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Apple Peel Polyphenol Extract Protects against Indomethacin-Induced Damage in Caco-2 Cells by Preventing Mitochondrial Complex I Inhibition

Catalina Carrasco-Pozo,[*,†,‡] Martin Gotteland,[†] and Hernán Speisky[‡,§]

[†]Laboratory of Microbiology and Probiotics, Institute of Nutrition and Food Technology, and [‡]Laboratory of Antioxidants, Institute of Nutrition and Food Technology, University of Chile, Av. Macul 5540, Santiago, P.O. Box 138-11, Chile

[§]Department of Pharmacological and Toxicological Chemistry, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, Santiago, Chile

ABSTRACT: The aim of this work was to investigate the role of mitochondrial dysfunction in the development of oxidative stress and cytotoxicity induced by indomethacin and to evaluate the potential of an apple peel polyphenol extract (APPE) in protecting against these events. Indomethacin induced, time-dependently, mitochondrial and oxidative perturbations which led to cell losses. An inhibition of complex I activity, shown for first time here, which resulted in a concomitant drop in cellular ATP and an increment in mitochondrial superoxide production, was observed after 10 min of exposure. These early cytotoxicity-triggering events were followed by an increase in the intracellular production of superoxide (20 min), an elevation in the activity of xanthine oxidase which led to an increased lipid peroxidation (30 min), and a decline in cell viability which manifested after 40 min. These events were selectively prevented using allopurinol, tempol and APPE (a standardized apple peel polyphenol extract). While the oxidative and cell lytic effects of indomethacin were equally prevented by the three agents, only APPE protected against complex I inhibition and its downstream oxidative consequences. Since tempol (a SOD mimetic) prevented the elevation in xanthine oxidase activity, and allopurinol (a xanthine oxidase inhibitor) totally abolished the increment in lipid peroxidation and loss of cell viability, it appears that a superoxide-dependent increase in xanthine oxidase activity is critical to trigger cytotoxicity. Thus, preventing the early increment in superoxide formation that, as a result of inhibiting complex I, takes place within mitochondria would be key toward protecting the cells against the oxidative and cytolytic effects of indomethacin. The ability of APPE in preventing the inhibition of complex I and the subsequent superoxide-dependent increase in XO activity warrants further studies to evaluate the mechanism involves in the protecting effect of APPE against the indomethacin-associated adverse effects in vivo.

KEYWORDS: indomethacin, apple polyphenols, mitochondrial complex I, xanthine oxidase, superoxide radicals

INTRODUCTION

Indomethacin (INDO) is a nonsteroidal anti-inflammatory drug (NSAID) widely used in the treatment of inflammatory disorders.[1] The mechanism of action underlying its anti-inflammatory effect involves the inhibition of both constitutive and inducible cyclooxygenases, COX-1 and COX-2, respectively.[1] The clinical use of INDO is, however, frequently associated with the development of gastrointestinal (GI) lesions.[2−4] Among the major cellular events believed to be involved in its GI toxicity are the ability of INDO to induce mitochondrial dysfunction[5−13] and to promote oxidative stress.[9−22] Regarding its mitochondria effects, INDO has been shown to uncouple the oxidative phosphorylation,[5−9,11] dissipate the mitochondrial membrane potential (MMP),[6,10,12,23] and diminish mitochondrial ATP levels.[6,8,23] With regard to its ability to induce oxidative stress, INDO has been shown, in vitro, to induce lipid peroxidation in RGM-1[10,21] and in Caco-2[23] cells, a gastric and intestinal cell line, respectively. INDO has been observed, in vivo, to enhance lipid peroxidation[9,11−19,22] and the production of reactive oxygen species (ROS)[12,13,19,20] in the GI mucosa of treated rodents. In addition, INDO has been reported to enhance the activity of pro-oxidant enzymes, such as NADPH oxidase,[16] myeloperoxidase,[9,17,24] and xanthine oxidase (XO),[11,15,18,22] and to diminish the activity of the antioxidant enzymes catalase,[11,18,22] superoxide dismutase (SOD),[15,18] and glutathione peroxidase[11,15,18,22] in the GI mucosa of treated rodents.

Although mitochondrial dysfunction and oxidative stress are involved in the development of cytotoxicity, it is still unknown how exactly these events are mechanistically related. Aiming to elucidate this problems, we have addressed, in terms of their temporality, the changes in mitochondrial function (complex I activity and ATP levels) and oxidative status ($O_2^{\bullet-}$ production, activity of pro-oxidant enzymes and lipid peroxidation levels) that follow the exposure of Caco-2 cells to INDO. To investigate their potential to act as cytoprotective agents, and as a strategy to possibly dissociate the above-referred changes in oxidative stress parameters from those affecting the mitochondria, we also included the use of tempol, a SOD mimetic,[25] and allopurinol, a XO inhibitor,[26] as antioxidants. In addition, since our laboratory recently described the ability of APPE, a polyphenol-rich apple peel extract,[27,28] to scavenge ROS and protect against the

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cytotoxicity induced by INDO,[23] we also evaluated its potential to protect Caco-2 cells from the mitochondrial dysfunction and the oxidative stress induced by this anti-inflammatory agent.

MATERIALS AND METHODS

Chemicals. Xanthine oxidase, xanthine, 2-thiobarbituric acid, indomethacin, MTT (thiazolyl blue tetrazolium bromide), and the protease inhibitor cocktail for use with mammalian cell and tissue extracts, containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatinA, E-64, bestatin, leupeptin, and aprotinin (P8340), were from Sigma. DHE (dihydroethidium) was from Calbiochem. CytoTox-ONE Homogeneous Membrane Integrity Assay kit and CellTiter-Glo luminescent cell viability assay kit were from Promega (Madison, WI, USA.).

APPE Preparation and Polyphenolic Content. APPE was prepared from Granny Smith apple peels and its polyphenolic composition confirmed as in Pastene et al.[27,28] Total polyphenols (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. Procyanidins accounted for 24% of TP. Flavan-3-ols, comprising 5% of TP, mostly consisted of epicatechin[27,28]

Cells and Cell Culture Conditions. The human colonic adenocarcinoma cell line, Caco-2 (ATCC HTB-37), was used at near 90% confluence; cells were maintained in a DMEM-F12 plus 10% fetal bovine serum medium and cultured at 37 °C (5% CO_2/95% air). All incubations were performed with 250 μM INDO based on results obtained in a previous study.[23] At this concentration, Caco-2 cells' mitochondrial and oxidative damage induced by INDO were already manifest after 60 min of incubation.[23]

Lipid Peroxidation Measurement. Lipid peroxidation was assayed measuring malondialdehyde (MDA) in cell lysates after reaction with thiobarbituric acid. Caco-2 cells were incubated (at 37 °C) for 10 to 60 min with INDO (250 μM, dissolved in ethanol, 1% v/v in PBS) in the absence or presence of tempol[25] (a SOD mimetic, dissolved in PBS) or allopurinol[26] (a XO inhibitor, dissolved in PBS) at 100 or 300 μM, or with APPE[27,28] (at 0.1 μg/mL, dissolved in PBS). MDA was separated by HPLC using an Agilent Eclipse XDB-C18 column (5 μM, 4.5 × 150 mm) eluted (0.6 mL/min) with a mixture of 25 mM methanol plus phosphate buffer, pH 6.5 (1:1, v/v), and detected using a fluorescent detector ($532_{Ex}/553_{Em}$). 1,1,3,3-Tetramethoxypropane was used as control standard. MDA levels are expressed as nanomoles of MDA per milligram of protein. Protein content was determined photometrically by the method of Bradford.

Xanthine Oxidase Activity Determination. XO activity was evaluated using xanthine as substrate and assaying uric acid production.[29] Caco-2 cells were incubated (at 37 °C) for 10 to 60 min with INDO (250 μM) in the absence or presence of tempol or allopurinol (each at 100 or 300 μM), or APPE (at 0.1 μg/mL). Cells were lysed in a 50 mM phosphate buffer (pH 7.4) solution containing 1 mM EDTA and a protease inhibitor cocktail. Cell lysates were incubated with xanthine (200 μM), and uric acid production was measured at 294 nm. Optical density (OD) was registered each 15 s, during 20 min, and normalized to the protein content. One unit of XO activity corresponds to the formation of 1 μmol of uric acid/min.[29]

NADPH Oxidase Activity Determination. NADPH oxidase activity was assayed in whole homogenates from Caco-2 cells incubated (37 °C; 10−60 min) with INDO (250 μM). The assay is based on the oxidation of DHE (an $O_2^{\bullet-}$-oxidizable probe) into its fluorescent product, hydroxyethidium ($470_{Ex}/590_{Em}$).[30] Then, the relative fluorescence units (RFU) were registered.

Cell Viability Assessment. Cell viability was assessed by the MTT reduction and LDH (lactate dehydrogenase) leakage assays. In the MTT assay, the ability of live cells to reduce thiazolyl blue was measured at OD_{540}. LDH released into the medium was measured fluorimetrically using the CytoTox-ONE kit ($560_{Ex}/590_{Em}$) and compared with Triton X-100-treated cells as control. Tempol or allopurinol (300 μM) or APPE (0.1 μg/mL) and INDO (250 μM) were simultaneously added to the cells, and these were incubated during 0 to 60 min at 37 °C.

Isolation of Mitochondria. Mitochondria were isolated from Caco-2 cultured cells, as described by O'Donnell et al.[31] Briefly, cells were harvested, washed with Ca^{2+}/Mg^{2+}-free PBS, and centrifuged (10 min; 500g; 4 °C). The pellet was resuspended in a buffer, pH 7.4, consisting of 250 mM sucrose, 1 mM EGTA (ethylene glycol tetraacetic acid), 10 mM HEPES, and 1 mg/mL BSA (fraction V), and homogenized in a Teflon homogenizer. The homogenate was centrifuged (10 min; 1500g; 4 °C), the supernatant kept aside, and the pellet re-extracted as above. Finally, the two supernatants were combined and centrifuged (10 min; 14000g; 4 °C).

Superoxide Radical Production. $O_2^{\bullet-}$ production was assessed measuring the oxidation of DHE ($470_{Ex}/590_{Em}$).[30] This assay was applied to experiments conducted in cells and in isolated mitochondria. Cells plated in 24-well plates or isolated mitochondria were incubated (at 37 °C) for 30 min with 10 μM DHE (dissolved in dimethyl sulfoxide, 0.2% v/v in PBS). After washing, cells or mitochondria were treated with INDO (250 μM) in the absence or in the presence of tempol or allopurinol (300 μM each) or APPE (0.1 μg/mL) for 10, 20, or 30 min. The cellular or mitochondrial protein contents were measured. DHE oxidation is expressed as RFU per milligram of protein.

ATP Quantification. Intracellular ATP levels were quantified by luminescence using a CellTiter-Glo kit. Cells were incubated (at 37 °C for 10−30 min) with INDO (250 μM) in the absence or presence of tempol or allopurinol (300 μM) or APPE (0.1 μg/mL). Results are expressed as relative luminescence units (RLU) per milligram of protein.

Complex I Activity Assay. The activity of complex I was determined in isolated mitochondria through the change in the OD_{340nm} due to the oxidation of NADH (ε 6.81 mM^{-1} cm^{-1}), as described by Birch-Machin et al.[32] Complex I activity was assayed in Caco-2 cells and in isolated mitochondria treated with INDO 250 μM in the absence and presence of rotenone (20 μM, a complex I inhibitor),[33] tempol (300 μM), allopurinol (300 μM), or APPE (0.1 μg/mL) for 10 or 20 min. The reaction solution consisted of 25 mM potassium phosphate buffer (pH 7.2) containing protease inhibitor cocktail, 5 mM $MgCl_2$, 2 mM KCN, 2.5 mg/mL BSA (fraction V), 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). The reaction was started after addition of NADH (0.13 mM) and the rate of NADH oxidation monitored at 340 nm for 3 min. Complex I activity was derived from the rotenone-sensitive NADH-ubiquinone oxidoreductase activity.

Complex II Activity Assay. The activity of complex II was determined in isolated mitochondria through the reduction of 2,6-dichlorophenolindophenol (DCIP) at 600 nm (ε 19.1 mM^{-1} cm^{-1}), as described by Birch-Machin et al.[32] Complex II activity was assayed in Caco-2 cell isolated mitochondria treated with INDO 250 μM. The mitochondrial homogenates were incubated in a solution containing 25 mM potassium phosphate buffer (pH 7.2), 5 mM $MgCl_2$, and 20 mM succinate for 10 min at 30 °C. 2 μg/mL antimycin A, 20 μM rotenone, 2 mM KCN, and 50 μM DCIP were then added, and the mixture was incubated for 1 min, followed by the addition of 65 μM coenzyme Q_1 to start the reaction. The decrease in OD_{600nm} was recorded for an additional 1 min and used for calculating the activity of complex II.

Complex III Activity Assay. The activity of complex III was determined in isolated mitochondria by measuring the rate of reduction of cytochrome c (III) at 550 nm (ε 20 mM^{-1} cm^{-1}) by the reduced form of coenzyme Q_1 (ubiquinol ε 19.1 mM^{-1} cm^{-1}), as described Birch-Machin et al.[32] Coenzyme Q_1 (ubiquinone) was reduced with borohydride, and ubiquinol was extracted by organic solvent (diethyl ether/cyclohexane 2:1 v/v). The assay medium contained 25 mM

Table 1. Effect of INDO on Lipid Peroxidation and Xanthine Oxidase Activity[a]

	lipid peroxidation (nmol of MDA/mg of protein) [xanthine oxidase act. (U/mg of protein)]					
	0[b]	10	20	30	40	60
control	28 ± 3.3 a [0.014 ± 0.004] a	30 ± 4.2 a [0.015 ± 0.002] a	28 ± 2.6 a [0.015 ± 0.003] a	33 ± 4.3 a [0.014 ± 0.002] a	30 ± 2.2 a [0.015 ± 0.003] a	32 ± 2.1 a [0.0014 ± 0.002] a
INDO 250 μM	27 ± 3.1 a [0.015 ± 0.003] a	29 ± 3.8 a [0.014 ± 0.002] a	35 ± 3.2 a [0.015 ± 0.004] a	48 ± 2.4 b [0.021 ± 0.003] b	66 ± 4.0 c [0.027 ± 0.002] c	90 ± 7.2 d [0.035 ± 0.004] d
INDO + tempol 100 μM					45 ± 4.0*,† [0.020 ± 0.001]*,†	
INDO + tempol 300 μM					29 ± 2.1† [0.014 ± 0.002]†	
INDO + allopurinol 100 μM					48 ± 3.2*,† [0.022 ± 0.003]*,†	
INDO + allopurinol 300 μM					31 ± 3.8† [0.013 ± 0.003]†	
INDO + APPE 0.1 μg/mL					30 ± 2.5† [0.015 ± 0.002]†	

[a] Cells were exposed to INDO (250 μM) from 10 to 60 min or in the presence of tempol or allopurinol (each at 100 or 300 μM) or APPE (0.1 μg/mL) for 40 min. Results are expressed as nanomoles of MDA per milligram of protein and as units of XO activity per milligram of protein. Values bearing different lowercase letters were significantly different at $p < 0.05$. The symbol * indicates significant difference ($p < 0.05$) between the marked value and that obtained with control cells. The symbol † indicates significant difference ($p < 0.05$) between the marked value and that obtained with 250 μM INDO-treated cells. Each incubation was carried out at least three times. [b] Exposure time (min).

potassium phosphate buffer (pH 7.2), 5 mM $MgCl_2$, 2.5 mg/mL BSA (fraction V), and 2 mM KCN. KCN was included in the assay medium to prevent the reoxidation of the product, cytochrome *c*, by cytochrome oxidase. Nonenzymatic activity was recorded for 1 min after the addition of 50 μM cytochrome *c*, 20 μM rotenone, 0.6 mM dodecyl-β-D-maltoside, and 35 μM ubiquinol. The complex III activity 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 recorded for 1 min.

Statistical Analysis. Data were analyzed using the GraphPad Prism 4 statistical software. Values represent the means of at least 3 independent experiments, each conducted in quadruplicate. Data was analyzed by ANOVA and Tukey's multiple comparison test. Values bearing different superscript letters were significantly different at $p < 0.05$.

RESULTS

Effects of Indomethacin on Lipid Peroxidation and Xanthine Oxidase Activity. Exposure of Caco-2 cells to INDO (thereafter 250 μM) led to a time-dependent increase in lipid peroxidation (assessed as MDA) (Table 1). The increase in MDA levels became significant after 30 min of exposure and tripled after 60 min. In cells exposed to INDO for 40 min, the addition of tempol led to a concentration-dependent inhibition of the increase in MDA, with total inhibition achieved at 300 μM (Table 1). A similar inhibitory effectiveness was observed using allopurinol. APPE at a concentration of 0.1 μg/mL completely abolished the increment in MDA induced by INDO (Table 1).

Prompted by previous reports indicating that the administration of INDO to rodents increases the activity of some pro-oxidant enzymes in the GI mucosa,[9,11,15−18,22,24] we investigated the effect of the in vitro exposure of Caco-2 cells to INDO on the activity of XO and NADPH oxidase. Cells exposed to INDO during 10 to 60 min showed a time-dependent increase in XO activity; compared with its basal activity, XO increased significantly by 45% after 30 min and by more than 100% after 60 min (Table 1). In cells exposed to INDO for 40 min, allopurinol and tempol concentration dependently prevented the increase in XO activity induced by INDO (Table 1); a total inhibition was attained with all agents at 300 μM, and with APPE at a concentration of 0.1 μg/mL (Table 1). Cells exposed to INDO for 10 to 60 min showed no effect in NADPH oxidase activity (data not shown).

Effects of Indomethacin on Cell Viability. Table 2 shows the changes in viability resulting from the exposure of Caco-2 cells to INDO during the 0−60 min period. INDO time-dependently decreased cell viability, as reflected by a drop in MTT reduction and an increment in the leakage of LDH. Such effects became significant only after 40 min. At a concentration of 300 μM, allopurinol and tempol totally prevented the loss of cell viability (observed after 40 and 60 min). APPE at a concentration of 0.1 μg/mL was totally effective in preventing such loss (Table 2).

Effects of Indomethacin on Superoxide Radical Formation. Results from the experiments illustrated in Table 1, in which tempol was shown to totally prevent the increase in XO activity induced by INDO, suggest that $O_2^{\bullet-}$ radicals are likely to be involved in this increase. Consequently, the ability of Caco-2 cells to produce $O_2^{\bullet-}$ anions was investigated after exposure to INDO during 10−30 min in which XO activation had not yet occurred. As shown in Figure 1A, exposure of the cells to INDO led to a time-dependent increase in the formation of $O_2^{\bullet-}$ which already had become significant after 20 min (reflected as a 30% increase in DHE oxidation). The increment in $O_2^{\bullet-}$ formation was prevented by the coincubation (20 min) of the cells with 300 μM tempol or 0.1 μg/mL APPE. The effect of allopurinol on the INDO-induced oxidation of DHE was also studied with 30 min of exposure time to cells, a time at which the increase by INDO on XO activity is significant. Allopurinol was only partially effective in preventing INDO-induced DHE oxidation after 30 min (Figure 1A). Since allopurinol only partially prevented DHE from oxidation of DHE, there may be a source of $O_2^{\bullet-}$ other than XO, such as the electron transport chain in

Table 2. Effects of INDO on Cell Viability[a]

	MTT reduction (% of control value)			LDH leakage (% over basal value)		
	30[b]	40	60	30	40	60
control	100 ± 3.2 a	100 ± 3.6 a	100 ± 3.3 a	2.1 ± 2.1 a	2.3 ± 1.5 a	2.5 ± 2.0 a
INDO 250 μM	96 ± 6.0 a	75 ± 6.2 b	75 ± 6.2 b	5.1 ± 2.3 a	13.7 ± 1.3 b	21.0 ± 3.0 b
INDO + tempol 300 μM	99 ± 3.2 a	96 ± 6.3 a	96 ± 6.3 a	3.8 ± 1.8 a	5.1 ± 1.2 a	3.6 ± 1.2 a
INDO + allopurinol 300 μM	98 ± 2.3 a	100 ± 9.3 a	100 ± 9.3 a	6.2 ± 3.2 a	3.6 ± 2.0 a	3.9 ± 1.4 a
INDO + APPE 0.1 μg/mL	100 ± 7.2 a	99 ± 4.3 a	99 ± 4.3 a	5.0 ± 1.1 a	3.9 ± 1.2 a	5.1 ± 1.7 a

[a] Cells were exposed to INDO (250 μM) alone or in the presence of tempol or allopurinol (300 μM) or APPE (0.1 μg/mL) for 30, 40, or 60 min. Cell viability was measured through MTT reduction and LDH leakage. The changes in MTT reduction and LDH leakage are expressed as percentage of the control value. Values bearing different lowercase letters were significantly different at $p < 0.05$. Each incubation was carried out at least three times. [b] Exposure time (min).

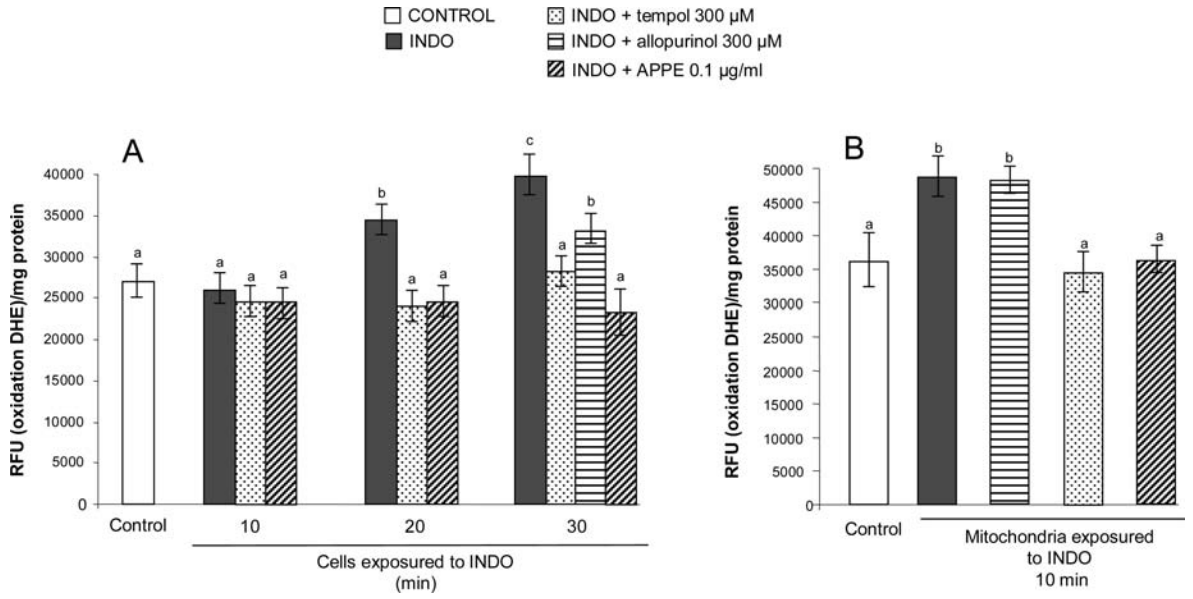

Figure 1. Effect of INDO on superoxide radical formation. (A) Cells were exposed to INDO (250 μM) alone or in the presence of tempol or allopurinol (300 μM) or APPE (0.1 μg/mL) for 10, 20, or 30 min. (B) Isolated mitochondria from Caco-2 cells were exposed to INDO (250 μM) alone or in the presence of tempol or allopurinol (300 μM) or APPE (0.1 μg/mL) for 10 min. Results are expressed as RFU (corresponding to oxidation of DHE) per milligram of protein. Values bearing different lowercase letters were significantly different at $p < 0.05$. Each incubation was carried out at least three times.

mitochondria.[33] In fact, we studied the effect of INDO on mitochondrial superoxide production and, as shown in Figure 1B, the 10 min exposure of isolated mitochondria to INDO led to a 30% increment in the basal formation of $O_2^{\bullet-}$. This latter effect of INDO was unaffected by allopurinol but totally abolished by the addition of tempol or APPE.

Effects of Indomethacin on Complex I Activity. The inhibition of mitochondrial complex I has been described to result in the increased formation of $O_2^{\bullet-}$ radicals;[33] thus, the possible inhibition of complex I by INDO as a mechanism that accounts for the above-described increase in mitochondrial $O_2^{\bullet-}$ was assessed in both Caco-2 cells and isolated mitochondria. Complex I activity was measured as rotenone-sensitive NADH-ubiquinone oxidoreductase activity. Rotenone inhibited the latter by over 90%, indicating that, under the present assay conditions, NADH oxidation largely reflects complex I activity. As shown in Figure 2A, when cells were treated with INDO, complex I activity decreased early and time-dependently by 25% at 10 min and 50% at 20 min. The complex I inhibiting effect of INDO was totally prevented by the exposure of Caco-2 cells to APPE, but was unaffected by either tempol or allopurinol (Figure 2B). Similar results in terms of time and intensity of complex I inhibition were obtained when INDO and the latter compounds were simultaneously added to mitochondria isolated from Caco-2 cells (data not shown). Interestingly, complex II and III activities were not affected by INDO incubation (data not shown).

Effects of Indomethacin on Intracellular ATP Levels. Since any alteration in the electron transport chain energy affects the cell energy, we studied the intracellular ATP levels in Caco-2 cells exposed to INDO. The early and time-dependent inhibition of complex I by INDO (seen after 10 and 20 min) was accompanied by a time-dependent drop in cellular ATP levels from 10 to 30 min (Figure 3). The latter effect (of 15%) was significant after 10 min and reached near 50% after 30 min. The decrease in ATP induced by INDO was totally prevented by APPE (0.1 μg/mL) but unaffected by tempol or allopurinol (300 μM each). APPE added alone had no effect on ATP levels.

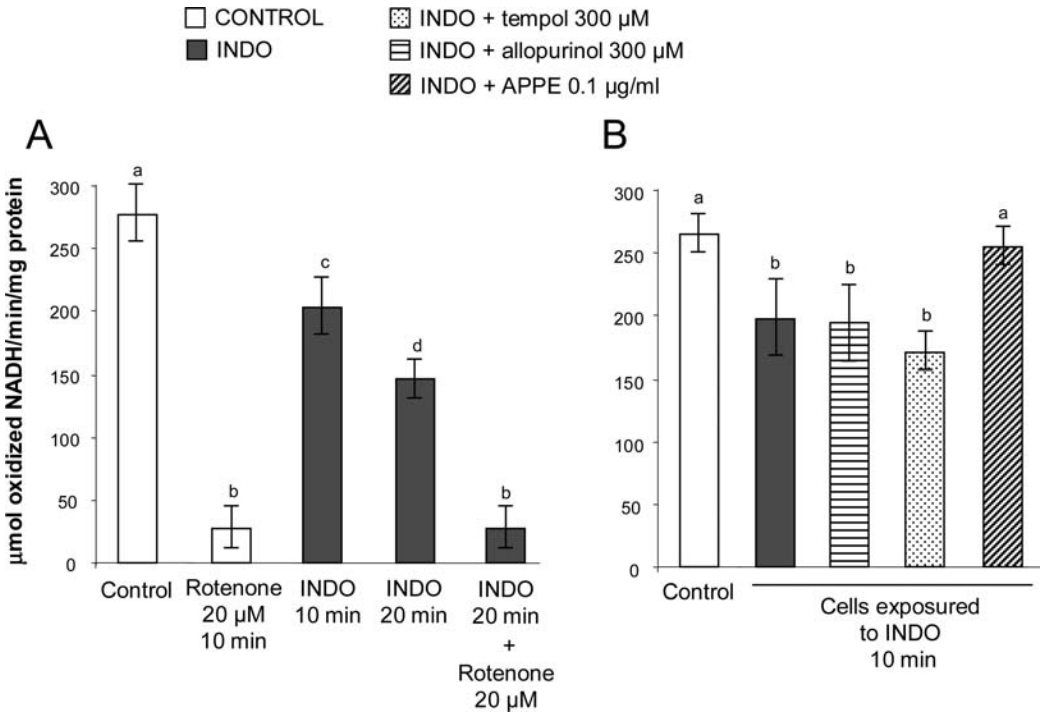

Figure 2. Effect of INDO on complex I activity. (A) Cells were exposed to rotenone 20 μM, or INDO (250 μM) for 10 or 20 min, or INDO (250 μM) for 20 min and after rotenone 20 μM. (B) Cells were exposed to INDO (250 μM) in the absence or presence of tempol or allopurinol (300 μM) or APPE (0.1 μg/mL) for 10 min. Complex I activity in mitochondria isolated from Caco-2 cells was assessed as described in Materials and Methods, using rotenone as a control. Results are expressed as micromoles of NADH oxidized per minute per milligram of protein. Values bearing different lowercase letters were significantly different at $p < 0.05$. Each incubation was carried out at least three times.

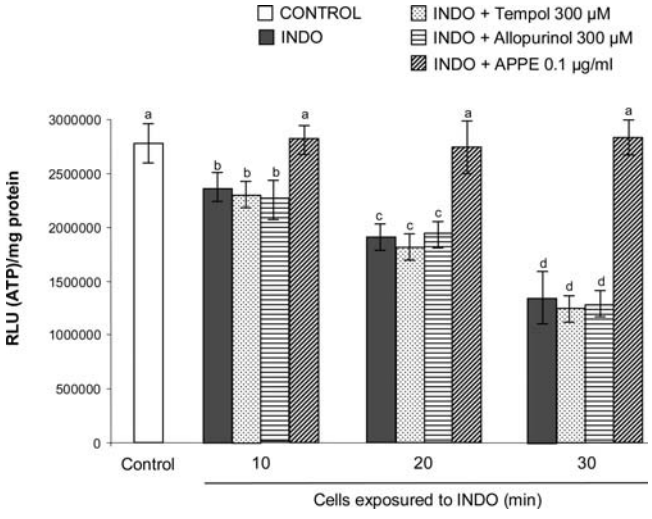

Figure 3. Effects of INDO on cellular ATP levels. Cells were exposed to INDO (250 μM) alone or in the presence of tempol or allopurinol (300 μM) or APPE (0.1 μg/mL) for 10, 20, or 30 min, and ATP levels were measured. Results are expressed as RLU per milligram of protein. Values bearing different lowercase letters were significantly different at $p < 0.05$. Each incubation was carried out at least three times.

DISCUSSION

This study describes and addresses the time course and the possible mechanistic link that would exist between the mitochondrial and the oxidative disturbances induced by INDO in Caco-2 cells. After exposure to INDO, these cells manifest early mitochondrial dysfunction consisting of the inhibition of complex I activity (but not of the complexes II or III) and a drop in ATP levels.

To our knowledge, this is the first observation showing that the INDO-induced mitochondrial changes[5−13] involve a direct inhibition of complex I, either in intact Caco-2 cells or in isolated mitochondria. Such inhibition of complex I by INDO has been previously suggested by Somasundaram et al., in rat liver mitochondria.[5] However these authors used indirect methods such as measuring oxygen consumption, showing that the glutamate/malate stimulated respiration in uncoupled mitochondria is inhibited by INDO. In our study, we directly assessed the activity of complex I by measuring the oxidation (rotenone-inhibited) of NADH.

In the present work, the inhibition of complex I and the decrease in ATP levels were associated with an enhancement of the oxidative status of the Caco-2 cells, as reflected by an increased formation of $O_2^{\bullet-}$, an increment in XO activity, and an enhancement of the rate of lipid peroxidation. Most likely, both the mitochondrial and the oxidative alterations contributed to the time-dependent loss of cell viability. From a time-course point of view, the inhibition of complex I and the decrease in ATP were early events (observed 10 min after exposure to INDO) that not only preceded but also presumably led to the oxidative changes and the loss of cell viability observed subsequently. Since only 25 to 45% of MTT reduction is mitochondrial-dependent,[34] INDO induces mitochondrial dysfunction (at 10 min of incubation time with cells) before diminishing MTT reduction (at 30 min of incubation time). Consistent with the interpretation that the inhibition of complex I can lead to oxidative stress

and cytotoxicity, rotenone[35] and MPP^+,[36] well-defined complex I inhibitors, are known to increase the production of $O_2^{\bullet-}$ and lower the levels of ATP, leading to cell death. Recently, Maity et al.[12] observed that mitochondria obtained from the gastric mucosa of rats treated with INDO show structural and functional alterations which include a severe impairment of their stage-3 and -4 respiration. In the same study, an increased intramitochondrial production of $O_2^{\bullet-}$ anions, upon releasing iron from the Fe−S cluster present in aconitase, was shown to lead to the production of hydroxyl radicals.[12] Although the inhibition of complex I and the drop in ATP may trigger cytotoxic events, we observed that removing of $O_2^{\bullet-}$ anions with tempol or preventing their formation with allopurinol prevented the oxidative and cytolytic damage induced by INDO although without protecting against any of the previously referred to mitochondrial perturbations. Consequently, $O_2^{\bullet-}$ anions would appear not to mediate the inhibition of complex I or the drop in ATP induced by INDO, but to play a role in inducing the oxidative perturbations caused by this agent. Since tempol and allopurinol prevented the loss of cell viability, it would appear that $O_2^{\bullet-}$ anions are involved (though not necessarily in a direct way) in initiating the peroxidation of lipids and the cytotoxicity in INDO-treated cells. In fact, the increase in the cellular production of $O_2^{\bullet-}$ (observed after 20 min) preceded the increase in lipid peroxidation (detected after 30 min).

On the other hand, since tempol prevented the increase in XO activity (observed only after 30 min), it is likely that the early (10 min) mitochondrial production of $O_2^{\bullet-}$ anions would be required to initiate the pro-oxidant event. Interestingly, while $O_2^{\bullet-}$ radicals induce the oxidation of sulfhydryl in proteins,[37] the conversion of xanthine dehydrogenase into XO has been shown to involve the oxidation of critical sulfhydryl moieties in the former.[38,39] Interestingly, enterocytes obtained from rats given INDO one hour earlier show an increase in XO which is concomitant with a decrease in xanthine dehydrogenase.[11] Since in our study allopurinol fully prevented cells from undergoing lipid peroxidation and the loss of cell viability induced by INDO, it would appear that, primarily, those $O_2^{\bullet-}$ generated by XO would be critical in initiating these deleterious events. Although lipid peroxidation and the loss of cell viability were prevented by allopurinol, the time-course studies revealed that the former event would occur previously. In line with the interpretation that a superoxide-dependent lipid peroxidation would be the primary cause of the loss in cell viability induced by INDO, we observed that tempol and APPE, in addition to allopurinol, were effective in preventing the two above-mentioned events: lipid peroxidation and loss in cell viability.

Compared with tempol and allopurinol, APPE was effective in preventing not only the oxidative events affecting Caco-2 cells (i.e., increased in $O_2\cdot^-$ production, XO activity and lipid peroxidation) but also mitochondrial dysfunction (decrease in ATP and complex I inhibition) induced by INDO. Since tempol failed to prevent the inhibition of complex I activity induced by INDO, it is possible that the protective effect of APPE on such inhibition would not reside in its antioxidant properties but go further. We recently observed that, on a per gallic acid equivalent basis, APPE exhibits a superoxide-scavenging capacity slightly lower than that of quercetin,[23] a polyphenol previously shown to protect rats from the gastric mucosal damage induced by INDO.[40] Taking this into account, and the major role that $O_2^{\bullet-}$ anions appear to play in enhancing the activity of XO, it seems reasonable to suggest that the protection afforded by APPE against the oxidative and cytolytic effects of INDO in Caco-2 cells would largely reside on its superoxide-scavenging capacity. Additionally, APPE could also exert its cytoprotective effects through its ability to prevent the early INDO-induced inhibition of mitochondrial complex I, avoiding the increment in $O_2^{\bullet-}$ formation that typically follows this phenomenon.[33] The ability of APPE to prevent the formation of $O_2^{\bullet-}$ anions arising from the inhibition of complex I, added to its ability to scavenge the $O_2^{\bullet-}$ radicals generated in the mitochondria and cytosol, warrants further study of its potential to protect against the GI lesions that frequently accompany the use of INDO and other NSAIDs. Interestingly, a polyphenolic extract obtained from whole Annurca apple has been shown to protect MKN28 cells in vitro against the cytotoxic effects of indomethacin and to prevent in vivo the injury induced by this agent[41] and by aspirin.[42] to the rat gastric mucosa. Since, in addition to their antioxidant properties, some polyphenols also display anti-inflammatory activity,[43] the use of extracts containing polyphenols may emerge as an interesting strategy to prevent the GI effects of NSAIDs. In fact, we recently reported that APPE protects against gastrointestinal mucosa alterations induced by indomethacin in rats;[44] however, the influence of APPE on the effectiveness of indomethacin remains to be established. Of note, the gastric lesions associated with the use of several anti-inflammatory agents are known to be exacerbated in subjects colonized with *Helicobacter pylori*. In this regard, our laboratory reported recently that APPE inhibits in vitro and in vivo the growth of *H. pylori*[45] and the respiratory burst of neutrophils activated by this pathogen.[28]

In conclusion, the prevention of the early increment in $O_2^{\bullet-}$ formation, that as result of inhibiting complex I takes place within mitochondria, would be key toward protecting cells against the oxidative and cytolytic effects of indomethacin. APPE, beyond its superoxide-scavenging capacity, could also exert its cytoprotective effects through its ability to prevent the early INDO-induced inhibition of mitochondrial complex I.

AUTHOR INFORMATION

Corresponding Author

*Phone: 56(2) 9781470. Fax.: 56(2) 978 1599. E-mail: ccarrasco@inta.cl.

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