



# Allergic sensitization increases the amount of extracellular ATP hydrolyzed by guinea pig leukocytes

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Received: 9 July 2018 / Accepted: 4 January 2019  
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## Abstract

Increased levels of ATP have been found in the bronchoalveolar lavage of patients with asthma, and subjects with this disease, but not healthy subjects, develop bronchospasm after nebulization with ATP. Because the main mechanism for controlling the noxious effects of extracellular ATP is its enzymatic hydrolysis, we hypothesized that allergic sensitization is accompanied by a decreased functioning of such hydrolysis. In the present study, peripheral blood leukocytes from sensitized and non-sensitized guinea pigs were used for determining the extracellular metabolism (as assessed by inorganic phosphate production) of ATP, ADP, AMP, or adenosine, and for detecting possible changes in the expression (qPCR and Western blot) of major ectonucleotidases (NTPDase1, NTPDase3, and NPP1) and purinoceptors (P2X<sub>1</sub>, P2X<sub>7</sub>, P2Y<sub>4</sub>, and P2Y<sub>6</sub>). Contrary to our hypothesis, we found that leukocytes from allergic animals produced higher amounts of inorganic phosphate after stimulation with ATP and ADP, as compared with leukocytes from non-sensitized animals. Although at first glance, this result suggested that sensitization caused higher efficiency of ectonucleotidases, their mRNA and protein expressions were unaffected. On the other hand, after sensitization, we found a significant increase in the protein expression of P2X<sub>7</sub> and P2Y<sub>4</sub>, two purinoceptors known to be responsible for ATP release after activation. We concluded that allergic sensitization increased the amount of ATP hydrolyzed by ectonucleotidases, the latter probably not due to the enhanced efficiency of its enzymatic breakdown, but rather due to an increased release of endogenous ATP or other nucleotides, partly mediated by enhanced expression of P2X<sub>7</sub> and P2Y<sub>4</sub> receptors.

**Keywords** Extracellular ATP · ATP hydrolysis · Ectonucleotidase · Purinoceptors · Allergic sensitization · Guinea pig asthma model

## Introduction

In addition to the well-known energetic role of intracellular adenosine 5'-triphosphate (ATP) in any cell that was described more than 80 years ago [1], when located extracellularly, ATP

and other related nucleotides produce diverse effects through activation of P2 purinoceptors. These receptors include the P2X and P2Y families of purinoceptors. The former is a group of ligand-gated ion channel receptors with seven sub-types (P2X<sub>1–7</sub>). The latter are G protein-coupled receptors with six members (P2Y<sub>1–6</sub>) [2–4].

Extracellular ATP, ADP, AMP, or adenosine may be involved in pathophysiological conditions such as platelet aggregation [5, 6], cardiovascular disease, immune response, chronic obstructive pulmonary disease, or asthma [7–11]. Mechanisms mediating extracellular ATP effects in human asthma or animal models of asthma include neurogenic bronchoconstriction [12], enhanced mast cell response to allergic stimuli [13], and TXA<sub>2</sub>-mediated bronchoconstriction [10]. Increased concentrations of ATP in the bronchoalveolar lavage of asthmatic subjects and sensitized mice have been found after an experimental antigenic challenge [14], and there is evidence that airways from asthmatic subjects are many times more sensitive to inhaled ATP than those from

**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s11302-019-09644-7>) contains supplementary material, which is available to authorized users.

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healthy subjects [15, 16]. Thus, extracellular ATP and related nucleotides pose a significant risk of noxious effects, and their concentrations are strictly regulated in all tissues through their active breakdown by a large family of ubiquitous enzymes called ectonucleotidases. Recently, we demonstrated that ectonucleotidases exhibited a protective role in a guinea pig model of allergic asthma, inasmuch as their inhibition greatly amplified the allergic bronchoconstriction [10]. Zhang et al. found in mice that ATP plays a crucial role in promoting the Th17-mediated cardinal features of neutrophilic allergic asthma [17].

Ectonucleotidases comprise three major families: the nucleoside triphosphate diphosphohydrolases (NTPDase), the nucleotide pyrophosphatase/phosphodiesterases (NPP), and the non-specific alkaline phosphatases. From these enzymes, only those that have their catalytic moiety in the outer part of the cell membrane are capable of metabolizing extracellular ATP/nucleotides, which include NTPDase1, NTPDase2, NTPDase3, NTPDase8, NPP1, NPP2, and NPP3 [18, 19]. With the exception of NTPDase8, these enzymes are all present in leukocytes from humans and other mammals [20, 21].

Recently, Montañó et al. reported that the rate of hydrolysis of exogenous ATP was reduced in total leukocytes from subjects suffering from severe asthma exacerbation, as compared with subjects with moderate or mild asthma exacerbation or with healthy blood donors [20]. A potential explanation of these results lies in the finding that mRNA expression of the *ENPP1* gene was notably reduced in leukocytes obtained during the severe asthma exacerbations. This study raised the possibility that the allergic process may interfere with the expression or functioning of ectonucleotidases, with an ensuing deficit in the hydrolysis of ATP/nucleotides. The aims of the present work were to explore whether allergic sensitization per se modifies the rate of hydrolysis of extracellular ATP or closely related molecules (ADP, AMP, and adenosine) by guinea pig peripheral blood leukocytes and to determine possible changes in mRNA or in the protein expression of major ectonucleotidases and purinoceptors.

## Materials and methods

### Animals

Male Hartley guinea pigs weighing ~320 g and raised in our laboratory animal facilities under conventional conditions, i.e., filtered air, 12/12 h light/dark cycles, drinking sterile water, and fed ad libitum with Guinea Pig Diet pellets (LabDiet, St. Louis, MO, USA) were used. Animal management was done according with the 2011 Guide for the Care and Use of Laboratory Animals. All procedures performed in guinea pigs were in accordance with the ethical standards of the institution, and the study protocol was approved by our institutional scientific and bioethics committees, with the approval number B23-12.

### Allergic sensitization procedure

Guinea pigs were sensitized to ovalbumin (OA) by administering 60 µg OA plus 1 mg Al(OH)<sub>3</sub>, both dissolved in 1 ml saline (0.9%) and equally distributed in the subcutaneous and intraperitoneal routes (day 0). This was followed by booster nebulization of 3 mg/ml OA in saline during 5 min (day 8) and of 1 mg/ml OA in saline during 10 s (day 15). Animals were studied at days 22–24. Healthy, non-sensitized guinea pigs with the same weight as experimental animals at the end of the sensitization procedures were used as control animals.

### Total leukocyte isolation

On the day of the study, animals were deeply anesthetized by means of the intraperitoneal administration of an overdose of sodium pentobarbital (~60 mg). A ~10–12-ml blood sample was obtained by transthoracic heart puncture. Blood was immediately transferred to heparinized tubes (Vacutainer®, Becton, Dickinson and Co., Franklin Lakes, NJ, USA), diluted in an equal amount of 0.9% saline, and mixed with Polymorphprep (Axis-Shield, Oslo, Norway) solution at a 1:1 (v/v) proportion. After centrifuging at 1800 rpm during 30–35 min at room temperature, a single band corresponding to total leukocytes was formed and harvested. This leukocyte-rich sample was washed twice by using culture media for leukocytes employed in the study of the kinetics of ATP hydrolysis (RPMI-1640, Lonza, USA) or 1% PBS for leukocytes utilized in qPCR. The remaining red blood cells were eliminated by a lysis solution (600 mg NaHCO<sub>3</sub>, 4.15 g NH<sub>4</sub>Cl, and 10 mg EDTA in 500 ml bidistilled water). Total leukocytes purified by this method were placed in a 1-ml working solution (0.5 mM CaCl<sub>2</sub>, 120 mM NaCl, 5 mM KCl, 60 mM glucose, 50 mM TRIS-HCl, pH 8.0) and, after reading in a Neubauer chamber, the total number of cells that we used in every experiment was normalized to 1 million cells in 1 ml of the same solution. Cell viability was tested by using Trypan blue, and it was corroborated to be >93%. The final fresh sample of leukocytes was immediately used for determination of the extracellular metabolism of nucleotides or adenosine, or frozen at –70 °C until qPCR or Western blot analyses.

### Determination of the extracellular metabolism of nucleotides and adenosine

Sixteen groups of experiments were performed by using ATP, ADP, AMP, or adenosine, each at concentrations of 0, 125, 250, or 500 µM. The number of experiments (each corresponding to a different guinea pig) was  $n = 5$  in the majority of cases, ranging from 3 to 7. To this aim, for every experiment, a total amount of 1 million total leukocytes in a 1-ml working solution was placed in a borosilicate glass tube that was maintained at 37 °C in a metabolic bath. Enzymatic

breakdown of nucleotides or adenosine was expected to occur progressively during the incubation of these compounds, with the ensuing release of inorganic phosphates in the case of ATP, ADP, and AMP. To monitor this metabolism, 70- $\mu$ l samples were taken from the warm tube at time 0 (immediately prior to adding the nucleotide or adenosine) and at 10, 20, 40, 60, 80, 100, and 120 min after the addition of the nucleotide or adenosine. In order to stop the enzymatic activity, each sample was immediately placed into a 1.5-ml plastic tube (MTC-150C, Axygen Inc., Union City, CA, USA) previously cooled by maintaining it for at least 30 min in ice. We corroborated that passive cooling is enough to reduce the enzymatic activity of extracellular ectonucleotidases by about 50% (data not shown). After the last sample was obtained, 32  $\mu$ l of the content of each tube was transferred onto a 96-well microplate, and then 125  $\mu$ l of a mixture of 0.84-mM malachite green and ammonium molybdate 5.72% in 6 N HCl were added to each well. Reaction of these compounds with inorganic phosphates produces a color change that was read in a spectrophotometer at 630 nm. The amount of inorganic phosphate production was calculated by comparing the readings in the sample with a standard curve performed with  $\text{KH}_2\text{PO}_4$  (0–100  $\mu$ M). Because the upper limit of detection was 100  $\mu$ M, all measurements above this threshold were considered as a concentration of 100  $\mu$ M inorganic phosphate. Total amount of inorganic phosphate production in each experiment was expressed as area under the curve.

### qPCR

After the total leukocyte sample was thawed, RNA was extracted by using the Direct-zol RNA MiniPrep kit (Zymo Research, Bentley, CA, USA), following the manufacturer's instructions. Isolated RNA was used as template to produce cDNA by means of a RevertAid First Stand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The resulting cDNA was stored at  $-80^\circ\text{C}$  until its assay.

qPCR was performed using custom-made Taqman probes for genes coding three ectonucleotidases (*Entpd1*, *Entpd3*, and *Enpp1*) and four receptors (*P2rx1*, *P2rx7*, *P2ry4*, and *P2ry6*), and for the internal control gene (*Gapdh*). DNA sequences of these genes were designed by using the Primer3web, v4.1.0, and this can be consulted in Supplemental Table 1. Briefly, 15  $\mu$ l cDNA was added to 10  $\mu$ l TaqMan Universal Master Mix II with UNG 2X (Applied Biosystems, Foster City, CA, USA), 1  $\mu$ l TaqMan Assay 20X, and RNA-free water sufficient for 20  $\mu$ l. Cycling reactions were programmed as follows: 2 min at  $50^\circ\text{C}$  (1 cycle), 10 min at  $95^\circ\text{C}$  (1 cycle), and 15 s at  $95^\circ\text{C}$  followed by 1 min at  $60^\circ\text{C}$  (40 cycles).

All measurements were performed in triplicate and the median was considered as final value. Results were normalized by logarithm transformation of individual values. The relative

expression of each target gene, as related to the expression of the control gene (*Gapdh*) in the same sample, was calculated by the  $2^{-\Delta\text{CT}}$  formula. Additionally, target/control relative expressions in leukocyte samples from sensitized guinea pigs were compared with those observed in non-sensitized animals by using the  $2^{-\Delta\Delta\text{CT}}$  method [22]. The number of experiments (each corresponding to a different guinea pig) was  $n = 6$  in all groups.

### Western blot

Total leukocyte samples were thawed, and 250  $\mu$ l of lysis solution containing 50 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.1 mM EGTA, 150 mM NaCl, 1% Igepal®, 1 mM  $\text{Na}_3\text{VO}_4$ , 5 mM NaF, 0.25% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml pepstatin, and a protease inhibitor cocktail (Set III, cat. 539134; Calbiochem, La Jolla, CA, USA, 1:250, v/v) was added. The sample was centrifuged at  $4^\circ\text{C}$  and 3000 rpm during 5 min. Total protein content was determined in the supernatant using a commercial kit (DC Protein Assay, Catalog 500-0116, Bio-Rad, Hercules, CA, USA). Forty micrograms of protein from each sample were loaded in different lanes of a 10% SDS-polyacrylamide gel and subjected to electrophoresis under reducing conditions. Afterward, proteins were transferred onto a nitrocellulose membrane and blocked with 5% nonfat dry milk in TBS-Tween (Tween 20, 0.1%) during 1 h at room temperature. Membranes were incubated overnight at  $4^\circ\text{C}$  with rabbit polyclonal antibody (Alomone Labs, Jerusalem, Israel) raised against  $\text{P2X}_1$  (cat. APR-001, 1:1000, v/v),  $\text{P2X}_7$  (cat. APR-004, 1:1000, v/v),  $\text{P2Y}_4$  (cat. APR-006, 1:300, v/v), and  $\text{P2Y}_6$  (cat. APR-011, 1:200, v/v) receptors, rabbit monoclonal antibody (Abcam, Cambridge, MA, USA) raised against NTPDase1 (CD39, cat. ab108248, 1:1000, v/v), or rabbit polyclonal antibody raised against NPP1 (cat. ab217368, 1:250, v/v) or NTPDase3 (cat. ab96335, 1:1000, v/v). Membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (cat. 12-348, Millipore, Temecula, CA, USA, 1:5000, v/v) as secondary antibody during 1 h at  $37^\circ\text{C}$ . Immunoblots were developed employing an enhanced chemiluminescent reactant (Super Signal West Femto Maximum Sensitivity Substrate, cat. 34096; Thermo Scientific, Rockford, IL, USA). GAPDH was blotted as a control for protein load by using a mouse monoclonal antibody (cat. 39-8600, Invitrogen, Frederick, MD, USA, 1:1000, v/v) with a horseradish peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (cat. ab97046, Abcam, Cambridge, MA, USA, 1:10,000, v/v). Immunolabeling specificity for each receptor was corroborated by utilizing the respective blocking peptides (1  $\mu$ g/1  $\mu$ g). Guinea pig tissues were used as positive controls in the NPP1 (kidney) and NTPDase1 and NTPDase3 (pancreas) assays.

Immunoblots were analyzed by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Data were expressed as the relative expression of target protein adjusted by control protein (GAPDH). We used the log-transformation of each relative-expression value to achieve better normality. The number of experiments (each corresponding to a different guinea pig) for each group was  $n = 4$  in most cases, ranging from 3 to 5.

## Data analysis

Normal distribution of variables was corroborated by the Shapiro-Wilk test. Due to the limited number of total leukocytes obtained from each animal, not all techniques were performed on the same sample; therefore, a non-paired analysis was mandatory. Thus, differences between sensitized and non-sensitized animals regarding phosphate production (area under the curve), and mRNA- and protein-expression levels were evaluated using the unpaired Student's  $t$  test. Data in the text and illustrations correspond to mean  $\pm$  standard error. Statistical significance was set at two-tailed  $p < 0.05$ .

## Results

As can be observed in Fig. 1, the evaluation of inorganic phosphate production as surrogate of nucleotide metabolism indicated that, even under baseline conditions, i.e., without any stimulation, there was a low spontaneous rate of phosphate production, suggesting that the metabolism of spontaneously released nucleotides was occurring. Addition of 125, 250, or 500  $\mu\text{M}$  ATP, ADP, or AMP to total leukocytes from non-sensitized animals produced time- and concentration-dependent phosphate production. Interestingly, 125, 250, or 500  $\mu\text{M}$  adenosine caused a somewhat similar concentration-dependent response, although at a much lower level than with ATP, ADP, or AMP. This adenosine-induced phosphate production suggests that adenosine (which lacks phosphates) induced the release of nucleotides or another phosphorylated molecule capable of being metabolized.

In leukocytes from sensitized guinea pigs, incubation with the same three concentrations of ATP or ADP produced higher amounts of phosphates, both with a  $p < 0.05$ , as compared with samples from non-sensitized animals. In contrast, incubation with AMP produced a lower amount of phosphates ( $p < 0.05$ ) in leukocytes from sensitized guinea pigs, as compared with non-sensitized ones, while adenosine did not reach statistically significant differences.

In general terms, the rank order of nucleotide and adenosine metabolisms can be described as  $\text{ATP} > \text{ADP} \gg \text{AMP} > \text{adenosine}$  in leukocytes from non-sensitized animals and as  $\text{ATP} > \text{ADP} \gg \text{AMP} = \text{adenosine}$  in leukocytes from sensitized guinea pigs.

With respect to leukocyte mRNA expression of purinergic receptors (Fig. 2), the rank order of expression was  $P2ry4 = P2ry6 > P2rx1 \gg P2rx7$ , but there were no differences between sensitized and non-sensitized animals. Regarding ectonucleotidases mRNA expression, the three enzymes analyzed (*Entpd1*, *Entpd3*, and *Enpp1* genes) were expressed to a similar degree and, as occurred with purinergic receptors, there were no differences between sensitized and non-sensitized samples.

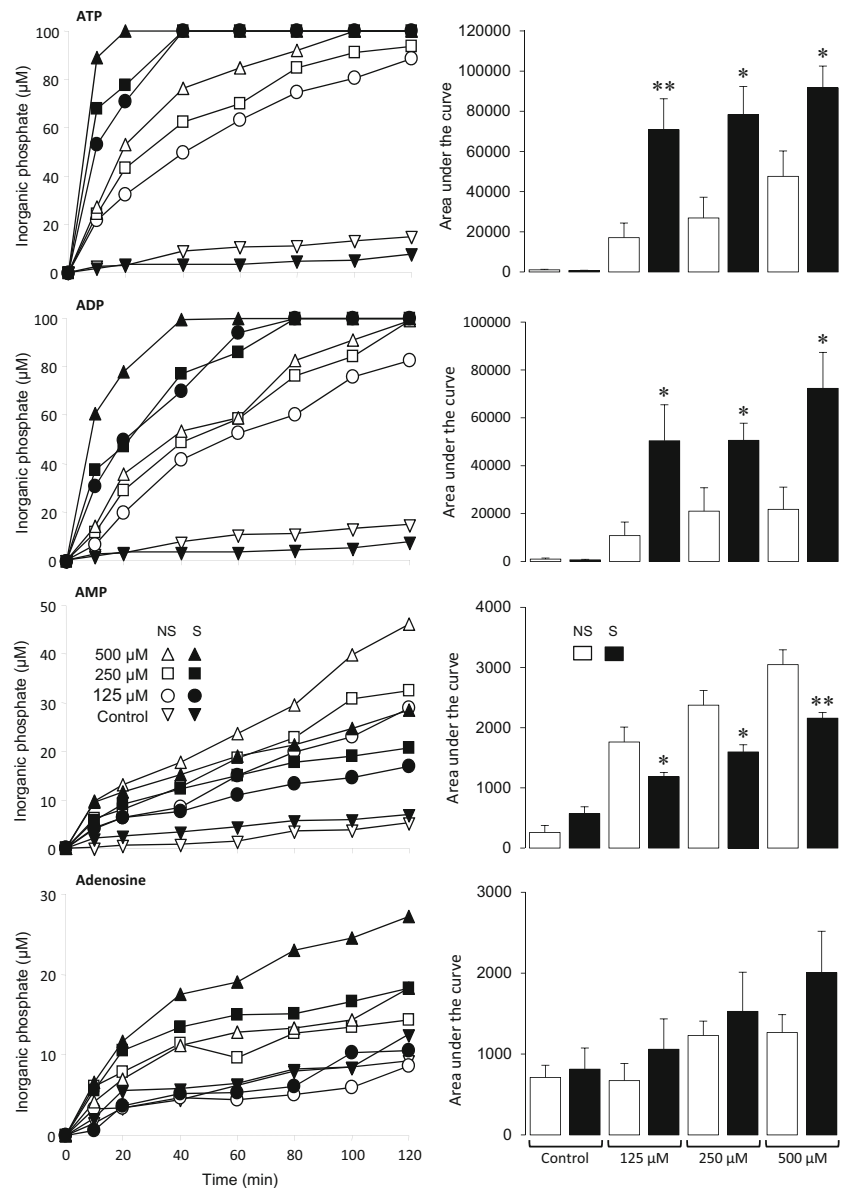
The protein expression of purinergic receptors, as assessed by Western blot assay (Fig. 3), showed that  $\text{P2X}_1$  was by far the most abundantly expressed receptor in leukocytes from either sensitized or non-sensitized animals. The sensitization procedure was accompanied by a statistically significant increase in protein expression of  $\text{P2X}_7$  and  $\text{P2Y}_4$ .

## Discussion

In the present study, we explored whether the rate of hydrolysis of extracellular ATP, ADP, or AMP was modified by the allergic sensitization of guinea pigs. Our hypothesis was that allergic sensitization would be associated with a *decreased* rate of metabolism of these nucleotides (i.e., a lower ectonucleotidase activity). If our results had corroborated this hypothesis, it could be speculated that under in vivo conditions, allergic subjects (humans or animals) may have higher amounts of extracellular nucleotides in their lungs, increasing the possibility of ATP-induced bronchospasm. In line with this possibility, Montañó et al. recently described that subjects with severe asthma exacerbation have a decreased expression of *ENPP1* mRNA, with no differences in the mRNA expression of other ectonucleotidases such as *ENTPD1*, *ENTPD2*, *ENTPD3*, *ENTPD8*, *ENPP2*, or *ENPP3* genes [20]. Contrary to our postulate, results from the present study showed that allergic sensitization apparently *increased* ATP and ADP hydrolysis, manifested by a greater amount of inorganic phosphate production. At first glance, these paradoxical results would suggest that sensitization caused higher expression of ectonucleotidases or enhancement of their enzymatic activity. However, this was not supported by the results from qPCR and Western blot experiments, inasmuch as we did not find modifications in the mRNA or protein expression of major ectonucleotidases (NTPDase1, NTPDase3, and NPP1).

In view of the aforementioned results, a second possibility to explain the increased production of inorganic phosphates (in the absence of modifications of the enzymatic activity of ectonucleotidases) is that the amount of extracellular purinergic nucleotides available for metabolism was increased. This hypothesis gains support with the phenomenon of “ATP-induced ATP/nucleotides release”, which was recently described in our laboratory in human total leukocytes [23]. Briefly, this phenomenon implicates that extracellular

**Fig. 1** Effect of allergic sensitization on the ectonucleotidases activity, as assessed by inorganic phosphate production, in guinea pig peripheral-blood total leukocytes. Left panels show that there was a time- and concentration-dependent increase in inorganic phosphates production after addition of 125, 250, or 500  $\mu$ M ATP, ADP, AMP, or adenosine, reaching higher amounts in leukocytes from sensitized animals (closed symbols), as compared with non-sensitized animals (open symbols); symbols correspond to median values (measures of dispersion are not shown for clarity). Right panels show the means  $\pm$  standard errors of the area under the curve from experiments with sensitized (closed bars) and non-sensitized (open bars) animals. The number of experiments (each made in different guinea pigs) was  $n = 5$  in most cases, ranging from 3 to 7. Upper limit of detection was 100  $\mu$ M, and all measurements above this threshold were set at 100  $\mu$ M. \*\* $p < 0.01$  and \* $p < 0.05$  vs non-sensitized group (non-paired Student's  $t$  test)



(exogenous) ATP added to total leukocytes isolated from peripheral blood induces the release of more ATP or other nucleotides by means of the activation of purinergic receptors P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub> and, perhaps, P2X<sub>1</sub> and P2X<sub>7</sub>. Therefore, it is possible that, in the present study, part of the inorganic phosphate production was due to endogenous ATP/nucleotides released from total leukocytes after stimulation of purinergic receptors by exogenous ATP.

In contrast with the lack of modifications in the relative mRNA expression of purinergic receptors after the sensitization procedure, it was evident that the protein expression of P2X<sub>7</sub> and P2Y<sub>4</sub> receptors was greater after the animal became allergic. Although at first glance this discrepancy between mRNA and protein expression could be surprising, such disagreement has been well described and explained through transcriptional regulatory mechanisms,

differences in protein half-lives, and the degree of mRNA stability [24–26]. Thus, allergic sensitization may modify some of these mechanisms, leading to higher expression of P2X<sub>7</sub> and P2Y<sub>4</sub> receptor protein despite the null effect on their mRNA expression. P2X<sub>7</sub> and P2Y<sub>4</sub> receptors are known to induce ATP release either via direct ATP efflux through P2X<sub>7</sub> receptor [27], which is a non-selective cation channel, or via ATP exocytosis secondary to P2Y<sub>4</sub> receptor activation [28]. The potential relevance of an increased expression of the P2X<sub>7</sub> receptor is outlined by the results of the study by Manthei et al. indicating that a low functionality of this receptor protected from asthma inception in a cohort of 172 children followed up from birth to 11 years of age [29]. Thus, one possible scenario is that allergic sensitization enhances these or other mechanisms leading to increased efficiency of the ATP-induced ATP/

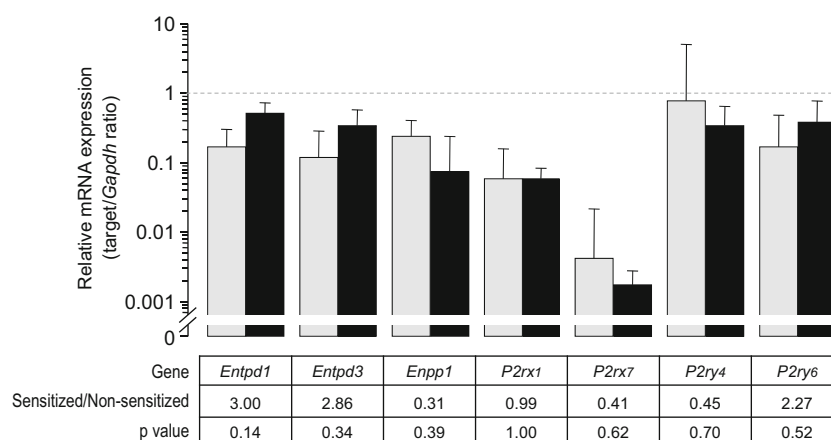


**Fig. 2** Lack of effect of allergic sensitization on the mRNA expression of major ectonucleotidases and purinoceptors in guinea pig peripheral-blood total leukocytes. Each bar corresponds to the expression the target mRNA in either non-sensitized (gray bars) or sensitized (closed bars) animals, adjusted by the constitutive gene (*Gapdh*) and calculated with the  $2^{-\Delta CT}$  method (geometric mean  $\pm$  standard error of  $n = 6$  animals, each by

triplicate). The horizontal broken line indicates the baseline level of expression of the constitutive gene. The inset table shows adjusted mRNA expression in sensitized guinea pigs as related to non-sensitized animals, calculated with the  $2^{-\Delta\Delta CT}$  method, and the  $p$  value on comparing both groups (Student's  $t$  test)

nucleotide release phenomenon, which in turn produces higher amounts of extracellular ATP available for its metabolism.

Interestingly, adenosine, which does not include any phosphate in its molecule, was also capable of producing the release of inorganic phosphates after being added to the total leukocyte preparation. This result strongly suggested that, as with ATP itself, adenosine also releases ATP or other nucleotides, which are then metabolized by ectonucleotidases and produce the time-dependent increase of inorganic phosphates. Although in a previous study we found that the pharmacological antagonism of  $A_1$ ,  $A_{2A}$ ,  $A_{2B}$ , or  $A_3$  receptors did not modify ATP-induced ATP/nucleotides release [23], adenosine has proven to be capable of releasing ATP in some cells via activation of  $A_1$  receptors [30, 31]; thus, the possibility that in our experiments adenosine

actively released ATP/nucleotides remains valid. Furthermore, we have performed preliminary experiments employing direct measurement of nucleotides (ATP, ADP, and AMP) by RP-HPLC/UV, as described by Graven et al. [32], and we found evidences that adenosine indeed enhanced the basal release of ATP (Supplemental Fig. S1).

We found that, even in the absence of any stimulus, there is a progressive production of low levels of inorganic phosphates, indicating the release and subsequent metabolism of nucleotides or of any other compound with one or more phosphates integrated into its molecule and susceptible to being hydrolyzed. This spontaneous or tonic release of ATP observed in peripheral blood leukocytes is in agreement with the same phenomenon observed in other non-excitable cells [33,

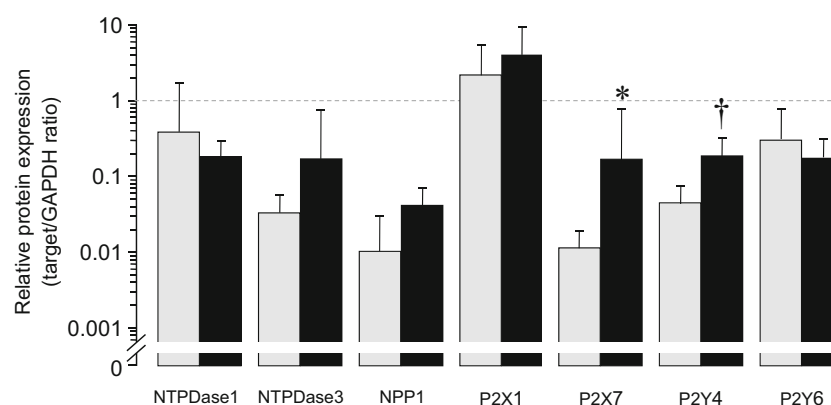


**Fig. 3** Effect of allergic sensitization on protein expression of major ectonucleotidases and purinoceptors in guinea pig peripheral blood leukocytes. While the protein expression of ectonucleotidases was not influenced by sensitization, in the purinoceptors analysis, only P2X<sub>7</sub> and P2Y<sub>4</sub> showed higher expression in sensitized animals, compared with control animals. Bars correspond to the geometric mean  $\pm$  standard

error of  $n = 4$  guinea pigs in most groups, ranging from 3 to 5, of sensitized (closed bars) or non-sensitized (gray bars) animals. The horizontal broken line indicates the baseline level of expression of the constitutive protein. GAPDH was used as constitutive protein. \* $p < 0.05$  and  $^{\dagger}p < 0.01$  (unpaired Student's  $t$  test)

34]. Preliminary results with direct measurement of nucleotides, as illustrated in the Supplemental Fig. S1, also support this spontaneous release.

Our experiments were performed in peripheral blood leukocytes, but asthma is a disease of the airways. Thus, it would be interesting to know whether constitutive or inflammatory cells in airways differ from peripheral blood leukocytes in terms of their nucleotide metabolizing potential. Experiments on this issue are ongoing in our laboratory, and we have indeed preliminary evidences that cells recovered from bronchoalveolar lavage (mostly macrophages) in non-sensitized guinea pigs had >40% larger production of inorganic phosphates after 500  $\mu$ M ATP than total leukocytes from peripheral blood (data not shown).

At first sight, our finding that sensitization did not induce changes in the mRNA levels of ectonucleotidase appears to disagree with a previous study from our laboratory in which blood leukocytes from patients with severe asthma exacerbations had reduced expression of *ENPP1* mRNA. Apart from differences in animal species, a possible explanation for this discrepancy is that, in the present study, we used leukocytes from guinea pigs that were sensitized but not antigenic challenged (which would resemble an asthma exacerbation). Thus, differences in animal species and severity of the condition could explain the contrasting results.

In conclusion, our results suggested that allergic sensitization increased the amount of ATP hydrolyzed by ectonucleotidases, probably not due to the enhanced efficiency of its enzymatic breakdown, but instead due to an increased release of endogenous ATP or other nucleotides, partly mediated by the enhanced expression of P2X<sub>7</sub> and P2Y<sub>4</sub> receptors. These findings might have implications in the clinical setting. Asthma is a disease with a strong background in allergy, either generalized or confined to lung tissue [35], and is highly prevalent worldwide, with an estimated amount of over 235 million subjects suffering from the disease [36]. Hypothetically, if our results could be transferred to humans, it would be possible to envisage a scenario in which patients with asthma have a trend toward a facilitated release of ATP. In well-controlled patients, the negative effect of extracellular ATP surely would be counteracted by its active breakdown by ectonucleotidases. However, in the eventual case of this enzymatic activity being impaired, for example after cold exposure or viral respiratory infections, the noxious effect of ATP could be expressed.

## Compliance with ethical standards

**Conflict of interest** Jaime Chávez declares that he has no conflict of interest.

Mario H. Vargas declares that he has no conflict of interest.

Jesús Martínez-Zúñiga declares that he has no conflict of interest.

Ramcés Falfán-Valencia declares that he has no conflict of interest.

Enrique Ambrocio-Ortiz declares that he has no conflict of interest.

Verónica Carbajal declares that she has no conflict of interest.

Rosa Sandoval-Roldán declares that she has no conflict of interest.

**Ethical approval** Animal management was done according with the 2011 Guide for the Care and Use of Laboratory Animals. All procedures performed in guinea pigs were in accordance with the ethical standards of the institution, and the study protocol was approved by our institutional scientific and bioethics committees, with the approval number B23-12.

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