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The epidermal growth factor receptor mediates allergic airway remodeling in the rat

Meiyo Tamaoka[1]*, Muhannad Hassan[1*], Toby McGovern*, David Ramos-Barbón[*], Taisuke Jo, Yasuyuki Yoshizawa[†], Barbara Tolloczko*, Qutayba Hamid[*] and James G. Martin[*]

[*]Meakins-Christie Laboratories, Department of Medicine, McGill University, Montréal, Québec, H2X 2P2 Canada

[†]Department of Integrative Pulmonology, Tokyo Medical and Dental University, Tokyo, Japan

Corresponding author: Dr. James G. Martin

Address: Meakins-Christie Laboratories, Department of Medicine, McGill University. 3626 St. Urbain Street, Montréal Québec H2X 2P2 Canada

Phone: 514-398-3864 **Fax:** 514-398-7483

E-mail address: james.martin@mcgill.ca

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[1] These authors contributed equally to the work

ABSTRACT

Rationale: The chronicity of bronchial asthma is attributed to persistent airway inflammation and to a variety of structural changes, or remodeling, that includes smooth muscle and goblet cell hyperplasia.

Objectives: To investigate the mechanisms of airway remodeling we used an established allergen (OVA)-driven rodent model (BN rat).

Methods: BN rats were sensitized to ovalbumin and challenged 3 times at 5 day intervals to evoke airway remodeling. The effects of an epidermal growth factor (EGF) receptor inhibitor, AG1478, and a cysteinyl leukotriene-1 receptor antagonist, montelukast, were tested on epithelial and ASM cell proliferation *in vivo* in response to repeated OVA challenge. Three challenges with leukotriene D_4 (LTD_4) were given to examine their effects on remodeling with and without AG1478 pre-treatment.

Measurements and Main Results: OVA challenges caused ASM hyperplasia with an increase in mass and epithelial cell proliferation and goblet cell proliferation. AG1478 prevented the changes as did montelukast. Multiple OVA challenges increased heparin-binding EGF-like growth factor (HB-EGF) but not EGF expression by airway epithelium. LTD_4 recapitulated the changes in remodeling induced by OVA and this was blocked by AG1478.

Conclusions: Allergen induced airway epithelial and ASM remodeling is mediated by cysteinyl leukotrienes via the cys-LT1R with downstream effects on the EGFR axis.

Abbreviations used: ASM, airway smooth muscle; Cys-LT, cysteinyl leukotriene; EGF, epidermal growth factor; EGFR, EGF receptor; HB-EGF, heparin-binding EGF-like growth factor; LTD_4, leukotriene D_4; OVA, ovalbumin, PCNA, proliferating cell nuclear antigen; α-SMA, smooth muscle α-actin.

INTRODUCTION

Asthmatic airways often show extensive and complex remodeling(1-4). The growth of smooth muscle has the potential to have the most significant pathophysiological consequences through excessive airway narrowing and airway hyperresponsiveness (5;6). Increase in airway smooth muscle ASM) in the airways has been associated with the severity of asthma (3;7) and when present in excess in the large airways is associated with mortality(8). It is known that hyperplasia of smooth muscle in animal models (9-11) and both hyperplasia and hypertrophy in airway specimens from human subjects (12;13) contribute to the increase in ASM mass. It has also been proposed that migration of subepithelial myofibroblasts may add to the tissue mass (7).

The mechanism of the growth response of muscle is quite uncertain although several descriptive studies of growth factor expression in human airway tissues have been reported (14-16) and many growth factors have been demonstrated to have mitogenic effects on ASM in culture (17-20). Cysteinyl-leukotrienes (cys-LTs) are known to be involved in allergen-induced ASM cell proliferation *in vivo* (21;22) but *in vitro* these substances are weak mitogens for ASM (23;24). In the sensitized mouse cys-LT_1 receptor antagonism prevents an increase in ASM thickening after repeated allergen challenge(25;26). It is possible that their effects *in vivo* are indirect and mediated by altering the expression of or by potentiating the effects of tyrosine kinase linked growth factors such as epidermal growth factor (EGF)(27) or by upstream effects on immune responses to allergen challenge (28).

EGF has been shown to be up-regulated in asthmatic human airways(16) and it stimulates ASM growth *in vitro* (23). Heparin-binding EGF-like growth factor (HB-

EGF), another ligand for the EGF receptor (EGFR) has also been reported to be present in the airway epithelium of asthmatic subjects (29) and it is mitogenic for vascular smooth muscle (30;31). Both of these factors are therefore plausible candidates for the ASM hyperplasia in asthma. EGFR has been implicated in goblet cell differentiation, in a murine model of allergic asthma (26). Stimulation of the EGFR causes goblet cell differentiation and up-regulation of MUC genes (32). We hypothesized that allergen challenge increases the mass of ASM and airway goblet cell numbers through EGFR activation *in vivo*. To test this hypothesis we used a rat model of allergen-induced airway remodeling(9-11), requiring only three allergen exposures for increase in ASM mass to occur(33). We wished to elucidate the possible role of the EGFR in the apparent mitogenic effects of cys-LTs on ASM (21;22) and in airway epithelial remodeling(26). In this study we report that inhibition of the EGFR or the cys-LT_1R completely abrogated allergen-induced ASM growth and goblet cell differentiation in the epithelium. Exogenous LTD_4 mimicked allergen challenges in causing ASM and goblet cell hyperplasia by mechanisms involving the EGFR, indicating that the cys-LT_1R is upstream of the EGFR in the remodeling cascade.

MATERIALS AND METHODS

Animal preparation and treatment

BN rats were sensitized with injections of 1mg of ovalbumin (OVA) and 100mg of alum subcutaneously and $2x10^9$ heat-killed *Bordetella pertussis* bacilli intraperitoneally. Anesthetized rats were challenged with either aerosolized OVA or saline delivered endotracheally on days 14, 19 and 24. The effects of AG1478, EGFR tyrosine kinase inhibitor, or montelukast, cys-LT_1R antagonist or appropriate vehicles given intraperitoneally or by gavage, respectively, 1 hour prior to each challenge were tested in independent samples. In a third sample, unsensitized rats were administered LTD_4 (100μg) intra-tracheally three times at 5 day intervals, with or without pre-treatment with AG1478. These groups were compared with vehicle-treated controls. Animals were killed two days after the final challenge for tissue collection.

The study protocol was approved by the Animal Care Committee of McGill University.

Immunostaining for HB-EGF, EGF and EGFR

The left lung was formalin-fixed at 25 cmH2O pressure and subsequently paraffin-embedded. Five μm mid-sagittal and para-hilar sections were cut. To localize and quantify HB-EGF and EGF protein expression in an airway, we performed immunostaining with rabbit polyclonal antibodies to human HB-EGF (R&D Systems) and to rat EGF (Biomedical Technologies, Inc.). EGFR was immunostained using a rabbit polyclonal IgG antibody (Santa Cruz, Biotechnology, Inc, CA). Negative controls were run using the same concentration of isotype control IgG (R&D systems) in place of each primary antibody. The signals were developed with Vector Red and followed by

methyl green counterstaining. In order to perform a morphometric analysis of the number of airway cells positive for HB-EGF and EGF we performed a dose-response curve of immunostained tissues to primary antibody and chose the concentration of antibody that produced definite but weakly positive staining in control tissues in order to allow the detection of an increase in immunostaining, if present. The number of immunoreactive cells in the epithelium was counted by an observer (MH), blinded as to group status and the results expressed per mm of P_{BM}.

Measurement of mass of ASM

The mass of ASM was estimated from the measurement of the area of smooth muscle specific α-actin (α-SMA) immunoreactivity as previously described(33). Briefly, sections were stained with a mouse monoclonal antibody to α-SMA (clone 1A4) (Sigma-Aldrich) and a biotinylated horse anti-mouse IgG, rat adsorbed (Vector Laboratories). The signal was detected with Vector Red (Vector Laboratories). The area of ASM was traced using a camera lucida side arm attachment to the microscope and digitized. The mass of ASM was standardized for airway size by dividing the area of ASM by the square of the perimeter of the basement membrane (P_{BM}^2).

Quantification of proliferating ASM cells *in vivo*

Proliferation of ASM cells was investigated using double-immunostaining for proliferating cell nuclear antigen (PCNA) and α-SMA using a monoclonal antibody to PCNA (Calbiochem) as previously described(33). Staining was preceded by high-temperature epitope unmasking in antigen retrieval solution (Vector Laboratories) and

permeabilization in 0.2% Triton X-100 (Sigma-Aldrich). Sections were then blocked with 20% horse serum (Vector Laboratories) in universal blocking solution (Dako Cytomation), and a monoclonal antibody to PCNA was detected with biotinylated horse anti-mouse IgG, avidin/biotin-alkaline phosphatase complex, and BCIP/NBT chromogen substrate (Vector Laboratories). Double-immunostaining with anti-α-SMA mAb was developed with Vector Red and followed by methyl green (Sigma-Aldrich) counterstain. The number of PCNA positive ASM cells was counted and corrected for airway size by dividing by P_{BM}^2

Quantification of epithelial remodeling

The turnover of epithelial cells was determined using PCNA staining. The goblet cells were enumerated on periodic acid Schiff (PAS) stained tissues and expressed as the number of PAS+ cells per mm P_{BM}**.**

Real-Time Quantitative PCR for growth factors in the lung and airway epithelial cells

To investigate the mRNA expression of HB-EGF and EGF in the whole lung and airway epithelium after treatment, we performed real-time quantitative PCR. The right lung was snap frozen in liquid nitrogen and kept at -80°C. Total RNA was extracted from the frozen lungs using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. Tracheas were dissected out and immersed in RNA*later* (QIAGEN) overnight and epithelial cells were obtained by scraping the lumen of the trachea with a cell scraper. Total RNA was extracted from the epithelial cells using RNeasy Mini Kit

(QIAGEN) according to the manual. Reverse transcription was performed using SuperScript II reverse transcriptase (Invitrogen). Real-time quantitative PCR was performed with the LightCycler® (Roche) using FastStart DNA Master SYBR Green I (Roche). The sequences of HB-EGF primers have been previously published(34). The primers for rat EGF, EGFR and ribosomal protein S9 were designed using web-based software, PrimerQuest (IDT, http://scitools.idtdna.com/Primerquest/). The sequences of each pair of primers are as follows. HB-EGF: 5'-ACTTGGAAGGGACCGATCTGGA-3', 5'-TAGGGTCAGCCCATGACACCTC-3', EGF: 5'-TGCCTTGCCCTGACTCTAC-3', 5'-AGCCAATGACACAGTTGCAC-3', EGFR: 5'-TCCCTTTGGAGAACCTGCAG ATCA-3', 5'-GTTGCTAAATCGCACAGCACCGAT-3', S9: 5'-AGGATTTCTTGGAGA GAAGGCTGC-3', 5'-CTTCTGAGAGTCCAGGCGAACAAT-3'. Standard curves were established for each growth factor and a housekeeping gene. Briefly, PCR products were extracted from agarose gel and purified with GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences). The amount of PCR product was calculated using densitometry. 10^1-10^{10} copies of standard were made by step dilution.

Effects of LTD_4, EGF and HB-EGF alone and in combination on proliferation of primary culture of rat airway smooth muscle cells

To assess the possibility that ASM growth following repeated OVA challenge involved interaction of the EGFR and LTD_4 at the level of the smooth muscle itself the effects of EGF and HB-EGF alone and in conjunction with LTD_4 were tested on cultured ASM. Rat tracheas were dissected and excess connective tissue was removed (35). Tissues were

digested in elastase at 37◦C for 30 minutes and subsequently placed on ice to stop the reaction. The tissues were allowed to stand and supernatant was collected. The tissue fragments remaining in the tube were washed with Hanks' balanced salt solutions (HBSS) and the supernatant was collected. We repeated this procedure 3 times. Finally cells were collected by centrifugation at 1400 rpm for 5minutes and re-suspended in 25cm^2 flasks in 1:1 Dulbecco's modified Eagle's medium (DMEM) -Ham's F12 medium supplemented with 10% fetal bovine serum (FBS), 0.224% NaHCO3 and 1% penicillin/streptomycin in the presence of 5% CO_2. Cell culture reagents were purchased from (GIBCO). The phenotype was confirmed as described previously(35).

Cells at passages 2-4 were used for the experiment. Cells were plated on 6-well plates (40,000 cells in 2ml medium/well) and starved in DMEM with 0.2% BSA when they reached at approximately 70% confluency. After 48 hours in DMEM with 0.2% BSA, cells were stimulated with either human HB-EGF (1-10ng/ml) (R&D Systems), LTD_4 (1μM) (Biomol International) or a combination of HB-EGF and LTD_4. In a separate series of experiments, cells were pretreated with montelukast (500nM) (Merck-Frosst Canada, Montreal, QC, Canada) for 30 minutes before administration of LTD_4. We trypsinized cells with HBSS containing 0.25% trypsin and 0.02% EDTA to detach them from the plate. Trypsin was neutralized by adding DMEM:F12 with 10% FBS and cells were collected by centrifuging at 1400rpm for 5minutes and re-suspended in 1ml of PBS. The cells were counted using a hemacytometer. For BrdU incorporation assay cells were starved and stimulated as described above and BrdU was added to each well 6 hours after stimulation. Cells were harvested 18 hours after BrdU (24 hours after stimulation) and were processed for the flow cytometric detection of incorporated BrdU

using an FITC-BrdU Flow Kit (BD Biosciences) according to the manufacturer's instructions.

Effects of LTD_4 on intracellular calcium responses of cultured ASM cells

To demonstrate the presence of functional cys-LT receptors on ASM we measured the changes in intracellular calcium in response to exposure to LTD_4 (1μM). Cytosolic calcium measurements were performed as previously described (36) ASM cells were incubated for 30 min at 37° C with Hanks' buffer (in mM: NaCl 137, $NaHCO_3$ 4.2, glucose 10, Na_2HPO_4 3, KCl 5.4, KH_2PO_4 0.4, $CaCl_2$ 1.3, $MgCl_2$ 0.5, $MgSO_4$ 0.8, N-2-hydroxyethylpiperazine-N'-ethane sulfonic acid [Hepes] 5) in the presence of 5 μM Fura–2-acetoxymethylester (Fura-2-AM). The loaded cells were washed and the cover slips were placed in a Leiden chamber (Medical Systems Corp, Greenville, NY, USA) containing 450 μl of Hanks' buffer on the stage of an inverted microscope equipped for cell imaging with 40x oil immersion objective (Nikon, Tokyo, Japan). The images of the cells before and after stimulation with LTD4 were obtained using an intensified camera (Videoscope IC 200) and PTI (Photon Technology International Inc., London, ON, Canada) software at a single emission wavelength (510 nm) with double excitatory wavelengths (345 and 380 nm). The fluorescence ratio (345/380) was measured in individual cells and the free Ca^{2+} was calculated using Grynkiewicz's formula (37).

Statistical analysis

Data are presented as mean ± standard error. One-way ANOVA followed by two tailed t-test was performed for the analysis. When data were not normally distributed log

transformation was applied. The Bonferroni correction was used to correct for multiple comparisons. P values less than 0.05 were considered significant.

RESULTS

Effects of allergen challenge on EGF and HB-EGF mRNA expression in whole lung and airway epithelium

We examined the effects of OVA sensitization and challenge on the expression of mRNA for the two EGFR ligands, EGF and HB-EGF as well as the EGFR and standardized their expression for the housekeeping gene S9. We confirmed that S9 did not vary significantly among study groups and furthermore was highly correlated with the expression levels of two other housekeeping genes, β-actin and cyclophilin (data are not shown). We quantified the expression of these growth factors in whole lung tissue using mRNA from lung homogenates and in the epithelium using cells harvested from the lower trachea by scraping. We observed that HB-EGF mRNA expression in tracheal epithelial cells was up-regulated 2-fold after multiple OVA challenges (0.0014 ± 0.00029 versus 0.0073± 0.00073 in controls, P=0.035) (Fig. 1 A), while EGF mRNA or EGFR did not show a change (0.013 ± 0.0011 versus 0.014 ± 0.0016 respectively for EGF, P=0.51; and 0.013 ± 0.0012 versus 0.015 ± 0.0016 for EGFR, P=0.43) (Fig. 1 B, C). There was no detectable alteration in the expression of any of the three genes in whole lung tissues (data not shown).

Immunolocalization and morphometric assessment of HB-EGF and EGF expression after OVA challenges

We performed immunostaining to localize the site of the expression of HB-EGF and EGF in the airways. Fig. 2 shows photomicrographs and data for immunoreactive HB-EGF and EGF. HB-EGF was mainly expressed in airway epithelium and the morphometric

assessment of HB-EGF immunoreactivity demonstrated a significant increase in OVA challenged animals (110.7 ± 4.15) compared to controls (28.2 ± 4.91; $P < 0.0001$) (Fig. 2 A-C). EGF was expressed strongly in airway epithelial cells and less strongly in ASM cells and in some alveolar cells. There was evidence of constitutive expression of EGF but there was no difference in the number of immunoreactive epithelial cells between the treatment groups (36.32 ± 3.23 cells/mm in OVA challenged animals versus 35.88 ± 2.86 in controls, P=0.92) (Fig. 2 D-F). These observations are consistent with the results of quantitative real-time PCR for mRNA obtained from tracheal epithelial cells (Fig 1D).

Effect of EGF receptor blockade on allergen induced ASM remodeling; mass of ASM

We investigated the effect of EGFR inhibition on OVA-induced increase in ASM. Contractile tissue was identified by immunohistochemistry for smooth muscle specific α-actin and is illustrated in Figure 3 (A,B). The mass of ASM was determined morphometrically and expressed as an area of tissue corrected for airway size (ASM/ P_{BM}^2) (Figure 3 C). The size corrected area of ASM showed a 2.8 fold increase after OVA inhalation challenges (0.0020 ± 0.0004) compared to saline challenged controls (0.0007 ± 0.0002) (P=0.0007). This increase in ASM mass was significantly suppressed by treatment with AG1478, an EGFR specific tyrosine kinase inhibitor (0.0009 ± 0.0002) (P=0.0011).

Effect of EGF receptor blockade on allergen induced proliferation of ASM cells

In order to confirm that ASM cells were undergoing hyperplastic growth we immunolocalized proliferating cell nuclear antigen (PCNA) positive nuclei to smooth muscle α-actin (α-SMA) positive cells and an illustrative example is shown in Figure 3 (D). The number of proliferating ASM cells detected by PCNA staining was 2.8-fold higher in OVA challenged animals (5.4 ± 0.69 cells/ mm^2) compared with saline controls (1.6 ± 0.46) (P=0.001) (Fig. 3 F), confirming proliferation of ASM in response to allergen challenge. The EGFR was expressed on epithelial cells and ASM as well as on alveolar macrophages. Epithelial expression of the EGFR and expression in subepithelial cells located in the smooth muscle layer are illustrated in Figure 3 D. AG1478 blocked the increase of proliferating ASM cells after multiple OVA challenges (P=0.01).

The effect of EGF receptor blockade on allergen induced airway epithelial remodeling

Allergic airway remodeling involves phenotypic changes of airway epithelial cells, most notably an increase in goblet cells. The increase in goblet cells evoked by allergen challenges is illustrated in figure 4 A and B. Quantification of the PAS+ cells showed an an 8-fold increase after OVA challenges (P=0.004) (Fig. 4 C). This increase was significantly reduced by AG1478 (P=0.006). The number of proliferating airway epithelial cells, identified by PCNA staining, also increased after multiple allergen challenges (17.4 ± 1.87 cells/mm) (P=0.001) (Fig. 4 D). This increase was also blocked by AG1478 (9.1 ± 2.1) (P=0.017)..

Effects of cys-LT_1R antagonist (montelukast) on ASM mass and hyperplasia after multiple OVA challenges

In order to confirm the role of the cys-LT_1R in airway remodeling we performed repeated allergen challenge in a separate series of animals, some of which were pre-treated with montelukast, a specific cys-LT_1R antagonist. We performed morphometric analysis of OVA-induced changes in ASM mass, with and without montelukast pre-treatment. As before the ASM mass increased in OVA challenged rats (0.0015 ± 0.00014; p=0.04) compared to saline challenged control animals (0.0011 ± 0.00016) and the OVA-induced increase in ASM mass was prevented by montelukast pre-treatment (0.0010 ± 0.0001; p=0.01), indicating that it was cys-LT_1R dependent. The number of PCNA positive ASM cells in OVA challenged rats, determined morphometrically, was also increased after OVA challenge and significantly reduced by montelukast (Fig. 5).

Effects of repeated LTD_4 challenge on ASM growth and goblet cell proliferation

To examine the potential for LTD_4 alone to induce airway remodeling in naïve rats we exposed animals to LTD_4 on three occasions at 5 day intervals mimicking the repeated OVA challenges. We found that there was significant remodeling of both the ASM and the goblet cells in these animals; ASM increased by more than 2-fold and goblet cell numbers increased by approximately 4-fold (Figure 6).

Effects of inhibition of the EGFR on LTD4 induced airway remodeling

To evaluate the possibility that LTD_4 was acting through the EGFR we pre-treated rats undergoing repeated LTD_4 challenges with AG1478, in the same doses that were

effective in preventing allergen-induced ASM and epithelial remodeling. The inhibition of the EGFR with AG1478 significantly reduced both goblet cell and ASM hyperplasia as assessed by PCNA immunoreactivity (Figure 6). Goblet cell numbers and ASM area were proportionately reduced.

Morphometric assessment of epithelial HB-EGF expression after LTD_4 challenges

The effects of repeated challenges with LTD_4 on epithelial expression of HB-EGF was assessed morphometrically and it increased significantly in the LTD_4 challenged animals compared to the controls (Figure 7). The effect was mediated by the cys-LT_1R because it was inhibited entirely by montelukast.

Effect of LTD_4 on ASM proliferation *in vitro*, alone and with concomitant EGFR stimulation

The ASM itself is a potential site of interaction of LTD_4 and the EGFR axis, through synergy or transactivation of the EGFR. First we confirmed that functional cys-LT receptors were present on ASM cells by demionstrating a rise in intracellular calcium in response to exposure of cells to LTD_4 (1μM; Figure 8A). To test the possibility that cys-LTs act in synergy with the EGFR in promoting smooth muscle cell growth we examined the mitogenic effects of HB-EGF and EGF alone and in combination with LTD_4 on ASM from BN rats in primary culture using BrdU incorporation and cell counting. HB-EGF 10ng/ml stimulated ASM proliferation significantly as measured both by BrdU incorporation (Fig. 8B) and cell counting (Fig. 8C). Exogenous LTD_4 did not increase the BrdU incorporation or cell number beyond the values observed after HB-EGF alone.

Similar results were obtained for EGF and EGF + LTD_4 (data not shown). AG1478 inhibited the growth response to EGF and HB-EGF whereas it did not reduce the response to PDGF or to the multi-mitogenic stimulus of FBS (10%) (data not shown).

DISCUSSION

Repeated allergen challenge of the actively sensitized BN rat caused hyperplasia of ASM that led to a substantial increase in ASM mass. It also caused the proliferation of airway epithelial cells and goblet cell differentiation. Increased expression of the growth factor HB-EGF in the airway epithelium, but not EGF, occurred after repeated OVA challenge. ASM hyperplasia and goblet cell differentiation were prevented by pre-treatment with a selective EGF receptor tyrosine kinase inhibitor indicating a primary role for the EGFR in these components of allergen-induced airway remodeling. Montelukast also blocked allergen induced ASM hyperplasia, confirming the role of the cys-LT_1R in the process. Intra-tracheal instillation of LTD_4 caused remodeling similar to OVA challenge when administered alone to naïve rats and these remodeling changes were inhibited by AG1478, indicating that LTD_4 mediates remodeling by indirect means through the EGFR. LTD_4 did not have detectable direct mitogenic effects on rat ASM in culture and did not interact with the EGFR in inducing ASM growth.

Although a number of growth factors have the potential to increase ASM growth, based on activity *in vitro*, we chose to focus on the EGFR ligands, EGF and HB-EGF because of the recent evidence that some of these ligands may be up-regulated in human asthmatic tissues(29). Increased immunoreactivity of EGF has been shown in the submucosa of asthmatic subjects(16) and in bronchial epithelium, glands, and smooth muscle in asthmatic human airways(29). The functional significance of EGFR and its ligands in the airways of asthmatic subjects is not clear. Our data shed light on the role of the EGF receptor and suggest a possible candidate ligand for the receptor in allergen-induced remodeling of epithelium and ASM *in vivo*. The inhibition of ASM growth *in*

vivo by the specific EGF receptor inhibitor AG1478 was virtually complete and goblet cell differentiation was also reduced to basal levels. EGFR has been demonstrated to be involved in goblet cell metaplasia following allergen challenge using a murine model but its role in ASM remodeling has not been examined in any detail *in vivo*(26). We did not find EGF or EGF receptor up-regulation at the mRNA level in either whole lung specimens or isolated epithelial cells, suggesting that regulation of proliferation does not result from alterations in the levels of EGFR or of EGF itself. The up-regulation of HB-EGF provides an alternative mechanism to account for the observed importance of the EGFR in remodeling although our data do not exclude a role for alternative EGFR ligands.

The relationship between the EGFR and cysteinyl-leukotrienes in mediating airway remodeling is not clear. The completeness of the inhibition of remodeling of ASM and epithelium by the specific antagonists for the EGFR and cys-LT_1R suggested a possible interaction of the pathways mediating their effects. Cysteinyl-leukotrienes are the principal mediators of allergic airway narrowing(36-41). Our data are consistent with their role in remodeling, as demonstrated in several previous studies(21;22;25;26). We expected that inhibition of the EGFR might diminish the remodeling effects of LTD_4 itself because of the reported role of mechanical stresses placed on the epithelium, such as would be caused by bronchoconstriction in releasing HB-EGF from airway epithelial cells(42). Consistent with these reported findings the inhibition of the EGFR by AG1478 prevented LTD_4-induced remodeling. Additionally repeated exposures of the airways to LTD_4 caused an up-regulation of HB-EGF, suggesting the possibility that this ligand may

be responsible for the observed remodeling mediated via the EGFR. Direct evidence of release of HB-EGF by LTD_4 is required to confirm this hypothesis.

We looked for an interaction of LTD_4 and the EGFR on the ASM itself. LTD_4 been shown to cause EGFR transactivation in cultured human ASM cells(43). In human fibroblasts LTD_4 also synergizes with the EGFR but by a mechanism that does not involve receptor transactivation(27). In contrast to data on human ASM (23;43) and fibroblasts(27) there was no evidence of a mitogenic effect of LTD_4 nor was an interaction demonstrable between LTD_4 and either HB-EGF or EGF in the rat ASM, despite the demonstrable presence of functional cys-LT receptors on the ASM in culture. The reason for the discrepancy between the rat and human ASM cells is not clear.

It is possible that, in addition to remodeling mediated by downstream effects on the EGFR axis, there may be effects of cys-LTs released following OVA challenge at upstream sites involving the inflammatory cascade. The cys-LT_1R has been implicated in the genesis of Th2 inflammation through effects on dendritic cell function(28). Consistent with an immunomodulatory action of the cys-LTs, montelukast reduces airway eosinophilia and interleukin-5 in the allergen challenged BN rat indicating an anti-inflammatory action involving T cells(44).

In conclusion, our data indicate that both the EGFR and the cys-LT_1R are necessary for some of the characteristics of airway remodeling following allergen challenge. However the cys-LT_1R is upstream of the EGFR in the remodeling process. The ligand for the EGFR is possibly HB-EGF because it is up-regulated by allergen and LTD_4 challenges. Cysteinyl-leukotrienes released by allergen challenge may release, in

turn, HB-EGF which then mediates the epithelial and ASM cell remodeling. However whether this phenomenon indeed occurs requires to be studied.

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The authors report no conflict of interest.

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Figure 1

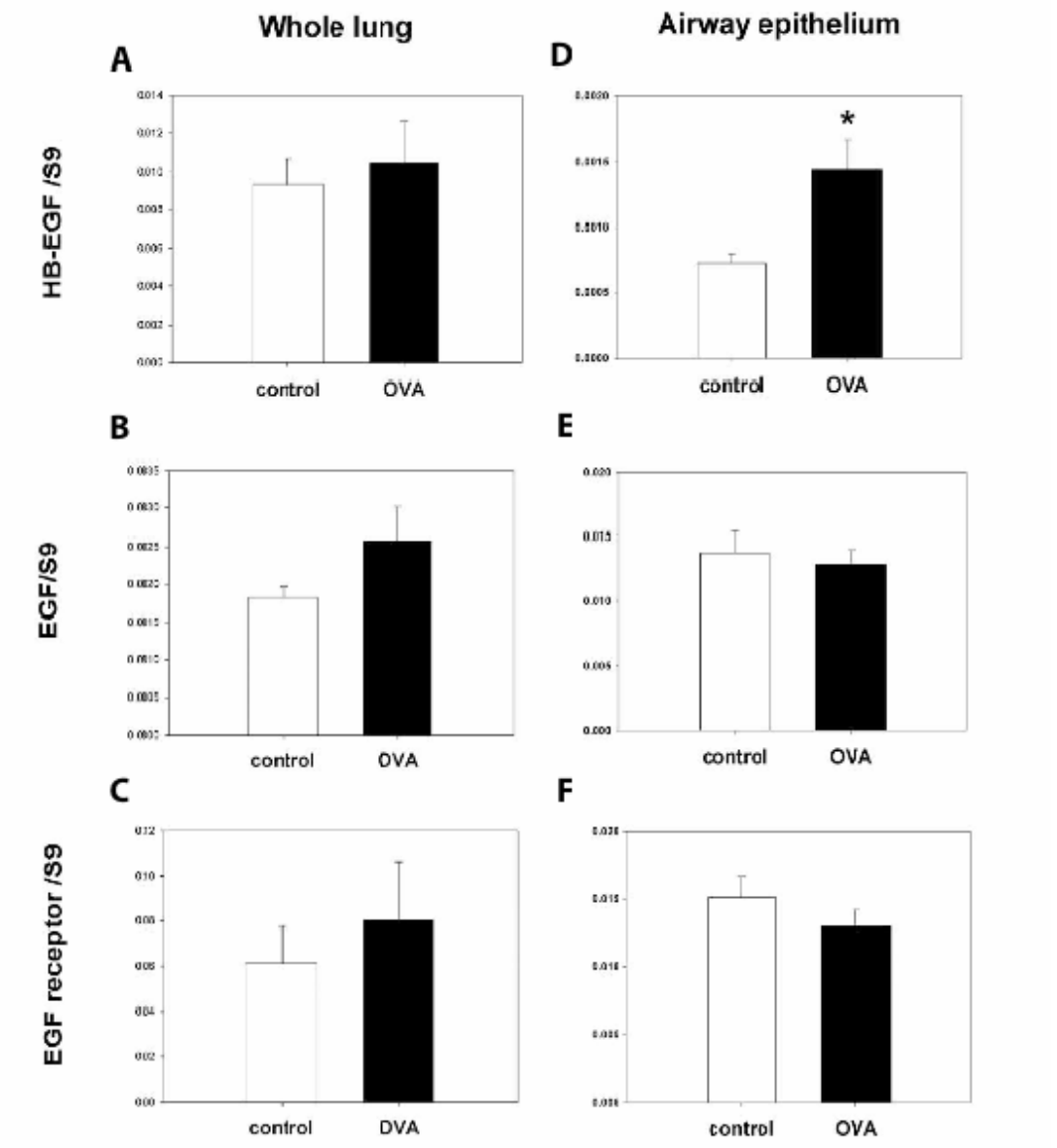

Whole lung
Airway epithelium
A
D
HB-EGF /S9
control
OVA
*
B
E
EGF/S9
control
OVA
C
F
EGF receptor /S9
control
OVA
control
OVA

Figure 2

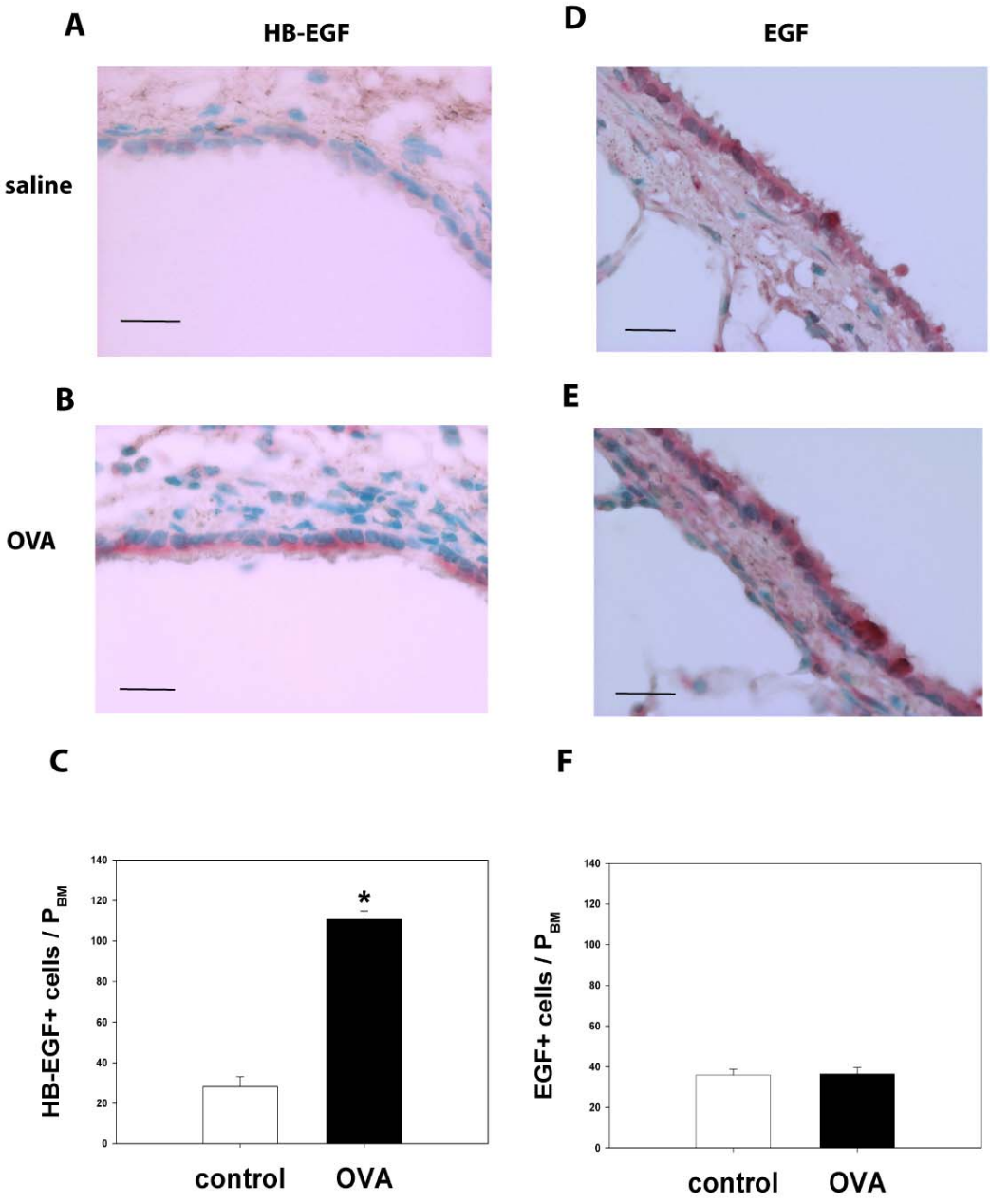

A
HB-EGF
D
EGF
saline
B
E
OVA
C
F
HB-EGF+ cells / P BM
EGF+ cells / P BM
0
20
40
60
80
100
120
140
*
control
OVA
control
OVA

Figure 3

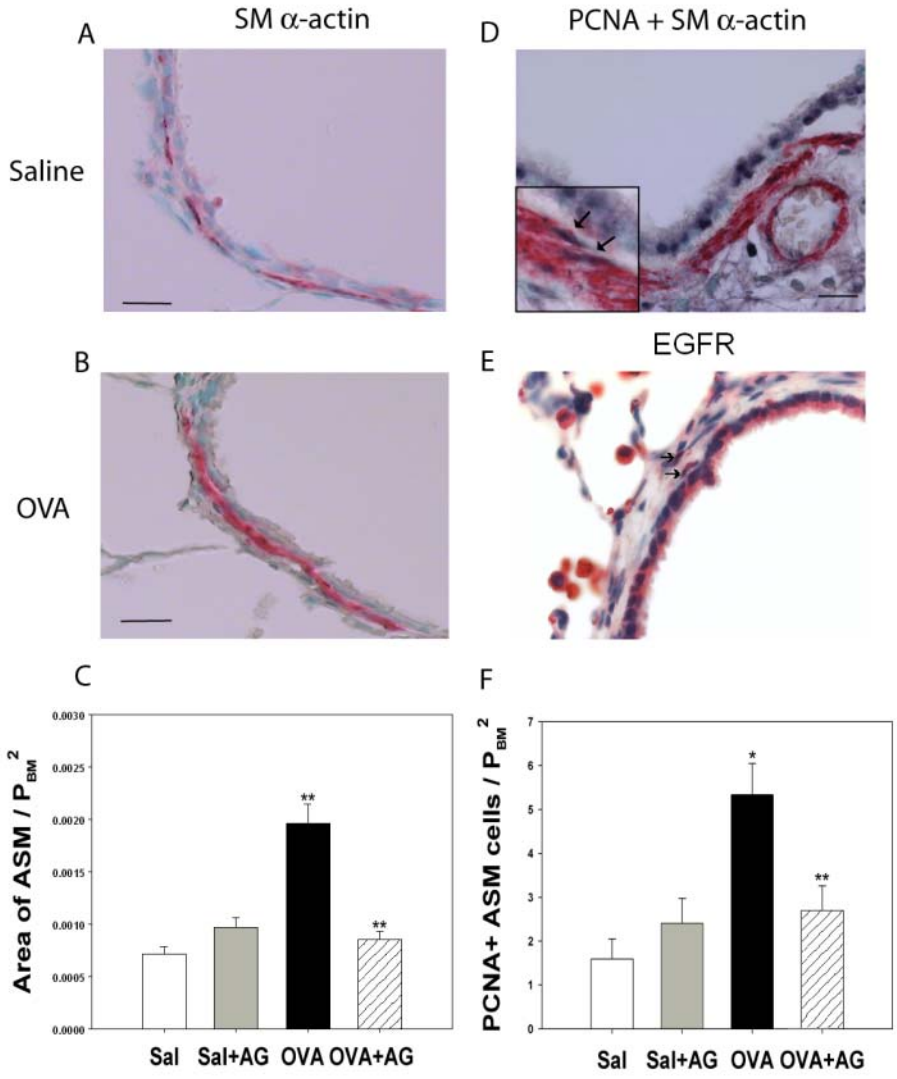

A
SM α-actin
Saline
D
PCNA + SM α-actin
B
OVA
E
EGFR
C
Area of ASM / P BM 2
0.0030
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
**
**
Sal
Sal+AG
OVA
OVA+AG
F
PCNA+ ASM cells / P BM 2
7
6
5
4
3
2
1
0
*
**
Sal
Sal+AG
OVA
OVA+AG

Figure 4

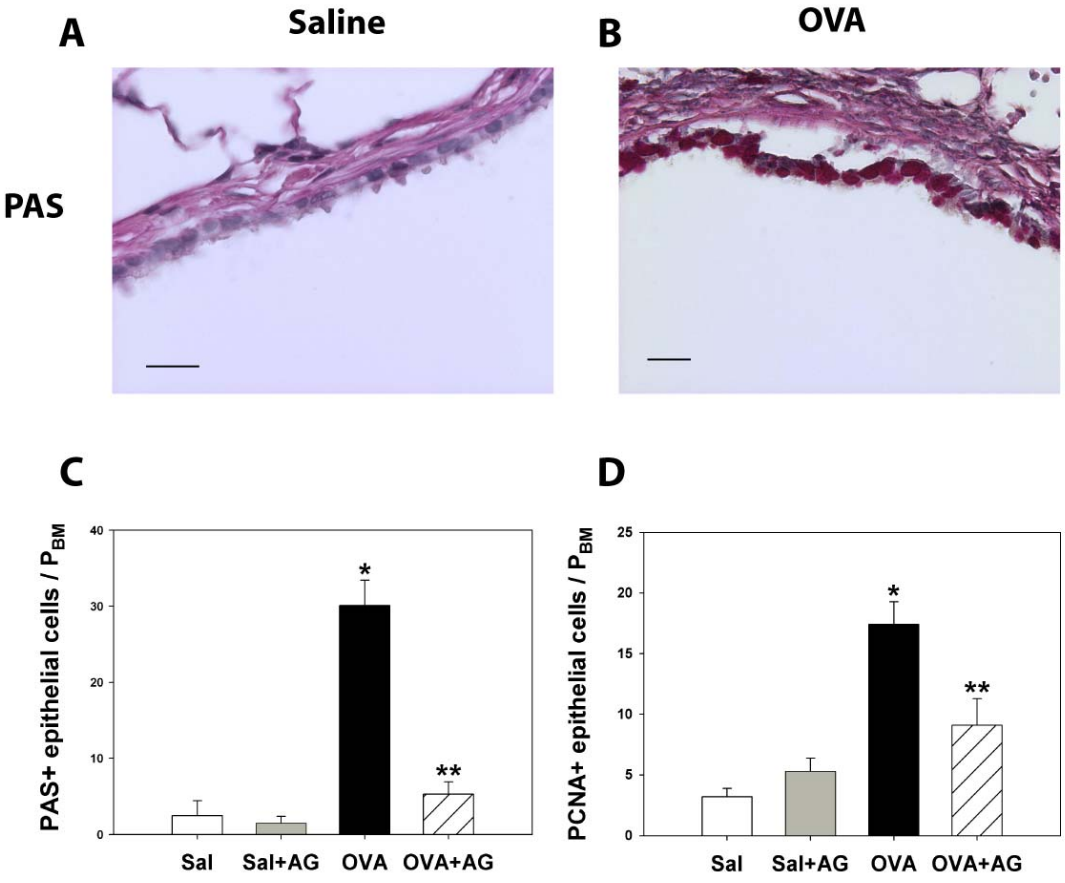

A
Saline
B
OVA
PAS
C
D
PAS+ epithelial cells / P_BM
PCNA+ epithelial cells / P_BM
Sal
Sal+AG
OVA
OVA+AG
*
**

Figure 5

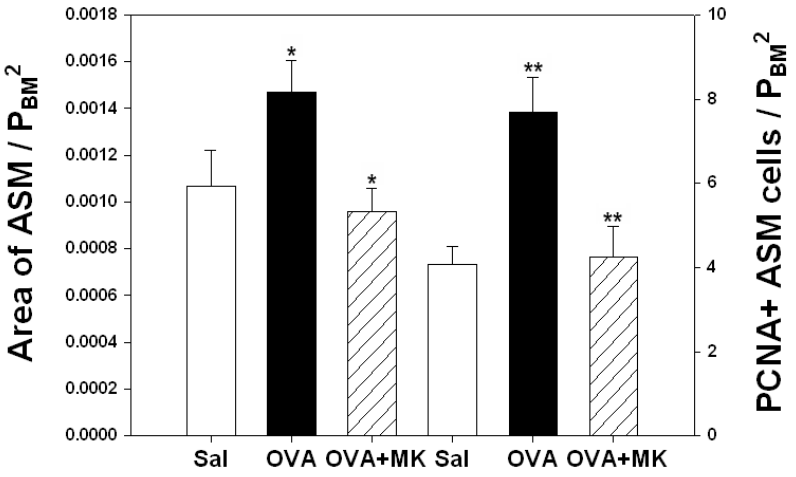

Area of ASM / P_{BM}^2
PCNA+ ASM cells / P_{BM}^2
0.0018
0.0016
0.0014
0.0012
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
10
8
6
4
2
0
*
*
**
**
Sal
OVA
OVA+MK
Sal
OVA
OVA+MK

Figure 6

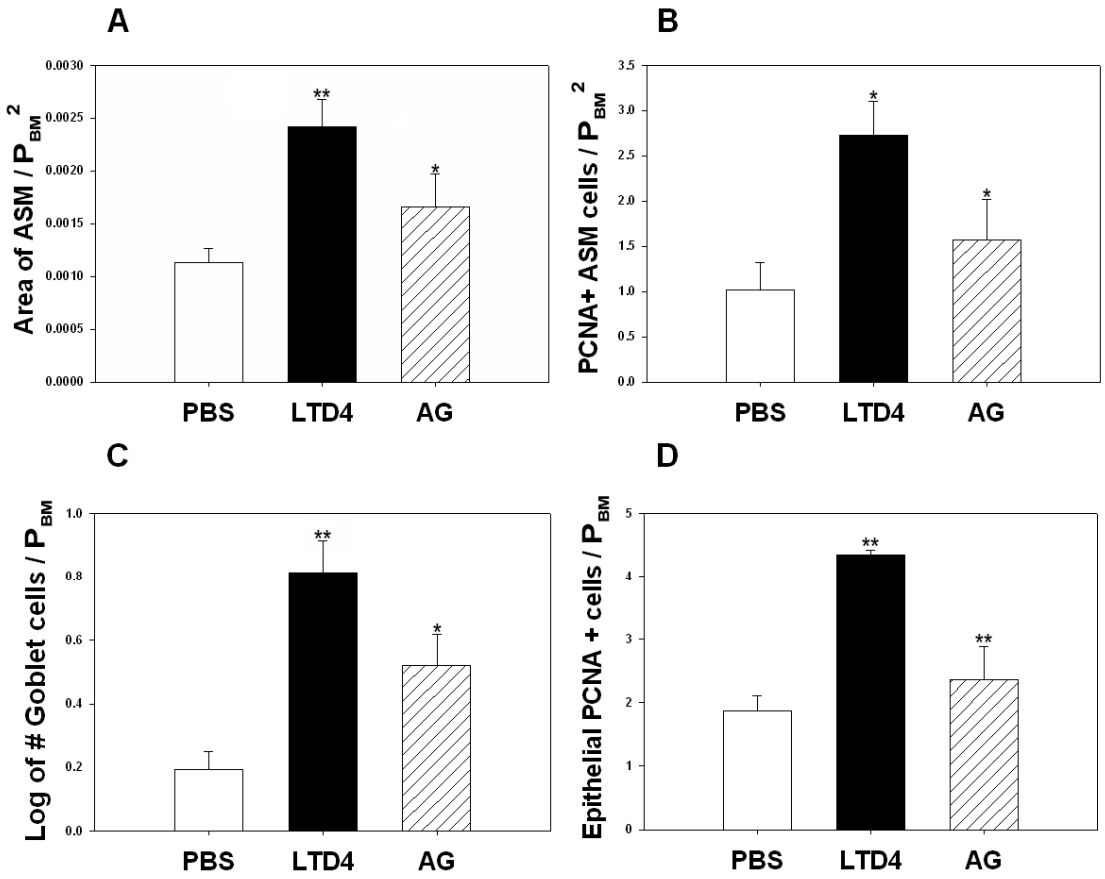

A
Area of ASM / P BM 2
0.0030
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
**
*
PBS
LTD4
AG
B
PCNA+ ASM cells / P BM 2
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
PBS
LTD4
AG
C
Log of # Goblet cells / P BM
1.0
0.8
0.6
0.4
0.2
0.0
**
*
PBS
LTD4
AG
D
Epithelial PCNA + cells / P BM
5
4
3
2
1
0
**
**
PBS
LTD4
AG

Figure 7

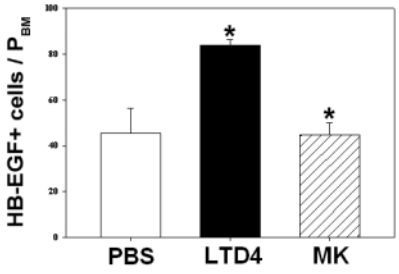
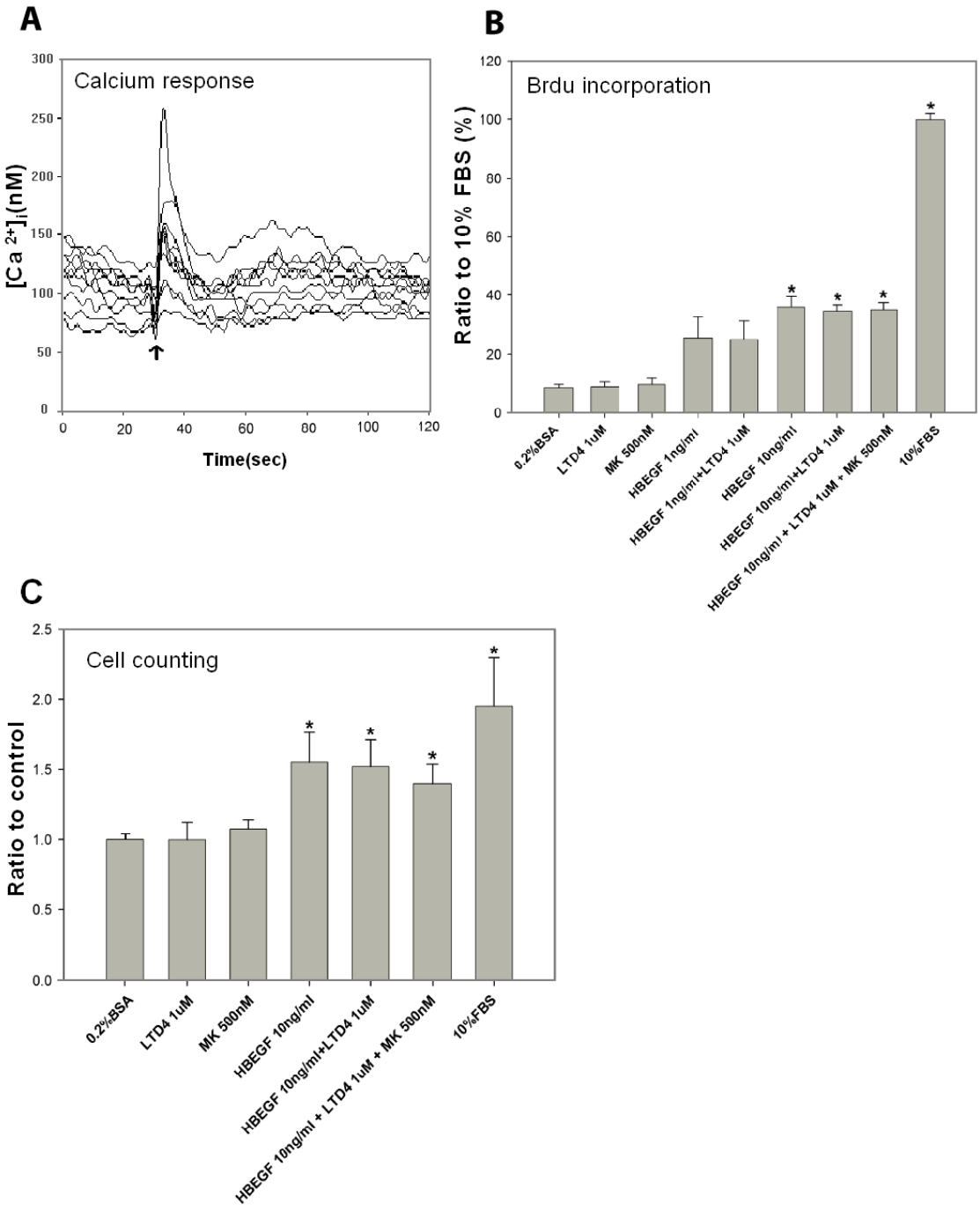


Figure 8


A
Calcium response
[Ca 2+]i(nM)
Time(sec)
B
Brdu incorporation
Ratio to 10% FBS (%)
0.2%BSA
LTD4 1uM
MK 500nM
HBEGF 1ng/ml
HBEGF 1ng/ml+LTD4 1uM
HBEGF 10ng/ml
HBEGF 10ng/ml+LTD4 1uM
HBEGF 10ng/ml + LTD4 1uM + MK 500nM
10%FBS
C
Cell counting
Ratio to control
0.2%BSA
LTD4 1uM
MK 500nM
HBEGF 10ng/ml
HBEGF 10ng/ml+LTD4 1uM
HBEGF 10ng/ml + LTD4 1uM + MK 500nM
10%FBS