ABSTRACT
Neuroinflammation has been implicated in the pathogenesis of neurodegeneration and is now accepted as a common molecular feature underpinning neuronal damage and death. Palmitic acid (PA) may represent one of the links between diet and neuroin- flammation. The aims of this study were to assess whether PA induced toxicity in neuronal cells by modulating microglial inflammatory responses and/or by directly targeting neurons. We also determined the potential of oleic acid (OA), a monoun- saturated fatty acid, to counteract inflammation and promote neuroprotection. We measured the ability of PA to induce the secretion of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), the induction of the nuclear factor kappa-light-chain- enhancer of activated B cells (NF-κB) signalling pathways, as well as the phosphorylation of c-Jun, and the expression of inducible nitric oxide synthase (iNOS). Finally, to determine whether PA exerted an indirect neurotoxic effect on neuronal cells, we employed a microglia-neuron co-culture paradigm where microglial cells communicate with neuronal cells in a paracrine fashion. Herein, we demonstrate that PA induces the activation of the NF-κB signalling pathway and c-Jun phosphorylation in N9 microglia cells, in the absence of increased cytokine secretion. Moreover, our data illustrate that PA exerts an indirect as well as a direct neurotoxic role on neuronal PC12 cells and these effects are partially prevented by OA. These results are important to establish that PA interferes with neuronal homeostasis and suggest that dietary PA, when consumed in excess, may induce neuroinflammation and possibly concurs in the development of neurodegeneration.

Introduction

Recently, several studies have established robust links be- tween neurodegenerative diseases and neuroinflammation [1–3]. Central inflammation occurs in multiple neurodegener- ative diseases with peculiar pathology and symptoms such as Alzheimer's disease (AD) [4] and Parkinson's disease (PD) [5], where neuroinflammation is largely mediated by activated microglia [6].

Several cell types have been implicated in contributing to neuroinflammation-mediated neurodegeneration; however, it is well known that activation of glial cells is a major compo- nent of the inflammatory responses underlying brain injury and neurodegeneration [7]. Particularly, microglial cells, de- rived from embryonic macrophages, mediate, among other actions, the innate immune responses of the central nervous system (CNS) and respond to endogenous and exogenous challenges by proliferating, changing morphology, and pro- ducing inflammatory mediators, including cytokines and nitric oxide (NO) and prostanoids [8]. Sustained or uncon- trolled activation of microglial cells leads to chronic neuro- inflammation that can prompt significant neuronal damage, leading, for example, to a gradual loss of dopaminergic neurons in the nigrostriatal pathway or the slow damage of cholinergic neurons typically occurring in PD and AD, respectively [7, 9, 10].

In parallel, specific dietary components, and particularly long-chain saturated fatty acids (SFA), have been shown to trig- ger inflammatory responses in a variety of cell types and tissues [11–16]. However, the inflammatory response elicited by SFA, also termed metabolic inflammation, differs from the classical inflammation triggered by injury of infections and represents a major etiological factor linking obesity with its comorbidities, including type-2 diabetes mellitus (T2DM) [17] which has been associated with both PD and AD [18, 19]. Particularly, palmitic acid (PA), the most prevalent SFA in highly processed foods typical of the Western diet, represents a key mediator linking diet, metabolic disorders, inflammation, and neurodegeneration [20–22]. For example,

according to a report by Klein-Platat et al. [23], overweight adolescents display higher levels of circulating PA compared to their lean counterparts, with the polyunsaturat- ed fatty acid: saturated fatty acid ratio being inversely associated with interleukin-6 (IL-6), thus further supporting the pro- inflammatory role of PA. PA has also been reported to activate systemic inflammatory signalling pathways and induce the se- cretion of pro-inflammatory cytokines in cultured peripheral im- mune cells, such as macrophages and monocytes [24–26]. While the effects of SFA on neuroinflammation remain to be fully characterized, there is compelling evidence that SFA are able to alter CNS physiology [12, 27, 28]. This includes the ability of PA to trigger the release of the pro-inflammatory cy- tokines tumor necrosis factor-α (TNF-α) and IL-6 in both pri- mary astrocytes [29] and microglia [14], with this response be- ing toll-like receptor 4-dependent in astrocytes [29]. Besides its ability to elicit inflammatory response, PA may also affect the differentiation of neurons, astrocytes, and oligodendrocytes from neural stem cells in vitro [30]. Furthermore, another study has recently illustrated possible non-genomic mechanisms by which extra-cellular PA regulates beta-amyloid peptide (Aβ) production, further supporting the link between SFA and the occurrence of neurodegenerative diseases [31]. Importantly, fat- ty acids have long been known to cross the blood–brain barrier [32], with the uptake of PA into the brain increasing in individ- uals affected by the metabolic syndrome [33], which emphasizes the physiological relevance of the deleterious effects of PA on CNS health. However, not all fatty acids are detrimental to CNS health. Indeed, the mono- and omega-3 polyunsaturated fatty acids are well known for their beneficial effects on human health [34, 35]. Specifically, the omega-9 mono-unsaturated fatty acid oleic acid (OA), particularly abundant in olive oil, has been widely described as one of the mediators of the health-promoting effects ascribed to the Mediterranean diet [34]. In
support of this, dietary patterns rich in extra virgin olive oil are associated with a reduced incidence of overall mortality, cardiovascular mortality, cardiovascular events, cancer mortality, and incident PD and AD [36–38]. Besides its well-established peripheral effects, in the brain, OA may prevent oxidative stress–mediated injury in vivo [39] and reduces palmitate-induced neuroinflam- mation and endoplasmic reticulum (ER) stress in hypothalamic neurons [12, 40]. In further support to its beneficial effects of brain health, OA supplementation may decrease Aβ secretion in both cells overexpressing amyloid precursor protein and in an early-onset AD transgenic mouse model [41].
Thus, the main aim of this study was to investigate the ability of microglial cells, activated by PA, to induce neuronal cytotoxicity in a microglia-neuron co-culture system as compared to the bacterial endotoxin lipopolysaccharide (LPS). We also characterized the direct neurotoxic and neuroinflammatory potential of PA in microglial and dopaminergic cells lines and elucidated whether these effects may be prevented by OA. These results provide further insights into the potential role of the long-chain SFA, PA in the development of glial-mediated neuroinflammation, highlighting the pro-inflammatory and neurotoxic role of PA as well as the neuroprotective effects of the mono-unsaturated fatty acid OA.

Materials and Methods

Drugs and Chemicals

All reagents and chemicals were purchased from Sigma- Aldrich (St. Louis, MO) unless stated otherwise.

Fatty Acid–BSA Conjugation

Free fatty acids (FA) were complexed to low-endotoxin fatty acid–free bovine serum albumin (BSA) as described previous- ly by Cousin et al. [42] with some minor modifications [12], with albumin serving as a vehicle for the FA [43]. Briefly, PA (C16:0)

and OA (C18:1) were dissolved in 0.1 M NaOH in a water bath at 70 °C or 37 °C, respectively, to yield a final concentration of 20 mM. A 0.5 mM fatty acid–free BSA solution was obtained by dissolving BSA in deionized water in a water bath at 55 °C. The BSA and the FA solutions were then mixed in order to obtain a 1:4 BSA to fatty acid molar ratio (0.5 mM BSA, 2 mM fatty acid) [44]. The fatty acid BSA mixtures were vortexed for 10 s and successively incubated for10mininawaterbathat55°Cor37°CforPAandOA, respectively. The fatty acid-BSA complexes were cooled to room temperature and filter-sterilized using 0.22-μm pore size membrane filters. All fatty acid-BSA complexes were stored at −20 °C prior to use.

N9 Microglial Cell Culture

The N9 microglial cells are a well-known model to study inflammation in neuronal cells [45–47].The microglial cell line N9 was routinely grown in DMEM nutrient mixture F12 Ham (DMEM-F12) supplemented with 10% horse serum (HS), in a humidified environment at 37 °C and 5% CO2 atmosphere, as already reported [48]. For the present experi- ments, N9 were used at 80–90% confluence and seeded at 30,000 cells/cm2 in 96-well plates and at 60,000 cells/cm2 onto inserts for co-cultures (Fig. 1A), as detailed below.

Neuronal PC12 Cell Cultures

PC12 cells, obtained from the American Type Culture Collection (ATCC, Rockville, MD), were maintained in a humidified environment at 37° C and 5% CO2 atmosphere and routinely grown in Roswell Park Memorial Institute me- dium 1640 (RPMI 1640) supplemented with 5% (v/v) heat- inactivated fetal bovine serum (FBS), 25 U/ml penicillin G, and 25 mg/ml streptomycin. Naïve PC12 cells were used at 60–80% confluence and seeded at a density of 20,000 cells/ cm2. Differentiation was evoked by administration of nerve growth factor 2.5S (NGF, 30 ng/mL, MilliporeSigma, Oakville Ontario, Canada) in RPMI 1640 supplemented with 1% FBS, for 8 days, as described previously [49, 50]. NGF-differentiated PC12 neurons, hereafter referred to as neuronal PC12, express a neuronal phenotype (Fig. 1B) as well as do- pamine and dopamine transporter as already reported [51, 52].

Cytotoxicity was assessed using LDH and MTT assays, as detailed below.

Microglial-Neuronal Co-culture System in the Absence of Cell-Cell Contact

Neuronal PC12 cells and N9 microglia were co-cultured to study the impact of PA-activated microglia on the survival of neuronal PC12 cells. In this co-culture system, microglial cells communicate with PC12 neurons through a semiperme- able membrane, in the absence of a direct contact between the two cell lines (Fig. 1) [47]. N9 microglial cells were seeded at a density of 60,000 cells/cm2 onto co-culture inserts (pore size 0.4 μm; BD Falcon, Oakville, Ontario, Canada) and were challenged with either PA, OA, PA+OA, LPS, LPS+OA, or respective controls for 6 h as described in “Microglia and neuronal treatments” below. Then, inserts containing N9 cells were transferred on differentiated neuronal PC12 cells for ad- ditional 24 h in treatment-free media (Fig. 1) (for more details see [47]). PC12 cytotoxicity was assessed after 24-h exposure to the inserts containing N9 microglia cells using LDH and MTT assays, as detailed below.

Microglia and Neuronal Treatments

In all experiments, Escherichia coli 0111:B4 LPS (MilliporeSigma, Oakville Ontario, Canada), a potent inducer of inflammation and activator of microglia [6, 47, 53], was administered as an internal control, to illustrate the inflamma- tory response in our experimental model.

N9 microglial cells were challenged with PA (100 μM), OA (100 μM), PA+OA (100 μM each) LPS (2 μg/ml), and LPS+OA (2 μg/ml and 100 μM, respectively), for 3.5, 6, or 24 h in DMEM-F12 containing 1% HS. A pre-incubation of 6 h was used to stimulate N9 cell prior co-culture. The con- centration of PA was chosen as it falls within the range of systemic concentration for this FA, as already reported [54]. The concentration of LPS used in these experiments did not affect cell density, as we already reported [47]. Medium (DMEM-F12 with 1% HS) served as control for LPS, and fatty acid–free BSA was used as control for fatty acids. OA was co-administered at the same time as PA or LPS. Neuronal PC12 were challenged with LPS, fatty acids, or respective controls, as described above for N9 cells.

Cytotoxicity and Metabolic Activity

Cytotoxicity was evaluated by the measurement of lactate deshydrogenase (LDH) activity released from damaged cells into the supernatant, as described previously [50]. LDH, a stable cytoplasmic enzyme present in all cells, is rapidly released into the cell culture supernatant upon plasma mem- brane damage. Enzyme activity in the culture supernatant correlates with the proportion of damaged cells [55]. Briefly, the cell-free supernatant is collected and incubated with the reaction mixture from the kit (cytotoxicity detec- tion kit, Roche Diagnostic, Laval, Qc., Canada). The LDH activity is determined as follows: NAD+ is reduced to NADH/H+ by LDH-catalyzed conversion of lactate to py- ruvate, then the diaphorase transfers H/H+ from NADH/H+ to the tretrazolium salt which is reduced to formazan. LDH activity was quantified by measuring absorbance at 490 nm using a microplate reader (Synergy H1, BioTek). Total cel- lular LDH was determined by lysing the cells with 1% Triton X-100 (high control); the assay medium was employed as a low control for LPS-treated cells, whereas BSA served as a control for the experiments involving PA. Cytotoxicity was calculated as follows:

Cytotoxicity ð%Þ

ðExperimental value−Low controlÞ 100 ðHigh control−Low controlÞ

In parallel, cell metabolic activity was measured using MTT (3-(4,5-dimethyltrazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. After the treatments, cells were incubated for 3 h at 37 °C with MTT dye (5 mg/ml); insoluble formazan crystals were then solubilized in DMSO. The absorbance of the formazan solution was measured at the 595 nm with a microplate reader (Synergy H1, BioTek, Canada).

Cytokines Secretion by ELISA

The secretion of pro-inflammatory cytokines IL-6 and TNF-α into the cell culture media was measured by ELISA (BioLegend, San Diego, CA). Following incubation with ei- ther, PA, OA, PA+OA, LPS, LPS+OA, or respective controls, N9 microglia cell supernatants were collected after 6 and 24 h and tested for the presence of selected cytokines by ELISA, according to the manufacturer protocol. Briefly, the superna- tants were collected following the fatty acid or LPS challenges. 96-well plates were coated with mouse-specific monoclonal antibody (IL-6 and TNF-α) and after an overnight incubation, standards and samples were added to the wells for 2 h. The plates were then incubated in the presence of a biotinylated anti-mouse detection antibody for 1 h, followed by 30-min incubation with an avidin horseradish peroxidase solution. Finally, a tetramethylbenzidine solution was added to the wells for 15 min in the dark to reveal the presence of the cytokines. The reaction was terminated by the addition of 2N sulfuric acid and resulting absorbance recorded at 450 nm using a microplate reader (Synergy H1, BioTek, Canada).

Proteins Expression by Western Blot

N9 microglial cells were seeded at 30,000 cells/cm2 in 24 wells. After a 3.5-h incubation with fatty acids, total proteins were extracted (Nuclear Extraction Kit, Active Motif, Carlsbad, CA, USA) and concentrations were determined by bicinchoninic acid quantification (BCA protein assay kit, Pierce Biotechnology Inc., Rockford, IL, USA). Twenty mi- crograms of proteins was loaded onto a 12% SDS- polyacrylamide gel. After electrophoretic separation (200 V, for 1 h), proteins were transferred onto PVDF membranes (0.22-µm pore size, BioRad) at 25 V overnight. The mem- branes were blocked for 30 min in TBST (TBS + Tween 0.05 %) with 5% non-fat dry milk and incubated overnight at 4 °C with primary antibodies anti-phosphorylated (P)-NF-κB p65 (Pp65), anti-P-c-Jun (ser73) (Santa Cruz Biotechnology, Mississauga, ON, Canada), and anti-β-actin (Sigma) (dilu- tion: 1:200, 1:200, and 1:4000, respectively) as described pre- viously [56]. The blots were then incubated with the appro- priate peroxidase-conjugated secondary antibody (1:10,000) (Santa Cruz Biotechnology, Mississauga, ON, Canada) for 2 h at room temperature and finally revealed using an en- hanced chemiluminescence substrate solution (ThermoFisher Scientifics, Ottawa, ON, Canada). All antibodies were diluted in 1% non-fat powdered milk, 1% BSA, 0.05% Tween 20, and 0.5 mg/mL sodium azide, in TBS. Immunopositive chemilu- minescent signals were visualized and analyzed with a ChemiDoc Imaging System (Biorad, Canada). Densitometric blot analyses were performed using ImageJ (https://imagej. nih.gov/ij/) software packages. β-actin blots served as a loading comparative standard and all immunoblotting results have been normalized to β-actin levels.

RNA Extraction, cDNA Synthesis and iNOS Gene Expression by qRT-PCR

N9 microglial cells were grown and treated with LPS, OA, PA, or PA+OA for 3.5 h, as described above. Cells were lysed and RNA extracted with 400 µl TRIzol (Invitrogen, Canada) following the manufacturer's protocol. One microgram of RNA was reverse transcribed following SensiFASTTM cDNA Synthesis Kit protocol (Biolines, London, UK). qPCR was performed with Sensifast SYBR no-ROX kit (Biolines, London, UK): 5 µl of SYBR mix, 4 µl of cDNA, and 1 µl of each forward and reverse primer in a total volume of 10 µl. Primer sequences were as follows: iNOS (5′-AGGA GGAGAGAGATCCGATTTAG-3′ and 5′-TCAG ACTTCCCTGTCTCAGTAG-3′), β Actin (5′-TCCC TGGAGAAGAGCTATGAG-3′ and 5′-CAGG ATTCCATACCCAAGAAGG-3′), TBP (TATA-box-bind- ing protein) (5′-AGTGCCCAGCATCACTATTTC-3′ and 5′-CTGGTCCATGATTCTCCCTTTC-3′). Primers were synthetized at Integrated DNA Technologies (Coralville, Iowa, USA). The standardized amplification protocol was car-ried out in a Bio-Rad Thermal cycler T100 (BioRad, Mississauga, Ont., Canada) consisted of initial denaturation at 95° C for 2 min, followed by 40 sequential cycles at 95° Cfor5s,61°Cfor10s,and72°Cfor8s.Thecomparative delta-Ct method was used for quantification as all data are expressed as fold differences in Ct between control and treat-ment after normalization to the reference genes TBP and β actin. An internal control without RT was also performed to detect genomic DNA impurities.

Statistical Analysis

Statistical difference was assessed using one-way ANOVA followed by Tukey's post hoc test, or Student's t-test when comparing two groups. All data were analyzed at the 95% confidence interval and expressed as mean ± standard error of mean (SEM) of 3–6 separate experiments. A p-value < 0.05 was considered as significant.

Results

Microglial N9 Cells Survival After LPS or PA Treatment

To determine whether LPS or PA may induce toxicity in microglial cells, we treated N9 microglial cells with LPS or PA for 6 or 24 h and measured cell metabolic activity by MTT, which indirectly reflects cell viability (Fig. 2a–d). Neither LPS nor PA affects N9 cellular metabolic activity compared to medium (Fig. 2a) or BSA (Fig. 2b) respective- ly, thus suggesting that LPS or PA did not induce cellular death when administered for 6 h. However, a 24-h PA chal- lenge resulted in a marked decrease in cell metabolic activ- ity, indicating a substantial increase in cellular death (Fig. 2d). This effect was absent in cells challenged with LPS for 24 h.

LPS and PA-Treated N9 Microglial Cells Induces Neuronal PC12 Cellular Death in Co-culture

Co-culture of microglial N9 cells with neuronal PC12 demon- strated that the humoral environment of activated N9 microglial with LPS or PA can disrupt neuronal PC12 homeo-stasis, after 24 h of co-culture. Indeed, LPS-activated N9 microglial cells for 6 h significantly ($p < 0.05$) decreased neu- ronal cell survival after 24 h in co-culture, as illustrated by a decrease in neuronal PC12 metabolic activity (Fig. 3a) and by the parallel increase ($p < 0.05$) in LDH release in the cell culture media (Fig. 3b). When N9 microglial cells were chal- lenged with OA in combination with LPS for 6 h, neuronal PC12 metabolic activity returned to control levels (Fig. 3a) and there was no evident neuronal cytotoxicity as measured by LDH assay (Fig. 3b). Similar to the effect exerted by LPS, when PA was used to activate N9 microglial cells for 6 h, which were then co-cultured with neuronal PC12 cells for additional 24 h, it induced a decline ($p < 0.001$) in neuronal PC12 metabolic activity (Fig. 3c) supported by a marked increase in ($p < 0.001$) LDH release from neuronal PC12 (Fig. 3d), compared with BSA, indicating that PA may induce neurotoxicity indirectly by modulating the microglial secretory milieu. Interestingly, OA, when ad- ministered with PA (OA+PA), prevented the decrease in metabolic activity induced by PA (Fig. 3c) and reduced ($p < 0.05$) neuronal cytotoxicity relative to PA-treated cells (Fig. 3d), supporting a protective role for OA in these co- culture systems.

Short-Term or Long-Term Treatment with LPS or PA Differently Affect IL-6 and TNF-α Secretion

To elucidate whether activated N9 microglial cells may re- lease pro-inflammatory mediators in response to LPS or fatty acids challenge, we quantified the secretion of IL-6 and TNF-α by ELISA assay after 6- or 24-h challenge. We select- ed IL-6 and TNF-α as they are the major effectors of the neuroinflammatory cascade.

LPS induced a significant release of both IL-6 and TNF-α after a 6-h challenge (Fig. 4a and 4b) from N9 microglial cells, compared with medium ($p < 0.001$). However, the co-administration of OA alongside LPS inhibited or tended to inhibit the release of IL-6 and TNF-α, respectively ($p< 0.005$ and $p=0.051$, respectively), compared with LPS alone. On the contrary, a 6-h fatty acid challenge did not affect the secretion of either IL-6 or TNF-α relative to BSA (Fig. 4c and d). In Fig. 5, the administration of LPS for a continuous 24-h period resulted in a strong release of IL-6 and TNF-α, with OA significantly reducing the level of IL-6 compared to LPS ($p < 0.05$) or preventing the upregulation of TNF-α release when co-administered with LPS (Fig. 5a and b). In addition, a 24-h continuous administration of PA tended to promote IL-6 secretion ($p = 0.0574$) and significantly upregulated the secre- tion of TNF-α ($p < 0.05$), compared with BSA (Fig. 5c and d), suggesting that PA promotes the secretion of cytokines. This effect was absent in cells treated with OA alone or PA+OA (Fig. 5c and d), confirming that OA does not promote cyto- kine secretion and can counteract the PA-triggered inflamma- tory stimulus.

PA Directly Induces Neuronal PC12 Cellular Death

PA also directly impacted upon neuronal PC12 homeostasis by inducing cellular death after 6 and 24 h of treatment (Fig. 6a and b). MTT experiments revealed a significant decrease in neuronal PC12 metabolic activity after 6 and 24 h of treatment with PA ($p < 0.001$) (Fig. 6a and b). These results support a direct neurotoxic effect of PA on neurons on a short period. Moreover, the administration of OA alone did not affect neu- ronal PC12 cellular metabolic activity, while it partially inhibited the neurotoxic effects exerted by PA when co- administered with it (PA+OA) (Fig. 6a and b), thus supporting a neuroprotective role for OA.

PA Induces Intracellular Inflammatory Pathways in Microglia Cells

Considering that in our model PA did not induce the secretion of pro-inflammatory cytokines after a 6-h treatment, we also investigated whether PA may modulate intracellular pro- inflammatory signalling pathways and pro-inflammatory en- zymes. Particularly, we assessed the activation of pro- inflammatory signalling pathways, P-p65 and P-c-Jun, and the expression of a major pro-inflammatory enzyme, iNOS, in N9 microglia cells treated with fatty acids for 3.5 h. c-Jun is an early gene which is activated transiently and rapidly in response to a wide variety of cellular stimuli. The expression of iNOS is also very rapid in N9 cells as we demonstrated [50]. NfK-B is also transiently activated in microglial cells [57, 58]. Particularly, p65 and c-Jun are key nodes in the NF-κB and JNK signalling pathways, respectively, and their phosphorylation is synonymous with the activation of these pro-inflammatory pathways. PA induced a significant in- crease of P-p65 ($p < 0.005$) (Fig. 7a) and c-Jun phosphoryla- tion compared to BSA-treated cells ($p < 0.001$) (Fig. 7b). Contrarily to PA, OA did not affect on these markers of in- flammation, suggesting that it does not activate these intracel- lular pro-inflammatory cascades as opposed to PA. Conversely, the presence of OA in the mixture PA+OA partially reduced these markers of inflammation compared to PA(Fig.7aandb)($p<0.05$forP-p65)and($p<0.001$forP-c- Jun), supporting the notion that OA acts as an anti- inflammatory molecule. PA, however, did not induce the expression of iNOS compared with BSA, with this effect being absent also in the cells treated with OA and PA in combination with OA (Fig. 7c). Nonetheless, LPS induced iNOS mRNA levels compared to the other treatments (Fig. 7c) (all $p < 0.005$).

Discussion

In this study, we provided evidence of the neurotoxic and pro- inflammatory effects that PA, the most abundant SFA in the Western diet, exerts on microglial N9 and neuronal PC12 cells directly exposed to this SFA. Furthermore, we demonstrated that the cytotoxic effects of PA on PC12 neurons also occur indirectly and are mediated by N9 microglial cells, which once activated by PA, induce neuronal death via a microglial- neuron co-culture system. Importantly, these effects overlap with those elicited by LPS, a well-known pro-inflammatory agent, and both the direct and indirect cytotoxic effects of PA are inhibited by the mono-unsaturated fatty acid OA which, instead, is known for its anti-inflammatory properties [12, 34, 59]. Then, despite inflammation may represent a key process in mediating the neurotoxic effects of PA, we highlighted the different inflammatory responses triggered by PA and LPS in N9 microglia cells proposing that the indirect cytotoxic effect exerted by PA on neuronal PC12 may be independent from IL-6 and TNF-α secretion from N9 microglial cells.

Although inflammatory responses may be protective at ear- ly stages, exacerbated and prolonged neuroinflammation is emerging as a pivotal pathogenetic factor promoting neurode- generation, with microglia playing a central role in this pro- cess [60]. Indeed, sustained or uncontrolled activation of microglial cells leads to chronic neuroinflammation, which can prompt significant neuronal damage and loss, and has been associated with

several neurodegenerative disorders, in- cluding AD and PD [61–63]. Although our in vitro model could not perfectly replicate in vivo microglia physiology, our data confirm the ability of LPS to induce neurodegeneration as indicated by the neurotoxicity elicited by LPS-activated microglial cells on PC12 neurons, with in- flammation being a hallmark of this process. This is in line with the ability of LPS to recapitulate PD pathophysiology in rodents leading to microglial inflammation, which, in turn, represents a key pathogenetic feature contributing to dopami- nergic neuronal degeneration [64]. The central role of inflammation in mediating the neurotoxic effect of LPS is further supported by our study, as indicated by the inflammatory re- sponse triggered by LPS in N9 microglia cells and marked by an increase in IL-6 and TNF-α secretion, two key pro- inflammatory cytokines. These pro-inflammatory mediators may be held responsible for neuron degeneration and loss in consideration of their ability to trigger the activation of the apoptotic cascade in downstream neurons [65]. Furthermore, the pro-inflammatory effect of LPS was inhibited by OA, which was paralleled by the prevention of the neurotoxic ef- fects exerted by LPS-activated microglia on neuronal PC12 cells, confirming the role of inflammation as a key contributor to neurotoxicity.

Long-chain saturated fatty acids, such as PA, are known to trigger inflammatory pathways in peripheral cells [16, 24, 26, 66] as well as in cells in the CNS, including microglia [13, 14, 67, 68] also in concert with sugar [69, 70]. Thus, once con- firmed the relationship between inflammation and neurotox- icity in our co-culture model, we investigated whether PA could recapitulate the neurotoxic effects of LPS. Actually, PA mirrored the neurotoxic effect of LPS on neuronal PC12 cells when it was used to activate N9 microglia in co-cultures. However, PA, contrarily to LPS, did not increase the secretion of pro-inflammatory cytokines by N9 cells after 6 h of treatment, suggesting that it may exert its indirect cytotoxic effect on PC12 neurons calling upon different mechanisms compared to LPS. These putative mechanisms may include the release of reactive oxygen species by microglia cells in response to PA, which represent a shared mediator of the cytotoxic effects of both LPS and PA. Indeed, increased oxi- dative stress has been reported to contribute to LPS-induced neurodegeneration [64] and to represent one of the manifestations of the lipotoxic effects of PA [63]. Nonetheless, despite PA challenge did not result in an increase in pro-inflammatory cytokine secretion by N9 cells after an acute stimulation in our cellular paradigm, it still induced the phosphorylation of c- Jun, a component of the transcription factor AP-1, and the activation of the NF-κB signalling pathway as indicated by the increase in p65 phosphorylation. Both transcription factors are activated in response to pro-inflammatory stimuli and pro- mote the expression of pro-inflammatory genes, including cy- tokines [71–74], confirming the ability of PA to trigger an inflammatory response in microglial cell, with the secretion of cytokines occurring at a later time point, as we demonstrat- ed after a 24h PA challenge. Furthermore, the inability of PA to induce the secretion of pro-inflammatory cytokines, at least after 6-h incubation or pre-stimulation, indicates that the in- flammatory response induced by this long-chain saturated fat- ty acid differs in nature from that elicited by LPS. Indeed, while LPS is a known activator of the innate immune re- sponse, being an endotoxin and a known component of the cell wall of Gram-negative bacteria, PA triggers a so-called metabolic inflammation which substantially differs from the classical inflammation activated by infection and injury [75, 76]. This difference is further confirmed by the ability of LPS but not PA to induce the expression of iNOS.

Nonetheless, treatment of N9 cells for 24 h with PA result- ed in an increase in IL-6 and TNF-α secretion, which however was paralleled by marked decrease in cellular metabolic activ- ity. Despite the levels of these constitutively secreted cyto- kines are predominantly regulated at the transcriptional and/ or translational level, their release from microglial cells

may also be dependent on the modulation of post-Golgi trafficking machinery [77] and may be differently regulated by PA and LPS. Indeed, the increase of cytokines in response to PA only appears evident when significant cell death occurs, suggesting this may be the result of cytokine release from death cells, in our in vitro model.

The cytotoxic effects we observed following the activation of microglia in culture by PA were fatty acid specific. Indeed, OA despite being used at the same concentrations as PA, not only failed to induce downstream neurotoxicity but also prevented the lipotoxic effect of PA mediated by the microg- lia. This neuroprotective effect may be ascribed to the anti-inflammatory effects of OA which have been previously de- scribed [12, 34, 78] as well as reported in the present study, with OA preventing the activation of the NF-κB pathway and

the phosphorylation of c-Jun by PA. Besides, OA, as de- scribed for PA, exerted a neutral effect on cytokines secretion at 6 h and it did not increase the phosphorylation of p65 and c- jun. Thus, while PA represents a metabolic insult able to dis- rupt cellular homeostasis and triggers the activation of inflam- matory pathways, this was not recapitulated by OA which instead exploit anti-inflammatory effects. The divergent effect of these fatty acids on inflammation may be related to their intracellular metabolic fate. OA compared to PA is β-oxidized more effectively [79] which prevents intracellular lipotoxicity typical of PA overload, characterized by oxidative stress, ac- cumulation of diacylglycerol as well as ceramides, and endo- plasmic reticulum stress, all of which have been implicated in the activation of pro-inflammatory pathways [12, 80–83]. Furthermore, PA compared to OA is poorly incorporated into triglycerides [84] causing excess PA to be shifted towards the synthesis of lipotoxic lipid species which are detrimental to normal cellular homeostasis and, not surprisingly, may con- tribute to neurodegenerative and neuroinflammatory disorders [85]. The modulation of the intracellular metabolic fate of PA may also be responsible for the ability of OA to counteract cytotoxic and the pro-inflammatory effects of this long-chain saturated fatty acid. Indeed, while PA has been reported to increase both intracellular diacylglycerol and ceramides, which, in turn, activate pro-inflammatory pathways [12, 83], OA has been reported to promote both PA incorporation into triglycerides [83] and its mitochondrial β-oxidation [86] thereby preventing the cytotoxic and pro-inflammatory effects linked with PA excess. Another potential mechanism respon- sible for the divergent effect of PA and OA on inflammation is dictated by the putative ability of PA to act as the Toll-like receptor 4 (TLR4) ligand [87], but this remains controversial, with other reports denying this possibility [12, 88, 89].

Our results also demonstrated that PA exerts direct neuro- toxic effects on PC12 neuronal cells, with this effect being specific to PA and absent in cells exposed to OA only. This further supports the notion that neurotoxicity is fatty acid spe- cific. Furthermore, PA-induced neurotoxicity is inhibited by equimolar co-administration of OA which is in line with our results obtained using the co-culture model system and agrees with the neuroprotective and neurotoxic effects exploited by OA [90] and PA [91, 92], respectively. Interestingly, the abil- ity of PA to decrease the reduction of MTT by neuronal mi-tochondria is more marked when administered directly to PC12 neurons compared to its indirect effects via the microg- lia, in our co-culture model. This is in agreement with the ability of PA to impair mitochondrial function and confirms the capacity of OA to prevent PA-induced mitochondrial dys- function [84]. Particularly, the potential of OA to prevent mi-tochondrial dysfunction may be instrumental in preventing the cytotoxic effects of PA by preserving mitochondrial oxidative capacity and allowing the cells to effectively β-oxidise PA. The catabolism of PA, in turn, prevents its lipotoxic effect, as previously reported in neurons, which includes the activation of inflammatory response, cytotoxicity, endoplasmic reticu- lum stress, oxidative stress, caspase activation, and mitochon- drial dysfunction [12, 40, 80, 81, 90, 93–95]. As already de- scribed for the indirect protective effect of OA,

channelling excess PA towards triglycerides synthesis represents another putative mechanism underlying the protective role of OA [84].

Despite describing both the direct and indirect cytotoxic effects of PA, this study does not address the molecular path- ways responsible for the direct and indirect effects of this long-chain saturated fatty acid. These effects may relay on the activation of key pro-inflammatory pathways such as NFκB and JNK, the interaction between PA and the TLR4, or the intracellular build-up of lipotoxic lipid species, includ- ing ceramides. Therefore experiments using specific inhibitors or KO models are required to shed the light on the mecha- nisms underpinning PA-induced toxicity. Another aspect that should be addressed in future works is the characterization of the N9 secretory milieu in order to pinpoint the molecules mediating the indirect effect of PA on PC12 cells. Nonetheless, to our knowledge, our study shows for the first time that PA can exert cytotoxic effects on PC12 neurons via microglia, with other studies conducted to date only focusing on the effect of PA on PC12 in isolation [96]. Indeed, the present results provide novel insights on the ability of PA to trigger cytotoxic response, not only by directly targeting neu- rons but also in a paracrine fashion via the microglia.

Importantly, despite our study reports on the effects of fatty acids in cellular cultures, these findings are physiologically relevant. Indeed, the concentrations of fatty acids used in the present study fall within the circulating concentrations of PA and OA reported in overweight/obese subjects affected by T2DM [97], with OA being associated with improved meta- bolic health, while PA exerting metabolically detrimental ef- fects [98]. In further support of the physiological relevance of the present study, PA can be taken up by the brain, its trans- port into the brain increases in individuals with the metabolic syndrome [33] and dopaminergic neurons are able to take up fatty acids [99]. Moreover, PA is enriched in the cerebrospinal fluid of obese humans and correlated with memory impair- ment, and in mice PA impaired memory and synaptic plastic- ity via a mechanism which involves the activation of microg- lia and the secretion of TNF-α [100]. These evidence also provide further support to the link, identified herein, which bridges the gap between PA, microglial activation, inflamma- tion, and neuronal damage.

To conclude, our results illustrate that PA exerts both direct and indirect neurotoxic effects, with the indirect effects being tightly linked with the activation of pro-inflammatory path- ways in microglia cells possibly independently of an increase in cytokines secretion. Thus, PA represents one of the putative nutrients, which, in the context of the Western diet, concur in the development of neurodegeneration. Finally, nutritional interventions aimed at decreasing PA while increasing OA intake should be strongly advocated as they may represent a promising strategy in the prevention of neurodegenerative disease.

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Figure Legends

Figure 1. Schematic representation of an insert co-culture system. A) Microglial N9 cells are grown and treated in an insert resting on the edges of a multiwell plate. The upper compartment is composed of the insert, which holds microglial N9, and its humoral environment. The lower compartment contains medium only. B) After the appropriate treatment, microglial N9 cells are transferred on a well containing neuronal PC12 differentiated for 8 days with NGF, as described in Material and Methods. Microphotographs of A) activated N9 microglia treated with LPS for 6 hours displays an amoeboid shape typical of the activated phenotype. B) Eight-day nerve growth factor-differentiated neuronal PC12 cells show obvious neuronal phenotypes such as long neurites and varicosities. Scale bar = 25 µm.
Figure 2. Microglial N9 metabolic activities after for 6 or 24 hours of treatment with LPS or FA as measured by MTT assay A) Histogram of N9 microglial cells survival after a 6-hour treatment with LPS. Data are presented as % of medium. B) Histogram of N9 microglial cells survival after a 6-hour treatment with FA. Data are presented as % of BSA. C) Histogram of N9 microglial cells survival after a 24-hour treatment with LPS. Data are presented as % of medium. B) Histogram of N9 microglial cells survival after a 24-hour treatment with FA. Data are presented as % of BSA. All data (N=9) are presented as mean ± SEM. *** represent significant variations, $p < 0.001$ versus BSA.
Figure 3. Effect of LPS- and FA-activated N9 microglial cells co-cultured on PC12 neuronal cells for 24 hours. Histograms of the MTT assay of A) LPS-activated N9 microglial cells co-cultured on PC12 neuronal cells, where data are represented as % of medium, or C) FA-activated N9 microglial cells co-cultured on PC12 neuronal cells, where data are represented as % of BSA. Histograms of the LDH assay of B) LPS-activated N9 microglial cells co-cultured on PC12 neuronal cells, where data are represented as % of medium, or D) FA-activated N9 microglial cells co-cultured on PC12 neuronal cells, where data are presented as % of BSA. All data (N=5-7) are presented as mean ± SEM. *, ** and *** represent significant variations, $p < 0.05$, $p < 0.005$ and $p < 0.001$ versus respective control, medium for LPS and BSA for fatty acids.
Figure 4. Effect of LPS and FA on IL-6 and TNF-α release from N9 microglial cells after 6 hours of treatment. Histograms of the release of A) IL-6 or B) TNF-α from N9 microglial cells 6 hours after treatment with medium, LPS or LPS+OA. Data are represented as % of medium. Histograms of the release of C) IL-6 or D) TNF-α from N9 microglial cells 6 hours

after treatment with BSA, PA, OA, or OA+PA. Data are represented as % of BSA. All data (N=9) are presented as mean ± SEM. ** and **** represent significant variations, $p < 0.005$ and $p < 0.0001$ versus respective control, medium for LPS and BSA for fatty acids.

Figure 5. Effect of LPS and FA on IL-6 and TNF-α release from N9 microglial cells after 24 hours of treatment. Histograms of the release of A) IL-6 or B) TNF-α from N9 microglial cells 24 hours after treatment with medium, LPS or LPS+OA. Data are represented as % of medium. Histograms of the release of C) IL-6 or D) TNF-α from N9 microglial cells 24 hours after treatment with BSA, OA, PA or OA+PA. Data are represented as % of BSA. All data (N=9) are presented as mean ± SEM. * and *** represent significant variations, $p < 0.05$ and $p < 0.001$, versus respective control, medium for LPS and BSA for fatty acids.

Figure 6. PA directly affects neuronal PC12 cell survival. Histograms of the MTT assay of A) PC12 neuronal cells 6 hours after treatment with PA, OA or PA+OA, or B) PC12 neuronal cells 24 hours after treatment with PA, OA or PA+OA. Data are represented as % of BSA. All data (N=9) are presented as mean ± SEM. *, ** and *** represent significant variations, $p < 0.05$, $p < 0.005$ and $p < 0.001$ versus BSA.

Figure 7. PA modulate intracellular pro-inflammatory signalling pathways. Immunoblotting analyses illustrating increases in expression of microglial pro-inflammatory markers of A) P-p65, B) P-c-Jun, as compared with β-actin expression in N9 microglial cells 3.5 hours after exposure to BSA, PA, OA or PA+OA. C) iNOS mRNA expression as revealed by qRT-PCR in N9 microglial cells 3.5 hours after exposure to BSA, PA, OA or PA+OA. Bottom panel: representative images of immunoblots. Data are represented as % of BSA. All data (N= 9) of 3 different experiments) are presented as mean ± SEM. from three separate experiments. *, ** and *** represent significant variations, $p < 0.05$, $p < 0.005$ and $p < 0.001$ versus BSA.