

Mitogenic Effect of Lysosomal Hydrolases on Bovine Tracheal Myocytes in Culture

D. Betty Lew* and Mario C. Rattazzi†

*Department of Pediatrics, University of Tennessee, Memphis, Tennessee 38163, and †Department of Pediatrics, North Shore University Hospital, Cornell University Medical College, Manhasset, New York 11030

Abstract

Studies were conducted to assess the mitogenic effect of lysosomal hydrolases, enzymes known to have an association with allergen- or ozone-induced airway hyperreactivity, on bovine tracheal myocytes in culture. Addition of purified human placental β -hexosaminidase and partially purified bovine liver β -glucuronidase resulted in the doubling of cell count after 4 d of incubation in medium M199 with 0.4% FBS. Unstimulated cells remained quiescent without a significant increase of cell count. Lysosomal hydrolases also selectively enhanced ^3H -thymidine incorporation four to seven times more than that in vehicle-treated cells or cells treated with endotoxin, a common contaminant of purified enzymes. Ovalbumin (glycoprotein control), pronase, and lysozyme caused a modest but statistically insignificant increase (up to twofold) in ^3H -thymidine incorporation. Elastase, collagenase and dialyzed *E. coli* β -glucuronidase had no effect. The mitogenic effect of hydrolases was equally seen in quiescent, serum-depleted cells as well as in those maintained in medium with 10% FBS, suggesting that it was independent of serum factors. The effect of lysosomal hydrolases was inhibited by exposure to yeast mannan, and mannosylated human serum albumin had a mitogenic effect, suggesting the involvement of a mannose receptor. We conclude that lysosomal hydrolases may play a role in the development of the hyperplasia/hypertrophy of respiratory smooth muscle. (*J. Clin. Invest.* 1991. 88:1969–1975.) Key words: proliferation • airway myocytes • lysosomal hydrolases • hexosaminidase

Introduction

Bronchial smooth muscle hyperplasia/hypertrophy is a well-recognized pathological change in asthma (1–3). The volume of smooth muscle positively correlates with the magnitude of contraction, measured isometrically, induced by histamine, and carbachol (4). Therefore, the changes in hyperplasia/hypertrophy of bronchial smooth muscle may play an important role in the development of airway hyperreactivity, the hallmark of asthma. Unfortunately, the pathogenesis of bronchial smooth muscle hyperplasia/hypertrophy is poorly understood.

An abstract of this paper was presented at the 59th annual meeting of the Society of Pediatric Research, Anaheim, CA, May 1990.

Address correspondence to D. Betty Lew, M.D., Pediatric Research, 956 Court Ave., Rm. B310, Memphis, TN 38163.

Received for publication 05 November 1990 and in revised form 09 August 1991.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/91/12/1969/07 \$2.00

Volume 88, December 1991, 1969–1975

Another important feature of asthma is airway inflammation (5). Recently, Panettieri and co-workers have shown that histamine, an inflammatory mediator associated with airway hyperreactivity, stimulated proliferation of canine airway smooth muscle cells (6). Lysosomal hydrolases, secretory products of inflammatory cells such as macrophages, eosinophils, neutrophils, and mast cells, are thought to be involved in the inflammatory process, particularly in allergen-induced asthma (7, 8). The activity of β -glucuronidase, a lysosomal hydrolase, was reported to be significantly increased in bronchoalveolar lavage fluid 2 h after allergen (*Dermatophagoides pteronyssinus*) inhalation challenge in sensitized human subjects compared to basal level. The elevated level of the enzyme in lavage fluid was accompanied by a decrease in the intracellular level of the enzyme in alveolar macrophages, thus suggesting that alveolar macrophages are a source of the secretion of the enzyme into airspace (7). Increased *N*-acetyl- β -D-glucosaminidase (EC 3.1.2.30; β -hexosaminidase) activity in bronchoalveolar lavage fluid also has been implicated in ozone-induced muscarinic hyperreactivity in the guinea pig model (9). However, the significance of elevated lysosomal hydrolase activity in airways in this animal model and in human subjects is unclear. In inflamed airways like in asthma, denudation of epithelium and an exposure of edematous basal membrane to airspace are seen (10). Moreover, basal membrane is infiltrated with inflammatory cells such as eosinophils, activated macrophages, and degranulated mast cells (11). Therefore, it is likely that bronchial smooth muscle may come in direct contact with mediators of inflammation such as lysosomal hydrolases on a chronic basis in such case.

Rokosova and Bentley in 1980, reported that crude lysosomal enzymes derived from mouse liver and dog alveolar macrophage augmented proliferation of cultured bovine aortic vascular smooth muscle cells and human fibroblasts (WI-38), respectively (12). However, the composition of these preparations was not characterized (12). As proliferation of respiratory smooth muscle cells may be directly related to the muscle contraction and airway hyperreactivity, we hypothesized lysosomal hydrolases released by airway inflammatory cells exert a mitogenic effect on respiratory smooth muscle cells. To test this hypothesis, we investigated the action of purified lysosomal hydrolases on bovine tracheal myocytes in culture. Because β -hexosaminidase is one of the most abundant lysosomal enzymes (13) and its biochemical, kinetic, and structural characteristics are well known (13), we utilized homogenous human placental β -hexosaminidase (Hex) A and Hex B for our experiments. The objectives of this study were to determine and characterize the mitogenic actions of lysosomal hydrolases on bovine tracheal respiratory myocytes in culture. The results of this study suggest that lysosomal hydrolases exert mitogenic action on bovine tracheal myocytes in culture. This effect was independent of the presence of serum and apparently selective

for lysosomal hydrolases. The effect was not abolished by heat inactivation of the enzymes, but strongly inhibited by yeast mannan. Although we cannot exclude an enzymatic mechanism, the data favor the hypothesis that ligand interactions with mannose receptors are involved.

Methods

Tracheal smooth muscle culture. Bovine trachea was obtained from a local slaughter house (Hernando Packing Co., Memphis, TN). The trachealis muscle was dissected, and primary cultures of tracheal myocytes were prepared by a modified method of Kotlikoff et al. (14). Approximately 1 g of dissected trachealis muscle was minced and digested by two successive 45-min incubations at 37°C in MEM with Earl's salt solution containing 0.2 mM CaCl₂, collagenase (Sigma Type IV, *Clostridium histolyticum*, 350 U/ml), protease (bovine pancreas, 0.3 mg/ml), trypsin inhibitor (soybean, 4.0 mg/ml), and globulin free crystalline BSA (2 mg/ml). Fluid containing the dissociated cells was filtered through 125- μ m nylon gauze and washed once by centrifugation for 15 min at 500 g. Cells were plated in tissue culture flasks (T-75; Costar Corp., Cambridge, MA) at a density of 10,000 cells/cm² in medium M199 with Earl's salt solution containing 0.1 μ g glutamine/ml, 100 U penicillin/ml, 0.1 μ g streptomycin/ml, and 0.25 μ g amphotericin B/ml (Sigma Chemical Co., St. Louis, MO) and 10% (vol/vol) FBS. Cells prepared by this method maintain the same doubling time (48 h) and morphology for several passages (tested up to passage 14).

Recognition of smooth muscle isoactin. Dry FITC was added to a solution containing ~ 10 mg protein/ml in 0.15 mol NaCl/liter and 10% by volume of carbonate buffer, pH 9.5. The dye (micrograms):protein (milligrams)- ratio was 2-4:1. The mixture was gently rotated for 2 h at room temperature and dialyzed overnight in the same buffer.

Monolayers of cells on a four-well chamber slide (Nunc Inc., Naperville, IL) were fixed with cold 100% methanol for 12 min and washed three times with 3% BSA in PBS. Direct immunofluorescent staining was performed by 45-min incubation with either FITC-conjugated preimmune mouse IgG₂ (Sigma Chemical Co.) or mouse FITC-conjugated MAb against smooth muscle isoactin (diluted in 3% BSA in PBS, final dilution 1:200; Sigma Chemical Co.) at room temperature in the dark. Stained cells were washed three times with PBS and examined by epifluorescence microscopy.

Assessment of proliferation. Cell counts were done by counting cell nuclei with a Coulter counter (Model ZH; Coulter Electronics, Hialeah, FL) in Zapoglobin (Coulter Diagnostics)-treated specimens.

DNA synthesis rates were measured by ³H-thymidine incorporation. Confluent monolayers of cells in T-75 flasks were detached by trypsinization (0.12% for 5-7 min) and were treated with appropriate amounts of trypsin inhibitor (soybean; Sigma Chemical Co.). Cells were then plated in a 96-well flat-bottom microtiter plates (25,000 cells/well; Linbro; Flow Laboratories Inc., McLean, VA) in M199 containing 10% (vol/vol) FBS and medium was changed 24 h later with serum-free medium 24-72 h before exposure to test substances and a ³H-thymidine pulse (1 μ Ci/well, sp act 5 Ci/mmol; Research Products Int. Corp., Mount Prospect, IL). Cells remained quiescent in the serum-free medium. Cells were then washed in Ca⁺⁺- and Mg⁺⁺-free M199 twice and were trypsinized (0.12% for 5-7 min) at the end of the pulse. The detached myocytes were aspirated onto a glass-fiber filter and washed with an automated harvesting system (Cambridge Technology, Inc., Cambridge, MA). Radioactivity was quantified in a liquid scintillation counter (2000 CA; Packard Instrument Co., Downers Grove, IL) as described by Dohleman et al. (15).

Enzyme preparations. The following enzyme preparations were evaluated as potential stimulants. Purified human placental lysosomal hydrolases Hex A and B were prepared by a modification of the method of Geiger and Arnon (16, 17), consisting essentially of Con A-Sepharose glycoaffinity chromatography, substrate analogue affinity chromatography on *N*-acetyl (*N*-caproyl) glucosaminide immobilized on Seph-

arose 4B and separation of the two isozymes by DEAE sephadex A50 ion exchange chromatography. Washing the diced placenta free of blood and plasma proteins before homogenization eliminated contaminants that required additional ion exchange chromatography and gel filtration steps in the original method (16). Both Hex A and Hex B are homogenous by electrophoresis on cellulose acetate gel (18) and on polyacrylamide gel electrophoresis in denaturing (19) and nondenaturing conditions. The purified enzymes have a specific activity of 150-170 U/mg protein (1 U = 1 μ mol substrate cleaved/min at pH 4.5, 37°C) using 4-methylumbelliferyl-*N*-acetylglucosaminide as a substrate (20). Purified Hex A retains activity against the natural substrate, GM₂ ganglioside (21, 22). For our experiments, β -hexosaminidase activity was routinely determined by colorimetric assay as previously described (23). *p*-Nitrophenyl-2-deoxy- β -D-glucosaminide (5 mM) in 0.1 M citrate/phosphate buffer, pH 4.5, was used as a substrate with an incubation time of 1 h. The *p*-nitrophenol released by the enzyme-dependent hydrolysis of the substrate was quantified spectrophotometrically using an enzyme immunoassay plate reader (Bio-Tek Instruments, Inc., Burlington, VT) fitted with a 405-nm wavelength filter. Units of activity were defined as micromoles of substrate cleaved per minute. Partially purified β -glucuronidase (*E. coli* and bovine liver), pronase (bovine pancreas), lysozyme (human milk), collagenase (Sigma type IV, calf skin), and elastase (human leukocyte lysosomal) were obtained from Sigma Chemical Co.

Miscellaneous testing compounds. Ovalbumin, endotoxin (LPS), yeast mannans, mannose-6-phosphate and phorbol ester (12-*O*-tetradecanoylphorbol-13-acetate, TPA) were obtained from Sigma Chemical Co. Mannose-BSA (ManBSA)-gold colloid conjugate (particle size, 10 nm) was purchased from E. Y. Laboratories, Inc., San Mateo, CA, and Man₃Glc NAC-derivatized human serum albumin (Man₃Glc NAC HSA; 10 haptens per 65 kD, Biocarb) was purchased from Accurate Chemical and Scientific Corp., Westbury, NY.

Analysis of the data. Data were subjected to analysis of variance followed by Dunnett's and Student-Neuman-Keuls tests to determine the significance of the differences between the control and test samples. A *P* value < 0.05 was considered to represent a significant difference between the two samples.

Results

Identification for smooth muscle isoactin with monoclonal antibody. To demonstrate the homogeneity of the cell population, direct immunofluorescent staining of bovine tracheal myocyte monolayers for smooth muscle specific isoactin was performed. A uniform bright fluorescent staining of the cytoskeleton of all cells stained with monoclonal IgG₂-FITC antibody specific for smooth muscle isoactin is shown in Fig. 1 B. In marked contrast, there was only a faint fluorescent staining outlining control cells stained with preimmune IgG₂-FITC (Fig. 1 A).

Mitogenic effect of lysosomal hydrolases. To determine whether lysosomal hydrolases stimulate the proliferation of bovine tracheal myocytes, monolayers (250,000 cells/16 mm-well) in M199 with 0.4% FBS were incubated with either purified human Hex A (0.25 U/ml, 0.5 U/ml) or partially purified bovine liver β -glucuronidase (0.25 U/ml, 0.5 U/ml) for 4 d. As shown in Table I, both hydrolases effectively stimulated cell proliferation, resulting in an approximately doubled cell count at a 0.5 U/ml concentration compared to that in vehicle-treated cells. In vehicle-treated cultures at this minimum amount of FBS, cells remained quiescent and there was virtually no increase in cell numbers. The rate of DNA synthesis was measured by ³H-thymidine incorporation in microtiter wells (25,000 cells/well, serum-starved for 24 h) up to 24 h as shown in Fig. 2A. The onset of ³H-thymidine incorporation in

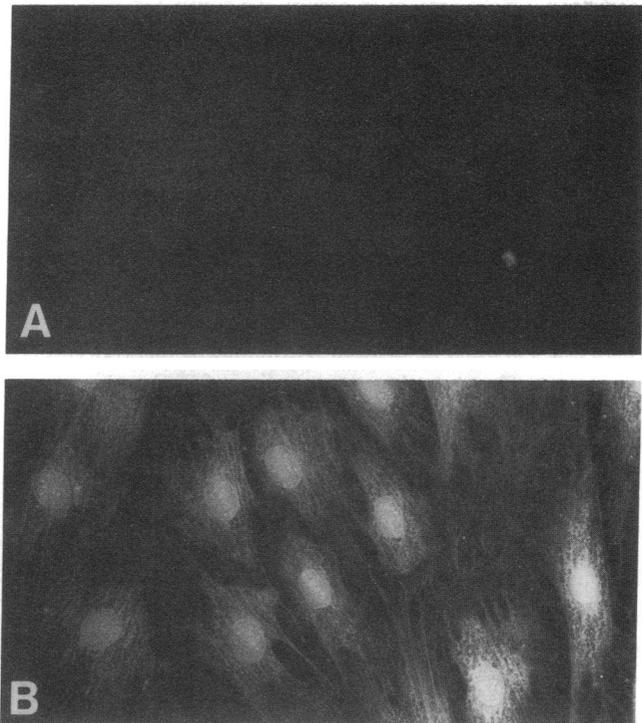


Figure 1. Direct fluorescent immunostaining of bovine tracheal myocyte monolayer in culture. Monolayers were fixed with 100% MeOH and stained with FITC-conjugated mouse IgG₂ (magnification, 500). (A) Preimmune mouse IgG₂-FITC conjugation. (B) Anti-smooth muscle isoactin-mouse monoclonal IgG₂-FITC conjugation.

response to Hex A occurred in between 2–4 h after the exposure, and the maximal effect occurred at 6 h. Unstimulated cultures remained quiescent up to 4 h and showed a mild increase of ³H-thymidine incorporation at 6 h. In contrast, the time course of ³H-thymidine incorporation in cells depleted of serum for 72 h is shown in Fig. 2 B. The onset of ³H-thymidine incorporation in response to Hex A (0.5 U/ml) and TPA (10 ng/ml) occurred at 18 h after the exposure. At 48 h, greater responses to both TPA and Hex A were seen (848±81 and 1,282±211% of vehicle control, respectively). In most trials, using the protocol of 24 h stimulation and ³H-thymidine pulse

Table 1. Effect of Lysosomal Hydrolase on Bovine Tracheal Myocyte Proliferation

Stimuli		Cell count × 10 ⁻³
Vehicle		264.9±4.7
Hex A	0.25 U/ml	400.4±7.0*
	0.5 U/ml	505.3±8.3*
β-Glucuronidase	0.25 U/ml	374.2±6.6*
	0.5 U/ml	472.3±0.5*

**P* < 0.05 (compared to vehicle treated group). 1 U = 1 μmol substrate cleaved/min. 250,000 cells were seeded in 16-mm wells in M199 with 0.4% FBS. 4 d later, nuclei count was done by Coulter Counter. Results are mean±SEM of triplicate cultures. Experiments were repeated three times and similar results were obtained.

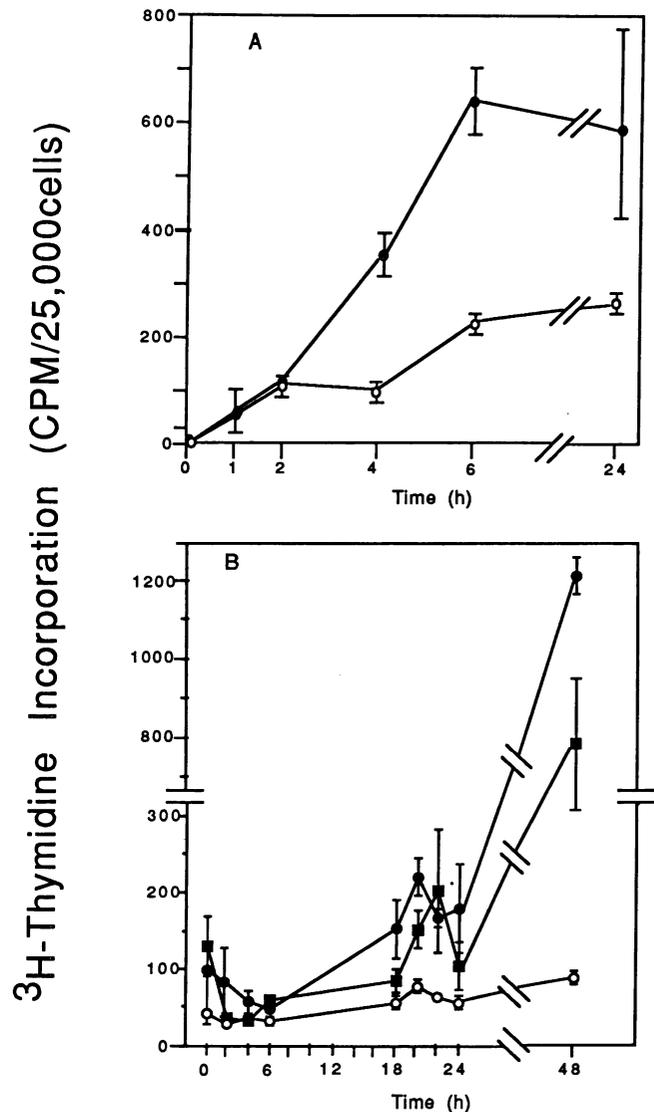


Figure 2. Time course of ³H-thymidine incorporation in response to the exposure to Hex A. Tracheal myocytes were plated in microtiter wells (25,000 cells/well) in medium M199 without serum and incubated for 24 h (A) and 72 h (B). Cells were then treated with Hex A 0.5 U/ml; (●), vehicle; (○) or TPA; (■) and simultaneously pulsed with ³H-thymidine (1 μCi/well). Cells were harvested at intervals following the ³H-thymidine pulse at given times. Results are means±SEM of quadruplicate cultures. Experiments were repeated three times.

for the last 6 h of stimulation period, Hex A caused four and sevenfold increase in ³H-thymidine incorporation compared to vehicle controls. Hex A effect was further increased consistently 10–30-fold increase from control values when cells were incubated for 48 h (six trials). These variabilities were much diminished when cells were incubated in media containing 0.4% FBS for 48 h to reach a quiescent state before the exposure to Hex A. To compare the mitogenic effect of hydrolases to that of other enzymes, we measured ³H-thymidine incorporation under the former experimental condition (serum depletion for 24 h, stimulation for 6 h) in response to various enzymes such as Hex A, Hex B, partially purified bovine liver β-glucuronidase, elastase, collagenase, bovine pancreas pronase, and hu-

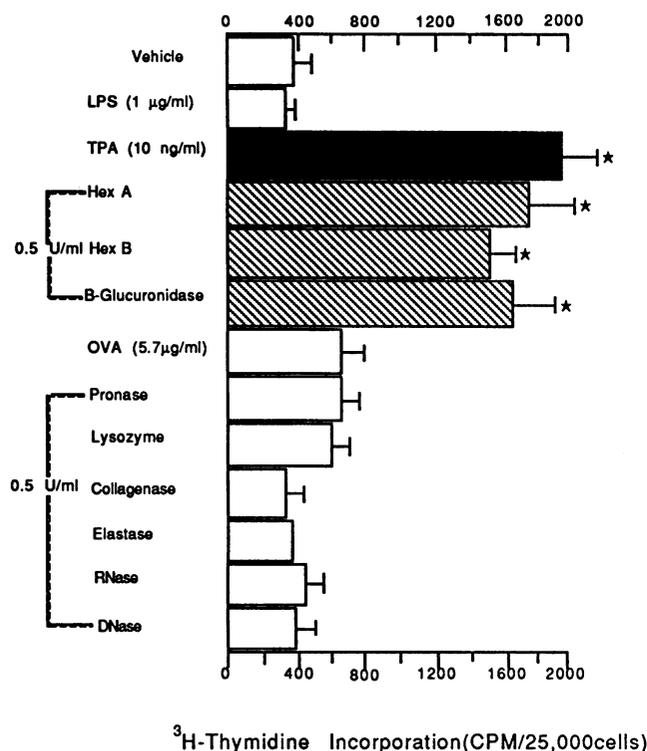


Figure 3. Comparative effects of lysosomal enzymes on ^3H -thymidine incorporation in bovine tracheal myocytes. Cells (25,000 cells/well) were seeded in microtiter wells in 0% serum for 24 h before stimulation. Cells were exposed to enzymes and pulsed with ^3H -thymidine for the following 6 h. Protein contents of enzymes tested are as follows; Hex A (5.7 $\mu\text{g}/\text{ml}$), Hex B (3.8 $\mu\text{g}/\text{ml}$), β -glucuronidase (3.0 $\mu\text{g}/\text{ml}$), pronase (55.6 $\mu\text{g}/\text{ml}$), lysozyme (5 ng–5 $\mu\text{g}/\text{ml}$), elastase (185.2 $\mu\text{g}/\text{ml}$), collagenase (416.7 $\mu\text{g}/\text{ml}$), RNase (100 $\mu\text{g}/\text{ml}$) and DNase (0.2–2 $\mu\text{g}/\text{ml}$). Results are mean \pm SEM of quadruplicate cultures. Experiments were repeated three times. * $P < 0.05$; significantly different from vehicle control.

man milk lysozyme. As shown in Fig. 3, ^3H -thymidine incorporation was significantly enhanced not only upon treatment with Hex A, but also upon exposure of the cells to Hex B and bovine β -glucuronidase. The magnitude of the response to any one of three hydrolases was comparable, and similar to that obtained with TPA (10 ng/ml, used as a positive control). *E. coli* β -glucuronidase (0.5 U/ml) caused sixfold increase in ^3H -thymidine incorporation. However, enzyme preparation dialyzed overnight against PBS at 0°C had no stimulatory effect while maintaining 50% of enzymatic activity suggesting that the effect was due to small molecular weight contaminant(s). *E. coli* endotoxin (1 $\mu\text{g}/\text{ml}$), a common contaminant of purified enzymes, had no effect on ^3H -thymidine incorporation. Notably, elastase and collagenase were effective in digesting the intercellular matrix observed by microscopic examination (data not shown). The glycoprotein control, ovalbumin (5.7 $\mu\text{g}/\text{ml}$: equivalent to protein content of Hex A, 0.5 U/ml), as well as bovine pancreas pronase and human milk lysozyme (0.5–500 U/ml: 1 $\mu\text{mol}/\text{min}$) induced a modest, statistically insignificant increase of ^3H -thymidine incorporation.

Serum effect. To test for the possibility that hydrolases might exclusively act indirectly by activating theoretical growth factors or inactivating a variety of theoretical growth

inhibitory factors present in serum, we compared concentration-dependent ^3H -thymidine incorporation in response to Hex A in the presence and the absence of 10% FBS. As shown in Fig. 4, a similar pattern was seen in both conditions. The maximum effective enzyme concentration (0.5 U/ml) and the relative magnitude of response were comparable in these experimental conditions; sixfold vs. fourfold increase from the baseline at maximum concentration in 10 and 0% FBS, respectively. We also tested various concentrations of serum (2, 5, 10, and 50% vol/vol) on vehicle-treated as well as Hex A (0.5 U/ml)-treated cultures. Maximum ^3H -thymidine incorporation occurred in both Hex A and 10% FBS-treated cultures, and there was a gradual increase in ^3H -thymidine incorporation in response to Hex A as the concentration of FBS was increased up to the concentration of 10% FBS (data not shown). However, at a FBS concentration of 50% (vol/vol), the Hex A effect was abolished (data not shown).

Inhibition of the mitogenic effect of Hex A/Hex B. To test whether the mitogenic effect of hydrolases was due to their hydrolytic action, we inactivated Hex A activity by incubating the enzyme at 50°C for 3 h, which resulted in a 94.4% loss of enzyme activity. When tested on serum-free cultures at concentrations of 5.7 $\mu\text{g}/\text{ml}$, heat-inactivated Hex A produced 41% of the Hex A effect on ^3H -thymidine incorporation (Fig. 5). The lack of near complete abolition of the mitogenic effect by heat inactivation of enzyme activity raised the possibility that these hydrolases might act through receptor-ligand interactions, specifically through the mannose/*N*-acetyl glucosamine (Man/Glc NAc) receptor or the cation independent mannose-6-phosphate/insulinlike growth factor II (MPR^{CI}/IGF II) receptor, that recognize lysosomal hydrolases (24, 25). To test these possibilities, we then exposed cultures to Hex A in the presence of yeast mannan (0.1 and 1.0 mg/ml) and mannose-6-phosphate (M6P: 10 mM) which we added 30 min before the exposure to Hex A. Mannan inhibited Hex A-induced ^3H -thymidine incorporation effectively (92 and 100% inhibition at 0.1 and 1.0 mg/ml concentrations, respectively), whereas M6P did not significantly inhibit the cellular response to Hex A (Fig. 5). Mannan and M6P at these concentrations did not cause a significant change of ^3H -thymidine incorporation from their vehicle control. Similar results were obtained with Hex B and bovine β -glucuronidase.

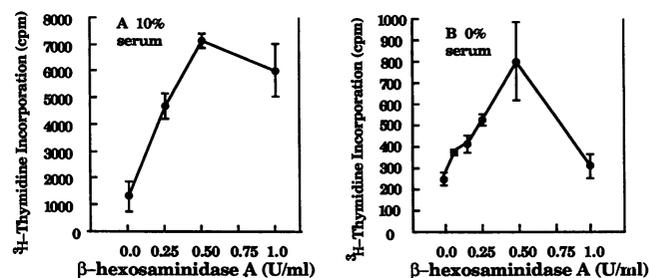


Figure 4. Concentration-dependent ^3H -thymidine incorporation in BTM stimulated by hexosaminidase-A in 10% vs. 0% FBS. Cells were plated in microtiter wells in either 10% (A) or 0% (B) FBS. Cells were stimulated 72 h later with given concentrations of Hex A for 24 h. ^3H -Thymidine incorporation from 6 to 24 h is shown. The scales of y axis were drawn differently due to the marked difference in baseline control values in two conditions. Results are means \pm SEM of quadruplicate cultures. Experiments were repeated three times.

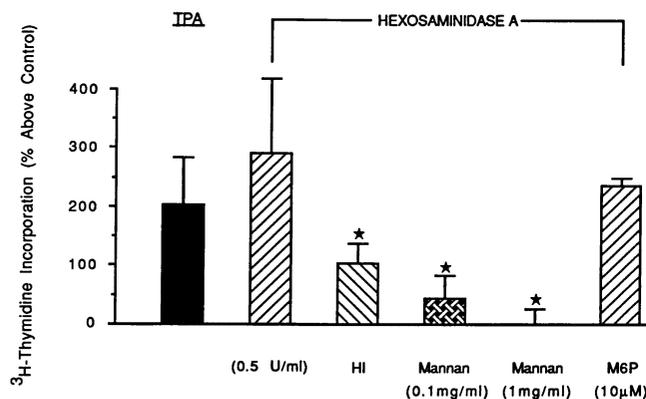


Figure 5. Inhibition of Hex A-induced mitogenic effect. ³H-Thymidine incorporation was measured in cells depleted of serum for 48 h in the presence of heat-inactivated Hex A, mannan, and mannose-6-phosphate. ³H-Thymidine was added at 18 h and cells were harvested at 24 h. Results are means±SEM of quadruplicate cultures (control; 327±69 cpm/25,000 cells). Experiments were repeated three times. HI, heat inactivated. **P* < 0.05; significantly different from Hex A effect.

Effect of D-mannose, Man₃ Glc NAc HSA, and BSA on ³H-thymidine incorporation. Experiments were done under the same conditions as previously described. Cells were exposed to either D-mannose, Man₃ Glc NAc HSA, BSA, or vehicle at given concentrations. D-Mannose up to 1,000 μg/ml (Fig. 6 shows only up to 100 μg/ml) had no effect on ³H-thymidine incorporation, whereas Man₃ Glc NAc HSA enhanced ³H-thymidine incorporation (168±22% above vehicle control at 10 μg/ml concentration). BSA had a mild effect, but only at a much higher concentration (77±19% above control at a 125 μg/ml concentration). The effect of Man₃ Glc NAc HSA, but not the effect of BSA (125 μg/ml), was blocked by mannan (0.1 mg/ml, data not shown).

Discussion

The results of this study suggest that lysosomal hydrolases exert a potent mitogenic effect on bovine tracheal myocytes in cul-

ture, as shown both by the cell numbers and by an enhanced rate of DNA synthesis determined by ³H-thymidine incorporation. Although a larger number of lysosomal and nonlysosomal enzymes should be tested, the mitogenic effect appears to be specific to lysosomal hydrolases because other enzymes were ineffective. The variable onset of ³H-thymidine incorporation in response to Hex A (2 vs. 18 h) seems to be associated with cell cycle stage because the longer the serum depletion, the slower the onset of the action. This effect did not require enzymes from a particular species and was independent of the presence of serum at concentrations normally used for cell culture. However, the effect of Hex A on ³H-thymidine was inhibited by a high concentration of serum (50% vol/vol). In view of the apparent mannose receptor involvement, one possible explanation is that mannose-binding protein present in human serum (26) may also be present in FBS and may act as a lectin, inhibiting the interaction between Hex A and the cell surface. Another possible explanation is that cells might have reached the maximum potential for ³H-thymidine incorporation; thus, the additive effect of Hex A and FBS (50%) could not be appreciated. The lack of Hex A effect at 50% FBS could also be explained by a toxic effect of high serum concentration.

An earlier report by Rokosova and Bentley in 1980 described a proliferative effect of mouse liver and dog alveolar macrophage-derived, mixed lysosomal enzymes (1.7–6.8 μg protein/ml) on bovine vascular smooth muscle cells and diploid human fibroblasts (WI-38), respectively (12). The range of protein content of the mixed lysosomal enzyme preparations used in their experiments is comparable to that of purified Hex A (5.7 μg/ml) and Hex B (3.8 μg/ml) used in our experiments. These authors, however, did not analyze the specific enzyme content and activity of their crude lysosomal preparation, and the specific compound(s) responsible for the mitogenic effect remained unidentified. The availability of highly purified human placental lysosomal hydrolases (Hex A, Hex B) enabled us to extend their findings to respiratory smooth muscle cells in culture under more controlled conditions.

In considering the mechanism(s) responsible for the stimulating effects of lysosomal hydrolases, there are several possible explanations. First, a significant reduction of the mitogenic effect by heat inactivation of Hex A may suggest that either the enzyme activity at the level of plasma membrane itself or a

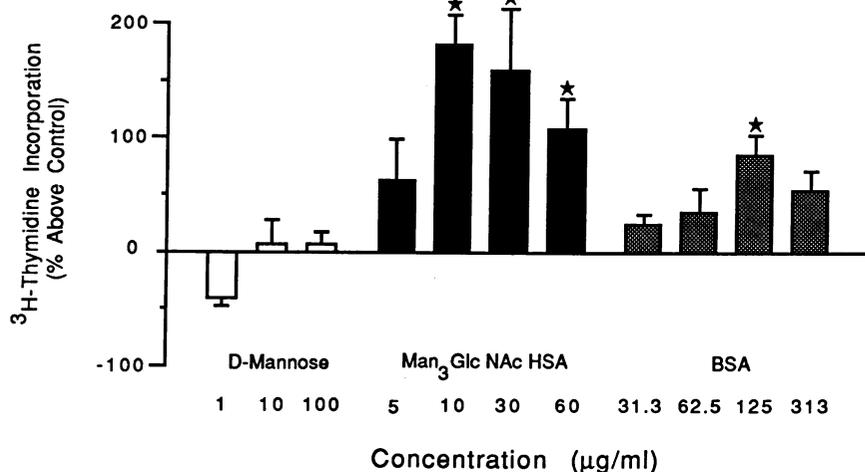


Figure 6. Effect of D-mannose, Man₃ Glc NAc HSA and BSA on ³H-thymidine incorporation. Cells depleted of serum for 48 h were exposed to either D-mannose (0–1,000 μg/ml) Man₃ Glc NAc HSA (0–60 μg/ml), BSA (0–313 μg/ml), or vehicle for 24 h. ³H-Thymidine incorporation for the last 6 h was measured. Results are means±SEM of quadruplicate cultures (control; 252±53 cpm/25,000 cells). Experiments were repeated three times.

product of hydrolytic activity may be responsible for the mitogenic action. Although a pH of 4.5–5.0 is generally optimum for lysosomal acid hydrolase activity, a small (~ 30% by our measurement) but significant activity may be present at the physiologic pH of the medium (pH 7.3). If this were the case, one could speculate that the mitogenic effect may be a consequence of the β -chain catalytic activity, resulting in water-soluble *N*-acetylglucosamine and *N*-acetylgalactosamine residues cleaved from plasma membrane neutral glycolipids or glycoproteins. The activity of α -chain on GM₂ ganglioside would not necessarily be involved because Hex B, that does not cleave this substrate (21, 22), is equally effective in mitogenic action. These residues may then trigger diverse enzymatic reactions such as phosphorylation and generation of intracellular messengers involved in cell growth. Several findings, however, appear inconsistent with this explanation. First, it is not clear why bovine β -glucuronidase should have the same effect as Hex A and Hex B, as it would act on different substrates and generate different products. Second, neither *N*-acetylglucosamine nor *N*-acetylgalactosamine (up to 100 μ M) increased ³H-thymidine incorporation in our system. Third, dialyzed catalytically active *E. coli* β -glucuronidase had no effect. Fourth, and most important, the stimulatory activity of Man₃ Glc NAc HSA cannot result from such a mechanism, as this compound has no enzymatic activity.

An intriguing possibility is that enzymatic activity on cell membrane components may take place in an endocytic, prelysosomal, acidified compartment (27). Whereas this could result in greater activity of lysosomal hydrolases because of the lower pH, it would also require that the enzymes be endocytosed effectively, i.e., by glycosyl-specific, receptor-mediated endocytosis. In this case, the nonglycosylated *E. coli* β -glucuronidase would not be active.

Two receptor systems are known to recognize high mannose-type lysosomal enzymes, the MPR^{Cl}/IGF II receptors and Man/Glc NAc receptors (28–32). The MPR^{Cl}/IGF II receptor is an attractive candidate, as it may not only be involved in endocytosis for catalytic activity in a prelysosomal compartment, but also possibly trigger mechanisms normally activated by the binding of IGF II, resulting in cell proliferation (31, 32). Involvement of the MPR^{Cl}/IGF II receptor, however, seems unlikely in our case because M6P at a concentration that effectively prevents lysosomal enzyme binding (20) did not significantly inhibit Hex A effect (Fig. 5). Furthermore, “mature” lysosomal enzymes in organ extracts have little or no M6P residues (33). Hex A and Hex B in our preparation are not significantly taken up by MPR^{Cl} rich cultured skin fibroblasts (data not shown). It is, however, theoretically possible that M6P-rich lysosomal enzymes may exert a mitogenic effect through MPR^{Cl}.

The second system, Man/Glc NAc-specific receptor, is known to be present on activated macrophages and nonparenchymal hepatic phagocytic cells (28–30). Although there is no formal proof that this system is present on tracheal myocytes, our initial data of enhanced ³H-thymidine incorporation induced by Man₃ Glc NAc HSA (Fig. 6) strongly suggest that this is the case. The optimum concentration required for Man₃ Glc NAc HSA effect was comparable to that of Hex A (10.0 vs. 5.7 μ g/ml).

Furthermore, preliminary experiments have shown that Man BSA-gold colloid conjugate binds to the surface of myocytes, and that this binding is nearly completely inhibited by a

30 min preincubation of cells with mannan (1 mg/ml) as observed by microscopic examination (data not shown). The Man/Glc NAc receptor is thought to be important for scavenging potentially harmful lysosomal enzymes released by macrophages during phagocytosis (28); while this may indeed be a function of this receptor system, it may not be the only one. Analogous to the MPR^{Cl}/IGF II receptor, the Man/Glc NAc receptor may have a trophic function unknown at this time. Inhibition of the mitogenic effect of Hex A and Hex B by mannan strongly argues for its direct involvement, rather than as a carrier/anchor for enzymes that may catalytically activate a mitogenic signal (34). This is further suggested by the retention of mitogenic activity by catalytically inactive Hex A, and by the same cellular response upon exposure to different hydrolases (β -hexosaminidase, bovine β -glucuronidase) and to a nonenzymatically active ligand (Man₃ Glc NAc HSA). This problem is currently under investigation in our laboratories.

Within the limitations of using a tissue culture system derived from bovine trachealis muscle, the results of this study suggest that lysosomal hydrolases may play a role in the pathogenesis of hyperplasia/hypertrophy of respiratory smooth muscle, as seen in asthma.

Acknowledgments

The authors wish to acknowledge Dr. K. U. Malik and Dr. William Walker for their helpful advice, Mr. Scott Ferguson and Anthony Calabro for technical assistance, Ms. Karen Willard and Ibby MacDonnell for manuscript preparation, and Mr. James G. Karas for statistical analysis.

This work was supported in part by LeBonheur Small Grant (73233-39), research grants from American Lung Association of Tennessee and American Lung Association (to D. B. Lew), NIH-R29 HL47022 (to D. B. Lew), NIH-NINCDS NS 21404-06 (to M. C. Rattazzi), and GCRC grant (5M01 RR00211).

References

- Dunnill, M. S., G. R. Massarella, and J. A. Anderson. 1969. A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis, and in emphysema. *Thorax*. 24:176–179.
- Heard, B. E., and S. Hossain. 1973. Hyperplasia of bronchial muscle in asthma. *J. Pathol.* 110:319–331.
- Takizawa, T., and W. M. Thurlbeck. 1971. Muscle and mucous gland size in the major bronchi of patients with chronic bronchitis, asthma and asthmatic bronchitis. *Am. Rev. Respir. Dis.* 104:331–336.
- Armour, C. L., L. M. Diment, and J. L. Black. 1988. Relationship between smooth muscle volume and contractile response in airway tissue. Isometric versus isotonic measurement. *J. Pharmacol. Exp. Ther.* 245:687–691.
- Barnes, P. J. 1987. Inflammatory mediator receptors and asthma. *Am. Rev. Respir. Dis.* 135:S26–S31.
- Panettieri, R. A., P. A. Yadavish, A. M. Kelly, N. A. Rubinstein, and M. I. Katlikoff. 1990. Histamine stimulates proliferation of airway smooth muscle and induces c-fos expression. *Am. J. Physiol.* 259(Lung Cell. Mol. Physiol. 3):L365–L371.
- Tonnel, A. B., M. Joseph, P. H. Gosset, E. Fourmer, and A. Capron. 1983. Stimulation of alveolar macrophages in asthmatic patients after local provocation test. *Lancet*. i:1406–1408.
- Murray, J. J., A. B. Tonnel, A. R. Brash, L. J. Roberts, P. Gosset, R. Workman, A. Capron, and J. A. Oates. 1986. Release of prostaglandin D₂ into human airways during acute antigen challenge. *N. Engl. J. Med.* 315:800–804.
- Lew, D. B., V. Chodimella, and C. G. Murlas. 1990. Guinea pig O₂-induced airway hyperreactivity is associated with increased *N*-acetyl- β -glucosaminidase (NAGA) activity in bronchoalveolar fluid. *Lung*. 168:273–283.
- Laitinen L. A., K. E. Heino, and K. Glich. 1982. Damage of the airway epithelium and bronchial reactivity in patients in asthma. *Lancet*. ii:11–16.
- Bousquet, J., P. Chanez, J. Y. Lacoste, G. Barneon, N. Ghavanian, I. Epander, P. Venge, S. Ahlstedt, J. Simony-Lafontaine, P. Godard, and F. Michel. 1990. Eosinophilic inflammation in asthma. *N. Engl. J. Med.* 323:1033–1039.
- Rokosova, B., and J. P. Bentley. 1980. The effect of lysosomal enzymes on

the proliferation of aortic smooth muscle cells and fibroblasts. *Pathol. Biol.* 28:493-500.

13. Davies, P., and A. C. Allison. 1976. Secretion of macrophage enzymes in relation to the pathogenesis of chronic inflammation. In *Immunobiology of the Macrophages*. D. S. Nelson, editor. Academic Press, Inc., New York. 427-461.

14. Kotlikoff, M. I., R. K. Murray, and E. E. Reynolds. 1987. Histamine-induced calcium release and phorbol antagonism in cultured airway smooth muscle cells. *J. Physiol. (Lond.)* 253(Cell Physiol. 22):C561-C566.

15. Dohlman, J. G., D. G. Payan, and E. J. Goetzl. 1984. Generation of a unique fibroblast-activating factor by human monocytes. *Immunology* 52:577-584.

16. Geiger, B., and R. Arnon. 1976. Purification of hexosaminidase. *Methods Enzymol.* 50:549-551.

17. Mahuran, D., and J. A. Lowden. 1980. The subunit and polypeptide structure of hexosaminidase from human placenta. *Can. J. Biochem.* 58:287-294.

18. Rattazzi, M. C., P. J. Carmody, and R. G. Davidson. 1975. Studies on human lysosomal β -D-N-acetyl hexosaminidase and arylsulfatase isozymes. In *Isozymes II. Physiological Function*. C. L. Markert, editor. Academic Press, Inc., New York. 439-468.

19. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.

20. Vladutiu, G. D., and M. C. Rattazzi. 1979. The excretion-reuptake route of β -hexosaminidase in normal and I-cell disease cultured fibroblasts. *J. Clin. Invest.* 63:595-601.

21. Li, S. C., Y. Hirabayashi, and Y. T. Li. 1981. A protein activator for the enzymic hydrolysis of GM₂ ganglioside. *J. Biol. Chem.* 256:6234-6240.

22. Conzelmann, E., and K. Sandhoff. 1979. Purification and characterization of an activator protein for degradation of glycolipids GM₂, hexosaminidase A. *Hoppe-Seyler's Z. Physiol. Chem.* 360:1837-1849.

23. Lew, D. B., C. C. Leslie, D. W. H. Riches, and P. M. Henson. 1986. Induction of macrophage lysosomal hydrolase synthesis and secretion by β -1,3-glucan. *Cell Immunol.* 100:340-350.

24. Von Figura, K., and A. Hasilik. 1986. Lysosomal enzymes and their receptors. *Annu. Rev. Biochem.* 55:167-193.

25. Kornfeld, S. 1987. Trafficking of lysosomal enzymes. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 1:462-468.

26. Clokisy, D. R., J. C. Chappel, and S. L. Teitelbaum. 1989. Bone marrow derived mononuclear phagocytes autoregulate mannose receptor expression. *J. Biol. Chem.* 264:5370-5377.

27. Kornfeld, S., and I. Mellman. 1989. The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* 5:483-525.

28. Stahl, P. D., J. S. Rodman, M. J. Miller, and P. H. Schlesinger. 1978. Evidence for receptor-mediated binding of glycoproteins glycoconjugates, and lysosomal glycosidases by alveolar macrophages. *Proc. Natl. Acad. Sci. USA.* 75:1399-1403.

29. Stahl, P., P. H. Schlesinger, E. Sigardson, J. S. Rodman, and Y. C. Lee. 1980. Receptor-mediated endocytosis of mannose glycoconjugates by macrophages: characterization and evidence for receptor recycling. *Cell.* 19:207-215.

30. Warr, G. A. 1980. A macrophage receptor for mannose/glucosamine-glycoproteins of potential importance in phagocytic activity. *Biochem. Biophys. Res. Commun.* 93:737-745.

31. Morgan, D. O., J. C. Edman, D. N. Standring, and V. A. Fried. 1987. Insulin-like growth factor II receptor as a multifunctional binding protein. *Nature (Lond.)* 329:301-307.

32. Waheed, A., T. Braulke, U. Sunghaus, and K. Von Figura. 1988. Mannose 6-phosphate/insulin-like growth factor II receptor: the two types of ligands bind simultaneously to one receptor at different sites. *Biochem. Biophys. Res. Commun.* 152:1248-1258.

33. Glaser, J., K. J. Rosen, P. E. Brut, and W. S. Sly. 1975. Multiple isoelectric and recognition forms of human beta glucuronidase activity. *Arch. Biochem. Biophys.* 166:536-542.

34. Sung, S. J., R. S. Nelson, and S. C. Silverstein. 1983. Yeast mannans inhibit binding and phagocytosis of zymosan by mouse peritoneal macrophages. *J. Cell Biol.* 96:160-166.