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Evidence for autocrine and paracrine regulation of allergen-induced mast cell mediator release in the guinea pig airways[☆]

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ABSTRACT

Mast cells play an essential role in immediate type hypersensitivity reactions and in chronic allergic diseases of the airways, including asthma. Mast cell mediator release can be modulated by locally released autacoids and circulating hormones, but surprisingly little is known about the autocrine effects of mediators released upon mast cell activation. We thus set out to characterize the autocrine and paracrine effects of mast cell mediators on mast cell activation in the guinea pig airways. By direct measures of histamine, cysteinyl-leukotriene and thromboxane release and with studies of allergen-evoked contractions of airway smooth muscle, we describe a complex interplay amongst these autacoids. Notably, we observed an autocrine effect of the cysteinyl-leukotrienes acting through cysLT₁ receptors on mast cell leukotriene release. We confirmed the results of previous studies demonstrating a marked enhancement of mast cell mediator release following cyclooxygenase inhibition, but we have extended these results by showing that COX-2 derived eicosanoids inhibit cysteinyl-leukotriene release and yet are without effect on histamine release. Given the prominent role of COX-1 inhibition in aspirin-sensitive asthma, these data implicate preformed mediators stored in granules as the initial drivers of these adverse reactions. Finally, we describe the paracrine signaling cascade leading to thromboxane synthesis in the guinea pig airways following allergen challenge, which occurs indirectly, secondary to cysLT₁ receptor activation on structural cells and/ or leukocytes within

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the airway wall, and a COX-2 dependent synthesis of the eicosanoid. The results highlight the importance of cell-cell and autocrine interactions in regulating allergic responses in the airways.

Key words: histamine; leukotrienes; thromboxane; cyclooxygenase; asthma; prostaglandin

1. Introduction

Immediate hypersensitivity reactions evoked by allergen challenge or by IgE receptor crosslinking can be studied in the intact airways and in isolated airway preparations from multiple species, including humans (Adams and Lichtenstein, 1977; Lamm et al., 1984; Björk and Dahlen, 1993; Ellis et al., 1994; Nagase et al., 1995; Roquet et al., 1997; Eum et al., 1999; Ressmeyer et al., 2006; Cyphert et al., 2009). Resident mast cells are thought to be the source of the resulting mediator release and primary drivers of the end organ effects (Boyce, 2007; Weigand et al., 2009). The responses evoked, which include airway smooth muscle contraction, mucus secretion, plasma exudation and altered mucosal blood flow, are primarily attributable to the actions of preformed biogenic amines (histamine in humans and guinea pigs, serotonin in rats and mice), cysteinyl-leukotrienes, and cyclooxygenase products including prostaglandin D₂ (PGD₂) and thromboxane A₂ (TxA₂). These end organ effects acutely mimic the pathophysiological features of asthma and eventually lead to inflammation, airways hyperresponsiveness and exaggerated respiratory reflexes that ultimately drive the symptoms of upper and lower airway diseases (Roquet et al., 1997; Bradding and Arthur, 2016). An understanding of the molecular, biochemical, pharmacological and physiological processes underpinning these reactions has and will continue to contribute significantly to the development of more effective therapies for asthma and other allergic diseases of the airways.

Multiple hormones and autacoids regulate mast cell mediator release in the airways. These regulatory mechanisms include inhibitory effects of β_2 adrenoceptor agonists (Undem and Buckner, 1984; Undem et al., 1988; Drury et al., 1998), prostaglandin E_2 (PGE_2 acting via EP_2 receptors (Saad and Burka, 1983; Adams and Lichtenstein, 1985; Undem et al., 1987a; Undem et al., 1987b; Kay et al., 2006; S  fholm et al., 2015)) and nitric oxide (Larsson et al., 2005), but also excitatory effects mediated by agonists of adenosine A_3 receptors (Ramkumar et al., 1993; Tilley et al., 2003) and the G-protein coupled MRGPRX2 (McNeil et al., 2015). It would thus not be surprising if mast cell-derived mediators act via autoreceptors to modulate their own release. Circumstantial evidence both for and against autoregulation has been described in studies using purified mast cells (and basophils) in culture through lipoxigenase products, $cysLT_1$ and $CRTH2$ receptor activation (Peters et al., 1981; Peters et al., 1982; Cohan et al., 1989; Larsson et al., 1999; Glitsch et al., 2002; Jiang et al., 2006; Di Capite et al., 2009; Moon et al., 2014), but such actions have not been convincingly documented in the target tissues of the airways and lungs.

Guinea pigs closely resemble humans in their acute response to mast cell activation in the airways, with airway smooth muscle contractions occurring primarily through the activation of histamine H_1 receptors and leukotriene $cysLT_1$ receptors (Joiner et al., 1974; Adams and Lichtenstein, 1977; Lamm et al., 1984; Bj  rk and Dahlen, 1993; Ellis et al., 1994; Roquet et al., 1997; Ressmeyer et al., 2006; Canning and Chou, 2008). This differs substantially from rats and mice, where the contractions are due primarily to serotonin as well as a secondary activation of parasympathetic cholinergic nerves (Nagase et al., 1995; Eum et al., 1999; Cyphert et al., 2009; Weigand et al., 2009). No involvement of acetylcholine has been documented in the acute response to allergen or crosslinking of IgE receptors in airways isolated from guinea pigs or

humans (Joiner et al., 1974; Canning and Chou, 2008). Human and guinea pig mast cells also store and release little if any serotonin (Joiner et al., 1974; Kushnir-Sukhov et al., 2007).

Because of these similarities, we set out to further characterize the autacoid receptors regulating allergen-induced mediator release and smooth muscle contractions in the airways and lungs of actively sensitized guinea pigs. We describe extensive cross talk between the autacoids within these tissues, with both excitatory and inhibitory effects on histamine, cysteinyl-leukotriene and thromboxane release. We also provide the first evidence for a differential role for COX-2 in regulating mast cell degranulation and the delayed synthesis of eicosanoids by mast cells and resident cells modulated by mast cell derived mediators.

2. Materials and methods

Our institutional animal care and use committee approved all of the experiments described in this study. Male Hartley strain guinea pigs (100-200g; Charles River) were purchased pathogen free and maintained in accredited animal facilities with appropriate light cycles and with food and water provided *ad libitum*. These guinea pigs were actively sensitized to ovalbumin with intraperitoneal injections of the antigen (10 mg/ ml dissolved in saline and administered; 10 mg/ kg in each injection) delivered on days 1, 3 and 7 of a 28-day sensitization period. No adjuvants were used. Once the sensitization period had been completed, some of these animals were used for *in vivo* experimentation while the majority of the guinea pigs were used for tissue harvest and *in vitro* studies. To harvest the trachea and lungs from these animals, the guinea pigs were killed by asphyxiation in a vessel filled with carbon dioxide followed by exsanguination. Nonsensitized guinea pigs were also used in a similar manner.

2.1 Muscle contraction studies performed *in vitro*.

Tracheae from the sensitized guinea pigs were prepared as tracheal strips for tissue bath experiments. Segments of 2-3 adjacent cartilage rings were taken from the trachea, sutured to a tissue holder and an isometric force transducer positioned over a 10ml organ bath filled with Krebs bicarbonate buffer (composition (mM) NaCl (118), KCl (5.4), NaHPO₄ (1), MgSO₄ (1.2), CaCl₂ (1.9), NaHCO₃ (25) and dextrose (11.1), pH=7.4). The tissues were suspended in the organ baths with an initial baseline tension of ~1.5 grams (overall mean±S.E.M. after the development of spontaneous tone (no differences amongst treatment groups): 1.79±0.02 grams (range: 1.23-2.85 grams); 52 tracheal strips from 9 animals). After a 60 min equilibration period with fresh buffer added at 15 min intervals, the tissues were pretreated with various drugs (or relevant vehicles for control experiments) prior to ovalbumin challenge. Ovalbumin was administered in cumulatively increasing concentrations (1 ng/ ml – 1 µg/ ml), or as a single concentration (0.3 µg/ ml) to study the kinetics of the evoked contractions. The single dose of ovalbumin used in the kinetic studies was selected to achieve a near maximal contraction (≥80% of maximum), but not a supramaximal dose that would have hindered assessments of the response kinetics. Ovalbumin was not administered until the effects (if any) of the pretreating drugs on baseline tone had reached equilibrium (~45 mins). At the end of each experiment, 300 mM barium chloride (BaCl₂) was administered to establish the maximum/ reference contraction for each tissue. In some experiments, we evoked comparable responses in the trachealis of nonsensitized guinea pigs using the MRGPRX2 selective and mast cell selective agonist compound 48/80. The data were recorded digitally using a Biopac MP data acquisition system.

2.2 Bronchospasm studied *in vivo*.

The effects of ovalbumin inhalation on pulmonary inflation pressure (PIP) in anesthetized, paralyzed, mechanically ventilated guinea pigs were also studied. Animals were anesthetized with urethane (1.5g/ kg i.p.), with supplemental anesthetic administered as needed. The trachea was cannulated and attached to a ventilator (60 breaths/ min, 6 ml kg), generating a peak pulmonary inflation pressure of ~10 cmH₂O at baseline. A positive end-expiratory pressure of 3-5 cmH₂O was maintained to limit airway closure. Pulmonary inflation pressure was measured using a transducer attached to a side port adjacent to the tracheal cannula. Once mechanical ventilation had started, the guinea pigs were paralyzed with succinylcholine (2.5 mg/ kg sc). The lower abdomen was then exposed for cannulations of the vena cava and abdominal aorta for drug administration and for monitoring of heart rate and blood pressure, respectively. Ovalbumin (10 mg/ ml) was delivered as an aerosol using an ultrasonic nebulizer for 5 mins or until PIP had increased 25% over baseline.

2.3 Mediator release assays.

Mediator release evoked by ovalbumin challenge was studied in parallel with the functional studies performed on the tracheae. We used lung instead of the extrapulmonary airways to increase our tissue yield thus increasing the number of interventions that could be studied in each animal. Lungs were quickly removed from the animals after euthanasia, placed in chilled, oxygenated Krebs buffer and trimmed of all large airways and visceral pleura. The remaining tissue was then cut into progressively smaller pieces until each tissue fragment was 1-2 mm in thickness, length and width. These pieces were then impaled in groups of 2-3 onto fine tungsten wires and placed in test tubes containing warmed, oxygenated Krebs buffer. The tissues were moved sequentially from test tubes with solutions of differing composition for an equilibration

period (30 mins), baseline and pretreatment (30 mins in the absence or presence of various drugs or vehicle), ovalbumin challenge (1 mg/ ml with the same drugs present during the baseline and pretreatment), and finally into 0.4M perchloric acid to extract histamine for measurement of total histamine content. Mediator release is shifted ~100-fold to the right of smooth muscle contractile responses evoked by ovalbumin (Undem et al., 1987). Consequently, the dose of allergen used to study mediator release was selected to evoke maximal release, a response that functionally (if not pharmacologically) approximated the maximal/ near-maximal contractions evoked in the kinetic studies of smooth muscle contraction.

Histamine was measured fluorometrically and the results were expressed as a percentage of total histamine. The cysteinyl-leukotrienes (LTC₄ and LTD₄) and thromboxane (measured as the stable metabolite thromboxane B₂ (TxB₂)) were measured by immunoassays (Cayman) and expressed as pg/ mg tissue. Tissue supernatants were analyzed immediately for histamine, or frozen for subsequent measurements of the eicosanoids. Tissue pieces averaged 32.4±0.7 mg and contained 451±20ng histamine (14.7±0.3 ng histamine/ mg tissue). These sizes and histamine content did not on average differ amongst treatment groups (P>0.1; multiple tissues from n=12 guinea pigs). Lung histamine content varied little within tissues taken from the same lung (1.5-2.8 fold) but by more than 8-fold (3 vs. 25 ng histamine/ mg tissue) between animals.

Dilutions of the supernatants were made as needed to achieve autacoid concentrations in the measurable range for each assay. Data were further normalized as a percentage of control for analysis, with the results presented either as a mean ± S.E.M. or as a median with intraquartile ranges depending on whether the data were normally distributed.

2.4 Pharmacological analyses.

As stated in the introduction, we designed the *in vitro* experiments to evaluate potential autocrine and paracrine effects mast cell derived mediators on mast cell mediator release and the functional responses resulting from their actions in the airways. As these experiments were tissue-based assays, we relied on pharmacological approaches to address our hypotheses. Cyclooxygenases were targeted using the nonselective inhibitors indomethacin (3 μ M), meclofenamic acid (10 μ M), and the COX-2 selective inhibitor NS398 (10 μ M). Leukotriene cysLT₁ receptors were targeted using the cysLT₁ receptor antagonists montelukast (1 μ M), pranlukast (1 μ M) and ICI198615 (3 μ M). Histamine H₁ receptors were blocked using pyrilamine (1 μ M). Thromboxane TP receptors were blocked using ICI 192605 (1 μ M). Voltage gated sodium channels on nerves were blocked using tetrodotoxin (2 μ M). The concentrations used for each drug were selected based on their established potency in guinea pigs and/ or based on precedence in previously published studies (Canning and Chou, 2008). Vehicle control experiments were carried out in parallel such that in all *in vitro* experiments, all tissues were exposed to similar amounts of each vehicle used (DMSO, ethanol).

2.5 Statistical analyses.

Comparisons amongst group means were achieved by analysis of variance, with p-values of 0.05 considered statistically significant. Specific group means were compared using Scheffe's f-test for unplanned comparisons, or Mann-Whitney rank tests.

We evaluated the effects of various interventions on the sensitivity of the preparations to allergen-evoked contractions by comparing the contractile responses evoked by 0.03 μ g/ ml ovalbumin. We selected this concentration retrospectively, as 0.03 μ g/ ml ovalbumin approximated an EC₅₀ value in control tissues (50 $\pm$ 12% of the maximum response to ovalbumin;

n=7). We did not compare ovalbumin EC₅₀ values, in part because of the many unmeasurable variables (e.g. tissue mast cell content, allergen-specific antibody affinity, Fc receptor occupancy) that contribute to this value, but also because several of the interventions studied markedly altered (increased or decreased) the magnitude (in grams tension or when expressed as a % of the max. contraction evoked by BaCl₂) of the allergen evoked contractions. The effects of the interventions on the peak efficacy of the ovalbumin were evaluated by comparing the maximum contractions evoked by allergen and expressed as a percentage of the max. contraction evoked by BaCl₂.

2.6 Reagents.

Compound 48/80, dexamethasone, indomethacin, meclofenamic acid, NS398, ovalbumin, pyrilamine and urethane were purchased from Sigma. ICI 192605 and montelukast were purchased from Tocris and Cayman, respectively. Tetrodotoxin (TTX) was purchased from Alamone Labs. Glaxosmithkline and AstraZeneca provided pranlukast and ICI198615, respectively. UCB Pharma provided zileuton. Ovalbumin was dissolved in normal saline. Stock solutions of compound 48/80, pyrilamine, TTX and urethane were prepared in water. Indomethacin was prepared in an ethanol stock solution. All other stock solutions used DMSO. Vehicle control experiments (ethanol, DMSO) were carried out in parallel.

3. Results

3.1 Allergen-induced contractions of the airways in actively sensitized guinea pigs.

Ovalbumin challenge of tracheal strips isolated from actively sensitized guinea pigs induced contractions of the airway smooth muscle in a concentration-dependent manner. A concentration of 0.01 µg/ ml ovalbumin approximated a threshold dose for evoking contractions, with half-maximal and maximal contractions achieved at concentrations near 0.03 and 1 µg/ ml,

respectively. The kinetics of the ovalbumin-induced contractions could be studied administering a single concentration of 0.3 $\mu\text{g}/\text{ml}$ (Fig. 1A, Table 1). In control (untreated) tissues, peak contractile effects of $89\pm5\%$ of the maximal attainable contraction were achieved in 94 ± 7 seconds after challenge, and steadily declined but persisted even 60 mins after dosing ($15\pm6\%$ of the maximum at 60 mins).

Consistent with many previous studies performed using isolated airways from guinea pigs and humans (Adams and Lichtenstein, 1977; Björck and Dahlen, 1993; Ellis et al., 1994; Ressmeyer et al., 2006), kinetic studies of the contractions evoked by 0.3 $\mu\text{g}/\text{ml}$ ovalbumin confirmed the roles of histamine and the cysteinyl-leukotrienes in the immediate and sustained components of these evoked contractions, respectively (Fig. 1A, Table 1). Thus, the histamine H_1 receptor antagonist pyrilamine (1 μM) significantly delayed the time to peak contraction, while the cysLT_1 receptor antagonist montelukast (1 μM) decreased the duration of evoked contractions and the magnitude of contractions 60 mins after allergen challenge. Measuring the area under the curve from the raw data further documented the marked inhibitory effects of montelukast on the magnitude and duration of the evoked contractions ($46\pm11\%$ of control; $n=7$; $P<0.01$), while pyrilamine failed to significantly inhibit contraction area under the curve ($172\pm35\%$ of control; $n=9$; $P>0.05$).

Rightward (2 to >100 -fold) and downward shifts in ovalbumin concentration response curves also occurred after pretreatment with pyrilamine (1 μM), the cysLT_1 receptor antagonist ICI 198615 (3 μM) and/ or the 5-lipoxygenase inhibitor zileuton (10 μM). The $-\log EC_{50}$ value for control tissues averaged 7.7 ± 0.1 ($n=5$), but could not be calculated in many tissues with either histamine and/ or leukotriene actions blocked. Thus, peak contractile effects (expressed as a percentage of the maximum contraction induced by BaCl_2) evoked during ovalbumin

concentration response curves averaged $91\pm5\%$, $55\pm15\%$ and $41\pm17\%$ in control tissues and tissues where the actions of histamine or the cysteinyl-leukotrienes were prevented, respectively ($n=5$ / treatment group; $P<0.05$).

The effects of allergen on isolated airway preparations were predictive of whole lung responses to allergen inhalation *in vivo* (Fig. 1B). An aerosol challenge with 10 mg/ ml ovalbumin solutions substantially increased pulmonary inflation pressure recorded in anesthetized, mechanically ventilated guinea pigs that had been sensitized to the allergen. This bronchospasm was diminished in magnitude by pretreating the animals with pyrilamine and zileuton (1 mg/ kg each, administered intravenously).

Differing from the responses to allergen evoked in the airways of actively sensitized mice (Eum et al., 1999; Cyphert et al., 2009; Weigand et al., 2009), the contractions caused by ovalbumin in the airways of sensitized guinea pigs were insensitive to the neurotoxin tetrodotoxin (2 μ M), suggesting the lack of involvement of parasympathetic-cholinergic nerves in this acute, *in vitro* response (Fig. 2A). Rather, the functional responses to ovalbumin challenge are likely due primarily to the activation of resident mast cells. This assertion is supported in part by the observation that the allergen-induced contractions of trachealis taken from sensitized guinea pigs were mimicked by challenge of airways from nonsensitized guinea pigs with the MRGPRX2-selective and mast cell selective agonist compound 48/80 (McNeil et al., 2015). Like the responses to ovalbumin evoked in airways from sensitized animals, the contractile responses to compound 48/80 were sensitive to H_1 and $cysLT_1$ receptor antagonism (Fig. 2B).

Neither pyrilamine nor the $cysLT_1$ receptor antagonists modulated the cyclooxygenase-dependent baseline tone in tracheal strips isolated from naïve or sensitized guinea pigs (Fig. 3).

3.2 Cyclooxygenase products modulate allergen-induced contractions

Cyclooxygenase inhibition by the nonselective inhibitor indomethacin (3 μ M) and the COX-2 selective inhibitor NS398 (10 μ M) reversed intrinsic tone in the tracheal strips isolated from actively sensitized guinea pigs (Fig. 3). Notably, however, while indomethacin and NS398 both greatly enhanced the sustained, leukotriene-dependent phase of the allergen-induced contractions and markedly prolonged the duration of the contractions, indomethacin but not NS398 also accelerated the onset of contractile effects assessed over the first 40 seconds of challenge, a kinetic parameter largely dictated by histamine release during degranulation (Fig. 3, Table 1). Overall, both indomethacin ($292 \pm 79\%$) and NS 398 ($323 \pm 98\%$) markedly increased the area under the curve for these contractions relative to control ($n=9$; $P<0.01$). These effects of cyclooxygenase inhibition suggest a differential enhancing function on the histamine and leukotriene-dependent components of the allergen-induced response, an assertion we confirmed in subsequent studies of mediator release (see below). But we found little evidence for thromboxane/ TP receptor dependent involvement in these responses, as the TP receptor antagonist ICI 192605 (1 μ M) was without effect on baseline tone or on the magnitude and kinetics of the trachealis contractions evoked by allergen (Fig. 4; Table 1).

The effects of the cyclooxygenase inhibitors on the ovalbumin-induced contractions suggested the presence of a tonic inhibitory prostanoid that either limits mast cell activation or functionally antagonizes the resulting contractions. It is possible these inhibitory prostanoids are formed secondary to histamine H_1 receptor activation, as pyrilamine profoundly blunted the initial, histamine-dependent contractions evoked by ovalbumin but consistently potentiated the sustained, leukotriene-dependent contraction (Fig. 1A; Table 1).

3.3 Modulation of allergen-induced histamine and cysteinyl-leukotriene release from lung.

TxB₂ and the cysLTs were detectable in lung fragments studied *in vitro* at baseline (8.6±4.2 and 5.3±0.5 pg/ mg tissue, respectively). Both eicosanoids increased in concentration upon 1 mg/ ml ovalbumin challenge. Histamine was also released in response to ovalbumin, but only a fraction of the total histamine was released from the lungs by the antigen, indicating the presence of viable mast cells in these tissues. There was little correlation ($r^2=-0.346$) between tissue histamine content (which is proportional to mast cell numbers) and %total histamine released upon allergen challenge. But the amount of histamine release evoked by ovalbumin was predictive of LTC₄/ LTD₄ release ($r^2=0.80$), suggesting a mast cell origin for the leukotrienes (Fig. 5).

Consistent with previous studies (Saad and Burka, 1983; Adams and Lichtenstein, 1985; Undem et al., 1987a; Undem et al., 1987b), indomethacin markedly potentiated the release of both histamine and the cysteinyl-leukotrienes (Fig. 6). This effect of indomethacin was likely attributable to its actions on COX enzymes. Thus, in 12 paired experiments, potentiation of histamine release by indomethacin was strongly correlated ($R^2=0.75$) to the potentiation of histamine release induced by the structurally unrelated but similarly nonselective COX inhibitor meclofenamic acid (10 μ M), with a correlation line slope approximating unity (1.02). This effect of COX inhibition was predicted by the marked potentiation of the allergen-induced contractions observed following pretreatment with this nonselective cyclooxygenase inhibitor (Fig. 4). The COX-2 inhibitor NS398 mimicked the effects of indomethacin on LTC₄/ LTD₄ release. Notably, however, similar to its lack of effect on the initial, histamine-dependent phase of the allergen-induced contractions (Fig. 4B), NS398 did not potentiate evoked histamine release (Fig. 6).

Also consistent with some but not all previous studies (Schleimer et al., 1987; Guhlmann et al., 1989), the steroid prednisone (1 μ M) was without acute effect on allergen-induced release of histamine or LTC₄/ LTD₄ from guinea pig lung fragments (Table 2, Fig. 7). TP receptor antagonism with ICI192605 and histamine H₁ receptor antagonism with 1 μ M pyrilamine also failed to modulate histamine release.

Studies performed in purified cultures of mast cells and basophils indicate that products of lipoxygenase including the cysLT₁ receptor agonists LTC₄ and LTD₄ may provide feedback facilitation of mast cell activation (Peters et al., 1981; Peters et al., 1982; Cohan et al., 1989; Larsson et al., 1999; Glitsch et al., 2002; Jiang et al., 2006; Di Capite et al., 2009; Moon et al., 2014). A limitation of these previous studies is that by studying purified cultures of these high affinity IgE receptor-bearing cells, the experiments may have exaggerated cell-cell interactions by artificially producing a proximity of the cells not often observed in tissues. No studies prior to ours have attempted to validate these cell-based results in tissues. We saw a modest but consistent and statistically significant effect of the cysLT₁ receptor antagonist montelukast on LTC₄/ LTD₄ release (Table 2; Fig. 7). Pranlukast had a comparable inhibitory effect on leukotriene release (5-90% reduction in 4 of 6 experiments (58 $\pm$ 16% inhibition on average)) but it failed to achieve statistical significance (p=0.064). Both montelukast and pranlukast also reduced (10-58%) histamine release in 7 of 10 separate experiments (14 out of 20 tissues from 10 animals when compared to control tissues from these animals),, but in each instance these modest effects failed to achieve statistical significance.

3.4 Modulation of allergen-induced thromboxane release from lung.

While ovalbumin-induced histamine and cysteinyl-leukotriene release varied less than 6-fold from experiment to experiment, TxB_2 release was volatile but consistently robust, with levels measured over a 100-fold range and the lowest quantity following allergen challenge more than 15-times the highest amount of cysLTs recovered (314 vs. 19.9 pg/ mg tissue). As expected, indomethacin essentially eliminated detectable TxB_2 release evoked by allergen challenge (Table 2; Fig. 8). Unlike at least one previous study (Selg et al., 2008), however, we also saw a marked inhibition of TxB_2 release following NS398 pretreatment (Table 2; Fig. 8). This implicates COX-2 involvement in allergen-induced TxB_2 release. COX-2 expression is induced by allergic inflammation and can be prevented or eventually reversed by corticosteroids (Pang and Knox, 1997; Oguma et al., 2002). In the present study we observed that acute (30 min) prednisone pretreatment prior to allergen challenge generated some evidence for marked inhibition of TxB_2 release (measured at <10% of that recovered in control tissues in 6 out 9 experiments), but ultimately the results were too variable and failed to achieve statistical significance (Table 2).

Several previous studies have indicated that thromboxane release evoked by allergen challenge may occur secondary to mast cell activation and downstream from cysLT_1 receptor engagement (Krell et al., 1989; Cheng et al., 1990). We provide further evidence in support of this signaling cascade for TxB_2 release, as both montelukast and pranlukast greatly reduced allergen-induced TxB_2 synthesis in actively sensitized lungs (Table 2, Fig. 8).

4. Discussion

4.1 Autocrine and paracrine regulation of mast cell mediator release in the airways

Mast cells are the likely primary drivers of immediate hypersensitivity reactions driven by allergen. Mediators released acutely from human and guinea pig airway and lung mast cells

by allergen or by MRGPRX2 activation include histamine, LTC₄, PGD₂, and perhaps thromboxane (Saad and Burka, 1983; Schleimer et al., 1983; Schleimer et al., 1987; Ellis et al., 1994; Boyce, 2007; Bradding and Arthur, 2016). Granule proteins/ contents including tryptase are also released. Within the tissues, mast cell derived mediators act upon adjacent cells to induce the synthesis and subsequent release of additional autacoids, including PGE₂, PGF_{2α}, prostacyclin and possibly bradykinin and nitric oxide (Proud et al., 1983; Schleimer et al., 1983; Schleimer et al., 1987; Ellis et al., 1994; Ricciardolo et al., 1994; Martin and Broadley, 2002; Larsson et al., 2005). It is our belief that the interplay between mast cells and mast cell derived mediators and the structural cells surrounding the mast cells is critical to the pathogenesis of respiratory diseases including asthma, and this interplay was the focus of the research presented in the present study. We describe 3 previously unreported autocrine and paracrine effects regulating immediate hypersensitivity in the airways.

An example of the interactions between mast cell derived mediators and the structural cells surrounding them is apparent from studies showing the marked potentiation of immediate hypersensitivity responses by aspirin and other cyclooxygenase inhibitors/ non-steroidal anti-inflammatory drugs (NSAIDs). Cyclooxygenase inhibition potentiates mast cell mediator release and allergen-induced contractile responses, accounting in part for the exacerbations of upper and lower airways disease (e.g. aspirin-sensitive asthma) resulting from NSAID therapy (Saad and Burka, 1983; Adams and Lichtenstein, 1985; Undem et al., 1987a; Undem et al., 1987b; Kay et al., 2006; S  fholm et al., 2015). It is thought that inhibition of cyclooxygenase removes the tonic inhibitory paracrine effects of COX-derived PGE₂ in the airways, which, via EP₂ receptor activation, can inhibit mast cell mediator release. We confirmed the enhancing effects of COX inhibition in the present study using indomethacin and meclofenamic acid.

Notably, however, we found that the effects of indomethacin were only partially mimicked by the COX-2 selective inhibitor NS398. This COX-2 inhibitor potentiated cysteinyl-leukotriene release and the leukotriene (slow reacting substance of anaphylaxis; SRS-A) –dependent, slowly developing but sustained contractile phase of the immediate hypersensitivity reactions, but failed to potentiate histamine release, a result consistent with the lack of effect of NS398 on the initial, histamine-dependent kinetics of the allergen induced contractions of the airways.

The profound effects of NS 398 on baseline tone in the trachea and on allergen-induced cysteinyl-leukotriene release leaves little doubt that we have adequately dosed the drug, and the consistent lack of effect on histamine release, in contrast to the potentiation induced by indomethacin and meclofenamic acid in paired experiments, leaves little doubt about the differential effects of COX-1 and COX-2 on allergen-induced degranulation. These differential effects of nonselective and COX-2 selective inhibitors on histamine and cysteinyl-leukotriene release and on the associated phases of the allergen-induced contractions have not been previously reported (and the effects would have been difficult to document using analog equipment and chart recorders for the physiological studies). It is, however, noteworthy that COX-2 inhibitors are in general less effective at inducing symptoms in aspirin sensitive asthmatics (West and Fernández, 2003; Gyllfors et al., 2003; Harrington et al., 2008). We speculate that the differential effects of the nonselective and COX-2 selective inhibitors on histamine and cysLT release reflects their differential and perhaps compartmentalized effects on tonically produced inhibitory prostanoids (e.g. PGE₂) and/ or a differential effect on those inhibitory prostanoids produced as a consequence of mast cell activation. The data also suggest that mediators acutely released upon mast cell degranulation are the primary initial drivers of

aspirin/ NSAID-induced pseudo-allergic reactions (although leukotrienes likely contribute at later stages in the reaction to the pathophysiological effects of COX-1 inhibition).

Possible sources of PGE₂ in the airways include the epithelium and airway smooth muscle (Krell et al., 1989; Undem et al., 1987a; Undem et al., 1987b; Undem et al., 1987c; Undem et al., 1988; Charette et al., 1995; Martin et al., 2012). Epithelium removal has been shown to reduce PGE₂ release evoked during allergen challenge in guinea pig trachea and human bronchi, and in guinea pigs at least the PGE₂ may be a downstream product of histamine H₁ receptor activation. But epithelium removal is reported to have little effect on allergen-induced histamine and LTC₄ release (Undem et al., 1988). Although further work is warranted, these data suggest that H₁ receptor antagonists might have opposing influences over immediate hypersensitivity reactions, preventing the end organ effects driven by histamine H₁ receptor activation and perhaps working synergistically with cysLT₁ receptor antagonists in asthma (Lamm et al., 1984; Roquet et al., 1997; Bartho and Benko, 2013), but also preventing the H₁ receptor dependent release of inhibitory prostanoids, an effect that may in turn promote enhanced mast cell degranulation.

Thromboxane release and TP receptor activation has also been described during acute responses to allergen in the airways (Schleimer et al., 1987; Krell et al., 1989; Cheng et al., 1990; Ellis et al., 1994; Sundström et al., 2003; Selg et al., 2008; Liu et al., 2012; Säfholm et al., 2013; Liu et al., 2015). The source of thromboxane released during the acute phase of allergic responses in the airways has not been firmly established. Purified human mast cells do release modest amounts of thromboxane upon IgE receptor crosslinking (Schleimer et al., 1983), but many other cells that are activated and/ or recruited to the airways during an acute allergic response can produce thromboxane. These other potential sources include platelets, eosinophils,

macrophages and perhaps airway smooth muscle and epithelial cells. We confirmed previous reports implicating cysLT_1 receptor activation as an essential upstream paracrine signal promoting thromboxane release in response to allergen in guinea pigs (Krell et al., 1989; Cheng et al., 1990). Unlike Selg and colleagues (Selg et al., 2008), however, who saw absolutely no effect of a COX-1 selective inhibitor on thromboxane release and an ~50% inhibition of thromboxane release following COX-2 inhibition that failed to achieve statistical significance, we found that the COX-2 inhibitor NS398 essentially abolished allergen-induced thromboxane release evoked from the lungs of sensitized guinea pigs. We speculate that the thromboxane formed during the acute response to allergen in guinea pigs is derived primarily from non-mast cell sources, but we acknowledge the circumstantial evidence we are using to support this assertion. Given the involvement of COX-2, we also speculate that preventing this secondary release of thromboxane following allergen exposure may be an additional therapeutic benefit of chronic corticosteroid therapy.

Like Selg and colleagues (Selg et al., 2008), we found allergen-evoked thromboxane release far in excess (by weight) of the $\text{LTC}_4/\text{LTD}_4$ released. Through TP receptor activation, thromboxane and other TP receptor agonists (e.g. PGD_2) can cause profound bronchospasm *in vivo* and airway smooth muscle contraction *in vitro*. Thromboxane has thus been implicated as an important mediator of airway allergic responses and perhaps asthma (Sundström et al., 2003; Liu et al., 2012; Säfholm et al., 2013; Liu et al., 2015). But unlike Selg et al., who describe TP receptor dependent constrictor responses to allergen challenge of perfused whole lungs (Selg et al., 2008), we found no evidence for TP receptor involvement in the allergen-induced contractile responses evoked in the guinea pig trachea. Our results are consistent with most other investigators that have studied only tracheal and larger airway preparations in guinea pigs and

humans (Adams and Lichtenstein, 1977; Björk and Dahlen, 1993; Ellis et al., 1994; Säfholm et al., 2013), while the results of Selg et al. are consistent with the results from experimenters that have studied more peripheral airways in guinea pigs and in other species (Ressmeyer et al., 2006; Selg et al., 2008; Martin and Broadley, 2002; Sundström et al., 2003). We thus suspect that the difference between our studies and those of Selg et al. is likely explained by the different airway preparations used to study the end organ effects of acute allergen challenge.

A modulatory autocrine role for leukotrienes in regulating mast cell activation has been described in studies of mast cells maintained in culture (Jiang et al., 2006; Di Capite et al., 2009). It has also been reported that lipoxygenase inhibitors can diminish degranulation of human basophils and purified human lung mast cells (Peters et al., 1981; Peters et al., 1982). Comparable results have been reported in studies of cultured rat basophilic leukemia (RBL) cells (Glitsch et al., 2002). In the present study, we observed a modest but consistent autocrine regulation of lung mast cell activation by the cysteinyl-leukotrienes, with both montelukast and pranlukast partially reducing histamine release and cysteinyl-leukotriene release evoked by ovalbumin. Such feedback regulation is common in neural systems but has not been well described for mast cells or other immune cells that regulate allergic reactions within tissues (Ramilowski et al., 2015). It will be important to confirm these novel results in human airway preparations and with full concentration response curves for the allergen (and not the single dose studies we performed), but the effects seemed modest at best and probably of limited physiologic significance. Indeed, the effects of the cysLT_1 receptor antagonists on histamine release failed to reach statistical significance (even though release was reduced (10-58%) following cysLT_1 blockade in 22 out of 30 separate tissues from 10 animals). Comparable tonic modulation by 5-LO products/ cysLT_1 receptor activation has been described in studies of intrinsic tone in human

airways and neuropeptide release from capsaicin-sensitive nerves in guinea pigs (Ellis and Undem, 1991; Ellis and Undem, 1994). Perhaps the LTC₄/ LTD₄ measurable even in the absence of allergen challenge is sufficient to account for these tonic effects of cysLT₁ receptor activation on mast cells, smooth muscle and airway sensory nerves.

4.2 Implications and Clinical Relevance

The intricate crosstalk between the autacoids acutely released from the airways upon allergen challenge described here highlights the many advantages of studying these processes within tissues and not exclusively using purified cells of interest. Without question, there are advantages to purified cell based studies, which provide more definitive descriptions of signal transduction pathways than is feasible in tissue or whole animal based studies, where it is often difficult if not impossible to implicate specific cell types as regulators of the end organ effects that can be evoked. But no cells function in isolation, and purified cell preparations may exaggerate the potential for autocrine regulation. The airways are a functional unit comprised of multiple cell types, and purified cell based assays are likely to miss the important paracrine interactions described here, including the inhibitory effects of prostanoids on mast cell mediator release, and the critical role of cysLT₁ receptor activation in promoting thromboxane release. This extends further to the whole animal, with isolated tissues failing to account for the influence of blood flow and of reflexes in modulating the end organ effects that ultimately drive the pathophysiology in models of human conditions such as asthma (Csete et al., 1991; Mazzone et al., 2010).

With the emergence of transgenic mouse models purporting to recapitulate the pathophysiological features of asthma and the almost exclusive use of mice as tools for drug discovery in respiratory disease, the results of studies such as ours highlight yet again the many

limitations of murine models of respiratory disease and the great predictive value of guinea pigs (Canning and Chou, 2008). We have previously described the many advantages of guinea pigs in studying these end organ effects and the many disadvantages of murine models (Canning and Chou, 2008). The results of this tissue based study of the immediate hypersensitivity reaction further establishes the utility of the guinea pig as a preclinical model of human respiratory and allergic diseases.

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Fig. 1. Kinetic and pharmacological characterization of ovalbumin-induced contractions of tracheal strips isolated from actively sensitized guinea pigs *in vitro* and bronchospasm evoked *in vivo*. A) The involvement of histamine and the cysteinyl-leukotrienes in the early and late phases of ovalbumin-evoked contractions is apparent in kinetic studies when comparing responses evoked by 0.3 µg/ ml ovalbumin in control tissues and following H₁ (1 µM pyrilamine) or cysLT₁ (1 µM montelukast) receptor blockade. B) Bronchospasm evoked by ovalbumin inhalation is mediated by histamine and the cysteinyl-leukotrienes. Anesthetized, paralyzed, mechanically-ventilated guinea pigs were challenged by aerosol with either saline or ovalbumin (10 mg/ ml) for 5 mins or until pulmonary inflation pressure increased by 20% above baseline. Prior to ovalbumin challenge, animals were pretreated with vehicle (saline) or a

combination of pyrilamine and zileuton (1 and 10 mg/ kg, respectively). The duration (range: 48-300 seconds; median: 240 seconds) of aerosol challenges did not differ amongst the treatment groups ($P>0.1$). Results are the mean $\pm$ S.E.M. of 5-9 experiments. An asterisk (*) indicates a statistically significant difference relative to the other treatment groups ($P<0.05$). See table 1 for comparisons of the kinetic properties of these responses.

Fig. 2. This graphs summarize evidence against the involvement of nerves but in favor of a role for mast cells in the acute contractile effects of allergen challenge in guinea pigs. A)

Contractions were evoked by 0.1 μ g/ ml ovalbumin in control tissues and in tissues pretreated with the voltage-gated sodium channel tetrodotoxin (TTX; 2 μ M; n=12). TTX failed to significantly reduce the magnitude of the evoked contractions and did not delay the attainment of peak contractile effects (16.1 ± 1.3 vs. 15.1 ± 1.7 mins in control and TTX-treated tissues, respectively; $P>0.1$) nor shorten the duration of the evoked contractions (63 ± 9 vs. 67 ± 16 mins in control and TTX-treated tissues, respectively; $P>0.1$). B) The MRGPRX2 selective and mast cell selective agonist compound 48/80 (1 mM) mimicked the effects of ovalbumin when administered to tracheal strips isolated from non-sensitized guinea pigs. Like those evoked by allergen, the contractions evoked by compound 48/80 were reduced in magnitude by H_1 (1 μ M pyrilamine) and $cysLT_1$ (1 μ M montelukast) receptor antagonism. Results in panel B are presented as the median and intraquartile ranges (25, 75) of 5 separate experiments. An asterisk (*) indicates a statistically significant difference relative to the contro response ($P<0.05$).

Fig. 3. The cyclooxygenase inhibitors indomethacin (3 μ M) and NS 398 (10 μ M) reverse the intrinsic tone present in tracheal strips isolated from actively sensitized guinea pigs. By contrast, H_1 (1 μ M pyrilamine), $cysLT_1$ (1 μ M montelukast) or thromboxane TP (1 μ M ICI 192605)

receptor antagonists were without effect on intrinsic tone. Data are presented as the mean $\pm$ S.E.M. of 7-9 experiments.

Fig. 4. The nonselective COX inhibitor indomethacin (3 μ M) and the COX-2 selective inhibitor NS 398 (10 μ M) enhance 0.3 μ g/ ml ovalbumin-induced contractions of tracheal strips isolated from actively sensitized guinea pigs. Both COX inhibitors greatly enhanced the sustained, leukotriene-dependent phase of ovalbumin-induced contractions (panel A) while indomethacin but not NS 398 accelerated the rate of the initial, histamine-dependent contractions evoked by the allergen (panel B). These results are presented as the mean $\pm$ S.E.M. of 9 experiments. See table 1 for comparisons of the kinetic properties of these responses. The data used in panel B are extracted from panel A to illustrate the different kinetics observed during the initial responses to ovalbumin challenge. An asterisk (*) indicates a statistically significant difference relative to the control groups ($P<0.05$).

Fig. 5. Histamine, cysteinyl-leukotrienes (LTC₄, LTD₄ and LTE₄), and thromboxane (measured as the stable metabolite thromboxane B₂) are released acutely in response to ovalbumin challenge (1 mg/ ml) of lung fragments isolated from actively sensitized guinea pigs. The results are presented as the mean $\pm$ S.E.M. (panels A and B) or median and intraquartile ranges (25, 75) from 6-10 separate experiments.

Fig. 6. Effects of the cyclooxygenase inhibitors indomethacin and NS 398 on 1 mg/ ml ovalbumin-induced A) cysteinyl-leukotriene and B) histamine release from lung fragments isolated from actively sensitized guinea pigs (see Table 2 for analysis). The paired results from each of 4-10 experiments performed with both drugs are presented. Red-shaded lines and symbols indicate an increase in mediator release relative to control, while blue shading indicates decreases. Both COX inhibitors potentiated leukotriene release, a result consistent with their

effects on the sustained phase of ovalbumin-induced contractions of guinea pig trachealis (see Fig. 4A). By contrast, only indomethacin potentiated the allergen-induced histamine release (panel B), a result that is consistent with the differential effects of these COX inhibitors on the initial (histamine-dependent) rate of ovalbumin-induced contractions (Fig. 4B). Indeed, NS 398 modestly but consistently inhibited the release of histamine ($P < 0.05$). The structurally unrelated COX-1 and COX-2 inhibitor meclofenamic acid (10 μM) mimicked the effects of indomethacin on histamine release (see Results for details; $n=12$). Median values are normalized to control values.

Fig. 7. The cysLT_1 receptor antagonists montelukast and pranlukast (1 μM each) modestly but consistently (31 out of 42 separate measurements) inhibit A) leukotriene and B) histamine release evoked by ovalbumin challenge (1 mg/ ml) of lung fragments taken from actively sensitized guinea pigs. In contrast to the effects of the cysLT_1 receptor antagonists, neither C) prednisone or ICI 192605 nor D) pyrilamine consistently modulated evoked histamine release. Note in panel D, however, that combining pyrilamine with montelukast resulted in a consistent albeit modest inhibition of histamine release in 8 of 10 experiments. The paired results from each of 6-10 experiments performed with each are presented. Red-shaded lines and symbols indicate an increase in mediator release relative to control, while blue shading indicates decreases. Median values are normalized to control values. See Table 2 for further analysis.

Fig. 8. Cyclooxygenase inhibitors and cysLT_1 receptor antagonists inhibit thromboxane release evoked by ovalbumin challenge (1 mg/ ml) of lung fragments taken from actively sensitized guinea pigs. A) Both the nonselective cyclooxygenase inhibitor indomethacin and the COX-2 selective inhibitor NS 398 essentially abolished evoked thromboxane release. B) Pranlukast and montelukast also markedly inhibited the allergen-induced release of thromboxane from the lung. The paired results from each of 6-10 experiments performed with each drug are presented. Red-shaded lines and symbols indicate an increase in mediator release relative to control, while blue shading indicates decreases. Median values are normalized to control values. See Table 2 for further analysis.

Table 1. Pharmacology, kinetics and magnitude of 0.3 µg/ ml ovalbumin-induced contractions of tracheal strips isolated from actively sensitized guinea pigs.

	Control	Pyrilamine	Montelukast	ICI192605	Indomethacin	NS 398
Max (%Ba++)	88.6±4.7	65.5±6.3 ^b	83.7±4.6	87.9±3.6	93.7±3.2	97.4±1.3
T_{max}	68±5s	290±66s ^b	50±4s	83±13s	185±10s ^b	127±18s ^a
T_{1/2}	418±112s	889±237s	174±30s ^a	432±121s	>60 min ^b	>60 min ^b
40s (%max)	5.4±3.3	1.4±0.6	30.0±10.9 ^a	5.5±3.4	29.7±8.0 ^b	8.0±3.8
60 min (%max)	19.7±4.6	53.3±4.5 ^b	9.0±7.3 ^a	22.8±4.4	76.6±3.1 ^b	77.7±5.7 ^b
AUC (%control)	-	172±35%	47±11% ^b	147±51%	292±79% ^b	323±98%

The results are presented as the mean±S.E.M. of 7-9 experiments in tissues pretreated with vehicles (DMSO, ethanol; control), pyrilamine (1 µM), montelukast (1 µM), ICI192605 (1 µM), indomethacin (3 µM) or NS 398 (10 µM), with parameters calculated including the %maximum attainable contraction (Max (%Ba++), relative to a reference contraction evoked by BaCl₂), the time (in secs (s)) taken to attain peak contractions (T_{max}), the time (in secs (s) or mins (min)) to 50% reversal from peak (T_{1/2}), the magnitude (relative to their own maximum) of the contractions 40 secs (40s (%max)) and 60 mins (60 min (%max)) after ovalbumin administration (0.3 µg/ ml), and the contraction area under the curve (AUC), expressed as a percentage of the control response. The P-values for comparisons amongst the treatment groups to control tissues were significant at <0.05 (^a) and <0.01 (^b).

Table 2. Pharmacological modulation of 1 mg/ ml ovalbumin-induced mediator release evoked from lung fragments isolated from actively sensitized guinea pigs.

Treatments	Histamine		cys-LTs		Thromboxane	
	%Total	%Control	pg/mg tissue	%Control	pg/mg tissue	%Control
Indomethacin	9.3±1.1	159±20 ^a	32.3±4.0	392±105 ^a	22±14	3±3% ^a
NS 398	4.7±0.6	80±9	12.5±2.1	289±71 ^a	68±34	1±0.4%
Prednisone	5.9±0.7	104±14	4.4±1.1	96±32	3624±2623	208±207
Montelukast	5.1±0.7	91±19	4.3±1.2	63±30 ^a	682±315	25±16 ^a
Pranlukast	5.0±0.7	83±8	6.3±1.7	80±28	386±100	28±16 ^a
Pyrilamine	6.9±1.1	117±14	NA		NA	
ICI 192605	5.5±0.7	101±18	NA		NA	

The results are presented as the mean±sem of 4-10 experiments in tissues pretreated with indomethacin (3 µM), NS 398 (10 µM), prednisone (1 µM), montelukast (1 µM), pranlukast (1 µM), pyrilamine (1 µM) or ICI 192605 (1 µM). Leukotriene and thromboxane release data are presented as absolute values or expressed as a percentage of the release measured in paired control tissues from the same animals. Histamine release was normalized to total histamine content, with baseline values subtracted from the evoked amounts. ^aIndicates a statistically significant difference relative to the control groups (P<0.05).

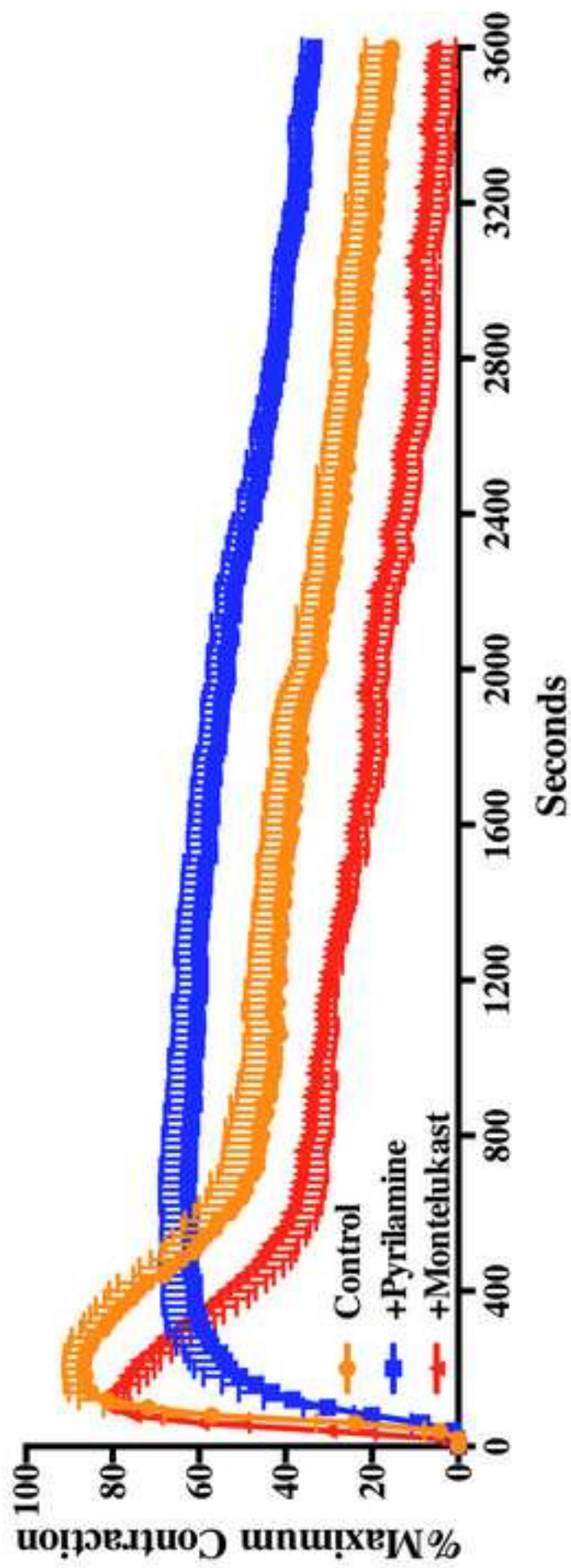
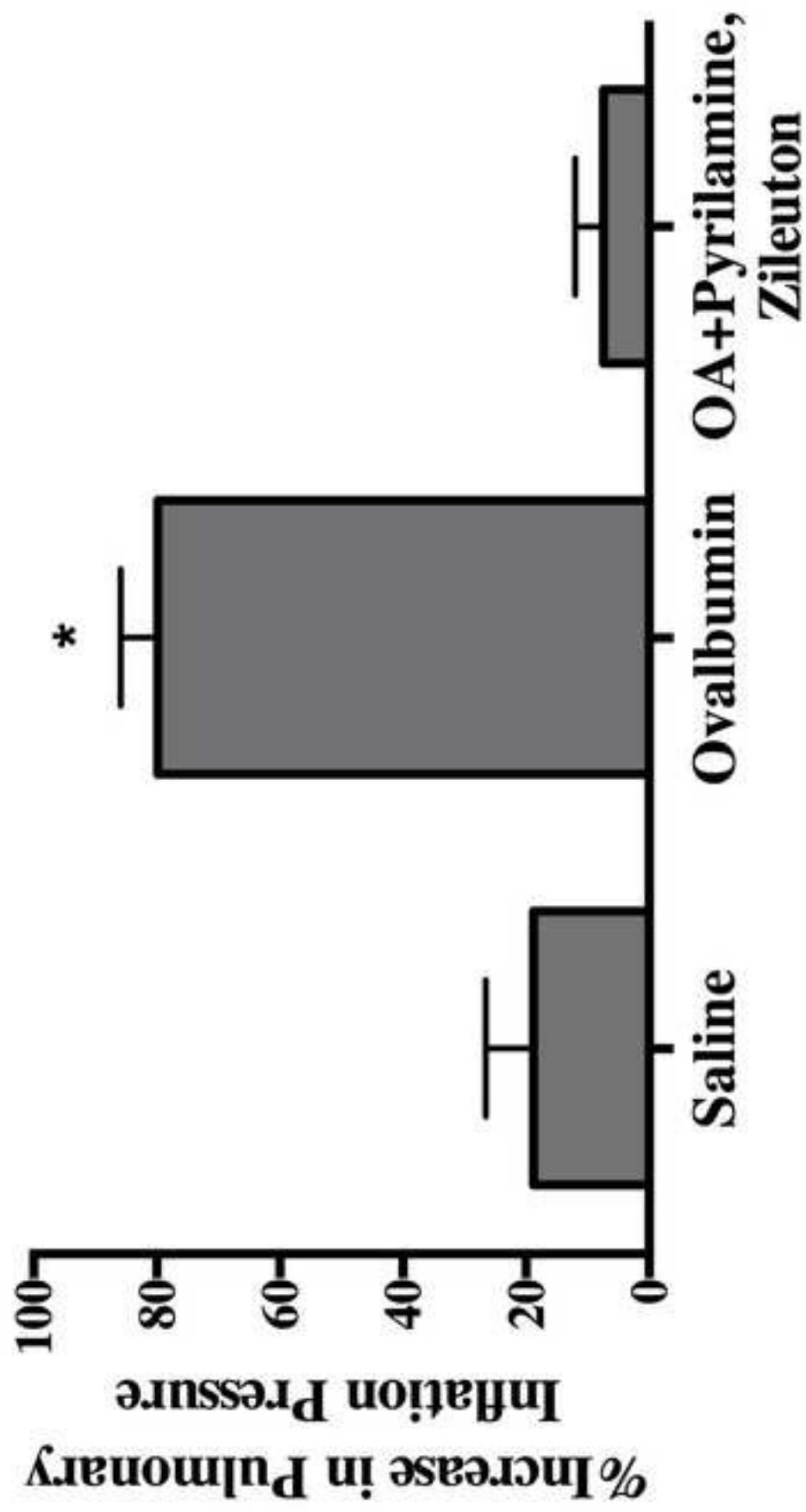
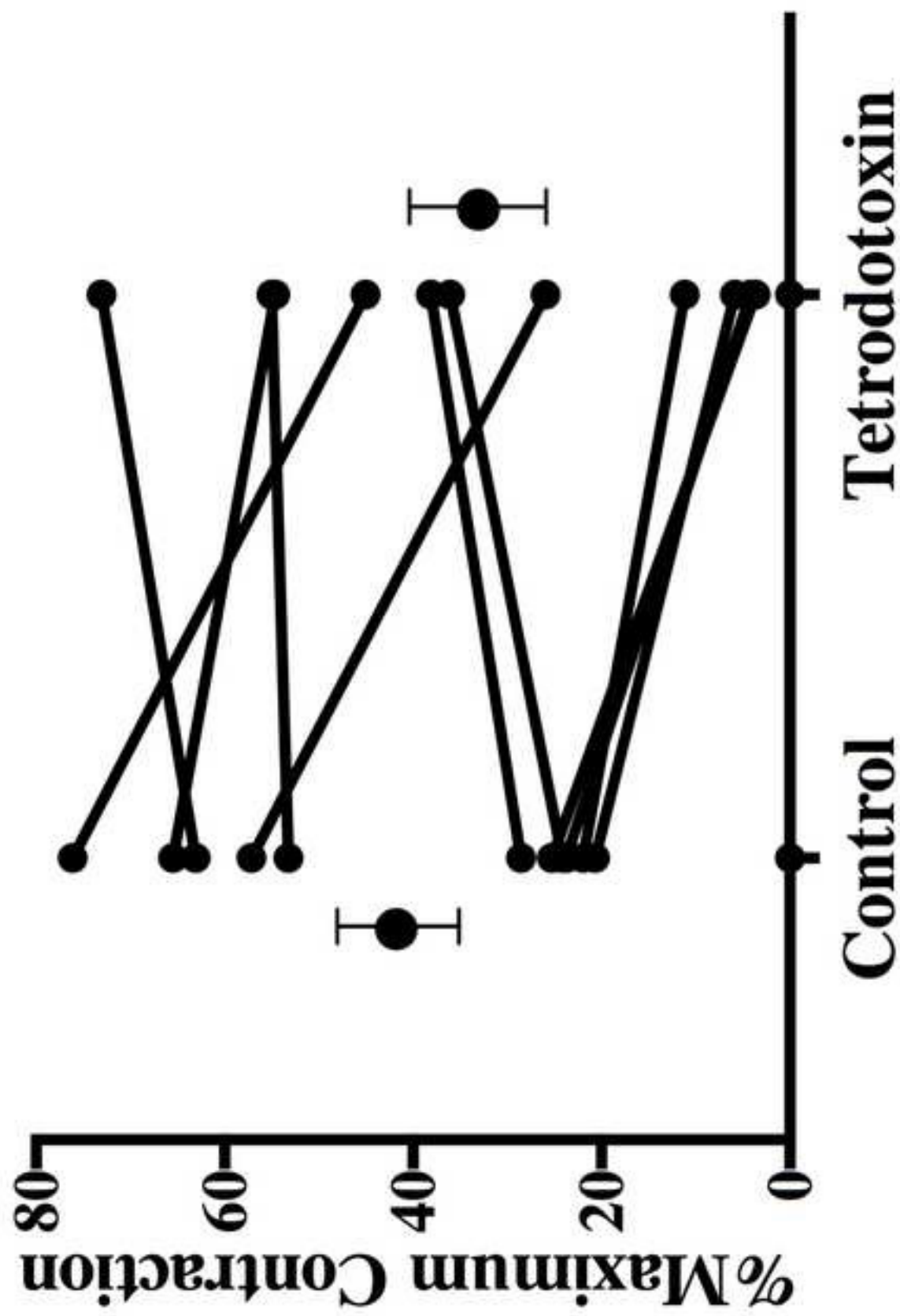


Fig. 1A





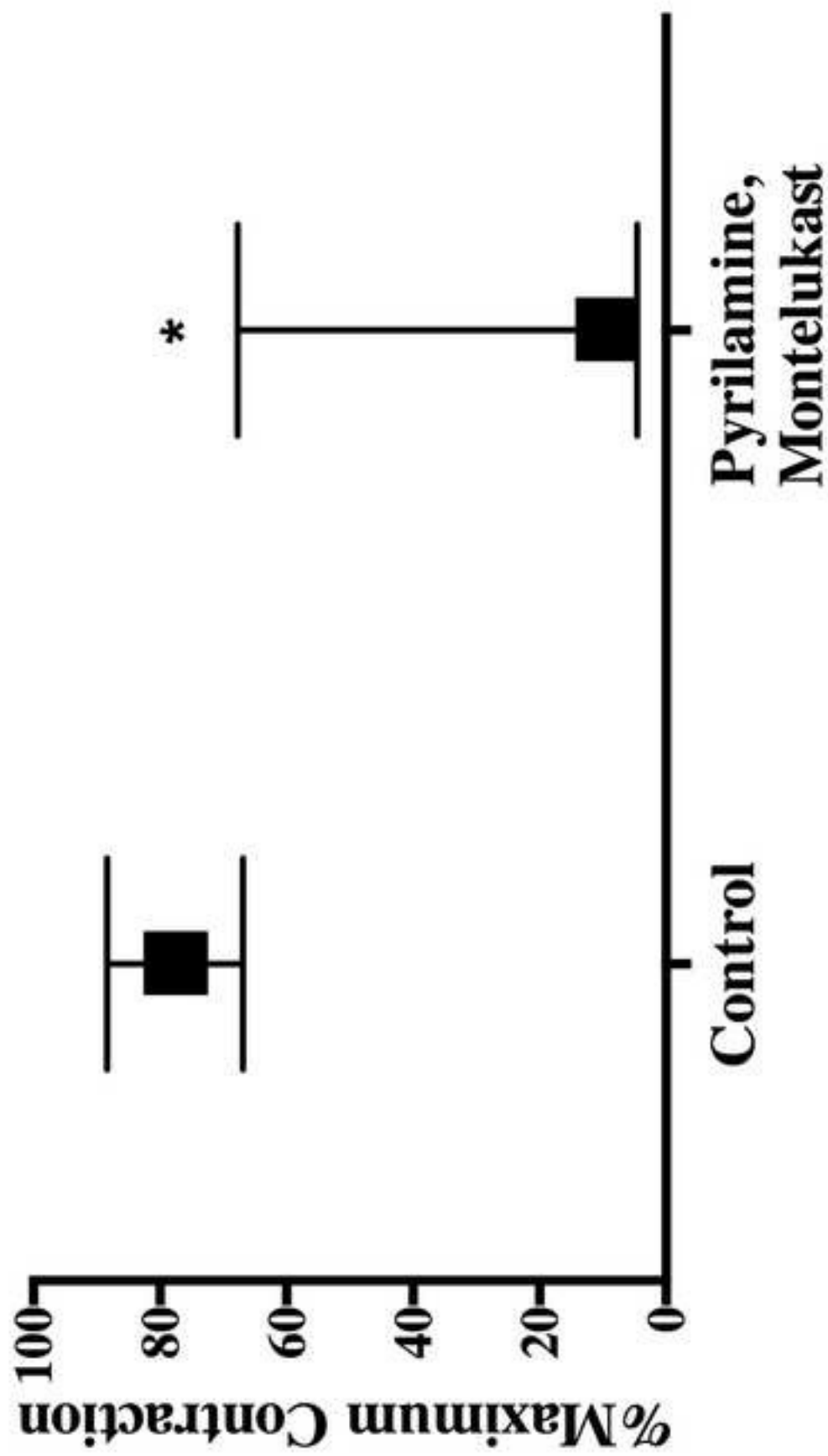
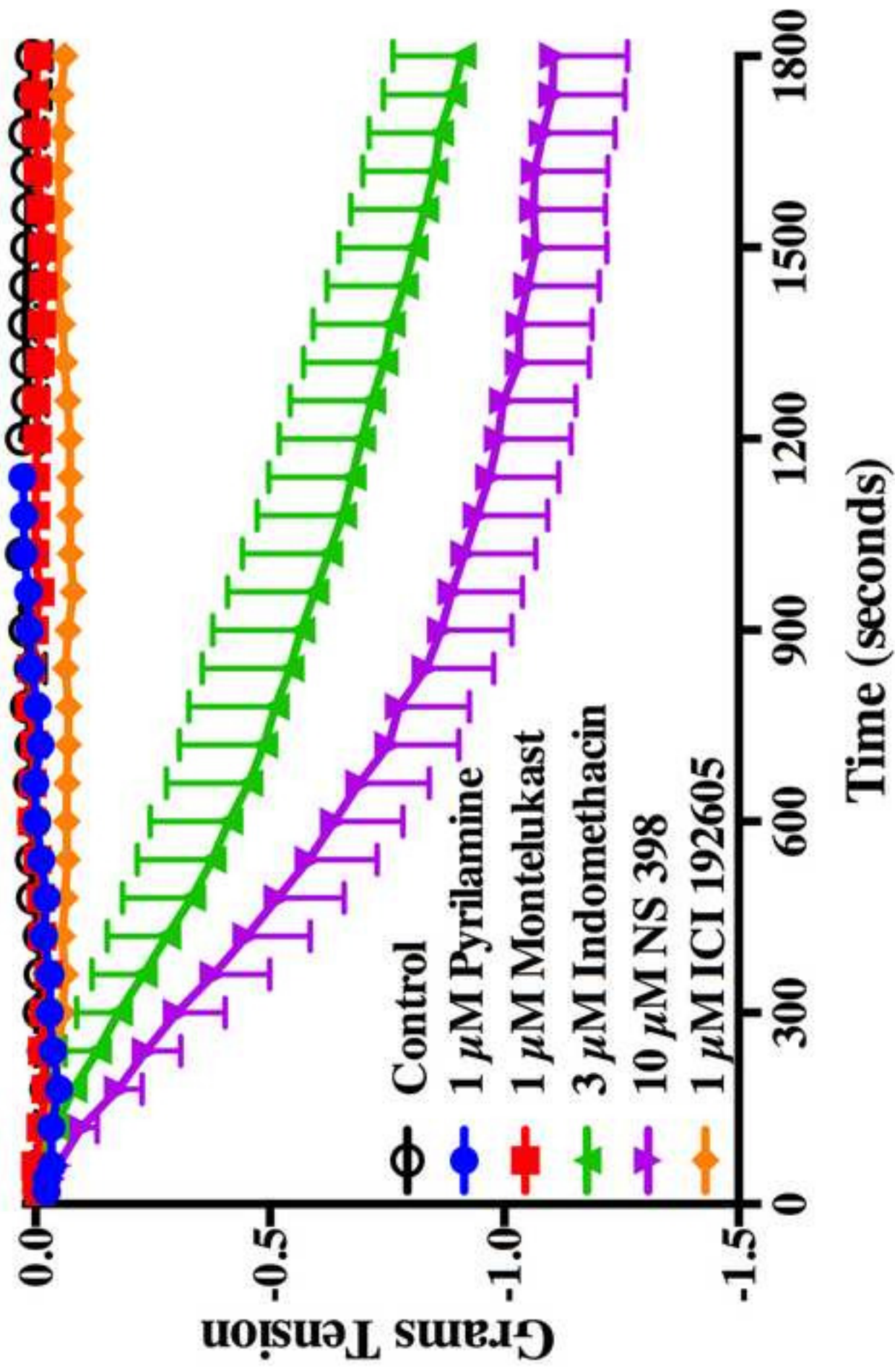


Fig. 2B



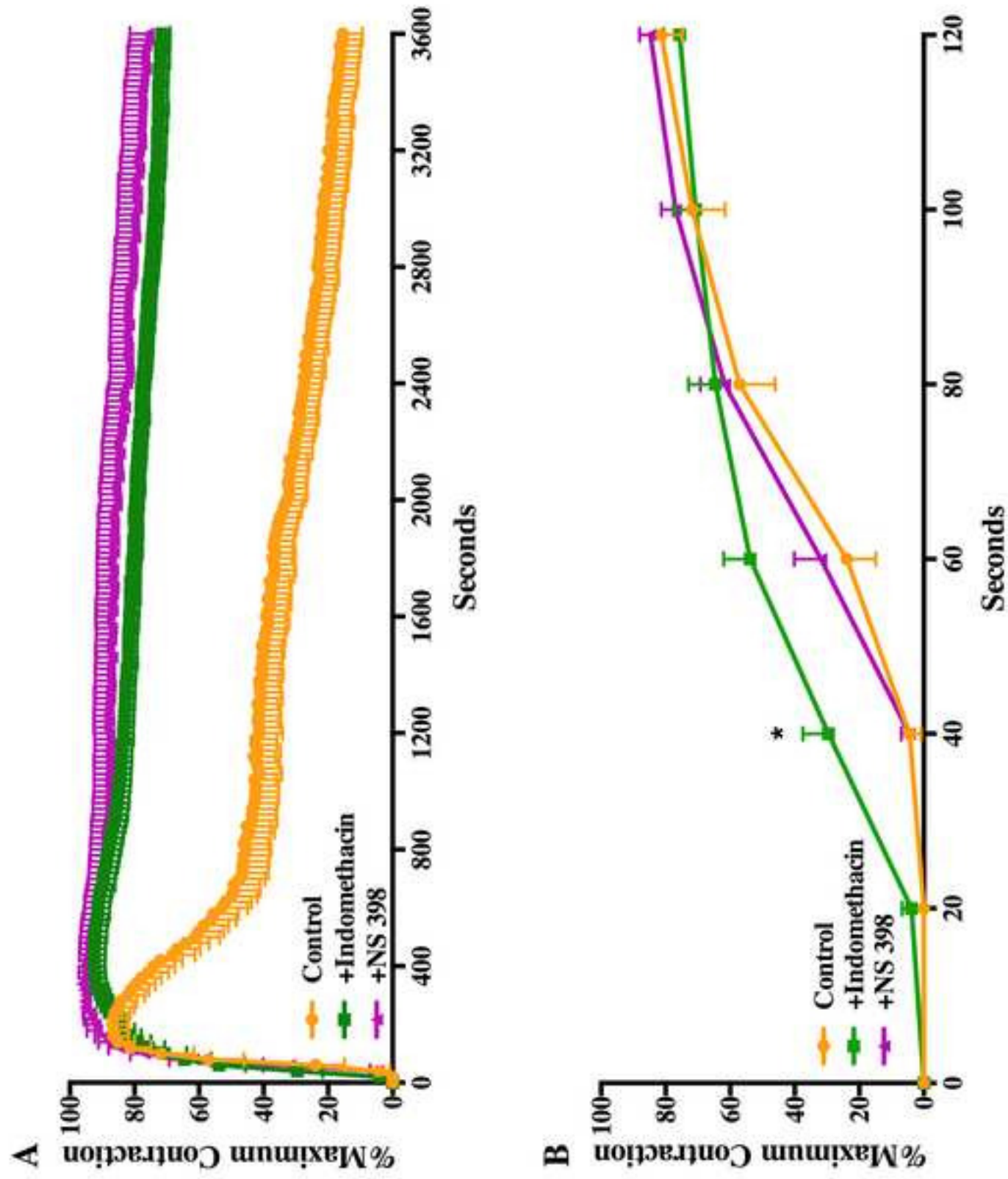
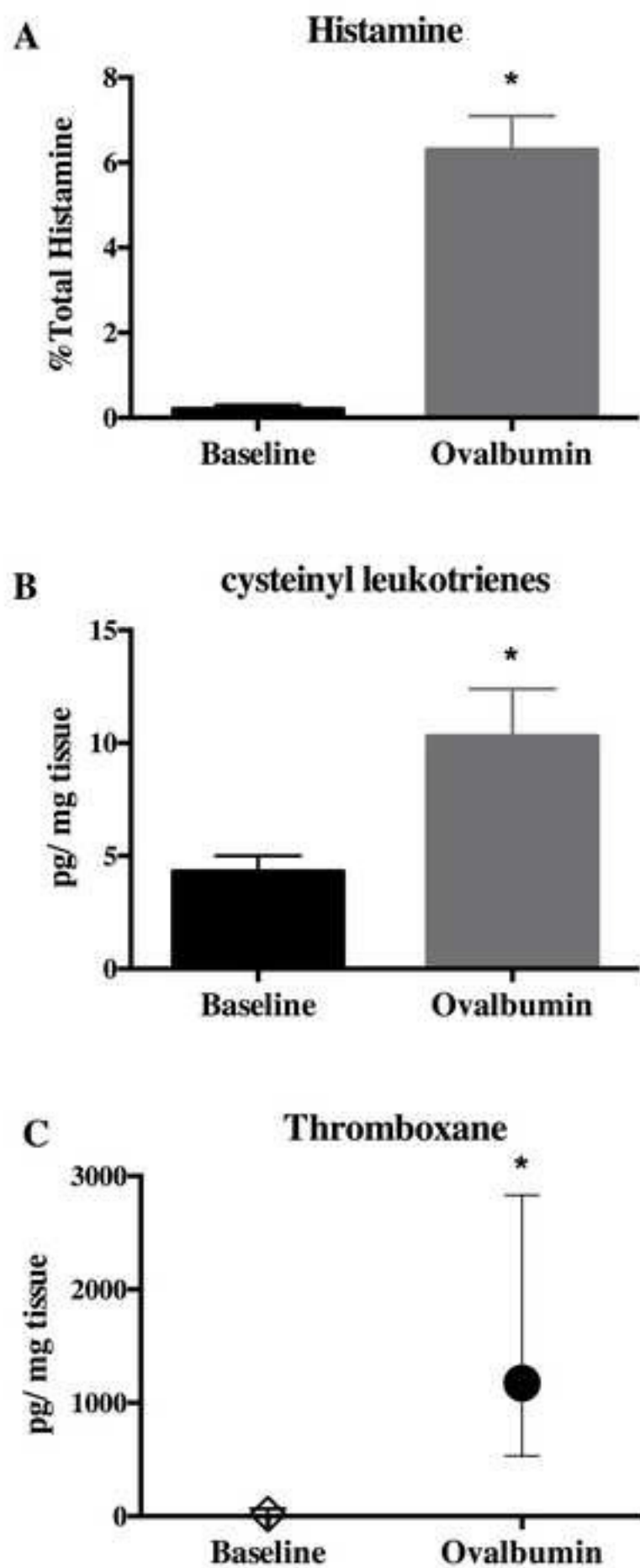
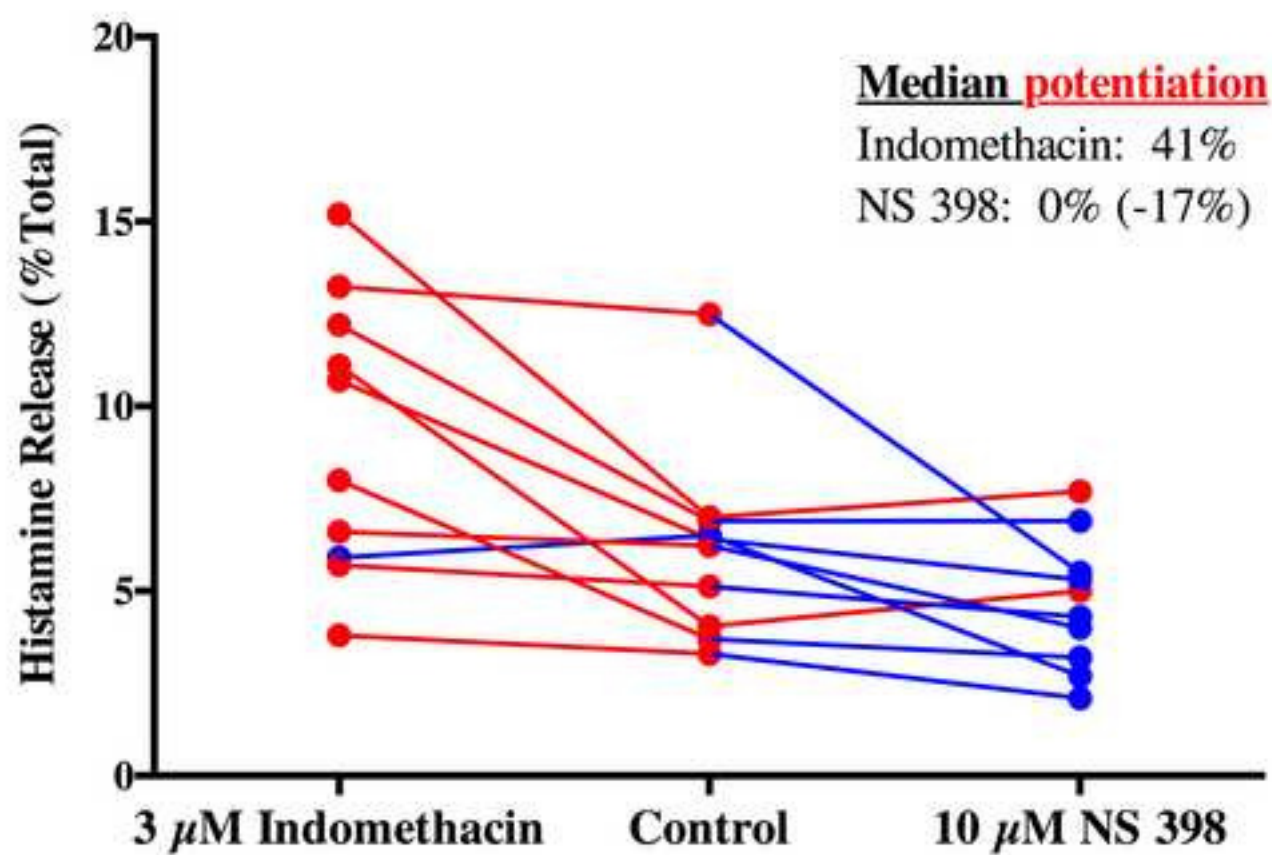
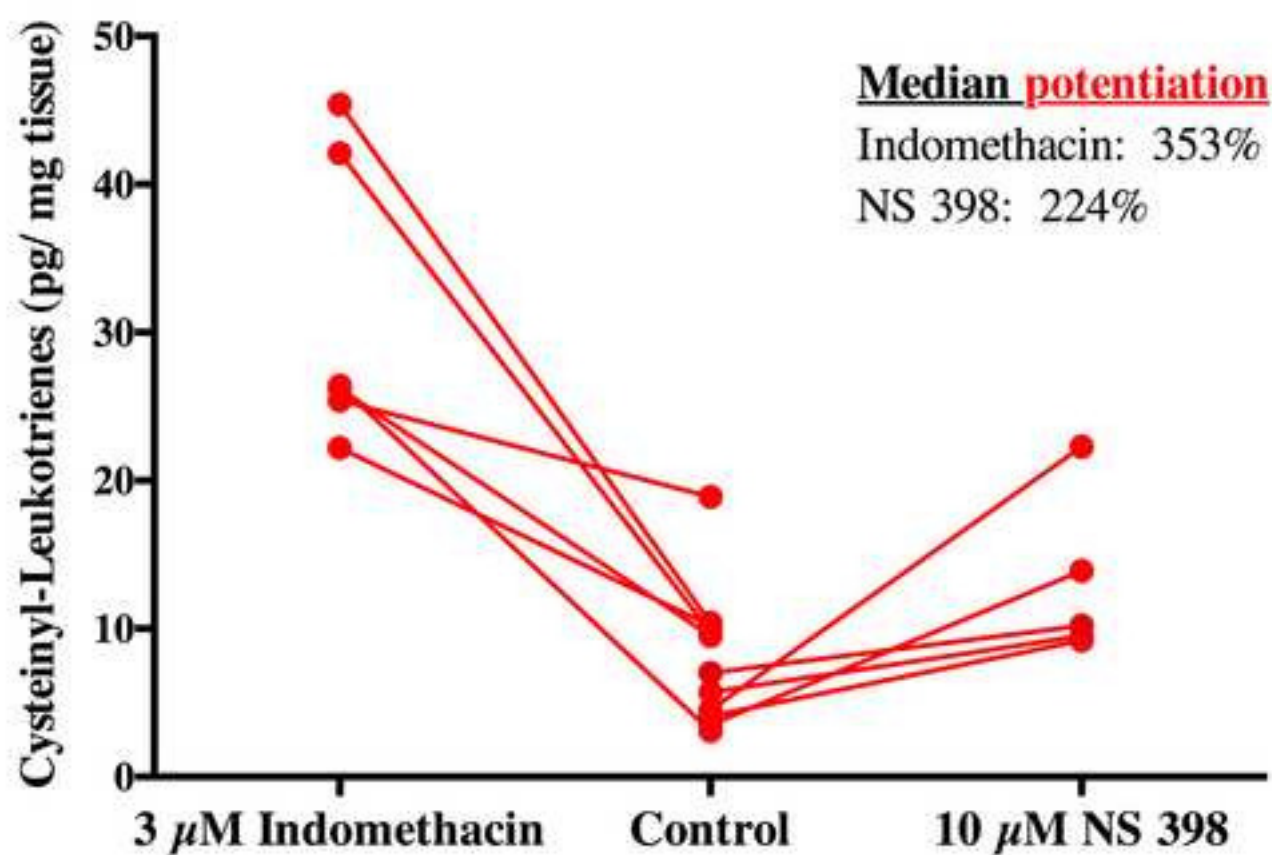


Fig. 4





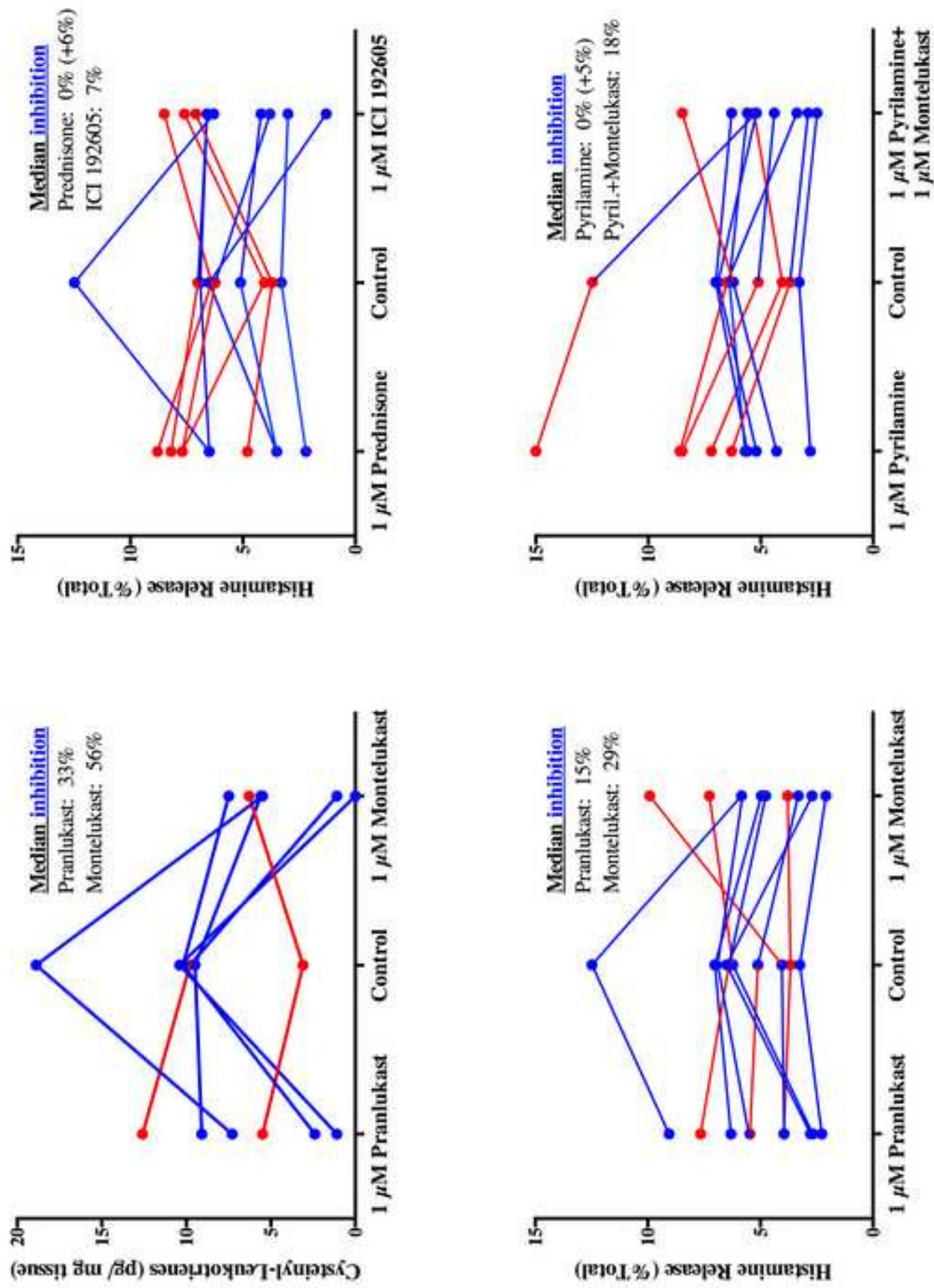


Fig. 7

