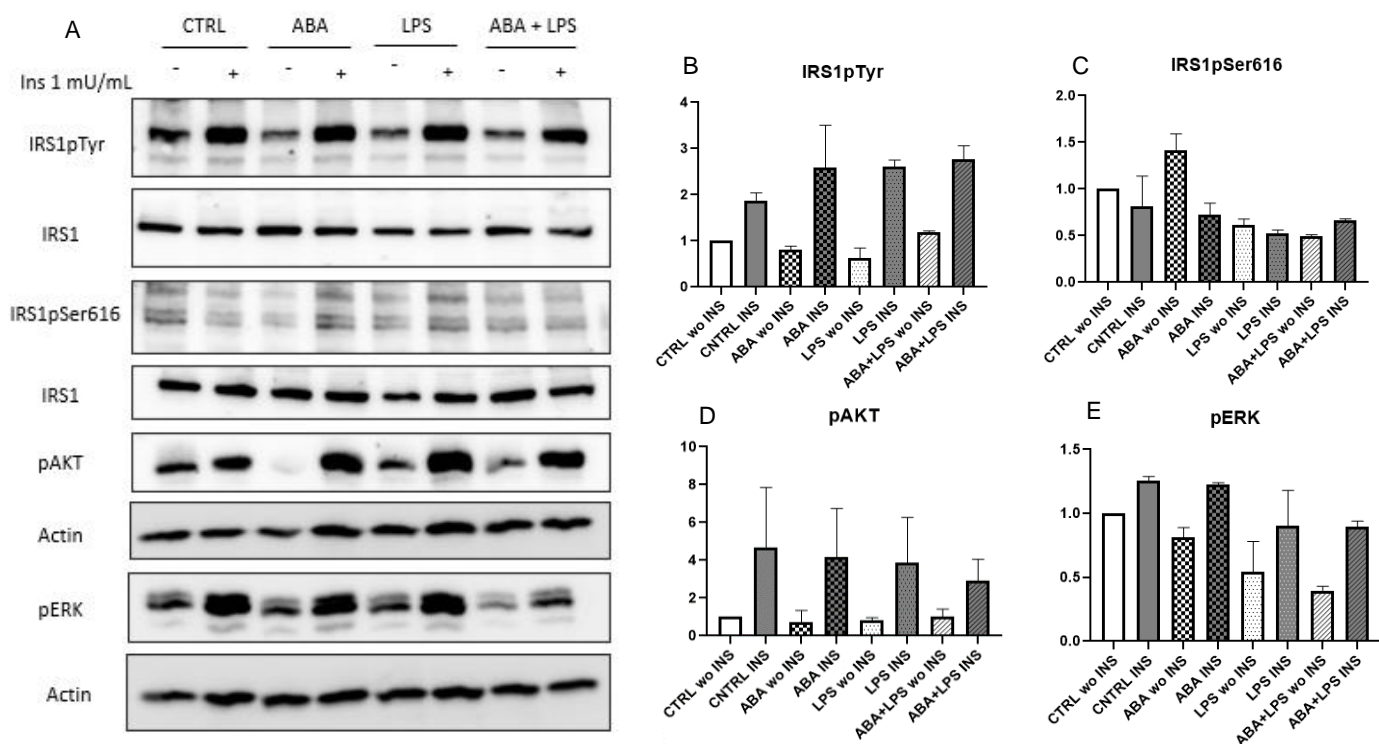
Figure 5. Western blot analysis showing the effect of chronic administration of DMSO (control), LPS, ABA and LPS+ABA on insulin signaling proteins in SH-SY5Y cells. Panel A shows the images obtained by western blot, and panels B-E show the quantification and graphical representation (mean $\pm$ SEM) of the activation of proteins involved in insulin signaling, such as IRS1pTyr (B), IRS1pSer$_{616}$ (C), pAkt (D) and pERK (E). Data were analyzed by Mann-Whitney U test (n = 2).
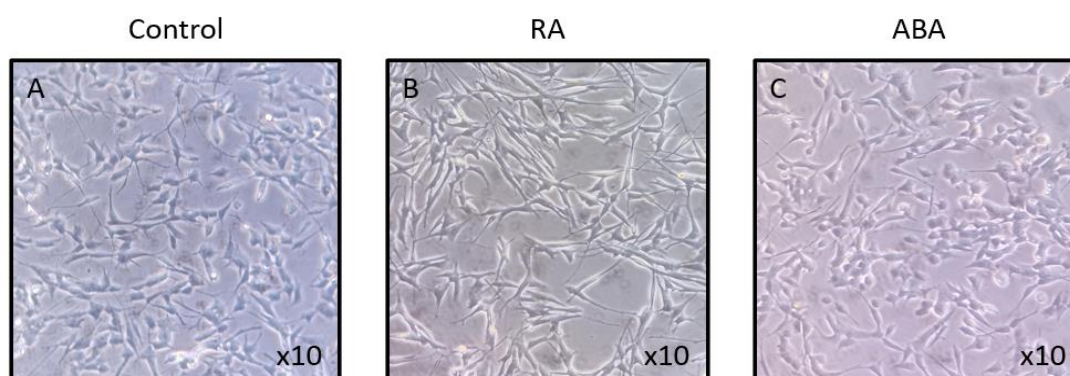

Figure 6. Overall images taken by inverted light microscopy showing the general morphology of SH-SY5Y cells treated with different conditions. Cells treated with DMSO (A, negative control) and those treated with ABA (C) show undifferentiated morphology. However, cells treated with RA (B, positive control) show a differentiated morphology.

DISCUSSION

To our knowledge, this is the first study to test the effects of LPS direct administration on insulin signaling in SH-SY5Y cell line. It should be noted that our results are preliminary, we have a small sample size so we cannot draw definitive conclusions. However, our results suggested that the administration of LPS, both acutely and chronically, had no effect on the insulin signaling pathway in the undifferentiated cell line SH-SY5Y. Likewise, ABA administration had also shown no effects on such activation, neither did it appear to function as a neuronal differentiating stimulus in that cell line.

As mentioned previously in the introduction, IRS1 is a scaffold protein which is phosphorylated in tyrosine residues by the insulin receptor kinase activity when insulin binds to its receptor under normal conditions. Once IRS1 is phosphorylated activates the PI3K/Akt pathway, which mediates several neuronal homeostatic and metabolic functions (41). Evidence have shown that under neuroinflammatory situation, phosphorylation of IRS1 serine residues hampers the phosphorylation of tyrosine residues, thereby reducing the PI3K/Akt signaling and resulting in impaired neuronal homeostasis (8,10). Specifically, the phosphorylated $IRS1Ser_{616}$ is increased in postmortem brains of AD patients (12), in rodent models of Alzheimer's disease such as APP/PS1 mice (42,43) and in high fat diet-fed mice, which is a well-stablished model of insulin resistance (44). Similarly, prolonged peripheral LPS administration in rodents also increased $IRS1pSer_{616}$ and decreased tyrosine phosphorylation in neurons of the hypothalamus (45). We confirmed that insulin induced IRS1 phosphorylation at tyrosine residues and downstream Akt and ERK phosphorylation. However, the proinflammatory condition induced by acute or chronic LPS-treatment did not exert a reduction in IRS1 activation. Consistently, we did not find an increase in $IRS1pSer_{616}$ with LPS administration.

Akt is a central serine/threonine protein kinase that participates in several functions within cells, including survival, growth, metabolism, proliferation and other functions involved in neuronal homeostasis. Akt deregulation has been found associated with pathologies like cancer, diabetes mellitus and inflammatory disorders (46). IRS1 activates the PI3K/Akt pathway upon insulin/IGF1 stimulation (Fig. 1), so the downregulation of IRS1 is expected to reduce Akt activation by insulin/IGF1 (10). In previous reports, central and peripheral administration of LPS in rodents reduced Akt activation (45,47,48), in the same way that rodents fed with high-fat and high fructose diet (a model of insulin resistance) (49). However, our data showed that acute and chronic LPS treatment did not hamper the ability of insulin to stimulate Akt, which is coherent with the fact that LPS treatment did not hamper insulin induced-IRS1pTyr. Hence, these results suggested that LPS does not affect PI3K/Akt insulin pathway directly on neuronal cells.

On the other hand, the MAPK/ERK$_{1/2}$ pathway can be independent of IRS1 activation (Fig. 1). This pathway, together with the PI3K/Akt are both activated by insulin/IGF1 and similarity exerts a central role in functions like neuronal survival and proliferation. Interestingly, an imbalance of both signaling pathways occurs in pathological situations, and the MAPK pathway is potentiated compared to the PI3K/Akt. This imbalance has been shown to exacerbates AD's onset (10,50). Because PI3K/Akt pathway is IRS1 dependent and ERK$_{1/2}$ is not, we could expect that IRS1 inactivation would greatly contribute to the imbalance of both pathways. Our results confirmed that ERK$_{1/2}$ activation is dependent on insulin stimulus and that LPS did not prevent insulin action, however, LPS alone increased basal pERK, which could be in agreement with previous reports where pERK was found elevated in rodents treated with LPS (48).

One of the most common models for testing the inflammatory status of SH-SY5y cells *in vitro* is the treatment of these neurons with conditioned culture media from LPS-stimulated microglia (or similar pro-inflammatory stimuli), as well as co-culture systems with BV-2 microglial cells. These treatments affect SH-SY5Y cells by increasing neuronal oxidative stress death (51–55). LPS-stimulated microglia secrete ROS and many proinflammatory cytokines, including IL-1β, IL-6 and TNFα, which severely affect neurons. Nevertheless, we have chosen a model of direct LPS treatment on neuronal cells to elucidate the direct effect on insulin signaling independently of the microglial action. This would help to understand if targeting microglial inflammatory status would be sufficient of LPS can damage the cells directly. Although this model has not been used as widely as the model of neurons co-cultured with microglia, acute treatment of undifferentiated SH-SY5Y with LPS reduced the expression of synaptophysin and neurotrophic factors such as BDNF and NGF, increased the production of ROS and proinflammatory cytokines like TNF-a, IL-1β and IL-6, while reducing the activity of antioxidant enzymes such as glutathione peroxidase (GPx) and superoxide dismutase (SOD) (25). In addition, both acute and chronic treatment of differentiated SH-SY5Y with LPS also increased the expression of pro-inflammatory cytokines and neuronal nitric oxide synthase (nNOS), which is a marker of inflammation (24). These data indicate that LPS can directly damage both differentiated and undifferentiated SH-SY5Y cells, supporting direct effect on neuronal cells, independently of the microglia action.

LPs is recognized by the toll-like receptor 4 (TLR4) (56), which are very abundant in immune cells. They have also been reported in neurons, although in lower levels (57). TLR4 activation promotes the activation of protein kinases such as IKK and JNK, and transcription factors like the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the signal transducer and activator of transcription 3 (STAT3). As a result, this inflammatory pathway leads to the expression of pro-inflammatory cytokines and has been shown to promote IRS1 phosphorylation at serine residues. Indeed, TLR4 is related to peripheral insulin resistance (58). Hence, we hypothesized that LPS/TLR4 activation would be a good model of insulin resistance induced by

inflammation to test the effect of ABA molecule. However, in our study, acute and chronic treatment with LPS had no effect on the insulin/IRS1 pathway in the undifferentiated SH-SY5Y cell line. This could be due to the fact that insulin signaling in undifferentiated cancerous neuroblastoma cells are resistant to LPS stimuli. Cancer cells are usually more resistant to inflammatory environments, so that LPS alone may not be enough to promote insulin resistance in those cells. Interestingly, Amine *et al*. recently reported that SH-SY5Y cells treated with palmitic acid exhibited a reduction in insulin-dependent Akt phosphorylation and up-regulation of the TLR4 levels and pro-inflammatory cytokines (IL-6 and TNFα) (59). This result suggests that co-treatment of palmitic acid may increase LPS-effect in this neuroblastoma model.

Several controls would help to further understand direct-LPS effects on SHSY5Y cells. One could be to use differentiated SHSY5Y cells (neuronal model). In addition, important controls include to treat these cells with conditioned medium of LPS-stimulated microglia, which it has been seen to exert greater neuronal damage, and the use of palmitic acid treatment and the evaluation of TLR4 activation by LPS. Of course, an important control is to test the parameters that have been reported on LPS directly on SHSy5y cells (i.e., cytokine secretion and nitric oxide in cell media, and oxidative markers). In fact, we have tested GRP78, a biomarker of endoplasmic reticulum stress, but no difference was observed.

Our group and others have shown the neuroprotective effects of ABA (34–36). In addition, ABA protects against prediabetes and T2DM improving insulin sensitivity (28-33), as well as ABA can restore neuroinflammation-induced alterations in hippocampal IRS1 and IRS2 expression (37). Therefore, ABA is a promising therapeutic molecule, although its mechanism of action in neurons have not been yet elucidated. Since we have not found alterations induced by LPS treatment, we cannot ascertain ABA is neuroprotective, but we can confirm that ABA administration did not represent a noxious factor for neurons.

Concerning the qualitative study on ABA as a neuronal differentiating stimulus of the SH-SY5Y cell line, we observed no visual phenotypic differences between ABA-treated cells and negative controls, while cells treated with RA showed a characteristic differentiated phenotype. Whereas undifferentiated neuroblastoma cells show epithelial-like morphology, differentiated cells show more elongated morphology, with visible projections and neurites (40). Cells treated with ABA showed epithelial-like morphology, indicating that ABA did not work as differentiating stimuli. Zhou *et al*. reported that ABA induced differentiation to astrocytic phenotype and exercised anti-tumoral effects against human glioblastoma cell line (39). ABA signaling pathways in mammalian cells are not full characterized, but it is speculated that its effect is due to the peroxisome proliferator-activated receptor (PPAR-γ) and/or the LanC Like 2 (LanCL2) binding. Often, PPAR-γ forms heterodimers with the retinoic X receptor γ (RXR-γ), which is commonly

activated by RA (27). These authors found that ABA increased RXR-γ expression, so they argued that ABA differentiates glioblastoma cells to astrocytes using RA pathway. However, our data showed that while RA act as a differentiating stimulus on the SH-SY5Y cell line, ABA did not exert this effect. Nonetheless, our study was qualitative, we have to further analyze this effect by immunohistochemistry and western blot to evaluate neuronal differentiation proteins.

STUDY LIMITATIONS AND FUTURE CONCERNS

This is a preliminary study, so it is required to increase the sample size with more cell cultures and different conditions to confirm a proinflammatory state and the mechanism by which ABA exerts its effects, at least the effects observed in rodent models and macrophage cell lines. ABA direct effect on neuronal cultures has never been reported up to date. Another limitation of this study is the lack of a positive control showing a pro-inflammatory effect of LPS on this cell line. Some potential options are to quantify the levels of TNFα, IL-1β and NO in the medium culture, as well as proteins of the TLR4 cascade such as MyD88, NF-kB or IKK and oxidative stress by GPx. Regarding the study of ABA as a differentiating molecule, it is a qualitative work, so we cannot draw conclusions until we perform more exhaustive biochemical and morphological analyses.

With future perspectives, it would be pertinent to perform the same experiments on differentiated SH-SY5Y cells to see the potential effect of differentiation to LPS, and the effects on insulin signaling. Another possibility would be to perform the studies with conditioned medium of microglial cells stimulated with LPS, in order to establish an inflammation model that would allow us to test the neuroprotective properties of ABA.

CONCLUSIONS

Neuroinflammation and insulin resistance are pathophysiological features present in neurodegenerative diseases such as AD. Understanding the relationship between these processes is really relevant to explore potential therapeutic strategies against these impairments. Interestingly, several nutraceuticals have shown beneficial anti-inflammatory and anti-oxidant effects, (i.e. ABA), thus they may be promising therapeutic approaches. LPS is a common pro-inflammatory molecule present in AD patients, so understand how LPS affects the onset of insulin resistance in neurons may be essential to prevent neuronal damage. At this point, our preliminary data suggest that LPS does not affect insulin signaling in neurons, specifically the PI3K/Akt pathway. Regarding ABA, while we could not test its neuroprotective effect in this model of inflammation, we could test ABA's role as a neuronal differentiating molecule. In this case, our qualitative data suggest that ABA does not play a role as a neuronal differentiating stimulus.

It is important to continue investigating how neuroinflammatory mechanisms and their relationship with insulin signaling may affect neuronal functions. Likewise, the identification of therapeutic targets may be essential to prevent and reverse this neuronal damage, providing new therapeutic approaches against neurodegenerative diseases.

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