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Extracellular ATP induces cell death in human intestinal epithelial cells

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ABSTRACT

Background: Extracellular ATP is an endogenous signaling molecule released by various cell types and under different stimuli. High concentrations of ATP released into the extracellular medium activate the P2X7 receptor in most inflammatory conditions. Here, we seek to characterize the effects of ATP in human intestinal epithelial cells and to evaluate morphological changes in these cells in the presence of ATP.
Methods: We treated human intestinal epithelial cells with ATP and evaluated the effects of this nucleotide by scanning and transmission electron microscopy analysis and calcium measurements. We used flow cytometry to evaluate apoptosis. We collected human intestinal explants for immunohistochemistry, apoptosis by the TUNEL approach and caspase-3 activity using flow cytometry analyses. We also evaluated the ROS production by flow cytometry and NO secretion by the Griess technique.
Results: ATP treatment induced changes characteristic of cell death by apoptosis and autophagy but not necrosis in the HCT8 cell line. ATP induced apoptosis in human intestinal explants that showed TUNEL-positive cells in the epithelium and in the lamina propria. The explants exhibited a significant increase of caspase-3 activity when the colonic epithelial cells were incubated with IFN-gamma followed by ATP as compared to control cells. In addition, it was found that antioxidants were able to inhibit both the ROS production and the apoptosis induced by ATP in epithelial cells.
General significance: The activation of P2X7 receptors by ATP induces apoptosis and autophagy in human epithelial cells, possibly via ROS production, and this effect might have implications for gut inflammatory conditions.

1. Introduction

Different stimuli may induce the release of extracellular nucleotides by several cell types, resulting in effects such as cell differentiation, proliferation, cell death and the production of cytokines [1]. ATP is a potential candidate for signaling cell damage because it is found in the intracellular compartment at millimolar concentrations. Additionally, ATP can function as an endogenous signaling molecule to control inflammation and the immune response [2,3]. Extracellular nucleotides are recognized by two families of nucleotide receptors in mammals: the P2Y family of G protein-coupled receptors and the P2X family, which are intrinsic ion channel receptors [4,5]. In particular, the P2X7 receptor subtype is a well known P2 receptor involved in inflammation that is present in the cell membranes of most inflammatory cells, such as macrophages, mast cells, dendritic cells, and lymphocytes [6]. The concentrations of extracellular ATP can increase considerably in the microenvironment of damaged and dead cells. Once in the extracellular environment, ATP is presented as an endogenous adjuvant that may initiate inflammation [6]. This step is followed by cellular damage and involves the activation of the P2X7 receptor, which is ubiquitous to most inflammatory conditions [7–9]. The P2X7 receptor has a longer carboxy-terminal domain than the other P2X receptors, which offers binding sites for different intracellular signaling molecules. The activation of the P2X7 receptor in transfected HEK 293 cells induces disturbances in the plasma membrane that result in blebs, small semi-spherical protrusions 1 to 10 μm in diameter [10]. In macrophages, the binding of ATP to the P2X7 receptor also opens a reversible pore in the membrane, which allows the inflow and outflow of molecules up to 900 Da [11]. The prolonged activation of the P2X7 receptor in macrophages by millimolar concentrations of ATP triggers cell

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death by apoptosis and necrosis [12–14]. Recently, it has been reported that ATP plays an important role in regulating the responses of intestinal T cells [15]. The authors have shown that the P2X7 receptor is highly regulated in the CD8 T cells of the intestinal epithelium and is functional and sensitive to the induction of apoptosis by ATP, identifying the P2X7 receptor as a novel regulatory element for the T cell responses of the intestinal mucosa [15]. The role of purinergic receptors in the enteric nervous system has also interested many groups. Sensory neurons appear to play an important role in inflammatory processes in the gut. Signal transduction appears to increase in the presence of ATP during inflammation, and several P2 receptors are positively regulated in chronic inflammatory conditions [16,17]. In 2005, our group demonstrated the functional expression of different subtypes of P2 receptors in two lines of human intestinal epithelial cells. We have shown that the stimulation or dysfunction of the P2X1-7 receptors $P2Y_1$ and $P2Y_2$ may contribute, at least in part, to the modulation of apoptosis and cell proliferation in the epithelial cells of human carcinoma [18]. It was proposed that purinergic signaling could act in the regulation of intestinal physiology [19,20] and that ATP is involved in the sensory/mechanical transduction of the inflamed colon in a model of rat colitis [21]. Therefore, the present study was undertaken to better characterize the effects of ATP in human intestinal epithelial cells and to reveal the morphological changes of these cells following incubation in the presence of ATP. The stimulus provided by ATP in intestinal cells and their consequent changes may indicate a key role for ATP in the initiation of primary immune responses in the gut.

2. Materials and methods

2.1. Cell culture HCT8

The human intestinal epithelial cell line HCT8 (human ileocecal adenocarcinoma cells) was obtained from an ATCC Cell Biology Collection stock culture (Manassas, VA). The cells were maintained in tissue culture flasks (TPP AG, Switzerland) at 37 °C in an atmosphere of 5% CO_2 in DMEM (Dulbecco's modified Eagle medium, Invitrogen) buffered with 3.7 g/L sodium bicarbonate and 5 g/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Sigma Aldrich, St. Louis, MO, USA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco/Invitrogen, Grad Island, NY, USA), 1% L-glutamine (Sigma Aldrich, St. Louis, MO, USA) and 10% fetal bovine serum (LGC Bio, São Paulo, Brazil). The medium was stored in fridge. Every 2 or 3 days, the cultures were expanded for use in experiments or maintenance in culture.

2.2. Primary cultures of macrophages

Murine macrophages were obtained from adult Swiss mice that were either untreated or treated with thioglycolate (Merck KGaA, Darmstadt, Germany) by harvesting the peritoneal cavity. The cell suspension was then plated in 24-well tissue culture plates (TPP AG, Switzerland) at a density of 4×10^5 cell per well and incubated in non-supplemented DMEM at 37 °C in a 5% CO_2 atmosphere for 1 h. The non-adherent cells were removed by washing with DMEM, and the adherent cells were cultured in DMEM complete medium.

2.3. Measurement of apoptosis by cytofluorometry

For the analysis of apoptosis, assays were performed by flow cytometry based on the identification of hypodiploid nuclei. HCT8 cells were plated in 96-well tissue culture plates (TPP AG, Switzerland) at a density of 5.0×10^4 cells per well. HCT8 cells were treated with various concentrations of ATP for different times at 37 °C in a 5% CO_2 incubator. In some experiments that investigated the effect of oxidants on the cellular response to ATP, the HCT8 cells were treated with 20 μM ascorbic acid (Sigma Aldrich, St. Louis, MO, USA) or 1 mM NAC (*N*-acetylcysteine; Sigma Aldrich, St. Louis, MO, USA) for 20 min before treatment with 2 mM ATP. After 4 h of ATP treatment in the presence of antioxidants, the medium was renewed, and the cells were kept in the CO_2 incubator for 24 h. The cells were then prepared for flow cytometry analysis as described below. At the end of the time specified for each experiment, the culture plates were centrifuged at 250 ×*g* for 5 min. Next, the cell cycle buffer (50 μg/ml ethidium bromide, 0.01 g of sodium citrate, 0.1% Triton X-100) was added to the cultures for 10 min. Later, with the aid of a pipette, the cells were homogenized in buffer, removed from the wells and transferred to flow cytometry tubes. The samples were stored in the dark at 4 °C until flow cytometry analysis. The fluorescence intensity was measured in at least 10,000 cells per sample using a FACScan flow cytometer (Becton-Dickinson) with an argon laser tuned to 488 nm. The cellular debris was excluded from analysis by raising the forward scatter (FS) threshold. The apoptotic cell nuclei containing hypodiploid DNA were enumerated as a percentage of the total population.

2.4. Lactate dehydrogenase (LDH) release assay

The cytoplasmic enzyme lactate dehydrogenase (LDH), which is constitutively expressed at high concentrations in eukaryotic cells, is highly stable and widely used as a marker of cell lysis resulting from the disruption of the plasma membrane that is characteristic of death by necrosis. HCT8 cells or macrophages obtained from the intraperitoneal lavage of Swiss mice were plated at concentrations of 5×10^4 and 4×10^5 cells per well, respectively, in 96-well plates and left to adhere for 1 h. The non-adherent cells were removed during the washing steps. Both cell types were treated or not with ATP for different times. At the end of the experiments, the culture supernatants were collected to determine the presence of LDH. LDH release was analyzed using a commercial kit based on a colorimetric assay (Doles Reagents, Goias, Brazil), and the resulting color variations were measured by an ELISA microplate reader (BIOTEC Mod Power Wazexs) with a filter to measure the absorbance at 490 nm. As a control for the comparison of cell damage caused by the treatments, 100% was assigned as the maximum value of LDH release in the supernatants of control cells disrupted by the use of 1% Triton X-100%.

2.5. Intracellular calcium measurements

HCT8 cell cultures were grown in 96-well plates (black well, clear bottom; BD Biosciences, NJ) until confluence. The cells were used after being plated at sub-confluence. The growth medium was then aspirated and replaced with 100 μl of loading medium (PBS containing 1 mM Fluo-4-AM, 10% pluronic acid, and 2.5 mM probenicid) and incubated for 30 min at room temperature. The cells were subsequently washed three times with PBS, and 100 μl of PBS supplemented with 1 mM $CaCl_2$ or with 5 mM EGTA was added to each well. The cells were then placed in a fluorometric imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA), and the changes in cellular fluorescence were recorded after the addition of 50 μl of the control buffer or 50 μl of the indicated concentration of the tested compounds in PBS.

In some experiments HCT8 cells were grown over glass coverslips on 35 mm plates for 24 h to subconfluence. The cells were loaded with 5 μM Fura 2-AM (Invitrogen) and 2.5 mM probenicide at 37 °C for 1 h in culture medium. The coverslips were washed in PBS and mounted in a three-compartment superfusion chamber attached to the stage of an inverted microscope (NIKON DIAPHOT 300 TMD) which base was formed by a coverslip containing the cells. The central chamber containing the cells had a volume of 300 μl with Ca^{2+}-containing saline (PBS supplemented with 1 mM $CaCl_2$) at room temperature. The intracellular calcium concentration of groups of 15–25 cells was monitored continuously with the use of a fluorescence photometer (Photon Technology; Princeton, NJ). Fura-2 was excited alternately at 340 and 380 nm, and the emission at 510 nm was measured. The ratio