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Polyphenols Protect the Epithelial Barrier Function of Caco-2 Cells Exposed to Indomethacin through the Modulation of Occludin and Zonula Occludens-1 Expression

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ABSTRACT: The aim of this study was to determine the protective effect of quercetin, epigallocatechingallate, resveratrol, and rutin against the disruption of epithelial integrity induced by indomethacin in Caco-2 cell monolayers. Indomethacin decreased the transepithelial electrical resistance and increased the permeability of the monolayers to fluorescein−dextran. These alterations were abolished by all the tested polyphenols but rutin, with quercetin being the most efficient. The protective effect of quercetin was associated with its capacity to inhibit the redistribution of ZO-1 protein induced in the tight junction by indomethacin or rotenone, a mitochondrial complex-I inhibitor, and to prevent the decrease of ZO-1 and occludin expression induced by indomethacin. The fact that the antioxidant polyphenols assayed in this study differ in their protective capacity against the epithelial damage induced by indomethacin suggests that this damage is due to the ability of this agent to induce not only oxidative stress but also mitochondrial dysfunction.

KEYWORDS: *indomethacin, polyphenols, quercetin, transepithelial electrical resistance, tight junction, rotenone, ZO-1, occludin*

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for their antipyretic, analgesic, and anti-inflammatory properties.[1] However, their chronic administration is frequently associated in humans with adverse effects that mainly affect the gastrointestinal (GI) mucosa. Indomethacin (INDO)-induced GI damage is a model widely used in animals to evaluate the gastro-protective activity of drugs and bioactive compounds. Some of the main cytotoxic events of INDO in the GI tract are linked to mitochondrial dysfunction, oxidative stress, and apoptosis, as recently reported in Caco-2 cells and animal models.[2−6]

INDO administration also strongly affects the integrity of the GI barrier function in vivo. The intragastric administration of INDO (5−20 mg/kg) to rats has been shown to increase their GI permeability to ^{51}Cr−EDTA;[7−9] sucrose;[7] and lactulose, mannitol, and sucralose.[10] In addition, INDO administration also increases the urinary excretion of sucrose as well as the lactulose/manitol ratio in human volunteers, suggesting an increased gastric and intestinal permeability, respectively.[11,12] It has been shown that INDO mainly affects paracellular permeability, which is mainly determined by tight junction (TJ) integrity; however, the mechanism underlying the disruption of GI epithelia induced by INDO has been poorly addressed. TJs are macromolecular complexes involving a great number of proteins. Zonula occludens (ZO)-1 is one of the major TJ-plaque proteins that directly binds to actin cytoplasmic filaments and to the transmembrane TJ protein occludin. These proteins play a crucial role as key molecules in cell-to-cell contact and in maintaining the structure of TJ and the epithelial barrier function.[13]

In vitro studies have shown that pro-oxidant molecules like hydrogen peroxide (H_2O_2) or the superoxide radical alter the distribution of some TJ proteins in monolayers of Caco-2 or T-84 cells.[14−18] In addition, it has been reported that ATP depletion induces the transient disruption and reassembly of TJs in kidney epithelial cells.[19−21] According to this evidence, both oxidative stress and mitochondrial dysfunction appear as important mechanisms underlying the disruption of the epithelial barrier function.

Polyphenols (PPs), including flavonoids and stilbenes, represent a ubiquitous group of secondary metabolites in fruits and vegetables and are part of the average human diet. In addition to their well-known antioxidant properties, dietary PPs display many other activities, such as antimicrobial, anti-inflammatory, antiproliferative, and mitochondrial protective activities.[2,3,5,22,23] However, most dietary PPs are found in plants in glycosylated forms that are poorly absorbed, favoring their accumulation in the GI tract, which may be considered as their main site of action.[24] Due to their antioxidant and mitochondrial protecting properties, dietary PPs represent a promising alternative to protect the GI mucosa against NSAID-induced side effects. Quercetin (QUE), epigallocatechingallate (EGCG), rutin (RUT), and resveratrol (RES) have been shown to prevent oxidative stress and the gastric damage induced by INDO in Caco-2 cells[5,6] and in rats.[25−27]

On the basis of these antecedents, we propose that mitochondrial dysfunction is a mechanism by which NSAIDs

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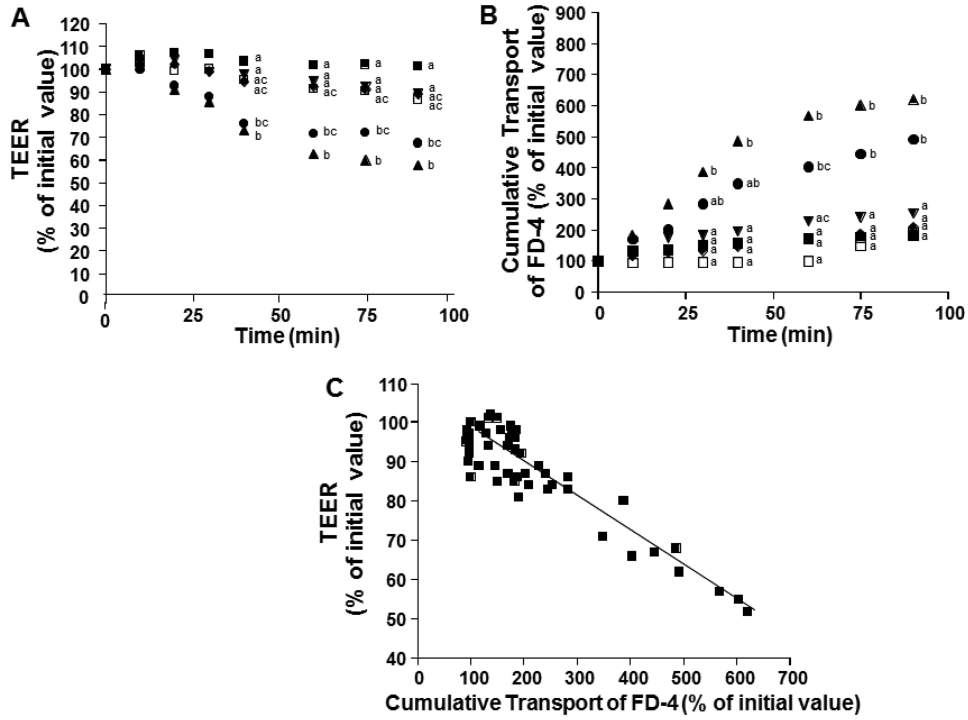

Figure 1. Effect of INDO on the barrier function of Caco-2 cell monolayers: protection by polyphenols. Cells were treated with vehicle (■, control); ▲, 250 μM INDO; ▼, 250 μM INDO + 33 μM (10 μg/mL) QUE; ◆, 250 μM INDO + 438 μM (100 μg/mL) RES; ●, 250 μM INDO + 164 μM (100 μg/mL) RUT; □, 250 μM INDO + 218 μM (100 μg/mL) EGCG. Before and after 10, 20, 30, 40, 60, 75, and 90 min of treatment, TEER (A) was regularly measured and FD-4 permeability across Caco-2 monolayer (B) was determined by measuring the fluorescence (λ excitation 490 nm/λ emission 520 nm) in aliquots from the basolateral compartment. Data were analyzed by two-way ANOVA ($P < 0.0001$) and the Bonferroni posthoc test. Values bearing different superscript letters are significantly different ($p < 0.05$). (C) Correlation between FD-4 transport and TEER. Kolmogorov–Simimov test, with a Pearson r value = −0.9282.

may alter the integrity of GI epithelia; its relationship with oxidative stress is also discussed. The protective effect of QUE, RES, EGCG, and RUT on the alterations of epithelia integrity induced by INDO has been assessed, using Caco-2 cell monolayers as an in vitro model.

■ MATERIALS AND METHODS

Chemicals. Fluorescein isothiocyanate–dextran, average mol wt 3000–5000 (FD-4), was from Sigma-Aldrich. ZO-1 monoclonal antibody mouse (clone ZO1-1A12) FITC conjugate was from Zymed (cat. no. 33–9111, Invitrogen). GeneJet Whole Blood RNA Purification Mini Kit was from Thermo Scientific, Fermentas (Vilnius, Lithuania). RQU1 RNase-free DNase (cat. no. M6101) and oligo(dT)15 primer (cat. no. C1101) were from Promega (Madison, WI). dATP (cat. no. 55082), dGTP (cat. no. 55084), dCTP (cat. no. 55083), dTTP (cat. no. 55085), RNaseOUT (cat. no. 10777019), and M-MLV reverse transcriptase (cat. no. 28025–013) were from Invitrogen. Brilliant II SYBR Green qPCR master mix kit (cat. no. ST. 600828) was from Stratagene, Agilent Technologies. All cell culture reagents were from Life Technologies.

Transepithelial Electrical Resistance (TEER) Assay in Caco-2 Cell Monolayers. Caco-2 cells were grown in transwell polycarbonate filters (diameter, 12 mm; pore size, 0.4 μm (Costar 3460, Corning Inc.) in DMEM-F12 with 10% fetal calf serum and antibiotics and incubated at 37 °C with 5% CO_2. The basolateral and apical compartments were filled with 1.5 and 0.5 mL of culture medium, respectively. The culture medium was changed three times per week, and cells became confluent after 10–14 days culture. Transepithelial resistance (TEER) was measured as described by Li et al., [28] using an ohm/voltmeter (EVOM, WPI). According to preliminary tests, cells were considered as confluent when basal TEER was higher than 600 Ω. The apical medium was then removed and 250 μM INDO was added to the culture medium without fetal calf serum, in the absence or presence of 33 μM (10 μg/mL) QUE, 438 μM (100 μg/mL) RES, 164 μM (100 μg/mL) RUT, or 218 μM (100 μg/mL) EGCG. TEER was regularly registered for 90 min (0, 10, 20, 30, 40, 60, 75, and 90 min) of treatment. Resistance values were calculated in $\Omega \cdot cm^2$ by multiplying the resistance values by the filter surface area. Results are expressed as the percentage of the basal (control) value. TEER changes (% of initial value) = $(RFU_{expl} \times 100)/RFU_{control}$

Fluorescein Isothiocyanate-Conjugated Dextran (FD-4) Transport. Caco-2 cells were cultured on Transwell filters as previously described. Immediately after adding 1 mg/mL FD-4 (MW 4.4 KD, dissolved in culture medium) in the apical compartment, 250 μM INDO was also added in the absence or presence of 33 μM (10 μg/mL) QUE, 438 μM (100 μg/mL) RES, 164 μM (100 μg/mL) RUT, or 218 μM (100 μg/mL) EGCG. The appearance of fluorescence in the basolateral compartment was regularly measured for 90 min (0, 10, 20, 30, 40, 60, 75, and 90 min), using a Multi-Mode Microplate Reader (Synergy HT, BioTek). The excitation and emission wavelengths were 490 and 520 nm, respectively. Results are expressed as percentage of the basal (control) value. Cumulative transport of FD-4 (% initial value) = $(RFU_{expl} \times 100)/RFU_{control}$

Immunofluorescence Staining of ZO-1. Caco-2 cells were grown on HCl-treated coverslips, and the medium was changed three times per week; cells formed a confluent polarized monolayer after 10–14 days of culture. After removing medium, cells were treated for 48 h with 500 μM INDO in the absence or presence of 66 μM (20 μg/mL) QUE, 438 μM (100 μg/mL) RES, 164 μM (100 μg/mL) RUT, or 218 μM (100 μg/mL) EGCG. Cells were also treated for 48 h with 40 μM rotenone, alone or with 20 μg/mL QUE. Cells were then washed three times with phosphate-buffered saline (PBS), pH 7.4, and fixed for 15 min at room temperature in freshly prepared 4% paraformaldehyde. The fixed cells were washed with PBS and permeabilized for 10 min at room temperature using 1% Triton-X-100 in PBS. Cells were washed with PBS and incubated overnight at 4

°C with 1 μg/mL anti-ZO-1−FITC. Cells were washed and counterstained for 2 min with 10 μg/mL DAPI. Fluorescence was visualized by using an epifluorescence microscope (Olympus) equipped with a digital camera.[29] The mean fluorescence intensity was obtained by using the ImageJ program taking into account the area of the culture under observation according to the magnification.

Expression of Zonula Occludens and Occludin by Quantitative RT-PCR. Caco-2 cells were grown in T75 flasks for 10−14 days until they formed a confluent, polarized monolayer. After removing the medium, cells were treated for 48 h with 500 μM INDO alone or with 66 μM (20 μg/mL) QUE, or with 66 μM QUE alone. Total RNA was extracted from cells using the GeneJet Whole Blood RNA Purification Mini Kit (Thermo Scientific, Fermentas) according to the manufacturer's instructions. RNA purity was spectrophotometrically checked (OD_{260}/OD_{280} nm, ratio ≥1.8). Triplicate samples of cells were collected from each treatment. After treatment with DNase (Promega, Madison, WI), total RNA (5 μg) was incubated with 1 μL of 500 μg/mL oligo $(dT)_{15}$ (Promega, Madison, WI), 1 μL of 10 mM dNTPs mix (10 mM each, dATP, dGTP, dCTP, and dTTP, Invitrogen, Promega), 4 μL of 5× first-strand buffer (Invitrogen), 2 μL of 0.1 M DDT (Invitrogen), and 1 μL of 40 U/μL RNaseOUT (0.5 μL) (Invitrogen) to synthesize single-stranded cDNA using 1 μL of 200 U/μL M-MLV reverse transcriptase (Invitrogen) in a total volume of 25 μL. The incubation was performed according to the manufacturer's indications for M-MLV reverse transcriptase. PCRs were carried out using Brilliant II SYBR Green qPCR master mix kit (Stratagene) according to the manufacturer's instructions using a Mx3000p Stratagene Real Time Thermocycler. Cycling conditions were as follows: 10 min at 95 °C, amplification for 45 cycles, with denaturation for 30 s at 95 °C, annealing for 1 min (60 °C for β-actin, used as housekeeping gene, 66 °C for ZO-1, and 68 °C for occludin), and extension for 30 s at 72 °C. The following primers were used:[30]

β-actin forward, 5′-ATTGCCGACAGGATGCAGAA-3′
β-actin reverse, 5′-AAGCATTTGCGGTGGACGAT-3′
ZO-1 forward, 5′-GAATGATGGTTGGTATGGTGCG-3′
ZO-1 reverse, 5′-TCAGAAGTGTGTCTACTGTCCG-3′
occludin forward, 5′-ATGAGACAGACTACACAACTGG-3′
occludin reverse, 5′-TTGTATTCATCAGCAGCAGC-3′

Samples were tested in triplicate and the average values were used for quantification using the $2^{-\Delta\Delta CT}$ method as previously described.[31]

Statistical Analysis. Data were analyzed using the GraphPad Prism 4 statistical software. Values represent the means of at least three independent experiments, each conducted in quadruplicate. Data were analyzed by ANOVA and posthoc Tukey's multiple comparison test. Values bearing different superscript letters were significantly different at $p < 0.05$.

RESULTS

Effect of Indomethacin on TEER in Caco-2 Cell Monolayers: Protection by Polyphenols. INDO time-dependently decreased TEER in Caco-2 cell monolayers. This change became statistically significant after 40 and 90 min of incubation, with decreased of almost 50%. QUE, RES, or EGCG totally protected against the decrease of TEER induced by INDO, while no protection was observed with RUT (Figure 1A). Lower concentrations of polyphenols also tested exerted a protective effect in a concentration-dependent manner; only the concentrations inducing the maximal effect of the polyphenol are shown.

Effect of Indomethacin on FD-4 Transport across Caco-2 Cell Monolayers: Protection by Polyphenols. INDO time-dependently increased the flux of FD-4 through Caco-2 cell monolayers. The decrease became significant after 30 and 90 min, with a 6 time increase of Caco-2 cell permeability to FD-4. QUE, RES, or EGCG totally protected against the alterations of permeability induced by INDO, while again, RUT failed to provide any protection (Figure 1B). As TEER and FD-4 transport were assayed in the same samples, a correlation was calculated between both parameters. As shown in Figure 1C, there is a strong negative correlation ($r = -0.9282$; $p < 0.0001$) between FD-4 permeability and TEER. Lower concentrations of polyphenols were tested, exerting a concentration-dependent protection; however, only those concentrations where the polyphenol effect was maximal are shown.

Effect of Indomethacin on ZO-1 Localization: Protection by Polyphenols. Polarized epithelial cells are joined together by the tight junction complex, which delimits the apical from the basolateral membrane. ZO-1 is localized in the cytoplasmic compartment of the TJs, at the points of cell-to-cell contact. In order to establish the implication of TJ integrity in the alterations of permeability induced by INDO in the Caco-2 cell monolayer, ZO-1 localization was assessed by immunofluorescence. As shown in Figure 2A,B, in control cells, ZO-1 formed a continuum between cells, which was homogeneously stained at the sites of contact [Figure 2A(a)]. The presence of 500 μM INDO or 40 μM rotenone altered the TJ distribution of ZO-1, which was detected as a punctate staining pattern [Figure 2A(b,c)]. INDO or rotenone decreased the fluorescence to 37 or 44% of the control, respectively (Figure 2B). This alteration was abolished by the presence of 66 μM (20 μg/mL) QUE, so that ZO-1 immunofluorescence was indistinguishable from that of control monolayers [Figure 2A(d,f),B]. On the contrary, 164 μM (100 μg/mL) RUT failed to exert a protective effect, showing a spotlike distribution of ZO-1 [Figure 2A(e),B]. Incubation times shorter than 48 h (4, 6, and 12 h) as well as INDO concentration of 250 μM for 48 h were also tested; however, at this lower concentration INDO did not cause any changes in the distribution of ZO-1 fluorescence, compared with control cells. As INDO concentration was doubled (compared with the concentration used in the TEER/FD-4 assay), QUE concentration was also doubled to evaluate its protective effect on further experiments.

Resveratrol (438 μM or 100 μg/mL) or a 10 times higher concentration of RUT (1640 μM or 1 mg/mL) was also tested, but when incubated with Caco-2 cells for 48 h, in the absence of INDO, these PPs induced the detachment of the cells, which made it impossible to carry out the experiments. When cells were incubated with 218 μM (100 μg/mL) of EGCG for 48 h in the absence of INDO, the cells developed a brown stain that masked the fluorescence under the microscope.

Effect of Indomethacin on ZO-1 and Occludin Expression: Protection by Polyphenols. As shown in Figure 3, 500 μM INDO decreased the expression of ZO-1 (Figure 3A) or occludin in Caco-2 cells (Figure 3B) to 25 and 20% of their basal levels, respectively. The presence of 66 μM (20 μg/mL) QUE totally protected against the decrease in the expression of these TJ proteins induced by INDO. QUE did not cause by itself any changes in the expression of the proteins.

DISCUSSION

Considering the high consumption of NSAIDs in the world (about 30 million people consuming them daily) and the high prevalence of adverse effects, particularly in the GI tract, there is considerable interest in elucidating the mechanisms underlying this toxicity and in evaluating strategies of prevention, for example, through the use of natural bioactive compounds with potential gastroprotective properties. Some of the cytotoxic events involved in the adverse effects of NSAIDs in the GI tract are linked to mitochondrial dysfunction, oxidative stress, and

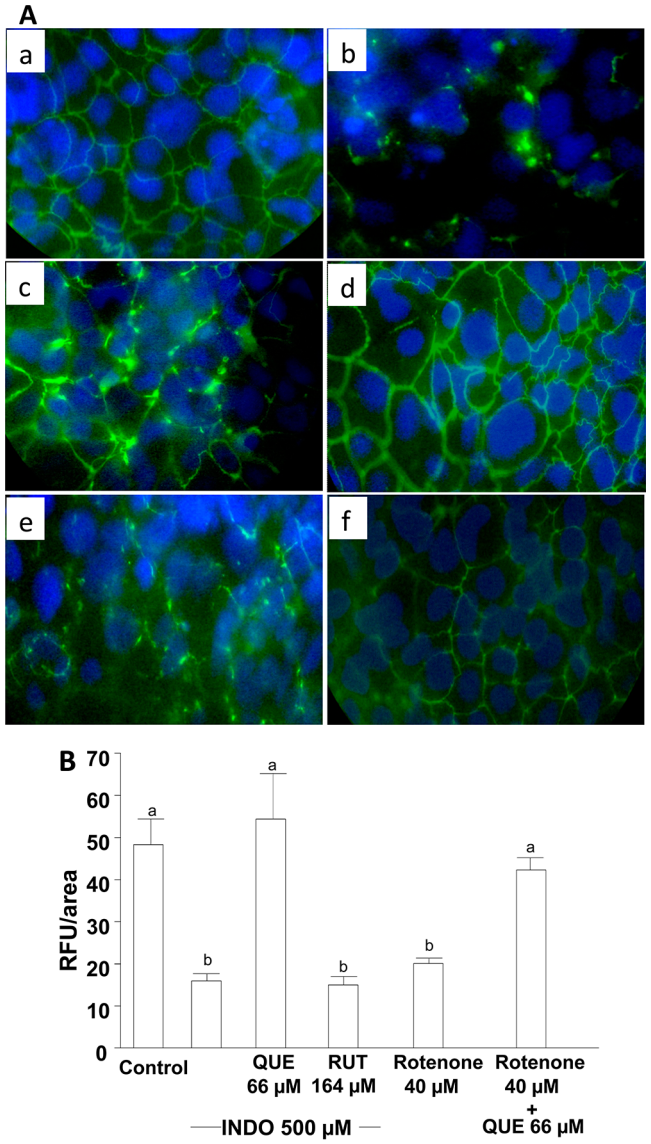

Figure 2. Effect of INDO and rotenone on the distribution of ZO-1 in Caco-2 cell monolayer: protection by polyphenols. (A) Cells were treated for 48 h with (a) control, (b) 500 μM INDO, (c) 40 μM rotenone, (d) 500 μM INDO + 66 μM (20 μg/mL) QUE, (e) 500 μM INDO + 164 μM (100 μg/mL) RUT, and (f) 40 μM rotenone + 66 μM (20 μg/mL) QUE. Magnification ×40. (B) Quantification of ZO-1 in Caco-2 cells. Data were analyzed by ANOVA ($P < 0.0001$) and Tukey's multiple comparison test. Values bearing different superscript letters were significantly different ($p < 0.05$).

apoptosis, as recently reported in Caco-2 cells and animal models.[2−6] However, the relationship between these cytotoxic events and the functional damage induced by NSAIDs, such as GI barrier dysfunction, has been poorly addressed. In the study reported here, we propose that mitochondrial dysfunction is a novel mechanism by which NSAIDs may alter the GI epithelium and its barrier function.

A major function of the intestinal epithelial cells is to provide a physical and functional barrier separating the intestinal lumen and the systemic compartment of the organism. This barrier function is mainly due to the TJs, which form a seal between the apical plasma membranes of adjacent enterocytes and block the penetration through the paracellular pathway of antigens, toxins, microorganisms, and LPS present in the intestinal lumen.[32] Degradation of the GI barrier function may facilitate the diffusion of potentially toxic luminal substances into the mucosa, initiating inflammatory processes and mucosal injury.[33−35] Available evidence suggests that alterations of this GI barrier function represent an important risk factor in the genesis of intestinal conditions such as Crohn's disease and ulcerative colitis, celiac disease, type 1 diabetes, NSAID-associated enteritis, and in the diarrheal syndromes caused by *Clostridium difficile*, *Vibrio cholerae*, and enteropathogenic *Escherichia coli*.[32,33,36,37]

The association between oxidative stress and alterations of the GI epithelium is well-known. Pro-oxidant molecules like H_2O_2 or free radicals such as the superoxide radical generated by the xanthine/xanthine oxidase system have been reported to alter the permeability of Caco-2 and T-84 cell monolayers,[14−18] widely used as in vitro models of intestinal barrier. In these studies, barrier function disruption has been evidenced by the increment of permeability to [^{14}C]inulin,[14] fluorescein,[17] or [^{3}H]manitol and by the decrease of TEER.[15,16,18] The pro-oxidant effect of INDO on epithelial cells has been demonstrated,[2,38,39] and it is possible that it may underlie the damage it induces to the epithelia.

Our results show that INDO increases Caco-2 permeability, as reflected by the decrease of TEER and the consecutive increase of FD-4 diffusion across the monolayer. These results confirm those reported by others in Caco-2 cells with the same NSAID.[40−42] In addition, these authors showed that pretreatment of Caco-2 with antioxidants inhibited the production of INDO-induced reactive oxygen species. They also suggest that the functional damage induced by INDO may rely on the oxidative stress induced by this NSAID; unlike our study, the effect of antioxidants molecules on INDO-induced permeability increase was not evaluated by the previously mentioned authors.

Among the proteins involved in the macromolecular complex forming the TJ, ZO-1, ZO-2, and ZO-3 are the major TJ-plaque proteins; ZO-1, which has been shown to directly bind to actin filaments and to the transmembrane TJ proteins occludin, plays a crucial role in maintaining the structure of the TJ and in regulating the permeability of the paracellular pathway of the epithelium and the barrier function.[43] It has been suggested that oxidative stress affects the integrity of the intestinal epithelium by promoting alterations (disassembling) of TJ proteins,[18,44,45] mainly of ZO-1,[18,45] and by affecting the integrity of the epithelial cells, especially through the oxidation of tubulin, a cytoskeleton protein.[46] However, oxidative stress not only alters TJ but also mitochondrial function. In fact, it has been reported that, in MDCK epithelial cell monolayers, the ATP depletion induced by antimicyn A (an inhibitor of mitochondrial complex III) alters the distribution of ZO-1 and occludin in TJ, without affecting their amounts, and leads to the formation of insoluble complexes that affect their interactions with the cytoskeleton proteins in the cytoplasm.[19,20] In glomerular epithelial cells, antimycin-induced ATP depletion also resulted in a rapid redistribution of ZO-1 from the cell membrane to the cytoplasm.[21]

In this study, we also observed that rotenone, an inhibitor of mitochondrial complex I, caused TJ disruption through ZO-1 delocalization, as evidenced by immunofluorescence. Additionally, we observed that INDO dramatically decreased the expression of ZO-1 and occludin. It is important to mention that we previously showed that INDO induces mitochondrial dysfunction with decreased concentrations of ATP and

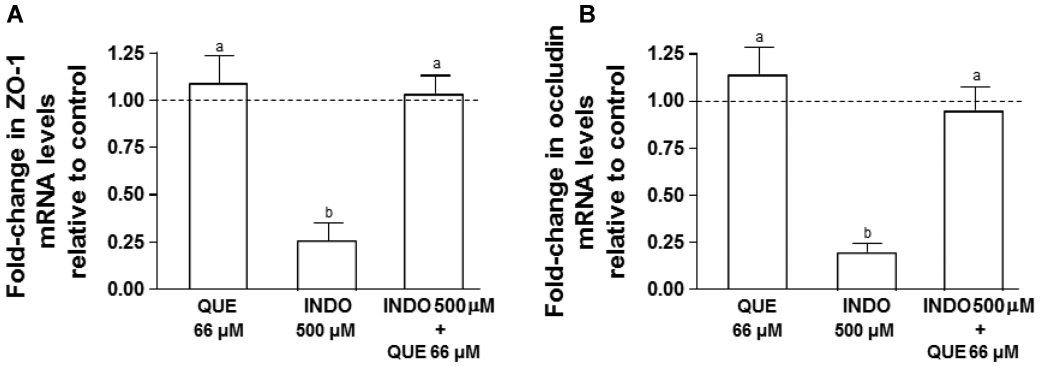

Figure 3. Effect of INDO on the expression of ZO-1 (A) or occludin (B) in Caco-2 cell monolayers: protection by polyphenols. Cells were treated for 48 h with control (expression = 1), 66 μM (20 μg/mL) QUE, 500 μM INDO, and 500 μM INDO + 66 μM (20 μg/mL) QUE. Target gene expression was normalized using β-actin as housekeeping gene. The absolute Ct (threshold) values from all the RT qPCR assays were used to calculate the mean fold change in target gene expression by the $2^{-\Delta\Delta CT}$ method. Data were analyzed by ANOVA ($P < 0.0001$) and Tukey's multiple comparison test. An asterisk represents a significant difference with respect to the control ($p < 0.05$).

decreased mitochondrial potential membrane, by inhibiting complex I activity.[3] As discussed above, any alteration in the ATP levels in epithelial cells as a consequence of mitochondrial dysfunction may affect TJ integrity and the GI barrier function. It is therefore possible that INDO, in addition to its oxidative promoting effects, may start TJ disassembly by inducing mitochondrial dysfunction and reducing ATP levels.[2,3,5] These alterations of TJ function could be explains by the decreased expression of ZO-1 and occludin.

Among all tested polyphenols, QUE was the most efficient in protecting the Caco-2 monolayer against the increase of permeability induced by INDO. Resveratrol and EGCG exerted the same protection as QUE when using around a 10 times higher concentration, which is clearly supraphysiological. RUT, even at the highest tested concentration, failed to protect against the damage induced by INDO, despite the fact that its antioxidant activity is similar to that of epicatechin, a flavanol closely related with EGCG.[2] The latter suggest that the effect exerted by the polyphenols against barrier function damage does not necessarily correlate with their antioxidant activities. The high concentrations tested were used only for comparative purpose and to highlight the efficacy of QUE against the damage induced by INDO. In fact, it is important to note that the concentrations of polyphenols in the human colon effectively tend to be lower than those currently used in our study, probably due to their hydrolysis by the commensal microbiota. For example, only 0.16−1.3 μM QUE or 0−0.12 μM RES were detected in fecal waters obtained from asymptomatic volunteers fed a standard diet (i.e., not enriched with polyphenol-rich foodstuffs).[47] In the present work, QUE at concentrations of 0.1 and 1 μM prevented almost by 10 and 40%, respectively, the increase of the permeability induced by INDO, while RES, however, failed to protect at a concentration of 0.1 μM (data not shown). In favor of QUE, we can argue that it can accumulate in the mitochondria and that the amount found in the colonic lumen may not reflect that occurring in the epithelial cells.[48] In addition, it is important to take into account that most of the damage induced by NSAIDs occurs at the proximal level of the gut (stomach/duodenum), where the concentrations of QUE or other polyphenols might be higher than in the colon, due to the much lower concentrations of bacteria at this level. Interestingly, dietary phenols are metabolized extensively in the GI tract by the microbiota into partly unknown compounds. In fact, intestinal bacteria play important roles not only in the flavonoid deconjugation but also in their further degradation, resulting in a high concentration of bacterial metabolites. For example, the ring fission affecting QUE and flavan-3-ols results in the formation of hydroxyphenylacetic acid and valerolactone, respectively, in amounts equivalent to 22 and 36% of intake of the rutinoside or EGCG-containing green tea.[49,50] In addition, the QUE released in the small intestine by cleavage of quercetin glucosides can be converted to glucuronide, sulfate, and methylated metabolites in the wall of the small intestine, but to a greatly lower extent that those formed after microbial degradation.[51] These metabolites may be absorbed in the colon and exert physiological effects after exerting local actions in the epithelial cells during their absorption. More studies are needed aimed at the biological relevance of the microbial degradation products of polyphenols, even more upon considering the high physiological concentration that they can reach in the colon with a common diet.[47]

It is important to note that QUE not only stands out for its free radical scavenging properties[2] but also for its mitochondrial-protecting activity.[5] In the present study, RUT did not protect against ZO-1 delocalization induced by INDO while QUE was effective. The latter might also be due to the fact that intracellular RUT is less available than QUE.[6] As we observed that QUE equally protects against the ZO-1 delocalization induced by either rotenone or INDO, we suggest that QUE can also protect against ZO-1 delocalization by preventing mitochondrial dysfunction, through the protection of complex I inhibition induced by INDO, in addition to its antioxidant effects. Supporting the antioxidant protection of QUE on TJ integrity, recently this flavonol was reported to protect ECV304 monolayers from TJ disruption and hyperpermeability induced by hydrogen peroxide by preventing the decreased expression of ZO-1 and occludin.[52] Our study shows, for the first time, the protective effect of PPs against molecules whose mechanism of damage induction involves mitochondrial dysfunction as well as oxidative stress. Therefore, molecules with mitochondrial-protecting capacities and antioxidant properties may be more efficient in protecting the integrity of the epithelium than those with only antioxidant properties. It is probable that the prevention by QUE of the decrease of ZO-1 and occludin expression induced by INDO resulted from its mitochondrial-protecting property. The beneficial effect of QUE on TJ proteins in the absence of any damaging agent was reported

previously by Amasheh et al.,[53] who found that QUE increased TEER of Caco-2 cell monolayers by increasing claudin-4. QUE was also reported to protect the integrity of HT-29/B6 cell monolayers disrupted by TNF-α, indicating that QUE exerts protective effects on cytokine-induced barrier damage.[54]

In conclusion, INDO altered the integrity of Caco-2 cell monolayer, as reflected by the decrease in TEER and the increase of FD-4 diffusion. Except for rutin, all the polyphenols tested protected against the disruption of the epithelium induced by this NSAID. INDO may alter the integrity of the epithelium by affecting TJ through a lower expression of ZO-1 and occludin and alterations of their localization, among other causes. As not all the polyphenols were able to protect against the functional damage induced by INDO, we propose that this damaging effect of the NSAIDs may not only be due to their ability to induce oxidative stress but also their capacity to induce mitochondrial dysfunction and subsequent ATP depletion. The latter is supported by the finding that QUE, a well-known mitochondrial protecting agent, was the most efficient polyphenol in protecting against the functional damage induced by INDO and by rotenone.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

EGCG, epigallocatechin gallate; FD-4, fluorescein isothiocyanate−dextran; GI, gastrointestinal; H_2O_2, hydrogen peroxide; INDO, indomethacin; NSAID, nonsteroidal anti-inflammatory drug; OP, optic density; PPs, polyphenols; QUE, quercetin; RFU, relative fluorescence unit; RES, resveratrol; RUT, rutin; TEER, transepithelial electrical resistance; TJ, tight junction; ZO-1, zonula occludens-1.

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