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Decreased expression of ectonucleotidase E-NPP1 in leukocytes from subjects with severe asthma exacerbation

Luis M. Montaña,^{1*} Mario H. Vargas,^{2*} Marlon De Ita-Ley,^{1, 3} Verónica Díaz-Hernández,³ Renata Kasakova,¹ Carlos Barajas-López,⁴

¹ Departamento de Farmacología, Facultad de Medicina, Universidad Nacional Autónoma de México, México DF, México

² Departamento de Investigación en Hiperreactividad Bronquial, Instituto Nacional de Enfermedades Respiratorias, México DF, México

³ Departamento de Embriología, Facultad de Medicina, Universidad Nacional Autónoma de México, México DF, México

⁴ División de Biología Molecular, Instituto Potosino de Investigación Científica y Tecnológica, San Luis Potosí, SLP, México

* Drs. Montaña and Vargas participated equally in this work.

Correspondence and reprint requests:

Dr. Luis M. Montaña

Departamento de Farmacología,

Edificio de Investigación, sexto piso, laboratorio 3,

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Facultad de Medicina, Universidad Nacional Autónoma de México,
Ciudad Universitaria, CP 04510, México DF, México
Tel/fax: (+55)56665868
E-mail: lmmr@unam.mx

Short title: Ectonucleotidases in severe asthma exacerbation

ABSTRACT

Several studies suggest that ATP and related nucleotides play a role in the pathophysiology of asthma. However, the functionality of ectonucleotidases in this disease has been scantily investigated. We studied total ectonucleotidase activity in leukocytes from patients suffering from asthma exacerbation and explored the expression of E-NTPDase 1, 2, 3, and 8, and E-NPP1, 2, and 3, in their polymorphonuclear cells by immunofluorescence and qPCR. Leukocytes from patients with mild or moderate asthma exacerbation had similar ectonucleotidase activity than leukocytes from healthy subjects, while in patients with severe asthma exacerbation, this activity was lower. Of the ectonucleotidases studied, only E-NPP1 displayed diminished immunofluorescence and a significant decrease in its mRNA expression, both in patients with severe asthma exacerbation. This reduced E-NPP1 expression could be responsible for increased amounts of ATP or other nucleotides, capable of worsening asthma exacerbation, and warranting further investigation.

Key words: Severe asthma exacerbation, ectonucleotidase, E-NPP1, ATP

Introduction

There is mounting evidence that adenosine 5'-triphosphate (ATP) and related nucleotides play a role in the pathophysiology of bronchoconstriction and asthma. Among the first studies on this issue, Pellegrino et al. found that inhaled ATP caused acute airway obstruction in healthy subjects, and that this obstruction was much more intense in patients with asthma (1). Later, Idzko et al. detected increased concentrations of ATP in bronchoalveolar lavage (BAL) fluid during the course of an antigenic challenge, either in subjects with asthma or in sensitized mice (2). In their study, the authors found that blocking the action of ATP with apyrase (an ATP-degrading enzyme) or suramin (a non-selective antagonist of purinergic receptors) significantly diminished eosinophilia, airway inflammation, and bronchial hyperresponsiveness. Finally, in a previous study, we found that inhibition of ectonucleotidases with ARL-67156 greatly intensified allergic bronchoconstriction in sensitized guinea pigs, suggesting that ectonucleotidases play a protective role against potential airway obstruction induced by ATP or other nucleotides (3).

Concerning the nucleotide-degrading enzymes, three major groups comprise eight types of ectonucleotide triphosphate diphosphohydrolases (E-NTPDase), seven types of ectonucleotide pyrophosphatase/phosphodiesterases (E-NPP), and five isoenzymes of alkaline phosphatases, respectively. Among these, E-NTPDase 1, 2, 3, and 8, and E-NPP1, 2, and 3 have their catalytic moiety located on the extracellular surface of the plasma membrane (4, 5), conferring the capability to metabolize extracellular ATP.

Li et al. (6) reported reduced expression of E-NTPDase 1 (CD39) in bronchial epithelial cells from a pulmonary allergy model in mice, and they found that this reduction was associated with a higher degree of allergic inflammation in the airways. This same research group demonstrated that in CD39^{-/-} knockout mice, airway inflammation after an antigenic challenge

was more intense than in the wild-type mice (7). These studies indicated that a relative deficiency in the extracellular ATP breakdown would worsen allergic airway inflammation. In this context, asthmatic airways have an underlying inflammatory process that greatly accounts for airway hyperresponsiveness and propensity to develop asthma exacerbations. At least hypothetically, this airway inflammation could be enhanced if a relative deficiency of ectonucleotidase activity were present.

Methods

Detailed methods are described in the Online Supporting Information (Appendix S1).

We studied patients with asthma exacerbation, which was categorized as mild, moderate, or severe according to international criteria. In order to explore whether asthma exacerbation was associated with a relative deficiency of the purine nucleotide metabolism, we determined total ectonucleotidase activity by the inorganic phosphate production technique in leucocytes from these patients and compared this activity with results obtained in a healthy control group (blood donors). Likewise, we explored the type and level of expression of ectonucleotidases in polymorphonuclear cells (PMNs) from all subjects by analyzing the protein and mRNA expression through immunofluorescence and quantitative PCR (qPCR), respectively.

Results

As can be observed in Fig. 1, after the addition of 500 μ M ATP to the leukocytes, there was a progressive, time-dependent increment of the inorganic phosphate concentration in all groups, suggesting the occurrence of an active breakdown of ATP by membrane ectonucleotidases. Cells from patients with mild or moderate asthma exacerbation showed a similar response to that in

healthy control subjects. In contrast, in patients with severe asthma exacerbation, ectonucleotidase activity was notably lower than in the control group, reaching a statistically significant difference at the end of the period studied (128 min, $p<0.01$) and in the whole area under the curve ($p<0.05$).

Concerning the expression of membrane ectonucleotidases, E-NTPDase 3 and 8 were not detected in PMNs by immunofluorescence (data not shown). Although qPCR was also unable to detect E-NTPDase 8 mRNA, in the case of E-NTPDase 3, the qPCR indeed detected the mRNA in PMNs from healthy subjects, but values were very close to the equipment's detection limit (data not shown), while it was absent in samples from subjects with asthma. Of the remaining ectonucleotidases, E-NPP1 was the enzyme exhibiting the most striking changes (Fig. 2). In this regard, immunofluorescence for E-NPP1 was clearly observed in cells from healthy controls, and this expression appeared to be similar in cells from patients with mild or moderate asthma exacerbations. In contrast, PMNs from patients with severe asthma exacerbation displayed a dramatic diminution of immunofluorescence to E-NPP1. These findings were in line with results from qPCR, in which mRNA expression was notably diminished in patients with severe asthma exacerbation ($p<0.005$ vs the control group).

With respect to E-NPP2 and 3 (Online Figure S1), as well as for E-NTPDase 1 and 2 (Online Figure S2), there were no apparent changes in immunofluorescence between healthy controls and patients with asthma. Likewise, there were no statistically significant changes in mRNA expression between the groups studied.

Discussion

We found that patients with severe asthma exacerbation had lower ectonucleotidase enzymatic activity in their peripheral blood leukocytes. This was accompanied by a significant decrease in the expression of E-NPP1 mRNA and a noticeable reduction of the protein immunolabeling of this ectonucleotidase in PMNs.

Currently, there is increasing awareness that ATP and related nucleotides are involved in a number of pathophysiological lung responses, such as bronchoconstriction, inflammation, and airway smooth muscle remodeling (2, 3, 8). Thus, an exquisite regulation controlling extracellular ATP levels through its enzymatic breakdown by ectonucleotidases must exist in order to avoid these harmful effects. Multiple types of stimuli are able to trigger the release of ATP from non-neuronal cells, including antigenic challenge, Th2-type cytokines, such as IL-4 and IL-13, tobacco smoke, receptor-mediated stimulation, physical perturbation, ischemia, hypoxia, and interaction with pathogens (9-13). Moreover, in the majority of living tissues, massive release of ATP acts as an indicator of cell damage. It is conceivable that many of these ATP-releasing mechanisms could be operating in the asthmatic airways, where an inflammatory process is well known to be established. In this context, a reduction of the ectonucleotidase activity in patients with severe asthma exacerbation could be associated with larger amounts of ATP in the lung.

The decrease of E-NPP1 immunoreactivity and mRNA expression suggests that probably this is one of the ectonucleotidases responsible for the lower enzymatic activity in PMNs from patients with severe asthma exacerbation. Some authors have already found that ectonucleotidases expression may be reduced in pathological states. Thus, Li et al. (6, 7) demonstrated that E-NTPDase 1 expression in bronchial epithelium was decreased in a mice

model of allergic airway inflammation. Recently, Wang et al. found that mRNA expression of CD39 (i.e., E-NTPDase 1) was diminished in peripheral blood mononuclear cells from 18 patients with persistent asthma and allergy to house dust mites (14). This last study was contrasting with our results, inasmuch as we did not find modifications of the E-NTPDase 1 mRNA, but this difference might be due to the different cell type that we studied (PMNs).

Asthma exacerbations can develop within a wide range of severity. Although severe asthma exacerbations are relatively uncommon, they often require admission to hospital or intensive care unit (15). Furthermore, severe asthma exacerbations are mainly responsible for deaths due to asthma. Thus, elucidation of mechanisms leading to this heightened severity is important. According to our results, decreased expression and activity of ectonucleotidases in leukocytes, especially E-NPP1, could be one such mechanism.

Some studies have shown that during severe asthma exacerbation, airway inflammation tends to be neutrophilic in nature, mainly if the crisis fully developed in a few hours in so-called sudden-onset fatal or near-fatal asthma (16). Neutrophils appear to play an additional role in asthma cases with persistent airway obstruction and relative insensitivity to inhaled corticosteroids (17). Some molecules have traditionally been considered major chemotactic agents for neutrophils, such as CXCL8 (IL-8) and leukotriene B₄ (LTB₄) (18, 19). However, it is increasingly evident that secondary release of ATP from these cells, with ensuing autocrine stimulation through P2Y₂ receptors, is highly relevant for the cellular orientation in the chemotactic gradient. Moreover, once ATP is metabolized by ectonucleotidases, the resulting adenosine molecule is important for enhancing cell-migration speed through the activation of A₃ receptors (20). In this regard, during asthma exacerbation, decreased activity and/or expression of ectonucleotidases in peripheral leukocytes (as found by our group in the present study, and by

Wang et al. (14)) or in other location, such as airway epithelium (as found by other authors (6)), accompanied by increased amounts of local extracellular ATP due to the inflammatory process, would explain the large number of PMNs infiltrating the airways in the most severe episodes.

Our finding that ectonucleotidase activity is decreased in severe asthma exacerbation raises the interesting speculation that administration of an exogenous ectonucleotidase such as apyrase may improve the therapeutic management of these patients. Apyrase has been shown to inhibit the development of airway inflammation in a murine model of allergic asthma (6), and we already found that its intratracheal administration greatly diminished allergic bronchospasm in guinea pigs (3).

One limitation of the study was that we were unable to follow up the patients after their severe asthma exacerbation subsided. Thus, we could not discern whether the reduced ectonucleotidase activity and E-NPP1 expression were due to severe asthmatic exacerbation or whether it was a long-standing feature of patients prone to develop severe exacerbations. If the latter possibility were true, then it would be interesting to investigate the origin of these ectonucleotidase abnormalities.

In conclusion, we found that patients with severe asthma exacerbation had reduced activity of leukocyte ectonucleotidases and reduced expression of E-NPP1 in PMNs. The biological relevance of these findings warrants further research.

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Authors' contributions

LMM contributed to the design and global supervision of the study, data analysis, and the writing of the manuscript. MHV contributed to the study design, data analysis, and writing of the manuscript. MDIL participated in the recruitment and blood sampling of control subjects and patients with asthma, leukocyte purification, qPCR experiments, and the writing of the manuscript. VD contributed to confocal experiments, mRNA extraction, and qPCR experiments. RK participated in the recruitment and blood sampling of control subjects and patients with asthma, and in the measurement of ectonucleotidase activity. CBL contributed to the critical revision of the manuscript. All authors read and gave the final approval of the version to be published.

Conflict of interest

The authors declare that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Detailed Materials and Methods.

Online Fig. S1.

Online Fig. S2.

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LEGENDS FOR FIGURES

Figure 1. Measurement of total ectonucleotidase activity in healthy control subjects and patients with a mild, moderate or severe asthma exacerbation. Time-dependent release of inorganic phosphates was measured by the malachite green method after adding 500 μ M ATP to polymorphonuclear + mononuclear cells isolated from peripheral blood. Symbols correspond to mean $\pm$ s.e.m. The inset shows the area under the curve (AUC, arbitrary units) of each group. * $p < 0.01$ and † $p < 0.05$ vs the healthy group (Dunnett test after ANOVA).

Figure 2. Diminished expression of ectonucleotidase pyrophosphatase/phosphodiesterase 1 (E-NPP1) in polymorphonuclear cells from patients with a severe asthma exacerbation. Images correspond to representative examples of E-NPP1 immunofluorescence staining (green) combined with nuclear staining with TOTO (red). The graph shows the relative expression of E-NPP1 mRNA as detected by qPCR analyzed with the $2^{-\Delta\Delta CT}$ method. * $p < 0.005$ vs healthy group (Student's t-test corrected by the Bonferroni method). Number of experiments in either technique was 6, 4, 11, and 11 for healthy controls and patients with mild, moderate, and severe asthma exacerbation, respectively. The horizontal bar in each panel corresponds to 10 μ m.

Fig. 1. Montañó et al

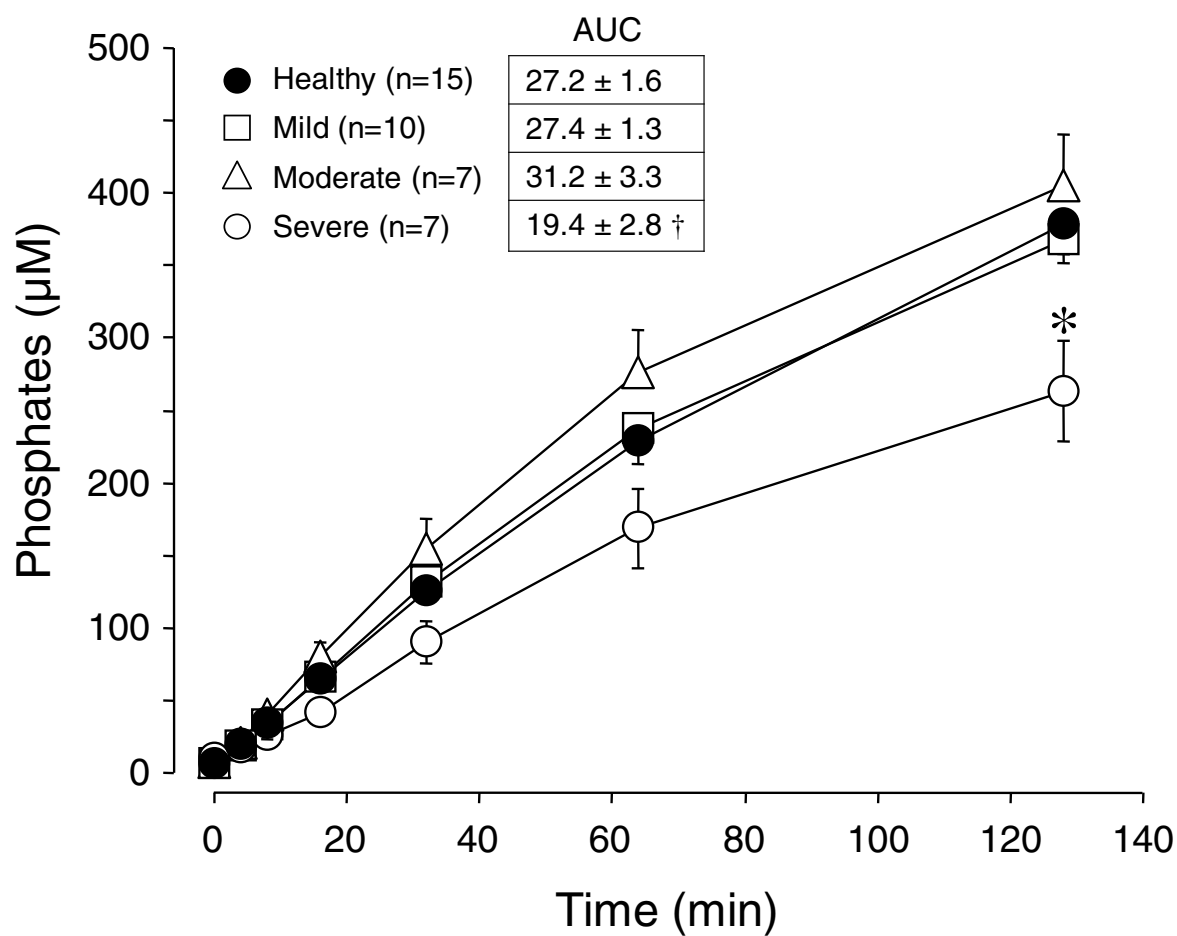
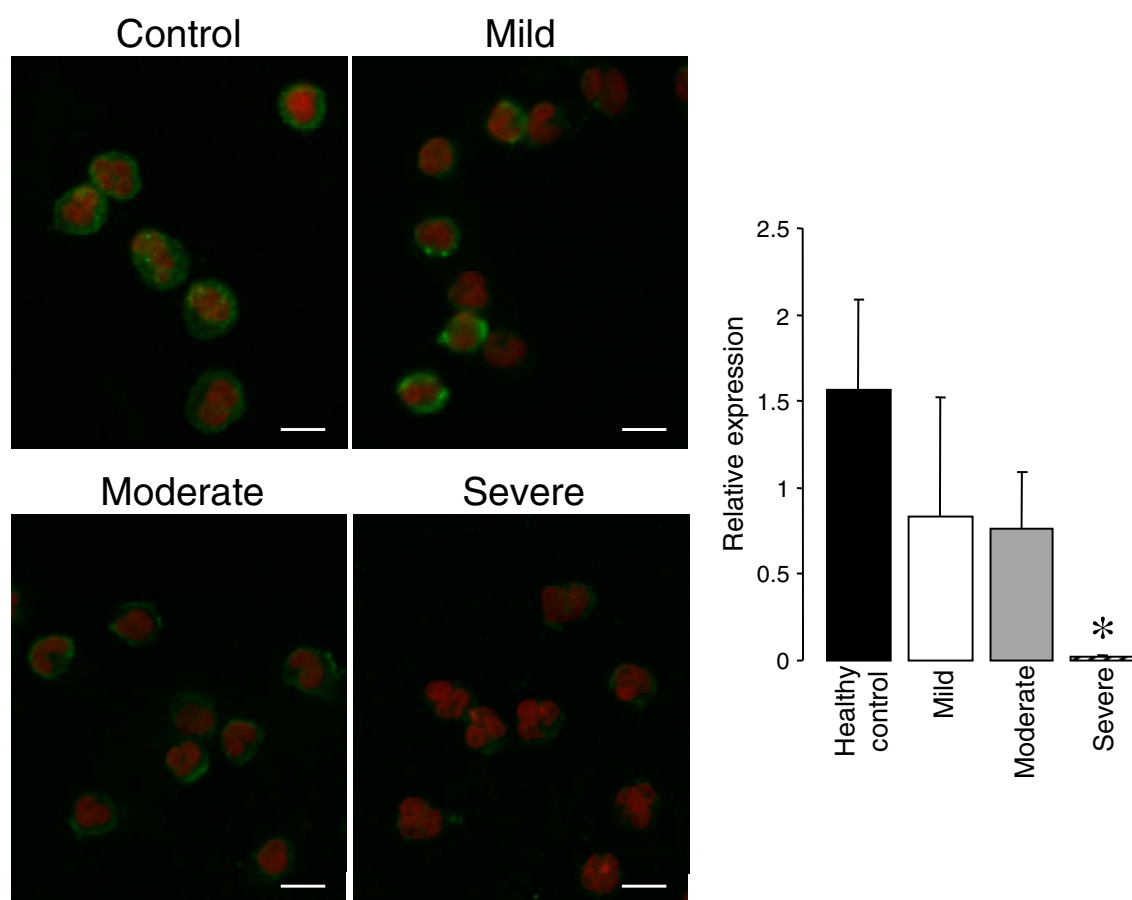
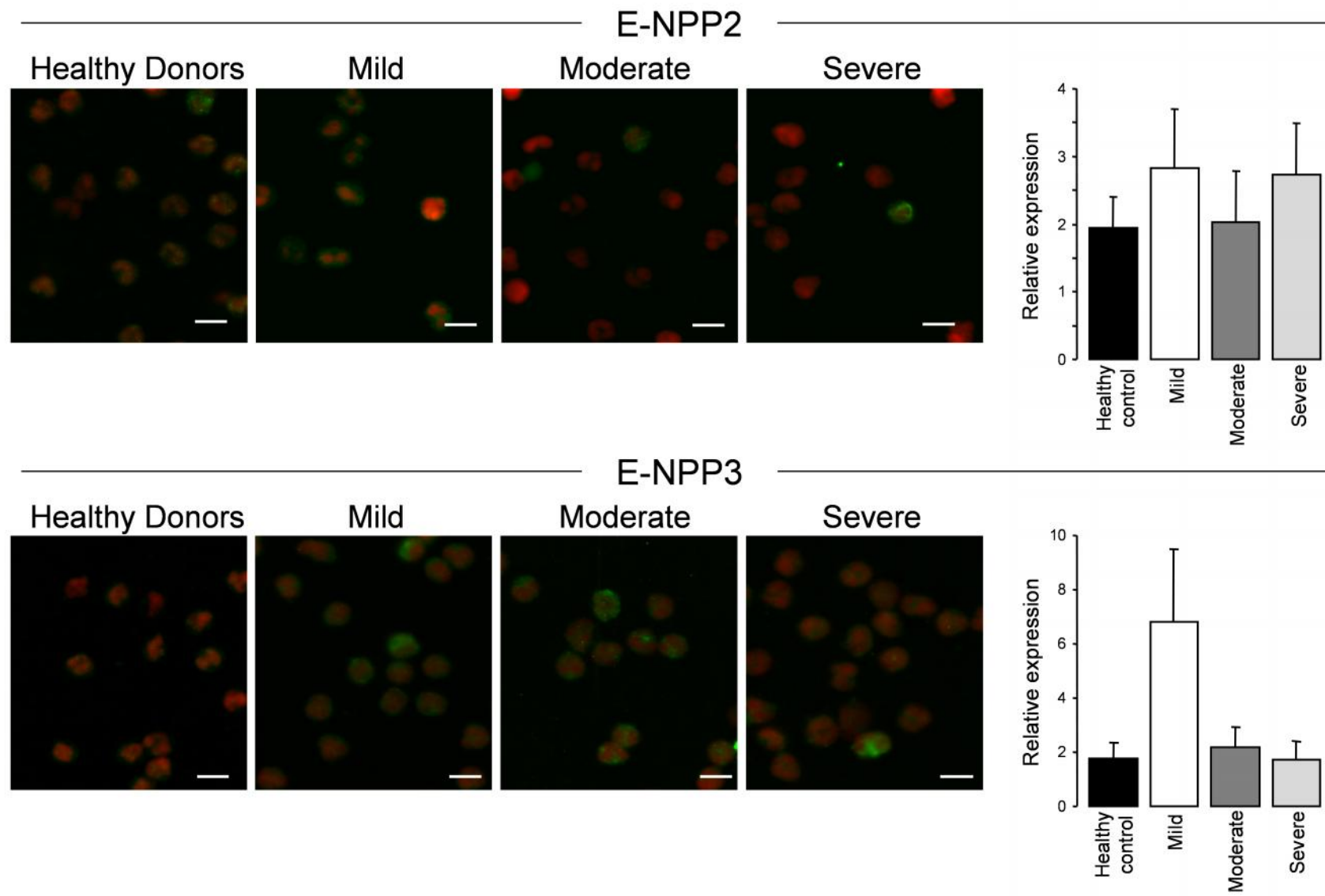


Fig. 2. Montañó et al

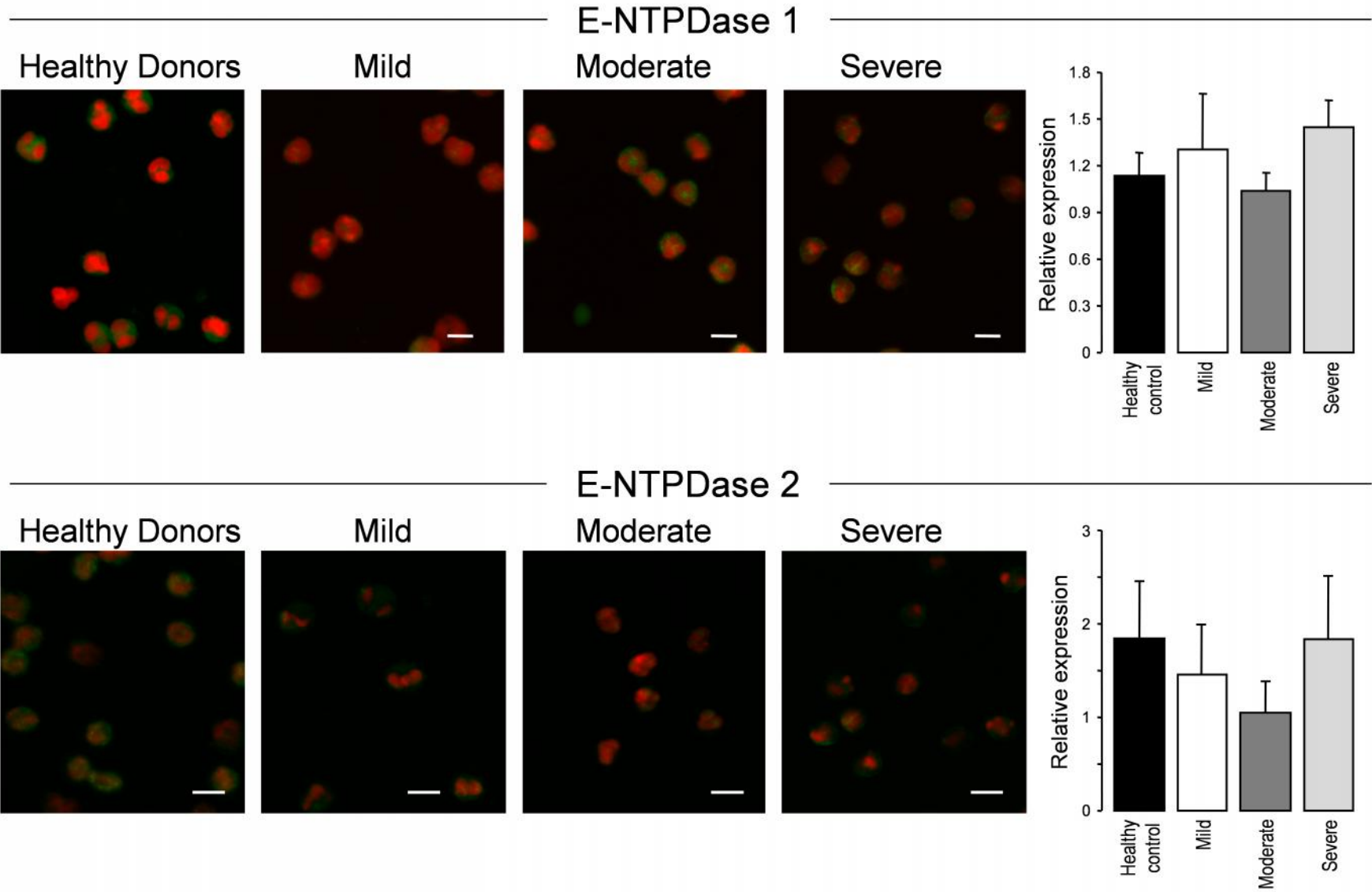
E-NPP1



Online FIG. S1



Online FIG. S2



Online Supporting Information

Appendix S1

Detailed Materials and Methods

Decreased expression of ectonucleotidase E-NPP1 in leukocytes from subjects with severe asthma exacerbation

Luis M. Montaña,¹ Mario H. Vargas,² Marlon De Ita-Ley,^{1,3} Verónica Díaz-Hernández,³
Renata Kasakova,¹ Carlos Barajas-López,⁴

Materials and Methods

Study design

The protocol was approved by the institutional review board of the Instituto Nacional de Enfermedades Respiratorias with approval number C09-12, and signed informed consent was obtained from all patients before entering into the study. Adult subjects admitted to the emergency room of this institute due to an asthma exacerbation were recruited. In all patients, a diagnosis of asthma was either already performed at the Asthma Clinic or during the emergency room visit, always according to international guidelines (1, 2). The severity of the asthma exacerbation was categorized as mild, moderate, or severe according to the classification published by the Global Initiative for Asthma (1). Major criteria in this classification included: 1) *Mild*: dyspnea when walking, can lie down, talks in sentences, no use of accessory respiratory muscles, moderate wheeze, heart rate <100 bpm, peak expiratory flow (PEF) >80% after initial bronchodilator, no hypoxemia or hypercapnia. 2) *Moderate*: dyspnea while talking, prefers sitting, talks in phrases, usually agitated, use of accessory respiratory muscles, loud wheeze, heart rate 100-120 bpm, pulsus paradoxus may be present, PEF 60%-80% after initial bronchodilator, no hypoxemia or hypercapnia. 3) *Severe*: dyspnea at rest, hunched forward, talks in words, usually agitated, respiratory rate often >30/min, use of accessory respiratory muscles, loud wheeze, heart rate >120 bpm, pulsus paradoxus often present, PEF <60 after initial bronchodilator, hypoxemia, hypercapnia and cyanosis may be present. Once the proper anti-asthma medication was initiated and the patient's clinical condition began to be stabilized, a 10-ml blood sample was drawn from a peripheral vein and immediately brought to the laboratory for its study. The control group was integrated by healthy blood donors recruited from the institute's blood bank.

First, the overall enzymatic activity of ectonucleotidases in polymorphonuclear cells (PMNs) plus mononuclear cells (MNC) was investigated. Then, the expression of the protein and mRNA of the ectonucleotidases known to be present in the cell membrane and to have their catalytic moiety faced extracellularly (E-NTPDase 1, 2, 3, and 8, and E-NPP1, 2, and 3) were analyzed in PMNs by immunofluorescence and real-time quantitative PCR (qPCR), respectively. Due to ethical constraints, not all determinations were performed in all patients, mainly because the amount of the blood sample was not sufficient for performing all of the techniques in the same patient.

PMNs and MNC purification

For PMNs and MNC separation, the blood sample was mixed 1:1 (v/v) with a commercial solution (Polymorphprep, Acurate, Oslo, Norway) and centrifuged at 1800 rpm during 35 min at 20°C. Each fraction was carefully collected and mixed with a 10-ml working solution (WS) containing NaCl 120 mM, KCl 5 mM, glucose 60 mM, CaCl₂ 0.5 mM, Tris-HCl 50 mM, pH 8.0 adjusted with HCl, in deionized water (3), and centrifuged at 1800 rpm during 10 min at 20°C. Supernatant was discarded and the last procedure was repeated again. Then, supernatant was discarded and the pellet was mixed with 5 ml erythrocyte lysis buffer solution containing 600 mg NaHCO₃, 4.15 g NH₄Cl, 18 mg EDTA in 500 ml H₂O and refrigerated during 10 min. After the addition of 8 ml WS, the tube was centrifuged at 1200 rpm during 10 min at 20°C. Supernatant was discarded, the pellet was re-suspended in 1 ml WS, and cells were counted in a Neubauer chamber.

Measurement of ectonucleotidase activity

Total ectonucleotidase activity was evaluated through a colorimetric assay with malachite green. This method quantifies the amount of inorganic phosphate released during the enzymatic breakdown of exogenous ATP by the cells (3-7).

Two million cells composed by PMNs+MNC [1:1] were suspended in 900 μ l WS during 10 min at 37°C. Exogenous ATP (500 μ M, Sigma-Aldrich Co., St Louis, MO, USA) was added and, after a brief vortexing, a first 100- μ l sample was obtained and immediately placed into an Eppendorf tube containing 100 μ l of 10% trichloroacetic acid and submerged in ice to stop any enzymatic reaction (time 0). This sampling was repeated at 4, 8, 16, 32, 64 and 128 min. After completion of the sampling, 32 μ l from each tube was transferred onto an ELISA plate, 124 μ l malachite green was added to each well and, during 7 min, the plate was shaken in a rocking platform (Daigger, Model AR100 117V 2A, IL, USA). Plates were then read at 650 nm in a spectrophotometer (Sinergy HT Microplate Reader, Biotek Instruments, Winooski, VT, USA). In each experiment, results were compared with a standard curve made with increasing concentrations of KH_2PO_4 (1-1000 μ M). The malachite green solution was prepared by mixing 0.0812 g malachite green dissolved in 100 ml deionized water, 5.72% ammonium molybdate in 6N HCl, and deionized water in a 2:1:2 proportion.

In a previous work, we corroborated that under the same protocol, the sole incubation of cells without the addition of ATP does not produce any noticeable inorganic phosphate release, suggesting that no spontaneous release and breakdown of nucleotides was occurring.

Immunofluorescence

Purified PMNs were fixed with 4% paraformaldehyde at 4°C during 10 min, washed with phosphate buffered saline (PBS), extended onto slides, and stored at 4°C until their study. The slides were treated with 0.05% Tween-20 in PBS as permeabilization buffer during 10 min at room temperature. Unspecific antibody binding sites were blocked with 10% horse serum plus 2% bovine serum albumin in 0.05% Tween-20 PBS. Antibodies against ectonucleotidases listed in Table 1 were eluted 1:50 in Da Vinci green diluent (Biocare Medical, Concord CA, USA) and incubated overnight at 4°C. Thereafter, the fluorescent antibodies (diluted 1:100) against the animal species in which ectonucleotidases antibodies were generated were incubated at room temperature during 20 min. For this purpose, Alexa Fluor 488 donkey anti-mouse (H+L) antibody (A21202, Life Technologies, Carlsbad CA, USA) was used for E-NTPDase 1, E-NTPDase 3, and E-NPP2; alexa Fluor 488 donkey anti-goat (H+L) antibody (A11055, Invitrogen, Carlsbad CA, USA) was used for E-NTPDase 8 and E-NPP1; and Alexa Fluor488 donkey anti-rabbit (H+L) antibody (A11055, Invitrogen, Carlsbad CA, USA) was used for E-NTPDase 2 and E-NPP3. Nucleus staining was performed by TOTO-3 iodide (Life Technologies) and washed with PBS afterward. Slides were fixed in 4% paraformaldehyde and coverslipped with Dako Fluorescence Mounting Medium (Dako, Carpinteria CA, USA). Immunostaining was observed and photodocumented in a confocal microscope (LSM 5 Pascal, Carl Zeiss AG, Jena, Germany). For the majority of ectonucleotidases, immunolabeling specificity was corroborated by using samples without the antibody against the enzyme as negative controls. In the case of E-NPP1, such specificity was corroborated in PMNs from healthy subjects by following the previously described protocol, but using the blocking peptide

(Ab45822 Abcam, Cambridge, UK) incubated overnight in a 2:1 ratio (blocking peptide:antibody) at 37°C.

mRNA extraction and quantitative PCR

The PMNs were submitted to lysis with 1 ml TRIzol[®] reagent according to the manufacturer's instructions (Life Technologies), and total RNA was purified using the RNeasy plus mini kit (Qiagen, Valencia, CA, USA). The samples were freed from DNA by RNase-free DNase Set digestion (Qiagen) and eluted with RNase-free water. Concentration of total RNA was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Barrington, IL, USA). RNA purity was evaluated by the absorbance ratios at 260:230 nm and 260:280 nm, where a 1.8 ratio was suitable for proceeding with cDNA synthesis. Total RNA was reverse-transcribed to cDNA using a SuperScript VILO cDNA synthesis kit (Life Technologies) and stored at -20°C for later use. qPCR reactions were performed in a StepOne Real Time PCR system (Foster City CA, USA). A final volume of 20-µl PCR reaction was employed containing 4 µl of water, 10 µl of 2XTaqMan Gene Expression Master mix (Life Technologies), 5 µl of diluted cDNA, and 1 µl of 20X mix PCR assay of specific probes for different ectonucleotidases (E-NTPDase 1, 2, 3, and 8, and E-NPP1, 2, and 3) (Table 1). Cycling conditions in the first phase were 2 min at 50°C and 10 min at 95°C, while for the second phase, these were 15 s at 95°C and 1 min at 60°C during 40 cycles. These ectonucleotidases were chosen because they are attached to plasma membrane with their catalytic moiety faced extracellularly (8-10). A series of normalizing genes (18S rRNA, beta actin, and GAPDH) was tested on the samples for their stable expression in PMNs, and eventually GAPDH was designed as housekeeping gene for

normalization (Table 1). The $2^{-\Delta\Delta CT}$ method was employed to analyze ectonucleotidase gene expression (11).

Data analysis

The amount of inorganic phosphate released by leukocytes from the control group was compared with each asthmatic group (mild, moderate, and severe) at each time period by means of analysis of variance followed by Dunnett test. The area under the curve of each experiment was also calculated, and the results were analyzed by these same statistical tests. qPCR results among groups were analyzed with unpaired Student's *t*-test corrected for multiple comparisons by the Bonferroni method.

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ONLINE Table S1. Probes and antibodies used to explore gene and protein expression of ectonucleotidases in polymorphonuclear cells

Ectonucleotide	qPCR probes *			Immunofluorescence primary antibodies †	
	Catalog number	Assay ID	Dye	Ab characteristics	Catalog number
E-NTPDase 1	4453320	Hs00969559_1	FAM	Mouse monoclonal	ab30422
E-NTPDase 2	4331182	Hs00154301_1	FAM	Rabbit polyclonal	ab102651
E-NTPDase 3	4331182	Hs00928977_1	FAM	Mouse polyclonal	ab67263
E-NTPDase 8	4331182	Hs01651150_m1	FAM	Goat polyclonal	sc-160609
E-NPP1	4331182	Hs01054040_m1	FAM	Goat polyclonal	ab40003
E-NPP2	4331182	Hs00905117_m1	FAM	Mouse monoclonal	ab77104
E-NPP3	4331182	Hs01038393_m1	FAM	Rabbit polyclonal	ab118445
GAPDH	4331182	Hs03929097_g1	FAM		
Beta actin	4331182	Hs99999903_m1	FAM		
18S rRNA	4318839		VIC-MGB		

* All purchased from Applied Biosystems (Foster City, CA, USA)

† All purchased from Abcam (Cambridge, UK), with the exception of the E-NTPDase 8 primary antibody, purchased from Santa Cruz Biotech (Dallas, TX, USA).

LEGENDS FOR ONLINE SUPPLEMENTARY FIGURES

ONLINE Figure S1. Expression of ectonucleotidase pyrophosphatase/phosphodiesterases 2 and 3 (E-NPP2 and E-NPP3, respectively) in polymorphonuclear cells from healthy controls and patients with an asthma exacerbation. Images correspond to representative examples of E-NPP2 and E-NPP3 immunofluorescence staining (green) combined with nuclear staining with TOTO (red). The graph depicts the relative expression of the enzyme mRNA as detected by qPCR analyzed with the $2^{-\Delta\Delta CT}$ method. There were no statistically significant differences among groups. Number of experiments in healthy controls and patients with mild, moderate, and severe asthma exacerbation was 10, 5, 13, and 11 for E-NPP2, and 12, 5, 11, and 12 for E-NPP3. The horizontal bar in each panel corresponds to 10 μ m.

ONLINE Figure S2. Expression of ecto-nucleotide triphosphate diphosphohydrolases 1 and 2 (E-NTPDase 1 and E-NTPDase 2, respectively) in polymorphonuclear cells from healthy controls and patients with an asthma exacerbation. Images correspond to representative examples of E-NTPDase 1 and E-NTPDase 2 immunofluorescence staining (green) combined with nuclear staining with TOTO (red). The graph shows the relative expression of enzyme mRNA as detected by qPCR analyzed with the $2^{-\Delta\Delta CT}$ method. There were no statistically significant differences among groups. Number of experiments in healthy controls and patients with mild, moderate and severe asthma exacerbation was 10, 5, 13, and 12 for E-NTPDase 1, and 10, 5, 11, and 12 for E-NTPDase 2. The horizontal bar in each panel corresponds to 10 μ m.