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Research Paper

Protection by apple peel polyphenols against indometacin-induced oxidative stress, mitochondrial damage and cytotoxicity in Caco-2 cells

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Abstract

Objectives Exposure of Caco-2 cells to indometacin can be a useful model to assess some of the cytotoxic events that appear to underlie the gastrointestinal lesions associated with the use of this anti-inflammatory agent. Using such a cellular model, we addressed here the cytoprotective potential of a recently standardized apple peel polyphenol extract, APPE.

Methods We firstly characterized APPE in terms of its free radical scavenging and antioxidant properties, and subsequently investigated its potential to protect Caco-2 cells against the deleterious effects of indometacin on cellular oxidative status (redox state, malondialdehyde, glutathione (GSH) and oxidized glutathione (GSSG) levels), mitochondrial function (ATP and mitochondrial membrane potential) and cell viability (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction and lactate dehydrogenase (LDH) leakage). For comparative purposes, the free radical scavenging properties and reducing capacity of quercetin, epicatechin and rutin were also estimated.

Key findings In the absence of APPE, indometacin induced mitochondrial perturbations (reducing ATP and the mitochondrial membrane potential), enhanced the oxidative status (decreasing the GSH/GSSG ratio and increasing dichlorofluorescein oxidation and malondialdehyde) and lowered the cell viability (decreasing MTT reduction and increasing LDH leakage). APPE, whether pre-added or co-incubated with indometacin, concentration-dependently prevented these mitochondrial, oxidative and cell viability alterations. Prompted by the recently recognized ability of indometacin to enhance the mitochondrial formation of reactive oxygen species, APPE was also characterized in terms of its free radical-scavenging capacity. APPE was found to actively scavenge $O_2^{\cdot -}$, HO· and peroxyl radicals. Such free radical-scavenging activity of APPE suggests that its ability to protect mitochondria and prevent the oxidative and lytic damage induced by indometacin arises from its potent antioxidant capacity.

Conclusions In Caco-2 cells APPE prevented mitochondrial oxidative and cell viability alterations induced by indometacin possibly through its ability to scavenge reactive oxygen species. These findings are of interest in view of the high prevalence of gastrointestinal side-effects associated with the use of conventional anti-inflammatory agents.

Keywords apple peel polyphenols; indometacin; mitochondrial dysfunction; oxidative stress

Introduction

Indometacin is one of the most widely used nonsteroidal anti-inflammatory drugs (NSAIDs). Unfortunately, however, its use is frequently associated with the development of erosions and ulcers in the gastrointestinal tract.[1,2] Among several mechanisms likely to be involved in the development of such lesions,[3] the inhibition of cyclooxygenase 1 (COX-1) has been frequently considered.[4] However, mechanisms other than (or additional to) COX-1 inhibition,[5] such as the ability of indometacin to promote apoptosis[6,7] or to induce mitochondrial dysfunction[1,3,7] are also likely to be involved. Regarding its mitochondria-perturbing effect, indometacin has been shown to uncouple the oxidative phosphorylation, inhibiting mitochondrial oxygen consumption,[1,3,8] dissipating mitochondrial membrane

potential (MMP)[7,8] and diminishing intracellular ATP.[8] Studies carried out in various cell lines[9] and in rodents[1,2,7,10,11] reveal an association between the oxidative stress and the cell damaging effects of indometacin. In fact, indometacin has been shown in rodents to increase the production of reactive oxygen species (ROS), such as superoxide ($O_2\cdot^-$) and hydroxyl radicals (HO·),[2,7] to induce lipid peroxidation,[1,2,10,11] and to decrease glutathione in gastrointestinal mucosa.[2,7] Besides increasing ROS production, indometacin decreases *in vivo* the activity of antioxidant enzymes such as catalase,[10] glutathione peroxidase[10,12] and superoxide dismutase.[10,12] In view of the putative role of oxidative stress in the genesis of the cytotoxic effects of indometacin, molecules with antioxidant properties, such as curcumin,[2] rebamipide[9] and melatonin[11] have all been evaluated as possible gastrointestinal-protective agents.

Besides single antioxidant molecules, an extract containing polyphenols, obtained from whole Annurca apple, has been shown *in vitro* to protect MKN28 cells against the cytotoxic effects of indometacin and *in vivo* to prevent the injury induced by this agent[13] and by aspirin[14] to the rat gastric mucosa. The use of extracts containing polyphenols to prevent the gastrointestinal effects of NSAIDs emerges as an interesting strategy since some polyphenols have anti-inflammatory properties.[15] Apples, which represent a major dietary source of polyphenols,[16] concentrate these compounds in the peel.[17] Recently, using the widely consumed Granny Smith variety, we prepared an apple peel extract (APPE) that, presenting a total polyphenols (TP) content a hundredfold higher than that of whole apple, exhibits a polyphenolic profile identical to that of the fresh apple peel used for its preparation.[18] The composition of APPE has been previously characterized through HPLC-DAD/FL and LC-MS analysis.[19]

Prompted by the accumulated evidence on the possible role of oxidative stress in the cytotoxicity induced by indometacin, in this study we firstly characterized APPE in terms of its free radical scavenging and antioxidant properties, to subsequently investigate its potential to protect Caco-2 cells against the deleterious effects of indometacin on cellular oxidative status (redox state, malondialdehyde (MDA), glutathione (GSH) and oxidized glutathione (GSSG) levels), mitochondrial function (ATP and MMP), and cell viability (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction and lactate dehydrogenase (LDH) leakage). This work reveals for first time that even before 60 min of exposure, indometacin induces mitochondrial and oxidative perturbations that lead to the loss of cell viability. A discussion on the possible mechanisms underlying these early cytotoxic effects of indometacin and the ability of APPE to afford cytoprotection against them is presented.

Materials and Methods

Chemicals

Fluorescein sodium salt, 2-deoxy-D-ribose, 2,4,6-tris(2-pyridyl)-S-triazine (TPTZ), L-ascorbic acid, xanthine oxidase, 2-thiobarbituric acid, indometacin, reduced and oxidized glutathione, MTT, (–)-epicatechin, rutin and quercetin were from Sigma (St Louis, USA). 2′,7′-Dichlorofluorescein diacetate (DCFD), 1,1,3,3-tetramethoxypropane and 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) were from Aldrich (St Louis, USA). Dihydroethidium (DHE) and Mitotracker Red CMX-Ros were from Calbiochem (San Diego, USA) and Molecular Probes (Eugene, USA), respectively. CytoTox-ONE Homogeneous Membrane Integrity Assay kit and CellTiter-Glo luminescent cell viability assay kit were from Promega (Madison, USA).

Preparation and polyphenol content of apple peel extract

APPE was prepared from Granny Smith apple peel and its polyphenolic composition confirmed as in Pastene *et al*.[18,19] TP amounted to 600 mg of gallic acid equivalents (GAE) per gram of dry weight. Flavonoids, accounting for about 60% of TP, were primarily quercetin and its glycosides. Flavan-3-ols, comprising 5% of TP, mostly consisted of epicatechin. Procyanidins accounted for 24% of TP.[18,19]

Determination of the free radical scavenging and ferric-reducing properties of apple peel extract

The free radical scavenging properties of APPE were addressed in terms of its ability to scavenge peroxyl, $O_2\cdot^-$ and HO·. To assess its peroxyl radical scavenging property, the ORAC (oxygen radical absorption capacity) assay was applied,[20] which measures the ability of the extract to react with AAPH-derived peroxyl radicals, using fluorescein as probe ($485_{Ex}/520_{Em}$) and Trolox as standard. To assess the superoxide scavenging activity of APPE, $O_2\cdot^-$ anions, generated by the hypoxanthine/xanthine oxidase system, were monitored by measuring the oxidation of DHE ($470_{Ex}/590_{Em}$). To assess the HO· radical scavenging property of APPE, the radicals were generated through an ascorbate-driven Fenton reaction and assayed using deoxyribose as substrate. Deoxyribose oxidation was assessed fluorometrically through formation of thiobarbituric acid reactive substances (TBARS; $532_{Ex}/553_{Em}$). The ferric-reducing antioxidant potential (FRAP)[21] of APPE was assessed as a form of quantifying its antioxidant capacity. The FRAP method is based on the reduction of the Fe^{3+}–TPTZ complex. The change in optical density at 593 nm (OD_{593nm}) was assayed after 3 min of reaction. For comparative purposes, the free radical scavenging properties and FRAP capacity of quercetin, epicatechin and rutin were also estimated.

Cell culture conditions and study design

The human colonic adenocarcinoma cell line, Caco-2, was used; these cells were maintained in a DMEM-F12 plus 10% fetal bovine serum medium, and cultured at 37°C (5% CO_2–95% air). Non-differentiated cells, at near 90% confluence, were plated at 10^5 cells/ml. The protective potential of APPE against the alterations in cell viability (MTT and LDH assays), oxidative stress (cellular redox status, lipid peroxidation, intracellular glutathione) and mitochondrial function (ATP quantification and MMP assessment) induced by indometacin was assessed in experiments carried out under both pre-incubation and co-incubation conditions. Details on the design of these two experimental conditions are provided

below and in the Results section, during the description of the corresponding results.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction

Cell viability was measured by the MTT assay, through cells' ability to reduce thiazolyl blue (OD_{540}). Cells were treated with APPE for 90 min, before their exposure to indometacin for 60 min. Cell viability (as percentage) was estimated as: $(OD_{Experimental} \times 100)/OD_{Maximum\ of\ control}$.

Lactate dehydrogenase release

Except for those experiments in which cell viability was assessed using MTT, all others were conducted under conditions in which cells were either pre-incubated with APPE for 90 min (pre-incubation experiments) before exposing (for 60 min) the cells to indometacin, or co-incubated by the simultaneous addition of APPE and indometacin to the cells, followed by a 60-min incubation (co-incubation experiments). LDH leakage, used as marker of cell toxicity, was assayed in cell supernatants using the CytoTox-ONE kit ($560_{Ex}/590_{Em}$). Cell injury (as percentage) was estimated as: $RFU_{Experimental} \times 100)/RFU_{Maximum\ LDH\ release}$.

Cellular redox status

Oxidative stress was assessed measuring the conversion of DCFD into a fluorescent dye. Plated cells were incubated with APPE for 90 min and with 50 μM DCFD for an additional 30 min. Finally, cells were exposed to indometacin for 60 min and fluorescence ($485_{Ex}/530_{Em}$) measured. DCF oxidation (as percentage over basal value) was estimated as: $RFU_{Experimental} \times 100)/RFU_{basal}$.

Determination of lipid peroxidation

Lipid peroxidation was assayed by measuring MDA in cell lysates after reaction with thiobarbituric acid. MDA was separated using an Agilent Eclipse XDB-C18 column (5 μM, 4.5 × 150 mM), eluted (0.6 ml/min) with a methanol–25 mM phosphate buffer, pH 6.5 (1 : 1, v/v) mixture and detected at $532_{Ex}/553_{Em}$. 1,1,3,3-Tetramethoxypropane was used as control standard.

Measurement of intracellular reduced and oxidized glutathione

GSH and GSSG were assayed at pH 8.0 and pH 12, respectively, using *O*-phthaldehyde as substrate ($350_{Ex}/420_{Em}$).

ATP quantification

Intracellular ATP levels were quantified by luminescence using a CellTiter-Glo kit. Results are expressed as: % ATP = $(RLU_{Experimental} \times 100)/RLU_{basal}$.

Mitochondrial membrane potential assessment

MMP was quantified using CMX-Ros, a cationic lipophilic fluorochrome ($530_{Ex}/590_{Em}$), which accumulates (500 nM for 30 min) in the mitochondrial matrix. Results are expressed as: % MMP = $(RFU_{Experimental} \times 100)/RFU_{basal}$.

Statistics

Data were analysed using the GraphPad Prism 4 statistical software. Values represented the means of at least three independent experiments, each conducted in quadruplicate. Data was analysed by analysis of variance and Tukey's Multiple Comparison Test.

Results

Free radical scavenging properties and ferric-reducing capacity of apple peel extract

Before evaluating the potential of APPE to protect Caco-2 cells against the oxidative damage and mitochondria- and cell-damaging effects induced by indometacin, we characterized APPE in terms of its free radical scavenging and antioxidant properties. The ability of APPE to scavenge peroxyl radicals, assessed here through the ORAC assay, reflects the capacity of the components of this extract to donate a hydrogen atom to such radicals. Table 1 compares, on a per GAE basis, APPE with quercetin, rutin and epicatechin; the three latter flavonoids, which occur in fresh apple peel,[18,19] have been shown to exhibit high ORAC values.[22] APPE exhibited an ORAC value that was half that of pure quercetin and almost identical to that of rutin. However, when compared with epicatechin, APPE was found to have only one-third of its peroxyl radical scavenging activity. Towards $O_2\cdot^-$ radicals, APPE exhibited a scavenging activity almost identical to that of epicatechin and rutin, but only two-thirds that of quercetin (Table 1). In the case of HO· radicals, APPE showed as much scavenging activity as epicatechin, one-third that of rutin and only one-quarter that of quercetin (Table 1). Regarding the antioxidant capacity of APPE, evaluated through its capacity to reduce ferric iron (FRAP assay), Table 1 also shows that APPE was as active as epicatechin, slightly more active than rutin and around ten-fold more active than quercetin.

Effect of apple peel extract on indometacin-induced cell toxicity in Caco-2 cells

MTT reduction and LDH leakage were assessed in cells pre-exposed to indometacin for 60 min. The possible protecting effect of APPE against indometacin-induced cytotoxicity was assessed either in cells pre-treated for 90 min with APPE (whose APPE-containing medium was subsequently removed) before indometacin treatment (pre-incubation experiments) or in cells to which APPE and indometacin were added simultaneously and incubated for 60 min (co-incubation experiments). Table 2 shows the percentages of MTT reduction and LDH leakage in cells exposed to indometacin alone, and in indometacin-exposed cells that had previously been treated with APPE. Exposure of cells to 50 and 250 μM indometacin led, respectively, to a 12% and a 62% decrease in the capacity to reduce MTT, and to a 12% and 17% increase in the leakage of LDH into the medium. Pre-exposure of cells to APPE prevented, concentration-dependently, the loss of MTT reduction capacity and the increase in LDH leakage induced by indometacin. At 50 μg/ml, APPE largely prevented the loss of cell viability induced by 250 μM indometacin and totally abolished that induced by 50 μM of indometacin. In co-incubation

Table 1 Free radical scavenging capacity and ferric-reducing capacity of apple peel extract

	Scavenging capacity			Ferric-reducing capacity
	Peroxyl radicals (mmol TE/g GAE)	Superoxide radicals (% protection against DHE oxidation)	Hydroxyl radicals (% protection against deoxyribose oxidation)	μmol $FeSO_4$/mg GAE
APPE	11 ± 2.3[a]	63 ± 3.5[a]	25 ± 6.5[a]	8.5 ± 0.3[a]
Rutin	12 ± 2.8[a]	63 ± 3.9[a]	75 ± 4.4[b]	6.2 ± 0.5[b]
Quercetin	24 ± 2.1[b]	100 ± 4.2[b]	100 ± 5.7[c]	0.9 ± 0.2[c]
Epicatechin	38 ± 3.2[c]	64 ± 5.0[a]	27 ± 3.9[a]	8.4 ± 0.6[a]

The ability of apple peel polyphenol extract (APPE) to scavenge peroxyl radicals was assessed through the ORAC assay. Results are expressed as millimoles of TE (trolox equivalents) per mg of GAE (gallic acid equivalents). The ability of APPE to scavenge $O_2^{\cdot -}$ or HO· radicals was assessed through DHE and deoxyribose oxidation, respectively. Results are expressed as percentage of protection against dihydroethidium (DHE) or deoxyribose oxidation. Values from assaying the ferric-reducing capacity of APPE represent μmoles of Fe^{2+} equivalents generated per mg of GAE. Values represent the mean ± SD, $n = 3$. Values bearing different superscript letters are significantly different ($P < 0.05$).

Table 2 Protective effects of apple peel extract on indometacin-induced loss of cell viability and oxidative stress in Caco-2 cells

		Cytotoxicity			Oxidative stress		
		Pre-incubation		Co-incubation	Pre-incubation		
Indometacin (μM)	APPE (μg/ml)	MTT reduction (% of control)	LDH leakage (% over basal value)	LDH leakage (% over basal value)	DCFD oxidation (% over basal value)	MDA level (% over basal value)	GSH/GSSG
0	0	100 ± 2.2[a]	1.0 ± 0.7[a]	1.0 ± 0.7[a]	3.1 ± 2.7[a]	2.1 ± 1.3[a]	26.1 ± 5.2[a]
50	0	88 ± 6.3[b]	13.1 ± 1.5[b]	13.1 ± 2.0[b]	32.3 ± 5.5[b]	63.0 ± 4.1[b]	–
	0.05	–	–	4.6 ± 0.9*[†]	–	–	–
	0.1	91 ± 5.8[†]	7.5 ± 1.0*[†]	4.0 ± 1.3*[†]	4.0 ± 2.9*	12.0 ± 3.3*[†]	–
	0.25	94 ± 6.1*	7.0 ± 1.8*[†]	–	3.0 ± 2.9*	10.0 ± 2.9*[†]	–
	5	100 ± 4.3*	3.0 ± 0.9*[†]	3.0 ± 1.0*[†]	–	–	–
	50	100 ± 6.2*	1.5 ± 1.3*	2.5 ± 2.3*	4.0 ± 3.2*	5.0 ± 3.0*	–
	100	–	–	–	4.8 ± 4.7*	4.0 ± 3.2*	–
250	0	38 ± 8.3[c]	18.3 ± 2.2[c]	18.3 ± 2.5[c]	75.3 ± 3.7[c]	100.0 ± 6.2[c]	14.1 ± 5.5[b]
	0.05	–	–	5.8 ± 2.9*[†]	–	–	–
	0.1	45 ± 6.5[†]	8.0 ± 1.9*[†]	5.7 ± 1.8*[†]	4.0 ± 3.3*	13.0 ± 4.3*[†]	18.3 ± 4.8*[†]
	0.25	55 ± 7.1*[†]	6.8 ± 1.3*[†]	–	3.0 ± 2.7*	12.0 ± 3.9*[†]	–
	5	73 ± 8.0*[†]	4.0 ± 1.7*[†]	4.8 ± 1.7*[†]	–	–	–
	50	88 ± 7.2*[†]	2.5 ± 2.1*	4.6 ± 1.2*[†]	3.0 ± 3.5*	9.0 ± 4.5*[†]	–
	100	–	–	–	2.8 ± 2.0*	5.0 ± 3.3*	27.2 ± 4.8*

Cell viability was assessed through the MTT reduction and the lactate dehydrogenase LDH leakage assays. Oxidative stress was assessed through DCFD oxidation, MDA levels and GSH/GSSG ratio. The change in MTT reduction is expressed as percentage of the control value. The changes in LDH leakage, DCFD oxidation and MDA levels are expressed as percentage over the basal values. Values represent the mean ± SD, $n = 3$. Values bearing different superscript letters are significantly different ($P < 0.05$). *$P < 0.05$ compared with APPE-untreated cells; [†]$P < 0.05$ compared with control cells. APPE, apple peel polyphenol extract.

experiments, LDH leakage was found to decrease substantially when APPE was added at 0.1 μg/ml. The protecting effect of co-exposure of cells to APPE and indometacin was almost two-fold that promoted in cells pre-treated with the same concentration of APPE 90 min earlier. This difference was no longer evident for concentrations of APPE equal to or higher than 5 μg/ml.

Effect of apple peel extract on indometacin-induced oxidative stress in Caco-2 cells

Table 2 shows the results from experiments conducted in cells that, after pre-exposure to APPE (for 90 min), were treated with indometacin for 60 min. Co-exposure experiments were also assayed (data not shown). The pro-oxidant effects of indometacin were evaluated determining: (1) the magnitude of the intracellular oxidation of DCF; (2) the degree of membrane lipid peroxidation (assessed as MDA accumulation); and (3) the levels (and ratio) of GSH and GSSG. Exposure of cells to 50 μM indometacin led to a 32% increase in DCF oxidation and a 63% elevation in the basal MDA levels (Table 2). Increasing the indometacin concentration to 250 μM further increased both oxidative parameters. The latter was accompanied by a decrease of almost 50% in the GSH/GSSG ratio. Pre-treatment of cells with APPE totally prevented the increment in DCF oxidation induced by 50 and 250 μM indometacin. This protective effect was already maximal at 0.1 μg/ml. The same concentration was effective in preventing (by nearly 90%)

the increment in MDA induced by indometacin (50 and 250 μM). The increase in MDA induced by 50 and 250 μM indometacin was totally abolished by APPE, at 50 μg/ml and 100 μg/ml, respectively. Regarding the decrease in the GSH/GSSG ratio induced by indometacin, APPE was partially effective, inhibiting it by over 50% at 0.1 μg/ml, and being totally effective at a concentration of 100 μg/ml (Table 2).

Effect of apple peel extract on indometacin-induced mitochondrial dysfunction

The mitochondria disturbing effects of indometacin and the possible preventing effect of APPE were addressed in intact Caco-2 cells employing the pre-incubation and co-incubation experimental protocols. The mitochondrial dysfunction was evaluated determining ATP levels and assessing the mitochondrial membrane potential. Figure 1 depicts the changes in ATP and MMP that, relative to control cells (e.g. neither indometacin nor APPE), were undergone by indometacin-treated cells that were either unexposed or pre-exposed to increasing APPE concentrations. Exposure of cells to 50 μM and 250 μM indometacin lowered the intracellular ATP levels to 72% and 60% of control values, respectively (Figure 1a). The same treatments lowered the MMP to 74% and 49% of control values (Figure 1b). Pre-exposure of cells to APPE, concentration-dependently prevented the decrease in both ATP (Figure 1a) and MMP (Figure 1b). At 50 μg/ml, APPE totally prevented the drop in ATP and MMP induced by 50 μM indometacin; its protective effect was over 90% when cells

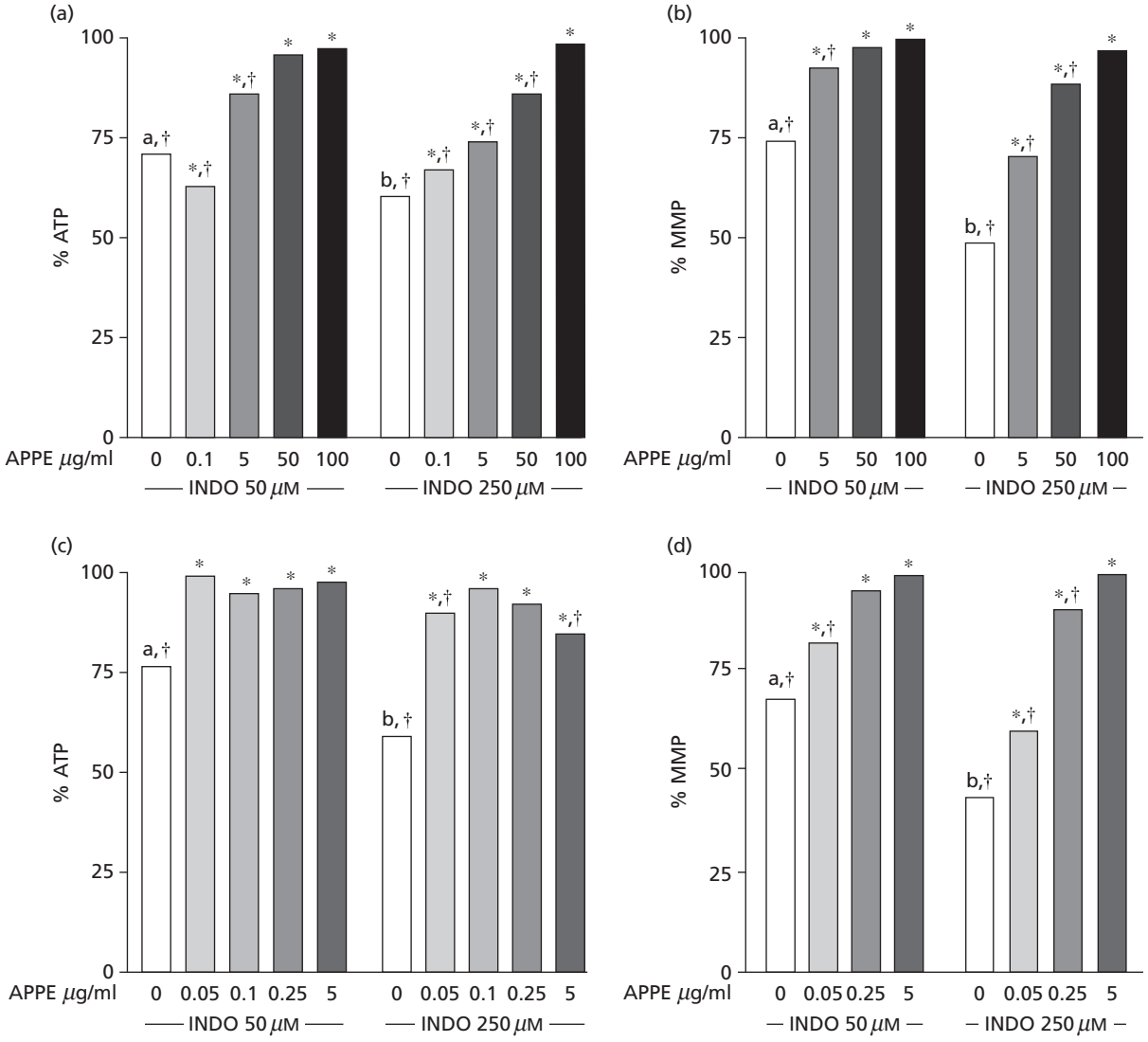

Figure 1 Protective effects of apple peel polyphenol extract on indometacin-induced mitochondrial dysfunction in Caco-2 cells. ATP levels were measured in experiments carried out under pre-incubation (a) and co-incubation (b) conditions. The mitochondrial membrane potential (MMP) was measured in experiments carried out under pre-incubation (c) and co-incubation (d) conditions. Changes in ATP and MMP are expressed as percentages of the control values. APPE, apple peel polyphenol extract; INDO, indometacin. Values bearing different superscript letters are significantly different ($P < 0.05$). *$P < 0.05$ APPE-untreated versus APPE-treated cells. $^{\dagger}P < 0.05$ vs control cells.

were exposed to 250 μM indometacin. Figure 1 also shows the results of co-exposure of cells to indometacin and APPE, and depicts the decreases in ATP and the drop in MMP (Figure 1c, d). At 0.05 μg/ml, APPE totally prevented the decrease in ATP (Figure 1c) and by over 80% the drop in MMP induced by 50 μM indometacin (Figure 1d). At 0.25 μg/ml, APPE fully prevented the ATP decrease (Figure 1c) and totally abolished the drop in MMP induced by 50 μM indometacin (Figure 1d).

Discussion

In view of the high incidence of gastrointestinal side effects associated with the use of NSAIDs, exploring pharmacological strategies that could prevent or minimize the consequences of the cellular events that lead to such effects is of particular interest. In the case of indometacin, one of such cytotoxicity-triggering events is the occurrence of oxidative stress. Although this has been extensively reported in animal experiments,[1–3,7,11] it should be noted that, in addition to a possible direct pro-oxidant effect of indometacin,[7,11,12] signs of oxidative stress affecting the gastrointestinal mucosa could be secondary to the inflammatory response that typically accompanies the occurrence of tissue damage.[1] In this regard, in-vitro studies conducted in cell lines relevant to the gastrointestinal mucosa can be of particular interest since they exclude the inflammation-mediated indirect pro-oxidant action of indometacin. Results from this study, conducted in Caco-2 cells, show that indometacin can enhance directly, and in a concentration-dependent manner, various parameters of oxidative stress. In fact, indometacin enhanced the intracellular oxidative status (assessed as an increased oxidation of DCF, most likely ROS-mediated), raised the rate of lipid peroxidation (assessed as MDA) and decreased the GSH/GSSG ratio (probably as a consequence of an elevation in the two former parameters). Such results are in line with the reported increase in lipid peroxidation (assessed as DPPP) induced by indometacin in RGM-1 cells, a gastric epithelial cell line.[9,23] The increase in the various oxidative stress parameters seen in our study was concomitantly and concentration-dependently associated with a drop in the intracellular ATP levels and MMP. These mitochondrial dysfunctioning effects of indometacin, which took place within one hour of exposure of Caco-2 cells, reveal that the alteration in the MMP reported to occur after treating RGM-1 cells with indometacin for 18 h[9] would be a much earlier mitochondrial-toxicity triggering event. The latter interpretation is coherent with the early (presumably within minutes) drop in oxygen consumption seen *ex vivo*, in mitochondria isolated from the intestine of rats pretreated (1 h) with indometacin,[1] and *in vitro* in mitochondria isolated from rat liver and directly incubated with indometacin.[3,8] In view of the reported ability of indometacin to directly induce mitochondrial dysfunction, it is likely that the drop in ATP levels and the alteration in MMP seen by us in whole Caco-2 cells arose from a direct action of this agent on the mitochondria.

Recently, the formation of ROS by mitochondria has been reported to be enhanced by the direct exposure of these organelles to indometacin.[7,11] Since ROS can permeabilize the mitochondrial inner membrane, possibly by collapsing its transmembrane potential, the enhanced production of ROS induced by indometacin may result in an efflux of such species towards the cytosol. The latter may account for the increase in the oxidative parameters seen by us in indometacin-exposed Caco-2 cells.

The mitochondrial dysfunctioning effects of indometacin were accompanied by an early (within 1 h) loss of cell viability (assessed as MTT reduction and LDH leakage). The latter effect is in line with the previously shown cytotoxic effect of indometacin in RGM-1,[23] AGS,[24] MKN 28,[13,24] HT 29[6] and Caco-2[25] cell lines. However, unlike these studies, which demonstrated cytotoxicity only after six or more hours, our study is the first to show that the cytotoxicity, as well as the oxidative stress and mitochondrial dysfunction, was already manifest within 1 h of exposure to indometacin. Therefore, we suggest that the loss of cell viability reported at 6 h or longer results from an early triggered and sustainably intracellular event.

APPE, which was investigated here for its potential to prevent some of the cellular events that could underlie the damage affecting the gastrointestinal mucosa, was found to concentration-dependently prevent each of the above-described cellular perturbations induced by indometacin. The effect of APPE was seen both in pre- and co-incubation experiments. The fact that the protective effect of APPE was evident even after its full removal from the incubation medium (pre-incubation experiments) suggests that some of the polyphenols present in APPE could have entered the cells (as suggested by Boyer *et al*.[26]) to preserve the intracellular antioxidant capacity (e.g. preventing the drop in the GSH/GSSG ratio) or to enhance it by exerting a direct antioxidant action within the cell (e.g. via a direct ROS-scavenging action). Comparatively, in the co-incubation experiments, lower concentrations of APPE were required to elicit any given cytoprotective effect, possibly because co-exposed cells are able to continually take up new polyphenol molecules from the medium.

This study does not allow us to elucidate which is the subcellular site of action of APPE relevant to its cytoprotective effects. However, based on the ability of indometacin to enhance mitochondrial ROS production,[7,11] it is likely that the cytoprotective effects of APPE arise, overall, from its capacity to directly scavenge ROS produced from within or outside the mitochondria. Results from DHE and deoxyribose oxidation and ORAC assay indicate that APPE actively scavenges $O_2\cdot^-$, HO· and peroxyl radicals. Its capacity to scavenge $O_2\cdot^-$ and HO· was two-thirds and one-third that of quercetin. Interestingly, quercetin, which accounts for near 60% of the TP in APPE, has been reported to be particularly active in scavenging $O_2\cdot^-$ and HO· radicals[27] and was shown to effectively protect against damage induced by indometacin in rat gastric mucosa.[28] In addition to scavenging ROS, quercetin is also able to inhibit their generation by chelating redox-active metals,[29] a mechanism which may contribute to the here-observed cytoprotective actions of APPE. On the other hand, the scavenging capacity of APPE towards peroxyl radicals, found here to be about one-half that of quercetin, is also of interest since APPE effectively protected Caco-2 cells against indometacin-induced lipid peroxidation, a process initiated by HO· but overall propagated by peroxyl radicals. In addition to its ROS-scavenging activity, APPE was

particularly effective as an antioxidant in the FRAP assay, often used to evaluate the electron transfer potential of antioxidant molecules; the ability of APPE to reduce iron (III) was nearly ten-fold higher that of quercetin, reportedly shown in FRAP studies to be one of the most active polyphenols.[30]

The above-discussed results indicate that APPE displays both a high capacity to donate a hydrogen atom, evidenced through the ROS scavenging assays, and a significantly high ability to donate an electron to such species, as demonstrated by the FRAP assay. Although further studies are needed to distinguish the extent to which such antioxidant activity accounts for the protective actions of APPE against the mitochondria-dysfuntioning and cytolytic effects of indometacin, the recently established ability of indometacin to enhance the mitochondrial production of ROS[7] strongly suggests that the mechanism by which APPE protects Caco-2 cells against this NSAID involves its antioxidant properties.

Conclusions

Exposure of Caco-2 cells to indometacin induced, within 60 min and in a concentration-dependent manner, alterations of various parameters of oxidative stress, mitochondrial function and cell viability. APPE, whether added before or simultaneously with indometacin, was found to concentration-dependently protect the cells against each of these cellular alterations. The high ROS scavenging capacity of APPE, mainly towards $O_2\cdot^-$, appears to be a key mechanism to explain its cytoprotective actions. Its efectiveness in preventing Caco-2 cells from undergoing mitochondrial and cellular damage induced by indometacin supports further studies to determine whether APPE can also protect against gastrointestinal damage induced by NSAIDs in animal models and humans. These findings may be of particular interest due to the high prevalence of gastrointestinal side-effects associated with the use of conventional NSAIDs.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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