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Enhancement of D-chiro-inositol transport across intestinal cells by alpha-Lactalbumin peptides

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Abstract. – OBJECTIVE: This study aims to characterize *in vitro* D-chiro-inositol intestinal absorption and identify factors able to improve its bioavailability. D-chiro-inositol, one of the natural occurring stereoisomer of myo-inositol, acts as a second messenger in insulin-regulated glucose metabolism in complementary mode with myo-inositol. Because of their insulin-mimetic activities and safety, both myo-inositol and D-chiro-inositol are often employed as supplements in insulin-resistance treatment.

MATERIALS AND METHODS: Trans-epithelial passage of D-chiro-inositol was evaluated in the human intestinal Caco-2 cell line differentiated on filter, a widely established *in vitro* model to study intestinal absorption. D-chiro-inositol transport was assayed in a concentration range corresponding to an estimated *in vivo* concentration following oral supplementation. α-Lactalbumin peptides, obtained by *in vitro* simulated gastrointestinal digestion, were tested as possible modulators of the intestinal permeability of D-chiro-inositol.

RESULTS: The absorption of this stereoisomer was relatively low and presumably due to passive diffusion, while it was greatly enhanced by the presence of α-Lactalbumin digest. α-Lactalbumin peptides induced an increase in paracellular permeability that was completely reversible, indicating lack of cytotoxicity. This effect involved temporary rearrangement of F-actin apical cytoskeleton and of the tight junction protein ZO-1.

CONCLUSIONS: Although further studies are required to identify and characterize the most effective peptides, the ability of α-Lactalbumin digest to act as absorption enhancers may have very interesting and promising applications in the fields of nutritional supplements and pharmacology.

Key Words:
Bioactive peptides, Trans-epithelial electrical resistance, Absorption enhancers, Intestinal permeability, Inositols.

Introduction

Inositols are a class of polyols composed of nine naturally occurring stereoisomers. Among these, myo-inositol (MI) is the most abundant form in nature, and is a key molecule in several biological processes, including signal transduction, osmoregulation and ion channel physiology; moreover, MI is a component of the cell membrane[1]. D-chiro-inositol (DCI) is one of the natural MI stereoisomers. Despite its low intracellular level as compared to MI, many studies indicate that both MI and DCI play a role in glucose and insulin metabolism, and that DCI intracellular imbalance is involved in the onset of insulin resistance (IR)[2,3], a clinical condition often associated with various diseases, such as cardiovascular pathologies, type 2 diabetes, hypertension, Polycystic Ovary Syndrome (PCOS) and nonalcoholic liver steatosis. These effects are related to the ability of both MI and DCI to form inositol-phosphoglycan derivatives that act as second messengers in insulin signaling[4,5]. The two glycan compounds exert different but complementary functions in insulin transduction pathway[6,7]. MI induces the translocation of GLUT-4 membrane transporter increasing glucose uptake[8-10], while DCI enhances glucose storage and conversion to ATP, accelerating glucose disposal[11]. Moreover, DCI phosphoglycans are able to specifically stimulate insulin secretion in pancreatic β-cells[12].

DCI has been detected in plants and the hypoglycemic effects of plant derived DCI have been demonstrated in streptozotocin diabetic rats[13,14]. In humans, DCI is synthesized from MI by the activity of a NAD-NADH epimerase that operates in insulin-dependent manner[15]. For this reason, in patients affected by IR, MI to DCI epimerization is impaired in several tissues (but

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not in the ovary), altering the relative intracellular concentrations of the two stereoisomers and affecting their coordinated signaling response. As a result, a pathological change in the relative concentrations of MI and DCI has been detected in tissues and in the urine of diabetic rat[16], as well as in diabetic patients[17]. In addition to systemic effects, DCI and MI exert specific local activities in the ovary. DCI facilitates the accumulation of testosterone by reducing aromatase activity in granulosa cells[18], and (as DCI phosphoglycan) directly stimulates the synthesis of testosterone, exerting an insulin-mimetic activity[19]. MI (as InsP3) is one of the second messengers of follicle-stimulating hormone (FSH) in the ovary[20] and it is noteworthy that MI concentration in the mammalian reproductive tract is substantially higher than in blood serum. Moreover, it has been shown that the hyper-insulinemic condition that accompanies PCOS, greatly affects MI/DCI ratio in ovary cells of patients. In these conditions, ovarian cells, that do not develop IR, undergo epimerase overstimulation that results in DCI increase, accompanied by higher androgen production, and a consequent MI depletion that may impair FSH signaling[15]. Consequently, MI/DCI imbalance may be very harmful for reproduction.

Therefore, based on their insulin-sensitizing activity and safety, MI alone or together with DCI in a 40:1 ratio are often employed for treating IR, diabetes or PCOS[5,21,22]. Of note, the 40:1 ratio, that mimics the relative physiological concentrantions of the two stereoisomers in plasma, was found to be the most effective in comparison to other MI/DCI ratios[5,23-26]. However, inositol supplementation was not always effective in all patients, stimulating further studies to improve its efficiency[27,28] especially in the so-called inositol-resistant subjects. Since the intestinal absorption of these compounds is not very efficient[29], its improvement represents a strategy to obtain better therapeutic results. Although intestinal bioavailability depends on several factors, passage across the epithelial barrier represents the most critical step in the absorption process. Specific transport mechanisms, when available for the substances of interest, have to be characterized in order to identify suitable approaches to enhance their absorption. Strategies for improving the absorption of pharmacological agents or bioactive molecules range from the use of permeation enhancers acting directly on membranes and facilitating the transcellular pathway, to those affecting tight junction permeability through intracellular signaling mechanisms and increasing paracellular passage[30].

While MI membrane carriers have been identified and characterized, DCI intestinal transport mechanisms have not been completely elucidated. Three different membrane carrier systems have been described for MI. Two of these, namely SMIT1 and SMIT2, are Na^+-coupled symporters with low affinity for glucose and similar tissue distribution; SMIT2 displays a lower affinity (Km 120 μM) for MI as compared to SMIT1 (Km 55μM), but it transports both MI and DCI with similar kinetic characteristics[31,32]. The third MI transporter (HMIT) is a proton dependent carrier, mainly expressed in the brain[33]. SMIT1 and HMIT probably do not contribute to the absorption of inositol orally administered since both transporters have not, so far, been detected in the intestine[34].

Recent *in vivo* and *in vitro* studies have shown that co-administration of α-Lactalbumin (*α-LA)* with MI leads to improvement of MI bioavailability[35] and *in vivo* efficacy in inositol-resistant PCOS patients[36].

In the present work, the intestinal absorption of DCI and the possible effects of *α-LA* digests on its transport have been investigated in the human intestinal Caco-2 *in vitro* model. Moreover, modulation of intestinal permeability by *α-LA* peptides and epithelial cell morphology, following the different treatments, have also been investigated.

Materials and Methods

Materials

DCI (purity 99%) and *α-LA* from cow milk (purity 96%) were provided by LoLiPharma Srl (Rome, Italy). All other reagents, unless otherwise stated, were from Sigma-Aldrich (Milan, Italy).

Cell Culture and Transport Experiments

The human intestinal Caco-2 cell line, obtained from INSERM (Paris, France), was routinely sub-cultured at 50% density[37] and differentiated on polycarbonate filters (Transwell, Corning Inc. Lowell, MA, USA) for 18-21 days[38]. Briefly, cells were maintained at 37°C in 90% air/10% CO_2 atmosphere in DMEM containing 25 mM glucose, 3.7 g/L $NaHCO_3$, 4 mM stable L-glutamine, 1% non-essential amino acids, 100 U/L penicillin, 100 μg/L streptomycin (complete medium), supplemented with 10% heat inactivated fetal bovine

serum (FBS; HyClone Laboratories, South-Logan, UT, USA). Cells were differentiated on polycarbonate filters, 12 mm diameter, 0.4 μm pore diameter (Transwell, Corning Inc. Lowell, MA, USA), in complete medium with addition of 10% FBS only in the BL compartment. Transport experiments were performed as previously reported[39], in saline buffer solution (BSS: 137 mM NaCl, 5.36 mM KCl, 1.26 mM $CaCl_2$, 1.1 mM $MgCl_2$, 5 mM D-glucose) adjusted to pH 6.0 in the apical (AP) compartment and to pH 7.4 in the basolateral (BL) chamber, in order to reproduce the pH conditions existing *in vivo*. To avoid possible transporter competition, the AP solution was deprived of glucose since the MI/DCI transporter SMIT2 is known to be inhibited by physiologic concentrations of D-glucose[40]. To deplete DCI intracellular stores, before transport cells were pre-equilibrated for 30 min in BSS. The transport of DCI in the concentration range 0.1-10 mM was assayed in the AP to the BL direction for 4 h. To investigate the effects of α-LA digests on DCI transport and on cell monolayer permeability, experiments were performed at two concentrations of DCI (5 and 10 mM) in the presence or absence of two concentrations of α-LA digested peptides (5 and 20 mg/ml). α-LA digest without DCI, as well as a digestion blank consisting of the mix of enzymes used in digestion at the same concentration without substrate protein, were also assayed in parallel.

At the end of DCI transport experiments, AP and BL media were collected and stored at -80°C for further analysis, and cells were transferred to complete cell culture medium and maintained in the incubator at 37°C for 24 h. Trans-Epithelial Electrical Resistance (TEER) was measured at the beginning and at the end of the transport experiment, and after the 24 h recovery period, using the voltmeter apparatus Millicell (Merk Life Science S.r.l., Milan, Italy). TEER was calculated as $\Omega \cdot cm^2$ after subtracting the resistance value of the supporting filter[41]. Moreover, the effects of α-LA peptides on monolayer paracellular permeability, were assayed by measuring the passage of the paracellular marker phenol red. Briefly, the AP to BL passage of 0.5 mM phenol red in BSS was evaluated after 4 h incubation in the presence of 5 and 20 mg/ml α-LA digested peptides[42].

In Vitro Gastrointestinal Digestion of α-Lactalbumin

Simulated gastrointestinal digestion of α-LA was performed *in vitro* according to a previously described method[43], with minor modifications. Briefly, for the gastric phase, α-LA (0.1 g/ml) was digested with pepsin (15,000 U/g α-LA) in PBS at pH 2.0 in a shaking water bath at 37°C for 2 h. Pepsin activity was stopped by adjusting the pH to 8.0 and intestinal digestion phase was started by adding a freshly prepared pancreatin solution (1,000 U/g α-LA) and incubating at 37°C for 3 h. To inhibit protease activities, samples were kept for 10 min at 100°C and subsequently centrifuged at 4,000 g for 100 min at 4°C in Centricon centrifugal filter devices (10,000 MW cut-off, Millipore Corporation, Bedford, MA, USA) to remove the digestive enzymes and undigested large MW peptides. Although this procedure provides for enzymes inactivation and removal, a control digestion was performed in the absence of α-LA protein, and the solution obtained was assayed on Caco-2 cells to verify the absence of adverse effects on the cell monolayer. The α-LA digest was subjected to 20% SDS-PAGE electrophoresis to verify complete digestion of α-LA into small (<5 kDa) MW peptides. Final concentration of α-LA peptides in digested sample was 55 mg/ml as determined by the Lowry method.

DCI Assay

Quantification of DCI levels (μmol/L) was performed by Mérieux NutriSciences Italia (Resana, Treviso, Italy) and was carried out by the following procedure. After extraction with organic solvents and derivatization, sample analysis was made by gas chromatography-mass spectrometry with Agilent 6890 (Agilent, Santa Clara, CA, USA). The injection (1.0 μl) was performed in a splitless mode at 270°C, using a capillary column Agilent 122-5532 DB-5 ms (0.25 mm x 30 m x 0.25 μm). The total run-time lasted 15 min: oven at 70°C from 0 to 1 min; 20°C/min to 150°C; 10°C/ min to 240°C; 4 min at 320°C post-run. The flow rate was fixed at 1.2 mL/min, and the results were analyzed by a MS 5973 Network Series detector in sim mode.

Morphological Studies

After incubation in the presence or in the absence of digested α-LA, cells where washed and fixed with 2% paraformaldehyde, permeabilized with 0.1% TRITON X-100 and filamentous actin (F-actin) was stained with 0.25 μM FITC-conjugated phalloidin[42]. For immunofluorescent staining of the tight junction (TJ) protein ZO-1, cells were fixed with 100% methanol at -20° for 5 min and incubated with monoclonal anti ZO-1 primary antibody (Thermo-Fisher Scientific, Milan,

Italy) followed by secondary goat anti-mouse IgG-TRITC antibody (Jackson Immuno Research Europe Ltd., Cambridge, UK)[42]. Cell nuclei were counter-stained by adding 300 nM 40,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich, Italy) directly to mounting medium (ProLong® Antifade Thermo-Fisher Scientific, Milan, Italy). Specimens were analyzed using an inverted laser-scanning confocal microscope equipped with a 40× oil-immersed objective (LSM 700; Carl Zeiss, Oberkochen, Germany). Serial optical sections were processed with ZEN 2009 software (Carl Zeiss, Jena, Germany). Section series were rendered in maximum intensity projections (MIP) and as z-stacks.

Statistical Analysis

All results are presented as mean ± standard deviation. Prior to statistical analysis, normal distribution and homogeneity of variance of all variables were controlled with Shapiro-Wilk and Levene's tests, respectively. Welch one-way ANOVA followed by Tamhane test was used when only normality was verified, while, when only homogeneity of variance was verified, Kruskal-Wallis and *post-hoc*. Steel-Dwass tests were used. Differences with p-values < 0.05 were considered significant. Statistical analysis was performed with Microsoft Office Excel 2011 upgraded with XLSTAT (ver. 4th March 2014).

Results

Trans-Epithelial Transport of DCI

Intestinal absorption of DCI was assayed in differentiated Caco-2 cells in the AP to BL direction over 4 h incubation time and in the concentration range 0.1-10 mM. As shown in Figure 1, in the concentration range tested, DCI passage rates increased linearly as a function of the initial concentration in the AP donor compartment. Linear regression analysis of the data resulted in the equation $y = 0.8749x$ with R^2 0.927, indicating that, under the conditions tested, DCI passed across the cell monolayer by a non-saturable passive diffusion mechanism. Under these experimental conditions, TEER values remained constant and unchanged compared to control, throughout the incubation time, indicating that monolayer integrity was not affected by the treatment and the observed passage of DCI was not due to perturbations in the monolayer permeability (as later discussed in Figure 4).

Effects of α-LA Peptides on DCI Intestinal Transport and Monolayer Permeability

In order to identify factors able to improve DCI intestinal absorption, the effects of α-LA on DCI intestinal *in vitr*o passage were tested. Prior to transport experiments, α-LA was subjected to simulated gastrointestinal digestion. The obtained digest contained a peptide concentration of 55 mg/ml, representing 50% of the original protein concentration. The α-LA digest was analyzed by SDS PAGE to verify complete digestion into peptides <5kDa, as previously shown[35].

To investigate the effects of α-LA digests on the AP to BL passage of DCI, filter-differentiated Caco-2 cells were exposed for 4 h to two concentrations of DCI (5 and 10 mM) in the presence of two concentrations of α-LA peptides (5 and 20 mg/ml). As shown in Figure 2, DCI transport was increased by almost 10 folds in the presence of 20 mg/ml α-LA peptides, while the lower α-LA peptides concentration (5 mg/ml) did not affect DCI transport compared to control. In addition, the passage of the paracellular marker phenol red (Figure 3) remained unaltered after treatment with 5 mg/ml α-LA peptides for 4 h, while it was greatly increased at the higher α-LA peptides concentration tested (20 mg/ml).

In order to further characterize the changes in monolayer permeability induced by DCI and α-LA peptides, TEER measurements were performed at the end of the treatment and after 24 h recovery

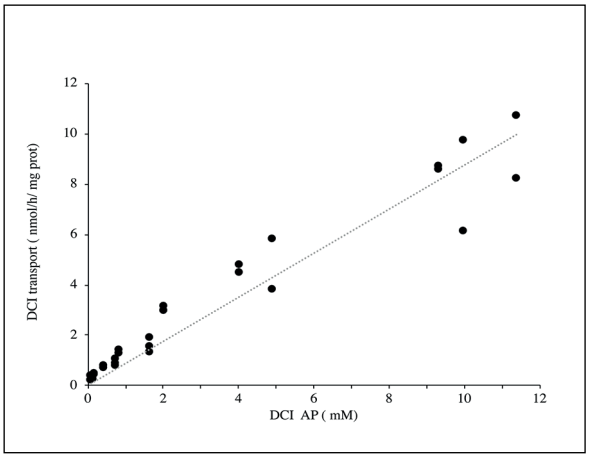

Figure 1. Kinetics of D-chiro-inositol (DCI) transport assayed in the AP to BL direction at the indicated AP concentrations for 4 h. Transport rates were expressed as nmol DCI /h/ mg protein in the BL compartment. Data represent single points obtained from 5 independent experiments performed in duplicate.

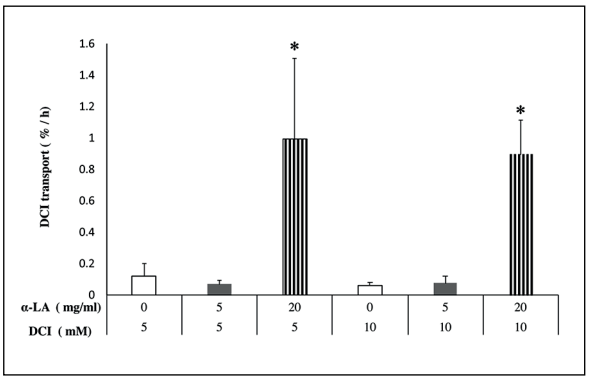

Figure 2. Trans-epithelial passage in Caco-2 cells of D-chiro-inositol (DCI) 5 and 10 mM, was assayed in the absence (white bar) or in the presence of 5 (grey bar) and 20 mg/ml (vertical lined bar) α-Lactalbumin (*α-LA*) peptides. DCI transport to the BL compartment after 4 h was expressed as % of the AP concentration /h. Data are the mean ± SD of 3 experiments performed in triplicate. Statistical analysis was performed by Kruskal-Wallis followed by Steel-Dwass test. Asterisks (*) indicate differences with $p<0.05$ within 5 and 10 mM AP DCI concentration.

in complete culture medium. An 80% decrease in TEER values was observed after 4 h in the presence of 20 mg/ml α-LA peptides, both with or without DCI, while, after removing the digest and returning the cells to complete medium, full recovery of TEER values was obtained within 24 h (Figure 4).

Fluorescence Imaging of F-actin and ZO-1

Since epithelial barrier function is maintained by the presence of the highly specialized TJ structure linked to the cell cytoskeleton, morphological studies were performed to better characterize the effects of **α-**LA peptides on cellular architecture and TJ organization. Cells were incubated in the presence of 5 and 20 mg/ml digests of α-LA and the TJ protein ZO-1 and F-actin were fluorescently stained after incubation and after the recovery phases. F-actin organization appeared already affected after 4 h of treatment with the lower peptides concentration of 5 mg/ml (α-LA 5); confocal MIP revealed an overall reduction in F-actin signal with respect to control cells. Microvillar F-actin staining by fluorescent phalloidin binding, appeared in scattered clusters, and was notably reduced in intensity compared to the strong punctuated pattern, corresponding to AP microvilli, observed in control cells (Figure 5, 4 h: CONT). A more pronounced reduction in F-actin AP staining was observed after treatment with 20 mg/ml α-LA peptide, frequently resulting in the staining being restricted to the cell periphery, with almost complete loss of microvillar signal (Figure 5, 4 h: α-LA 20). Remarkably, after 24 h in com-

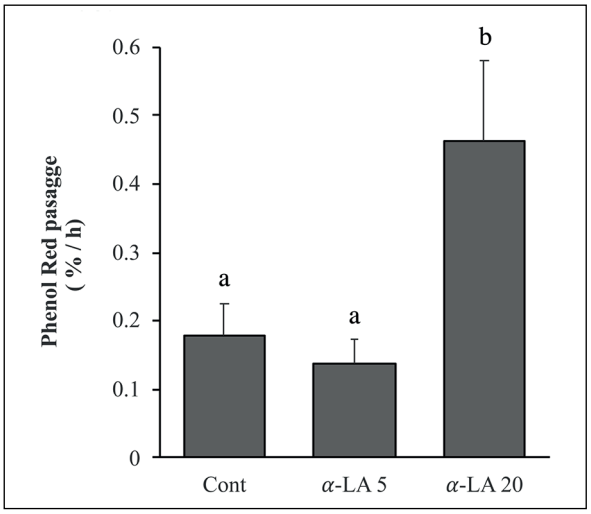

Figure 3. Trans-epithelial passage in Caco-2 cells of phenol red 5 mM, was assayed in the absence (Cont) or in the presence of 5 (*α-LA 5)* and 20 (*α-LA 20)* mg/ml α-Lactalbumin peptides. Phenol red transport to the BL compartment after 4 h was expressed as % of the AP concentration /h. Data are the mean ± SD of 3 experiments performed in triplicate. Statistical analysis was performed by Kruskal-Wallis followed by Steel-Dwass test. Different letters indicate differences with $p<0.05$.

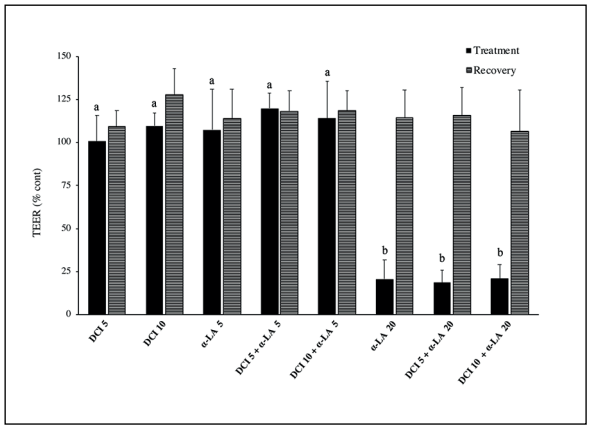

Figure 4. Monolayer permeability, assayed as TEER, was measured after 4 h of treatment (solid bar), and after 24 h of recovery in complete culture medium (horizontal lines bar). TEER values were expressed as % of control cells maintained in BSS. Cells were incubated in the presence of 5 (α-LA 5) and 20 (α-LA 20) mg/ml α-Lactalbumin (*α-LA*) peptides with or without D-chiro-inositol 5 (DCI 5) and 10 mM (DCI 10) as indicated. Data are the mean ± SD of 3 experiments performed in triplicate. Statistical analysis was performed by Welch one-way ANOVA followed by post hoc Tamhane's test. Different letters indicate differences with $p<0.05$ within Treatments and Recovery.

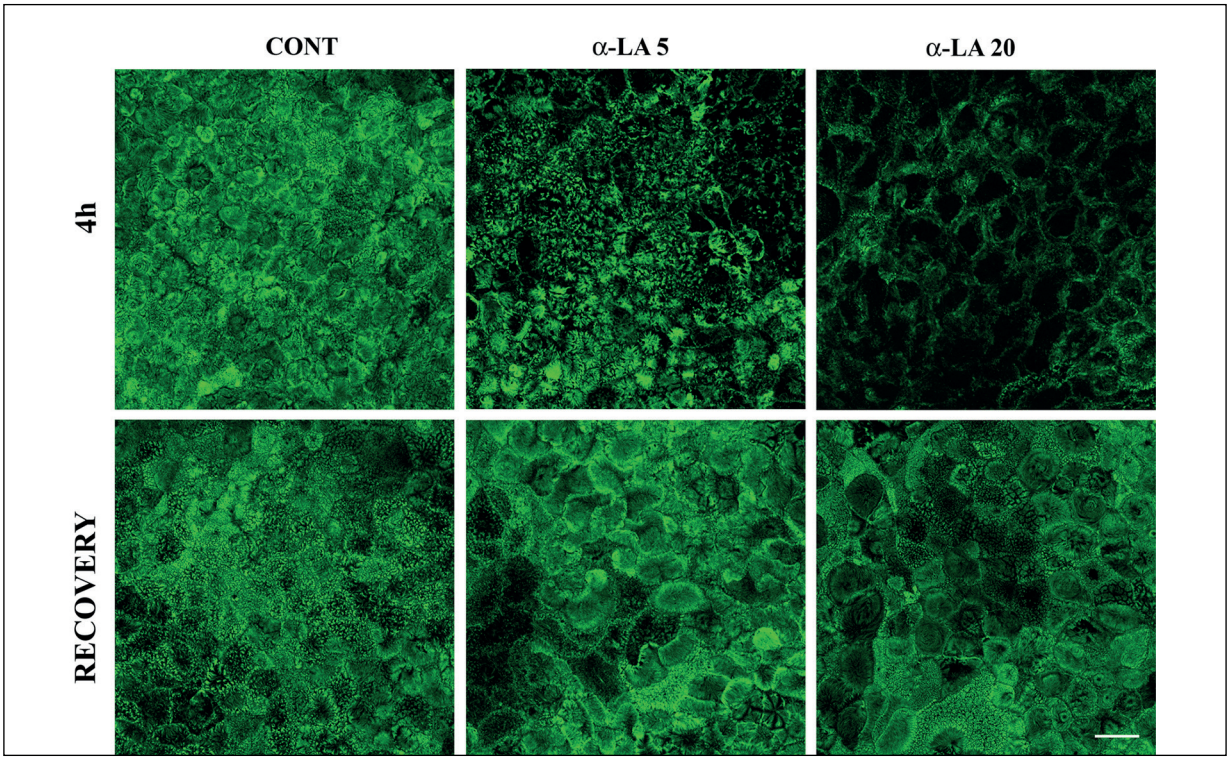

Figure 5. Confocal MIPs of F-actin staining in Caco-2 cells after 4 h treatment and after 24 h recovery in complete culture medium, in the absence (CONT) and in the presence of 5 (α-LA 5) and 20 (α-LA 20) mg/ml α-Lactalbumin (*α-LA) peptides*. Magnification bar 20 *μm*.

plete culture medium (Figure 5, RECOVERY), F-actin localization was restored and microvilli structures appeared well organized at the AP membrane in all experimental conditions.

The orthogonal view along the z-y axis of cells stained for F-actin and counterstained with DAPI for nuclear visualization (Figure 6), clearly showed that in control cells the F-actin was restricted to the AP region, depicting a well developed brush border. Conversely, increasing the concentration of α-LA peptides produced a gradual decrease of the AP F-actin signal (Figure 6, 4 h: α-LA5 & 20), and its organized reappearance after α-LA removal and recovery for 24 h in complete culture medium (figure 6, REC).

Immunolocalization of ZO-1 in control cells and after treatment with 5 and 20 mg/ml α-LA peptides (Figure 7), showed the peripheral local-

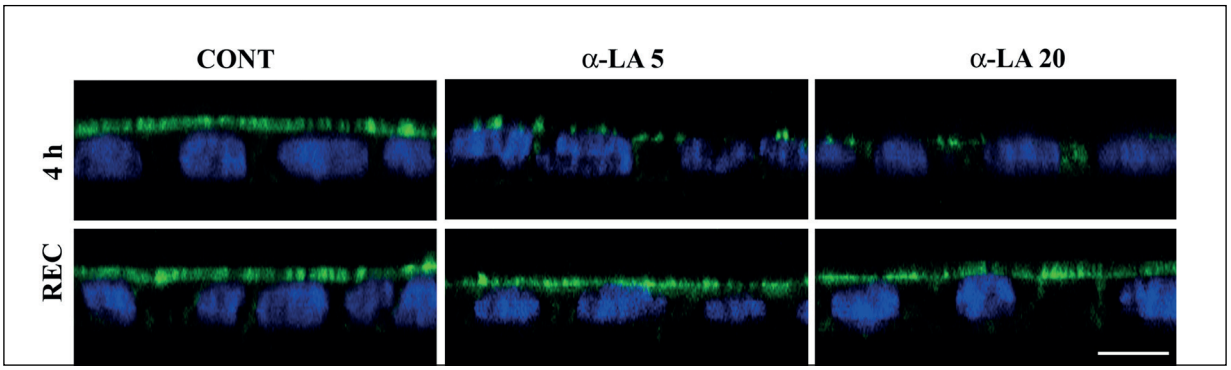

Figure 6. Cross-sectional view of z-stack (z–y axis) of Caco-2 cells stained for F-actin and nuclear DAPI after 4 h treatment and after 24 h recovery in complete culture medium, in the absence (CONT) and in the presence of 5 (α-LA 5) and 20 (α-LA 20) mg/ml α-Lactalbumin peptides. Magnification bar 20 *μm*.

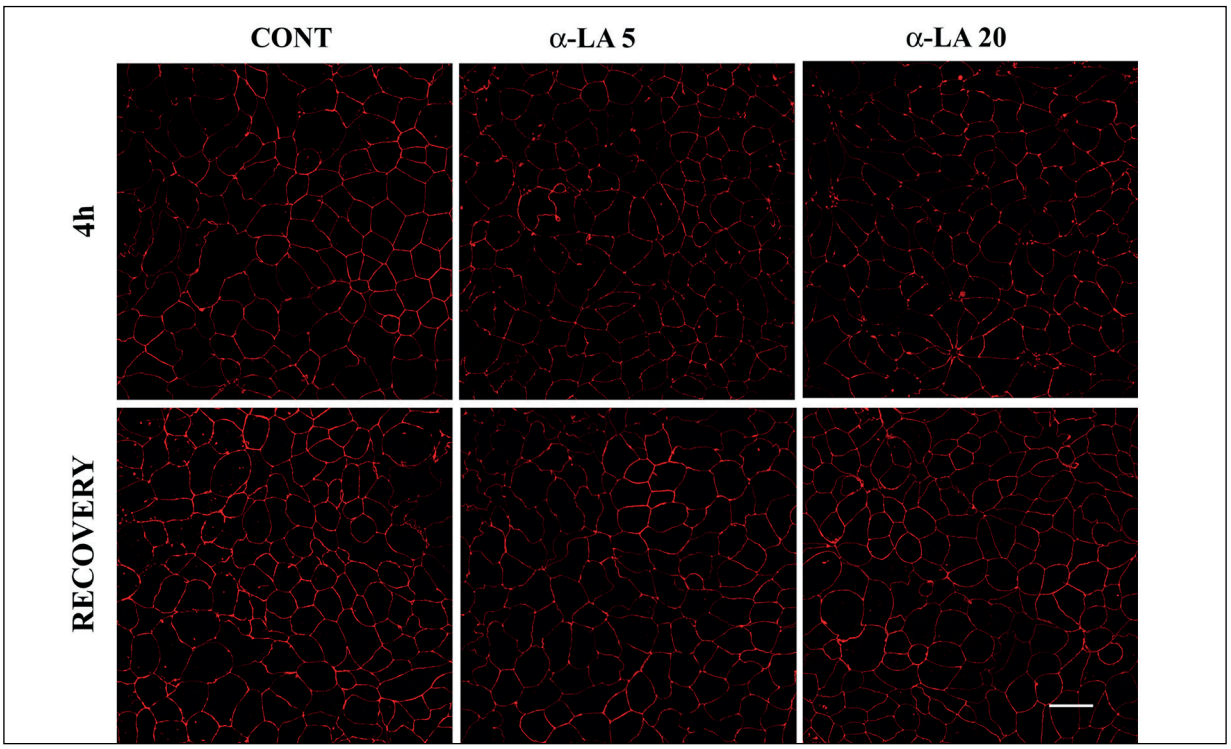

Figure 7. Confocal MIPs of Caco-2 cells stained for ZO-1 after 4 h treatment and after 24 h recovery in complete culture medium, in the absence (CONT) and in the presence of 5 (α-LA 5) and 20 (α-LA 20) mg/ml α-Lactalbumin peptides. Magnification bar 20 μm.

ization of the protein, clearly outlining cell borders in close apposition to one another. While treatment with the lower concentration of α-LA peptides (Figure 7, 4 h: α-LA 5) did not cause apparent changes in ZO-1 staining, the higher concentration produced some discontinuities in the regular peripheral staining, and signal disappearance in certain areas (Figure 7, 4 h: α-LA 20). The normal cellular outlines evidenced by ZO-1 staining were recovered after 24 h in complete medium (Figure 7, RECOVERY).

Discussion

DCI is a natural occuring inositol with insulin-mimetic activity, that operate in a complementary mode with MI, its more abundant stereoisomer. Humans can endogenously synthesize MI from glucose-6-phosphate (G6P), however a considerable amount of MI is of dietary origin. DCI is synthesized from MI by a specific insulin-dependent epimerase. Due to their interdependent actions, the relative balance of MI *vs.* DCI concentration is important to maintain a proper response to insulin stimuli; the relative MI/DCI ratio differs in various tissues, with estimated values that range between 40:1 in plasma to 100:1 in ovary cells. Because of their insulin-sensitizing properties and concomitant relative safety[5], MI and DCI are often employed as dietary supplements for insulin-related diseases[21,22]. Due to their interconnected metabolism, the appropriate dosage is still a debated topic, although 4 g inositol in the 40:1 MI:DCI ratio seems to be the best one[5]. However, commercially available supplement formulations may contain one or both steroisomers in different amounts and proportions[44]. Therefore, since DCI intestinal absorption has not been characterized, DCI transport mechanism and the modulating effects of α-LA peptides on intestinal permeability were investigated using the human intestinal Caco-2 cells, differentiated on permeable filter inserts, a suitable and largely utilized experimental system for intestinal transport studies[45]. Several active small intestinal transport carriers present *in vivo* are also expressed in differentiated Caco-2 cells, although their level of expression can be different, as shown for the main transporters involved in drug absorption, namely P-glycoprotein and breast cancer resistant protein[46].

Although the expression of possible DCI carriers SMIT1 and SMIT2 has not been evaluated in Caco-2 cells, current data suggest a linear diffusion kinetic for DCI passage across Caco-2 cells, not indicating any contribution by carrier mediated transport, at least at the tested concentrations. DCI exhibits low absorption across Caco-2 cells similarly to what previously observed for MI[31]. The only available data on inositol pharmacodynamics concern MI. They show that oral doses of 2 g twice a day (in the morning and in the evening) are required to reach and maintain suitable therapeutic levels in plasma. Of note, a single dose of 2 g MI normally does not cause side-effects[47]. Thus, permeation enhancers that are safe and of transient effect would greatly contribute to the improvement of their intestinal absorption. An increase in MI bioavailability was obtained in healthy human volunteers after administration of MI with α-LA, and was further confirmed *in vitro* in human intestinal differentiated Caco-2 cells treated with MI and digested α-LA[31].

In vitro gastrointestinal digestion is essential for bioavailability studies on cultured intestinal cells, to better mimic the molecules expected to reach the intestinal mucosa *in vivo*. A recent comparison[48] of peptides released after *in vivo* jejunum and *in vitro* simulated digestion of milk proteins, identified similar peptide patterns and common regions resistant to digestion, indicating that the *in vitro* protocol constitutes a good approximation to gastrointestinal digestion of these proteins. In differentiated Caco-2 cells, the presence of α-LA digests for 4 h altered monolayer permeability by decreasing TEER and increasing the passage of MI and of the paracellular marker phenol red[35]. While these results suggested that α-LA peptides could represent a suitable permeation enhancer, the effects of the digest on the cell monolayer had not been further investigated. A recent *in vivo* study[36] of PCOS patients has demonstrated that co-administration of MI and α-LA can increase MI absorption, determining an improvement in therapeutic efficacy.

The results of the present study indicate that the substantial increase in DCI passage induced by the presence of 20 mg/ml α-LA peptides, confirmed both by the the decrease in TEER and the increase in phenol red passage, can be attributed to an increase in paracellular permeability of the monolayer. Such permeability changes were accompanied by morphological perturbation of the F-actin AP cytoskeleton that is tightly linked to the TJ[49].

Notably, the study further established that these effects are fully reversible representing a modulation of epithelial permeability, rather than the consequence of cytotoxic damage to the monolayer.

A similar recovery of permeability changes in Caco-2 cells was reported to depend on the degree of TEER reduction achieved after treatment with Fe(II), where lower concentrations led to a reversible increase in TJ permeability, while higher concentrations produced irreversible cytotoxicity leading to necrosis and apoptosis[50,51]. Similarly, reversible effects on TJ permeability following a mild toxic stimulus were observed for ethanol[52], mycotoxins[53], and heat[54]. In addition, a number of peptides have been shown to reversible modulate TJ functions and enhance intestinal paracellular permeability of poorly absorbed compounds[55,56]. It is therefore conceivable that some peptides derived from α-LA digestion may exert similar effects on Caco-2 cellular monolayer, modulating the intestinal barrier permeability in a reversible manner.

Impairment of intestinal barrier and increased uncontrolled luminal passage of potentially toxic or immunogenic molecules is often observed in intestinal inflammatory and infective diseases. In inflammatory bowel disease (IBD) enhanced release of pro-inflammatory cytokines affects the epithelium and promotes a leakier barrier, a hallmark of the disease[57]. Similarly, several entero-pathogenic bacteria produce peptide toxins that are able to increase paracellular permeability by inducing F-actin perturbation or by specifically targeting TJ components, thus allowing host invasion[58]. A pivotal role of the actin cytoskeleton in maintaining TJ sealing has been demonstrated by using actin depolymerizing agents that reversibly increase paracellular permeability[59]. Another strategy of host invasion also involves microvillar effacement, that can be visualized by F-actin disappearance at the microvillar level[60,61].

Shen et al[62], however, indicate that intestinal permeability is a dynamic property of this epithelium and can be modulated also in healthy conditions. In our system, exposure to the lower concentration of α-LA peptides did not affect monolayer permeability but induced changes in F-actin expression and organization, while treatment with 20 mg/ml α-LA peptides, produced a substantial increase in monolayer permeability accompanied by TJ and cytoskeleton perturbation. In these conditions, F-actin staining was dramatically reduced, frequently resulting in re-

striction of staining at the cell periphery, with almost complete loss of microvillar signal, suggesting microvillar effacement.

The TJ protein ZO-1 belongs to the family of cytoplasmic plaque proteins with a scaffolding role to couple the trans-membrane membrane junctional proteins to the F-actin cytoskeleton[63]. Discontinuities in ZO-1 localization as visualized by immunofluorescent staining, were further evidence of perturbations of cytoskeleton and of TJ organization following exposure to α-LA peptides. Remarkably, both the effects on F-actin cytoskeleton and on ZO-1 completely disappeared after 24 h in complete culture media, together with recovery of initial TEER values. Among the toxin peptides acting as absorption enhancers is *Vibrio cholera* zot toxin (Zot)[64] that is not toxic but induces an increase in paracellular transport across the intestinal tissue by inducing the redistribution of the F-actin cytoskeleton in a time- and dose-dependent manner[65]. In addition, other peptides have recently been characterized as intestinal absorption enhancers, and among them are those able to transiently open the TJ responsible for cell-cell adhesion in a concerted manner, by acting directly on TJ proteins or indirectly on the cytoskeleton, as already discussed for Zot[66]. Thus, although the mechanisms that control intestinal permeability have not yet been fully defined, the possibility of modulating epithelial permeability in a safe and reversible manner is an appealing possibility, especially for improving intestinal absorption and targeting of drugs and nutritional supplements. It is therefore possible that, in normal physiological conditions, luminal peptides are produced by digestion processes that interact with TJ proteins and/or the F-actin cytoskeleton, modulating the overall permeability of the epithelial monolayer. These peptides might exert mild reversible effects on epithelial cells causing an increase of monolayer permeability as observed for α-LA digest on DCI passage and TEER reduction in Caco-2 cells.

Conclusions

Briefly, the present study has shown that the *in vitro* intestinal absorption of DCI is relatively low, also at concentrations comparable to those potentially reachable by oral supplementation. However, as already observed for MI transport, DCI passage can be significantly enhanced (almost 10 times) by the presence of ***α***-LA peptides obtained by a simulated gastrointestinal digestion protocol. These effects were not due to permanent damage to the epithelial monolayer, but to reversible modulation of paracellular permeability that likely involves transient F-actin rearrangement. Further characterization of α-LA peptide moiety could be useful to identify active peptides able to modulate intestinal barrier as possible absorption enhancer candidates. Low intestinal absorption of drugs and dietary supplements represents a great limitation for their oral administration. Although the bioavailability of a substance depends on several factors, its passage through the epithelial barrier represents the most critical step.

The *in vitro* data presented suggest that small peptides derived from α-LA digestion may act as modulators of intestinal permeability also *in vivo*. Although further researches are required to characterize the peptides involved in this effect, our results strengthen the potential use of α-LA as a new intestinal absorption enhancer. The use of α-LA offers the opportunity to safely improve the therapeutic use of compounds not sufficiently absorbed. Moreover, this study provides a suitable novel approach to achieve *in vivo* more effective and proper DCI dosages, by co-administrating this molecules with α-LA. This may offer a valid support in the field of inositols supplementation, that represents a very promising intervention strategy not only in pathological conditions related to insulin resistance, but also for other disorders.

Conflict of Interest

Vittorio Unfer is an employee at Lo.Li. Pharma srl. (Rome, Italy). The other authors declare no conflict of interest, financial or otherwise.

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