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Intraluminal nutrients acutely strengthen rat intestinal MRP2 barrier function by a glucagon-like peptide-2-mediated mechanism

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Short title: Nutrients and intestinal MRP2 barrier

Abstract

Aim: MRP2 is an intestinal ABC transporter that prevents the absorption of dietary xenobiotics. The aims of this work were: i) to evaluate whether a short-term regulation of intestinal MRP2 barrier function takes place in vivo after luminal incorporation of nutrients and ii) to explore the underlying mechanism.

Methods: MRP2 activity and localization were assessed in an in vivo rat model with preserved irrigation and innervation. Nutrients were administered into distal jejunum. After 30-min treatments, MRP2 activity was assessed in proximal jejunum by quantifying the transport of the model substrate 2,4-dinitrophenyl-S-glutathione. MRP2 localization was determined by quantitative confocal microscopy. Participation of extracellular mediators was evaluated using selective inhibitors and by immunoneutralization. Intracellular pathways were explored in differentiated Caco-2 cells.

Results: Oleic acid, administered intraluminally at dietary levels, acutely stimulated MRP2 insertion into brush border membrane. This was associated with increased efflux activity and, consequently, enhanced barrier function. Immunoneutralization of the gut hormone glucagon-like peptide-2 (GLP-2) prevented oleic acid effect on MRP2, demonstrating the participation of this trophic factor as a main mediator. Further experiments using selective inhibitors demonstrated that extracellular adenosine synthesis and its subsequent binding to enterocytic A2B adenosine receptor (A2BAR) take place downstream GLP-2. Finally, studies in intestinal Caco-2 cells revealed the participation of A2BAR/cAMP/PKA intracellular pathway, ultimately leading to increased MRP2 localization in apical domains.

Conclusion: These findings reveal an on-demand, acute regulation of MRP2-associated barrier function, constituting a novel physiological mechanism of protection against the absorption of dietary xenobiotics in response to food intake.

Keywords

MRP2, adenosine, GLP-2, intestinal barrier, intestine, oleic acid

1. Introduction

The small intestine constitutes a biochemical barrier between external and internal environments which depends on the proper localization and activity of ATP-binding cassette (ABC) transporters. ABC efflux pumps, particularly localized to the brush border membrane (BBM) of the enterocyte, are responsible for actively extruding xenobiotics back to the intestinal lumen. Among these transporters, the multidrug resistance-associated protein 2 (MRP2; also known as ABCC2) has a wide substrate specificity, limiting the absorption of numerous prescription drugs and food contaminants.[1,2] Intestinal MRP2 expression and localization were first described two decades ago. Enterocytes from the tip of the villus exhibited the maximal MRP2 expression with a decreasing pattern towards the cells from the crypt. A gradient along the intestinal tract was also found, with the highest MRP2 content in proximal jejunum decreasing towards the ileum.[3,4] Although these studies have been performed in rats, a similar distribution was subsequently demonstrated in humans.[5,6] MRP2 relevance as a key component of the intestinal barrier was demonstrated using TR- rats, in which this transporter is hereditarily defective. As result of oral bioavailability studies, TR- rats showed increased absorption, plasma concentration and tissue accumulation of the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) when compared to normal Wistar rats.[7]

Glucagon-like peptide-2 (GLP-2) is a 33 amino acid peptide produced by enteroendocrine L cells from distal jejunum, ileum and colon epithelia. Its vast repertoire of actions at the intestinal level includes both long-term and short-term effects focused on improving intestinal functions such as digestion and absorption. Among long-term effects are the increase in proliferation and the decrease in apoptosis of epithelial cells, eventually leading to intestinal hypertrophy and hyperplasia.[8-10] Among short-term effects, the most remarkable is the increase in intestinal blood flow after meals, also known as postprandial hyperemia.[11,12] GLP-2 exerts these biological functions by binding to GLP-2R, a G protein-coupled receptor expressed in enteric neurons, subepithelial myofibroblasts and enteroendocrine cells of the intestine.[13] Interestingly, the presence of GLP-2R was ruled out both in epithelial crypt cells and in differentiated enterocytes, i.e. the main targets of GLP-2 actions. In fact, the multiple actions of GLP-2 at the intestinal

level are exerted indirectly, through paracrine mediators produced by GLP-2R-expressing cells. While the polypeptide insulin-like growth factor-1 (IGF-1) was responsible for GLP-2 intestinotrophic actions,[14,15] the mediators behind GLP-2 acute effects are less known. However, indirect evidence suggests that enteric neurons and extracellularly generated adenosine could play a role in this regard.[16,17]

The main stimulus for GLP-2 release under physiological conditions is the presence of nutrients in the digestive tract. After food intake there is a biphasic increase in circulating levels of GLP-2. The first peak occurs within the first 15-30 min, when nutrients reach the duodenum. It is triggered by an indirect mechanism involving endocrine factors and the vagus nerve, ultimately resulting in muscarinic receptor-dependent L cell activation.[18] The second peak occurs approximately 60-90 min after intake and involves direct luminal stimulation of L cells by nutrients. Interestingly, this second peak of GLP-2 secretion depends on the specific composition of the diet, with lipids and carbohydrates being more potent stimulators than proteins.[19,20]

Our group provided the first experimental evidence supporting the regulatory role of GLP-2 on MRP2 function through transcriptional mechanisms.[21] Additionally, subcutaneous administration of GLP-2 produced a significant increase in cAMP levels evaluated in rat enterocytes after 2 h of treatment. Moreover, a selective adenylate cyclase inhibitor prevented such increase, demonstrating activation of this enzymatic effector.[21]

Recently, we have reported that intestinal MRP2 localization may be subjected to a dynamic equilibrium between BBM and intracellular domains, thus allowing for rapid regulation of MRP2 function.[22] This acute and bidirectional translocation was demonstrated for both rat and human orthologues using dibutyryl cAMP and estradiol-17β-D-glucuronide. Particularly, we described the ability of cAMP to stimulate the insertion of MRP2 into the BBM. This effect was mediated by PKA and associated with increased secretory activity of the model substrate 2,4-dinitrophenyl-S-glutathione (DNP-SG). In spite of all this evidence, it is unknown whether intestinal MRP2 acute regulation takes place *in vivo* as part of a physiological process.

The three main experimental findings described above can be summarized and sequentially linked as follows: (*i*) after intake of certain nutrients (such as oleic acid or glucose) GLP-2 is released from L cells;[23] (*ii*) GLP-2 triggers an increase in cAMP levels of rat enterocytes;[21] (*iii*) the increase in intracellular cAMP levels stimulates insertion of

MRP2 into BBM with consequent impact on MRP2 transport activity.[22] This sequence of events, although possible, needs experimental demonstration and constitutes our current working hypothesis. We here developed an appropriate *in vivo* model and demonstrated the occurrence of such events under physiological conditions. Moreover, we identified participation of additional and relevant extracellular mediators. These findings reveal a novel on-demand regulation of MRP2-associated barrier function, constituting an adaptative mechanism of protection against the absorption of dietary xenobiotics.

2. Results

2.1. *In vivo* studies: Effect of nutrients on MRP2 activity and localization and elucidation of the extracellular mechanism involved.

2.1.1. The *in vivo* model provides a useful tool to detect rapid changes in MRP2 activity.

First, we established whether the proinsertor agent dbcAMP acutely increases MRP2 transport activity in our *in vivo* model when administered intravenously. As shown in Figure 1a, dbcAMP (20 µmol/kg b.w., 20 min) produced a significant increase (+213 % *versus* control) in DNP-SG efflux, demonstrating that this model allows for detection of rapid changes in MRP2 activity, at least in response to a systemic treatment.

2.1.2. Oleic acid acutely increases MRP2 activity.

We next evaluated the acute effect of GLP-2 releasing agents on MRP2 activity. Figure 1b shows that, among the agents evaluated, only oleic acid and glucose were able to significantly modulate DNP-SG efflux in the proximal jejunum, suggesting a nutrient-specific effect. Specifically, oleic acid, administered as an emulsion at a concentration of physiological relevance,[24] increased MRP2 activity in comparison with its respective control group (+231 %). Intraluminal administration of saline + Tween 20 produced no effect on MRP2 activity when compared with saline alone (data not shown), ruling out any effect of the surfactant itself. In the case of glucose, an increase was observed at the 100 mM (+186 %) but not at the 10 mM concentration, suggesting a dose-dependent effect. The effect of glutamine did not differ from that of the vehicle administered to control rats. Considering that intraluminal glucose concentrations of 100 mM are unlikely reached after a regular meal,[25] we chose oleic acid as a prototype MRP2 modulating agent for the following studies aimed at elucidating the mechanism involved.

2.1.3. GLP-2 mediates oleic acid effect on MRP2 activity

The next step in testing our hypothesis was to evaluate the ability of GLP-2 to acutely regulate MRP2 activity. Figure 1c shows that intravenous administration of recombinant GLP-2 (125 µg/kg b.w.) significantly increases MRP2 activity (+210 %), evaluated after 20 min of treatment. Next, we assessed whether this hormone mediates oleic acid effects

on MRP2 activity. Using a previously established GLP-2 immunoneutralization strategy, we found that the effect of oleic acid was suppressed in the oleic acid + anti-GLP-2 group (Figure 1c), confirming GLP-2 participation. Pre-administration of anti-GLP-2 did not affect MRP2 activity in rats receiving oleic acid vehicle (data not shown), ruling out any effect of immunoneutralization procedure *per se*.

2.1.4. Extracellular adenosine synthesis takes place downstream GLP-2 and leads to increased MRP2 activity

After demonstrating GLP-2 role, we evaluated the participation of IGF-1 and adenosine as mediators of GLP-2 action. In the case of IGF-1, the experimental approach consisted of IGF-1 immunoneutralization. In the case of adenosine, it consisted of selective inhibition of the adenosine receptor A2BAR and inhibition of CD73, which is considered the most important enzyme in the generation of extracellular adenosine at the intestinal level.[26,27] As shown in Figure 1d, the effect of oleic acid was not prevented by anti-IGF-1 administration. In contrast, pre-treatment with MRS 1754 suppressed oleic acid effect on MRP2, confirming A2BAR participation. Moreover, APCP prevented the increase in MRP2 activity produced by both oleic acid and GLP-2 (Figure 1d), suggesting a role for extracellular adenosine in this effect and also establishing a sequence of events, with adenosine operating downstream GLP-2. Since pre-administration of anti-IGF-1, MRS 1754 and APCP did not affect MRP2 activity in rats receiving oleic acid vehicle (data not shown), we ruled out any effect by these agents themselves. Adenosine *per se*, administered intravenously (10 mg/kg b.w.), significantly increased DNP-SG efflux (+222 %; Figure 1d), confirming the ability of this nucleoside to modulate MRP2 activity when reaching the intestinal epithelium from the serosal side.

2.1.5. The stimulus generated by oleic acid is transmitted along the intestinal wall

After providing evidence supporting a role for specific mediators on the rapid action of oleic acid on MRP2 activity, the following question arose: how does the stimulus triggered by oleic acid in distal intestine reach the proximal jejunum? One possible transmission route is endocrine, with GLP-2 reaching systemic circulation after its release and finally acting at proximal jejunum. The other possibility is a transmission through the complex organization of neurons of the enteric nervous system, able to propagate stimuli in both

oral and caudal directions.[28] To distinguish between these possibilities, we sectioned the jejunum between the distal end of the intestinal sac and the site of administration of oleic acid, right before lipid administration. With this simple intervention, the neuronal connection between both regions of the jejunum is interrupted while the systemic connection remains unaffected. Figure 1e shows that jejunum sectioning limited the ability of oleic acid to enhance MRP2 activity. This finding is an unequivocal evidence of the participation of a transmission pathway that depends on the continuity of the intestine.

2.1.6. Oleic acid acutely increases apical MRP2 localization by a GLP-2-mediated mechanism

To assess whether these changes in MRP2 activity were correlated with changes in MRP2 subcellular localization, we performed a quantitative and qualitative confocal microscopy analysis. Figure 2 shows representative images of each group. In the upper panel images, channels corresponding to nuclei (blue) and MRP2 (red) are shown. The panels below show only the MRP2 channel in gray scale. The densitometric analysis is shown at the bottom. Acute, intraluminal treatment with oleic acid increased MRP2 sorting from intracellular compartments to the apical membrane, as evidenced by a less dispersed signal along of the enterocytic longitudinal axis and more focused signal on the BBM region when compared to control group. Consistent with this qualitative interpretation, analysis of densitometric curves results in significantly higher MRP2 peak height (+185%) compared to control group. This higher density of transporter at the apical membrane of the enterocyte is in agreement with the reported increase in transport activity after oleic acid treatment. On the other hand, immunoneutralization of GLP-2 30 min before treatment resulted in images, densitometric curve and MRP2 peak height similar to those from control group, demonstrating the participation of GLP-2 in the proinsertor effect of oleic acid (Figure 2).

2.2. *In vitro* studies: Elucidation of the intracellular mechanism involved in adenosine effects

2.2.1. Adenosine acutely increases MRP2 activity in Caco-2 cells through A2BAR and PKA activation

To elucidate the molecular mechanism downstream adenosine, we first confirmed the effect of this mediator on MRP2 activity through the evaluation of CDF efflux ratio across Caco-2 cell monolayers. Figure 3a shows that acute treatment with adenosine (50 µM) significantly increased CDF Papp in BA direction with respect to control cells, without changes in Papp in AB direction. This led to a significant increase in CDF efflux ratio, confirming that adenosine is able to increase MRP2 activity also in this model (Figure 3b). In addition, we demonstrated that this effect was mediated by the A2BAR receptor, since increases in both CDF Papp in BA direction and CDF efflux ratio were prevented by the selective inhibitor MRS 1754 (Figure 3). MRS 1754 itself had no effect on CDF efflux ratio (MRS group; data not shown).

Finally, we evaluated the participation of PKA in adenosine-mediated increase of MRP2 activity using KT5720. In the presence of this inhibitor, adenosine did not significantly modify CDF Papp in BA direction or CDF efflux ratio (Figure 3), confirming PKA mediation. However, because there were no differences between adenosine and adenosine + KT groups (Figure 3), PKA may not be the only mediator involved in the effects of adenosine. It is worth noting that KT5720 alone did not affect CDF efflux ratio, dismissing any possible unspecific effect of this agent in MRP2 activity (KT group; data not shown).

2.2.2. Adenosine acutely increases apical MRP2 localization through A2BAR activation

The next step was to assess whether the adenosine-dependent increase in MRP2 activity is associated to an increase in transporter apical insertion. Quantitative confocal microscopy studies showed that adenosine treatment (50 µM, 30 min) significantly increased MRP2 peak height, which was also found to be markedly displaced towards the apical surface of the monolayer (Figure 4). Images corresponding to the Z-stack adequately illustrate this finding, since adenosine-treated cells show apical MRP2 concentration with concomitant decrease of the signal from intracellular regions when compared to control cells, which show a much more homogeneous MRP2 distribution along the Z axis (Figure 4). Panels on the right show that MRS 1754 (1 µM; A2BAR inhibitor) was able to prevent adenosine-induced changes in MRP2 distribution. These findings demonstrate that acute treatment with adenosine was able to stimulate

translocation of MRP2 from intracellular reservoirs to the apical membrane, in an A2BAR-mediated fashion.

2.2.3. Adenosine increases intracellular cAMP levels

Finally, considering that A2BAR is coupled to Gs, an adenylate cyclase activating protein, we assessed the intracellular cAMP levels after adenosine treatment (50 µM; 15 min). As result, we found a significant increase in Caco-2 cell lysates (269.5 ± 21.9 pmol/mL *versus* 206.7 ± 6.4 pmol/mL, respectively; $p < 0.05$, $n = 4$).

3. Discussion

In this study we provide evidence supporting a novel physiological mechanism aimed at acutely regulating intestinal MRP2 activity in response to food intake, hence protecting the organism against the absorption of dietary toxicants. After our recent proof-of-concept article demonstrating the short-term regulation of intestinal MRP2,[22] we focused on the development of an *in vivo* model with preserved irrigation and innervation that allows for detection of changes in MRP2 activity and localization in response to intraluminal administration of nutrients (section 4.2.2). This model was validated using the proinsertor agent dbcAMP (Figure 1a), intravenously administered at a dose known to be effective in modulating MRP2 in another highly perfused organ such as the liver.[29] The differential effect of intraluminally administered agents on MRP2 activity (Figure 1b) is consistent with previous evidence demonstrating that GLP-2 secretion depends on the specific nutrient composition of the diet.[23] Glucose exerted a dose-dependent action, with no effect at 10 mM and a statistically significant increase at 100 mM concentration (Figure 1b). Considering that the intrajejunal glucose concentration after a meal ranges between 0.4 and 24 mM in rats and between 0.5 and 40 mM in humans, depending whether it is distal or proximal jejunum,[25] our finding at 100 mM may not have physiological relevance. Regarding glutamine, the work of Reimann *et al*.[30] demonstrated the ability of a 10 mM concentration to stimulate GLP-1 secretion in an *in vitro* model. Since GLP-1 is co-secreted with GLP-2 in equimolar amounts, this finding provided a rationale for inclusion of glutamine in the list of nutrients to be evaluated. However, the negative results (Figure 1b) are rather aligned with *in vivo* studies ruling out a stimulatory effect of proteins and amino acids on GLP-2 secretion.[20,31] Of the highest significance is the maximal response on MRP2 activity registered after oleic acid treatment (Figure 1b) at a physiologically relevant concentration.[24] This led us to select this nutrient as the prototype to be used for the subsequent *in vivo* studies. This increase in transport activity was associated with MRP2 insertion on the apical membrane, evidenced by a higher MRP2 peak localized to the apical end of enterocytes and concomitant weaker signal from intracellular regions (Figure 2).

The participation of GLP-2 in oleic acid effect was demonstrated using an immunoneutralization method, originally reported in a work linking GLP-2 with adaptative

intestinal growth secondary to streptozotocin-induced diabetes.[32] This finding, supported by the effect of intravenously administered GLP-2 on MRP2 activity (Figure 1c), constitutes a new item in the increasing list of GLP-2 biological functions at the intestinal level.[8,11,12,33-36] Regarding the molecular mechanism that follows GLP-2 secretion, we first evaluated the participation of IGF-1. This growth factor, although closely linked to GLP-2 actions, was mainly associated with long-term effects such as proliferation and intestinal growth.[14] Immunoneutralization of IGF-1 was unable to block the stimulating effect of oleic acid on MRP2 activity (Figure 1d). This would mean either that IGF-1 is not involved downstream GLP-2 or that the treatment was not effective enough to completely abolish IGF-1 activity. Additional experiments are required to properly distinguish between these two possibilities. In contrast, we identified adenosine as a valid candidate based on the results obtained using APCP (Figure 1d). Moreover, the evidence strongly suggests that extracellular adenosine synthesis by CD73 is a prerequisite for the effect of both oleic acid and GLP-2 on MRP2 activity. CD73, also known as ecto-5′-nucleotidase, mediates the generation of extracellular adenosine at the intestinal level.[26,27] This process, of central role in both physiological and pathophysiological conditions, begins with ATP released by enteric neurons from submucosal and/or myenteric plexuses.[37] ATP is then hydrolyzed by different ecto-nucleoside triphosphate diphosphohydrolases yielding AMP, which is further dephosphorylated to adenosine by CD73.[38] cAMP was suggested as an alternative precursor to ATP in extracellular adenosine synthesis, in this case by tandem action of ecto-phosphodiesterase and CD73.[27] With our experimental strategy it is not possible to ascertain whether ATP or cAMP constitutes the initial substrate in the cascade that culminates with CD73-dependent adenosine synthesis (Figure 5). Although we cannot rule out the participation of additional mediators such as IGF-1, it is noteworthy that the administration of either APCP or MRS 1754 (Figure 1d) was capable of abolishing oleic acid stimulation of MRP2 activity. This is consistent with a major role for GLP-2/extracellular adenosine/A2BAR pathway in oleic acid effects. Finally, experiments involving sectioning of the intestine demonstrate that the effects reported for oleic acid totally depended on the preservation of the structure and continuity of the intestinal wall, indirectly ruling out the intervention of systemic circulation (Figure 1e). Considering both GLP-2R expression in enteric neurons[13] and the deep innervation of intestinal epithelial cells by these neurons,[39] the hypothesis of a transmission through the

multiple neurons from submucosal and/or myenteric plexuses seems the most likely. Figure 5 illustrates only one of the possibilities, since other more complex alternatives to the one represented, such as the participation of more than one neuronal type, cannot be ruled out. In this regard, signal transmission in caudal-oral direction may involve intrinsic primary afferent neurons, ascending interneurons and motor neurons.[28] Additional experiments including synaptic transmission blockades may contribute to clarify this route.

Due to the technical difficulties inherent in establishing a primary enterocyte culture, Caco-2 intestinal cells were chosen to evaluate the intracellular mechanism. Despite their colonic origin, Caco-2 cells undergo spontaneous differentiation after 21 days of culture to form a confluent monolayer of enterocyte-like polarized cells, which structurally and functionally resembles the small intestinal epithelium.[40-42] Regarding expression of the most relevant intestinal transporters (including MRP2), different studies demonstrated a similar pattern between differentiated Caco-2 cells and human small intestine.[43-45] Thus, Caco-2 cell monolayers grown on permeable supports constitute an optimal *in vitro* model system for drug transport studies.[40] In addition, using confocal microscopy and membrane fractionation followed by immunoblotting studies, rapid changes in MRP2 localization were already confirmed in these cells.[22] In the current work we demonstrated that acute treatment with adenosine led to a significant increase in MRP2 activity (Figure 3b) which was associated with apical insertion of the transporter (Figure 4). These findings complement those resulting from *in vivo* studies (Figure 1d), and demonstrate for the first time adenosine modulatory action on both human and murine intestinal MRP2. Interestingly, interstitial adenosine levels in the jejunum are increased up to two orders of magnitude during post-intake periods, a situation so far associated with postprandial jejunal hyperemia.[46,47] Based on our findings, we suggest that postprandial adenosine boost not only leads to increased intestinal blood flow but also contributes to strengthen the intestinal barrier function provided by MRP2.

The effect of adenosine on MRP2 activity and localization in Caco-2 cells was mediated by A2BAR (Figure 3 and Figure 4). Within adenosine receptors, A2BAR is the most relevant in the intestine and is expressed, among other sites, at epithelial level.[48,49] Since it is coupled to G stimulatory protein, an increase in cAMP synthesis by adenylate cyclase after activation is expected. In fact, studies using T84 intestinal epithelial cell

monolayers demonstrated that activation of A2BAR/cAMP/PKA axis by adenosine stimulates polarized apical secretion of the proinflammatory cytokine IL-6.[50] Considering that several compounds with proinflammatory activity such as prostaglandins or leukotrienes are MRP2 substrates,[1,2] it is possible that this regulation takes place also under pathophysiological conditions, e.g. to deal with intraluminal pathogens. In our experimental conditions, intracellular levels of cAMP were also significantly increased in response to adenosine stimulation after a 15-min treatment (section 2.2.3). This result provides evidence in support of adenosine/A2BAR/adenylate cyclase axis functionality in our experimental model. We also found that PKA participation is necessary to explain, at least in part, modulation of MRP2 activity by adenosine (Figure 3). These findings agree with previous evidence in hepatic and intestinal models demonstrating the ability of cAMP/PKA axis to acutely increase MRP2 activity.[22,51] However, it is important to note that cAMP increase not only leads to PKA activation, but can also increase the activity of other kinases such as exchange protein directly activated by cAMP, phosphoinositide 3-kinase or p38 mitogen-activated protein kinases, all of them also able to acutely modulate MRP2 localization in hepatic models.[51] Additional studies are necessary to further determine the eventual participation of kinases other than PKA.

Our *in vivo* model allows for evaluation of the effect of GLP-2 produced only by direct stimulation of L cells by nutrients, and does not consider the effect of GLP-2 produced by indirect mechanisms in response to the presence of nutrients in the duodenum. This mechanism involving participation of vagal fibers and nicotinic synapses[18] results in earlier L cell stimulation and complements the effect here described. Regarding the time of permanence of nutrients in the small intestine, a transit time between one and four hours has been estimated, regardless of whether they are originated in solid or liquid foods.[52] Sustained secretion of GLP-2 along the whole digestive process would provide an enhanced barrier function in order to deal with the exposition to potentially harmful components of the diet.

Because of its preferential localization at the proximal region of the small intestine, MRP2 exerts a significant function in reducing the body load of potentially harmful compounds entering the digestive tract. Although with different distribution along the intestine, other ABC transporters expressed at the BBM of the enterocyte provide a similar barrier function.[53] In the current study, we have only explored the regulation of MRP2 by

nutrients and cannot ascertain whether other members of the ABC family would be affected in a similar fashion. To our knowledge, there is no information in the literature describing such effect. If demonstrated, it would represent a regulation complementary to MRP2 since a broader substrate specificity and a more extensive intestinal region will be covered. In addition to the barrier function, this physiological mechanism could also improve nutrient absorption during the course of a meal if demonstrated for intestinal uptake proteins such as solute carrier transporters. In this regard, Cheeseman made a substantial contribution by reporting increased sodium-dependent glucose uptake in rat jejunum BBM vesicles after 30 min of GLP-2 intravenous treatment. This was associated with increased SGLT-1 localization in BBM. Based on the results obtained using wortmannin, the author proposes phosphoinositide 3-kinase as the mediator of this acute GLP-2 effect.[54]

The main conclusion of this work is summarized in Figure 5. After intake, the presence of nutrients such as oleic acid in the intestinal lumen triggers GLP-2 release by enteroendocrine L cells, presumably by both indirect and direct mechanisms. GLP-2, secreted through the basolateral membrane into the interstitial space, would activate its receptor in enteric neurons. As a consequence, the generated stimulus would be transmitted along the intestinal wall, eventually involving participation of more neurons or other cellular types. AMP precursors such as ATP or cAMP are likely secreted by these neurons, although the intervention of some other cell type in the proximity of the enterocyte cannot be ruled out. AMP is then hydrolyzed by CD73 yielding adenosine, which directly activates A2BAR at the basolateral membrane of the enterocyte. This activation results in increased intracellular cAMP concentrations, ultimately stimulating MRP2 insertion from intracellular reservoirs to BBM in a process mediated, at least in part, by PKA. This increase in MRP2 density at the apical membrane is associated with increased transport activity and thus increased membrane barrier function. An additional contribution of our study is that, during fasting periods, GLP-2- MRP2 interaction is expected to decrease due to absence of stimuli on intestinal L cells. This event, also physiologic, would result in decreased MRP2 apical localization and activity, saving metabolic energy and avoiding the unnecessary luminal secretion of valuable substrates such as glutathione, essential for cellular homeostasis maintenance.

4. Materials and Methods

4.1. Chemicals

Adenosine, adenosine 5′-(α,β-methylene) diphosphate (APCP), 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDFDA), 1-chloro-2,4-dinitrobenzene (CDNB), dbcAMP, glucose, glutamine and MRS 1754 were purchased from Sigma-Aldrich (Missouri, USA); anti-GLP-2 (C-20) and KT5720 were purchased from Santa Cruz Biotechnology (Dallas, USA); anti-IGF-1 (M23) was purchased from ThermoScientific (Illinois, USA); rat recombinant GLP-2 was purchased from Abcam (Cambridge, UK) and double-distilled oleic acid was purchased from Ballester Productos Químicos S.A. (Villa Ballester, Argentina). DNP-SG was synthesized using 1-fluoro-2,4-dinitrobenzene and glutathione as described by Sokolovsky *et al.*[55] All other chemicals were of analytical grade purity or higher.

4.2. *In vivo* studies

4.2.1. Animals

Adult male Wistar rats (310 - 330 g) were supplied by Centro de Medicina Comparada (UNL - CONICET, Esperanza, Argentina). They were maintained with a standard diet and tap water *ad libitum* in a room with controlled temperature and humidity and a 12-12 h light-dark cycle. The rats were fasted for 12 hours before the experiments. At the time of the experiments, they were anesthetized using ketamine + xylazine (ketamine: 100 mg/kg b.w; xylazine: 15 mg/kg b.w.; i.p) and, after that, their body temperature was monitored and maintained between 37°C and 37.5°C using heating lamps. All procedures involving animals were in accordance with the ‘European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes’ and were approved by the Bioethical Committee for the Management of Laboratory Animals from the Faculty of Biochemical and Pharmaceutical Sciences of the National University of Rosario (Res. 410/2014).

4.2.2. *In vivo* model

The *in vivo* model used in this work allows for direct stimulation of GLP-2-producing L cells in the intestinal region in which their presence is maximal (distal jejunum, ileum and

colon) with simultaneous evaluation of MRP2 activity and localization in the intestinal region exhibiting the highest MRP2 expression (proximal jejunum) (see Figure 6a). In addition, this model preserves intestinal irrigation and innervation.

4.2.3. Intraluminal and intravenous treatments

To evaluate the effect of GLP-2 secretion stimulators on MRP2 activity and localization, 2.5 mL of oleic acid (10 % V/V in saline + 3 % Tween 20; pH = 6.5),[24] glucose (10 and 100 mM in saline, pH = 6.5),[56] glutamine (10 and 100 mM in saline, pH = 6.5),[30] saline + 3 % Tween 20 or saline alone were gently injected into distal jejunum (approximately 19 cm after the ligament of Treitz) using a 25G needle. During this procedure, the region of the intestine proximal to the site of administration was occluded with a thread to avoid retrograde flow of the agents. After 20 min of treatment, MRP2 activity studies were performed.

For systemic treatments, the femoral vein was catheterized with polyethylene tubing (PE-40) and dbcAMP (20 µmol/kg b.w. in 0.4 mL of saline), GLP-2 (125 µg/kg b.w. in 0.4 mL of saline), adenosine (10 mg/kg b.w. in 0.4 mL of saline) or saline alone were administered.[29,57,58] After 20 min, MRP2 activity studies were performed. In the case of adenosine, the total volume was administered in four doses of 0.1 mL each, with 5-min intervals. All these procedures are illustrated in Figure 6b.

To gain insight into the mechanism of stimulation by oleic acid, immunoneutralization of GLP-2 and IGF-1 was performed by intraperitoneal injections of anti-GLP-2 (20 µg in 1 mL of saline) or anti-IGF-1 (50 µg in 1 mL of saline) respectively, 30 min before administration of this nutrient.[32] Identical experiments of immunoneutralization with anti-IGF-1 and anti-GLP-2 were performed with animals receiving only the vehicle of oleic acid. To evaluate the participation of extracellular adenosine in the signaling mechanism, the selective CD73 inhibitor APCP (2 mg/kg b.w. in 1 mL of saline) and the selective A2B adenosine receptor (A2BAR) inhibitor MRS 1754 (0.5 mg/kg b.w. in 1 mL of saline) were intraperitoneally administered 30 min before administration of oleic acid or GLP-2.[59,60] Identical experiments of inhibition with APCP and MRS 1754 were performed replacing oleic acid and GLP-2 by the respective vehicles. Finally, to evaluate the participation of the local nervous system, the intestine was sectioned 2 cm proximal to the site of nutrient

administration (i.e. approximately 17 cm after the ligament of Treitz), just before intraluminal treatment with oleic acid. All these procedures are illustrated in Figure 6c.

4.2.4. MRP2 activity studies

During the 20-min treatments, the 15-cm proximal jejunum segment for evaluation of DNP-SG (i.e. model substrate of MRP2) efflux was delimited. The proximal and distal ends of this segment (located 1 cm and 16 cm after the ligament of Treitz, respectively) were identified with pre-assembled, untied knots. Once the treatment was completed, the distal end knot was tied, the segment was filled with 2.6 mL of a 100 µM CDNB solution in Krebs-Henseleit buffer and finally the proximal end knot was tied, completely closing the intestinal sac. The incubation time under these conditions was 30 min. Afterwards, the animal was sacrificed by exsanguination and the intestinal sac was removed. After sac length measurement, the content was collected in a graduated tube and its volume was recorded. After deproteinization, the solution was subjected to quantification of DNP-SG and its dinitrophenyl-cysteinyl glycine derivative (DNP-CG) by HPLC-UV as previously described.[61] DNP-SG + DNP-CG mass was calculated using their concentration and the volume measurements of the intestinal sac content. DNP-CG is produced by the action of γ-glutamyltransferase on DNP-SG at the BBM after this compound reaches the mucosal compartment.[62] Therefore, the excretion rate of DNP-SG + DNP-CG adequately estimates MRP2 activity. These procedures are summarized in Figure 6d.

4.2.5. MRP2 localization studies

MRP2 localization studies were performed by immunostaining followed by quantitative confocal microscopy. Rats were treated with intraluminal saline (control group), intraluminal oleic acid (oleic acid group) or intraluminal oleic acid with anti-GLP-2 pretreatment (oleic acid + anti-GLP-2 group) as previously described. After sacrifice by exsanguination, a segment from proximal jejunum was carefully washed and frozen in liquid 1,1,1,2-tetrafluoroethane (Electroquímica DELTA SRL, Buenos Aires, Argentina). The segment was cut into slices of 5 µm thick with a microtome cryostat (HM 500, Microm International GmbH, Walldorf, Germany) and mounted on slides. Preparations were fixed, permeabilized and blocked as previously described.[63] MRP2 was detected

using anti-MRP2: M2III-6 (1:100) from Enzo Life Sciences (Farmingdale, USA) followed by incubation with anti-mouse IgG conjugated with Alexa Fluor 555 (1:300) from ThermoScientific (Illinois, USA). Nuclei were detected using Hoechst 33258 from Santa Cruz Biotechnology (Dallas, USA). At least 8 images per group were captured using a Nikon C1+ confocal system built onto a Nikon TE2000 inverted microscope with a 60X Plan Apo NA 1.4 objective. Images were then analyzed using ImageJ software (NIH, Bethesda, USA). To establish the relative MRP2 distribution with respect to the nuclei, MRP2 and nuclei channels were separated and the distribution of the fluorescence intensity along the longitudinal axis of the enterocyte was evaluated (see Figure 7). The total area under the curves were assigned the same arbitrary value and the maximum value of MRP2 fluorescence intensity (peak height) was compared between groups as a quantitative measurement of MRP2 distribution. For data presentation, maximum fluorescence intensity values of the nuclei were aligned to qualitatively reveal eventual changes in MRP2 cellular distribution in response to treatments.

4.3. *In vitro* studies

4.3.1. Caco-2 cell culture

Caco-2 cells were obtained from the American Tissue Culture Collection (Manassas, USA) and grown in DMEM (Gibco; Carlsbad, USA) supplemented with 10% V/V fetal bovine serum (Natocor; Córdoba, Argentina), 1 % V/V non-essential amino acids, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco; Carlsbad, USA). Cells were grown at 37°C in a humid atmosphere containing 5 % CO_2.

4.3.2. MRP2 activity studies

For MRP2 activity studies, 3 × 10^5 cells/well were seeded in 12-well Transwell® permeable supports (cat. No. 3401; Corning Inc., Corning, USA) and cultured for 21 days as described by Hubatsch *et al*.[40] Culture medium in both apical and basolateral compartments was replaced every second day. To evaluate monolayer integrity, transepithelial electrical resistance (TEER) was monitored every second day using an epithelial voltohmmeter (EVOM; World Precision Instruments, Sarasota, USA). All cell monolayers used in the experiments had TEER values at 37°C above 300 Ω cm^2, indicating a continuous monolayer.[40]

MRP2 activity was evaluated by assessing the ratio between the apical-to-basolateral (AB) and the basolateral-to-apical (BA) directional transport of the prototypical MRP2 substrate 5(6)-carboxy-2′,7′-dichlorofluorescein (CDF) as previously described.[61] The CDF non-fluorescent promoiety, CDFDA, is taken up passively by Caco-2 cells and immediately hydrolyzed by intracellular esterases to the fluorescent CDF.[64] Briefly, prior to the activity measurement, cell monolayers were washed with Hank's balanced salt solution (HBSS) for 15 min at 37°C. Afterwards, cells were treated with culture medium alone (control group) or culture medium containing 50 µM adenosine (adenosine group), 50 µM adenosine + 10 µM MRS 1754 (adenosine + MRS group), 50 µM adenosine + 1 µM KT5720 (adenosine + KT group), 10 µM MRS 1754 (MRS group) or 1 µM KT5720 (KT group).[22,65,66] After 20 min of treatment, cells were washed with HBSS alone (control group) or HBSS containing 50 µM adenosine (adenosine group), 50 µM adenosine + 10 µM MRS 1754 (adenosine + MRS group), 50 µM adenosine + 1 µM KT5720 (adenosine + KT group), 10 µM MRS 1754 (MRS group) or 1 µM KT5720 (KT group) for 15 min at 37°C. Then, filter inserts were transferred to new plates and CDFDA (10 µM, in HBSS) was added to the donor compartment (apical for AB direction experiments, basolateral for BA direction experiments). The receiving compartment (basolateral for AB direction experiments, apical for BA direction experiments) was filled with pre-warmed HBSS. Plates were then placed in a 37°C incubator and samples from the receiving compartment were withdrawn with volume replacement every 30 min up to 120 min. CDF was quantified using a DTX 880 fluorometer (Beckman Coulter; Brea, USA) set at 485 nm/520 nm for excitation and emission wavelengths, respectively. Apparent permeability coefficients (P_{app}, $cm.s^{-1}$) of CDF across cell monolayers in both AB and BA directions were calculated using the following equation:

$$P^{app} = \frac{1}{C^0 A} \times \frac{dQ}{dt}$$

where dQ/dt is the appearance rate of CDF on the receiving compartment, C_0 is the initial CDFDA concentration in the donor compartment (10 µM) and A is the Transwell® support surface area (1.12 cm^2). CDF efflux ratio was obtained by dividing P_{app} in the BA direction by P_{app} in the AB direction.

4.3.3. MRP2 localization studies

To detect MRP2 localization in Caco-2 cells, a sterile coverslip was placed in each well of 6-well plates; 2.5×10^5 cells were seeded on each coverslip and cultured for 21 days as described. Then, differentiated cells were treated with culture medium alone (control group) or culture medium containing 50 µM adenosine (adenosine group) or 50 µM adenosine + 10 µM MRS 1754 (adenosine + MRS group). After 30 min of treatment, immunostaining of MRP2 and nuclei was performed as described earlier in this section. Optical sections (23-31 z-series) were collected with a step size of 0.4 µm. Images are shown as top and 3D orthogonal views. The densitometric study was similar to that performed for intestinal segments, except that the determination of fluorescence intensity was performed along the z axis, using a 10 µm line.

4.3.4. Quantification of intracellular cAMP

To quantify intracellular levels of cAMP, 2.5×10^5 cells/well were seeded in 6-well plates and cultured for 21 days. Then, they were incubated with culture medium alone (control group) or culture medium containing 50 µM adenosine (adenosine group) for 15 min. Immediately afterwards, cells were washed with PBS and lysed in HCl (0.1 M, 20 min) at room temperature in order to stop phosphodiesterase activity and stabilize the released cAMP.[67,68] Finally, cAMP was quantified using the cAMP Select ELISA kit (Cayman Chemical, Ann Arbor, USA) according to the manufacturer's instructions.

4.4. Statistical analysis

All results were expressed as mean ± standard deviation. Statistical comparisons were performed through the Student's t-test (two experimental groups) or through ANOVA followed by Tukey (more than two experimental groups). Statistical significance was set at $p < 0.05$.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1 *In vivo* studies: Effect of nutrients on MRP2 activity and elucidation of the extracellular mechanism involved.

a. The increase in MRP2 activity after intravenous administration of dbcAMP (20 µmol/kg b.w.; 20 min) validated the current model. *b*. Effect of intraluminal administration of glucose (Glc), oleic acid (OA) and glutamine (Gln) on MRP2 activity. *c*. Modulation of MRP2 activity after intravenous GLP-2 administration and participation of this peptide in oleic acid effect. *d*. Evaluation of the mechanism downstream GLP-2, including the mediators IGF-1 and adenosine. *e*. Jejunum sectioning before oleic acid treatment confirmed that the stimulus triggered by the lipid is transmitted along the intestinal wall. Data are presented as % of the corresponding control group and expressed as mean ± standard deviation (n = 4). *: $p < 0.05$

Fig. 2 *In vivo* studies: Oleic acid acutely increases apical MRP2 localization by a GLP-2-mediated mechanism.

MRP2 was labeled with Alexa Fluor 555 (red fluorescence) and nuclei were stained with the blue dye Hoechst 33258 (images from the top row). For the sake of clarity, grayscale images are included showing only the MRP2 channel. Fluorescence intensity distribution along a 30 µm line parallel to the longitudinal axis of the enterocyte (distance represented in x-axis) was evaluated for red and blue channels (n = 20 per group). To detect changes in the distribution of MRP2 curve, all data sets were scaled to the same arbitrary area under the curve value and the resulting curves were aligned using the nuclei channel as a reference. Additionally, MRP2 peak height was calculated, expressed as mean ± standard deviation and compared between groups. *: $p < 0.05$ *versus* the two remaining groups. White scale bar represents 10 µm

Fig. 3 Studies in Caco-2 cells: Adenosine acutely increases MRP2 activity through A2BAR and PKA activation.

a. Permeability coefficients (Papp) of the MRP2 substrate CDF after 120 min, in both apical to basolateral (AB) and basolateral to apical (BA) directions. *b*. The CDF efflux ratio represents the quotient between both permeabilities and properly estimates MRP2

activity. MRS: 10 µM MRS 1754. KT: 1 µM KT5720. Data are expressed as mean ± standard deviation (n = 4). *: $p < 0.05$

Fig. 4 Studies in Caco-2 cells: Adenosine acutely increases apical MRP2 localization through A2BAR activation.

Main images represent the top view of the cell monolayer. Images created by stacking along the Z axis are shown on the right and at the bottom. A small region of the Z-axis images from each group is shown with higher magnification as an inset. MRP2 was labeled with red fluorescence whereas the nuclei, in blue, were stained with Hoechst 33258. Fluorescence intensity distribution along a 10 µm line parallel to the longitudinal axis of the cells (distance represented in x-axis) was evaluated for red and blue channels (n = 20 per group). To detect changes in the distribution of MRP2 among groups, all data sets were scaled to the same arbitrary area under the curve value and the resulting curves were aligned using the nuclei channel as a reference. Additionally, MRP2 peak height was calculated, expressed as mean ± standard deviation and compared between groups. * $p < 0.05$ *versus* the two remaining groups. White scale bar represents 10 µm

Fig. 5 Sequence of events in the regulation of MRP2 by oleic acid.

Taken together, the data allow us to propose the following sequence of events. After ingestion, intraluminal oleic acid stimulates intestinal L cells to release GLP-2 towards the basolateral pole (*1*). GLP-2 in turn binds to its receptor in enteric neurons (*2*) which, either by themselves or through activation of other cell types, release one or more of the possible adenosine precursors (*3*). CD73 catalyzes the hydrolysis of extracellular AMP to adenosine (*4*), a nucleoside able to increase MRP2 apical localization and activity in the enterocyte via the A2BAR/cAMP/PKA axis (*5*)

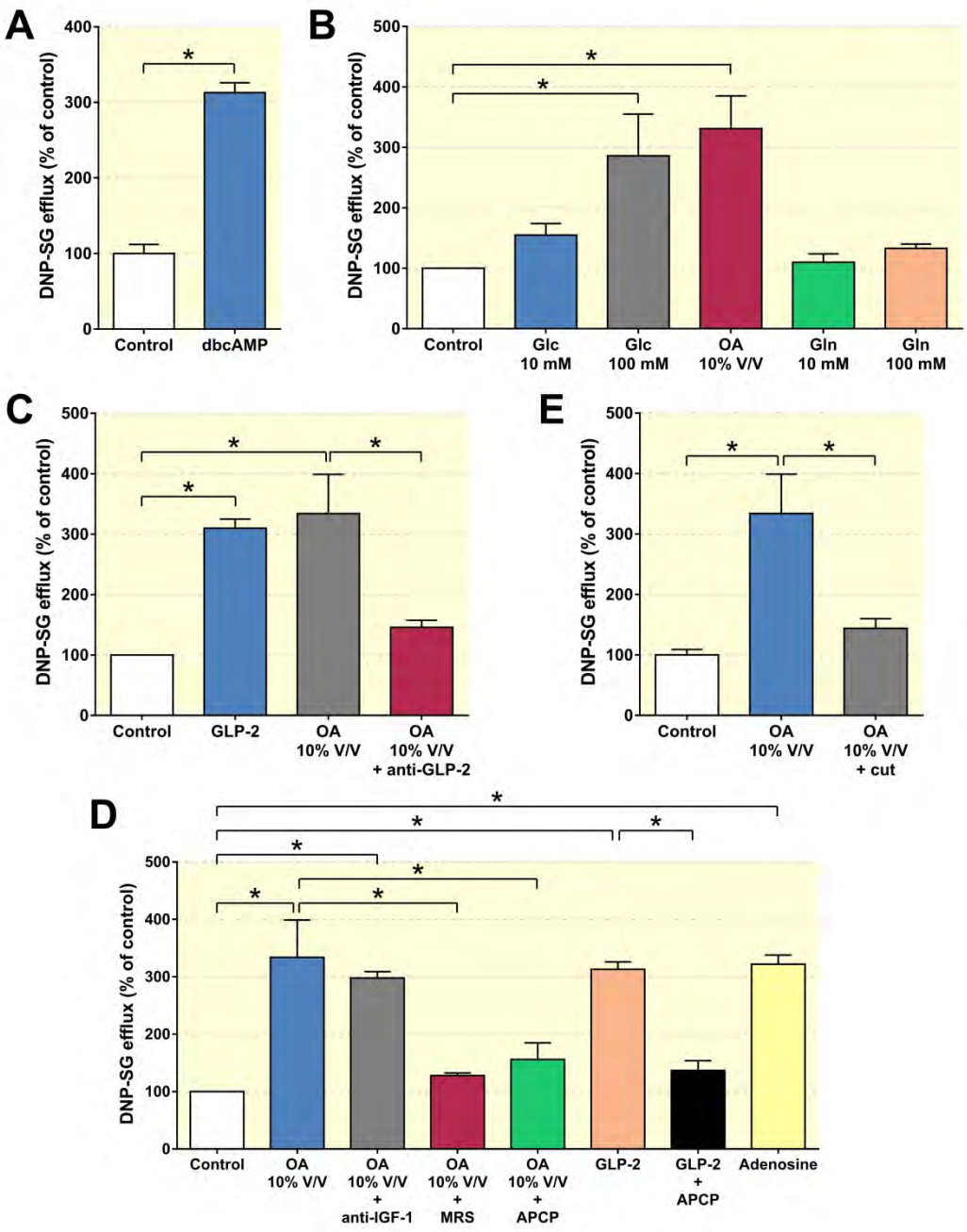
Fig. 6 *In vivo* model for assessment MRP2 activity and localization.

a. Studies were performed in two well identified and separated regions based on the highest MRP2 expression (proximal jejunum) and the highest presence of GLP-2 producing L cells (distal jejunum and ileum). *b*. For direct stimulation of L cells, nutrients were intraluminally incorporated into distal jejunum (left panel). In contrast, and due to their action from the serosal side of the epithelium, dbcAMP, GLP-2 and adenosine were

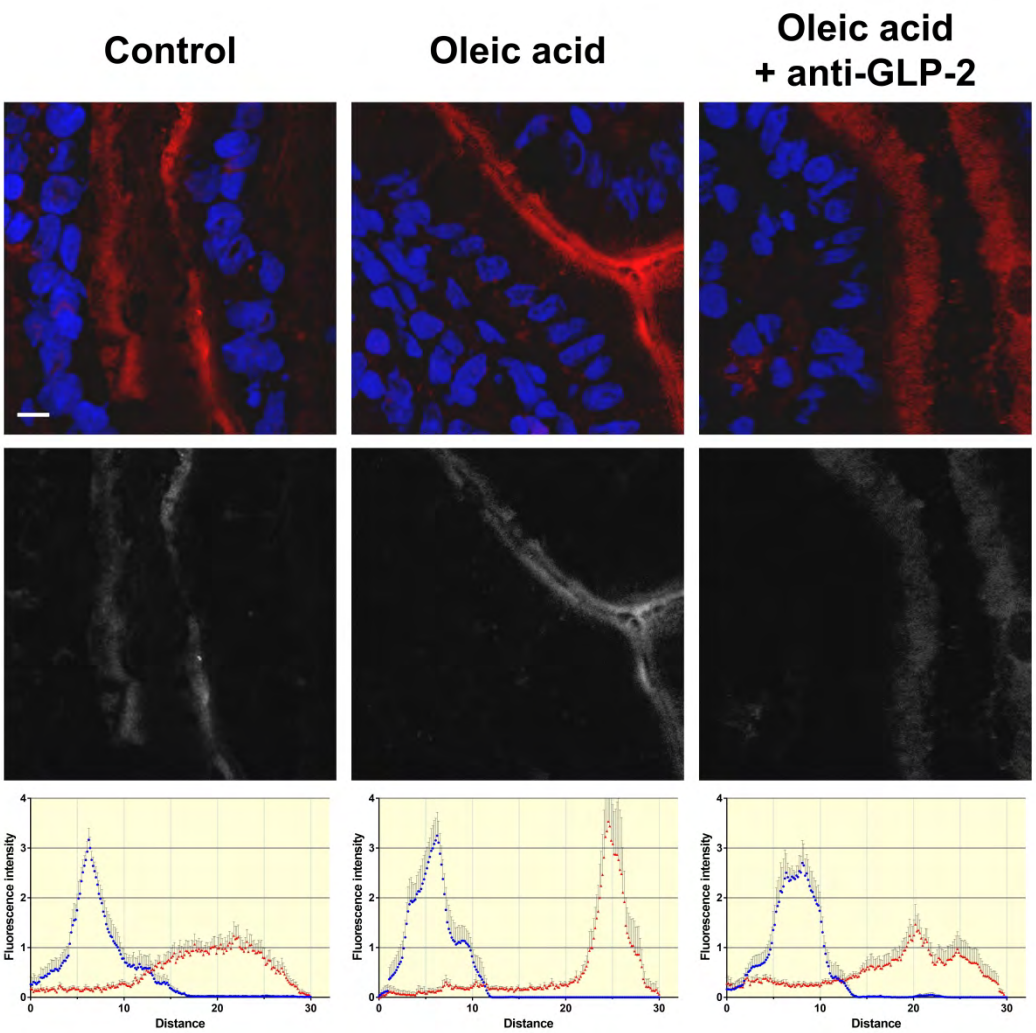
systemically administered into the femoral vein (right panel). After 20 min of treatment, MRP2 activity was determined. *c*. Pretreatments to assess mechanistical aspects underlying oleic acid effect consisted of both the immunoneutralization of GLP-2 or IGF-1 and the inhibition of CD73 or A2BAR by intraperitoneal injection of antibodies or selective inhibitors, respectively (left panel). To assess whether the signal triggered by oleic acid is transmitted along the intestinal wall, the jejunum was sectioned to physically separate the two regions referred above (right panel). All these procedures were followed by oleic acid intraluminal treatment. *d*. After treatments, DNP-SG efflux was determined to estimate MRP2 activity. For that purpose, a 15 cm intestinal sac was created right after the ligament of Treitz (proximal jejunum) and filled with a solution of the parent compound CDNB. After 30 min, the amount of DNP-SG secreted to the luminal compartment was quantified by HPLC-UV

Fig. 7 MRP2 localization studies: densitometric data collection.

All procedures were performed using ImageJ software on images obtained by confocal microscopy. *a*. Distribution of fluorescence intensity was evaluated along a 30 µm line parallel to the longitudinal axis of the enterocyte. *b*. Next, the channels corresponding to the nuclei (graph on the left) and MRP2 (graph on the right) were separated and plotted. This procedure was repeated until obtaining 20 pairs of graphs for each experimental group



apha_13514_f1.jpg



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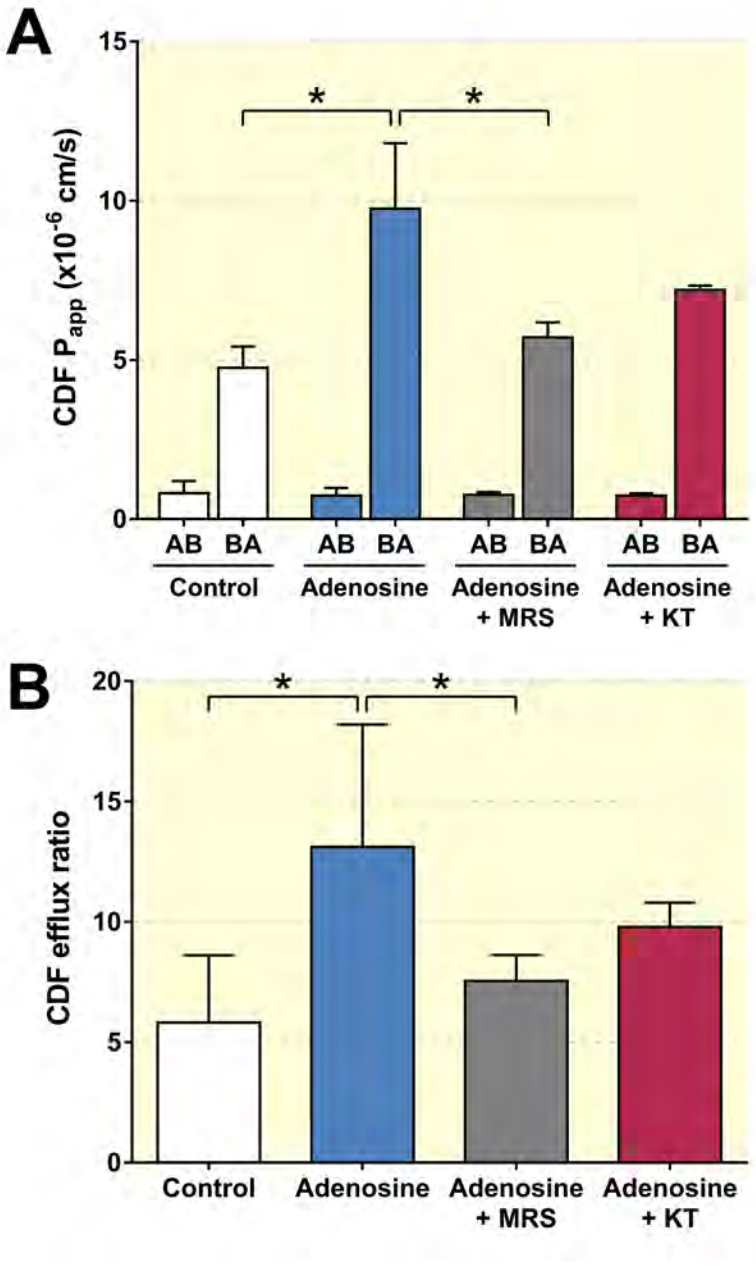
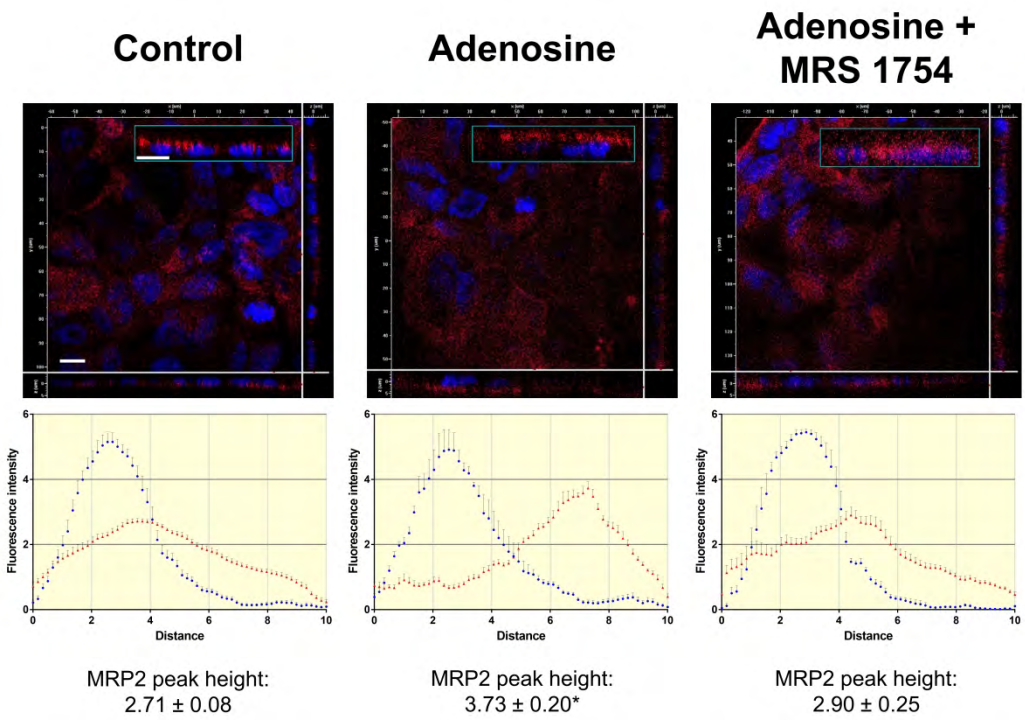


apha_13514_f3.jpg



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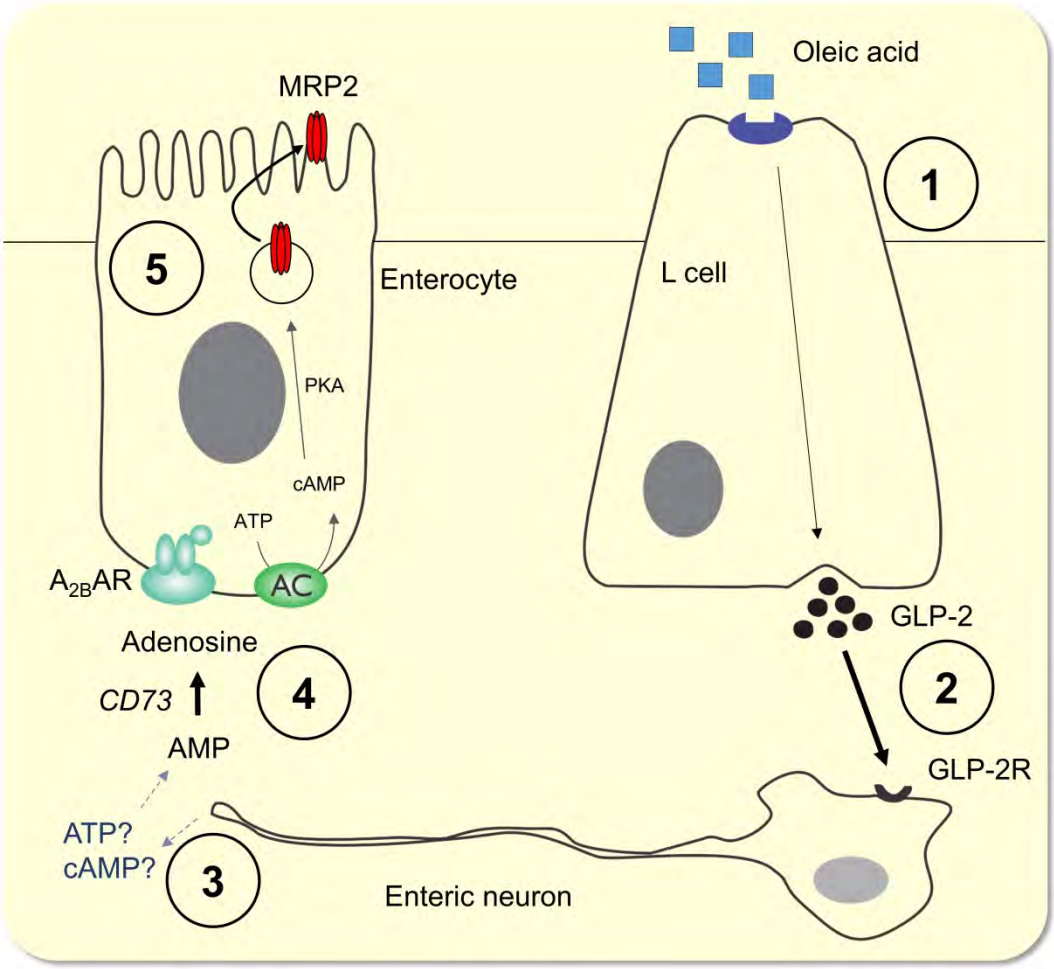

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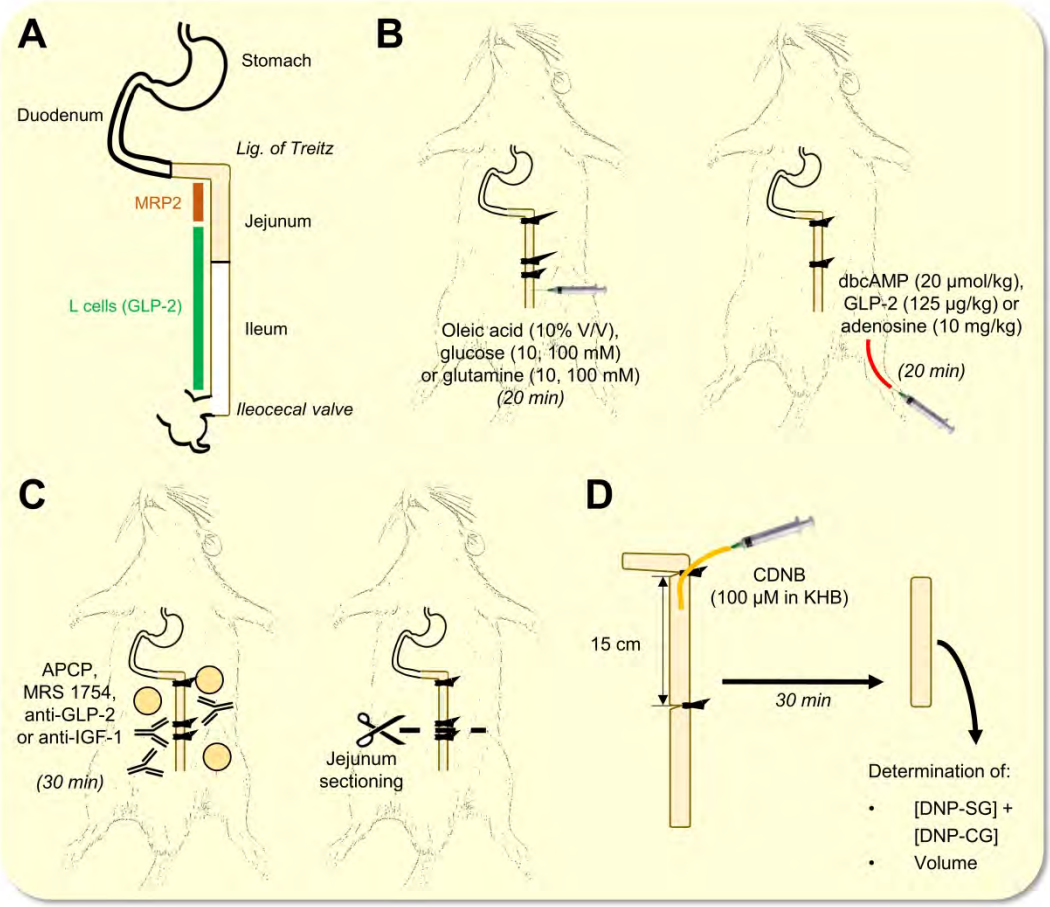

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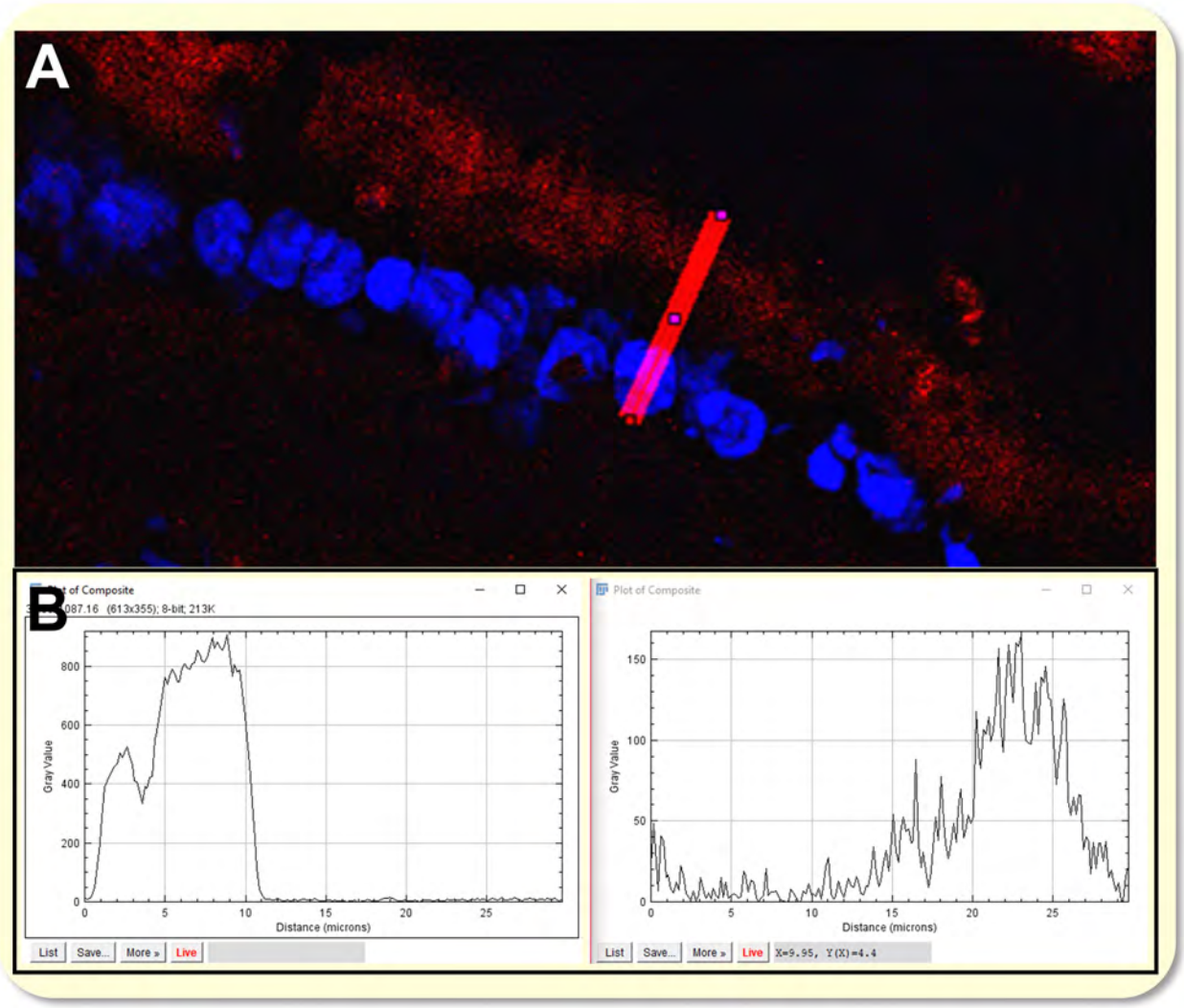

apha_13514_f7.jpg