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Research paper

Histamine stimulates secretion of extracellular vesicles with nucleotidase activity in rat submandibular gland

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ABSTRACT

Background: Extracellular vesicles released by different cells have been isolated from diverse fluids including saliva. We previously reported that rat submandibular glands secrete nanovesicles that catalyze hydrolysis of ATP, ADP and AMP, which are actors of the purinergic signaling system along with adenosine. Extracellular nucleotides like ATP and adenosine are involved in the regulation of inflammatory processes and apoptosis. Histamine, a widely distributed biogenic amine, is involved in inflammatory response.
Objective: To test if activation of histamine receptors in rat submandibular gland promotes changes in the release of vesicles with nucleotidase activity that could modulate purinergic signaling.
Methods: Rat submandibular glands were incubated in the absence or presence of histamine and JNJ7777120, an antagonist for H_4 receptors. Extracellular vesicles were isolated from incubation media by differential centrifugation. Vesicular nucleotidase activity was measured following Pi release by 3 mM MgATP, MgADP or MgAMP.
Results: Histamine increased the release of vesicles with nucleotidase activity in a concentration dependent manner. JNJ7777120 significantly reduced this effect. Vesicular nucleotidases obtained in the absence or presence of histamine promoted Pi production from ATP, ADP and AMP.
Conclusion: The results show a relationship between histamine and the regulation of purinergic signaling, which could be important in the modulation of inflammatory processes.

1. Introduction

Extracellular vesicles (EVs) released by different cells have been isolated from diverse fluids including blood, urine, saliva, breast milk, amniotic fluid, ascites, cerebrospinal fluid, bile and semen, and intense research is being directed to know their roles in the modulation of pathophysiological processes including cancer spread. They are released by direct budding from the cell plasma membrane (PM) or by fusion of internal multivesicular bodies (MVB) with the PM. Small (30–100 nm) vesicles of endosomal origin are called exosomes. However, most cells can probably release both PM- and endosome-derived vesicles. Although EVs secretion can be found as constitutive or regulated secretion depending on the cell type, little is known about the specific mechanisms that activate the EVs release (Colombo, Raposo, & Théry, 2014).

We previously reported that rat submandibular glands (SMG) incubated *in vitro* secrete exosome-like nanovesicles containing ectoenzymes that catalyze the hydrolysis of ATP, ADP and AMP which are actors of the purinergic signaling system along with adenosine, the final product of ATP hydrolysis (González et al., 2015). EVs from SMG could be released into salivary ducts to regulate luminal nucleotide concentrations. Purinergic metabotropic (P2Y1, P2Y2) and ionotropic (P2X4, P2X7) receptor subtypes were found in rat SMG, in both basolateral and luminal membranes in acinar and ductal cells (Burnstock & Knight, 2004; Lee et al., 1997; Turner, Landon, Gibbons, & Talamo, 1999). Particularly, it was shown that P2X7 activation by ATP regulates ionic currents, saliva volume, and also induces reactive oxygen species production, inflammatory responses and apoptosis in rat or mouse (Fontanils et al., 2010; Nakamoto et al., 2009; Woods et al., 2012). It is accepted that cell stress or apoptosis trigger the release of ATP, ADP and other nucleotides to the extracellular space, further advancing inflammatory responses mediated by purinergic P2 receptors. These

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extracellular nucleotides can be metabolized to adenosine, leading to a reduction in P2 signaling and increasing anti-inflammatory signaling by the interaction of adenosine with purinergic P1 receptors (Cekic & Linden, 2016). It is interesting to note that EVs released by salivary glands could arrive at the oral cavity where gingival epithelial cells, gingival fibroblasts, cells of the periodontal ligament, nerve cells and pulp cells, present purinergic receptors (J. C. Lim & Mitchell, 2012).

Histamine is a widely distributed biogenic amine that acts as an intermediary in several pathophysiological processes through activation of four receptor subtypes, of which H_1 and H_4 are related to inflammation. H_4 receptors have almost 10000-fold higher affinity for histamine than conventional H_1 receptors (e.g., pKi is 8.1 for hH_4R and 4.6 for hH_1R) (Panula et al., 2015). Little is known about the relationship between histamine and purinergic signaling. Histamine effects mediated by ATP release and activation of P2 receptors have been reported in guinea pig airways (C. Weigand, Ford, & Undem, 2012) and human subcutaneous fibroblasts (Pinheiro et al., 2013). Regarding the SMG, there are reports showing the secretagogues and anti- apoptotic effects of histamine that could be linked with the EVs secretion. The binding to H_1R promotes an increase in amylase secretion in rat SMG (Borda, Stranieri, & Sterin-Borda, 2002) and it activates aquaporin translocation in human submandibular cells, explaining the anti-histamine induced xerostomia (Kim et al., 2009). It was shown that histamine, binding to H_4R, has anti-apoptotic properties in human salivary cells (Stegajev et al., 2014). Previously, the application of subcutaneous histamine had completely prevented enhanced apoptosis of acinar and ductal rat SMG cells induced by experimental periodontitis or irradiation (Medina et al., 2011; Prestifilippo et al., 2012). Recently JNJ7777120, an H_4R antagonist, was paradoxically able to ameliorate periodontitis-induced injury on SMG, gingival tissue and bone structure (Prestifilippo et al., 2016).

The increase of extracellular ATP in oral tissues can cause apoptosis, bone loss, changes in the proliferation of the periodontal ligament and pain associated with inflammation. On the other hand, adenosine has an opposite role, as an analgesic, anti-inflammatory and tissue protector (J. C. Lim & Mitchell, 2012). Given that histamine −a pro inflammatory molecule- was shown to modulate SMG secretion and to provide protective effects to the gland, the aim of this study was to test whether the activation of histamine receptors in the rat SMG modifies the secretion pattern of EVs with nucleotidase activities that could regulate ATP and adenosine concentrations.

2. Materials and methods

2.1. Reagents and solutions

Unless otherwise noted, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Submandibular glands extracellular vesicles

Animals were treated in accordance with European Commission guidelines concerning the care and use of laboratory animals. Male, 3 months, 250–300 g Wistar-rats, were sacrificed by exposure to CO_2. For each experiment, 3 or 4 rats of the same age, reared in the same cage, were used. The SMG were removed and separated from the surrounding connective tissue and sublingual glands. To induce secretion *in vitro*, each gland was cut into pieces of 30–40 mg that were first washed by immersion during 15 min in cold physiological solution (PS) (115 mM NaCl, 5 mM KCl, 5 mM glucose, 10 mM Hepes, 25 mM $NaHCO_3$, pH 7.4), and then incubated in PS at 37 °C with carbogen bubbling (95% O_2:5% CO_2), with different additions according the experiment. On each experiment two conditions were compared, assigning them one of the two SMG of the same rat. SMG fragments were removed after 30 min and incubation media were centrifuged for 15 min at 2000*g* to separate cells or debris. Then, supernatants were centrifuged for 60 min at 27000*g* and the resulting pellets were washed in PS, centrifuged again and resuspended in 300 mM saccharose + 20 mM Hepes or in phosphate buffered solution (PBS), pH 7.2. Protein concentration was measured with Bradford assay, with bovine seroalbumin as standard.

2.3. Nucleotidase activity by extracellular vesicles

EVs nucleotidase activity was measured at pH 7.2 and 37 °C following Pi release. The incubation media contained 50 mM MOPS-Tris, pH 7.2, 100 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$. Reactions were started by addition of 3 mM ATP, ADP or AMP. At the selected times, the reactions were stopped by adding equal volume of 10% cold trichloroacetic acid. Pi production was determined with the colorimetric method of Baginski, based on the formation of a complex between Pi and ammonium molibdate (C. Baginski, Foà, & Zak, 1967).

2.4. Transmission electron microscopy

To analyze ultrastructure, SMG was cut into 2 × 2 mm fragments and immediately fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.5, for 4 h at 4 °C, washed with 0.32 M sucrose/phosphate, followed by postfixation with 1.5% osmium tetroxide. After washing with distilled water, the fragments were contrasted with 2% uranyl acetate overnight, dehydrated with a graded alcohol series, and then embedded in Spurr resin (Electron Microscopy Sciences, Hatfield, PA 19440, USA). Ultrathin (approximately 60 nm thick) slices obtained with a manual ultramicrotome Sorvall MT1 were collected using a 200-mesh grid and contrasted with 2% uranyl acetate solution and lead citrate 0.4%.

Extracellular vesicles resuspended in PBS were seeded (5 µl) on a grid covered by acrylic membrane and allowed to settle for 20 min at room temperature. Excess PBS was removed by wicking with filter paper before fixation with 2% paraformaldehyde and 2% glutaraldehyde in PBS for 2 min. Grids were washed 3 times with distilled water prior to application of 1% phosphotungstic acid counterstain for 1 min. Excess liquid were allowed to dry overnight at room temperature. Grids were analyzed with a Zeiss EM 109T transmission electron microscope equipped with digital camera Gatn ES1000W.

2.5. Statistical analysis

Data shown are mean ± standard error of the mean (SEM). Student's *t*-test for unpaired values was used to determine the levels of significance. Differences between means were considered significant if $p < 0.05$. The linear association was performed with the Pearson correlation analysis.

3. Results

3.1. Extracellular vesicles

To characterize EVs, rat SMG were incubated under physiological conditions to isolate nanovesicles released to the incubation media. An image obtained by TEM is shown in Fig. 1A; size distribution indicated that EVs could be a mixture of exosomes and PM derived vesicles (Fig. 1B). Fig. 1C and D, shows that the lumen next to the apical side of acinar cells contained vesicles which size and morphology are compatible with isolated EVs.

3.2. Histamine increases EVs-associated nucleotidase activities

The nucleotidase activities of the EVs isolated from SMG incubation media with or without histamine were evaluated. To study the ATPase activity, EVs were incubated with 3 mM MgATP. Fig. 2A shows that ATP hydrolysis rates decayed along incubation time, which was attributed to a decrease in NTPDase2 activity, an ecto-ATPase that is