Effect of a Soluble Guanylate Cyclase Stimulator on the Purinergic Pathway in an Animal Model of Emphysema Induced by Cigarette Smoke

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Abstract

Purinergic and nitric oxide (NO) signaling pathways appear to be involved in the development of emphysema and vascular remodeling of chronic obstructive pulmonary disease (COPD). In an animal model, we evaluate the gene and protein expression of *ENTPD1*/CD39 and *NT5E*/CD73, and the effect on this expression of a guanylate cyclase (GC) stimulator. Forty-two guinea pigs underwent sham exposure (SHAM) or cigarette smoke (CS) exposure from three to six months. They were divided into six groups (sham-exposed, smokers or former smokers) and treated with vehicle (VH) or BAY 41-2272 (GC stimulator) for three months. Immunohistochemistry, western blot and qPCR assays were performed on lung tissue samples. Compared to SHAM + VH, *ENTPD1* and *NT5E* were downregulated in the CS + VH group (1 ± 0 vs. 0.78 ± 0.51, $p > 0.05$, and 1 ± 0 vs. 0.45 ± 0.27, $p = 0.027$ respectively). Treatment with BAY 41-2722 increased *ENTPD1* and *NT5E* expression to 1.06 ± 0.4 and 0.71 ± 0.35, respectively. No changes in the Ex-CS + BAY group were found. *NT5E*/CD73 was downregulated in the lungs of an animal model of emphysema. Treatment with a soluble GC stimulator tended to restore the gene and protein expression of *ENTPD1/*CD39 and *NT5E/*CD73 in smoke-exposed animals, suggesting its implication in a new mechanism for preventing emphysema.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by chronic inflammation in the small airways. It causes structural changes in the bronchus and vessels, as well as systemic manifestations, leading to the development of comorbidities [1–3]. Exposure to cigarette smoke (CS) is the major risk factor, but host genetic factors, immune dysregulation or alterations in repair mechanisms can also lead to the development of COPD [4, 5].

Purinergic signaling appears to be involved in the pathogenesis of COPD, since extracellular ATP can promote the chronic inflammation that characterizes the disease [6–11]. Different families of hydrolysing enzymes, alone or acting sequentially, are responsible for hydrolysing ATP into adenosine diphosphate (ADP) and adenosine monophosphate (AMP); the NTPDase1/CD39 ectoenzyme is the most frequently expressed in human lungs [11]. Finally, Ecto-5'-NT/CD73 dephosphorylates AMP, generating adenosine [12].

An association has been observed between extracellular ATP levels in plasma samples and the patient's condition, quality of life and disease progression, suggesting that extracellular ATP could be a possible diagnostic or prognostic biomarker [13]. Moreover, a previous study demonstrated that CD39 deficiency induces the progression of emphysema and lung inflammation in mice, and that the administration of an external functional NTPDase1/CD39 analogue (apyrase) can limit their development [14]. Furthermore, reduced expression and activity of NTPDase1/CD39 and differences in the expression of genes in the purinergic pathway have been described in the lung tissues of moderate-mild stable COPD patients compared to non-smokers [15, 16], suggesting that this may be a mechanism that underlies the development of COPD.

COPD is associated with early vascular changes in pulmonary arteries. It is well established that patients can develop pulmonary hypertension due to vascular remodeling, emphysema and chronic hypoxemia in the advanced stages of COPD [17–20]. Nitric oxide (NO) signaling is involved in many physiological processes, such as vascular homeostasis, through its ability to stimulate soluble guanylate cyclase (sGC) [21, 22]. NO signaling is altered in COPD patients, with a downregulation of sGC expression in the lungs correlating with disease severity [23, 24]. Interestingly, chronic administration of the sGC stimulators BAY 41-2272 and BAY 63-2521 in guinea pigs (GP) and mice respectively prevented vessel remodeling and emphysema [24]. Although additional studies demonstrated that sGC stimulation in GPs prevented the oxidative stress induced by CS and attenuated inflammatory responses in the lung [25], the molecular mechanisms underlying the reduction in emphysema in this animal model were not fully clarified [26]. We hypothesized that one of the mechanisms involved in this improvement might be related to the restoration of purinergic enzyme levels.

Accordingly, the aims of this study were to evaluate the gene and protein expression of *ENTPD1*/CD39 and *NT5E*/CD73, the main enzymes involved in purinergic signaling, in lung samples from our previous animal model of CS-induced emphysema, and to analyse the potential effect of BAY 41-2272 (which stimulates sGC) on their expression [26].

2. Results

2.1 *ENTPD1* and *NT5E* gene expression in lung samples

The relative expression (RQ) of *ENTPD1* and *NT5E* per group is detailed in Table 1 and Fig. 1. Compared to the control group (SHAM + VH), the expression of *ENTPD1* and *NT5E* was downregulated in the group exposed to CS for six months (CS + VH) (1 ± 0 vs. 0.78 ± 0.51, $p > 0.05$, and 1 ± 0 vs. 0.45 ± 0.27, $p = 0.027$ respectively). In the CS + BAY group, after sGC stimulation, the expression levels of *ENTPD1* and *NT5E* increased to 1.06 ± 0.4 and 0.71 ± 0.35 respectively, bringing them closer to those of the control group. *ENTPD1* gene expression did not vary between the control and the Ex-CS + VH group (1 ± 0 vs. 0.9 ± 0.35) and its expression remained stable after treatment with BAY 41-2272 (1.05 ± 0.77). *NT5E* gene expression was also downregulated in the group of former smokers (1 ± 0 vs. 0.66 ± 0.25) compared to the control group and did not change after treatment with BAY 41-2272 (0.63 ± 0.33).

A significant decrease in the expression of *NT5E* in relation to tobacco was observed (two-way ANOVA, $p = 0.007$). The expression of *ENTPD1* was not significantly decreased by the tobacco factor ($p = 0.35$), but a numerical trend was observed. The two-way ANOVA did not show a statistically significant effect of the BAY treatment on the gene expression.

Table 1
Gene expression of *ENTPD1* and *NT5E* in guinea pigs exposed to cigarette smoke or sham and with or without treatment with BAY 41-2272, a stimulator of soluble guanylate cyclase. Relative expression (RQ) compared to the control SHAM + VH group. *p < 0.05.

	ENTPD1	*NT5E*
	RQ ± SD	**RQ ± SD**
SHAM + VH (n = 6)	1 ± 0	1 ± 0
SHAM + BAY (n = 6)	1.29 ± 0.74	1.03 ± 0.47
Ex-CS + VH (n = 7)	0.9 ± 0.35	0.66 ± 0.25
Ex-CS + BAY (n = 8)	1.05 ± 0.77	0.63 ± 0.33
CS + VH (n = 8)	0.78 ± 0.51	0.45 ± 0.27*
CS + BAY (n = 7)	1.06 ± 0.4	0.71 ± 0.35

2.2 Western blot of NTPDase1/CD39 and Ecto-5'-NT/CD73 in GP lung samples

Western blot assays were performed in order to evaluate NTPDase1/CD39 and Ecto-5'-NT/CD73 expression in GP lung samples. The expression of CD39 and CD73 did not differ statistically between the group exposed to CS between three and six months and the control group; however, in the Ex-CS + VH and CS + VH groups, a trend to downregulation was observed. Furthermore, after sGC stimulation, the expression levels of CD73 increased, bringing them closer to those of the control group; those of the CS + BAY group were even higher (Fig. 2).

2.3 Immunolabeling of NTPDase1/CD39 and Ecto-5'-NT/CD73 in GP lung samples

NTPDase1/CD39 and Ecto-5'-NT/CD73 were expressed in the lung parenchyma, bronchus and pulmonary arteries. Both proteins were expressed in all the tunics of the pulmonary arteries (endothelial and muscular cells), with no differences observed between them. Therefore, we used the immunoexpression in the muscular layer for our analysis of pulmonary arteries (Table 2). Semiquantitative NTPDase1/CD39 analysis showed that its expression was reduced in the parenchyma in the untreated CS and Ex-CS groups compared to the SHAM + VH group, but not in the bronchus and pulmonary arteries. BAY 41-2272 treatment showed a tendency to raise NTPDase1/CD39 expression in the parenchyma and bronchus of the CS and Ex-CS groups and in the pulmonary arteries of the CS group.

Ecto-5'-NT/CD73 immunolabeling was weaker in the parenchyma, bronchus and pulmonary arteries of the untreated CS and Ex-CS groups compared to the SHAM + VH group. In the control group, BAY 41-2272 treatment enhanced Ecto-5'-NT/CD73 expression in the pulmonary arteries. The lungs of the CS and Ex-CS groups treated with BAY 41-2272 showed increased expression of Ecto-5'-NT/CD73 in all the structures analysed (parenchyma, bronchus and pulmonary arteries) compared to the untreated groups.

No statistically significant differences were found in immunolabeling of NTPDase1/CD39 and Ecto-5'-NT/CD73 in GP lung samples compared to the SHAM + VH group. Representative images of the CD39 and CD73 immunolabeling and the effect of BAY 41-2272 treatment are shown in Fig. 3.

Table 2
NTPDase1/CD39 and Ecto-5′-NT/CD73 immunostaining intensity by groups. Data are expressed as n (%). No statistical differences were found between group $p > 0.05$).

NTPDase1/CD39							Ecto-5′-NT CD73					
Parenchyma	SHAM + VH	SHAM + BAY	ExCS + VH	ExCS + BAY	CS + VH	CS + BAY	Parenchyma	SHAM + VH	SHAM + BAY	ExCS + VH	ExCS + BAY	CS + VH
	N = 6	N = 6	N = 7	N = 8	N = 8	N = 7		N = 5	N = 6	N = 7	N = 8	N = 8
+++	4 (66.67%)	1 (16.67%)	1 (14.29%)	2 (25%)	0 (0%)	3 (42.86%)	+++	3 (60%)	3 (50%)	2 (28.6%)	4 (50%)	2 (25%)
++	1 (16.67%)	2 (33.33%)	3 (42.85%)	6 (75%)	5 (62.5%)	2 (28.58%)	++	2 (40%)	3 (50%)	4 (57.1%)	2 (25%)	2 (25%)
+	1 (16.67%)	3 (50%)	3 (42.86%)	0 (0%)	2 (25%)	2 (28.58%)	+	0 (0%)	0 (0%)	1 (14.3%)	2 (25%)	2 (25%)
-					1 (12.5%)		-					2 (25%)
Bronchus	SHAM + VH	SHAM + BAY	ExCS + VH	ExCS + BAY	CS + VH	CS + BAY	Bronchus	SHAM + VH	SHAM + BAY	ExCS + VH	ExCS + BAY	CS + VH
	N = 6	N = 6	N = 7	N = 8	N = 8	N = 7		N = 5	N = 6	N = 7	N = 8	N = 8
+++	3 (50%)	4 (66.67%)	5 (71.43%)	7 (87.5%)	5 (62.5%)	5 (71.43%)	+++	4 (80%)	5 (83.3%)	2 (28.6%)	5 (63%)	1 (13%)
++	3 (50%)	2 (33.33%)	1 (14.29%)	1 (12.5%)	3 (37.5%)	1 (14.29%)	++	1 (20%)		4 (57.1%)	3 (37%)	5 (63%)
+	0 (0%)	0 (0%)	1 (14.29)	0 (0%)	0 (0%)	1 (14.29%)	+	0 (0%)	1 (16.7%)	1 (14.3%)	0 (0%)	1 (13%)
-							-					1 (13%)
Pulmonary artery	SHAM + VH	SHAM + BAY	ExCS + VH	ExCS + BAY	CS + VH	CS + BAY	Pulmonary artery	SHAM + VH	SHAM + BAY	ExCS + VH	ExCS + BAY	CS + VH
	N = 6	N = 6	N = 7	N = 8	N = 8	N = 7		N = 5	N = 6	N = 7	N = 8	N = 8
+++	1 (16.67%)	2 (33.33%)	3 (42.86%)	1 (12.5%)	0 (0%)	3 (42.86)	+++	2 (40%)	5 (83.3%)	2 (28.6%)	5 (63%)	1 (12%)
++	4 (66.67%)	2 (33.33%)	3 (42.86%)	7 (87.5%)	6 (75%)	2 (28.58%)	++	3 (60%)		4 (57.1%)	3 (37%)	5 (63%)
+	1 (16.67%)	2 (33.33%)	0 (0%)	0 (0%)	2 (25%)	2 (28.58%)	+	0 (0%)	1 (16.7%)	1 (14.3%)	0 (0%)	2 (25%)
-							-					

2.4 Morphometry and inflammatory cells in lung samples

Data on lung morphometry and inflammatory infiltrates in this animal model have been previously reported [25]. No significant correlation was found between inflammatory cell counts (macrophages and neutrophils) and the gene expression of *ENTPD1* and *NT5E*, based on the analysis of individual data (data not shown).

3. Discussion

The focus of the present study was to analyse the gene and protein expression of *ENTPD1*/CD39 and *NT5E*/CD73 and the effect of BAY 41-2272, a stimulator of sGC, in lung tissue in an animal model of emphysema. To the best of our knowledge, this is the first study to analyse the effect of an sGC stimulator on the expression of genes and proteins associated with purinergic signaling in experimental COPD.

In our animal model of emphysema, we demonstrated a lower gene expression of both *ENTPD1* and *NT5E* in the lungs of both smokers (CS group) and former smokers (Ex-CS group) compared to the control group (SHAM group). Although our findings were not statistically significant, except for *NT5E* in the CS + VH group, the results are in line with those of other studies indicating that purinergic signaling is involved in the development of COPD. Differences in the expression pattern of certain genes associated with the purinergic pathway have been reported in the lung tissues of COPD patients and smokers without obstruction [15]. These changes may be responsible for the pulmonary inflammation that characterizes this disease.

Our group previously observed reductions in lung expression and activity of CD39 in patients in the initial stages of COPD [16]. Consistent with our findings, Kratzer et al. [27] found a significant downregulation of CD39 in the lungs of rats with emphysema caused by CS exposure. In contrast, other studies have reported an upregulation in CD39 in peripheral blood samples from COPD patients with an acute exacerbation compared to patients with stable COPD and healthy patients [28]. Lazar et al. also found increased cell levels of CD39 in BALF and sputum cells from COPD patients compared to those from smokers and non-smokers [14]. These contradictory findings may be attributable to the differences in the samples used in the studies, or to the fact that patients were at

different stages of the disease. The influence on CD39 expression of an acute condition such as a respiratory infection in the exacerbation of COPD or active tobacco consumption may be different from that of chronic lung damage.

The role in COPD of CD73, another key enzyme in purinergic signaling, is still not well understood. Its encoding gene, *NT5E*, seems to be overexpressed in the lungs of smokers without obstruction but not in COPD patients, suggesting that adenosine generated by the hydrolysis of AMP by CD73 may contribute to a decrease in the inflammatory environment in the lungs [15]. These findings for *NT5E*/CD73 support the idea that the upregulation of *ENTPD1*/CD39 and *NT5E*/CD73 could be a compensatory mechanism in acute lung damage (active smoking and acute exacerbation) [15, 16].

A very interesting finding in the present study was the fact that treatment with a sGC stimulator tends to restore the expression of *ENTPD1* in all groups, including the control group (SHAM + VH vs SHAM + BAY). These results suggest that treatment with BAY 41-2272 could act by enhancing *ENTPD1* expression and could revert the downregulation of *ENTPD1* and *NT5E* observed after CS exposure. On the other hand, the effect of BAY 41-2272 treatment on the expression of *NT5E* remains unclear since its expression remained almost unchanged in the SHAM and Ex-CS groups after treatment. In contrast, the expression of *NT5E* was partially restored in the smoker group (CS + VH vs CS + BAY). These differences in *NT5E* may be due to the length of exposure to CS (3 months in Ex-CS group vs 6 months in CS group). The results of the protein expression analysed by immunohistochemistry and western blot were in line with those of the gene expression studies, as the protein expression of NTPDase1/CD39 and Ecto-5'-NT/CD73 increased after treatment with BAY 41-2272.

Treatment with a sGC stimulator has been previously studied in COPD, where it has been shown to reduce oxidative stress and attenuate the inflammatory response in the lungs induced by CS [24–26]. The sGC stimulator treatment also has beneficial effects on lung vasculature, reducing pulmonary arterial pressure and right ventricular hypertrophy. Our findings may suggest a new beneficial effect of treatment with an sGC stimulator in COPD that upregulates *ENTPD1*/CD39 and *NT5E*/CD73 expression in lung tissues, thus reducing ATP levels and inflammation. However, further studies of this issue are required.

Data on lung morphometry and inflammatory infiltrates in lung tissue from this animal model have been reported previously [26]. In our study, we failed to find a significant correlation between lung morphometry, inflammatory cell counts and the gene expression of *ENTPD1* and *NT5E* based on the analysis of individual data. However, Paul et al. [26] reported an increase in inflammatory cells in the lung tissue of animals exposed to tobacco smoke which had developed emphysema as well as an inhibitory effect of BAY 41-2272 on inflammatory cell infiltration.

Several studies have described an interaction between nitric oxide (NO) and ATP. For example, Ruiz-Stewart et al. showed that NO and sGC are involved in ATP supply/demand as mediators, with changes in ATP levels regulating NO signaling through sGC [29]. Moreover, ATP and ADP have been shown to induce endothelial nitric oxide synthase (eNOS) via P2 receptors (also involved in the purinergic pathway), increasing NO generation [30].

Although it is not well established how purinergic and NO signaling interact in modulating ATP levels, or whether the two pathways share some signaling mechanisms, our findings suggest that sGC stimulation could activate the purinergic pathway, thereby enhancing *ENTDP1*/CD39 and *NTE5*/CD73 expression in lung tissues and consequently reducing ATP levels and leading to a decreased inflammatory environment in COPD. More studies are required to shed more light on the relationship between the purinergic and NO signaling pathways in COPD.

The main limitation of this study was the large number of groups, which meant that each group produced only a limited number of samples. This made it difficult to draw conclusions about the effect of BAY 41-2272 on the genes and proteins associated with purinergic signaling. Secondly, we only analyzed the gene and protein expression of the main purinergic enzymes. In future work it would be interesting to extend the study to other molecules of the purinergic pathway, using new molecular techniques to better understand the interaction of this complex signaling pathway.

4. Conclusions

In summary, this study showed a downregulation in the gene expression of *NT5E*/CD73 in the lungs of an animal model of CS-induced emphysema, with a guanylate cyclase stimulator that tends to upregulate the expression of *ENTPD1* and *NT5E*, thereby partially reversing the effects of CS. The findings suggest the involvement of a new mechanism in the prevention of emphysema with this treatment. We observed, for the first time, an interaction between the NO and purinergic signaling pathways in COPD. More studies are needed to better understand how the NO and purinergic signaling pathways interact with ATP and inflammation in COPD pathophysiology so as to identify new possible therapeutic strategies.

5. Materials And Methods

5.1 Animal model and samples

Lung samples from animals from our previously established animal model of emphysema induced by CS were used in this study [26]. Briefly, Dunkin Hartley guinea pigs (GPs) were sham-exposed (control group) or exposed to CS (smokers, or the CS group) for a total of six months, or exposed to CS for three months before sham exposure for another three months (former smokers, or the Ex-CS group) (Fig. 4). From the third to the sixth month, animals were daily treated with a vehicle (VH) or BAY 41-2272 (an sGC stimulator) (at a dose of 3 mg/kg administered by oral gavage) and divided into six groups: (1) sham-exposed treated with a vehicle (SHAM + VH; n = 6); (2) sham-exposed treated with BAY 41-2272 (SHAM + BAY; n = 6); (3) former smokers treated with a vehicle (Ex-CS + VH; n = 7); (4) former smokers treated with BAY 41-2272 (Ex-CS + BAY; n = 8); (5) smokers treated with a vehicle (CS + VH; n = 8); and (6) smokers treated with BAY 41-2272 (CS + BAY; n = 7). At the end of the experiment, animals were anesthetized with urethane (2g/kg i.p.; SigmaAldrich, Steinheim, Germany) and euthanized by exsanguination. The lungs (n = 42) were removed and processed for histological, protein and RNA studies following standard protocols. All procedures were approved by the local ethics committee for animal experimentation of the University of Barcelona (registry: 2009/5026). All experiments were performed in accordance with relevant guidelines and regulations including ARRIVE guidelines.

5.2 Quantitative real-time polymerase chain reaction (qPCR) analysis of *ENTPD1* and *NT5E*

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed as previously described [15]. Custom TaqMan gene expression assays (Thermo Fisher Scientific, Massachusetts, USA) for guinea pig *ENTPD1* and *NT5E* were designed as follows: for *ENTPD1* gene, forward primer, 5'-CTTCATCTGGGAACCCCATTCTT-3', reverse primer, 5'-GTTAATGTCTGCTTTTATGCTTGGATCTT-3' and probe, 5'-CAGGAATGCTGGTATTTAG-3'; and for *NT5E* gene, forward primer, 5'-CTTCATCTGGGAACCCCATTCTT-3'; reverse primer, 5'-GTTAATGTCTGCTTTTATGCTTGGATCTT3' and probe, 5'-TCTAAATACCAGCATTCCTGAAGAT-3'. GAPDH (Cp03755742_g1, Thermo Fisher Scientific) was used as endogenous control for normalization. Data are expressed as a relative quantification (fold change ratio) of mRNA.

5.3 Western blot for NTPDase1/CD39 and Ecto-5'-NT/CD73

Briefly, 20-µg of protein was loaded onto pre-cast 4–20% Mini-Protean TGX stain free gels (Bio-Rad, Hemel Hempstead, Great Britain) for electrophoresis and then transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% non-fat milk (Nestle, Vevey, Switzerland) in tris-buffered saline (TBS) containing 0.1% Tween® 20 (TBS-T) at pH 7.4, for 1 hour at room temperature (RT). Anti-CD73 (ab175396) or anti-CD39 (ab178572) primary antibodies (1/1000) were incubated overnight at 4˚C. After 3 washes with TBS-T, the appropriate secondary antibody horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse (1/8000; Sigma-Aldrich) was incubated at RT for 1 hour. Immunoreactive bands were detected using ECL SelectTM Western Blotting Detection Reagent (GE Healthcare, Chicago, Illinois) and digitized using the ChemiDocTM Touch Imaging System (Bio-Rad). Band density was quantified by densitometry using Image J software 2.1 and normalized to β-actin levels (4970L, Cell Signaling).

5.4 Immunohistochemistry for NTPDase1/CD39 and Ecto-5'-NT/CD73

The procedure used for the immunohistochemistry was performed as previously described [16]. Briefly, formalin-fixed, paraffin-embedded lung section were underwent dewaxing, dehydration, antigen retrieval, and quenching of endogenous peroxidase activity. After three rinses in phosphate buffered saline (PBS), tissue sections were pre-incubated for 1 hour at room temperature in 10% normal goat serum (Sigma-Aldrich, Sant Louis, Missouri, MO, USA). The following primary antibodies were incubated overnight at 4°C: anti-CD39 antibody [IMG17B5F11] (ab178572) at 1:400 and anti-CD73 antibody (ab175396) at 1:50 (Abcam, Cambridge, UK). Nuclei were counterstained with haematoxylin. Human lung tissue samples were used as positive control. The intensity of the labeling in the samples from the parenchyma, bronchus and pulmonary arteries was semi-quantitatively evaluated by two investigators blinded to study conditions following a visual scale previously defined as negative (–), weak (+), intermediate (++), or strongly positive (+++).

5.5 Lung morphometry and inflammatory cells

For the purposes of correlation only, in this study we used previously published data on emphysema and inflammatory cells [26]. Emphysema was assessed by histological hematoxylin staining, measuring the mean linear intercept (MLI). Inflammatory cells (intraseptal neutrophils and alveolar macrophages) were counted on histological sections. The methodology used for lung histological assessments has been described elsewhere [24].

5.6 Statistical analysis

Gene expression data are shown as means ± standard deviations (SD) and were statistically analysed by two-way analysis of variance (ANOVA), considering CS exposure and treatment with BAY 41-2272 as independent factors. If necessary, the data were transformed to natural logarithm to achieve normal distribution. Comparisons between the groups were evaluated by one-way ANOVA or the Kruskal-Wallis test, as appropriate, and an overall p-value was calculated. Chi^2 test was used for qualitative variables. Spearman's correlation was also used to assess the relationship between morphometry, inflammatory cells in the lungs and *ENTPD1* and *NT5E* gene expression. Statistical analysis was performed using IBM SPSS version 19.0 (IBM Corp., Armonk, NY, USA). A p-value < 0.05 was considered statistically significant.

Declarations

Data availability: The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Author Contributions: EC: participated in the design of the study, gene expression experiments, immunohistochemistry experiments, data analysis, statistical analysis, figure preparation, manuscript drafting and obtaining funding. **EA:** participated in the gene expression experiments, immunohistochemistry experiments, data analysis, statistical analysis and figure preparation. **TP:** participated in data analysis and interpretation. **YPG:** participated in the data analysis and interpretation. **MLS:** participated in the data analysis and interpretation. **JAB:** participated in the data interpretation and critical revision of the manuscript for important intellectual content. **JD:** participated in the data interpretation and critical revision of the manuscript for important intellectual content. **VIP:** the co-corresponding author contributed to the development of the study, obtaining funding, data analysis and interpretation and critical revision of the manuscript for important intellectual content. **SS:** the corresponding author contributed to the development of the study concept and design, obtaining funding, study supervision, data interpretation, manuscript drafting, and critical revision of the manuscript for important intellectual content. All the authors have read and approved the final manuscript.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

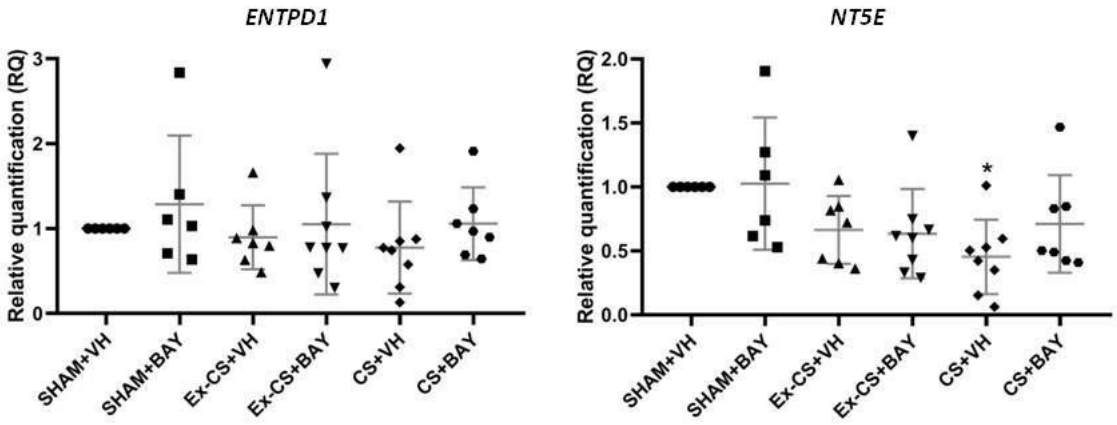

Figure 1

Gene expression of *ENTPD1* and *NT5E* in CS and Ex-CS guinea pigs with or without BAY 41-2272 treatment compared to the control group (SHAM + VH). Differences between groups were evaluated using the Kruskal-Wallis test followed by Dunn's multiple comparison tests, in which six conditions were simultaneously tested. P values were considered significant when the mean differed from control (SHAM+VH) group (*p < 0.05). *ENTPD1* and *NT5E* expression was downregulated in the CS and Ex-CS groups after CS exposure; BAY 41-2272 treatment increased their expression in both groups compared to controls.

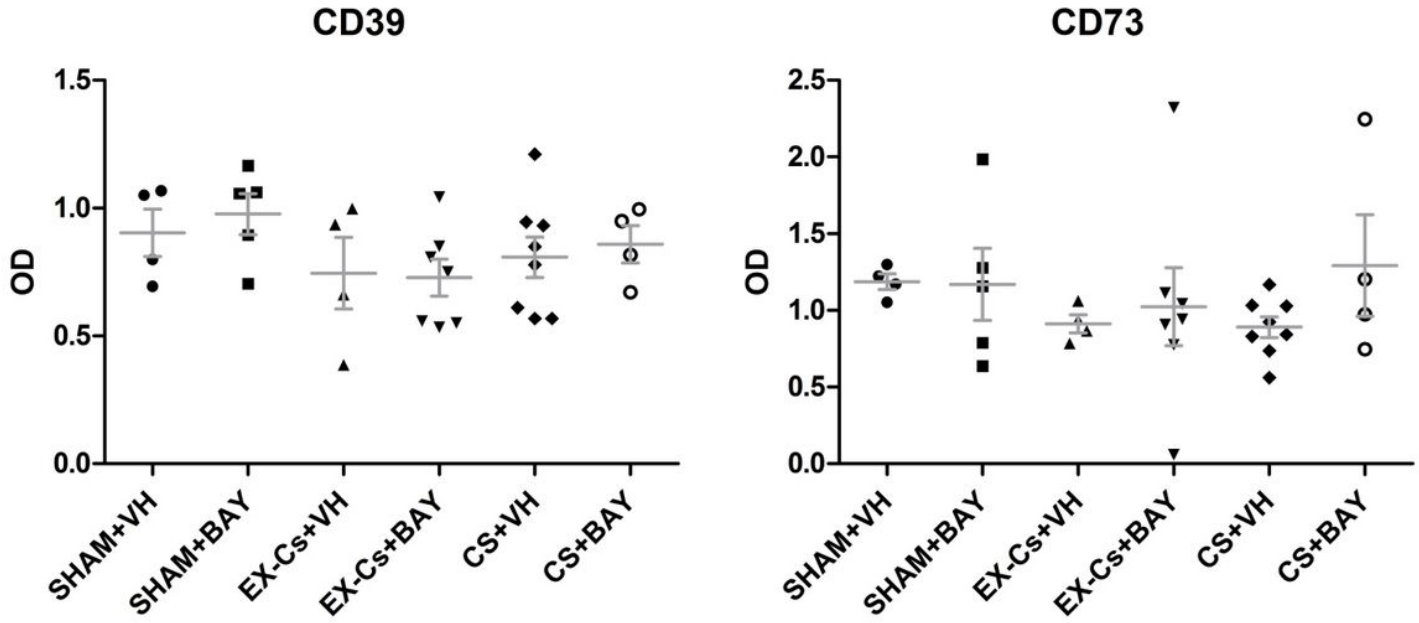

Figure 2

Protein expression analysis of NTPDase1/CD39 and Ecto-5'-NT/CD73 in CS and Ex-CS guinea pigs with or without BAY 41-2272 treatment compared to the control group (SHAM + VH) by western blot. Although NTPDase1/CD39 protein expression was similar between groups, the Ecto-5'-NT/CD73 protein expression was downregulated in the CS and Ex-CS groups after CS exposure; BAY 41-2272 treatment increased CD73 expression in both groups compared to controls.

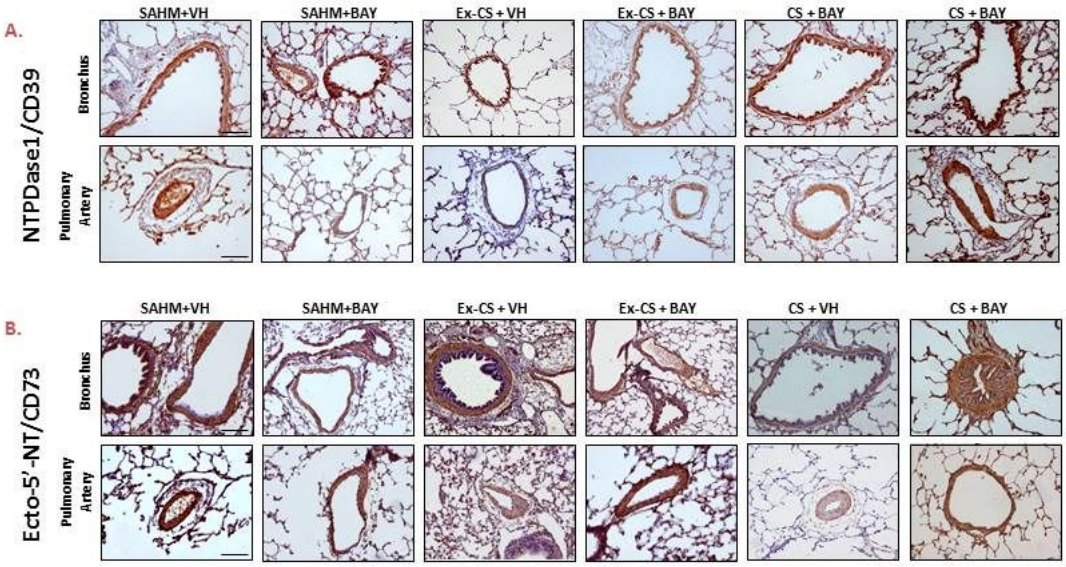

Figure 3

NTPDase1/CD39 (3A) and Ecto-5'-NT/CD73 (3B) immunostaining by groups in the bronchus (upper panels) and pulmonary arteries (lower panels). (3A) In the parenchyma, NTPDase1/CD39 expression was decreased in the untreated CS and ex-CS groups compared with control group. Treatment with BAY 41-2272 increased the NTPDase1/CD39 expression in the parenchyma and bronchus of the CS and Ex-Cs groups compared with the control group. In the pulmonary arteries, NTPDase1/CD39 immunolabeling remained unchanged between groups. (3B) Ecto-5'-NT/CD73 expression was weaker in all structures in untreated CS and Ex-CS groups than in with controls; treatment with BAY 41-2272 showed increased expression of Ecto-5'-NT/CD73 in parenchyma, bronchus and pulmonary arteries. Bar size is 100 µm.

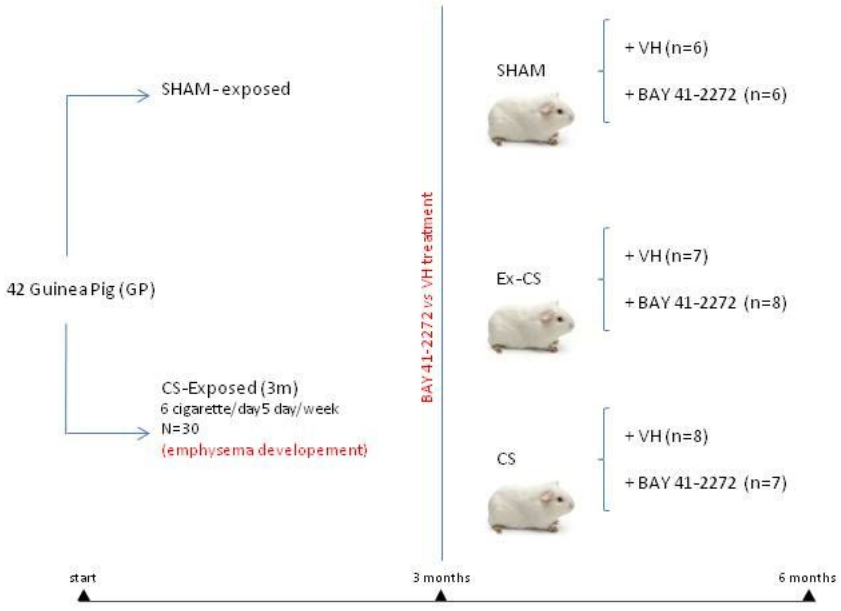

Figure 4

Study design and groups. CS, cigarette smoke; Ex-CS, former smoker; VH, vehicle. Adapted from Paul et al. [26].