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Potential of omega-3 and conjugated fatty acids to control microglia inflammatory imbalance elicited by obesogenic nutrients

A.S. Salsinha [a,b], R. Socodato [b], A. Rodrigues [b], R. Vale-Silva [b,d], J.B. Relvas [b,c], M. Pintado [a,*], L. M. Rodríguez-Alcalá [a,*]

[a] Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina, Laboratório Associado, Escola Superior de Biotecnologia, Rua de Diogo Botelho, 1327, 4169-005 Porto, Portugal
[b] Instituto de Investigação e Inovação em Saúde (i3S) and Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, 4200-135 Porto, Portugal
[c] Departmento de Biomedicina, Faculdade de Medicina da Universidade do Porto (FMUP), 4200-319 Porto, Portugal
[d] Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, 4050-313 Porto, Portugal

A B S T R A C T

High-fat diet-induced obesity detrimentally affects brain function by inducing chronic low-grade inflammation. This neuroinflammation is, at least in part, likely to be mediated by microglia, which are the main immune cell population in the brain. Microglia express a wide range of lipid-sensitive receptors and their activity can be modulated by fatty acids that cross the blood-brain barrier. Here, by combining live cell imaging and FRET technology we assessed how different fatty acids modulate microglia activity. We demonstrate that the combined action of fructose and palmitic acid induce Ikβα degradation and nuclear translocation of the p65 subunit nuclear factor kB (NF-κB) in HCM3 human microglia. Such obesogenic nutrients also lead to reactive oxygen species production and LynSrc activation (critical regulators of microglia inflammation). Importantly, short-time exposure to omega-3 (EPA and DHA), CLA and CLNA are sufficient to abolish NF-κB pathway activation, suggesting a potential neuroprotective role. Omega-3 and CLA also show an antioxidant potential by inhibiting reactive oxygen species production, and the activation of LynSrc in microglia. Furthermore, using chemical agonists (TUG-891) and antagonists (AH7614) of GPR120/FFA4, we demonstrated that omega-3, CLA and CLNA inhibition of the NF-κB pathway is mediated by this receptor, while omega-3 and CLA antioxidant potential occurs through different signaling mechanisms.

1. Introduction

The prevalence of obesity and overweight is increasing worldwide, having almost tripled since 1975. Even though new strategies devised to fight obesity emerged in the last few years, data from the Organization for Economic Co-operation and Development (OECD) show a steady increase in obesity rates until 2030 [1]. Indeed, epidemiologic studies have identified obesity (high body mass index, BMI > 30) as a risk factor for cardiovascular disease [2,3], diabetes mellitus, chronic kidney disease [2], cancer [4], and several musculoskeletal disorders [5,6]. Obesity poses a high economic burden closely connected to the associated health effects – cardiovascular diseases present an estimated burden of €210 billion/year, and diabetes will have an economic burden of US$ 2,1 trillion by 2030 [7]. Thus, a growing number of resources are being applied to develop anti-obesity drugs (*e.g.* orlistat, Sibutramine, Lorcaserin/Belviq, *etc.*). However, such efforts have been associated

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with systematic failure as most of the treatments are not effective and the sufficiently efficient ones are related to severe health side effects, being the most common ones flatulence, oily spotting, fecal urgency, fatty/oily stool, oily defecation, increased defecation and fecal incontinence and other adverse effects such as nephrotoxicity, hepatotoxicity, nephrolithiasis and pancreatitis [8]. Moreover, sibutramine and Lorcaserin/Belviq were withdrawn from the European market by the European Medicine Agency (EMA). The Food and Drug Administration (FDA) has also withdrawn Sibutramine from the United States' market and requested the withdrawal of Lorcaserin/Belviq due to the associated side effects [7]. One reason behind such failures might be the lack of knowledge of how obesity impacts other organs, besides adipose tissue, especially the brain [9], in which the hypothalamus has a fundamental role in regulating hunger and satiety. Indeed, lesions in specific regions of rat's hypothalamus lead to cessation of feeding and subsequent death by starvation [10–12]. Thus, there is a need to better understand the effects of obesity, namely high-fat diets (HFDs) on hypothalamus.

Processed foods, rich in saturated fats, are a significant part of the diet in western countries. Such diet – the western pattern diet (WPD) – is considered a relevant factor for the progression of obesity [13,14]. Besides consumption of high-glycemic/high-insulinemic carbohydrates, including sugars (mainly sucrose and fructose), WPD is also characterized by high-fat levels, primarily saturated fatty acids (SFAs) and trans fats [13,15]. Several *in vivo* studies show that HFD-induced obesity detrimentally affects brain function, including synaptic plasticity and cognitive performance. In models of diet-induced obesity, the consumption of an HFD increased cytokine expression, c-Jun N-terminal kinases (JNK), and inhibitor of kappa B kinase (IKK) activation in both the liver and hypothalamus [16,17]. Besides, obesity-induced oxidative stress causes inflammatory reactions resulting in abnormalities in protein, lipid, DNA function, brain aging, and cognitive impairment. Indeed, HFD disrupts intracellular cascades involved in synaptic plasticity and insulin signaling/glucose homeostasis, increases corticosterone levels, and activates the innate immune system [18]. As a result, HFD is commonly associated with chronic, low-grade inflammation in the adipose tissue and in the central nervous system (CNS) [19–21]. High-fructose feeding has been used as an efficient method to establish metabolic syndrome. Indeed, in rodent models, high fructose-fed rats often exhibit hypertension, insulin resistance, impaired glucose tolerance, dyslipidemia and obesity. Interestingly, compared to glucose, fructose-fed rats show worse features of metabolic syndrome [22,23]. Identical results are observed in overweight humans where the administration of fructose containing-beverages causes more visceral obesity and insulin resistance compared to glucose groups [24]. Moreover, fructose has been shown to increase tumor necrosis factor (TNF)-α concentration and to activate cellular stress responses and reactive oxygen species production (ROS) in animal models [25,26]. Interestingly, in contrast to glucose, CNS delivery of fructose in rodent models promotes feeding behavior and it seems to be an endogenous production of fructose from glucose in CNS, suggesting that fructose effects in CNS may extend beyond its direct dietary consumption [27]. Moreover, it has been reported that a potential action of fructose on brain can be manifested, specifically, in alterations in neuronal and glia cells [28] and one study has reported that short-term consumption elevated GLUT5 levels in the hippocampus [29].

Importantly, microglia are the principal immune resident cells of the CNS, representing 5 to 10 % of the total brain cells [30]. In response to external stimuli, microglia can initiate a neuroinflammatory response which, similar to peripheral inflammation, includes the production of cytokines such as TNF-α and IL-1β, and several chemokines. Bioactive fatty acids resulting from nutrition can cross the blood-brain barrier (BBB) and reach the CNS, specifically the hypothalamus, with the potential to modulate microglial activity [31,32]. Microglia directly respond to LC-SFAs (*e.g.*, palmitic acid, PA), functioning as a sensor capable of initiating inflammation in response to increased saturated fatty acids (SFAs) in the hypothalamus [21,33,34]. Accordingly,

microglia express a wide range of lipid metabolism-related genes and lipid-sensitive receptors, including toll-like receptors (TLRs) [35]. Fatty acids act predominantly through TLR 4 [33] and hippocampal TLR 4 expression is increased under chronic HFD exposure [18].

The harmful effects presented by SFAs may be reversed by other fatty acids, including polyunsaturated fatty acids (PUFAs). Recent studies showed that supplementing rats' diet with fish oil has beneficial effects by modulating hypothalamic inflammation and attenuating SFA-induced abnormal behavior, inflammatory response, oxidative reactions and neuronal apoptosis [36–38]. Moreover, the intracerebroventricular administration of docosahexaenoic acid (DHA), reduces energy intake, body weight gain and HFD-induced hypothalamic inflammation [39]. Several receptors, including G-protein coupled receptors (GPCR) GPR40/FFA1 and GPR120/FFA4 (from now on simply mentioned as GPR120), can be activated by free fatty acids, namely LC-PUFAs [40,41]. By signaling through GPR120, omega-3 fatty acids, DHA, and eicosapentaenoic acid (EPA) produce anti-inflammatory effects [36,41–46]. Attention has been given to other PUFAs that may induce similar effects. For instance, the dietary fatty acids conjugated linoleic acid (CLA) and conjugated linolenic acid (CLNA) can decrease the production of several pro-inflammatory agents, including TNF-α, PGE2, nitric oxide (NO), IL-1, and IL-6 in adipose tissue [47,48].

CLA, mostly *cis*-9, *trans*-11 (C18:2 *c*9,*t*11) and *trans*-10, *cis*-12 (C18:2 *t*10,*c*12), and CLNA (punicic acid - PUA- C18:3 *c*9,*t*11,*c*13) isomers, have anti-inflammatory properties mediated, at least in part, by the nuclear hormone receptor peroxisome proliferator-activated receptor (PPAR)-γ in adipocytes [48–52]. PPARs are members of the nuclear receptor superfamily of ligand-dependent transcription factors, and PPAR-γ is highly expressed in adipose tissue, adrenal gland, colon, and macrophages [53]. Despite the increasing knowledge of conjugated fatty acids (CFA) beneficial effects, little is known about their mechanism of action. Although several studies show evidence supporting the anti-inflammatory properties of CLA and CLNA, it remains elusive how it affects the immune system, and very few studies have specifically addressed their role on hypothalamic inflammation [49,54,55].

As stated, fatty acids, namely SFAs and omega-3 fatty acids, can exert pro- and anti-inflammatory effects in the hypothalamus, respectively. Nevertheless, the exact mechanisms behind such effects are incomplete and the role of other fatty acids, such as CFAs is still elusive. In this work, using an human microglia cell model, through live cell imaging and Fluorescence resonance energy transfer (FRET) technology, we studied the potential role of omega-3 fatty acids and CFAs - C18:2 *c*9,*t*11 and C18:2 *t*10,*c*12 CLA isomers and C18:3 *c*9,*t*11,*c*13 CLNA isomer - in modulating microglia inflammation triggered by obesogenic nutrients (SFAs and fructose).

2. Material and methods

2.1. Reagents

PA, D-(-)-Fructose and Rumenic acid (RA, C18:2 *c*9*t*11) were purchased from Sigma-Aldrich (Missouri, USA); EPA, DHA, the C18:2 *t*10*c*12 CLA isomer and PUA (C18:3 *c*9*t*11*c*13) were purchased from Larodan AB (Solna, Sweden). The cell culture reagents: Dulbecco's modified Eagle's medium (DMEM) + GlutaMAX™-I, fetal bovine serum (FBS), penicillin, streptomycin and HBSS supplemented with CaCl₂ and MgCl₂ and the PrestoBlue reagent were purchased from Thermo Fisher Scientific (Massachusetts, USA). The transfection reagent jetPRIME® was purchased from Polyplus-Transfection SA (Illkirch, France). The ethanol absolute anhydrous, used as the fatty acid's vehicle, was purchased from Carlo-Erba (Barcelona, Spain). Both GPR120 agonist, TUG-891 (*ortho*-biphenyl ligand 4-{[4-fluoro-4′-methyl(1,1′-biphenyl)-2-yl] methoxy}-benzenepropanoic acid), and antagonist, AH7614 (4-methyl-N-9H-xanthen-9-yl-benzenesulfonamide), were purchased from Sigma-Aldrich (Missouri, USA). Regarding the western-blot reagents, both RIPA buffer (R0278), protease inhibitor cocktail (P8340) and

Ponceau S dye (78376) were from Sigma-Aldrich. The primary antibodies used were as follows: GPR120 (extracellular) Polyclonal Antibody (PA5-111778) from Sigma-Aldrich and Anti-GLUT5 antibody (ab279363) from Abcam. The 0.45 μm nitrocellulose blotting membranes (10600002) were purchased from Amersham Protran. The SuperSignal West Pico Plus Chemiluminescent Substrate (34580) was from ThermoFisher.

2.2. Human microglia clone 3 cell line

The human microglia clone 3 (HMC3) cell line (ATCC CRL-3304) was obtained from primary cultures of human embryonic microglial cells and immortalized through transfection with a plasmid encoding for the large T antigen of SV40 [56]. These cells were cultivated with DMEM + GlutaMAX™-I and supplemented with 10 % FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were maintained at 37 °C, 95 % air and 5 % CO_2 in a humidified incubator [57–59].

2.3. Fatty acids solutions

The fatty acids stock solutions (PA and Fructose, RA, EPA, DHA, the C18:2 *t*10*c*12 CLA isomer and PUA) were prepared at 100 mM and dissolved in ethanol absolute anhydrous. The stock solutions were maintained up to a month at -20 °C and were stored under a nitrogen atmosphere. The work solutions were freshly prepared according to Table 1 in HBSS with $CaCl_2$ and $MgCl_2$ and added to the cells as a final concentration of 100 μM. The selected fatty acids' concentrations were based on previous *in vitro* studies. For instance, a 100 μM PA concentration has been reported by several studies, as inducing the pro-inflammatory responses without presenting cell toxicity [34,35,60–63]. Regarding PUFAs, 50-200 μM has been reported as a bioactive concentration for omega-3 fatty acids, specifically EPA and DHA [41,44,62,64,65], for CLNA isomers (C18:3 *c*9,*t*11,*c*15 and C18:3 *c*9,*t*13,*c*15 isomers) [66] and for CLA isomers (including C18:2 *c*9,*t*11 and C18:2 *t*10,*c*12 isomers) [48]. Thus, 100 μM was selected as the studied concentration for both PA and PUFAs (omega-3 and CLA) and 50 μM for the CLNA isomer. Fructose has been reported with no

Table 1-
Fatty acids solutions composition.

Solution ID	Fatty acids	Other Components	Final Proportion	Final concentration added to the cells
Stimulus solution (western pattern diet)	Palmitic Acid (C16:0) (≥99 % purity)	D-(-)-Fructose (≥99 %)	2:1	
Omega 3	EPA (C20:5 n-3) (90 % purity)	–	1:1	
	DHA (C22:6 n-3) (≥99 % purity)	–		100 μM
CLA	Rumenic acid (C18:2 *c*9*t*11) (≥96.0 % purity)	–	1:1	
	C18:2 *t*10*c*12 CLA isomer (>98 % purity)	–		
CLNA	Punicic Acid C18:3 *c*9*t*11*c*13 (>98 % purity)	–	–	50 μM

cytotoxic effects in higher doses (5-50 mM) in several different cell lines: caco-2 cells [67], cholangiocyte and cholangiocarcinoma cell lines [68] and macrophages [69]. In microglia there are reports of use of glucose in similar doses (17.5 or 25 mM) [70].

2.4. Cytotoxicity analysis

The cytotoxicity of the fatty acids solutions (PA and Fructose, RA, EPA, DHA, the C18:2 *t*10*c*12 CLA isomer and PUA) was evaluated using the PrestoBlue reagent, according to the manufacturer's instructions. The cells were seeded at a concentration of 1×10^4 cells/well in 96-well plates for 24 h. Afterwards, cells were exposed to the fatty acids solutions according to the concentrations presented in Table 1. Since our experiment for FRET analysis has a 45 mins duration, for the cytotoxicity assay we exposed the cells for 5 and 24 h to the study fatty acids. Cells treated with 10 % DMSO were used as a negative control. After the two incubation time periods (5 and 24 h), PrestoBlue reagent was added to the medium and it was incubated for 1 h in the dark. The fluorescence signal was read in a Synergy H1 microplate reader.

2.5. Western blot to confirm the expression of GPR120 and GLUT5 membrane receptors in microglia cell line

To confirm the expression of both GPR120 and GLUT5 transmembrane receptors in the study cell line, HMC3 cell lysates from 3 independent cultures were collected in RIPA Buffer (150 mM NaCl, 1.0 % IGEPAL® CA-630, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris, pH 8.0) containing 1:1000 of a phosphatase inhibitor and DTT (Dithiothreitol – Promega, V3151, 1 M) and 1:100 of a protease inhibitor. Protein concentrations were determined using a BCA kit (Pierce BCA Protein Assay Kit, Thermo Scientific). The samples were stored at -80 °C until use. The cell lysates (10 μg) were processed using SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and western-blot. For the SDS-PAGE, 1 % DTT and 1× GLB (Gel loading buffer, 150 mM Trizma Base – Fisher Scientific, BP152 –, 6 % SDS – Sigma Aldrich, L4390 –, 0.05 % Bromophenol Blue – Alfa Aesar, A18469 –, 30 % glycerol – Merck, 1.04092.1000 – and 6 nM EDTA pH 8.8 – Merck, 1.08452.1000) were added to each cell lysate and a 10 mins 95 °C denaturation process was performed, before addition of the samples to a 10 % polyacrilamide electrophoresis gel. After, the proteins were blotted from the gel into nitrocellulose blotting membrane by a semidry transfer process, using the Trans-Blot Turbo System (Biorad). The protein transfer was confirmed by Ponceau S staining. The membranes were blocked with 5 % milk and were further incubated overnight with the GPR120 and GLUT-5 primary antibodies. After incubation with appropriate secondary antibodies the immunodetection was performed with Chemiluminescent Substrate solution and using a ChemiDoc XRS+ (Biorad).

2.6. GPR120 receptor chemical activator and inhibitor

TUG-891 (*ortho*-biphenyl ligand 4-{[4-fluoro-4′-methyl(1,1′-biphenyl)-2-yl]methoxy}-benzenepropanoic acid) was the chosen selective agonist for the long-chain free fatty acid receptor GPR120. The stock solution was prepared at 20 mg/mL (≈55 mM) in DMSO. It was added to the cells as a final concentration of 30 μM, considering previous studies [71–73]. AH7614 (4-methylN-9H-xanthen-9-yl-benzenesulfonamide) was the selected negative allosteric modulator of the long-chain free fatty acid receptor GPR120 [74]. The stock solution was prepared at 100 mM in DMSO and it was added to the cells as a final concentration of 100 μM. Indeed, *in vitro* studies (including neuronal cells) reported AH7614 GPR120 antagonistic effect on concentrations ranging from 10 to 100 μM [62,73,75]. The stock solutions were maintained at -20 °C.

2.7. Biosensors

2.7.1. Cytosolic ROS production

The generation of cytosolic ROS, mainly superoxide radical and hydrogen peroxide, was detected in microglia using the pFRET-HSP33 cys biosensor (mentioned in this paper as HSP biosensor; addgene plasmid #16076). This is a FRET probe consisting of a CFP/YFP proteins linked by a 69 amino acid cysteine-containing regulatory domain from the redox-regulated heat-shock protein HSP-33 [76]. Since the oxidation of the CFP and YFP fluorophores increases the CFP/YFP HSP-FRET ratio, an increase in donor to FRET fluorescence ratio is translated into the increased generation of ROS.

2.7.2. Src tyrosine kinase activation sensor

To measure the specific activity of Src at the plasma membrane of microglia, the LynSrc (WT) YPet FRET probe was used (herein mentioned as LynSrc biosensor). This Src reporter is composed of a CFP protein, the SH2 domain, a flexible linker, a Src substrate peptide – derived from a primary *in vivo* c-Src substrate molecule - and an YFP protein [77]. An increase in the activation of Src is detected by an increase in donor to FRET fluorescence ratio.

LynSrc and HSP biosensors were previously validated in microglia cells giving reliable FRET and donor signals within the dynamic range of each probe [58,78–80].

2.7.3. Ikβα reporter for the canonical activation of NF-κB pathway

The canonical activation of NF-κB pathway was studied using a monomeric near-infrared fluorescent protein probe: the miRFP703-Ikβα reporter (excitation 673 nm, emission 703 nm; addgene plasmid #80005). Ikβα is a known member of a family of proteins that inhibit the NF-κB transcription factor. Canonical activation of NF-κB therefore, depends on induced phosphorylation Ikβα degradation. In resting cells, Ikβα sequesters NF-κB dimers in the cytoplasm. After a certain stimulus, the IKK kinase is activated and phosphorylates Ikβα marking it for degradation and as a result NF-κB is released to the nucleus [81]. Consequently, the activation of NF-κB is detected by a decrease in the fluorescent signal of Ikβα.

2.7.4. Nuclear translocation of the GFP-p65 subunit for NF-κB pathway activation

The measurement of the nuclear accumulation of the p65 subunit of NF-κB as a functional indicator of NF-κB activation was achieved by using the NF-κB GFP-tagged p65 (here defined and GFP-p65; addgene plasmid #23255) [82]. The nuclear accumulation of NF-κB was assessed by an increased nuclear fluorescence of the p65-GFP construct.

To correctly analyze the live cell imaging of the NF-κB's nuclear translocation, the GFP-p65 probe was co-transfected (1:1) with the mneptune2-H2B-6 (addgene plasmid #56146), which is specific for the nucleus/histones and thus allows the cellular nucleus staining.

2.8. Live cell imaging and FRET

HMC3 human microglia were plated on plastic bottom culture dishes (μ-Dish 35 mm, iBidi, Gräfelding, Germany) at a density of 20,000 cells/dish with DMEM + Glutamax® (supplemented and maintained as previously described). Cells were transfected with the different biosensors (miRFP703-Ikβα, GFP-p65, HSP and LynSrc, previously described in Section 2.5) using the jetPRIME® DNA transfection reagent according to manufacturer's instructions: in a proportion of 2 μL of reagent per 1 μg of DNA. Total medium was changed 4 h after transfection. Imaging was performed 48 h post transfection using a Leica DMI6000B inverted microscope as previously described [57,58,79,80]. The cell preparation and the experimental protocol was specifically designed for each experiment as described in the following sections.

Live cell imaging was performed using a fully-monitorized DMI6000B inverted microscope (Leica Microsystems, Wetzlar,

Germany) equipped with high-speed low vibration external excitation/emission filters wheels equipped with filter cubes for cyan fluorescent protein (CFP) (BP427/10) and yellow fluorescent protein (YFP) (BP504/12) working with specific dichroic (CG1 440-520 nm) mounted into a microscope filter carrousel (Leica fast filter wheels). The excitation light source was a mercury metal halide bulb, integrated with an EL6000 light alternator. Microglia cells were observed with a PlanApo 63 × 1.3 NA glycerol immersion objective with a correction ring. Images were acquired with a 2 × 2 binning with an exposure of 200 ms using a digital CMOS camera (ORCA-Flash 4.0 v2, Hamamatsu Photonics, Japan). The LAS X software (Leica Microsystems Wetzlar, Germany) controlled all microscope parameters. For FRET biosensors and at each time-point, CFP and FRET images were sequentially acquired using different filter combinations: CFP excitation plus CFP emission (CFP channel), and CFP excitation plus YFP emission (FRET channel). A digital small-stage incubation (iBidi, Gräfelding, Germany) was used for real-time monitoring of temperature. For quantification purposes, images were exported as 16-bit tiff files and processed in Fiji software. Background was dynamically subtracted from all frames from both channels. For FRET biosensors, segmentation (on a pixel-by-pixel basis) and generation of 32-bit ratiometric images were achieved using the precision FRET (pFRET) plugin for Image J. A whole cell/subcellular domain analysis was performed and the mean grey intensity for each time point were extracted. Values were normalized to the control untreated group (ethanol or ethanol+DMSO) and plotted.

2.9. Omega 3, CLA and CLNA effect on microglia activation

To assess the preventive effect of the studied fatty acids' solutions (Omega 3- EPA and DHA-, CLA (C18:2 *c*9,*t*11 and C18:2 *t*10,*c*12) and CLNA (PUA- C18:3 *c*9,*t*11,*c*13) the cells were transfected with miRFP703-Ikβα reporter, GFP-p65, HSP and LynSrc probes. A pre-incubation with the mentioned fatty acids (testing solutions) was performed as described in Fig. 1. An ethanol control (0.2 % (*v/v*)) was used as the untreated control since ethanol was used as the fatty acids' vehicle. In summary, the cells were recorded for 5 min (baseline reading) in the presence of the testing solutions or ethanol. Then all cell groups were recorded in the presence of the stimulus solution (PA + Fructose) or ethanol (negative control) for 15 min (stimulation period). During the assay, cells were kept under 37 °C in HBSS with CaCl$_2$ and MgCl$_2$.

2.10. GPR120 as fatty acids receptor in microglia

To elucidate the role of GPR120 as HMC3 cellular receptor/sensor of omega 3, CLA and CLNA, a chemical agonist (TUG-891) and antagonist (AH7614) were used. The cells, previously co-transfected with miRFP703-Ikβα reporter or HSP probe, were incubated overnight with 0.1 % (*v/v*) DMSO (agonist and antagonist solvent) and 0.2 % (*v/v*) absolute ethanol (fatty acids vehicle) in DMEM + GlutaMAX™-I culture medium supplemented with 10 % FBS and 100 U/mL penicillin and 100 μg/mL, as mentioned. This overnight incubation intended to decrease the DMSO and ethanol influence on microglial cells' response. Before microscope analyses, a 30 min pre-incubation with the mentioned fatty acids and TUG-891 or AH7614 was performed as described in Figs. 2 and 3, respectively. Thus, the overnight solution was replaced by the HBSS testing solution. An ethanol and DMSO control were used as the untreated control. In summary, the cells were recorded for 5 min (baseline reading) in the presence of the testing solutions or ethanol. Then all cell groups were recorded in the presence of the stimulus solution (PA + Fructose) or ethanol and DMSO (negative control) for 15 min (stimulation period). During the assay, cells were kept under 37 °C in HBSS with CaCl$_2$ and MgCl$_2$ (Thermo Fisher Scientific, Massachusetts, USA).
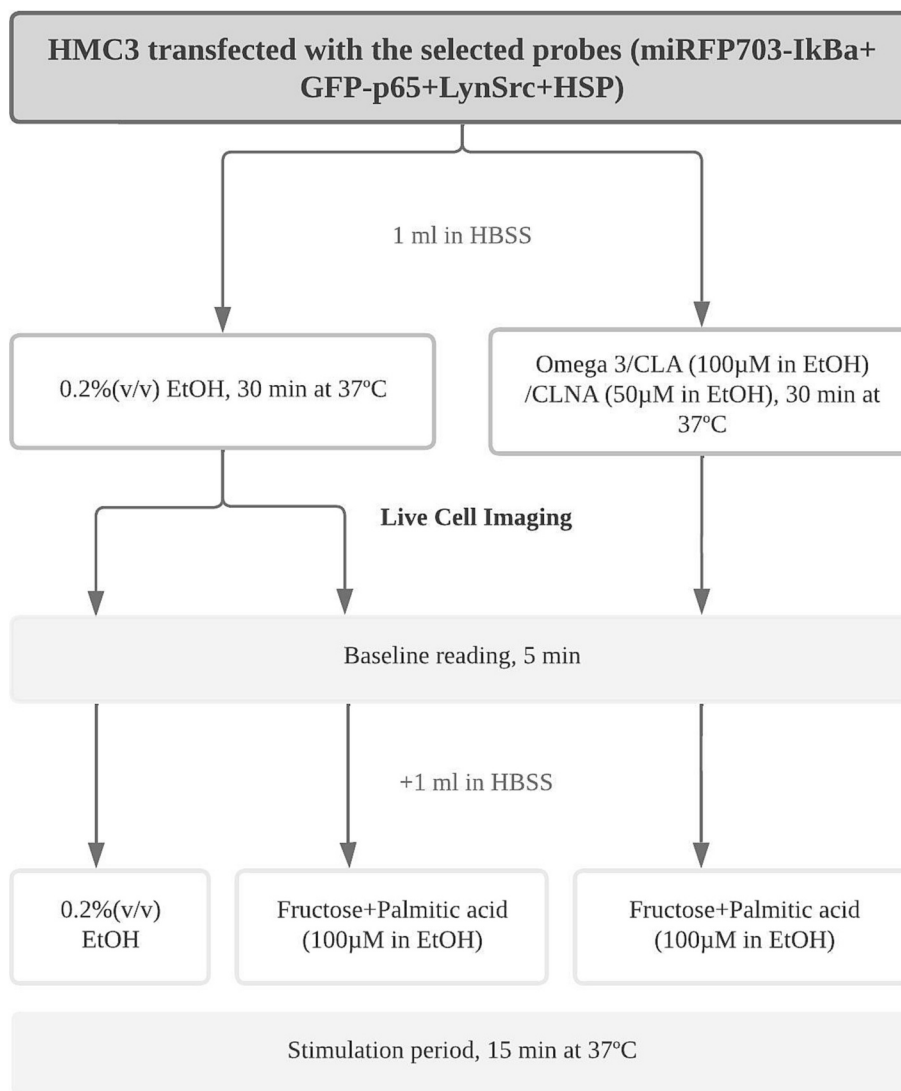
Fig. 1. Schematic representation of the experimental protocol for the evaluation of omega-3 and conjugated fatty acids (CLA and CLNA) effect on microglia activation.

2.11. Statistical analysis

Experimental units in individual biological replicates were evaluated *a priori* for Gaussian distribution using the D'Agostino & Pearson omnibus normality test. For live-cell imaging experiments when comparing two or more groups with two independent variables (fatty acid treatment and time), a two-way ANOVA with Bonferroni's multiple comparison test was used to evaluate the statistical significance of all groups. In the specific case of the GFP-p65 experiments four groups were compared considering only one factor: fatty acid treatment in the end of the experimental time. Thus, in this specific case, an ordinary One-way ANOVA followed by the Bonferroni's multiple comparison test was used for data with normal distribution. All quantifications were performed using Graph Pad Prims 6.0 (Graphpad® software, San Diego, California, USA). A 95 % confidence interval was used and $p < 0.05$ was considered as a statistically significant difference between the analyzed groups. Experimental groups were randomly assigned, and all quantifications were performed blindly. More details on statistical analysis are indicated in figure legends.

3. Results

3.1. Cytotoxicity

Exposure of HMC3 cells to 100 μM of PA + Frut and 100 μM of omega-3 and CLA did not affect the cell viability after 5 and 24 h exposure. In the case of the CLNA isomer, exposure of 50 μM of a PUA solution did not affect the cell viability after 5 h exposure (Fig. 4). Nevertheless, 24 h exposure to this PUFA seems to induce cytotoxicity in the experimental cell line. Since our experiment for FRET analysis involved a 45 mins exposure to this fatty acid and we demonstrated that was no cytotoxicity observed after a 5 h exposure, we decided to use the 50 μM concentration since, as discussed, it was described as a bioactive concentration. The results are reported as the percentage of cell viability as compared with the control (cells without treatment).

3.2. Omega 3, CLA, and CLNA prevent NF-κB pathway activation by palmitic acid and fructose in microglia

Studies show that PA activates microglia and triggers a pro-inflammatory response, partially through the NF-κB pathway [34,63]. In addition, short-term fructose ingestion affects the brain without
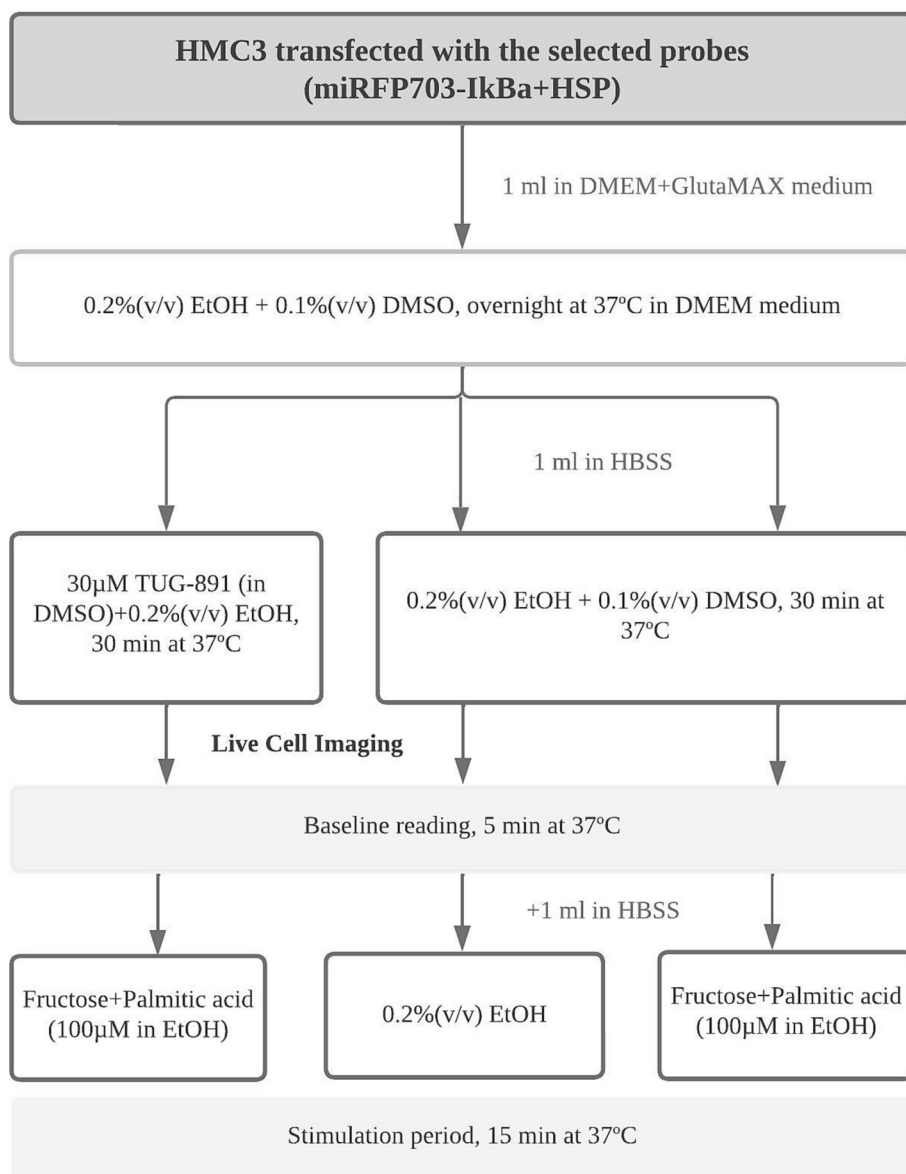
Fig. 2. Schematic representation of the experimental protocol for the evaluation of GPR120/FFA4 activation, by using TUG-891 GPR120/FFA4 agonist, in microglia activation.

significant involvement of peripheral tissues [29]. However, the effect of the combination of fructose and PA, the two major constituents of a WPD, on microglia activation and inflammation is entirely elusive. Glucose transporter 5 (GLUT5) is a hexose transporter involved in fructose transport which is primarily expressed in microglia within the CNS. Such transporter shows greater affinity to fructose than to glucose. Fructose is known to activate NF-κB pathway, similarly to PA [28]. The expression of GLUT5 was confirmed by Western Blot (Fig. 1A supplementary material). The presence of the GLUT5 in the Western blot was confirmed by the presence of a band with 100 kDa. Two other bands were detected with ≈75 kDa and ≈45 kDa. Previous works have reported that GLUT5 protein presents a 45-70 kDa molecular weight [83–87].

To evaluate the role of PA and fructose in microglia inflammation, we assessed the activation of the canonical NF-κB pathway (the primary pro-inflammatory driver in myeloid cells, including microglia). We visualized NF-κB activity using a biosensor-based approach coupled to live-cell imaging in cultured microglia expressing the miRFP703-Ikβα nanosensor (to detect the amounts of the canonical NF-κB inhibitor), and the GFP-p65 probe (to detect the nuclear accumulation of the catalytic

p65 subunit of the NF-κB complex). Exposure to a WPD-mimicking solution (PA + Fructose; 2:1; 100 μM) caused a fast and sustained decrease in the signal of the miRFP703-Ikβα reporter in living microglia (Fig. 5A), suggesting increased Ikβα degradation and consequent NF-κB pathway regulation, and increased the nuclear accumulation of GFP-tagged p65 NF-κB catalytic subunit (Fig. 5B). We concluded that exposure to a WPD-mimicking solution leads to the activation of NF-κB pathway in microglia.

Although SFAs induce microglia activation, other fatty acids, such as PUFAs, can normalize the inflammatory effects caused by SFAs [64,88]. Because omega-3 fatty acids, EPA, and DHA, mostly through fish oil supplementation studies, were shown to have a significant positive impact in reversing HFD-induced inflammation in hypothalamus, we hypothesized that other PUFAs could display similar effects. Thus, we explored the modulatory effect of omega-3 and CFAs, CLA (specifically RA and C18:2 *t*10,*c*12 CLA isomer) and CLNA (PUA) in cultured microglia. In this study, we intended to approach these PUFAs action on microglia as a potential preventive strategy to the negative PA + Fructose-induced effects. Thus, we used them as a pre-treatment. Interestingly, the omega-3 (EPA and DHA; 100 μM) combination prevented the
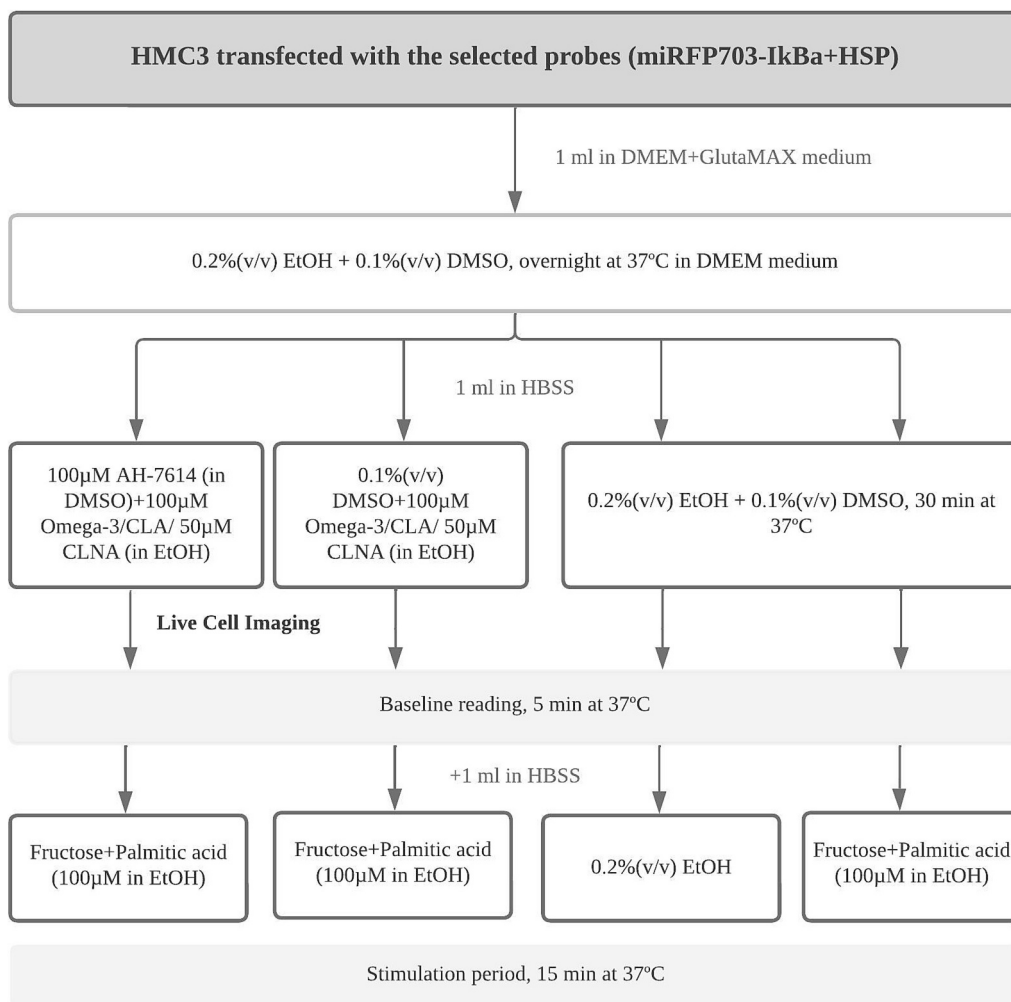
Fig. 3. Schematic representation of the experimental protocol for the evaluation of omega-3, CLA and CLNA action in GPR120/FFA4 chemical inhibition, by using AH7614 GPR120/FFA4 antagonist, in microglia activation.
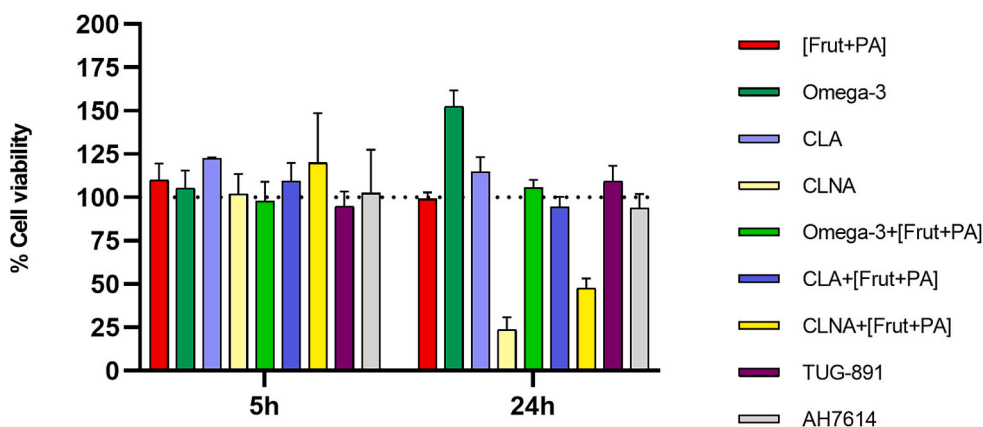

Fig. 4. Cytotoxicity of HMC3 microglia cell after 5 and 24 h exposure to the study PUFAs. Bars represent cell viability, measured by the PrestoBlue assay in percentage after treatment.

PA + Fructose-induced nuclear accumulation of GFP-p65 NF-κB catalytic subunit (Fig. 5B) but did not prevent the PA + Fructose -induced Ikβα degradation (Fig. 5A). Thus, the omega-3 prevention of NF-κB pathway activation was observed with GFP-p65 probe but not with the miRFP703-Ikβα nanosensor. Such results might be related to the short pre-incubation time used in the assays, suggesting that a more prolonged

pre-incubation period might be required to detect omega-3 effects in NF-κB pathway through Ikβα degradation with the miRFP703-Ikβα reporter. Moreover, we found that pre-incubation of microglia with either CLA (100 μM) or CLNA (50 μM), significantly prevented the PA + Fructose-induced canonical NF-κB pathway activation (using the miRFP703-Ikβα nanosensor) by restoring Ikβα levels and the nuclear accumulation of the
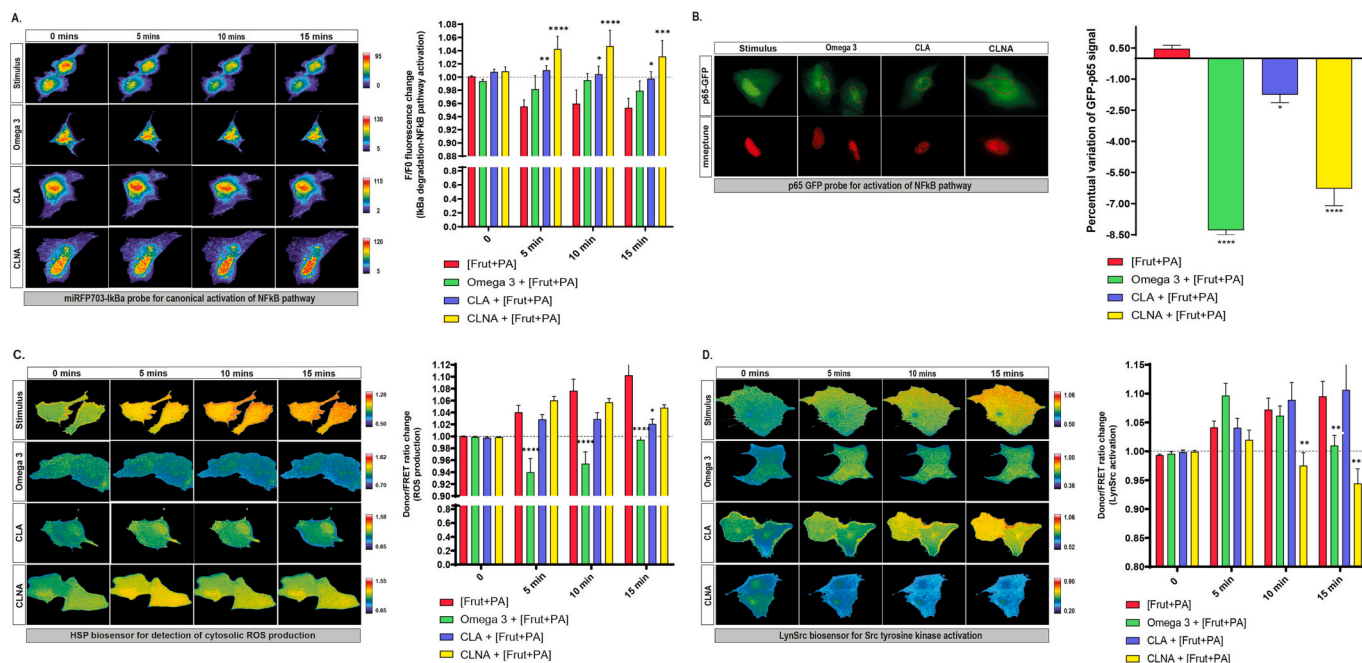
Fig. 5. Omega-3, CLNA and CLA fatty acids can revert Fructose and Palmitic acid-induced microglia inflammatory imbalance elicited by NF-κB pathway activation, ROS production and Src Tyrosine activation.

(A) Fluorescence imaging and results of the quantification of human microglia cell line (HMC3) expressing the miRFP703-Ikβα sensor in cells subjected to the stimulus solution (Palmitic acid+Fructose) and in cells pre-incubated with the studied fatty acids (omega-3, CLA and CLNA) for the selected time points (0, 5, 10 and 15 min). A decreased signal means a bigger Ikβα degradation and higher NF-κB pathway activation. Error bar represents the SEM calculated from $n > 10$ cells from two independent cultures. Two-way ANOVA in relation to Palmitic acid+Fructose (PA + Frut), where no pre-incubation with the selected fatty acids was performed. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

(B) Fluorescence imaging and results of the quantification of human microglia cell line (HMC3) expressing the GFP-p65 and mneptune biosensor in cells subjected to the stimulus solution (Palmitic acid+Fructose) and in cells pre-incubated with the studied fatty acids (omega-3, CLA and CLNA). The mnpetune probe allows nucleus staining. The results correspond to n of the sensor signal of human microglia expressing the GFP-p65 sensor after the 15 min incubation time. This variation is in relation to the baseline measured for each experiment. A negative signal means a decreased signal in relation to the baseline, meaning that the catalytic p65 subunit of the NF-κB complex migration to the nucleus decreased in cells exposed to a pre-incubation with the studied fatty acids. In cells only subjected to the stimulus solution the signal increased after baseline measure, indicating catalytic p65 subunit translocation to the nucleus. Error bar represents the SEM calculated from $n > 10$ cells from two independent cultures. One-way ANOVA in relation to Palmitic acid+Fructose (PA + Frut), where no pre-incubation with the selected fatty acids was performed. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

(C) Fluorescence imaging and results of the quantification of human microglia cell line (HMC3) expressing the ROS FRET sensor HSP in cells subjected to the stimulus solution (Palmitic acid+Fructose) and in cells pre-incubated with the studied fatty acids (omega-3, CLA and CLNA) for the selected time points (0, 5, 10 and 15 min). Error bar represents the SEM calculated from $n > 15$ cells from two independent cultures. Two-way ANOVA in relation to Palmitic acid+Fructose (PA + Frut), where no pre-incubation with the selected fatty acids was performed. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

(D) Fluorescence imaging and quantification of human microglia cell line (HMC3) expressing the LynSrc FRET sensor in cells subjected to the stimulus solution (Palmitic acid+Fructose) and in cells pre-incubated with the studied fatty acids (omega-3, CLA and CLNA) for the selected time points (0, 5, 10 and 15 min). Error bar represents the SEM calculated from $n > 15$ cells from two independent cultures. Two-way ANOVA in relation to Palmitic acid+Fructose (PA + Frut), where no pre-incubation with the selected fatty acids was performed. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

catalytic p65 subunit of the NF-κB complex (using the GFP-p65 probe) (Fig. 5A and B). The inhibitory effect was more pronounced in microglia pre-incubated with the CLNA isomer, PUA, than in microglia exposed to CLA (Fig. 5A and B).

3.3. Omega 3 and CLA display antioxidant capacity

Microglia response to tissue damage and infection is characterized by the production and release of ROS to the surrounding CNS milieu [89,90], which directly changes cell metabolism, transcription, and the secretion of bioactive molecules such as cytokines, chemokines, and neurotransmitters. Studies in BV-2 microglia-like cells and primary microglia showed that besides increasing microglial secretion of pro-inflammatory cytokines, SFAs promote ROS production [61]. Thus, we assessed the PA + Fructose impact on ROS production, as well as omega-3 (EPA and DHA), CLA (RA and C18:2 t10,c12 CLA isomer), and CLNA (PUA) antioxidant potential in HMC3 human microglia. We found that PA + Fructose stimulation caused a time-dependent increase in ROS production in living microglia (Fig. 5C). However, pre-incubation of

microglia with 100 μM omega-3 abrogated the PA + Fructose -induced ROS generation in living microglia, suggesting that, under these experimental conditions, omega-3 displays an antioxidant potential. Pre-incubation of microglia with 100 μM CLA also significantly prevented the ROS generating effect by PA + Fructose (Fig. 5C). Although CLNA treatment promoted a noticeable tendency to suppress ROS generation triggered by PA + Fructose, the inhibitory effect of CLNA on ROS production, under the tested experimental conditions, did not reach statistical significance (Fig. 5C). These results indicate that omega-3 and CLA attenuate the oxidative stress generated by PA + Fructose and are potential regulators of the cytosolic redox balance in microglia.

3.4. CLNA and omega-3 prevent Src tyrosine kinase activation by fructose and palmitic acid

Activation of the cytosolic tyrosine kinase Src is essential for the production of inflammatory mediators by microglia [58,80]. Thus, we tested whether PA + Fructose could also modulate Src in microglia. To answer this question, we used the LynSrc FRET sensor, which reports

specifically the activity of c-Src, but not other Src family kinases (SFKs), at the plasma membrane of living cells. We observed that PA + Fructose leads to a time-dependent increase of Src activity in living microglia (Fig. 5D). Interestingly, whereas the pre-incubation of microglia with CLNA or Omega 3 significantly suppressed the activation of Src triggered by PA + Fructose, no inhibitory effect was observed by pre-incubating the cells with CLA (Fig. 5D).

3.5. Chemical activation of GPR120/FFA4 receptor does not suppress NF-κB pathway activation but inhibits fructose and palmitic acid-induced ROS production

Some PUFAs, such as the omega-3 fatty acids, ALA, DHA, and EPA, are known to activate the GPR120 receptor [41]. Moreover, activation of GPR120 is considered a significant mediator of omega-3 anti-inflammatory actions [91]. The *ortho*-biphenyl ligand 4-{[4-fluoro-4′-methyl(1,1′-biphenyl)-2-yl]methoxy}-benzenepropanoic acid (TUG-891) is the first selective agonist of GPR120 [92], with reported biological effects in the range of 10 μM [71–73]. This is considered a significant selective agonist ligand for GPR120, showing a good potency at both human and mouse GPR120 [92–94].

The expression of GPR120 in our cell line was confirmed through Western Blot by the detection of a band of approximately 70 kDa (Fig. 1B supplementary material), which agrees with previous studies reporting molecular weights bands ranging from 42 kDa to 90 kDa [95]. Indeed, the detection of higher molecular weight receptor dimers and oligomers are features commonly found after SDS-PAGE in G-Protein coupled receptors in general [96]. Since GPR120 was confirmed to be expressed in HMC3 cell line, TUG-891 was assayed to activate GRP120 in microglia following exposure to PA + Fructose. The results suggest that TUG-891-modulation of GPR120 did not prevent the PA + Fructose -mediated activation of the canonical NF-κB pathway, during the assayed time (Fig. 6A) but completely suppressed the generation of ROS elicited by PA + Fructose (Fig. 6B). These results may suggest that activation of GRP120 alone may modulate the redox balance without affecting the classical inflammatory status of microglia exposed to a WPD-mimicking solution.
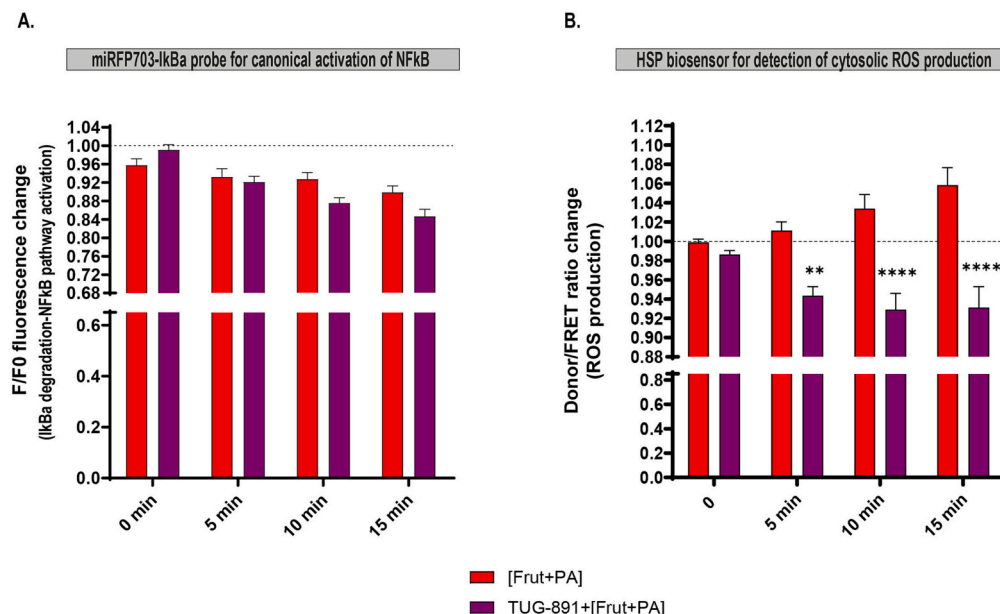
3.6. Omega-3, CLA, and CLNA anti-inflammatory, but not antioxidant, action occurs through GPR120 receptor in microglia

Considering that some studies have demonstrated that omega-3 is able to activate GPR120 [89,95], we hypothesized whether CLA (RA and C18:2 *t*10*c*12 CLA isomer), and CLNA (PUA) may modulate NF-κB pathway and ROS production through GPR120, in microglia. Thus, we tested AH7614, a non-competitive antagonist of the GPR120 receptor [74], in microglia exposed to PA + Fructose. We found that the pharmacological blockade of GPR120 by 100 μM AH7614 abolished the inhibitory effect on the NF-κB pathway triggered by omega 3 (Fig. 7 A1), CLA (Fig. 7 A2), and CLNA (Fig. 7 A3) following exposure to PA + Fructose. Interestingly and opposing to the results obtained with TUG-891 (GPR120 agonist), treatment with AH7614 did not block the effect of omega 3 (Fig. 7 B1) or CLA (Fig. 7 B2) on ROS production upon PA + Fructose exposure. Thus, these data strongly suggest that distinct pathways control the anti-inflammatory and antioxidant potential of omega-3 and CFAs (CLA and CLNA) in microglia exposed to a WPD-mimicking solution.

4. Discussion

PA is an important SFA, indeed its average dietary intake is around 20-30 g/day being found in many dietary sources, with levels of 20-30 % in animal lipids and 10-45 % in vegetable oils [97]. Significantly, as reported by Nadjar et al. [35] dietary fatty acids can cross the BBB and modulate CNS biochemistry. Different lipids, mainly SFAs and PUFAs, have proved to induce microglia activity. Indeed, chronic or acute exposure of microglia in culture to SFAs activates the NF-κB pathway [34] and classical downstream inflammatory cascades [60]. Although the exact mechanisms through which fatty acids exert their action is elusive, microglia express a wide range of lipid-sensitive receptors [35] that could potentially initiate microglia inflammation. Therefore, SFAs entry into the CNS could be a potential nutritional trigger to hypothalamic inflammation in the context of diet-induced obesity [34]. Importantly, high-fructose consumption has also been associated with metabolic syndrome development and obesity development, in part related with inflammatory pathways activation (including NF-κB) [26,98]. Fructose consumption has been increasing and been highly associated with obesity prevalence. In fact,since 1970s fructose intake has increased to a mean of around 7.5 % of total energy intake, in USA

Fig. 6. Chemical activation of GPR120 by TUG-891 enhances NF-κB pathway activation and inhibits ROS production by Fructose and Palmitic acid.

(A) Results of the quantification of human microglia expressing the miRFP703- Ikβα sensor for the selected time points (0, 5, 10 and 15 min). A decreased signal means a bigger Ikβα degradation and higher NF-κB pathway activation. Error bar represents the SEM calculated from $n > 12$ cells from two independent cultures. (B) Results of the quantification of human microglia expressing the HSP-FRET sensor for the selected time points (0, 5, 10 and 15 min). Error bar represents the SEM calculated from $n > 10$ cells from two independent cultures. (A) and (B) Two-way ANOVA in relation to Palmitic acid+Fructose (PA + Frut). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.
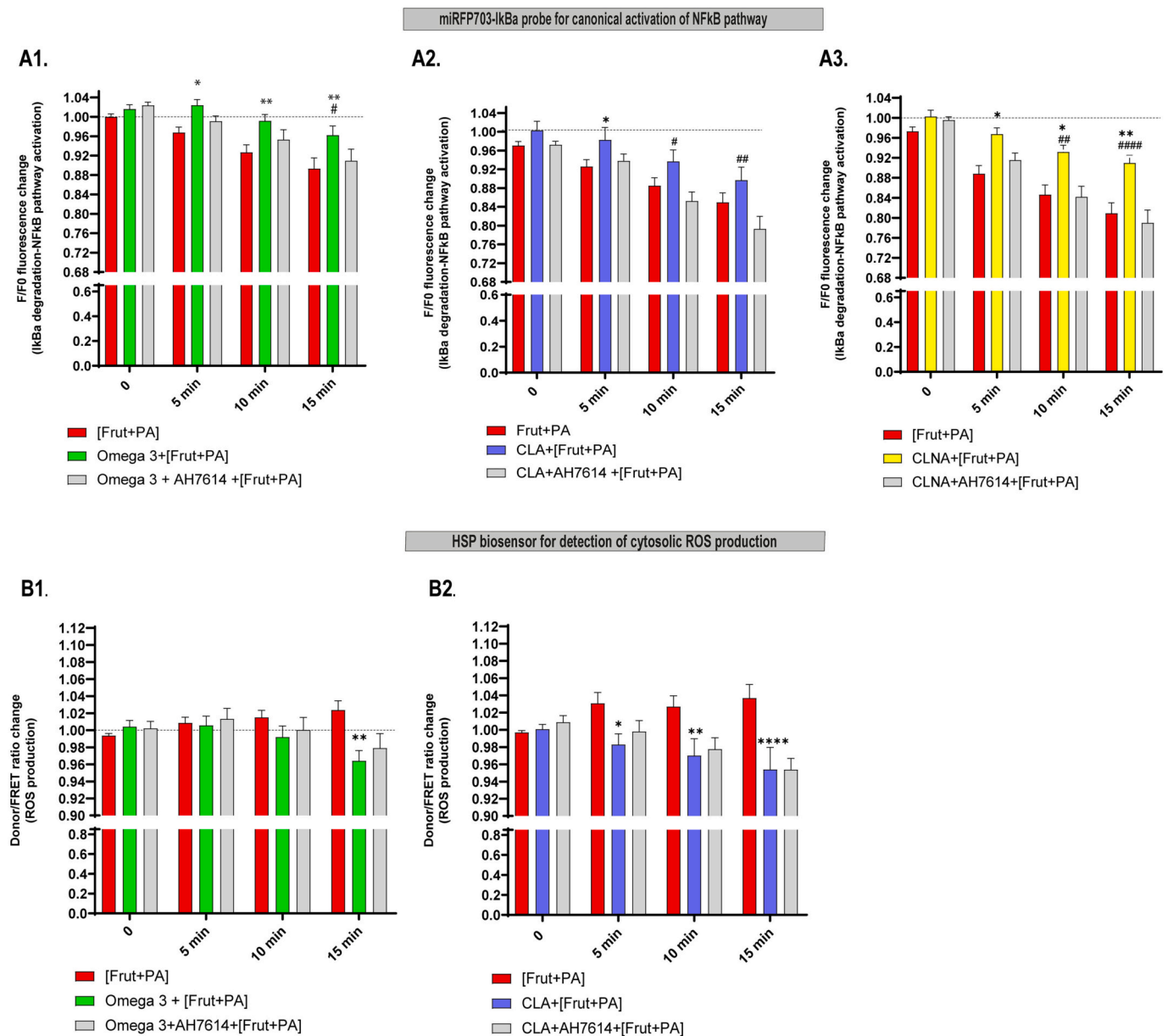
Fig. 7-. Chemical inhibition of GPR120 by AH7614 prevents omega-3, CLA and CLNA anti-inflammatory action but is not involved in omega-3 and CLA antioxidant action.

(A) Results of the quantification of human microglia expressing the miRFP703- Ikβα sensor for the selected time points (0, 5, 10 and 15 min). A decreased signal means a bigger Ikβα degradation and higher NF-κB pathway activation. Error bar represents the SEM calculated from $n > 20$ cells from two independent cultures. AH7614 inhibition of GPR120/FFA4 in cells exposed to (A1) omega-3, (A2) CLA (rumenic acid and C18:2 t10c12 CLA isomers) and (A3) CLNA (PUA isomer). Two-way ANOVA in relation to Palmitic acid+Fructose (PA + Frut), where no pre-incubation with the selected fatty acids was performed: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ and in relation to cells which were pre-incubated with omega 3/CLA/CLNA and AH7614, a GPR120 inhibitor: #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, ####$p < 0.0001$.

(B) Results of the quantification of human microglia expressing the HSP-FRET sensor for the selected time points (0, 5, 10 and 15 min). Error bar represents the SEM calculated from $n > 12$ cells from two independent cultures. AH7614 inhibition of GPR120/FFA4 in cells exposed to (B1) omega-3 and (B2) CLA (rumenic acid and C18:2 t10c12 CLA isomers). Two-way ANOVA in relation to Palmitic acid+Fructose (PA + Frut) (*), where no pre-incubation with the selected fatty acids was performed: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ and in relation to cells which were pre-incubated with omega 3/ CLA and AH7614 (#), a GPR120 inhibitor: #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, ####$p < 0.0001$.

[99]. Interestingly, high fructose consumption has been associated with cognitive dysfunction in rats. Moreover, such effects may be amplified by diets low in omega-3 fatty acids, suggesting the importance of these fatty acids in fructose-induced effects. In addition, combining fructose with saturated fats (coconut oil) causes worse memory defects than when combined with fats rich in omega-6 polyunsaturated fats [100]. Recently, it was suggested that neuroinflammatory and neurodegenerative diseases, such as Alzheimer's development may be related to the

activation of the fructose survival pathway by eating excess sugar and fructose [100]. Suggesting a mechanism of endogenous fructose production in the brain.

Mammalian NF-κB functions as a dimer composed of p50 (NF-κB1) and p65 (RelA). Under normal conditions, NF-κB remains inactive in the cytoplasm by binding to a set of proteins from the IκB inhibitory protein family. In the canonical pathway, following cellular stimulation, activation of IKKβ induces IκB phosphorylation by IKKβ, which results in

proteasomal degradation, NF-κB nuclear translocation, and transcription of its target genes, including TNF-α, IL-1β, and cyclooxygenase-2 (COX-2) [101,102]. PA induces the phosphorylation and nuclear translocation of the p65 subunit of NF-κB in BV-2 microglia, resulting in proinflammatory activation [61]. Recently, it was demonstrated that PA induced a time- and dose-dependent lipotoxicity in BV-2 microglia cells, since it decrease cell viability and increase cell death [103]. Our data suggest that the combined action of PA + Fructose induces Ikβα degradation and p65 subunit nuclear translocation in HCM3 human microglia. PA + Fructose exposure also leads to ROS production and Src activation (critical regulators of microglia inflammation), suggesting that a WPD may trigger neuroinflammatory and neurotoxic effects in the CNS.

In the brain, PUFAs are esterified to phospholipids and largely located at the membranes of neurons, glial cells, and endothelial cells [35]. PUFAs account for 35 % of total lipids in the adult brain [104]. AA and DHA, which make up 50 % and 40 % of brain PUFAs, are essential to brain development and function. Although SFAs act deleteriously on microglia (*i.e.* inflammation trigger), PUFAs can normalize these harmful effects. The anti-inflammatory effect of the omega-3 fatty acids EPA and DHA is thought to involve the inhibition of the phosphorylation of the inhibitory subunit of NF-κB – Ikβ [105]. In this work, exposure to omega-3 (EPA and DHA) blocked the NF-κB pathway activation and the production of ROS triggered by PA + Fructose. These results agree with others reported elsewhere showing a reduction in ROS production in BV-2 microglia supplemented with PUFAs [106]. Omega-3 also prevented the activation of Src in microglia. Indeed, Src tyrosine kinase is recognized as a potential therapeutic target for neuroinflammation-related diseases, including Parkinson's [107] and Alzheimer's disease [108]. Besides EPA and DHA, CLA (RA and C18:2 *t*10,*c*12) inhibited NF-κB pathway activation and ROS production by PA + Fructose. Exposure to CLNA (PUA) also inhibited the NF-κB pathway and Src activation. This is the first demonstration of a potential modulatory role for CFAs in microglia to the best of our knowledge. Interestingly, the inhibitory effect on NF-κB pathway was more pronounced in microglia pre-incubated with the CLNA isomer, PUA, than in microglia exposed to the CLA isomers. Importantly, we have reported that microglia activation requires c-Src and that its activation *per se* is sufficient to trigger a classical proinflammatory signature following acute LPS or hypoxia challenging [58,59]. In addition, we have also demonstrated that overexpression of a constitutively active c-Src was sufficient to increase the nuclear accumulation of p65 suggesting that c-Src promotes NF-κB activation during microglia activation [109]. These observations may explain the difference in NF-κB pathway regulation capacity when comparing CLA and CLNA isomers. The CLNA (PUA) effect on Src activation, which is not observed with the CLA isomers, may contribute, in part, to CLNA capacity to strongly regulate NF-κB pathway.

SFAs activate TLR 4 and this one regulates the myeloid differentiation factor 88 (Myd88), TAK1/TAB1 modulation, and NF-κB activation. On the other hand, omega-3 fatty acids, such as EPA and DHA, activate GPR120. This interaction recruits β-arrestin 2, leading to the internalization of the GPR120-β-arrestin 2 complex. Such complex interacts with TAB1, explicitly inhibiting TAK1 phosphorylation and activation [42,110]. Indeed, GPR120 is known to bind some omega-3 fatty acids, namely DHA and EPA [41] and α-linolenic acid (ALA) [40]. GPR120 is thought to be expressed predominantly in microglia cells, whereas GPR40/FFA1 in POMC and NPY neurons [111]. GPR120 activation by the synthetic agonist TUG-891 failed to block NF-κB pathway activation by PA + Fructose. Indeed, contradictory results have been reported: in mouse intestinal epithelial endocrine cell line, STC-1, exposure to TUG-891 for 30 min did not induce GPR120 internalization and presented no effects on NF-κB, while the opposite was reported in Caco-2 cells [112]. On the other hand, in our study, inhibiting GPR120 with AH7614 abolished the effect of omega-3 (EPA and DHA) on the NF-κB pathway, confirming that omega-3 anti-inflammatory action occurs *via* GPR120 activation as reported by previous studies [91,111]. There is a lack of

information about the interaction of CFAs with GPR120, especially in microglia. The CLA isomer RA, but not the C18:2 *t*10,*c*12 isomer, was found to enhance GPR120 expression [113]. Here, CLA (RA and C18:2 *t*10,*c*12) and CLNA (PUA) actions on the NF-κB pathway inhibition was mediated through GPR120 signaling in HMC3 microglia. CLA and CLNA may have a mechanism of action similar to that of omega-3 on microglia, probably *via* GPR120 activation and modulation of NF-κB-associated inflammatory pathways. Moreover, our results, using TUG-891 agonist and AH7614 antagonist may suggest that although omega-3, CLA and CLNA fatty acids action on NF-κB pathway is mediated by GPR120 other mechanisms may be linked and are necessary for such action. Indeed, TUG-891, a selective agonist for GPR120, failed to suppress NF-κB pathway activation in our study. In a different study it was suggested that the ability of GPR120 to couple to Gαq or to β-arrestin-2 may explain the divergence observed in different cell types, since GPR120 stimulation could induce distinct signaling pathways [112]. In addition, some studies have reported that attenuation of microglial activation by lauric acid (a medium-chain saturated fatty acid) may occur *via* the GPR40-dependent pathway [114]. Moreover, other study demonstrated that exposure of murine primary hepatocytes to DHA for 12 h increased both GPR40 and GPR120 mRNA levels [115]. Thus, a combined role of GPR40 and GPR120 on NF-κB pathway regulation cannot be disregard. Thus, further studies should focus in describing the ligand-receptor interaction to fully understand if response is dependent of the fatty acids. Regarding ROS production, interestingly, GRP120 activation by TUG-891 prevented the oxidative stress generated by PA and fructose. However, omega-3 and CLA antioxidant capacity was not mediated by GPR120. These results indicate that the anti-inflammatory action and antioxidant potential of omega-3, CLA, and CLNA occur through different signaling mechanisms, as previously reported elsewhere for omega-3 DHA in macrophages [116]. This is highly relevant since ROS production has been suggested to be intrinsically connected to NF-κB pathway activation in microglia [102]. Indeed, the redox state is widely accepted to control NF-κB nuclear levels [90], and inhibition of NF-κB associates antioxidant and anti-neuroinflammatory effects of specific bioactive molecules on microglia [117]. Other antioxidants, such as ascorbate [58] and piperlongumine [118] also exert their effects by suppressing NF-κB activity in microglia. Our data suggest that the antioxidant potential of omega-3 and CLA isomers was not related to NF-κB pathway inhibition. For instance, it has been reported that CLA can directly scavenge ROS in the human neuroblastoma cell line SH-SY5Y [118] and a modest scavenge potential was demonstrated for omega-3 DHA [116]. Nevertheless, this is a controverse theme considering that some concerns are present due to their potential pro-oxidant effect, having detrimental effects in several tissues, especially in the CNS. On the other hand, the action of oxidized omega-3 fatty acids is known to be directed against kelch-like ECH-associated protein 1 (keap1). This protein is the negative regulator of the nuclear factor erythoid 2-related factor 2 (Nrf2) [119]. Interestingly, tiliroside, a natural dietary glycosidic flavonoid, protects BV-2 microglia from LPS/IFN-γ-induced neuroinflammation *via* Nrf2 antioxidant mechanisms [120]. An Nrf2-dependent antioxidant role for omega-3 fatty acids EPA and DHA may also occur in rat primary astrocytes [121]. Thus Nrf2-dependent antioxidant mechanism for omega-3, and eventually for CLA and CLNA, cannot be excluded. The biological mechanisms proposed in this work are illustrated in Fig. 8.

Despite their relevance and novelty, these results must be carefully considered since a cell line was used. In fact, cell lines are incredibly advantageous due to their ease, especially considered the performed assays using FRET technology. Nevertheless, it must be point out that a major disadvantage is their susceptibility to be dedifferentiate and the possibility that the immortalization process may alter the microglial phenotype. Indeed, recent studies have pointed out that microglia cell lines differ genetically and functionally from primary microglia and *ex vivo* microglia. Despite such limitations, microglia cell lines are still suitable and a relevant tool for biochemical and molecular approaches
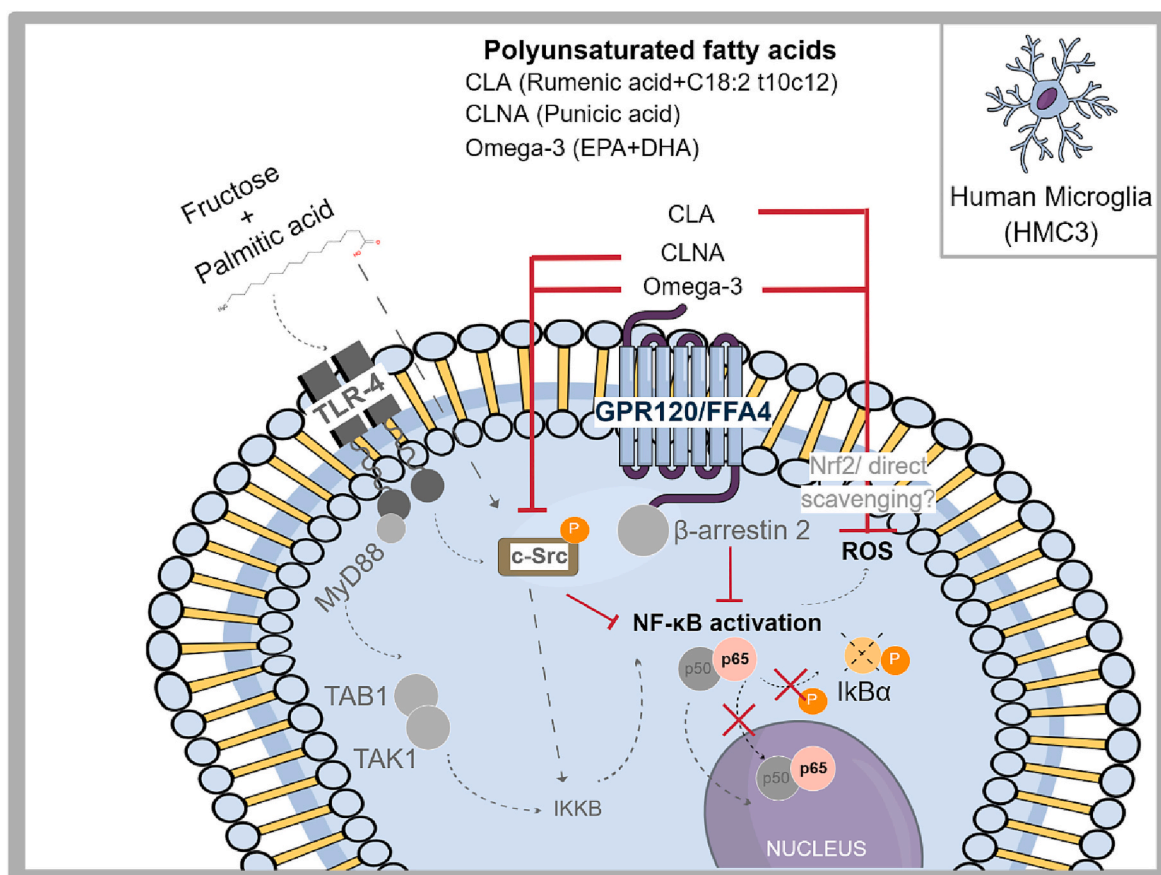
Fig. 8. Proposed molecular mechanisms involved in Omega-3, CLA and CLNA isomers inhibitory action on microglia activation induced by Fructose and Palmitic acid.

In our proposed mechanism Fructose and Palmitic acid combined action activate NF-κB through TLR-4 binding. The activation of myeloid differentiation factor (MyD88) leads to TAK1 activation and consequent interaction with TAB1. Such effects result in IKKβ activation which ultimately leads to Ikβ phosphorylation and NF-κB nuclear translocation. Fructose and Palmitic acid are also able to induce oxidative stress through reactive oxygen species production (ROS) and Src tyrosine kinase activation. Omega-3 (EPA and DHA), CLA isomers (Rumenic acid and C18:2 t10c12) and CLNA isomer (Punicic acid) stimulate GPR120/FFA4 receptor which recruits β-arrestin 2. Their internalization inhibits NF-κB nuclear translocation and consequently its activation. Moreover, CLA and Omega-3 show antioxidant potential that might be a result of direct scavenging or be mediated by Nrf2 antioxidant mechanisms. CLNA and Omega-3 inhibit c-Src activation, which may also have an effect on NF-κB pathway activation. Figure created in the Mind the Graph platform (www.mindthegraph.com).

as well as for high-throughput screenings studies due to high cell number requirements such as in this experiment. Moreover, although primary cells are often used, they present several limitations regarding transfection processes, for instance. Moreover, rodent primary microglia face a major limitation due to their evolutionary divergence from humans. On the other hand, human primary microglia, besides ethical constraints, present limited availability of (healthy) human brain tissue, limited control over the *ante mortem* conditions and *post-mortem* delay, which affect the microglia phenotype [122,123]. Despite the limitations of using a cell line, HMC3 cells present the advantage of being authenticated by ATCC in terms of morphology evaluation, karyotyping, and PCR-based approaches to confirm their identity and to rule out contaminations. Significantly, recent studies have also shown that HMC3 (ATCC CRL-3304) cells retain most of the original antigenic properties [122].

5. Conclusion

By a combination of live cell imaging and FRET technology we were able to describe some of the mechanisms involved in PA + Fructose induced-microglia activation. We have demonstrated that Fructose and PA combined action induces an immediate pro-inflammatory response in the human microglia cell line (HMC3). In fact, exposure to the WPD-mimicking solution caused increased IkBα degradation and nuclear

accumulation of GFP-tagged p65 NF-κB catalytic subunit, strong indicators of NF-κB pathway activation. Moreover, this stimulus caused a time-dependent increase in ROS production. For the first time we have demonstrated that this stimulus increased Src activity in living microglia. Importantly, both ROS production and Src activation are critical regulators of microglia inflammation. Such results suggest a neuroinflammatory and neurotoxic effect of WPD in brain. In contrast, we have demonstrated that omega-3 and for the first time, CLA and CLNA isomers, prevented the induced-NF-κB pathway activation. However, only omega-3 and CLA displayed antioxidant potential by inhibiting ROS production, protecting the cells against the generated oxidative stress. Furthermore, only omega-3 and CLNA isomer PUA demonstrated an effect on Src activation. The effect of CLNA on Src tyrosine kinase activation may contribute for its strong regulatory action on NF-κB pathway regulation, when compared to CLA. Interestingly, using a GPR120 antagonist (AH7614) it was observed that although a role between ROS production and NF-κB pathway activation is widely accepted, under these experimental conditions, while omega-3, CLA and CLNA regulatory action on NF-κB pathway is mediated by GPR120, the antioxidant potential of omega-3 and CLA isomers is mediated by different cellular mechanisms. Importantly, we have demonstrated that in microglia cells and under our experimental conditions, the anti-inflammatory action of CFAs was mediated by GPR120 and not by the widely reported PPARs.

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CRediT authorship contribution statement

A.S. Salsinha: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **R. Socodato:** Conceptualization, Methodology, Validation, Formal analysis, Writing – review & editing. **A. Rodrigues:** Methodology, Investigation, Formal analysis. **R. Vale-Silva:** Methodology, Investigation, Formal analysis. **J.B. Relvas:** Conceptualization, Validation, Resources, Writing – review & editing, Supervision. **M. Pintado:** Conceptualization, Validation, Resources, Writing – review & editing, Supervision, Project administration. **L.M. Rodríguez-Alcalá:** Conceptualization, Validation, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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