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Stimulation of cytosolic and mitochondrial calcium mobilization by indomethacin in Caco-2 cells: Modulation by the polyphenols quercetin, resveratrol and rutin

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ABSTRACT

Background: The effect of indomethacin (INDO) on Ca^{2+} mobilization, cytotoxicity, apoptosis and caspase activation and the potential protective effect of quercetin (QUE), resveratrol (RES) and rutin (RUT) were determined in Caco-2 cells.
Methods: Caco-2 cells were incubated with INDO in the presence or absence of QUE, RES or RUT. The concentrations of Ca^{2+} in the cytosol (Fluo-3 AM) and mitochondria (Rhod-2 AM) were determined as well as the cytotoxicity (MTT reduction and LDH leakage), apoptosis (TUNEL) and caspase-3 and 9 activities.
Results: INDO promoted Ca^{2+} efflux from the endoplasmic reticulum (ER), resulting in an early, but transient, increment of cytosolic Ca^{2+} at 3.5 min, followed by a subsequent increment of intra-mitochondrial Ca^{2+} at 24 min. INDO also induced cytotoxicity, apoptosis, and increased caspase activities and cytochrome c release. All these alterations were prevented by the inhibitors of the IP3R and RyR receptors, 2-Aminoethoxydiphenyl borate (2-APB) and dantrolene. QUE was the most efficient polyphenol in preventing Ca^{2+} mobilization induced by INDO and all of its consequences including cytotoxicity and apoptosis.
Conclusions: In Caco-2 cells, INDO stimulates ER Ca^{2+} mobilization, probably through the activation of IP3R and RyR receptors, and the subsequent entry of Ca^{2+} into the mitochondria. Polyphenols protected the cells against the Ca^{2+} mobilization induced by INDO and its consequences on cytotoxicity and apoptosis.
General significance: These results confirm the possibility of using polyphenols and particularly QUE for the protection of the gastroduodenal mucosa in subjects consuming NSAIDs.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for their antipyretic, analgesic and anti-inflammatory properties [1]. However, their chronic administration in humans is frequently associated with adverse effects, mainly in the gastrointestinal (GI) mucosa. Indomethacin (INDO)-induced GI damage in animals is a model widely used to evaluate the gastro-protective activity of drugs and bioactive compounds. During their absorption NSAIDs accumulate in the epithelial cells, where they may induce mitochondrial dysfunction [2]. This occurs because of the uncoupling of oxidative phosphorylation resulting in the decrease of ATP/ADP ratio, the dissipation of the mitochondrial membrane potential (MMP) [3–5] and the induction of mitochondrial permeability transition pores (mPTP) [6] that results in the release to the cytosol of cytochrome c from the mitochondrial intermembranous space; finally apoptosis occurs. However, mPTP opening occurs not only when the ATP/ADP ratio is depressed but also when mitochondrial Ca^{2+} levels are high [7,8]. Calcium mobilization provides the signal for a number of cellular events including apoptosis. However, Ca^{2+} modulation by NSAIDs as a possible underlying mechanism in apoptosis, mucosal restitution or cell renewal (phenomena where Ca^{2+} also plays a key role) has been poorly addressed [6].

Polyphenols (PPs) represent a ubiquitous group of secondary metabolites present 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 and anti-proliferative activities [9,10]. It

Abbreviations: 2-APB, 2-Aminoethoxydiphenyl borate; QUE, quercetin; RES, resveratrol; RUT, rutin; PPs, polyphenols; IP3R, inositol trisphosphate receptors; RyR, ryanodine receptors; EGTA, ethylene glycol tetraacetic acid; GI, gastrointestinal; INDO, indomethacin; MMP, mitochondrial membrane potential; NSAID, nonsteroidal anti-inflammatory drug; RFU, relative fluorescence unit; mPTP, mitochondrial permeability transition pore; ER, endoplasmic reticulum; DMSO, dimethylsulphoxide; SOCI, store-operated Ca^{2+} influx; SOCC, Store-Operated Ca^{2+} Channel; SERCA, Sarcoplasmic reticulum Ca^{2+} ATPase

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is noteworthy, for example, that a recent study failed to confirm the biological relevance of polyphenols in the prevention of cardiovascular diseases in humans when only their antioxidant property was addressed, concluding that the beneficial effects of PPs on cardiovascular health reside on a number of different biological properties [11]. The GI tract may be considered as the main site of action of PPs [12]. Quercetin (QUE), rutin (RUT) and resveratrol (RES) as well as some polyphenol-rich extracts have been shown to prevent oxidative stress, mitochondrial dysfunction and cell death induced by INDO in Caco-2 cells [4,5,13].

Based on these antecedents, the aim of this study was to evaluate the effect of INDO on the mobilization of cytosolic and mitochondrial Ca^{2+} and its repercussion on caspase-3 activation and apoptosis. The protecting effect of QUE, RES and RUT on the alterations of cytosolic and mitochondrial Ca^{2+} induced by INDO was also assessed as a possible mechanism underlying their protective effects against cell death induced by the NSAID.

2. Materials and methods

2.1. Chemicals

Indomethacin, QUE, RUT, RES, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and the protease inhibitor cocktail (4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, bestatin, leupeptin and aprotinin) were purchased from Sigma. Fluo-3 AM and Rhod-2 AM were provided by Molecular Probes (Eugene, OR); DeadEnd™ Colorimetric TUNEL System and CytoTox-ONE® Homogeneous Membrane Integrity Assay kit were from Promega (Madison, WIS, E.U.). Colorimetric caspase-3 substrate I and caspase-9 substrate II, were from Calbiochem. Cytochrome c was measured using a Cytochrome c Kit ELISA assay (Invitrogen, CA). All cell culture reagents were from Life Technologies (Grand Island, USA).

2.2. Cell culture conditions and study design

The human intestinal epithelial cell line Caco-2 was maintained in DMEM-F12 plus 10% fetal calf serum and cultured at 37 °C (5% CO2/95% air). Experiments were carried out using cells at near 90% confluence.

2.3. Measurement of cytosolic Ca^{2+} levels

Changes in cytosolic Ca^{2+} levels were measured as previously described [14,15] using Fluo-3 (dissolved in DMSO, 0.2% v/v in Ca^{2+}-free buffer) as Ca^{2+} indicator. Cells grown in 24-well plates were incubated at 37 °C for 30 min with 4 μM Fluo-3 AM (dissolved in DMSO, 0.2% v/v in Ca^{2+}-free HEPES). After washing, cells were treated with increasing concentrations (0–500 μM) or a fixed concentration (250 μM) of INDO in the absence or in the presence of the PPs: QUE (0.1, 1 or 10 μg/mL), RES (1, 10 or 100 μg/mL) or RUT (10, 100 or 1000 μg/mL). In some experiments, cells were also incubated in the presence of 2-APB (100 μM), dantrolene (50 μM), or 2-APB plus dantrolene to explore the receptors implicated in the INDO-induced Ca^{2+} mobilization. These treatments were conducted using Ca^{2+}-buffer (10 mM HEPES pH 7.4, 137 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 0.33 mM Na_2HPO_4 and 2 mM $CaCl_2$) or Ca^{2+}-free buffer (10 mM HEPES buffer (pH 7.4), 137 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 0.33 mM Na_2HPO_4 and 2 mM EDTA). The time-course of Fluo-3-Ca^{2+} fluorescence was measured at $488_{EX}/526_{EM,}$ using a Multi-Mode Microplate Reader (Synergy HT, BioTek). The values were normalized to the amount of protein, as determined by Bradford's assays and the results are expressed as F/F0.

2.4. Measurement of mitochondrial Ca^{2+} levels

The cationic Ca^{2+}-sensitive dye Rhod-2 AM (dissolved in DMSO, 0.2% v/v in Ca^{2+}-free buffer) was first incubated for 10 min in the presence of $NaBH_4$ to produce dihydro Rhod-2 AM, the membrane-permeable form of the dye which selectively accumulates in the mitochondria [15]. Cells grown in 24-well plates were incubated at 37 °C for 1 h in 100 nM dihydro-Rhod-2 AM. After washing, cells were treated with increasing concentrations (0–500 μM) or with a fixed concentration (250 μM) of INDO, in the absence or presence of QUE (0.1, 1 or 10 μg/mL), RES (1, 10 or 100 μg/mL), RUT (10, 100 or 1000 μg/mL). These treatments were conducted using Ca^{2+}-buffer or Ca^{2+}-free buffer. The fluorescence of dihydro Rhod-2-Ca^{2+} was measured along time at $552_{EX}/581_{EM}$, using the Synergy HT Microplate Reader. The values were normalized to the amount of protein as determined by Bradford's assay and the results are expressed as F/F0.

2.5. Cell death detection assay

Cells were plated in HCl-treated coverslips and grown until 90% confluent in 24-well plates. They were subsequently treated with 250 μM INDO or 20 μM rotenone for 1 h, in (an inhibitor of mitochondrial complex I) the absence or presence of 10 μg/mL QUE. Cell death was evaluated by terminal deoxynucleotide transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) using a commercial kit. Briefly, after washing, cells were fixed with 4% paraformaldehyde for 25 min at room temperature. Cell membranes were permeabilized using 0.2% Triton x-100 for 5 min. 3% H_2O_2 was then used to block endogenous peroxidase. The TUNEL reaction mixture was applied to the cells and incubated for 1 h. After incubation with streptavidin conjugated-horseradish peroxidase (HRP), diaminobenzidine was then used as chromogen and developing agent. Cells treated with culture medium without INDO were used as negative controls whereas cells treated with DNase I (RQ1 RNase-Free DNase, Promega, Madison, WI, USA, Cat. M6101) served as positive controls. Apoptosis was analyzed with a light microscope by counting the number of apoptotic (cell nuclei were stained in dark brown) and non-apoptotic cells present in twelve randomly selected fields per coverslip. Results are expressed as Apoptotic index (AI = ((number of apoptotic cells)/(number of apoptotic + non-apoptotic cells)) × 100).

2.6. Enzymatic assay for caspase-3-and caspase-9-like proteases

The catalytic activity of caspase-3 and caspase-9 was measured independently by using a commercial colorimetric assay according to the manufacturer's instructions (Calbiochem). The determination is based on the detection of the chromophore *p*-nitroanilide at 405 nm ($\varepsilon = 9160\ cm^{-1}\ M^{-1}$) after its caspase-3 or caspase-9 enzymatic cleavage from the labeled substrate DEVD- or LEHD-*p*-nitroanilide, respectively [16]. In brief, cells grown in 24-well plates were incubated at 37 °C for 1 h with 20 μM rotenone, 20 μM rotenone plus 10 μg/mL QUE, 250 μM INDO, 250 μM INDO plus 10 μg/mL QUE; 250 μM INDO plus 50 μM dantrolene; 250 μM INDO plus 100 μM 2-APB; 250 μM INDO plus 50 μM dantrolene plus 100 μM 2-APB. Cells were washed twice with cold PBS, lysed in extraction buffer containing 50 mM HEPES, 100 mM NaCl, 10 mM EDTA, 10 mM DTT, 0.1% CHAPS, pH 7.4 and centrifuged at 12,000 *g* at 4 °C for 10 min to recover the supernatant. Twenty micrograms of cytosolic protein extract was incubated at 37 °C in assay buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 10 mM EDTA, 10% glycerol) for 10 min to carry out the proteolytic reaction. Freshly prepared DEVD-pNA (200 μM) or LEHD-pNA (200 μM) for assessing caspase 3 or caspase 9 activity (as independent experiments), each was subsequently added to the mixture; the samples were mixed and their optic density was recorded at 37 °C every 30 min for a total of 4 h. Cells without drug treatment were