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RESEARCH ARTICLE

3, 4-Dihydroxyphenyl Acetic Acid, A Microbial Metabolite of Quercetin, Protects Intestinal and Pancreatic Beta Cell Lines from the Cytotoxicity Induced by Rotenone

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ABSTRACT

Quercetin (QUE) and its glycosylated derivatives are poorly absorbed in the intestine and accumulate in the gut lumen. In the colon, they are degraded by the microbiota, generating different metabolites including 3, 4-dihydroxyphenylacetic acid (DOPAC) as one of the most abundant. In this exploratory study, the cytoprotective effect of DOPAC against the cytotoxicity induced by rotenone, an inhibitor of complex I and an inducer of mitochondrial dysfunction, was determined in intestinal (Caco-2) and β- pancreatic (Min-6) cells. In a concentration-dependent manner, DOPAC protected the cell lines against the rotenone-induced cell death, as evaluated through the reduction of 3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide and Lactate Dehydrogenases (LDH) leakage. Moreover, DOPAC did not alter the epithelia integrity, assessed through transepithelial electrical resistance, and no cytotoxicity effect was observed when DOPAC was added to the basolateral compartment in a Caco-2 cell monolayer transwell-model. In consequence, the health-promoting effects of QUE in vivo could be due in part to the mitochondrial protective effect of its metabolite.

Introduction

The flavonol Quercetin (QUE), one of the most abundant polyphenol present in fruit and vegetables, is mainly found in its glycosylated forms which are few absorbable in the gastrointestinal tract and accumulate in the lumen. Intestinal bacteria from the colonic microbiota have an important role in the degradation of QUE and flavonoids in general. As a result of this degradation process, flavonoid bacterial metabolites are generated which can be absorbed through the colonic mucosa and exert biological effects in the organism. The major microbial metabolite of quercetin and its glycosylated derivatives is 3, 4 Dihydroxyphenylacetic Acid (DOPAC) [1-6]; this metabolite may be detected in plasma and in urine. For example, after standard diet, the mean concentration in urine is 4.2 µM in humans [7] and after quercetin-3-rutinoside supplementation (660 µmol/d for 7 days), 52 µmol/24 h can be excreted in urine in humans [8]. In addition, DOPAC among other 2 microbial-derived metabolites, showed the largest increment in 24 h urine after cocoa consumption, increasing its concentration in a 76%, 1.44 µM/24 h. In plasma,

DOPAC showed an increment of 10% after cocoa consumption, enhancing its concentration to 0.11 µM [9]. The present study was carried out to determine the in vitro cytoprotective properties of DOPAC in intestinal and endocrine pancreatic cells that could contribute to explain some of the health benefits associated with QUE intake, taking into account the low bioavailability of this compound.

Materials and Methods

Cell culture conditions and study design

The Human colonic adenocarcinoma cell line, Caco-2, and the murine β-pancreatic cell line, Min-6, were maintained in DMEM-F12 or DMEM-High glucose medium, respectively, supplemented with 10% Fetal Bovine Serum (FBS) at 37°C (5% CO2/95% air). Cells were cultured in 25 mL flasks in medium with rotenone (25 µM), a selective inhibitor of mitochondrial complex I, in the presence or absence of DOPAC (0.1-100 µM) for 120 min. Cell viability was assessed by the colorimetric detection at ʎ540nm of thiazolyl blue after 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) reduction and by the fluorimetric detection at Excitation ʎ560nm/Emission ʎ590nm of lactate deshidrogenase (LDH) release, using the CytoTox-ONE kit (Promega, Madison, WI, USA). The absorbance and fluorescence were measured with a Multi-Mode Synergy HT Microplate Reader (BioTek Instruments, Inc; USA).

To differentiate the apical and basolateral effect of DOPAC on the epithelial Caco-2 cells, these cells were also grown in transwell polycarbonate filters (diameter: 12mm; Pore size: 0.4 µm (Costar 3460, Corning Inc) in DMEM-F12 with 10% FBS and antibiotics at 37°C with 5% CO2. The basolateral and apical compartments were filled with 1.5 and 0.5 mL culture medium, respectively. The culture medium was changed three times per week and cells became confluent after 10–14 days culture. Transepithelial Electrical Resistance (TEER) was measured as described elsewhere using an ohm/voltmeter (EVOM, WPI) [10]. According to preliminary tests, cells were considered as confluent when basal TEER was higher than 600 Ω. The basolateral medium was replaced by culture medium without FBS and containing increasing concentrations of DOPAC (0-1000 µM). Cell viability was assessed by the fluorimetric detection of LDH release in the apical and basolateral compartment.

Statistical analysis Values represent the means of at least 3 independent experiments, each conducted in quadruplicate. Data were analyzed by using the Graph Pad Prism 4 statistical software by using ANOVA and Tukey's Multiple Comparison Test.

Results and Discussion

Rotenone acts as a selective inhibitor of the mitochondrial complex I, inducing a decrease of the ATP levels and promoting mitochondrial dysfunction and cell death [11].Our results confirm that rotenone significantly decreased cell viability in Caco-2 and Min-6 cells after 2 h incubation, as reflected by the decreased MTT reduction and the increased LDH leakage, compared with the control cells (Fig. 1A and 1B). DOPAC alone did not affect cell viability at the tested concentrations (data not shown). DOPAC is released in the colonic lumen from QUE metabolism by the microbiota and is subsequently absorbed across the colonic epithelium to the systemic circulation. In consequence, colonocytes may be exposed to DOPAC either on their apical or their basolateral sides. For this reason we evaluated the effect of DOPAC in Caco-2 cell monolayers grown on polycarbonate inserts, which allows accessing both sides of the epithelium. Our results indicate that DOPAC (up to 1 mM) does not induce cell toxicity in Caco-2 cells monolayer when added into the basolateral compartment (Fig. 2A and Fig. 2B) and that it does not alter monolayer TEER. (data not shown). As DOPAC is also the major metabolite of dopamine in the central nervous system, most of the studies evaluating the cellular effect of DOPAC have been carried out in culture of neuronal cells [12]; studies carried out in other type of cells are scarce. DOPAC has shown a potent anti proliferative effect on colonic cancer cell line HCT116, with an IC50 value of 90 µM after 24 h of incubation [13]. Skrbe et al. showed that the growth of HT29 colonic cells was decreased by 60% after 72 h of exposition to 100 µM

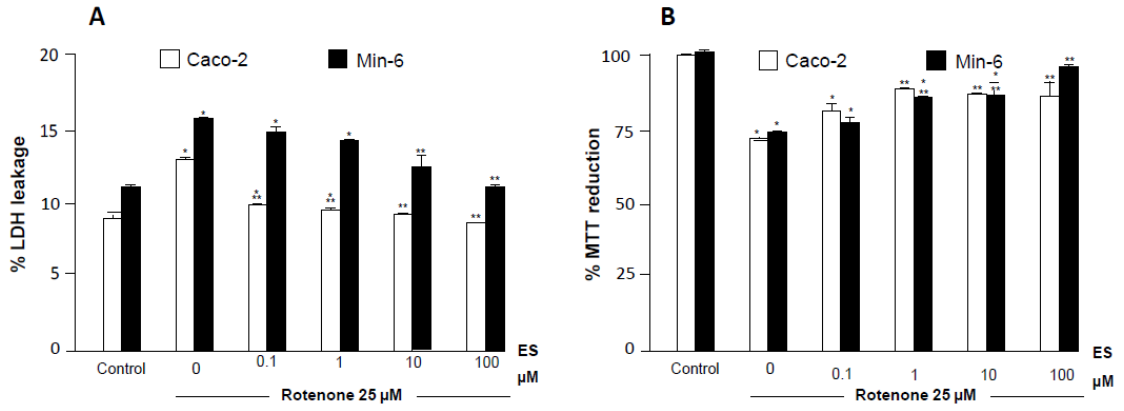

Figure 1: ES protects Caco-2 and Min-6 cells against the decrease of cell viability induced by rotenone.
Cells were incubated with rotenone 25 μM in the presence or in the absence of ES (0.1, 1, 10 or 100 μM) for 2 h, and cell viability was assessed through LDH leakage (Fig. 1A) and MTT reduction (Fig. 1B). The LDH leakage was expressed as percentage of the Triton X-100- induced maximum LDH release and was estimated as: $(RFU_{Experimental} \times 100)/RFU_{Maximum}$ LDH release. The changes in MTT reduction was expressed as percentage of the value of control (no-treated) cells and was estimated as: $OD_{Experimental} \times 100)/OD_{control\ cells}$(*distinct to control, $p < 0.05$; ** distinct to rotenone, $p < 0.05$).

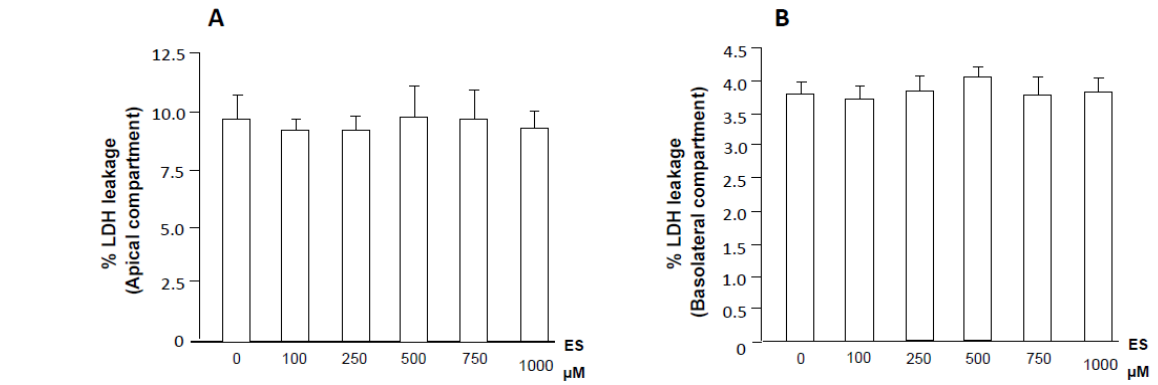

Figure 2: The presence of ES at the basolateral compartment does not affect the cell viability of Caco-2 cell monolayers.
Caco-2 cell monolayers were incubated with ES (100, 250, 500, 750 or 100 μM) for 2 h, and cell viability was assessed through LDH leakage towards apical (Fig. 2A) and basolateral compartment (Fig. 2B). The LDH leakage was expressed as percentage of the Triton X-100- induced maximum LDH release and was estimated as: $RFU_{Experimental} \times 100)/RFU_{Maximum}$.

DOPAC [14]. Such effect was reduced in the presence of catalase, suggesting the involvement of hydrogen peroxide (H_2O_2). Recently, DOPAC synthesized by an Aspergillus species isolated from marine alga was shown to decrease the viability of HeLa cells exposed to epidermal growth factor (EGF) by inhibiting EGF receptor phosphorylation/activation and the corresponding downstream signaling cascade; this effect was observed after 24 h of incubation with 300 ȝM DOPAC [15]. In these studies, the times of incubation were higher than that used in the present study. Our results indicate that DOPAC, at a concentration of 10 μM protected Caco-2 and Min-6 cells against the loss of viability induced by rotenone, as reflected by the total prevention of LDH leakage and decrease of MTT reduction. It is interesting that the protection exerted by DOPAC in Caco-2 cells occurred at concentrations (10 μM) physiologically observed in the colonic lumen. In fact, Jennner et al. determine the concentrations of different polyphenols and their bacterial metabolites in the fecal water of human volunteers fed a standard diet (i.e. not particularly high in polyphenol-rich foodstuffs) and they detected DOPAC in concentrations ranging between 0.47 and 16.4 μM [16]. It has also been reported that 40% of the QUE initially present in pig caecum contents is metabolized to DOPAC after 10 h

incubation [17]. DOPAC at the same concentration also protects the pancreatic Min-6 cells, suggesting that this molecule may exert its beneficial effects after its colonic absorption. Recently, we reported that DOPAC protected against cytotoxicity induced by cholesterol in Min-6 cells [18]. It is noteworthy that urinary DOPAC has been proposed as a marker of QUE intake in humans, with concentrations higher than 4 µM being detected in subjects consuming their normal diet.

The protective effect of DOPAC in the present model of cytotoxicity may rely in its free radical scavenging properties [17], since rotenone may affect cell viability by promoting the increment of the O_2•- status in the mitochondria, as a consequence of the inhibition of complex I [19]. Another possible mechanism should be through the ability of this metabolite to prevent the rotenone-induced inhibition of mitochondrial complex I, avoiding the production of O_2•- as we previously showed with QUE and a QUE-rich apple peel polyphenol extract [20].

In conclusion, DOPAC totally protected Caco-2 and Min-6 cell lines against the cell death induced by rotenone, a damaging agent of mitochondrial function and an inhibitor of complex I. In addition, DOPAC did not induce cytotoxicity in the basolateral side, nor in apical side of Caco-2 cell monolayer. Taking these preliminary results in consideration, the healthy promoting effects of QUE in vivo may be due in part to the antioxidant activity or/and the mitochondrial protective effect of its metabolite.

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