EPA blocks TNF-α-induced inhibition of sugar uptake in Caco-2 cells via GPR120 and AMPK

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The aim of the present work was to investigate in Caco-2 cells whether eicosapentaenoic acid (EPA), an omega-3 polyunsaturated fatty acid, could block the inhibitory effect of tumor necrosis factor-α (TNF-α) on sugar transport, and identify the intracellular signaling pathways involved. After pre-incubation of the Caco-2 cells with TNF-α and EPA for 1 hr, EPA prevented the inhibitory effect of the cytokine on α-methyl-D-glucose (αMG) uptake (15 min) and on SGLT1 expression at the brush border membrane, measured by Western blot. The ERK1/2 inhibitor PD98059 and the AMPK activator AICAR also prevented the inhibitory effect of TNF-α on both αMG uptake and SGLT1 expression. Interestingly, the AMPK inhibitor, Compound C, abolished the ability of EPA to prevent TNF-α-induced reduction of sugar uptake and transporter expression. The GPR120 antagonist, AH7614, also blocked the preventive effect of EPA on TNF-α-induced decrease of αMG uptake and AMPK phosphorylation.

In summary, TNF-α inhibits αMG uptake by decreasing SGLT1 expression in the brush border membrane through the activation of ERK1/2 pathway. EPA prevents the inhibitory effect of TNF-α through the involvement of GPR120 and AMPK activation.

KEYWORDS
AMPK, EPA, GPR120, SGLT1, TNF-α

1 INTRODUCTION

The Inflammatory Bowel Disease (IBD) is characterized by chronic inflammation of the gastrointestinal mucosa due to a dysregulation of the immune system (Tabas & Glass, 2013). Genome, diet, and intestinal microbiota are implicated in the pathogenesis of IBD (Albenberg, Lewis, & Wu, 2012). In IBD patients, the gastrointestinal mucosa contains high concentrations of pro-inflammatory molecules such as the tumor necrosis factor α (TNF-α) (Tabas & Glass, 2013). TNF-α is a cytokine produced by activated T-lymphocytes and macrophages that acts in an autocrine and paracrine way by binding to its receptors TNFR1 and TNFR2. This binding induces the activation of the nuclear factor NF-κB which, in turn, stimulates the production of other cytokines, including TNF-α itself (Rossol et al., 2007). It has been found that the levels of TNF-α are a hundred times higher in the intestine and blood of IBD patients than in healthy people (Komatsu et al., 2001).

During intestinal inflammation, the expression and activity of many nutrients and electrolytes transporters can be modified (Bertolo, Barrenetxe, Barber, & Lostao, 2002; Foley, Pantano, Ciolino, & Mawe, 2007; Sharma et al., 2005), which may explain the characteristic malabsorption and diarrhea found in IBD patients. As a consequence, patients may lose weight and suffer from vitamins, micronutrients and protein deficiency (Hébuterne, Filippi, Al-Jaouni, & Schneider, 2009). In this sense, we have previously demonstrated in the human intestinal epithelial cell line Caco-2, that TNF-α inhibits sugar uptake by decreasing the expression of the Na+-glucose cotransporter SGLT1 in the brush border membrane (Barrenetxe et al., 2013).
The omega-3 long-chain polyunsaturated fatty acids (n-3 PUFAs) have been proposed to exert beneficial actions in a great variety of human diseases such as asthma, hypertension, myocardial infarction, some cancers, and inflammatory diseases such as obesity, atherosclerosis, and rheumatoid arthritis (Figueras, Olivan, Busquets, López-soriano, & Argilés, 2011; Hur et al., 2012; Lorente-Cebrián et al., 2013, 2015). In patients with IBD, it has been shown that treatment with n-3 PUFAs produces protection against development of illness, improves the intestinal mucosa histology, reduces inflammatory markers (TNF-α), and protects against the effects of oxidative stress (Barbalho, de Ávares Goulart, Quesada, Dib Bechara, & de Cássio Alves de Carvalho, 2016). Furthermore, it has been found a strong correlation between the incidence of Crohn’s disease and the decrease in n-3 PUFAs in the diet (Issa & Saeian, 2011; Shoda, Matsueda, Yamato, & Umeda, 1996).

The eicosapentaenoic acid (EPA) is an n-3 PUFA found in fish oil. In high-fat-fed rats, it has been observed that EPA blocks the increase of TNF-α production in adipose tissue (Pérez-Matute, Pérez-Echarri, Martínez, Martí, & Moreno-Aliaga, 2007). In murine cultured adipocytes, EPA directly inhibits the stimulation of lipolysis induced by TNF-α through mechanisms that include inhibition of the pro-inflammatory pathways ERK1/2 and NF-κB, and stimulation of the AMPK pathway (Lorente-Cebrián, Bustos, Martí, Martínez, & Moreno-Aliaga, 2009; Lorente-Cebrián et al., 2012).

GPR120, also known as free fatty acid receptor 4, is a cell-surface receptor, member of the G protein-coupled receptors (GPCRs) superfamily, which can bind unsaturated medium-to-long chain fatty acids (Hirasawa et al., 2005; Ichimura, Hara, & Hirasawa, 2014). GPR120 is widely expressed in several organs, including intestine, and it is involved in several homeostatic functions such as inflammation, glucose homeostasis, and insulin sensitivity (Ichimura et al., 2014). Furthermore, it has been observed that the anti-inflammatory effects of EPA and DHA are mediated by GPR120 acting as a sensor/receptor (Oh et al., 2010).

The goal of the present study was to analyze the ability of EPA to block the inhibitory effect of TNF-α on intestinal sugar transport. Moreover, we aimed to unravel the potential involvement of ERK1/2, AMPK, and GPR120 in EPA actions.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell culture

Caco-2 cells (RRID: CVCL_Z580) were maintained at 37°C and 5% CO₂ in a humidified atmosphere. The cells were grown in Dulbecco’s Modified Eagles medium (DMEM [1X] + GlutaMAX, Gibco, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific), 1% non-essential amino acids (NEAA 100×, LONZA, Basilea, Switzerland), 1% penicillin (10,000 U/ml) + streptomycin (10,000 µg/ml) (Gibco, Thermo Fisher Scientific), and 1% amphotericin B (250 µg/ml, Gibco, Thermo Fisher Scientific). The culture medium was changed every 2 days. When cells reached 80% confluence, confirmed by microscopic observance, they were dissociated with 0.05% trypsin-EDTA (0.25% trypsin 1X, Gibco, Thermo Fisher Scientific) and subcultured on 75 cm² plastic flasks at a density of 25 x 10⁶ cells/cm². For the uptake studies, the cells were seeded at a density of 6 x 10⁴ cells/cm² in 24-well culture plates. Experiments were performed 15–19 days post-seeding.

### 2.2 | Sugar uptake in the presence and absence of TNF-α and EPA

Cells were grown in 24-well culture plates. The cells were pre-incubated for 1 or 24 hr in Dulbecco’s Modified Eagles Medium without glucose (DMEM, Gibco, Thermo Fisher Scientific), supplemented with 1% BSA free fatty acids (Sigma-Aldrich, Saint Louis, MO) in control conditions and in the presence of 10 ng/ml TNF-α (PeproTech, Inc., Rocky Hill, NJ) without or with 10 or 100 µM EPA (Cayman, Ann Arbor, MI). This EPA concentration range has been reported to be comparable to plasma concentrations of this fatty acid after intake of dietary EPA (Murata et al., 2000; Perez-Matute et al., 2005). After the pre-incubation period, cells were incubated for 15 min with 0.1 mM α-methyl-α-glucose (aMG) and traces of [14C]-α-methyl-glucoside (0.3 µCi/ml). After incubation, the reaction was stopped by adding 500 µl of cold Phosphate Buffered Saline with calcium and magnesium (PBS, Sigma–Aldrich). Cells were then washed three times with PBS and solubilized by adding 500 µl 1% Triton X-100 in 1 M NaOH for 1 hr 30 min at 37°C. After this time, samples were analyzed in the scintillation counter.

### 2.3 | Identification of the intracellular signaling pathways and the implication of GPR120 on EPA effect

Cells were grown in 24-well culture plates. For the intracellular signaling pathways studies, the cells were pre-incubated for 1 hr with 10 ng/ml TNF-α in the absence or in the presence of 100 µM EPA plus 1 mM AICAR (AMPK activator), 50 µM PD98059 (ERK inhibitor), or 20 µM Compound C (CC, AMPK inhibitor). To allow the permeability of CC and PD, cells were incubated with each of these compounds for 30 min before starting the pre-incubation period. For the studies about the implication of GPR120 on EPA effect, the cells were pre-incubated for 1 hr with 10 ng/ml TNF-α in the absence or in the presence of 100 µM EPA and the GPR120 antagonist AH7614 (Tocris, Bristol, UK) at 100 µM. After the pre-incubation, cells were incubated for 15 min with 0.1 mM aMG and traces of [14C]-α-methyl-glucoside and treated as described in section 2.2. All experiments were performed in DMEM supplemented with 1% BSA free fatty acids.

### 2.4 | Western blot analysis

Cells grown on 75 cm² plastic flasks were treated as described in section 2.3. After the incubation period, brush border membrane vesicles (BBMV) were isolated using the method of Shirazi-Beechey et al. (1990) with some modifications (Garriga, Rovira, Moreto, & Planas, 1999), we also obtained the cytosolic fraction with this technique. All the manipulations were carried out at 4°C to avoid protein degradation of
the samples. The protein content of the vesicles was determined by the standardized method of Bradford (Bio-Rad Protein Assay; Bio-Rad Laboratories, Hercules, CA). Solubilized proteins from BBMV or cytosolic fractions (20 μg) were resolved by electrophoresis on 12% SDS–PAGE mini-Protean TGX gels (Bio-Rad). The resolved proteins were transferred to a PVDF membrane (Hybond P, GEHealthcare, Little Chalfont, UK) which was then blocked in TBS-Tween 1X buffer (TBS-T 1X) with 10% of milk (Nestle, Sveltesse, Vevey, Switzerland) for 2 hr at room temperature and incubated overnight at 4°C with the corresponding primary rabbit antibodies used at 1:1,000. The primary antibodies were anti SGLT1 (Santa Cruz Biotechnology, Dallas, TX, Cat# sc-98974, RRID: AB_2191582), anti AMPKα (Cell Signaling Technology, Danvers, MA, Cat# 2532, RRID: AB_330331), anti Phospho-AMPKα (Cell Signaling Technology, Cat# 2535, RRID: AB_331250), anti-ERK1/2 (Cell Signaling Technology Cat# 4695, RRID: AB_390779), and anti-Phospho-ERK1/2 (Cell Signaling Technology Cat# 4370, RRID: AB_2315112). The β-actin (Santa Cruz Biotechnology Cat# sc-47778, RRID: AB_626632) mouse antibody was also used at 1:1,000. After the incubation with the corresponding antibody, the membranes were washed out four times in TBS-T 1X and incubated for 1 hr at room temperature with the corresponding peroxidase conjugated secondary antibody, goat anti-rabbit (Santa Cruz Biotechnology, Cat# sc-2004, RRID: AB_631746), and goat anti-mouse (Santa Cruz Biotechnology, Cat# sc-2005, RRID: AB_631736) at 1:10,000. The immunoreactive bands were detected by enhanced chemiluminescence (Super Signal West Dura; Thermo Scientific, Waltham, MA) and quantified by densitometry analysis (Image Studio Lite, Li-cor, Lincoln, NE, RRID: SCR_014211). The results were expressed in percentage of the control value, which was set to 100.

2.5 Statistical analysis

Statistical analysis was performed using the program Stata v12 (Stata, RRID: SCR_012763). Parametric or non-parametric tests (One-way ANOVA, Kruskal–Wallis test, Median test) followed by the corresponding post hoc test (Tukey, SNK, Bonferroni) were run depending on the sample size and the normality of the data. Results were expressed as mean ± Standard Error of the Mean (SEM), and differences were considered significant at a p-value <0.05.

3 RESULTS

3.1 Sugar uptake in the presence of TNF-α and EPA

As presented in Figure 1, 10 ng/ml TNF-α (1 hr) decreased 0.1 mM αMG uptake in Caco-2 cells by ~30%, similar as shown by Barrenetxe et al. (2013). Pre-incubation of the cells for 1 hr with 100 μM EPA totally blocked TNF-α inhibition of αMG transport. EPA alone did not have any effect on sugar uptake (Figure 1). A similar preventive effect on TNF-α action was observed after chronic treatment (24 hr) with 100 μM EPA, while 10 μM EPA for 1 hr was not able to significantly counteract the actions of the cytokine on αMG uptake (data not shown).

3.2 Implication of the ERK1/2 intracellular pathway in the preventive effect of EPA on TNF-α inhibition of αMG uptake

As previous studies in adipocytes have shown that EPA acts through the inhibition of the ERK1/2 pathway (Lorente-Cebrián et al., 2012), we first decided to investigate whether ERK1/2 intracellular pathway was involved in the TNF-α inhibition of αMG uptake. As shown in Figure 2a, the incubation of the cells with 10 ng/ml TNF-α was accompanied by an increase of ERK1/2 phosphorylation (activation). As expected, the ERK1/2 inhibitor PD98059 (50 μM) alone or together with TNF-α, induced a clear decrease on ERK1/2 phosphorylation. Moreover, the pre-incubation of the cells with PD98059 blocked TNF-α-induced decrease of sugar uptake (Figure 2b). In agreement with these results, TNF-α reduced the amount of SGLT1 in Caco-2 cells BBMV, but the ERK1/2 inhibitor PD98059 prevented this reduction (Figure 2c). PD98059 alone did not have any effect on αMG uptake or SGLT1 expression in the apical membrane (Figures 2b and 2c). All these results demonstrated that TNF-α decreases αMG uptake by diminishing SGLT1 expression in the apical membrane through ERK1/2 activation.

Next, we studied whether EPA could act through the ERK1/2 pathway to prevent TNF-α-inhibition of sugar uptake. As shown in Figure 2d, EPA alone did not alter ERK1/2 phosphorylation with respect to control. On the other hand, the incubation of the cells with 100 μM EPA in the presence of TNF-α did not modify the increase on ERK1/2 phosphorylation levels observed after TNF-α treatment (Figure 2d). In addition, αMG uptake in the presence of EPA, TNF-α and PD98059 was not altered when compared to control (Figure 2e),

![FIGURE 1](image-url)  
**EPA blocks TNF-α inhibition of αMG uptake.** Caco-2 cells were pre-incubated for 1 hr with 10 ng/ml TNF-α in the absence or in the presence of 100 μM EPA before measuring the uptake of 0.1 mM αMG for 15 min. Data (n = 12) are represented as mean ± SEM. *p < 0.05 versus control; &&p < 0.01 versus TNF-α.
which further suggests that the ERK1/2 pathway is not involved in the EPA blocking effect of TNF-α inhibition of sugar uptake.

3.3 Implication of AMPK activation in the preventive effect of EPA on TNF-α inhibition of αMG uptake

It is known that EPA activates the AMPK intracellular pathway (Wu et al., 2012). Moreover, AMPK is entailed in the EPA blocking effect of lipolysis induced by TNF-α (Lorente-Cebrián et al., 2009, 2012). Therefore, we decided to investigate whether AMPK could be also involved in the preventive effect of EPA on TNF-α inhibition of αMG uptake. Our data showed that the phosphorylation (activation) of AMPK was diminished by the incubation of the cells with 10 ng/ml TNF-α (Figure 3a). Interestingly, in the presence of 100 μM EPA, TNF-α did not modify AMPK phosphorylation levels compared with the control condition (Figure 3a). To deepen in the role of AMPK in the effect of EPA, we performed a new set of experiments using the AMPK inhibitor CC (20 μM) and its activator AICAR (1 mM). As expected, the AMPK inhibitor alone reduced the phosphorylation of AMPK and also reversed the ability of EPA to prevent the inhibition of AMPK phosphorylation induced by TNF-α (Figure 3a). On the other hand, AICAR increased AMPK phosphorylation and this stimulatory effect was maintained in the presence of TNF-α (Figure 3a).
decreased the expression of the sugar transporter, whereas in the presence of EPA or AICAR, the inhibition induced by TNF-α on SGLT1 expression was not observed (Figure 3c). However, CC totally abolished the ability of EPA to prevent the reduction of SGLT1 expression in the apical membrane induced by TNF-α (Figure 3c).

As expected, EPA diminished the inhibitory effect of CC on AMPK phosphorylation, sugar uptake and SGLT1 expression in the plasma membrane (supplementary Figure S1).

All these results demonstrated that the AMPK intracellular pathway is implicated in the insertion of SGLT1 in the plasma membrane and that this pathway is regulated by both, EPA and TNF-α.

To investigate if ERK1/2 activation is involved in TNF-α effect on AMPK we analyzed the phosphorylation of AMPK in the presence of PD98059 (ERK1/2 inhibitor) alone and with TNF-α. As shown in Figure 4, the decrease induced by TNF-α on AMPK phosphorylation was partially prevented by PD98059. In the presence of PD98059 alone, AMPK phosphorylation level was similar than in the control condition (Figure 4). These results suggest the involvement of GPR120 stimulation in the effect of EPA on TNF-α-induced inhibition of sugar uptake.

### 3.4 | Implication of GPR120 in EPA effect on TNF-α-induced inhibition of αMG uptake

Finally, we investigated whether EPA action on αMG uptake was mediated through the EPA receptor GPR120. Our data showed that in the presence of the GPR120 antagonist AH7614 (100 μM), EPA was not able to block either TNF-α inhibitory effect on αMG uptake (Figure 5a) or TNF-α-induced inhibition of AMPK phosphorylation (Figure 5b). These results suggest the involvement of GPR120 stimulation in the effect of EPA on TNF-α-induced inhibition of sugar uptake.

### 4 | DISCUSSION

Our group previously demonstrated in Caco-2 cells that TNF-α, via TNFR1 receptor, decreases galactose and αMG uptake by down-regulating the expression of the Na+/glucose cotransporter SGLT1 in the apical membrane (Barrenetxe et al., 2013).

In the present work, we show that TNF-α reduces sugar transport in Caco-2 cells through the activation of ERK1/2 that would inhibit the AMPK pathways which, in turn, would induce the recruiting of SGLT1 from the apical membrane into intracellular compartments. Other authors have demonstrated that the activation of intracellular MAPKs-dependent signaling pathways (ERK, P38 and JNK) regulates the expression of rabbit SGLT1 (Castaneda-Sceppa, Subramanian, & Castaneda, 2010). Also, it has been shown in muscle cells that endoplasmic reticulum stress activates ERK which, in turn, decreases AMPK activity. The ERK inhibitor U0126, recovered AMPK phosphorylation (Hwang et al., 2013). Regarding the role of AMPK in the regulation of intestinal sugar transport, we show that activation of AMPK by AICAR enhances SGLT1 expression in the brush border membrane of Caco-2 cells, as it has been previously observed (Sopjani et al., 2010). Moreover, AMPK inhibition by CC reduces the levels of the other hand, AICAR prevented the decrease of αMG transport induced by TNF-α treatment (Figure 3b), as occurred with EPA.

Regarding SGLT1 expression in the brush border membrane, as previously shown (Figure 2c), pre-incubation of the cells with TNF-α
SGLT1 in the brush border membrane, supporting a role of AMPK in the regulation of this sugar transporter. In this context, we have recently shown that Cardiotrophin-1, a cytokine of the IL-6 family, decreases sugar transport by reducing SGLT1 expression in Caco-2 cells apical membrane through the inhibition of AMPK (López-Yoldi et al., 2016).

In the last years, the potential beneficial effects of n-3 fatty acids on intestinal inflammation have been widely studied (Almallah et al., 1998; Belluzzi et al., 1996; Calder, 2008). At the molecular level, EPA and DHA show anti-inflammatory properties in premature rat pups by regulating eicosanoid- and nuclear factor-κB-related metabolite expression (Ohtsuka et al., 2011). Here, we demonstrate for the first time, that EPA prevents the inhibitory effect of TNF-α on sugar uptake by activating AMPK. In line with these results, glucose transport in weaning piglets’ jejunum is up-regulated when feeding n-3 PUFA during gestation and lactation via activation of AMPK (Gabler, Radcliffe, Spencer, Weibel, & Spurlock, 2007). The implication of AMPK on the physiological effect of EPA has already been demonstrated in 3T3-L1 adipocytes, where EPA blunts the lipolytic effect of TNF-α through the stimulation of this intracellular pathway (Lorente-Cebrián et al., 2012). Also, in diet-induced obese mice, diet containing n-3 PUFAs increases AMPK phosphorylation levels in several tissues (González-Pérez et al., 2009; Kopecky et al., 2009).

It has been proved that GPR120 stimulation by n-3 fatty acids inhibits inflammatory responses mediated by TNF-α (Calder, 2015; Oh & Lagakos, 2011). Moreover, it is widely known that GPR120 usually acts as EPA receptor (Oh & Lagakos, 2011). GPR120 is expressed in Caco-2 cells, where binding of n-3 and n-6 PUFAs induces its activation, which in turn activates Goq leading to an increase of cytosolic Ca²⁺ and activation of ERK1/2 (Moberaten, Haug, Kleiveland, ...
& Lea, 2013). Also, it has been demonstrated that ligand activation of GPR120 in intestinal epithelial Caco-2 cells exert anti-inflammatory effects via inhibition of NF-κB activation (Anbazhagan et al., 2016). All this data supports our current results which strongly suggest that the ability of EPA to reverse the inhibitory effect of TNF-α on aMG uptake and AMPK phosphorylation is mediated by the binding to the receptor GPR120. Other authors have shown that DHA, another marine n-3 PUFAs, stimulates glucose uptake in skeletal muscle through the phosphorylation of AMPK via GPR120 (Kim et al., 2015).

5 CONCLUSION

We have demonstrated that TNF-α decreases sugar transport in Caco-2 cells by the activation of ERK1/2 and the inhibition of AMPK pathways. Most importantly, we have shown that EPA prevents TNF-α inhibition of SGLT1 expression in the plasma membrane and sugar uptake by activating the AMPK signaling pathway, probably through GPR120 (Figure 6). These results open perspectives to investigate the use of EPA as a beneficial fatty acid for the treatment of nutrients absorption disorders in patients suffering intestinal inflammation.

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CONFLICTS OF INTEREST

The authors have declared no conflicts of interest.

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Figure 6. TNF-α decreases sugar transport via the activation of ERK1/2 and inhibition of AMPK pathways in Caco-2 cells. The inhibition of SGLT1 expression in the plasma membrane and sugar uptake by activating the AMPK signaling pathway, probably through GPR120.


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