Chemico-Biological Interactions 195 (2012) 199–205

Contents lists available at SciVerse ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Differential protective effects of quercetin, resveratrol, rutin and epigallocatechin gallate against mitochondrial dysfunction induced by indomethacin in Caco-2 cells

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ARTICLE INFO

Article history:
Received 6 October 2011
Received in revised form 19 December 2011
Accepted 20 December 2011
Available online 27 December 2011

Keywords:
Quercetin
Rutin
Resveratrol
Epigallocatechin gallate
Mitochondrial dysfunction
Mitochondrial complex I

ABSTRACT

The beneficial effects of dietary polyphenols on health are due not only to their antioxidant properties but also to their antibacterial, anti-inflammatory and/or anti-tumoral activities. It has recently been proposed that protection of mitochondrial function (which is altered in several diseases such as Alzheimer, Parkinson, obesity and diabetes) by these compounds, may be important in explaining the beneficial effects of polyphenols on health. The aim of this study was to evaluate the protective effects of dietary polyphenols quercetin, rutin, resveratrol and epigallocatechin gallate against the alterations of mitochondrial function induced by indomethacin (INDO) in intestinal epithelial Caco-2 cells, and to address the mechanism involved in such damaging effect by INDO, which generates oxidative stress. INDO concentration dependently decreases cellular ATP levels and mitochondrial membrane potential in Caco-2 cells after 20 min of incubation. INDO also inhibits the activity of mitochondrial complex I and causes accumulation of NADH; leading to overproduction of mitochondrial $O_2^{\cdot -}$, since it is prevented by pyruvate. Quercetin (0.01 mg/ml), resveratrol (0.1 mg/ml) and rutin (1 mg/ml) protected Caco-2 cells against INDO-induced mitochondrial dysfunction, while no protection was observed with epigallocatechin gallate. Quercetin was the most efficient in protecting against mitochondrial dysfunction; this could be due to its ability to enter cells and accumulate in mitochondria. Additionally its structural similarity with rotenone could favor its binding to the ubiquinone site of complex I, protecting it from inhibitors such as INDO or rotenone. These findings suggest a possible new protective role for dietary polyphenols for mitochondria, complementary of their antioxidant property. This new role might expand the preventive and/or therapeutic use of PPs in conditions involving mitochondrial dysfunction and associated with increased oxidative stress at the cellular or tissue levels.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for their antipyretic, analgesic and anti-inflammatory properties [1]. However, the chronic administration of these agents is frequently associated with adverse effects, mainly in the gastrointestinal (GI) mucosa. Indomethacin (INDO)-induced GI lesions in animals are widely used as a model to evaluate the gastro-protective activity of drugs and bioactive compounds. NSAIDs accumulate in epithelial cells during their absorption and one of the mechanisms underlying their "topical" toxicity involves the induction of mitochondrial dysfunction [2]. INDO has been shown to uncouple oxidative phosphorylation, resulting in inhibition of oxygen consumption, dissipation of the mitochondrial membrane potential (MMP) and decrease of intracellular ATP [3–5]. Additionally, INDO induces oxidative stress by increasing reactive oxygen species (ROS) production and lipid peroxidation in Caco-2 and RGM-1 [5–7] cells as well as in the GI mucosa of rodents [3,4,8–10]. The gastrointestinal side effects of NSAIDs may be minimized through the administration of prostaglandin analogs and proton pump inhibitors [11]; however, these agents only diminish the clinical manifestations without inhibiting their causes. The administration of selective COX-2 inhibitors is

Abbreviations: QUE, quercetin; RES, resveratrol; RUT, rutin; EGCG, epigallocatechin gallate; PPs, polyphenols; DHE, dihydroethidium; EGTA, ethylene glycol tetraacetic acid; GI, gastrointestinal; INDO, indomethacin; MMP, mitochondrial membrane potential; NSAID, nonsteroidal anti-inflammatory drug; $O_2^{\cdot -}$, superoxide anion; ROS, reactive oxygen species; RFU, relative fluorescence unit; RLU, relative luminescence unit; ETC, electron transport chain.

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doi:10.1016/j.cbi.2011.12.007

limited by their high cost and by their increasingly recognized cardiotoxicity. Taking into consideration these side effects and the fact that NSAIDs are one of the most prescribed drugs in the world wide, it is important to develop new strategies to allow their use in a safer context.

Polyphenols (PPs), including flavonoids and stilbenes, represent an ubiquitous group of secondary metabolites present in fruits and vegetables and are therefore part of the average human diet. The interest in dietary PPs has increased considerably recently due to their antioxidant properties and their probable role in preventing pathologies associated with oxidative stress, such as tumoral, cardiovascular and neurodegenerative diseases [12,13]. In addition to their antioxidant properties, some dietary PPs also display antiviral, antibacterial, anti-inflammatory and anticarcinogenic activities [12,13]. However, most of the dietary PPs are found in plants in glycosylated forms which are poorly absorbed, favoring their accumulation in the GI tract which, in consequence, may be considered as their main site of action [14]. Due to their antioxidant properties, dietary PPs represent a promising alternative to protect the GI mucosa against the NSAID-induced side effects. Quercetin (QUE), rutin (RUT) and resveratrol (RES) have been shown to prevent oxidative stress and the gastric damage induced by INDO in rats [15–17]; similar results have also been reported with PP-rich extracts [18].

On the light of the above-mentioned studies, the aim of this study was to evaluate whether structurally different PPs: QUE (a flavonol abundant in onions), RUT (a quercetin-rhamnoglucoside found in tomatoes), RES (a stilbene present in grapes and red wine) and epicatechin gallate (EGCG) (a flavan-3-ol abundant in green tea) may prevent the mitochondrial dysfunction induced by INDO in Caco-2 cells.

2. Materials and methods

2.1. Chemicals

Indomethacin, QUE, RUT, RES, EGCG and the protease inhibitor cocktail (4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, bestatin, leupeptin and aprotinin) were from Sigma. DHE (dihydroethidium) and Mitotracker Red CMX-Ros® were purchased from Calbiochem and the CellTiter-Glo® luminescent cell viability assay kit from Promega (Madison, WIS, EU). 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% CO_2/95% air). Experiments were carried out using cells at near 90% confluence.

2.3. ATP quantification

Intracellular ATP levels were quantified in metabolically active cells using the CellTiter-Glo® kit. Cells were incubated with INDO (50–750 μM) in the presence or absence of each PP (10–1000 μg/ml) for 20 min, at 37 °C. Finally, 100 μl of CellTiter-Glo® reagent was added to each well and luminescence was recorded using a Multi-Mode Microplate Reader (SpectraMax M2, Molecular Devices). Results were expressed as% ATP = $(RLU_{Experimental} \times 100)/RLU_{control}$.

2.4. Mitochondrial membrane potential (MMP)

The MMP was assessed using CMX-Ros, which accumulates in the negatively charged mitochondrial matrix. Dye uptake is dependent upon the magnitude of the MMP ($530_{Ex}/590_{Em}$). Cells were incubated with 500 nM CMX-Ros in PBS for 30 min at 37 °C in the dark. After washing, cells were incubated with INDO (50–750 μM) in the presence or absence of each PP (10–1000 μg/ml) for 20 min, at 37 °C. Finally, cells were lysed with 0.03% Triton X-100 and the fluorescence was measured. Results were expressed as% MMP = $(RFU_{Experimental} \times 100)/RFU_{control}$.

2.5. Isolation of mitochondria

Mitochondria were isolated from Caco-2 cultured cells, as described by O'Donnell et al. [19]. Briefly, cells were harvested, washed with a Ca^{2+}/Mg^{2+}-free PBS and centrifuged (10 min; 500*g*; 4 °C). The pellet was resuspended and homogenized in a buffer solution (pH 7.4) consisting of 250 mM sucrose, 1 mM EGTA, 10 mM HEPES and 1 mg/ml BSA (fraction V). The homogenate was centrifuged (10 min; 1500*g*; 4 °C), the supernatant kept aside and the pellet re-extracted as above. Finally, the two supernatants were combined and centrifuged (10 min; 14,000*g*; 4 °C).

2.6. Complex I activity assay

Complex I activity was measured in isolated mitochondria through the changes of OD_{340nm} due to NADH oxidation ($\varepsilon = 6.81\ mM^{-1}\ cm^{-1}$), as described by Birch-Machin et al. [20]. Mitochondria isolated from Caco-2 were treated with INDO 250 μM in the absence or presence of rotenone (20 μM, a complex I inhibitor) [21] or QUE (10 μg/ml or ~30 μM) for 20 min. Complex I activity was measured in the treated, homogenized mitochondria, by using a potassium phosphate buffer solution (25 mM, pH 7.2) containing the protease inhibitor cocktail, 5 mM $MgCl_2$, 2 mM KCN, 2.5 mg/ml BSA, 65 μM coenzyme Q_1 and 2 μg/ml antimycin A. The mitochondrial homogenates were added to the reaction solution and incubated in the presence or absence of 20 μM rotenone (10 min; 30 °C), at 37 °C. The reaction was started by the addition of NADH (0.13 mM) and the rate of NADH oxidation was monitored at 340 nm for 3 min. Complex I activity was derived from the rotenone-sensitive NADH–ubiquinone oxidoreductase activity.

2.7. Complex II activity assay

The activity of complex II was quantified in INDO-treated mitochondria by the increase in 2,6-dichlorophenolindophenol (DCIP) reduction of at 600 nm ($\varepsilon = 19.1\ mM^{-1}\ cm^{-1}$), as described by Birch-Machin et al. [20]. The decrease in OD_{600nm} was recorded for an additional 1 min and used to calculate the activity of complex II.

2.8. Complex III activity assay

The activity of complex III was measured in INDO-treated mitochondria as the rate of reduction of cytochrome *c* (III) at 550 nm ($\varepsilon = 20\ mM^{-1}\ cm^{-1}$) by the reduced form of coenzyme Q_1 (ubiquinol $\varepsilon = 19.1\ mM^{-1}\ cm^{-1}$), as described by Birch-Machin et al. [20]. The assay was initiated by the addition of the mitochondrial homogenate in the presence or absence of 2 μg/ml antimycin A and the rate of reduction of cytochrome *c* was recorder for 1 min.

2.9. Superoxide radical production

The fluorogenic oxidation of DHE ($470_{Ex}/590_{Em}$) to ethidium was assessed to specifically measure $O_2^{\cdot -}$ production, as described by Benov et al. [22]. Mitochondria isolated from Caco-2 cells were incubated at 37 °C for 30 min with 10 μM DHE (dissolved in DMSO, 0.2% v/v in PBS). After washing, mitochondria were treated with

250 μM INDO in the absence or presence of 2 mM pyruvate for 20 min. The mitochondrial protein content was measured. DHE oxidation was expressed as RFU per milligram of protein.

2.10. Polyphenol determination by high-performance liquid chromatography (HPLC)

To rule out a possible direct interaction between INDO and the polyphenols, QUE (the PP with the highest protective effect in the current study) was incubated in the presence or absence of INDO and subsequently analyzed by RP-HPLC using an Agilent 1100 and a Lachrom instrument equipped with a 250 × 4.6 mm, 5 μm, Kromasil KR100-5C18 column (Eka Chemicals AB, Bohus, Sweden). Double distilled water containing 0.1% TFA, v/v (solvent A)) and 0.1% TFA in acetonitrile, v/v (solvent B) were used as solvents following the following gradient profile 0–25 min, 10–30% B; 25–30 min, 30–75% B; 30–35 min; 75–10% B at a flow rate of 1 ml/min. Detection was carried out at 280 nm using a diode array detector.

2.11. Statistical analysis

Data were analyzed using the GraphPad Prism 4 statistical software. The experiments were performed three times and in quadruplicate. Data were analyzed by ANOVA and Tukey's Multiple Comparison Test.

3. Results

3.1. Effects of polyphenols on the decrease of intracellular ATP induced by INDO

Fig. 1 depicts the effect of incubating Caco-2 cells with increasing concentrations of INDO on the intracellular levels of ATP (values are expressed relative to the baseline level). Exposure of the cells to INDO led to a concentration-dependent decrease in intracellular ATP levels; this effect became significant only after 20 min of incubation. Intracellular ATP levels were 62% and 28% of the control values after exposure to 250 and 750 μM of INDO, respectively.

The protective effect of PPs was addressed in cells exposed to 250 μM of INDO. Cells were simultaneously incubated for 20 min with increasing concentrations of each of the polyphenols QUE, RUT, RES and EGCG (from 0 to 1000 μg/ml) and INDO (Fig. 2). All the PPs tested, except EGCG, exerted a protective effect in a concentration-dependent manner against the decrease in ATP levels induced by INDO (Fig. 2D). QUE was the most efficient (Fig. 2A) as it was totally protective at a concentration of 10 μg/ml (30 μM) while RUT (Fig. 2B) and RES (Fig. 2C) attained a similar protective effect but at much higher concentrations: 1000 (1.5 mM) and 100 μg/ml (440 μM), respectively.

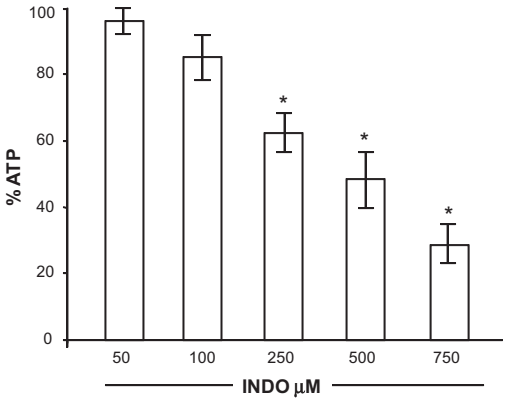

Fig. 1. Effects of INDO on cellular ATP levels. Cells were exposed to INDO (50–750 μM) for 20 min and ATP levels were measured. Control cells (cells treated neither with PPs nor INDO) correspond to 100% ATP level. The symbol * indicates a significant difference ($p < 0.05$) from the control. The experiments were performed three times and in quadruplicate. Values are expressed as mean ± standard deviation.

In another set of experiments, cells were simultaneously exposed for 20 min to increasing concentrations of INDO (250–750 μM) and to each PP at the concentration shown to be effective in preventing the decrease in ATP when confronted with 250 μM INDO. QUE (10 μg/ml) and RES (100 μg/ml) fully inhibited the drop in ATP levels induced by 250 or 500 μM INDO. ATP levels decreased to 32% with 750 μM INDO, while in the presence of QUE or RUT this decrease was only up to ~75% of the basal value. RUT (1 mg/ml), totally protected against the decrease in ATP levels induced by 250 μM INDO but failed completely to protect the mitochondria exposed to 500 or 750 μM INDO (Fig. 3).

3.2. Effects of polyphenols on the decrease in mitochondrial membrane potential induced by INDO

The alteration of MMP as an expression of the mitochondrial dysfunction induced by INDO, was also addressed. As shown in Fig. 4, a 20 min incubation with INDO decreased MMP in a concentration-dependent manner, the decrease becoming significant at 250 μM INDO. MMP values were 48% and 25% of the control values after exposure to 250 and 750 μM of INDO, respectively.

When the cells were treated with increasing concentrations of PP (from 0 to 1000 μg/ml) and INDO at the fixed concentration of 250 μM (Fig. 5), QUE, RUT and RES were shown to protect in a concentration-dependent manner against the decrease in MMP induced by INDO while EGCG was ineffective. QUE, RUT and RES were totally protective at concentrations of 10, 1000 and 100 μg/ml, respectively.

Cells were then simultaneously exposed to increasing concentrations of INDO (250–750 μM) and to fixed concentrations of QUE, RUT and RES and EGCG. QUE (10 μg/ml) and RES (100 μg/ml) protected against the drop in MMP induced by 250 and 500 μM INDO (Fig. 6). However, RUT (1000 μg/ml) protected only partially against the MMP-disturbing effect of 500 μM INDO. All these PPs tested protected by about 50% against the decrease in MMP induced by 750 μM INDO.

3.3. Quercetin protection against complex I alterations induced by INDO

The mechanism implicated in the mitochondrial dysfunction induced by INDO involves the capacity of this agent to alter the electron transport chain (ETC) [2]. The activities of complex I, II and III involved in the ETC were individually determined in mitochondria isolated from Caco-2 cells and treated with INDO (250 μM). INDO inhibited by 50% the activity of complex I (Fig. 7A) without affecting complexes II and III (data not shown). Complex I activity was measured as rotenone-sensitive NADH–ubiquinone oxido-reductase activity. Rotenone (20 μM) inhibited the NADH–ubiquinone oxido-reductase activity by almost 90%, indicating that under the assay conditions reported here, NADH oxidation largely reflects complex I activity (Fig. 7A). The inhibitory effects of INDO and rotenone on complex I were totally prevented by the addition of 10 μg/ml QUE for 20 min.

3.4. Effects of INDO on mitochondrial superoxide production

The inactivation of complex I has been proposed as an important cause of increased superoxide radical ($O_2^{\cdot -}$) production by mitochondria [21]. Fig. 7B depicts the increase in DHE oxidation,

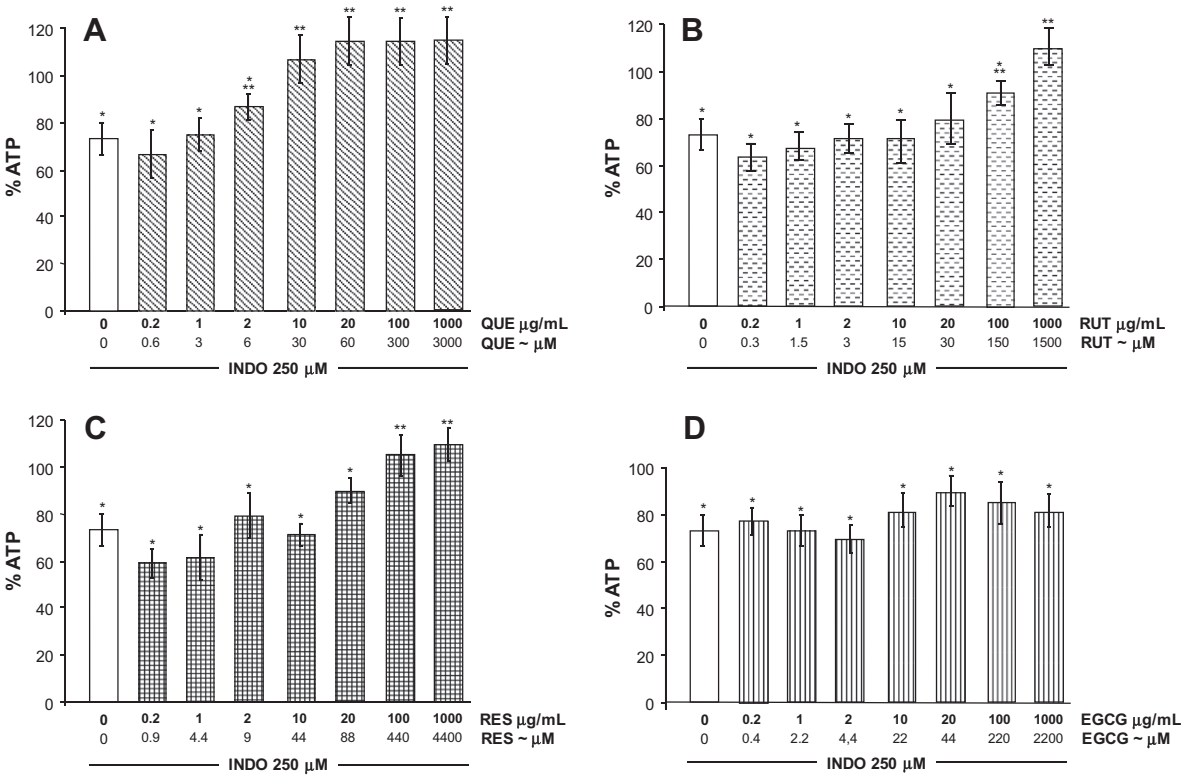

Fig. 2. Effects of increasing concentrations of polyphenols on the decrease of ATP levels induced by INDO. Cells were exposed – for 20 min – to INDO (250 µM) in the presence of increasing concentrations of polyphenols QUE (A), RUT (B), RES (C) and EGCG (D). Control cells (cells treated neither with PPs nor INDO) correspond to 100% ATP level. The symbol * indicates a significant difference ($p < 0.05$) from the control. The symbol ** indicates a significant difference ($p < 0.05$) from cells treated only with INDO. The experiments were performed three times and in quadruplicate. Values are expressed as mean ± standard deviation.

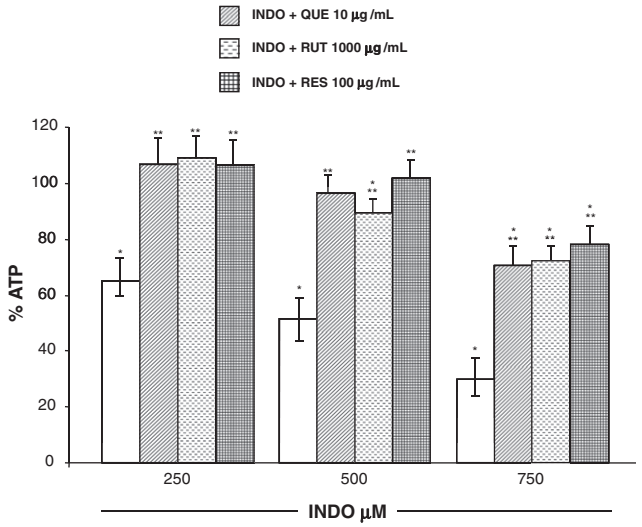

Fig. 3. Effect of a fix concentration of each polyphenol on the decrease of ATP levels induced by increasing concentrations of INDO. Cells were exposed – for 20 min – to INDO (250–750 µM) in the presence of QUE (10 µg/ml), RUT (1000 µg/ml), or RES (100 µg/ml). Control cells (cells treated neither with PPs nor INDO) correspond to 100% ATP level. The symbol * indicates a significant difference ($p < 0.05$) from the control. The symbol ** indicates a significant difference ($p < 0.05$) from cells treated only with INDO. The experiments were performed three times and in quadruplicate. Values are expressed as mean ± standard deviation.

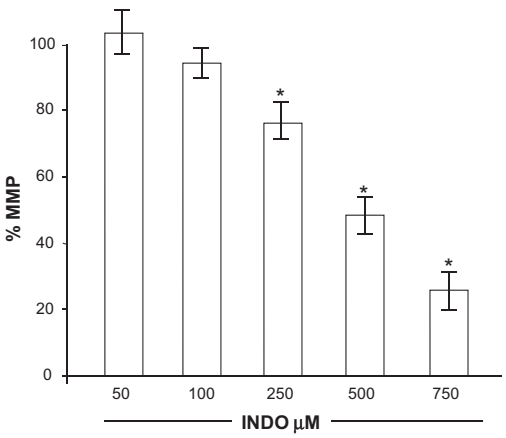

Fig. 4. Effects of INDO on MMP. Cells were exposed to INDO (50–750 µM) for 20 min and MMP were measured. Control cells (cells treated neither with PPs nor INDO) correspond to 100% MMP. The symbol * indicates a significant difference ($p < 0.05$) from the control. The experiments were performed three times and in quadruplicate. Values are expressed as mean ± standard deviation.

a superoxide-sensitive probe, after exposure of Caco-2 mitochondria to 250 µM INDO. As complex I inactivation results in the accumulation of NADH and in a higher NADH/NAD^+ ratio within the mitochondria [21], we evaluated the influence of decreasing this ratio on the mitochondrial production of $O_2^{\cdot -}$ (dependent on complex I) induced by INDO. For this purpose, we used pyruvate, a substrate of lactate dehydrogenase for the production of lactate, to decrease the NADH/NAD^+ ratio. As shown in Fig. 7B, the incubation of mitochondria with INDO in the presence of pyruvate completely abrogated the overproduction of $O_2^{\cdot -}$. This effect of pyruvate was not due to prevention of the inhibition of complex I induced by INDO, since pyruvate failed to protect against inhibition of mitochondrial of complex I by INDO (data not shown).

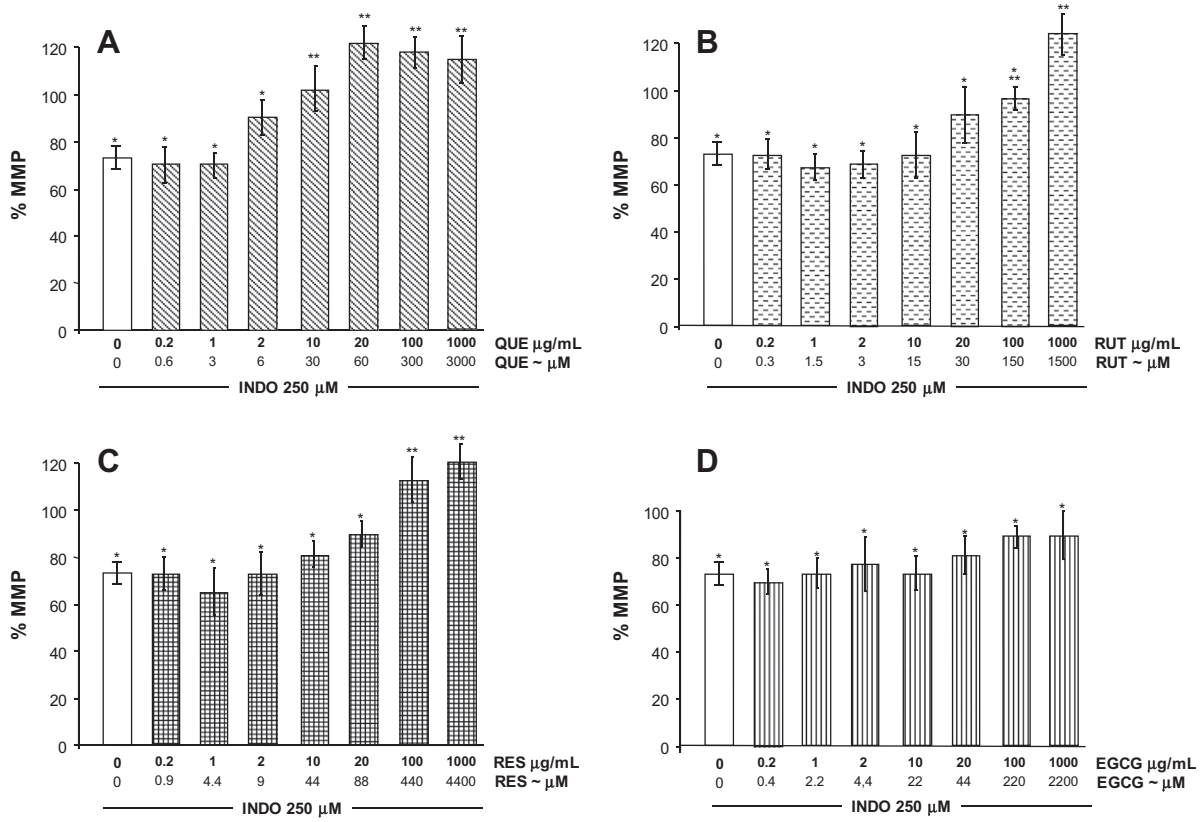

Fig. 5. Effects of increasing concentrations of polyphenols on the decrease of MMP induced by INDO. Cells were exposed – for 20 min – to INDO (250 µM) in the presence of increasing concentrations of polyphenols QUE (A), RUT (B), RES (C) and EGCG (D). Control cells (cells treated neither with PPs nor INDO) correspond to 100% MMP. The symbol * indicates a significant difference ($p < 0.05$) from the control. The symbol ** indicates a significant difference ($p < 0.05$) from cells treated only with INDO. The experiments were performed three times and in quadruplicate. Values are expressed as mean ± standard deviation.

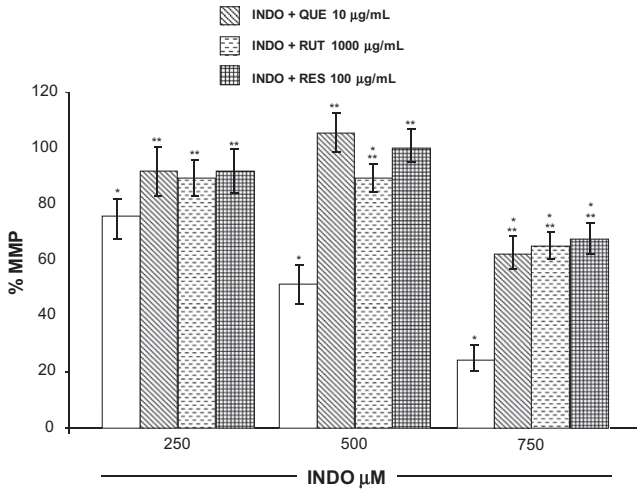

Fig. 6. Effect of a fix concentration of each polyphenol on the decrease of MMP induced by increasing concentrations of INDO. Cells were exposed – for 20 min – to INDO (250–750 µM) in the presence of QUE (10 µg/ml), RUT (1000 µg/ml), or RES (100 µg/ml). Control cells (cells treated neither with PPs nor INDO) correspond to 100% MMP. The symbol * indicates a significant difference ($p < 0.05$) from the control. The symbol ** indicates a significant difference ($p < 0.05$) from cells treated only with INDO. The experiments were performed three times and in quadruplicate. Values are expressed as mean ± standard deviation.

3.5. Possible direct reaction of INDO with QUE

In order to rule-out a direct interaction of INDO with PPs which might explain the protective effect of QUE against the mitochondrial dysfunction, QUE profiles were examined by reverse HPLC after incubation in presence or absence of INDO. No differences in HPLC profiles (retention times or peak areas) were observed under both conditions (data not shown).

4. Discussion

Given the high prevalence of GI adverse effects of NSAIDs and the fact that 30 million people consume them daily, the development of strategies to prevent their adverse effects, using natural bioactive compounds with potential gastroprotective effects is of particular interest. Some of the main cytotoxic events involved in the GI adverse effects of NSAID are linked to mitochondrial dysfunction and oxidative stress. A number of studies suggest that phenolic compounds with antioxidant activity play an important role in the prevention of oxidative damage occurring in GI cells and tissues [5,23–25]. However, the protective effect of PPs against NSAID-induced GI damage, beyond their direct antioxidant properties, has been poorly addressed. It is noteworthy that a recent study from ILSI-Europe failed to confirm the biological relevance of the direct antioxidant effects of polyphenols in the prevention of cardiovascular diseases in humans, concluding that the beneficial effects of PPs on cardiovascular health may reside in other biological properties, probably barely addressed to date [26].

The ETC couples the electron transfer between a donor, such as NADH, and an acceptor such as O_2 with the transfer of protons across the mitochondrial membrane, generating an electrochemical gradient indispensable for the synthesis of ATP. ETC alterations appear to be one of the mechanisms underlying the decrease of ATP induced by INDO; in fact, as shown in the present study, INDO

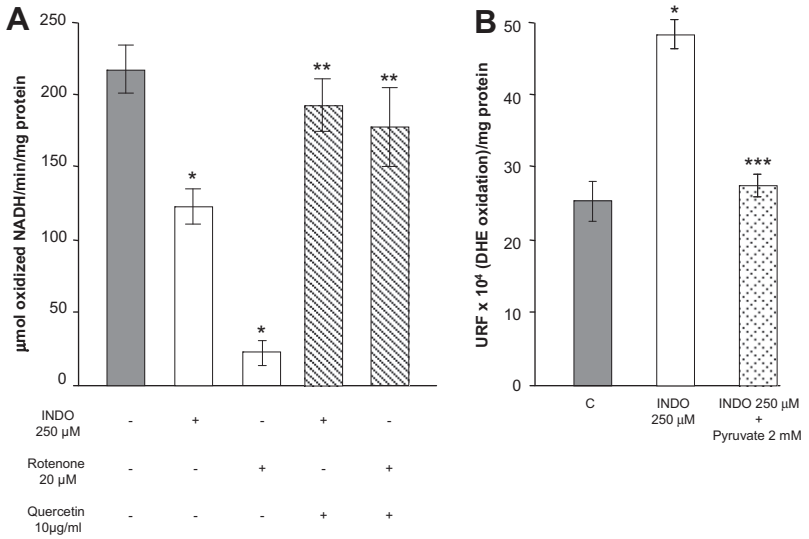

Fig. 7. Effect of QUE on complex I inhibition by INDO (A). Mitochondria isolated from Caco-2 cells were exposed to rotenone (20 μM) or INDO (250 μM) for 20 min in the absence or presence of QUE (10 μg/ml or 30 μM). Results are expressed as μmole of NADH oxidized per min per mg of protein. Effect of INDO on mitochondrial superoxide radical formation (B). Mitochondria isolated from Caco-2 cells were exposed to 250 μM INDO in the absence or presence of 2 mM pyruvate for 20 min. Results are expressed as RFU (corresponding to oxidation of DHE) per mg of protein. The symbol * indicates a significant difference ($p < 0.05$) from the control. The symbol ** indicates a significant difference ($p < 0.05$) between QUE-treated and QUE-untreated cells. The symbol *** indicates a significant difference ($p < 0.05$) from cells treated only with INDO. The experiments were performed three times and in quadruplicate. Values are expressed as mean ± standard deviation.

inhibits the activity of complex I. This effect is coherent with the decrease in intracellular ATP levels described in human cell lines treated with an inhibitor of complex I [28] or complex II [29]. As the MMP is generated by the electrochemical gradient produced by the ECT and its maintenance depends on ATP, it is likely that the inhibitory effects of INDO on complex I activity and ATP synthesis are the main mechanisms involved in the decrease of MMP induced by this agent. In fact, other ATP depleting agents, like cyanide and oligomycin, have been reported to decrease MMP in cultured hepatocytes, leading to cell death [30], and the glycolytic production of ATP from fructose has been shown to maintain the MMP and to prevent the cyanide-induced cell death [30].

Our results are in an apparent contradiction with those of Rezin et al. who observed the inhibition of complex II with INDO; however, these authors measured this parameter in homogenates of gastric mucosa from rats pre-treated for 6 h with 2.5 mg INDO [31] instead of isolated mitochondria. As the cytoplasm is considered as an extremely reducing media rich in NADPH [21] it is possible that the higher 2,6-dichloroindophenol reduction interpreted by these authors as an increase of complex II activity was only a consequence of the direct transfer of electrons from the cytosolic NADPH to the 2,6-dichloroindophenol present in the homogenates [32]. In our study, each complex activity was measured independently in a more specific manner and probably with fewer artifacts.

On the other hand, the inhibition of complex I could increase the availability of reducing equivalents (*i.e.*, NADH) in the mitochondria and the formation of redox active semiquinone intermediates within complex I, favoring a more "reduced" state and thereby facilitating the reduction of O_2 to $O_2^{\cdot -}$ [21]. In fact, in this study the basal production of mitochondrial $O_2^{\cdot -}$ doubled after a 20 min incubation with INDO, as reflected by increased DHE oxidation. The accumulation of NADH is supported by the observation that adding pyruvate prevents DHE oxidation, probably due to the fact that pyruvate decreases NADH concentrations when reduced to lactate [33]. On the other hand, the lack of effect of pyruvate on complex I inhibition (data not shown) suggests that INDO inhibits this activity through a mechanism independent of mitochondrial NADH availability, and that the increased production of mitochondrial $O_2^{\cdot -}$ induced by INDO is consequence and not cause of the increased NADH/NAD^+ ratio. These findings suggest that the mitochondrial dysfunction induced by INDO is an inducing factor for the subsequent oxidative stress at the cellular and tissue level.

The interest on dietary PPs has grown during the last decades due to the recognition of their antioxidant properties and their probable role in the prevention of a number of pathologies associated with oxidative stress [12,13]. Nevertheless, the bioavailability of these compounds is generally considered as low and they tend to accumulate in the GI tract, which has been proposed as their main site of action [14].

The partition coefficient (XLOGP3, a lipophilicity index) and topological surface area (TPSA, a polarity index) are considered as good predictors of molecule absorption [34]. According to the PubChem database, the XLOGP3/TPSA values for QUE and RUT (1.5/128 *vs* −1.3/266, respectively), indicate that the latter is more hydrophilic than QUE. In the intestinal lumen, the hydrophilic moiety of RUT, the disaccharide rhamnoglucoside must be removed to allow the absorption of QUE across the apical membrane of enterocytes. This process may be carried out in the colon by some bacterial populations with rhamnosidase activity [35]. This process does not occur *in vitro* and may explain the higher concentration of RUT required for protection of mitochondrial function.

The fact that EGCG failed to protect mitochondrial function despite its more favorable XLOGP3 and TPSA values (1.2/197) compared with RUT, and that the protective effect of RES was lower than that of QUE despite exhibiting the best XLOGP3 and TPSA conditions (3.1/60.7) of all the tested PPs, indicates that the ability to enter into the cell is a necessary but not sufficient condition for their biological activities. PPs must also enter the mitochondria side-stepping the electrochemical gradient between the inner membrane and inter-membrane space and have to remain inside the organelle to exert their protective effect, probably by interacting with the specific subunit of complex I targeted by INDO. As EGCG can enter into the cell without losing its galloyl moiety [35], it probably does not "fit" in the key site of complex I to induce a protective effect against rotenone or INDO inhibition. It is possible that QUE binds to complex I without acting as an inhibitor, thus protecting against the effects induced by rotenone and INDO [36]. The structural similarity between QUE, rotenone and INDO seems to be an important factor in determining the protective effect of this flavonoid. In fact, the stilbene RES, without flavonoid structure, exhibits a very weak protective effect despite its high lipophilicity. QUE

not only diffuses easily across biological membranes but also concentrates 100-fold in mitochondria [37]. The high lipophilicity of QUE, with its rotenone-like structure (flavonoid skeleton preserved without substituent groups), its high capacity for accumulating in mitochondria and its high affinity for the rotenone binding site in complex I (without inhibiting it) gave it exceptional characteristics for mitochondrial protection. A direct interaction between INDO and QUE which may result in a lower absorption of INDO into the enterocyte may be excluded as no changes in the chromatographic profile of the PP was detected after incubation with the NSAID. In conclusion, mitochondrial dysfunction represents at the cellular level launching factor of the oxidative stress that follows INDO exposure. Quercetin is the most efficient PP exerting a protective effect against mitochondrial dysfunction probably due to its ability to enter cells and accumulate in mitochondria. In addition, its structural similarity with rotenone could favor its binding to the ubiquinone site of complex I, protecting it from inhibitors such as INDO or rotenone. These findings suggest a new mitochondrial protective function for dietary PPs, independent of their antioxidant properties. This function expands the spectrum of preventive and/or therapeutic uses of PPs in pathological conditions where mitochondrial dysfunction is involved.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

The present study was supported by Fondecyt Postdoctoral Grant 3110177. We thank Dr. Oscar Brunser for its editorial assistance.

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