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Short-Term Hyperprolactinemia Reduces the Expression of Purinergic P2X7 Receptors during Allergic Inflammatory Response of the Lungs

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Keywords

Purinergic receptor · Asthma · Prolactin · Domperidone · Lung

Abstract

Purpose: We have previously shown that domperidone-induced short-term hyperprolactinemia reduces the lung's allergic inflammatory response in an ovalbumin antigenic challenge model. Since purinergic receptor P2X7R activity leads to proinflammatory cytokine release and is possibly related to the pathogenesis of allergic respiratory conditions, the present study was designed to investigate a possible involvement of purinergic and prolactin receptors in this phenomenon. **Methods:** To induce hyperprolactinemia, domperidone was injected intraperitoneally in rats at a dose of $5.1 \text{ mg} \times \text{kg}^{-1}$ per day for 5 days. P2X7 expression was evaluated by lung immunohistochemistry while prolactin receptor expression in bronchoalveolar lavage leukocytes was analyzed through flow cytometry. **Results:** Previous reports demonstrated that rats subjected to short-term hyperprolactinemia exhibited a decrease in leukocyte counts in bron-

choalveolar lavage, especially granulocytes. Here, it is revealed that hyperprolactinemia promotes an increased expression of prolactin receptors in granulocytes. Also, increased expression of purinergic P2X7R observed in allergic animals was significantly reduced by hyperprolactinemia. **Conclusions:** Both purinergic and prolactin receptor expression changes occur during the anti-asthmatic effect of hyperprolactinemia.

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Introduction

Asthma is defined as a chronic inflammatory disease of the airways, in which several structural and inflammatory cells, among them T lymphocytes, eosinophils, and mast cells, play an important role [1–3]. The inflammatory process is associated with bronchial hyperresponsiveness, plasma exudation, pulmonary cell infiltration [4], and several stimuli, which are partially reversible, spontaneously or after treatment [5]. Asthma results from an inappropriate Th2 response against innocuous

airborne antigens. Exposure to allergen is necessary for disease development and onset of symptoms [6]. Infiltration by eosinophils and CD4⁺ Th2 lymphocytes into the airways are the main characteristics [2, 7]. The hypersecretion of mucus from the bronchial walls is also a hallmark of the disease [2, 3]. In asthma, inflammatory mediators are released into the lungs by alveolar macrophages, neutrophils, eosinophils, and mast cells that result in its typical pathophysiological changes [1, 4].

Prolactin is a hormone with multiple biological actions including osmoregulation, lactation, reproduction, growth and development, brain and behavior, and immunomodulation [8]. Prolactin action patterns in the immune system vary [9]. The prolactin receptor (PRLR) can be found in many cell types, including monocytes and lymphocytes. Physiological levels of pituitary prolactin may be required to maintain the basal function of activated lymphocytes [10]. During the immune response, prolactin stimulates T cells, B cells, natural killer cells, macrophages, neutrophils, CD34-hematopoietic cells, and antigen-presenting dendritic cells [11, 12]. Previously, our group has demonstrated that both domperidone-induced and lactation hyperprolactinemia are capable of attenuating allergic lung inflammation in rats [13, 14] by decreasing leukocyte counts in bronchoalveolar lavage, reducing mucus production, and increasing the expression of IL-4, IL-6, IL-10, TNF- α , and IFN- γ in the lungs [15].

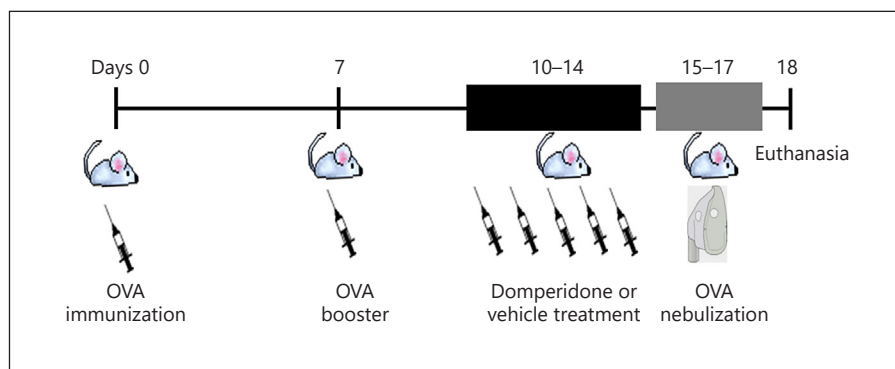
PRLR have been localized to a wide variety of cells. They are distributed throughout the immune system and belong to the superfamily, which includes prolactin, growth hormone, leptin, IL-2, IL-3, IL-4, IL-6, IL-7, erythropoietin, and leukemia inhibitory factors [16–18]. Expression of the PRLR can be regulated by various stimuli, among which are the inflammatory mediators. A differential expression of PRLR mRNA was observed in a murine model of acute inflammation. Following an intraperitoneal LPS injection, PRLR mRNA was found to be increased in the thymus; however, it was decreased in the liver, prostate, seminal vesicle, kidney, heart, and lung tissues, while no significant mRNA changes were observed in the spleen [19].

P2X7R was initially identified and characterized by Cockcroft and Gomperts [20] in rat mast cells, and named the “ATP4-receptor”. The effect of extracellular purines on immunosuppressive pathways is an expanding field of interest. Findings by Bianchi et al. [21] revealed that extracellular ATP might also have an important role in immunosuppression since mouse myeloid-derived suppressor cells overexpress P2X7R, which, in this cell type, is

coupled to release of immunosuppressive factors, such as arginase-1 and TGF β .

ATP is a signaling molecule in the extracellular medium that activates nucleotide receptors [22]. Both ATP and UTP bind to purinergic receptors 2. The purinergic P2X7 receptor located on hematopoietic cells is a key intermediary in signal transduction initiated at Toll-like receptors resulting in release of the mature IL-1 β . These purinergic receptors 2 are ionotropic. They are selective plasma membrane channels for monovalent cations activated by ATP. When activation of the P2X7R receptor occurs, there is Ca²⁺ and Na⁺ entry, and K⁺ output, with consequent depolarization of the membrane. The P2X7R receptor functionally alters the cation channel to an extensive pore, allowing transmembrane flow of hydrophilic molecules with a mass of approximately 507 kDa. Expression of the P2X7R receptor is increased during inflammation of the acute and chronic asthmatic airways and is highly expressed in immune cells [23, 24]. When exposed to high concentrations of ATP, it can form a large pore permeable to hydrophilic solutes [25, 26]. P2X7R receptors are expressed on various immune cells such as monocytes, macrophages, and dendritic cells [27]. In addition, the expression of P2X7R in lung tissue of mice with allergic inflammation is increased. Signaling of P2X7R receptors in structural cells may be related to the pathogenesis of allergic respiratory diseases [26]. Activation of P2X7R is associated with the production of proinflammatory cytokines IL- β and inflammatory mediators, such as NO and ROS [27]. The airway epithelium has been recognized as an important modulator of inflammatory events and remodeling of the airways in asthma. This epithelium secretes many inflammatory mediators such as cytokines, chemokines, eicosanoids, growth factors, free radicals and nucleosides, and nuclear factor κ B. The production of cytokines has been attributed to asthmatic airway epithelia related to the chemotaxis of eosinophils and Th2 lymphocytes [28]. Epithelial cells represent sources of cytokines and chemokines, which participate in localized inflammatory responses. Many of them stimulate eosinophils. Thus, the interactions of eosinophil-epithelial cells probably represent a central regulatory mechanism in modulating local immune responses in lung tissue. Epithelial cells express various immunomodulators, cytokines, and chemokines, causing the proliferation, recruitment, and activation of eosinophils and all proinflammatory leukocytes [29]. So far, to our knowledge, no study has evaluated the role of P2X7R expression in the mechanisms involving the effects of hyperprolactinemia on the immunomodulatory function of

Fig. 1. Experimental design of the effect of short-term hyperprolactinemia, prior to antigen challenge, on ovalbumin (OVA)-induced pulmonary allergic inflammatory response.



epithelial cells in a murine model of lung allergic inflammation.

Purinergic signaling is being actively explored for the treatment of diseases of the airways, including asthma, where both A2A receptors and P2X7 in particular were shown to be involved in human studies. For the treatment of chronic obstructive pulmonary disease, P2Y2 and P2X7 receptor involvement is under investigation [30].

Since both prolactin and purinergic receptors can be modulated by immune responses, we tested how they would respond in a model for allergic pulmonary response in rats. Moreover, since this model is sensitive to inhibitory effects of acute hyperprolactinemia, we investigated how these two receptors would respond to a prolactin-induced decrease of allergic pulmonary response.

Methods

Animals

Male Wistar rats obtained from the Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo were used. The animals were kept in ventilated, humidity- and temperature-controlled rooms (22°–23 °C) and artificially illuminated with a 12-h dark-light cycle, with the light phase beginning at 7:00 a.m. Water and food were provided ad libitum throughout the experiment, being withdrawn during the experimental observations. All animals were used in accordance with the guidelines of the FMVZ-USP Department of Pathology, which were based on the Colegio Brasileiro de Experimentação Animal and National Institutes of Health Guides for the Care and Use of Laboratory Animals.

Allergic Lung Inflammation Model in Rats

Rats were sensitized with 10 µg ovalbumin (OVA; Egg Albumin Grade II; Sigma-Aldrich, St. Louis, MO, USA) [31, 32] and 10 mg aluminum hydroxide (EMS Pharmaceuticals, Brazil) dissolved in phosphate-buffered saline (PBS) and administered subcutaneously on day 0 at a dose of 0.1 mg × kg⁻¹. One week later (day 7), rats were boosted subcutaneously with another OVA dose. For the

challenge with OVA aerosol (1% w/v in PBS), animals were individually placed in an inhalation chamber connected to an ultrasonic nebulizer for 15 min per day for 3 consecutive days (days 15, 16, and 17) according to previous studies [4, 13, 33].

Experimental Hyperprolactinemia

Hyperprolactinemia was induced by the dopamine receptor blocker domperidone (Johnson & Johnson, Brazil). Although domperidone does not cross the blood-brain barrier [34], it acts on hypophysis and increases PRL secretion [35–37]. Domperidone was administered at a dose of 1.7 mg × kg⁻¹ (i.p.) 3 times per day (6:30 a.m., 2:00 p.m., and 9:00 p.m., therefore a total of 5.1 mg × kg⁻¹ per day) for 5 consecutive days from the 10th to 14th day after OVA immunization [13, 37, 38]. This treatment protocol has effectively produced stable hyperprolactinemia for more than 30 days [13, 39].

Experimental Outline

As previously described [13], male rats, 60–90 days of age, were randomly divided into four groups: naive group with no treatment and no lung allergy, control group, vehicle group, and domperidone group with induced lung allergy and respective treatments.

On day 0, the control, vehicle, and domperidone groups were injected with OVA and boosted on day 7. Between days 10 and 14, the domperidone group was treated with domperidone as described above, whereas the vehicle group was administered 0.9% NaCl. On days 15, 16, and 17, the control, vehicle, and domperidone groups received OVA in aerosol form to induce lung allergy. The experimental outline is depicted in Figure 1.

Evaluation of PRLR by Flow Cytometry

Bronchoalveolar lavage (BAL) cells were fixed in 4% paraformaldehyde in PBS for 30 min, washed, and maintained in PBS at 4 °C. The following day, a blockade for nonspecific binding with mouse serum 1:10 was carried out in PBS. Cells were then washed and labeled with anti-PRLR monoclonal antibody conjugated to fluorochrome allophycocyanin (APC) (Novus Biologicals), at the manufacturer's recommended concentration for immunocytochemistry of 5 µg × mL⁻¹. After incubation for 1 h, a further wash was performed and the cells were resuspended in PBS for reading on the flow cytometer model FACScalibur (BD Biosciences). BAL and bone marrow cells were first identified on a graph of size and internal complexity (FSC × SSC) and then each population was

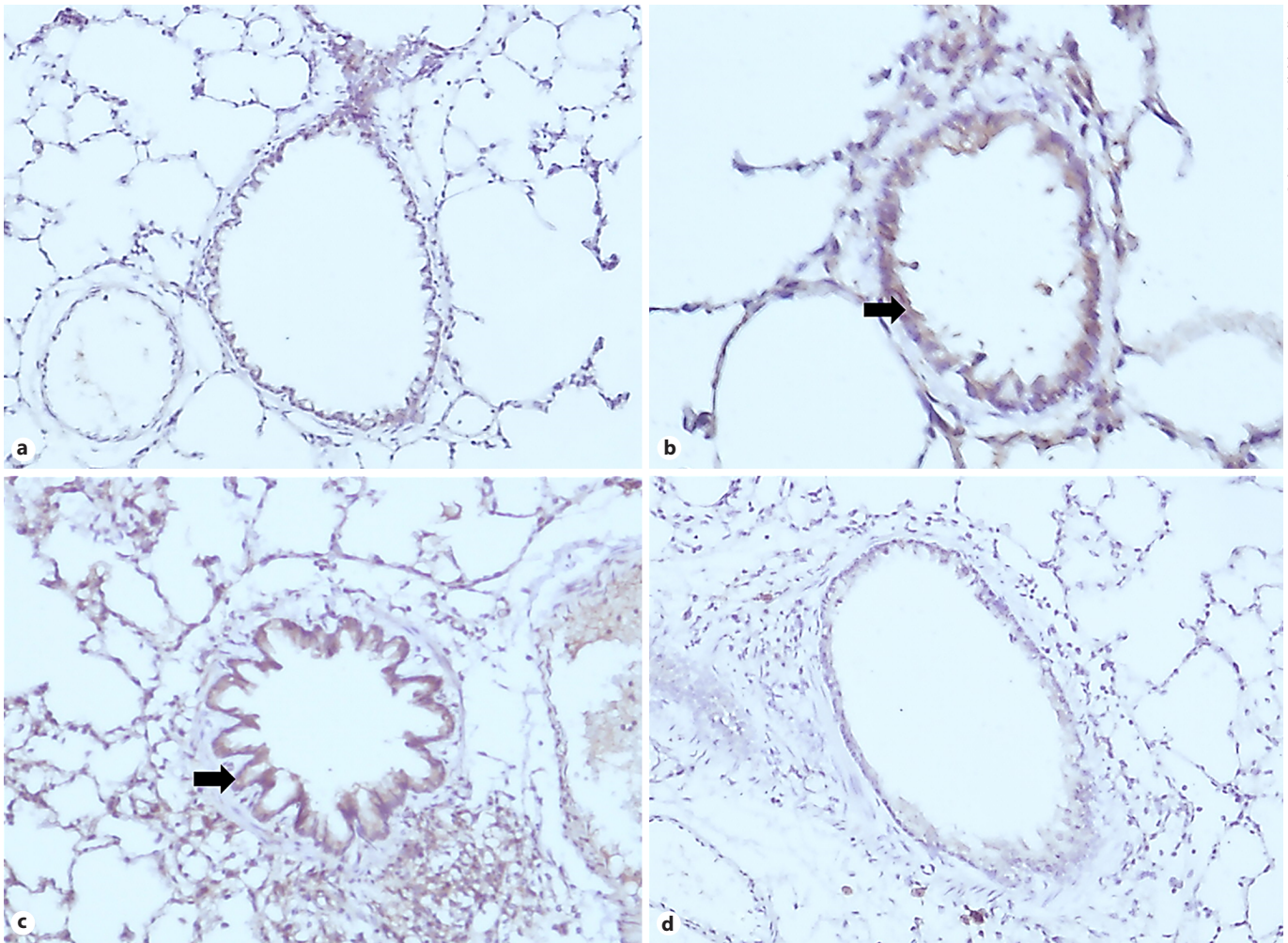


Fig. 2. Immunohistochemistry staining for P2X7 receptor in rat lungs. **a** Naive group. **b** Control group. **c** Vehicle group. **d** Domperidone group. DAB positivity for P2X7 receptor (brown) is indicated by black arrows.

evaluated for PRLR expression through APC positivity. The percentage of PRLR APC-positive cells and the number of receptors expressed per cell (geometric mean of APC fluorescence) were considered.

P2X7R Receptor Expression Analysis

Lungs were removed in block, fixed in buffered formalin, and embedded in paraffin. Sections of 5- μ m thickness were cut from lung tissue. Immunohistochemistry was performed with antibody to purinergic receptor 7 (P2X7R) (Santa Cruz, CA, USA). An ABC Vectastin kit (Vector Elite PK-6105 or PK-6101) was used as the secondary antibody and 3,3'-diaminobenzidine (DAB; Sigma Chemical Co., St. Louis, MO, USA) was used as the chromogen. Sections were counterstained with Harris hematoxylin (Merck, Darmstadt, Germany). The epithelial area was measured, as was the antibody and DAB-positive area, using an image analysis program (Image-Pro Plus; Media Cybernetics, Silver Spring, MD, USA). The results were expressed as the percentage

of immunostained-positive area relative to the total area of the epithelium. Measurements were made for the complete epithelium of 5 bronchioles per animal with a magnification of 400 $\times$ [26].

Results

Purinergic receptors were evaluated directly in lung tissue more specifically in bronchiole epithelial cells because of the functional relevance of P2X7R receptor signaling in allergic airway inflammation. Immunohistochemical staining for the purine P2X7R receptor has presented differences among experimental groups, as shown by ANOVA ($p < 0.0001$; $F = 42.27$). The Newman-Keuls multiple comparison test revealed that the groups with a

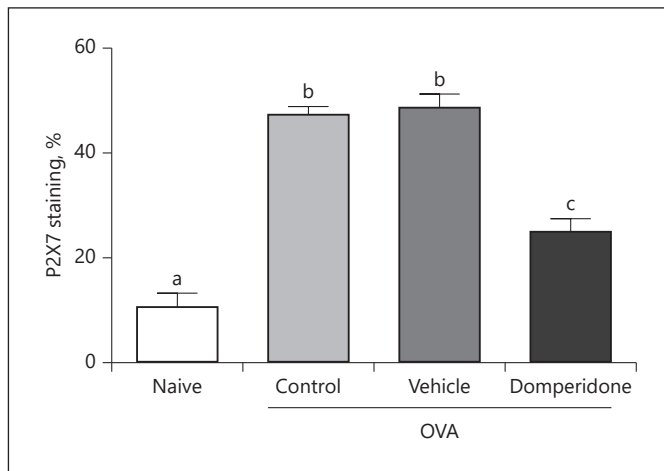


Fig. 3. Immunohistochemistry staining values for P2X7 receptor expression in lung of rats sensitized and challenged with ovalbumin (OVA). N, naïve, rats not sensitized, challenged, or treated; C, control rats immunized and challenged with OVA; V, vehicle, OVA-immunized and challenged mice treated for 5 days with physiological saline plus Tween 80 ($1 \text{ mL} \times \text{kg}^{-1}$); D, domperidone, OVA-immunized and challenged rats, treated for 5 days with domperidone ($5.1 \text{ mg} \times \text{kg}^{-1}$ body weight per day), divided into three daily injections (i.p.) at 6:30 a.m., 2:00 p.m., and 9:00 p.m. Different letters represent statistically significant differences.

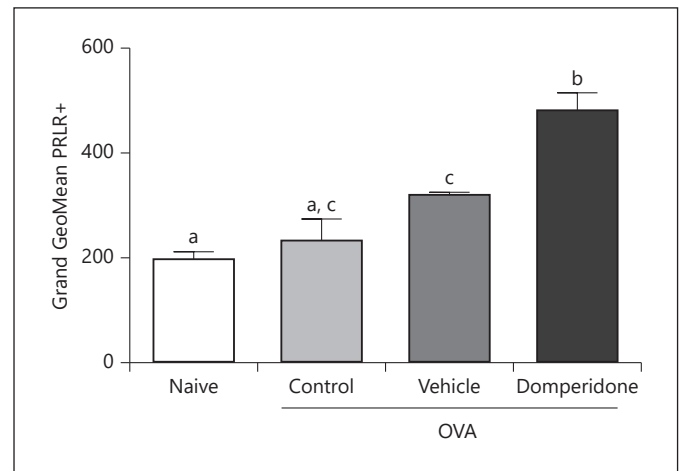


Fig. 4. Flow cytometry mean fluorescence intensity values of prolactin receptor (PRLR) expression in BAL granulocytes. N, naïve, rats not sensitized, challenged, or treated; C, control, rats immunized and challenged with OVA; V, vehicle, OVA-immunized and challenged mice treated for 5 days with physiological saline plus Tween 80 ($1 \text{ mL} \times \text{kg}^{-1}$); D, domperidone, OVA-immunized and challenged rats, treated for 5 days with domperidone ($5.1 \text{ mg} \times \text{kg}^{-1}$ body weight per day), divided into three daily injections (i.p.) at 6:30 a.m., 2:00 p.m., and 9:00 p.m. Different letters represent statistically significant differences.

higher expression of the purine receptor were the control and vehicle groups, and these groups were statistically different from the domperidone and naive groups. However, the domperidone group was still statistically higher in the expression of P2X7R receptor relative to the naive group (Fig. 2, 3).

There was a statistical difference in mean fluorescence intensity for PRLR in granulocytes among groups (ANOVA, $p = 0.0005$; $F = 19.63$). The Newman-Keuls multiple comparison test revealed that the domperidone group expresses more PRLR in relation to both the vehicle and control asthma groups and also in relation to the naive group ($p < 0.001$; Fig. 4). We found no significant difference between groups for the PRLR mean fluorescence intensity, that is, in the mean expression of PRLR by macrophages and lymphocytes (data not shown).

Discussion

The epithelial expression of the P2X7R receptor was evaluated in the context of the effects of hyperprolactinemia on the immunomodulatory function of epithelial cells in a rat model of pulmonary allergic inflammation.

The animals sensitized in the control and vehicle groups showed a significant increase in the epithelial expression of P2X7R, while the sensitized hyperprolactinemic animals reduced their expression, suggesting an inhibitory effect of hyperprolactinemia on OVA-induced overexpression of P2X7R.

Signaling of the P2X7R receptor contributes to the pathogenesis of pulmonary allergic inflammation [25, 26]. The expression of the receptor was increased in the lungs of animals in the control and vehicle groups. This increase was reversed in hyperprolactinemic animals. These results suggest that pulmonary allergy would have induced an increase in the expression of this receptor. Alternatively, the increase of this receptor may be important for the expression of pulmonary allergy. In addition, hyperprolactinemia appears to partially reverse this effect. Besides, the result speaks in favor of an increase in purinergic signaling as being important for the expression of the pulmonary allergic response.

Increased expression of the P2X7R receptor has already been described in eosinophils and macrophages during asthma [25]. P2X7R is expressed in pulmonary epithelial cells and related to the regulation of the immune system, including the control of proinflammatory

cytokines [28]. These receptors have a critical role in mediating inflammatory and immune responses, contributing to chronic inflammatory conditions in various disorders such as chronic inflammatory pulmonary disease, asthma, and inflammatory bowel disease [25]. Domperidone-treated animals showed a strong reduction in the intensity of pulmonary allergic inflammation, airway eosinophilia, mucus hypersecretion [13, 14], and P2X7R receptor expression. Consistent with the idea of an effect of prolactin in this circumstance, these hyperprolactinemic animals did not present a statistically significant difference from naive rats.

Mean intensity of immunofluorescence, which is an indicator of the number of receptors per cell, was higher in the domperidone group than in the other groups. This suggests that the domperidone group has greater expression of the receptors per cell for prolactin in granulocytes. Since eosinophils are primordial cells in the allergic response, it is possible that hyperprolactinemia caused granulocytes to express a larger number of PRLR per cell. In conclusion, our data provide evidence that a domperidone-induced increase in prolactin levels may contribute to fluctuations of both adenine and PRLR.

Several studies have shown that dopamine not only mediate interactions into the nervous system, but can also contribute to the modulation of immunity [40]. Despite the well-known role of dopamine in brain and behavioral functions, accumulating evidence indicates that immune cells also constitutively express tyrosine hydroxylase and secrete dopamine, as well as expressing dopamine receptors [15]. Components of the dopaminergic system have been identified not only in T cells and dendritic cells, but also in other immune cells such as B cells or monocytes/macrophages, which, like dendritic cells, constitute APCs for T cells. Interestingly, evidence has shown that the involvement of dopamine in the functioning of these immune cells is not limited to dopamine receptor expression, but that they also are capable of synthesizing and eventually releasing dopamine. Thereby, the release of dopamine during antigen presentation seems to be a general feature for APCs. The evidence presented suggests that dopamine and dopamine receptors play an important role in the regulation of the physiology of T cells and dendritic cells [40].

Part of the effect of domperidone may be independent of the effect of prolactin, because dopamine D₂ receptors are present in immune cells and it is known that dopamine regulates its physiology under inflammatory conditions. Besides controlling prolactin secretion, dopamine also has direct effects on immune function [15]. It has

been shown that dopamine D₂ receptor antagonist haloperidol treatment increases macrophage activity by enhancing cell spreading, phagocytosis, and nitric oxide (NO) release. In addition, dopamine protects against ventilation-induced lung injury, decreasing myeloperoxidase activity and neutrophil infiltration through inhibition of NLRP3 signaling pathways [15, 41–43].

Fibroblasts are important cells in lung inflammatory reactions and there is evidence that proinflammatory cytokines upregulate PRLR in lung fibroblasts (and in lung epithelial cells) where prolactin is anti-inflammatory [44]. Therefore, it is possible that in asthma PRLR are upregulated in lung fibroblasts and epithelial cells. Prolactin has been implicated as a modulator of immune function, and some of its actions may be linked to NO synthesis (NOS). It is known that NO acts as mediator of inflammation and during this prolactin could affect NO syntheses. Cytokines induce the expression of PRLR in pulmonary fibroblasts, allowing prolactin to inhibit NO production and the expression of induced NOS (iNOS). Thus, these might be mechanisms by which prolactin and its receptors modulate inflammatory immune processes [44].

In the lungs, prolactin stimulates cell maturation and the production of surfactant. Inflammatory cytokines such as TNF α , TGF β , IL-1 β , and IFN γ are the main regulators of NO production in lung epithelial cells. Endogenous prolactin modulates basal NO production in human alveolar epithelial cells (A549). Thus, fibroblasts produce prolactin and can locally process prolactin at a fraction of 16 KDa, enhancing their function as an autocrine factor in the lung [44]. As 16 KDa prolactin activates NF- κ B in primary lung fibroblasts, this effect may lead to the production of the iNOS gene [3, 45, 46]. It has been demonstrated experimentally that administration of 1,25(OH)₂D₃ alleviates the symptoms of the inflammatory response and decreased iNOS expression in the rat asthma model.

Additional studies are needed to assess whether targeting prolactin and cAMP in asthmatic patients has potential to improve the management of the worsening of asthma [47]. The present study shows that hyperprolactinemia decreases immunoreactivity for the purinergic P2X7R receptor in the lung and increases the number of PRLR in granulocytes. Thus, these results suggest a modulatory effect resulting from hyperprolactinemia induced after treatment with domperidone. Therefore, a relevant functional interaction among purinergic receptors and prolactin modulating the inflammatory response in the lungs seems likely.

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Disclosure Statement

The authors have no conflicts of interest to declare on the subject matter of this article.

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