

The selective blockade of metabotropic glutamate receptor-5 attenuates fat accumulation in an *in vitro* model of benign steatosis

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ABSTRACT

It has been previously found that the blockade of metabotropic glutamate receptor type 5 (mGluR5) protects against hepatic ischemia/reperfusion injury and acetaminophen toxicity. The role of mGluR5 in NAFLD has not yet been elucidated. Here, we evaluated the effects of mGluR5 blockade in an *in vitro* model of steatosis. HepG2 cells were pre-incubated for 12 h with an mGluR5 agonist, a negative allosteric modulator (DHPG and MPEP, respectively) or vehicle, then treated with 1.5 mM oleate/palmitate (O/P) for another 12 h. Cell viability was evaluated with the MTT assay; fat accumulation was measured using the fluorescent dye Nile red; SREBP-1, PPAR- α , iNOS and Caspase-3 protein expression were evaluated by Western blot; NFkB activity was evaluated as pNFkB/NFkB ratio. mGluR5 modulation did not alter cell viability in O/P-incubated cells; MPEP prevented intracellular lipid accumulation in O/P treated cells; MPEP administration was also associated with a reversion of O/P-induced changes in SREBP-1 and PPAR- α expression, involved in free fatty acid metabolism and uptake. No changes were observed in iNOS and caspase-3 expression, or in NFkB activity. In conclusion, mGluR5 pharmacological blockade reduced fat accumulation in HepG2 cells incubated with O/P, probably by modulating the expression of SREBP-1 and PPAR- α .

Key words: NAFLD; mGluR5; MPEP, oleate/palmitate; HepG2; SREBP-1; PPAR- α .

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is a liver disorder caused by intrahepatic fat overload, usually associated with obesity, insulin resistance, hypertension and dyslipidemia. NAFLD definition includes a vast array of hepatic conditions, such as uncomplicated fat accumulation or fatty liver, steatohepatitis (NASH), fibrosis and cirrhosis. In Western countries, NAFLD prevalence is about 20-40% in general population, rising up to 75% in obese or diabetic people, even though NASH is present in a limited portion of subjects.¹ The pathogenesis of NAFLD has been studied in *in vitro* models, animal models and in patients. According to the “two hit hypothesis”, the first hit consists in the hepatic free fatty acid (FFA) accumulation that, in turn, triggers inflammatory cascades (the second hit) resulting in a chronic inflammatory state and finally leading to NASH.^{2,3} Nowadays, the multiple-hit hypothesis is prevalent; in fact, a wide range of parallel processes are deemed to be involved in the first manifestation and in the progression of NAFLD, including insulin resistance, mitochondrial dysfunction, endoplasmic reticulum stress, activation of inflammatory pathways, adipose tissue dysfunction, genetic variants, epigenetic factors, nutritional habits and altered gut microbiome. However, at the liver level, FFA overflow is considered the triggering factor.⁴ The presence of metabotropic glutamate receptors (mGluRs) in peripheral tissues, including those not originating from neural crests, has been demonstrated in numerous studies.⁵ mGluRs have been found in organs and tissues, such as: pancreas, liver, stomach mucosa, human testes and ovaries.⁶⁻¹⁰ The presence of mGluRs in the liver has been demonstrated for the first time in 1997, when the mGluR competitive agonists 1-Amino-1,3-dicarboxycyclopentane (ACPD) and quisqualate were shown to induce inositol monophosphate formation in primary rat hepatocytes.¹¹ Successively, Storto and colleagues confirmed that mGluR5 is expressed in rat primary hepatocytes and hepatoma HepG2 cells using Western blot, immunohistochemistry and RT-PCR analysis; the homologue mGluR1 was not found in the same cells.⁷ The role of mGluRs in liver injury was also investigated: mGluR5 activation promoted ischemic damage in primary hepatocytes, while its pharmacological blockade using the selective mGluR5 negative allosteric modulator 2-methyl-6-(phenylethynyl)pyridine (MPEP), as well as mGluR5 gene depletion, provided primary hepatocytes a higher resistance to hypoxic injury.^{7,12} The blockade of mGluR5 protected also against oxidative injury. MPEP administration was associated with a reduced ROS production, lipid peroxidation and protein SH groups oxidation in isolated rat hepatocytes treated with 0.5 mM *tert*-butyl hydroperoxide. The improvement of oxidative stress was not matched by an increase in GSH stores, suggesting that the mGluR5 negative modulator did not act by scavenging the toxic acetaminophen metabolite but by modulating inflammatory pathways. In fact, the administration of MPEP to acetaminophen-intoxicated mice reduced iNOS activity in liver homogenates, suggesting that mGluR5 may be involved in the activation of inflammatory processes.¹³ More recently, it has been shown in *ex vivo* models of hepatic cold and warm ischemia that mGluR5 blockade stops mGluR5 hyperactivation and reduces the expression of inflammatory factors such as TNF- α and iNOS.¹⁴ The mechanism underlying the increase in glutamate release involved in mGluR5 hyperactivation has not yet been completely elucidated; however, an increase in glutamate release has been observed under hypoxic conditions in isolated rat hepatocytes⁷ and in a rat portal vein ligation model.¹⁵ The resulting glutamate accumulation was considered the culprit for mGluR5 hyperactivation involved in the promotion of liver ischemic injury.^{14,16} Recently, it has been shown that an increased glutamate secretion also occurs in an alcohol-induced steatosis model, resulting in mGluR5s hyperactivation and

abnormal fat accumulation in hepatocytes.¹⁷ No study is currently available on the mGluR5 role in the development of NAFLD; then, the aim of this work was to elucidate the role of mGluR5 in an *in vitro* model of steatosis in HepG2 cells. Since we have previously shown that mGluR5 blockade is associated with the downregulation of inflammatory proteins, we opted for an *in vitro* model in which fat accumulation was associated with low inflammatory levels, to focalize our attention on the role of mGluR5 in FFA metabolism modulation.

Materials and Methods

Substances

Sodium palmitate (#P9767) and sodium oleate (#O5701), fatty acid free bovine serum albumin (BSA, #A7030), cell lysis reagent (#C3228), protease inhibitor cocktail (#P8340), Nile red (#19123), minimum essential medium EAGLE (#M4655), sodium pyruvate (#S8636), penicillin-streptomycin-amphotericin antibiotic mixture (#A5955) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, #M2128) and (S)-3,5-dihydroxyphenylglycine (DHPG, #D3689) were purchased from Sigma-Aldrich (Milan, Italy). 2',7'-dichlorofluorescein diacetate (H₂DCF-DA, #D-399) and Hoechst 33342 (#H1399) were provided respectively from Molecular Probes and Life Technologies (Invitrogen, Thermo Fisher Scientific, Milan, Italy). 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP, #1212) was acquired by Tocris Bioscience (Bristol, UK). L-glutamine was from Biological Industries (Cromwell, CT, USA), while fetal bovine serum (FBS) from South America was purchased from Corning Life Science (#35-079-CV, Oneonta, NY, USA). The mouse monoclonal anti-tubulin (#T9096) was from Sigma-Aldrich, the rabbit polyclonal anti-mGluR5 (#AB5675) was from EDM Millipore (Merk Life Science, Milan, Italy). Santa Cruz Biotechnology (Dallas, TX, USA) provided us the rabbit polyclonal anti-actin (#sc-1616) and the following mouse monoclonal antibodies: anti-sterol regulatory element-binding protein 1 (SREBP-1, #sc-365513), anti-peroxisome proliferator-activated receptor alpha (PPAR- α , #sc-398394), anti-NF κ B p65 (#sc-514451), anti-p-NF κ B p65 (#sc-136548). The mouse monoclonal anti-cleaved caspase-3 was from Cell Signaling Technology (Leiden, The Netherlands). The rabbit polyclonal anti-inducible nitric oxide synthase (iNOS, #160862) was from Cayman Chemical (Ann Arbor, MI, USA).

Cell culture

HepG2 cells were grown in minimum essential medium EAGLE supplemented with 10% FBS, 1% L-glutamine, 1% sodium pyruvate and 1% antibiotic, at 37°C and 5% CO₂. HepG2 cells were seeded in 96-well plate at 50.000 cells/well and were used at 75% of confluence. For Western blot analyses, 2x10⁶ cells were seeded onto 10 cm Petri dishes.

Treatments

On the day of the experiment, cells were washed once with sterile phosphate-buffered saline (PBS; sodium phosphate dibasic anhydrous 40 mM, sodium dihydrogen phosphate monohydrate 10 mM, 700 mM sodium chloride, pH 7.4) and pre-treated for 12 h with the mGluR5 orthosteric agonist DHPG 100 μ M alone or in combination with the negative allosteric modulator MPEP at 0.3 μ M. Palmitic and oleic acids, provided as sodium salts, were dissolved in PBS (stock solution 50 mM). After the pharmacological pre-treatment, a mixture of oleate:palmitate (O/P, molar ratio 2:1) at final concentration of 1.5 mM was diluted in a serum-free medium supplemented with 1% fatty acid free BSA, with or without the

previous pharmacological treatments, and used to incubate HepG2 cells for 12 h. Control cells were incubated in serum-free medium supplemented with 1% fatty acid-free BSA. Three independent experiments were realized; each condition was reproduced in duplicate.

Cell viability

Viability of HepG2 cells treated with fatty acids and mGluR5 modulators was assessed by the colorimetric MTT assay, as previously described.¹⁸ Briefly, cells were washed once with PBS and MTT 1.2 mM diluted in serum and BSA free fresh medium was added to each well. For negative control, 4 μ l of 25% Tryton X per well were added prior to the treatment with MTT. After 2 h of incubation at 37°C at 5% CO₂, formazan crystals were dissolved with DMSO. The absorbance was measured at 540 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Lipid accumulation

The lipid content in HepG2 cells was determined employing the vital lipophilic dye Nile red, calculated in function of labeled dsDNA by means of Hoechst 33342. At the end of treatment, cells were washed once with PBS and incubated with 1 μ M Nile red and Hoechst, for 15 min. Then, cells were washed once with PBS and images were taken using ZOE (Bio-Rad). Images were then analyzed by ImageJ software, using the subtraction background method (50%).

Reactive oxygen species production

Oxidative stress in HepG2 cells was obtained using the conversion of the 2',7'-dichlorofluorescein diacetate (H₂DCF-DA) to a fluorescent molecule, the 2',7'-dichlorofluorescein (DCF), compared to Hoechst 33342 stained nuclei. Cells were incubated with a PBS solution containing H₂DCF-DA and Hoechst, both at 1 μ M, for 15 min at 37°C and 5% CO₂. Then, cells were washed once with PBS and observed at the fluorescent cell imager ZOE (BIO-RAD). Images were analyzed by ImageJ software, using the subtraction background method (100%).

Western blot

HepG2 cells were treated with 500 μ L of CelLytic buffer supplemented with protease inhibitor cocktail (10 μ L/mL) and centrifuged at 15000 g for 10 min. The protein concentration was determined by Lowry assay. Cell extracts containing the same amount of proteins were separated in SDS-PAGE on 7, 10 or 12% acrylamide gels, and transferred to PVDF membrane, as previously described.¹⁴ Unspecific sites were blocked for 2 h with 5% BSA

in tris-buffered saline (TBS, 20 mM Tris/HCl, 500 mM sodium chloride, pH 7.5, 0.1% Tween 20) at 4°C. The membranes were incubated with primary antibodies overnight at 4°C, under gentle agitation, while secondary antibodies were incubated at the same condition for 1 h. Primary antibodies against mGluR5, SREBP-1, PPAR- α , iNOS, NFkB p65 and phospho-NFkB p65, cleaved caspase-3, tubulin and actin were used at 1:1000 dilution. Mouse peroxidase-conjugated secondary antibody was used at 1:1000 dilution for SREBP-1, PPAR- α and NFkB, as well rat peroxidase-conjugated secondary antibody for cleaved caspase-3. Immunostaining was revealed with BIO-RAD Chemidoc XRS+. Bands intensity quantification was performed by Bio-Rad Image Lab software 5.

Statistical analysis

Statistical analysis was performed using R Statistical software (v. 3.3.0) and the graphical interface R Studio (v. 1.0.143). The normality and homogeneity of variances were verified employing Shapiro's test and Levene's test, respectively. In the majority of cases, data had normal distribution curves and ANOVA and Tukey's HSD Test used for multiple comparisons. The Kruskal-Wallis non-parametric test was used for data with non-homogeneous variances; in this case, Dunn's test was used for multiple comparisons.

Results

mGluR5 protein expression in HepG2

mGluR5s have been previously found in rat and mouse hepatocytes^{11,19} and in HepG2.⁷ However, considering that the mGluR5 expression on human hepatoma cells has not been extensively investigated, we decided to evaluate its expression in the hepatoma cell lines available in our laboratory (HepG2 and Huh7.5) in comparison with the receptor expression in rat cortex. As expected, mGluR5 is much more expressed in rat cortex than in hepatoma cells, even though it was significantly expressed in HepG2 cells (Figure 1). The mGluR5 expression in Huh7.5 cells was considered too scarce.

mGluR5 blockade reduces fat accumulation in HepG2 treated with 1.5 mM O/P mix

Based on mGluR5 expression levels, HepG2 were chosen for these experiments. HepG2 were pre-incubated for 12 h with 100 μ M of mGluR5 agonist DHPG alone (DHPG group) or with MPEP

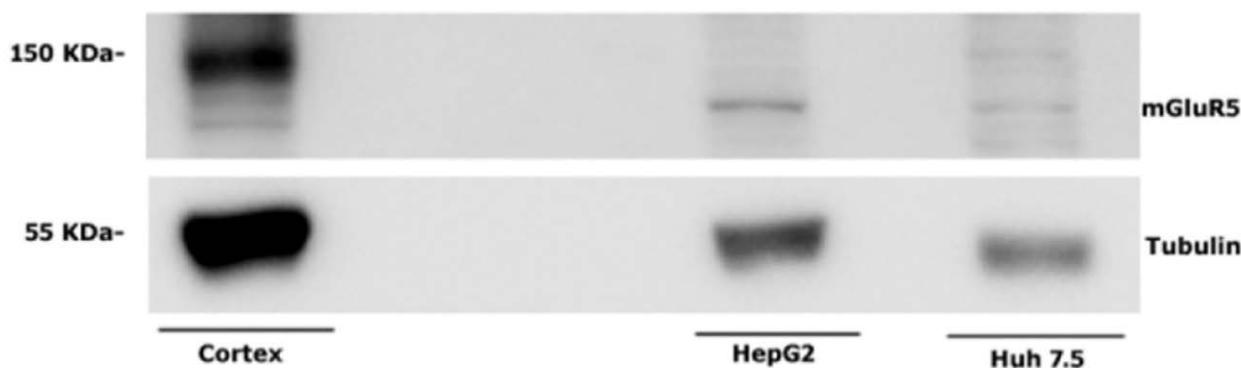


Figure 1. Expression of mGluR5 in rat cortex, HepG2 and Huh7.5 hepatoma cells.

0.3 μ M (MPEP group); then, O/P mixture was added to both DHPG and MPEP groups for 12 h. In MIX group, HepG2 were incubated with O/P mixture and received no drug treatment; CTRL group consisted in untreated control HepG2. A significant reduction in the MIX group cell viability, as evaluated with the MTT assay, was found in comparison with the CTRL group; no other significant difference was observed (Figure 2A). Fat accumulation was evaluated as Nile red/Hoechst ratio using Image J. The administration of 1.5 mM O/P mixture to HepG2 induced a significant increase in fat content in the MIX group compared to CTRL cells. The activation of mGluR5 in O/P-treated cells did not result in a further increase in fat accumulation when comparing the DHPG group with untreated MIX cells, suggesting that either mGluR5 or the downstream mechanism involved in fat accumulation was fully

activated by the O/P mixture alone. Finally, the mGluR5 blockade in MPEP group reduced significantly FFA accumulation when comparing MPEP cells to both MIX and DHPG groups (Figure 2 B-F). The expression of mGluR5 was also evaluated but no significant difference was found between the experimental groups (Supplementary Figure 1).

mGluR5 blockade is associated with a reversion of O/P mixture-induced changes in SREBP-1 and PPAR- α expression

SREBP-1 and PPAR- α were evaluated in association with the mGluR5 blockade to better clarify the changes in fat accumulation. SREBPs are involved in the modulation of cholesterol biosynthesis/uptake and fatty acid biosynthesis;²⁰ SREBP-1 mRNA has been

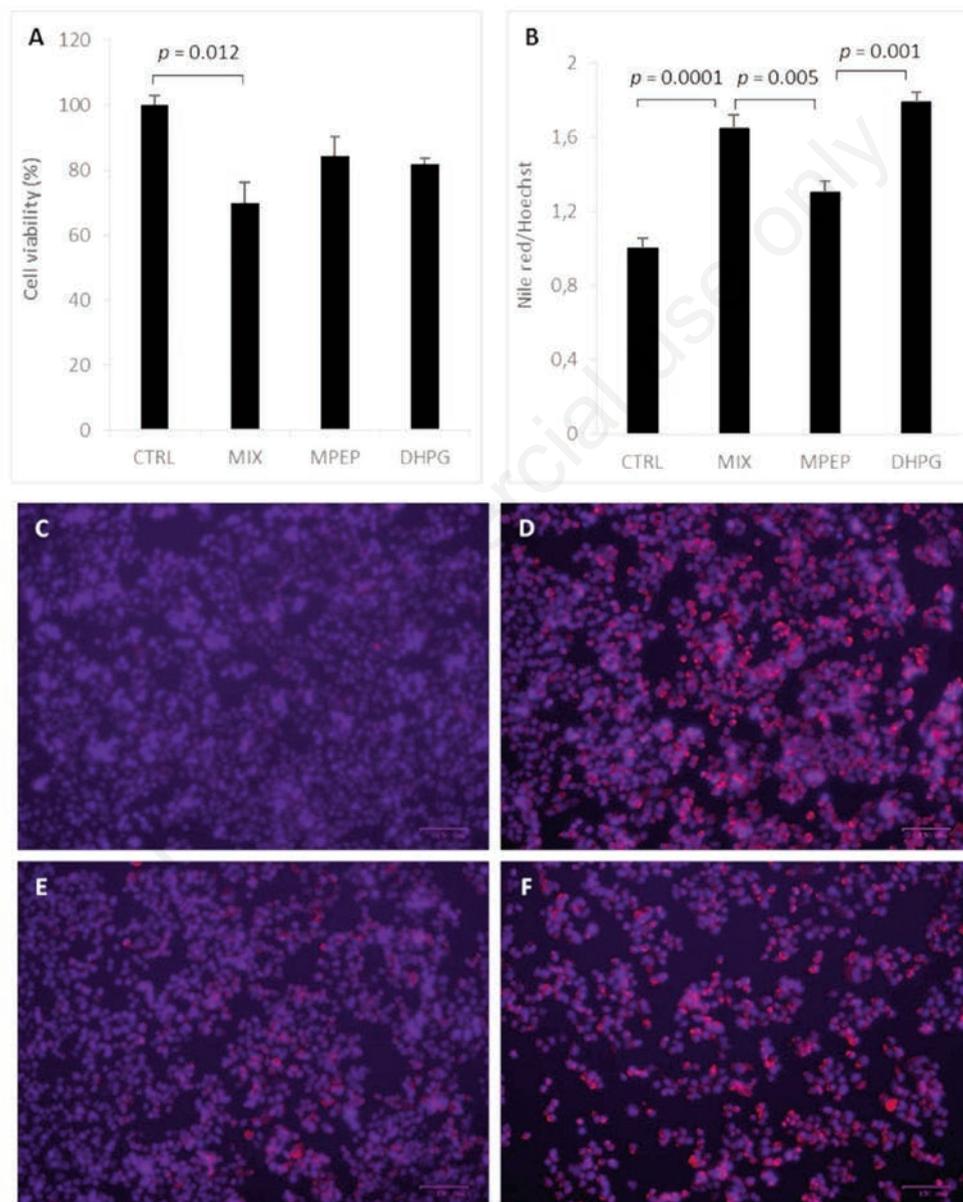


Figure 2. Effect of mGluR5 activation (DHPG) and blockade (MPEP) on cell viability and O/P mix-induced fat accumulation in HepG2 cells. A) 1.5 mM O/P mixture induced a significant decrease in cell viability in the MIX group in comparison with the CTRL group. B) FA content in HepG2 cells increased significantly when cells were incubated in presence of 1.5 mM O/P mixture; the blockade of mGluR5 receptor in O/P treated HepG2 cells prevented FA accumulation. C-F) Hoechst and Nile red uptake in HepG2 cells from CTRL, MIX, MPEP and DHPG groups, respectively; fluorescent probes uptake was evaluated using Image J. The error bars represent the standard error of measurements from three independent experiments each run in duplicate.

found to be upregulated in both *in vitro*^{21,22} and *in vivo*²³ models of hepatic steatosis. PPAR- α is also considered a major regulator of lipid metabolism in the liver;²⁴ OA-induced fat accumulation resulted in a reduced PPAR- α expression in HepG2.²⁵ In our model, mGluR5 blockade was associated with a reversion in SREBP-1 and PPAR- α expression changes observed in HepG2 treated with 1.5 mM O/P mixture. As expected, the incubation with O/P mixture resulted in a significant increase in SREBP-1 and decrease in PPAR- α expression; on the contrary, the mGluR5 blockade restored SREBP-1 and PPAR- α to levels similar to those observed in CTRL group. For both SREBP-1 and PPAR- α , the DHPG group was not significantly different from the MIX group, although it was significantly different from the MPEP group (Figure 3 A,B).

12-h incubation with O/P mixture does not affect iNOS and caspase-3 expression or NFkB activation

Hepatocytes, the most represented population of liver cells, are among the most important sources of iNOS. iNOS is almost undetectable in non-pathological conditions and its expression is usually induced in response to stress; it has also been found that hepatocyte-derived iNOS has protective effects on NASH involving the iNOS-mediated modulation of hemoxygenase (HO)-1 expression.²⁶ In our model of hepatocyte fat accumulation, the expression of iNOS was unaltered when comparing MIX and CTRL groups; moreover, the activation/blockade of mGluR5 did not affect iNOS expression levels (Figure 4A). To rule out the activation of inflammatory processes in the MIX group, we also evaluated NFkB activation; as a result, we found that in the MIX group

NFkB activation was not significantly different with respect to the CTRL group. However, the mGluR5 blockade was associated with a significant reduction of NFkB activation (Figure 4B). Caspase-3 activation is considered a prominent event in different experimental models of NAFLD; moreover, the pan-caspase inhibitor VX-166 reduced the development of fibrosis in murine NASH models.²⁷ We found no significant changes in caspase-3 expression after HepG2 incubation with O/P mixture, with or without mGluR5 modulation (Figure 4C). Finally, using H₂DCF-DA to evaluate oxidative stress, we observed that the administration of oleate and palmitate induced the production of reactive oxygen species (ROS), while MPEP treatment lowered ROS levels (Figure 5; Supplementary Figure 2). Considered together, these results suggest that, in our FFA accumulation model, inflammatory processes are not activated and the reduction in FFA accumulation may be related to a modulation of SREBP-1 and PPAR- α .

Discussion

Considering NAFLD rising prevalence at a global level, it is essential to find effective molecular targets suitable as therapeutic options for its treatment. Life style changes are currently among the recommended non-pharmacological therapies for treating NAFLD, even though their long-term sustainability is unsatisfactory due to poor patient compliance.²⁸ The recommended pharmacological treatment for patients with NAFLD currently involves the administration of glitazones, bile acids or bile acid analogues, fibrates, vitamin E, metformin, and betaine. However, no drug has

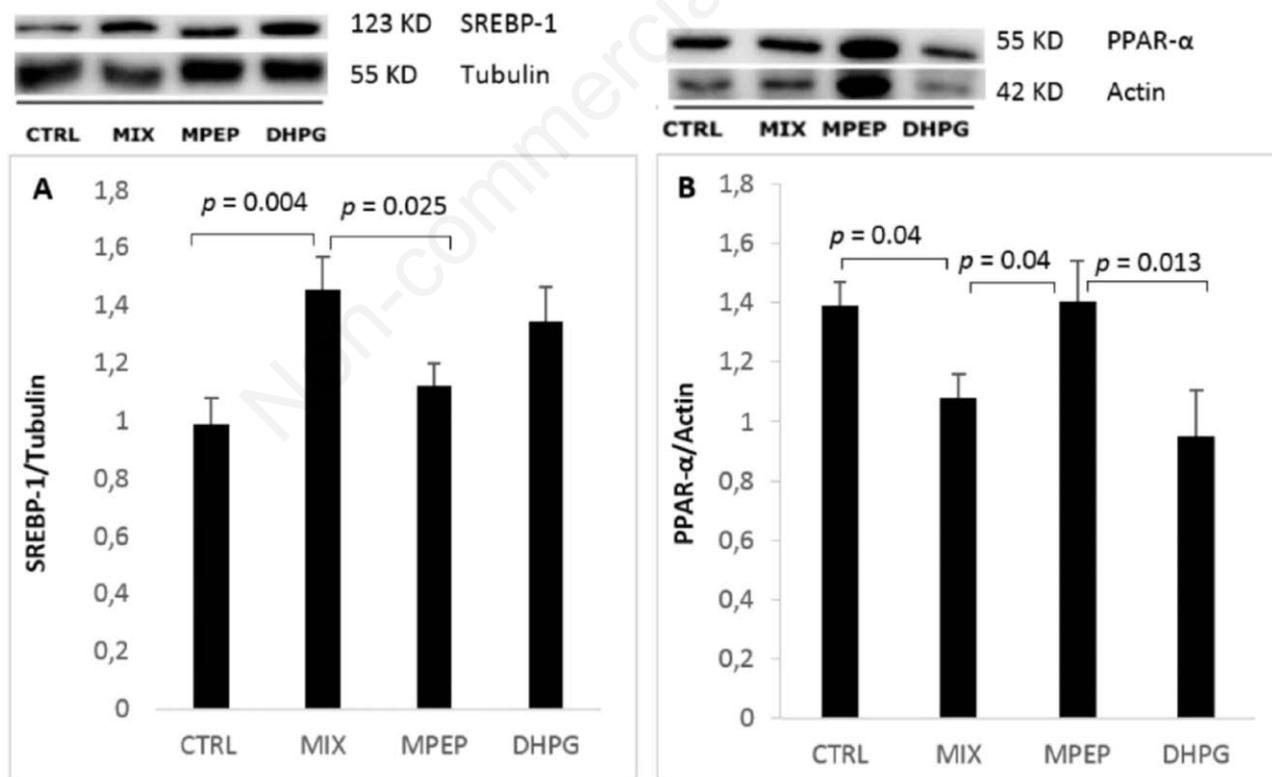


Figure 3. mGluR5 blockade reverts SREBP-1 and PPAR- α expression in HepG2 treated with 1.5 mM O/P mixture. A) The incubation of HepG2 cells with 1.5 mM O/P mixture induced a significant increase in SREBP-1 protein expression; the blockade of mGluR5 (MPEP group) reduced SREBP-1 to levels similar to the CTRL group; the DHPG group was not significantly different from the MIX group. B) In the MIX group, PPAR- α expression was significantly lower when compared to CTRL; the addition of MPEP to DHPG 100 μ M prevented the change in MPEP group. The error bars represent the standard error of measurements from three independent experiments each run in duplicate.

currently passed phase III trials and was approved for NASH by regulatory agencies.^{29,30} FFAs have a pivotal role in the pathogenesis of NAFLD and their hepatic levels are closely related to NAFLD severity.³¹ In this work, we showed for the first time that MPEP, a negative mGluR5 modulator, reduces FFA accumulation in HepG2 human hepatoma cells incubated with a 1.5 mM O/P mixture. We also found that MPEP administration is associated with a reversion of O/P-induced changes in SREBP-1 and PPAR- α expression and that these changes are independent from inflam-

matory processes. The primary goal of this study was to investigate the role of mGluR5 in FFA accumulation in HepG2 human hepatoma cells. The ability of certain substances to reduce FFA accumulation has been previously investigated, especially in models involving a significant degree of cytotoxicity. In a study on HepG2 cells, 24-h treatment with a 2:1 OP mixture was associated with FFA accumulation as well as the upregulation of inflammatory cytokines, such as TNF- α , IL-6 and IL-1 β ; a reduced activation of NFkB was also observed. In the same work, gardenoside treat-

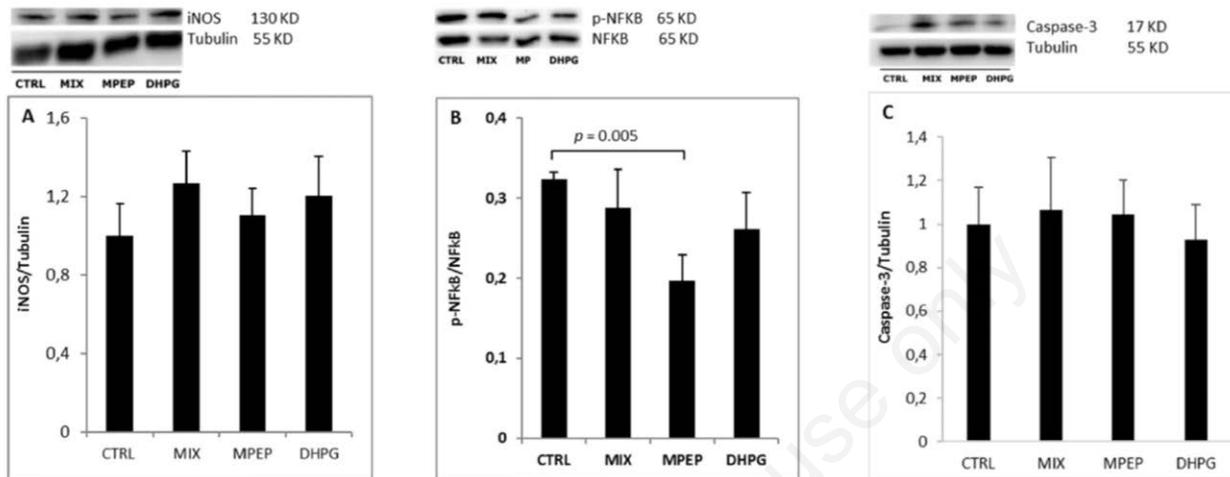


Figure 4. iNOS, NFkB and cleaved caspase-3 expression/activation does not change in HepG2 cells incubated with 1.5 mM O/P mixture. A) HepG2 incubation with 1.5 mM O/P mixture or mGluR5 agonist/negative modulator did not induce significant changes in iNOS expression levels. B) HepG2 incubation with 1.5 mM O/P mixture did not alter NFkB activation levels; mGluR5 blockade induced a significant reduction in NF-kB activation. C) No changes were observed in cleaved caspase-3 expression. The error bars represent the standard error of measurements from three independent experiments each run in duplicate.

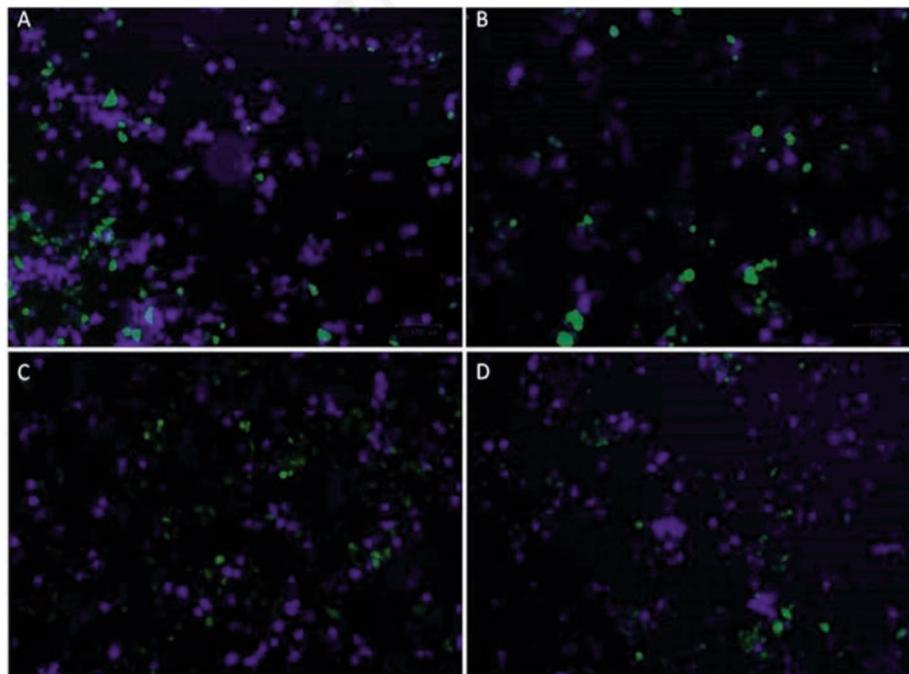


Figure 5. Effect of mGluR5 activation (DHPG) and blockade (MPEP) on O/P mix-induced ROS production in HepG2 cells. Hoechst and H₂DCF-DA uptake in HepG2 cells from CTRL, MIX, MPEP and DHPG groups, respectively. Fluorescent probes uptake was evaluated using Image J.

ment resulted in a 2-fold decrease in FFAs possibly related, according to the authors, to the anti-inflammatory effect of gardenoside itself.³² In a similar work, glycyrrhetic acid, a licorice extract credited of anti-inflammatory properties in hepatitis, was found to reduce FFA accumulation in HepG2 cells. Also in this work, the FFA-reducing effect was related by the authors to glycyrrhetic acid ability to stabilize lysosomal membranes, to inhibit cathepsin B and mitochondrial cytochrome c release and to reduce FFA-induced oxidative stress.³¹ In other studies, a higher proportion of palmitic acid is used to induce the inflammatory injury.³³ In our study, we selected a 12-h, 2:1 OP treatment; the reduced incubation time, associated with the low proportion of palmitic acid resulted in minor/absent lipotoxic effects, thus representing a cellular model of FFA accumulation mimicking benign asymptomatic chronic steatosis.³³ The incubation of HepG2 cells with 2:1 OP resulted in a 1.6-fold increase in FFA accumulation; however, iNOS and cleaved caspase-3 expression, and NFκB activation state did not change in comparison with control cells, suggesting that in our model inflammatory processes are not yet activated. The pharmacological blockade of mGluR5 by MPEP administration resulted in a significant reduction in FFA accumulation, so that MPEP-group FFA content was comparable to the control cells. Glutamate role at the central level in the metabolic homeostasis has long since been established; in a study, the injection of monosodium glutamate into newborn mice resulted in central obesity and moderate to severe NAFLD at 6-month age.³⁴ The current explanation is that high doses of monosodium glutamate have neurotoxic effects in the hypothalamic arcuate nucleus (ARC). ARC neurons are crucial for the regulation of metabolic homeostasis, including insulin secretion and action.³⁵ It has also been shown that mGluR5 modulates central reward pathways and modulates feeding stimulus: selective mGluR5 negative modulation reduced food intake in mice after food deprivation and in obese rats,³⁶ selective mGluR5 negative modulation also reduced high-palatable food intake in baboons.³⁷ Conversely, intracerebroventricularly injected CHPG, an mGluR5 agonist, induced a dose-dependent stimulation of food intake in rodents.³⁸ These studies prompted a Phase I clinical trial to test GET73, a selective mGluR5 negative modulator, as a novel therapeutic agent to treat neuropsychiatric disorders, including alcohol abuse and obesity.³⁹ If many studies have been published on the role of mGluR5 in central modulation of appetite and obesity, the same cannot be said on the investigation of mGluR5 role in peripheral organs such as the liver. At the best of our knowledge, only one paper investigated the role of mGluR5 in alcoholic steatosis; in this work, Choi and colleagues demonstrated the alcohol-induced selective expression of mGluR5 in hepatic stellate cells (HSCs), stimulating 2-arachidonoylglycerol (2-AG) production, a molecule promoting *de novo* lipogenesis in alcoholic liver disease.¹⁷ We have previously shown that mGluR5 is expressed in primary mice and rat hepatocytes and in human HepG2 cells^{7,12,13} Moreover, in this work, we have shown that FFA accumulation is inhibited by mGluR5 selective blockade in a HepG2-based model of non-inflammatory fat accumulation, showing that lipid metabolism and uptake may be regulated by this receptor without the intervention of HSCs.

SREBP-1 is a transcriptional factor known to regulate the expression of hepatic lipogenic enzymes reported to play an important role in the pathogenesis of NAFLD.⁴⁰ SREBP-1 upregulation has been associated to oleate or palmitate administration in *in vitro* NAFLD/NASH models^{21,22,41} and to fructose or leptin administration in *in vivo* NAFLD models.^{23,42} On the other hand, mice with genetic deletion of PPAR-α are more prone to hepatic triglycerides hyperaccumulation when fed with a high fat diet, developing a significantly higher NAFLD activity score compared to WT controls.⁴³⁻⁴⁵ PPAR-α knockout mice fed with a high fat diet also have

increased markers of oxidative stress, inflammation, and cell death.⁴⁶ A reduced PPAR-α expression was also observed in an oleic acid-induced steatosis in HepG2.²⁵ In this work, MPEP administration was associated to SREBP-1 downregulation and PPAR-α upregulation in OP-treated HepG2, restoring in both cases values not dissimilar to those found in CTRL group, in concomitance with a reduced FFA accumulation. It has been shown in NAFLD that mTOR, controlling the nuclear localization of both lipin1 and nuclear receptor corepressor 1 (NCoR1), promotes SREBP-1 upregulation and PPAR-α downregulation, respectively.⁴⁷⁻⁴⁹ To date, no work has been published demonstrating that the mTOR pathway is a downstream target of mGluR5 in the liver. However, mGluR5 has been found to activate the mTOR pathway in hippocampal slices, a mechanism that seems to be compromised in Fragile X syndrome,^{50,51} a condition associated with a loss of visceral adiposity.⁵² It is possible then that the same pathway might be replicated also in hepatocytes, thus justifying the effects on SREBP-1 and PPAR-α expression we observed in MPEP-treated fatty hepatocytes (Figure 6).

It is not possible to extrapolate from our data the exact modalities of mGluR5 activation. A glutamate accumulation may be responsible for the mGluR5 hyperactivation as already seen in the promotion of liver ischemic injury.^{14,16} Recently, serum and liver levels of glutamate/glutamine ratio have been shown to increase significantly in patients with NAFLD; the increase in glutamate/glutamine ratio significantly correlated with the severity of NAFLD and fibrosis.⁵³ The underlying mechanism for altered glutamate levels and the associated correlation with NAFLD severity is unclear. However, gene expression changes in NAFLD patients suggest that the liver may switch from a relatively low glutamine catabolism by healthy hepatocytes to a significantly higher glutamine consumption in NASH.⁵³ These changes may be responsible for a massive conversion of glutamine into glutamate, resulting in an increase in glutamate secretion and mGluR5 hyperactivation. Recently, it has also been shown that a ROS-mediated increase in glutamate secretion occurs in a model of alcohol-induced steatosis, resulting in mGluR5 hyperactivation on stellate cells and abnor-

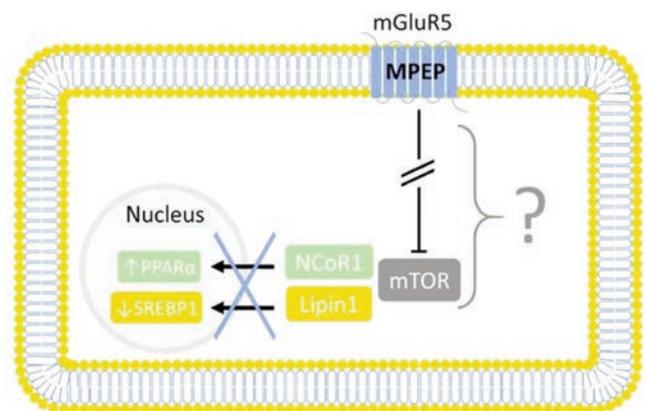


Figure 6. Schematic representation of the hypothetical mechanism proposed for the changes observed in SREBP-1 and PPAR-α protein expression. mTOR has a central role in lipid metabolism; dysregulation of mTOR signaling has been observed in obesity, diabetes, cancer and fatty liver diseases. The modulatory mTOR role involves the modulation of SREBP-1 and PPAR-α expression. mGluR5 has been found to activate the mTOR pathway in hippocampal slices, suggesting that the same pathway might be replicated also in hepatocytes.

mal fat accumulation in hepatocytes.¹⁷ In addition to fatty acid accumulation, in our model we also observed an increase in ROS production, which was reversed by the mGluR5 blockade. Surprisingly, also the activation of this receptor significantly reduced the oxidative stress. According to our results, in a mouse model of acetaminophen toxicity, the use of MPEP has been demonstrated to protect against oxidative stress.¹³ However, because of its structure, MPEP may form conjugates with GSH,¹⁹ minimizing its protective effect. On the other hand, DHPG is able to attenuate glutathione loss in oligodendrocyte precursors, thus attenuating ROS production.⁵⁴

In conclusion, we found for the first time that the pharmacological blockade of mGluR5 reduces FFA accumulation in an *in vitro* model of steatosis. We also found that the mGluR5 inhibition is associated with a reversion of FFA induced changes in SREBP-1 and PPAR- α expression, suggesting that mGluR5 hyperactivation may promote FFA accumulation through changes in the expression of enzymes involved in FFA metabolism and uptake. Glutamate has been found to have increased tissue and serum levels in NAFLD, so explaining mGluR5 hyperactivation. A limitation of the present work is that no precise mechanism can be demonstrated based on the presented data. However, considering that mTOR is the main actor in NAFLD progression and that mGluR5 controls mTOR activation in the CNS, we suggest that a similar mechanism may reasonably occur also in human hepatoma cells.

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