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CONTRIBUTORY ROLE OF LUNG PLEURA TO RELEASE OF ANAPHYLACTIC MEDIATORS FROM GUINEA PIG LUNG IN RESPONSE TO OVALBUMIN OR A23187

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Abstract—Previous findings revealed greater contractile responses of guinea pig lung pleural surface strips to antigen or A23187 challenge than denuded lung parenchymal strips (lung strip devoid of any pleura). Moreover, we have identified a high density of mast cells distributed throughout the lung pleura. The present study examined mediators released from guinea pig lung pleural surface and denuded lung parenchyma fragments in response to immunologic challenge with ovalbumin (OA) or non-immunologic challenge with the ionophore A23187. Histamine levels were measured radioenzymatically; leukotrienes (LTs), prostaglandins (PGs) and thromboxane B_2 (TXB_2), a stable metabolite of thromboxane A_2 (TXA_2), were quantitated using an enzyme immunoassay. Histamine release reached a maximal level 3–5 min after OA challenge, whereas A23187-induced histamine release increased gradually in a time-dependent manner. Similar kinetics were observed in the release of LTs, PGs and TXA_2. Pleural surface released a substantially ($P < 0.05$) greater amount of histamine to both challenges than denuded parenchyma. Moreover, histamine content in pleural surface was significantly ($P < 0.05$) higher than in denuded parenchyma. Pleural surface also released considerably ($P < 0.05$) more LTB_4, LTC_4, and LTE_4 in response to OA and A23187 than denuded parenchyma. In contrast, pleural surface and denuded parenchyma released equivalent amounts of PGD_2, PGE_2, $PGF_{2\alpha}$, and TXA_2 in response to both challenges. The rank order of leukotriene release was $LTC_4 > LTE_4 > LTB_4$, whereas that of prostanoid release was $TXA_2 \gg PGD_2 \geq PGF_{2\alpha} \gg PGE_2$. We conclude that pleural surface is the major source of histamine and leukotrienes released from guinea pig lung *in vitro* in response to OA and A23187, whereas both pleural surface and denuded parenchyma participate to the same extent in prostaglandin and TXA_2 production after such challenges.

Recently, Halonen *et al.* [1] identified multiple layers of smooth muscle cells in the visceral pleura of guinea pig lung, which are responsible for contraction of the lung parenchymal strips to platelet-activating factor. Our laboratory confirmed this histologic finding and extended the observation by showing that mast cells could be found regularly throughout the guinea pig lung pleura [2, 3]. Moreover, contractile responses of pleural surface strips to immunologic challenge with ovalbumin (OA)‖ or non-immunologic challenge with the ionophore A23187 were greater than those produced by denuded parenchymal strip, a lung preparation devoid of any pleural surface [3]. These findings suggested potential quantitative and/or qualitative differences in regional release of anaphylactic mediators from the lung. The purpose of the present investigation was to evaluate mediators released from pleural surface and denuded parenchyma in response to OA or the ionophore A23187.

Many studies using lung parenchymal strips *in vitro* [4, 5] or isolated lung fragments challenged with antigen or ionophore A23187 [6–8] have identified and quantified a wide range of released anaphylactic substances. Histamine, a preformed mediator, is released immediately after mast cell degranulation, whereas products of arachidonic acid metabolism including various leukotrienes (LTs), prostaglandins (PGs) and thromboxane A_2 (TXA_2) are synthesized *de novo* and thus released after the initial histamine release [9–13]. Anaphylactic mediators have been widely documented for their roles in asthma including airway smooth muscle contraction, increased mucus secretion and vascular permeability, airway hyperreactivity, and inflammation [9–13]. Although studies using dispersed guinea pig lung cells, purified human lung mast cells, and purified alveolar epithelial cells demonstrated production and release of these mediators in response to immunologic and non-immunologic challenges [14–17], lung tissue is such a complex structure that cellular sources of these mediators and the relative contribution of each distinct lung section to mediator release are still unresolved. The present study examined regional release of histamine, LTs, PGs and TXA_2 from guinea pig lung pleura and denuded lung parenchyma in response to either OA or A23187. We conclude that lung pleura is the major source of histamine and leukotrienes released from

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‖ Abbreviations: OA, ovalbumin; LTs, leukotrienes; PGs, prostaglandins; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; HNMT, histamine *N*-methyltransferase and [^{3}H]N-τ-MHm, tritiated *N*-τ-methylhistamine.

guinea pig lung in response to such challenges *in vitro*.

MATERIALS AND METHODS

Materials. Ovalbumin (grade V), histamine dihydrochloride, and bovine serum albumin (essential fatty acid free) were purchased from the Sigma Chemical Co. (St. Louis, MO). Rabbit IgG fraction against chicken ovalbumin was from the Organon Teknika Corp. (West Chester, PA). Tritiated *S*-adenosyl-L-[*methyl*-^{3}H]methionine (60–85 Ci/mmol), histamine *N*-methyltransferase (HNMT) and Econoflour were from New England Nuclear (Boston, MA), toluene and isoamyl alcohol from EM Science (Gibbstown, NJ), and bis-diethylhexylhydrogen phosphate from the Eastman Chemical Co. (Rochester, NY). The following enzyme immunoassay reagents were obtained from the Cayman Chemical Co. (Ann Arbor, MI): mouse monoclonal anti-rabbit IgG antibody, specific eicosanoid-acetylcholinesterase conjugate tracers, specific rabbit anti-eicosanoid antiserums, and Ellman's reagent. A23817 was prepared at Eli Lilly & Co. and was dissolved in dimethyl sulfoxide. All other reagents were of analytical grades and were dissolved in distilled H_2O.

Chopped lung preparations. Male Hartley guinea pigs (Charles River, Portage, MI) weighing 450–600 g were killed by CO_2 asphyxiation and subsequent decapitation. After thoracotomy, heart and lung were excised *en block* and perfused with 50 mL of oxygenated Krebs-bicarbonate solution through the pulmonary artery. The composition of the Krebs-bicarbonate solution in mmol/L was: NaCl, 118.2; KCl, 4.6; $NaHCO_3$, 24.8; $CaCl_2 \cdot 2H_2O$, 2.5; KH_2PO_4, 1.2; $MgSO_4 \cdot 7H_2O$, 1.2; and dextrose, 10.0. Only the lower lung lobes were used for the present study. Lung lobes were cut into longitudinal strips; these were designated intact lung parenchymal strips. Some were further dissected into pleural surface strips (~1 mm in thickness) and denuded lung strips (lung segment devoid of any pleural surface). For antigen-induced mediator release studies, lung strips were isolated from guinea pigs passively sensitized by a single intraperitoneal injection of 1 mg/kg rabbit IgG antibody against chicken ovalbumin. Animals were killed 2 days after injection. The three distinct lung strip preparations, intact parenchyma, pleural surface, and denuded parenchyma (Fig. 1), were then separately cut into approximately $1 \times 1 \times 1$ mm^3 pieces using a McIlwain tissue chopper (Brinkmann Instruments, Westbury, NY). Fragmented lung preparations were washed thoroughly with fresh Krebs buffer before incubation.

Mediator release studies. Duplicate or triplicate aliquots of 200 mg lung fragments from each chopped lung preparation were weighed and placed in scintillation vials containing 2 mL of oxygenated Krebs solution. Lung samples were then incubated in a shaker bath at 37° for 45 min before they were challenged with either 1×10^{-5} M A23187, a divalent cationic ionophore, for 5-, 15-, 30- and 60-min intervals or 1×10^{-5} g/mL OA for 5-, 15- and 30-min intervals. Since OA-induced histamine release

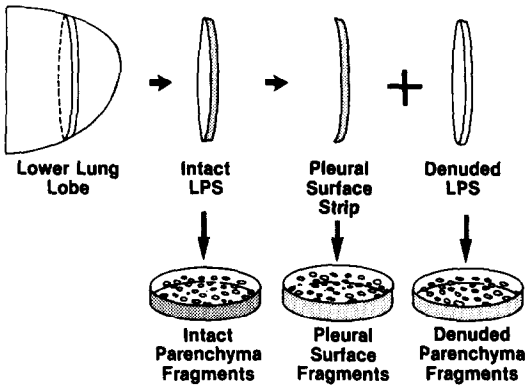

Fig. 1. Diagram illustrating the isolation of intact lung parenchymal strip (LPS), pleural surface strip, and denuded lung strip from guinea pig lung. These three distinct lung strips were then cut into lung fragments using a McIlwain tissue chopper.

appeared to have reached a plateau at 5 min, a separate experiment measuring histamine levels at three additional time intervals (0.5, 1, and 3 min) was conducted. Diffusates were then decanted into 12 × 75 mm polypropylene tubes and centrifuged at 3000 *g* for 10 min at 4°. The resultant supernatant fractions were collected and stored at −70° until assay. To assess total histamine content, 2 mL of Krebs buffer was added to scintillation vials containing lung fragments and boiled for 10 min. Diffusates were decanted, centrifuged, and stored at −70°. In the initial studies, we determined that for every 1 g of lung tissue, there was 0.53 ± 0.004 g of alveolar parenchyma and 0.47 ± 0.02 g of pleural surface tissue (N = 6 animals). Therefore, the amount of mediators including histamine, leukotrienes, and prostanoids released from fragmented denuded parenchyma or pleural surface chopped lung was subsequently multiplied by a factor of 0.53 and 0.47, respectively, to evaluate their relative contribution to total mediator release from the lung.

Eicosanoid assay. The release of LTB_4, LTC_4, LTE_4, PGD_2, PGE_2, $PGF_{2\alpha}$, and thromboxane B_2 (TXB_2) from lung preparations in response to either ionophore A23187 or OA challenge was quantitated using enzyme immunoassay [18], as outlined in the manufacturer's instructions. Briefly, flat-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with mouse monoclonal anti-rabbit IgG antibody overnight at room temperature, and subsequently saturated with 0.3% bovine serum albumin for another 24 hr at 4°. Plates were then washed and duplicate aliquots of biological samples (50 μL final) containing unknown concentrations of eicosanoids were systematically pipetted into the wells using a robotic sample processor (Tecan, model RSP 5051, Chapel Hill, NC). Aliquots (50 μL each) of a specific eicosanoid conjugated with acetylcholinesterase and rabbit IgG antisera specific for the corresponding eicosanoid were then added into each well. A competitive immunologic reaction was allowed to take place for at least 18–24 hr at

room temperature. Subsequently, plates were washed and developed by the addition of acetylcholinesterase substrate (Ellman's reagent). Colorimetric measurements of each well were performed using a microplate reader (UV_{max}, Molecular Devices Corp., Menlo Park, CA) at a wavelength of 405 nm and concentrations of eicosanoids were calculated from standard curves generated simultaneously. With the exception of LTC_4 antiserum, which exhibits 46% cross-reactivity toward LTD_4, all other antisera used in the present experiment were highly specific for their corresponding eicosanoids.

Histamine assay. Histamine release from each lung sample in response to A23187 or OA was determined using a radioenzymatic assay as described by Henry *et al.* [19, 20] utilizing highly purified HNMT. Briefly, total incubation volumes of 60 μL were generated by sequential addition of 25-μL biological samples (or H_2O for the blank), 10 μL H_2O (or H_2O containing 500 pg histamine for internal standards) and 25 μL reaction reagent in 12 × 75 mm borosilicate culture tubes. Reaction reagent for a 50-tube assay, prepared immediately before use, contained 1050 μL of 0.4 M potassium phosphate/0.1% bovine serum albumin, pH 7.8, 100 μL of HNMT, and 100 μL of tritiated *S*-adenosylmethionine (80 Ci/mmol). After a 1-hr incubation in a shaker bath at 2°, the enzymatic reaction was terminated by the addition of 75 μL of 2.5 M potassium borate, pH 11; 4 mL of toluene-isoamyl alcohol (3:1, v/v) was then added to each tube. After centrifugation for 5 min, 3.8 mL of the organic phase, which contained tritiated *N*-τ-methylhistamine ([^{3}H]N-τ-MHm) formed by the HNMT reaction, was transferred to another set of tubes containing 250 μL of potassium phosphate at pH 7.1 for back extraction of the [^{3}H]N-τ-MHm into the aqueous phase. Tubes were centrifuged for 5 min and the organic phase was aspirated. The residual aqueous phase was washed with an additional 1.25 mL of toluene-isoamyl alcohol and centrifuged. The organic phase was again aspirated and discarded, and 150 μL of the aqueous phase was transferred to scintillation vials containing 500 μL of potassium phosphate and 10 mL of Econoflour with 2% bis-diethylhexylhydrogen phosphate. Radioactivity was quantitated using liquid scintillation spectrometry. Samples were assayed in duplicate.

Data analysis. All release data are expressed as either pg or ng per 100 mg of the three distinct fragmented lung tissues (mean ± SEM). Each data point represents 3–4 experiments. Total histamine content was the sum of OA- or A23187-stimulated plus residual histamine obtained by boiling. Percent histamine release in response to OA or A23187 was obtained using (stimulated release/total histamine) × 100%. Statistical comparison among the three distinct chopped lung preparations was performed using ANOVA. Significance was determined at a level of $P < 0.05$.

RESULTS

All three chopped lung preparations released histamine, LTs, PGs and TXA_2 in response to ionophore A23187 or OA challenge. The kinetics of histamine release in response to A23187 or OA, however, were quite different. Histamine release reached a maximal level 3–5 min after OA challenge (Fig. 2, upper insert), whereas A23187-induced histamine release increased gradually in a time-dependent manner (Fig. 2). Pleural surface released significantly ($P < 0.05$) greater amounts of histamine than denuded parenchyma in response to OA or A23187. As expected, the combined histamine release at 30 min after OA challenge from pleural surface (128.6 ± 29.0 ng/100 mg lung tissue) and denuded parenchyma (56.4 ± 11.0 ng/100 mg lung tissue) was equivalent to the release from intact

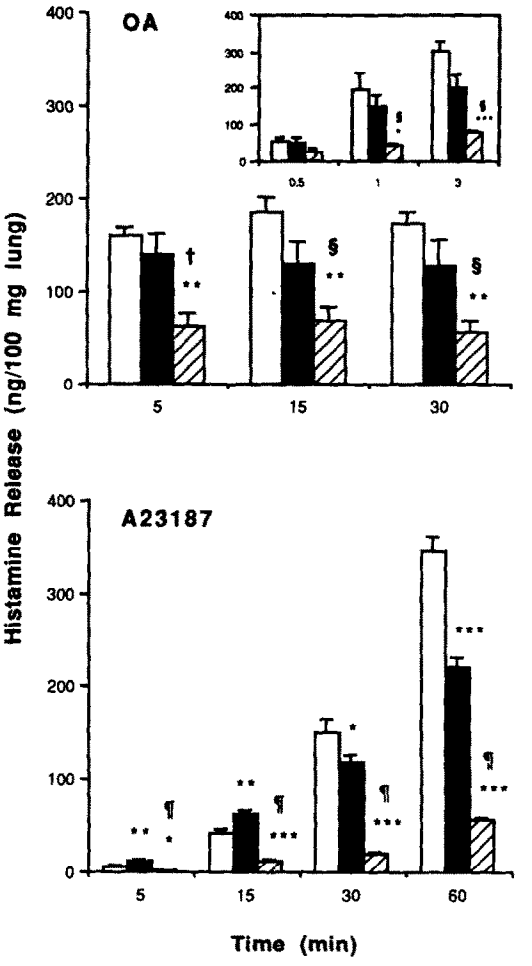

Fig. 2. Time course of histamine release from intact parenchyma (□), pleural surface (■) and denuded parenchyma (▨) after challenge with ovalbumin (upper histogram) or ionophore A23187 (lower histogram). Upper insert represents a separate experiment measuring histamine release at earlier time points. Each point is the mean ± SEM of 3–4 experiments. Five to six animals were used in each experiment. Spontaneous release of histamine from the three distinct fragmented lung tissues was negligible. Significant differences from the intact parenchyma are indicated as $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Significant differences from the pleural surface are designated as $\S P < 0.05$, $\dagger P < 0.01$, and $\P P < 0.001$.

parenchyma (173.0 ± 12.8 ng/100 mg lung tissue). Likewise, combined histamine release at 30 min after A23187 from pleural surface (118.2 ± 7.8 ng/100 mg lung tissue) and denuded parenchyma (21.0 ± 1.2 ng/100 mg lung tissue) was equivalent to the release from intact parenchyma (150.0 ± 14.4 ng/100 mg lung tissue). Total histamine content in pleural surface was significantly greater than that in denuded parenchyma in OA (1179.1 ± 51.7 vs 758.8 ± 41.9 ng/100 mg lung tissue, $P < 0.001$) and A23187 (1316.5 ± 41.9 vs 937.0 ± 36.3 ng/100 mg lung tissue, $P < 0.05$) treatment groups. Combined total histamine content in pleural surface and in denuded parenchyma was also similar to the amount of histamine contained in the intact parenchyma (1798.9 ± 125.0 ng/100 mg lung tissue in the OA-treated group and 2438.6 ± 192.6 ng/100 mg lung tissue in the A23187-treated group). Maximum percent histamine release values from intact parenchyma, denuded parenchyma and pleural surface chopped lung preparations in response to OA were 14.0 ± 1.7, 10.2 ± 2.0 and 17.5 ± 4.2%, respectively, and to A23187 were 16.0 ± 2.8, 6.6 ± 0.9 and 17.4 ± 0.9%, respectively.

Pleural surface produced substantially higher levels of LTB_4, LTC_4, and LTE_4 at almost all time intervals examined than the denuded parenchyma in response to either A23187 or OA (Fig. 3). Release of LTs in response to A23187 was slow and time dependent, and continued to increase at 60 min. In contrast, release of LTs by OA reached a maximum level between 5 and 15 min. Combined individual leukotriene release from pleural surface and denuded parenchyma in response to either OA or A23187 was comparable to the release from intact parenchyma. The rank order of LT released from these three fragmented lung preparations was $LTC_4 > LTE_4 > LTB_4$.

Unlike histamine and leukotrienes, spontaneous release of PGs and TXA_2 from the lung preparations was substantial. Thus, all PG and TXB_2, an index of TXA_2 production, data were corrected for spontaneous release. Overall, pleural surface and denuded parenchyma produced similar quantities of PGs and TXA_2 in response to OA or A23187 (Fig. 4). For the most part, the amount of PGs and TXA_2 pooled from these two lung fragments approached that from the intact parenchyma. Interestingly, at 60 min after A23187 challenge, PGs and TXA_2 released from the intact parenchyma were elevated markedly to a level which greatly exceeded the amount of prostanoid from pleural surface and denuded parenchyma combined (Fig. 4A). PGs and TXA_2 released from these three distinct lung preparations reached a plateau 5–15 min after OA challenge, and continued to increase 60 min after A23187 stimulation. The rank order of prostanoid generation from these three lung preparations in response to both A23187 and OA was $TXA_2 \gg PGD_2 \geq PGF_{2\alpha} \gg PGE_2$.

DISCUSSION

The role of lung pleura in pulmonary hypersensitivity is unknown. Identification of smooth muscle cells in the visceral pleura of guinea pig lung was first noted by Miller [21] in 1921 and then by Nagaishi [22] in 1972. More recently, Schmidt and Gown [23] described the subpleural tissue of lung pleura as submesothelial stromal cells, or modified smooth muscle cells capable of expressing smooth muscle α-actin. Recent findings by Halonen *et al.* [1] indicated that these multiple layers of pleural smooth muscle cells are the primary contractile elements for guinea pig lung parenchymal strip contraction in response to platelet-activating factor. Removal of this pleural surface rendered the lung strips unresponsive to platelet-activating factor. Our previous findings [2, 3] demonstrated that pleural surface strips not only exhibited higher responsiveness to histamine, LTD_4, and U46619, a TXA_2 mimetic, but also elicited greater contractile response to immunologic challenge with OA or non-immunologic challenge with the ionophore A23187 than the lung strips devoid of any pleura. Moreover, our histologic data revealed high density of mast cells distributed throughout the guinea pig lung pleura [3], which helps explain the greater contractile response of pleural surface strips to OA and A23187 challenges.

The present study demonstrated the ability of guinea pig lung pleura to release anaphylactic mediators in response to OA or the ionophore A23187. The results correspond well with our previous functional and histological findings in that pleural surface released substantially more histamine and LTs than the denuded parenchyma in response to these stimuli. This contrasts with the contribution of pleural surface and denuded parenchyma to PG and TXA_2 release which occurred to the same extent in response to OA or A23187.

OA and ionophore A23187 have been extensively documented to release anaphylactic mediators from lung tissue [6–8], dispersed lung cells [16], and purified lung mast cells [17]. A23187 also released various eicosanoids from purified alveolar epithelial cells [14, 15]. OA-induced mediator release is primarily a mast cell-mediated event, whereas A23187 is a non-selective agent stimulating release of a variety of mediators from many cellular sources [7, 14, 15, 17]. A major difference observed between OA and A23187 challenge was the kinetics of mediator release from the lung fragments. OA elicited a more rapid release of histamine, LTs, PGs, and TXA_2 than A23187. This is consistent with the results of Sautebin *et al.* [6] and Salari [7]. On the other hand, A23187 challenge of the lung fragments released more LTs, PGs, and TXA_2 than OA stimulation, suggesting participation of cellular sources other than mast cells to the production of these arachidonate metabolites [7, 14, 15]. A23187 caused a considerably larger amount of all PG and TXA_2 production from intact parenchyma than from its components, pleural surface and denuded parenchyma combined, at 60 min. This phenomenon may be due to the necessary presence of both pleural surface and denuded parenchyma, and suggests potential cellular interaction between these two different lung tissues. In contrast, combined histamine or LT release at 60 min from the pleural

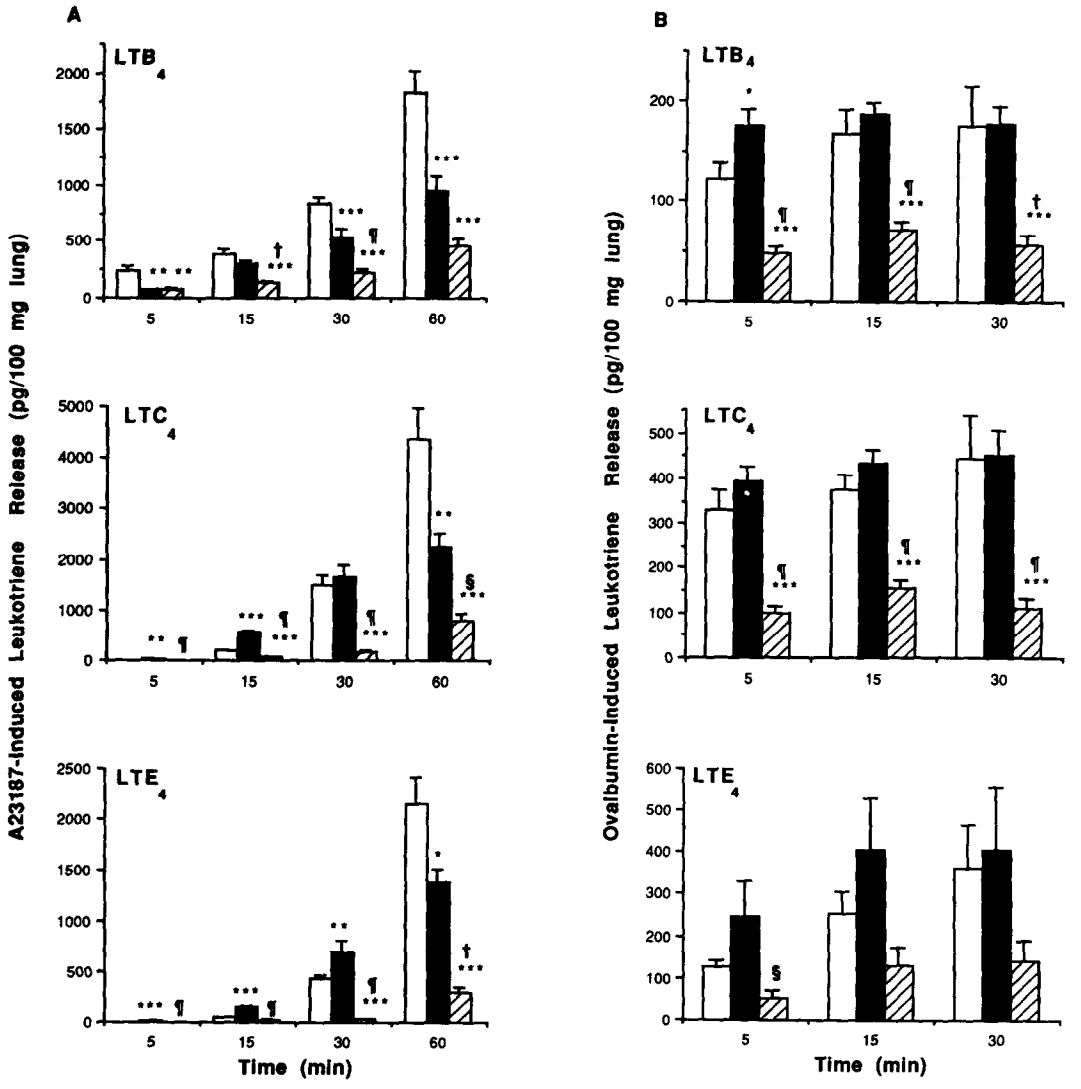

Fig. 3. Time course of leukotriene release from intact parenchyma (□), pleural surface (■), and denuded parenchyma (▨) after challenge with ionophore A23187 (A) or ovalbumin (B). Each point is the mean ± SEM of 4 experiments. Five to six animals were used for each experiment. Spontaneous release of leukotrienes from the three distinct fragmented lung tissues was negligible. Significant differences from the intact parenchyma are indicated as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Significant differences from the pleural surface are designated as §$P < 0.05$, †$P < 0.01$, and ¶$P < 0.001$.

surface and denuded parenchyma was comparable to that from the intact parenchyma. OA challenge produced the same quantities of histamine and PGD_2 as compared to A23187, suggesting that these mediators are primarily derived from mast cells [8–10, 12, 13]. Moreover, the relative proportion of various LTs and prostanoids released after challenge with either OA or A23187 was equivalent in that the rank order of LT release was $LTC_4 > LTE_4 > LTB_4$, and that of prostanoid release was $TXA_2 \gg PGD_2 \geq PGF_{2\alpha} \gg PGE_2$. These results are in line with other findings [4, 6, 16] that, in lung parenchyma, TXA_2 is the predominant cyclooxygenase metabolite of arachidonic acid and the amount of PGE_2 is minimal in response to antigen or A23187 challenge.

Among all the inflammatory mediators measured, histamine levels were the highest, which supports the findings of Salari [7] and Schleimer *et al.* [17]. Histamine is primarily released subsequent to degranulation of mast cells. Although basophils are another source of this biogenic amine [24], under the present experimental conditions, the contribution of basophils to histamine release would be negligible due to thorough lung perfusion with Krebs solution via the pulmonary artery. The pleural surface contained about 55% more histamine than the denuded parenchyma which was reflected in the

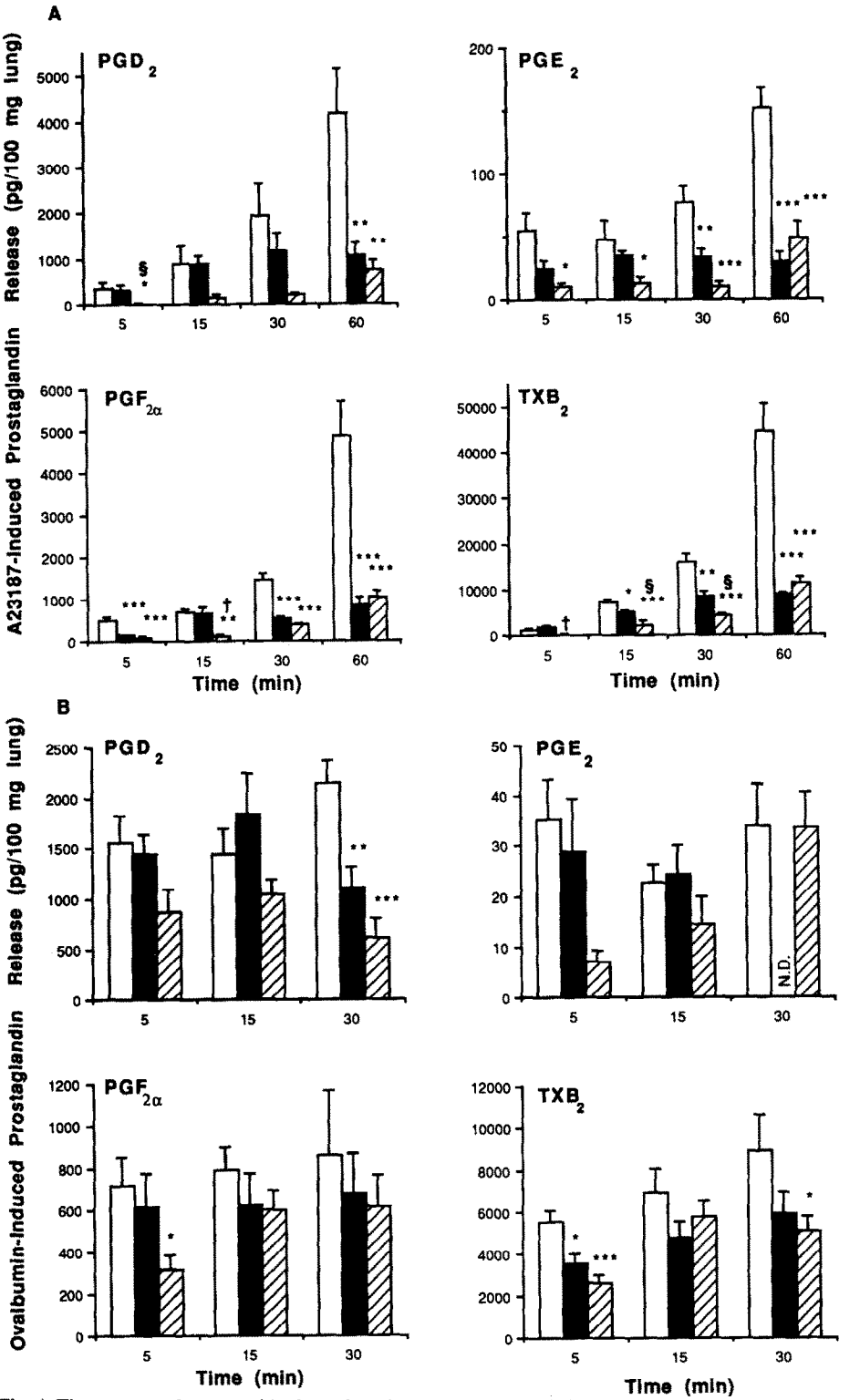

Fig. 4. Time course of prostanoid release from intact parenchyma (□), pleural surface (■), and denuded parenchyma (▨) after challenge with ionophore A23187 (A) or ovalbumin (B). Each point is the mean ± SEM of 4 experiments. Five to six animals were used for each experiment. N.D. = no data, basal PGE_2 release was too high to assess actual ovalbumin-induced release of this prostaglandin. Spontaneous release of various prostanoids in pg/100 mg lung tissue from intact parenchyma, denuded parenchyma, and pleural surface chopped lung preparations were: PGD_2, 709.3 ± 104.7, 528.9 ± 194.4 and 454.7 ± 167.1; PGE_2, 71.3 ± 7.2, 44.0 ± 5.4 and 45.9 ± 9.2; $PGF_{2\alpha}$, 793.2 ± 58.1, 580.8 ± 69.1 and 519.7 ± 97.0; and TXB_2, 6450.4 ± 683.9, 3392.4 ± 167.1 and 2915.3 ± 243.2, respectively. Significant differences from the intact parenchyma are indicated as $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Significant differences from the pleural surface are designated as §$P < 0.05$ and †$P < 0.01$.

substantially higher level of histamine release in response to OA or A23187. Together, these data indicate that pleural surface contains a higher concentration of mast cells than the denuded parenchyma where mast cells are distributed mainly in the peribronchiolar area and blood vessels. This finding further substantiates our previous histologic observation showing high density of mast cells throughout the lung pleura [2, 3]. Alternatively, our results may suggest heterogeneity of mast cells [25, 26] in these two regions of the lung.

With the exception of PGE_2 being a smooth muscle relaxant, LTs, PGD_2, $PGF_{2\alpha}$, and TXA_2 have been implicated in the pathogenesis of airway hypersensitivity reactions such as asthma [9–13]. They contract central and peripheral airways with greater potency than histamine, increase mucus secretion and vascular permeability, and initiate airway hyperreactivity and inflammation. Our results show that guinea pig lung pleura synthesized and released LTs, PGs, and TXA_2 in response to OA or A23187, with the level of LTs significantly higher than that produced by the denuded parenchyma. These results further indicate active involvement of guinea pig lung pleura in anaphylactic challenge.

In conclusion, lung pleura appears to be the major source of histamine and LTs from guinea pig peripheral lung in response to immunologic and non-immunologic challenges. The present study also established a potential role for the lung pleura in inflammatory mediator release, peripheral lung contraction, and possibly airway inflammation in pulmonary disorders such as asthma. However, since the presence of pleural smooth muscle (and possibly mast cells) in the lung is species dependent [1, 22], extrapolation of the present findings into human lung pleura requires further investigation.

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