Persistent Inactivation of Macrophage Cyclooxygenase-2 in Mycobacterial Pulmonary Inflammation

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The induction of cyclooxygenase-2 (COX-2) in tissue macrophages (MØ) increases prostaglandin E₂ (PGE₂) release, potentially down-regulating granulomatous inflammation. In response to *Mycobacterium*, local MØ express COX-2, which is either nuclear envelope (NE)-associated or NE-dissociated. Persistent mycobacterial pulmonary inflammation is characterized by alveolar MØ expressing NE-dissociated (inactive) COX-2 without release of PGE₂. In this study, we examined COX-2 in alveolar MØ after intranasal exposure to heat-killed *Mycobacterium bovis* BCG (HK-BCG). After administration, whole lungs of C57Bl/6 mice were lavaged with saline; COX-2 expression and PGE₂ release by alveolar MØ and tumor necrosis factor (TNF)- α and nitric oxide levels in the lung lavage were monitored. Normal alveolar MØ had undetectable levels of COX-2 on Western blots. However, 1 day after intranasal administration, almost all alveolar MØ had phagocytosed HK-BCG and expressed NE-dissociated COX-2 without any increase in the release of PGE₂. At 28 days after intranasal administration, 68% of alveolar MØ still contained both BCG and the NE-dissociated form of COX-2. NE-associated (active) COX-2 was not observed in alveolar MØ. In contrast, 7 days after intraperitoneal injection of HK-BCG, peritoneal MØ containing HK-BCG were no longer detected. At 28 days after intranasal administration, TNF- α and nitrite levels in the lung lavage fluid were significantly higher than those in controls. Our results indicate that mycobacterial pulmonary inflammation is associated with suppressed PGE₂ production by alveolar MØ, with expression of COX-2 dissociated from the NE.

Keywords: alveolar macrophages; PGE₂; COX-2; mycobacteria

Alveolar macrophages (MØ) secrete numerous chemical mediators in response to *Mycobacterium tuberculosis* and *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), a tuberculosis vaccine and adjuvant, thereby regulating persistent inflammatory responses in the lungs and causing granuloma formation. Granulomatous inflammation represents the pathological correlate of both protective immunity and inflammatory tissue destruction and repair (1, 2). Immunoregulatory effects of mycobacteria are demonstrated by increased resistance to infections and tumor growth, attenuation of the allergic response, and induction of autoimmune diseases in animal models (3–6). The pleiotropic effects of mycobacteria could be mediated by induction in MØ of not only Th1-mediated microbicidal MØ responses but also pathogenic Th2, Th17, and Treg responses (7–9). The exact

CLINICAL RELEVANCE

This study investigates the pathophysiology associated with mycobacterial pulmonary inflammation. A novel aspect of alveolar macrophage activation by mycobacteria is the persistent presence of inactive cyclooxygenase isoforms with suppression of prostaglandin E₂ production.

mechanisms regulating persistent mycobacterial inflammation in alveolar MØ still remain to be elucidated.

Previous studies with MØ demonstrated that mycobacteria induce expression of cyclooxygenase-2 (COX-2), an inducible enzyme, which increases prostaglandin E₂ (PGE₂) biosynthesis *in vitro* (10, 11). PGE₂ suppresses granuloma formation (12) by down-regulating bactericidal MØ activities, including iNOS-mediated synthesis of nitric oxide (NO), NADPH oxidase-mediated superoxide anion release, and synthesis of interleukin (IL)-12/IL-18/tumor necrosis factor (TNF)- α (13–15). In contrast, PGE₂ promotes production of IL-10, IL-23, and matrix metalloproteinase 9 by MØ (7, 8, 16, 17). Therefore, regulation of these responses at various stages of pulmonary inflammation may depend on the presence of COX-2⁺ MØ at appropriate locations.

Catalytically active COX-2 (and COX-1) are localized subcellularly in the nuclear envelope (NE) and endoplasmic reticulum (ER), where they mediate PGE₂ biosynthesis (18, 19). COX-2 dissociated from the NE is catalytically inactive (20–22). These forms of COX-2 have microscopically distinct cellular appearances and their associated activity in producing PGE₂ has been established in several studies including this one. Therefore, we have used the designations active or inactive interchangeably with NE associated or NE dissociated, respectively. Previously, we found that normal splenic and peritoneal MØ treated *in vitro* with heat-killed BCG (HK-BCG) express active COX-2 and release increased amounts of PGE₂ within 24 hours (20, 21). In sharp contrast, within 24 hours after intraperitoneal administration, HK-BCG induces inactive COX-2 transiently in splenic and peritoneal MØ, followed by active COX-2 in splenic MØ at 7 to 14 days after administration (20, 21). Splenic MØ with active COX-2 are derived from bone marrow (23). The formation of these COX-2⁺ MØ subsets seems to be dependent on the microorganism and its phagocytosis, the tissue, and the presence of bone marrow-derived precursors which become mature MØ expressing active COX-2 at inflammatory sites (20, 21, 23, 24). Thus, COX-2⁺ MØ could potentially either inhibit or promote PGE₂-sensitive antimicrobial immunity (7, 20, 21).

We have hypothesized that pulmonary inflammation in response to *Mycobacteria* is directly related to the activity of COX-2. To elucidate the role of MØ COX-2 in mycobacterial pulmonary inflammation, we administered HK-BCG intranasally and determined whether this resulted in expression of catalytically active or inactive COX-2, and the persistence of the effects on alveolar MØ for up to 28 days. The pulmonary inflammation induced by mycobacteria was also compared with that induced by

(Received in original form July 19, 2008 and in final form November 20, 2008)

This work was supported by NIH RO1 HL71711, DOD DAMD 17-03-1-0004, Bankhead-Coley Cancer Research Program 06BS-04-9615, the Charles E. Schmidt Biomedical Foundation (Y.S.), and Florida Atlantic University.

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Am J Respir Cell Mol Biol Vol 41, pp 146–154, 2009

Originally Published in Press as DOI: 10.1165/rcmb.2008-0230OC on December 18, 2008

Internet address: www.atsjournals.org

other selected microbes and fractionated mycobacterial components.

MATERIALS AND METHODS

Microbes

Cultured *M. bovis* BCG Tokyo 172 strain or *Propionibacterium acnes* (24) were washed, autoclaved, and lyophilized. This HK-BCG or HK-*P. acnes* powder was suspended in pyrogen-free saline at 10 mg/ml and dispersed by brief (10 s) sonication immediately before use. These preparations contained undetectable levels of endotoxin (< 0.03 EU/ml), as determined by the *Limulus* amebocyte lysate assay (Sigma Aldrich, St. Louis, MO) (25). *Listeria monocytogenes* 10403S serotype 1/2a and *Escherichia coli* MG1655 (obtained from Dr. Chris Burns, Florida Atlantic University) were grown in brain-heart infusion broth (Difco Laboratories, Detroit, MI) at 37°C. The bacteria were harvested in the logarithmic phase of growth, treated at 60°C for 60 minutes, washed three times with cold saline, re-suspended in saline at 10 mg/ml (5×10^8 bacilli/mg), and stored in aliquots at -80°C. HK-*M. tuberculosis* and HK-*Mycobacterium butyricum* were purchased from Difco Laboratories. Lysate of HK-BCG was prepared by sonication (20 s, 15 times) followed by filtration with a 0.22- μ m Millipore filter. Alternatively, HK-BCG was treated for 6 hours at 37°C with either 1 M hydrochloric acid or 1 M sodium hydroxide (26, 27). After this treatment, HK-BCG was washed six times with phosphate-buffered saline, pH 7.0 (PBS), and suspended in saline at 10 mg/ml. Delipidated HK-BCG was prepared by extraction with chloroform/methanol (3:1, vol/vol) 1 hour at 37°C followed by centrifugation to remove the supernatant. This process was repeated once. The pellet was washed with 70% ethanol six times followed by saline three times and resuspended in saline (26, 27).

Animals

Nonpregnant female C57Bl/6 mice, 8 to 10 weeks old, were obtained from Harlan Laboratory (Indianapolis, IN). Mice were maintained in barrier-filtered cages and fed Purina laboratory chow and tap water *ad libitum*. Experimental protocols employed in this study were approved by the IACUC at Florida Atlantic University.

Administration of HK Microbes

Groups of mice (3–5/group) received 0.5 mg of HK-BCG, HK-*P. acnes*, HK-*L. monocytogenes*, or HK-*E. coli* in 0.05 ml saline intranasally or 1.0 mg in 0.1 ml saline intraperitoneally on Day 0. Controls received 0.05 ml (intranasally) or 0.1 ml (intraperitoneally) saline. Mice were killed on Days 1, 7, 14, and 28.

Macrophage Preparations

To obtain alveolar MØ from mice, the trachea was cannulated and the whole lung lavaged with 1 ml saline. For peritoneal MØ, peritoneal lavage was performed with 5 ml of saline. MØ were prepared as previously described (24). Total cell counts were determined with a Coulter counter (Model Z1; Beckman Coulter, Brea, FL). Cell classification was performed on cytocentrifuged preparations stained with Diff-Quik (Baxter Healthcare, Miami, FL). To enrich plastic-adherent MØ for *in vitro* experiments, peritoneal or alveolar cells at 10^6 cells/ml suspended in RPMI 1640 plus 5% FBS were incubated in culture dishes (Falcon, Oxnard, CA) for 2 hours. Nonadherent cells were removed by washing with media. In some experiments, adherent cells were cultured with 20 μ g/ml HK-BCG, HK-*P. acnes*, HK-*L. monocytogenes*, or HK-*E. coli* for an additional 24 hours.

Western Blotting

Washed alveolar or peritoneal MØ were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 4 mM EDTA, 0.1% SDS, 1:500 protease inhibitor cocktail [P8340; Sigma Aldrich], 1% NP-40, and 1% sodium deoxycholate). After 10 minutes on ice, debris was eliminated by centrifugation (10 min, $10,000 \times g$). Protein concentration in the lysate was measured with a bicinchoninic acid assay (Pierce, Thermo Fisher Scientific, Rockford, IL) and bovine serum albumin as standard. For each sample, 1.5 μ g of lysate protein, unless stated otherwise, was separated by SDS-PAGE, and then transferred to PVDF membrane (Millipore,

Bedford, MA). The membrane was blocked with 10% nonfat dry milk, and incubated with antibodies (anti-COX-2, 1:2,000; anti-COX-1, 1:2,000 [Cayman Chemical]; anti- β -actin, 1:8,000 [Sigma Aldrich] for the detection of β -actin as constitutively expressed protein control) in 5% nonfat dry milk, overnight at 4°C. After incubation with peroxidase-conjugated secondary antibody (1:20,000; Jackson ImmunoResearch, West Grove, PA), proteins were detected by chemiluminescence (ECL plus; Amersham, Piscataway, NJ) following the manufacturer's instructions.

Subcellular Localization of COX-2 by Confocal Microscopy Analysis

Alveolar and peritoneal MØ prepared as described above were fixed in 4% paraformaldehyde in PBS, pH 7.5, for 30 minutes. The fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes and incubated in blocking buffer consisting of PBS with 10% FBS overnight at 4°C before incubation with anti-COX-2 or anti-COX-1 antibody (Cayman Chemical), 1:500 in blocking buffer, overnight at 4°C. Subsequently, cells were washed with PBS three times and incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch) for 1 hour at 22°C. For detection of nucleus and HK microbes, propidium iodide (PI; Sigma Aldrich) was mixed at 10 μ g/ml with the secondary antibody solution. After washing three times, cells were examined with a laser scanning confocal microscope (Bio-Rad Radiance 2100; Bio-Rad Laboratories, Hercules, CA). Images were processed with Adobe Photoshop software. We have demonstrated previously (20) that HK-BCG is co-stained by PI and anti-BCG anti-sera.

Evaluation of Apoptosis

The apoptosis of MØ was examined microscopically. After removal of the culture media from culture dishes, a fluorescent dye solution containing acridine orange (Sigma Aldrich) and PI at 10 and 20 μ g/ml, respectively, in culture media was added to the dishes (28). A coverslip was placed over the cells, and they were immediately examined by fluorescence microscopy.

ELISA

Levels of TNF- α and IL-10 in supernatants from lung lavage were measured by two-site ELISA specific for the respective cytokines, according to the manufacturer's instructions (BD Pharmingen, San Diego, CA).

Measurement of Nitrite Concentration

To evaluate the concentration of NO present in supernatants of lung lavage, the stable conversion product of NO, nitrite (NO_2^-), was measured by the Griess assay (29). Supernatants were added to an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% H_3PO_4) in a 96-well plate and incubated for 10 minutes at 22°C. Nitrite levels were determined colorimetrically at 540 nm from a standard curve prepared with known concentrations of sodium nitrite.

PGE₂ Assay

Plastic adherent MØ (10^6 cells/ml) were cultured in RPMI 1640 for 2 hours. PGE₂ levels in the culture supernatants were measured by competitive ELISA (Cayman Chemical, Ann Arbor, MI).

Immunodetection of PGE₂ at its Sites of Production

MØ were treated with 1% water-soluble 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDAC; Sigma Aldrich) to cross-link eicosanoid carboxyl groups to amines in adjacent proteins (30). After 30 minutes of incubation with EDAC at 37°C to promote both cell fixation and permeabilization, cells were stained with mouse anti-PGE₂ at 1:50 (Cayman Chemical). Presence of the primary antibody was determined with FITC-conjugated donkey anti-mouse IgG at 1:500 (Jackson ImmunoResearch). Cellular localization was determined by confocal microscopy.

Statistical Analysis

Lung lavage fluid isolated from at least three mice was pooled. Cytokines and nitrite in supernatants were measured in at least

triplicate in each group. Differences between mean values were analyzed by Student's *t* test. *P* < 0.05 is considered statistically significant.

RESULTS

Catalytically Inactive Forms of COX in Alveolar MØ

Both peritoneal and splenic MØ isolated 24 hours after intraperitoneal injection of 1 mg HK-BCG contain phagocytosed BCG and express increased levels of COX-2, which is catalytically inactive (20, 21). However, we noted in our earlier investigation that alveolar MØ isolated from these mice contained no BCG and expressed no COX-2. In the present studies, we have determined the effect of intranasal administration of HK-BCG on expression of COX-2 by alveolar MØ isolated 24 hours after administration of HK-BCG. A dose of 0.5 mg HK-BCG was selected to achieve an inflammatory response comparable to that associated with mycobacterial infections and models of autoimmune disease employing Freund's complete adjuvant (31, 32). In addition, pulmonary granulomatous inflammation with a polarized Th1 cytokine profile has been induced in mice treated with 0.5 mg HK-*P. acnes* (33).

One day after intranasal administration of HK-BCG, alveolar MØ had increased COX-2 protein (Figure 1A). Confocal microscopy revealed that MØ containing intracellular HK-BCG were

positive for COX-2. Catalytically inactive COX-2 was indicated by its localization in densely stained cytoplasmic structures dissociated from the NE. No NE associated COX-2 was detected (Figure 1B).

The response to HK-BCG was compared with that induced by intranasal administration of other microbes. As shown in Figures 1A and 1B, *in vivo* exposure to HK-*P. acnes* resulted in expression of NE-dissociated COX-2 at a lower level than that induced by HK-BCG. Active (NE-associated) COX-2 was not detected after exposure to HK-*P. acnes*. In contrast, HK-*E. coli* induced NE-associated COX-2 whereas HK-*L. monocytogenes* induced little or no COX-2 (Figures 1A and 1B).

The expression level of COX-1 by normal alveolar MØ was significantly lower than for peritoneal MØ. By Western blotting of 1.5 µg MØ protein, COX-1 was not detected (Figures 1A and 2A). When protein loading was increased to 12.5 or 25 µg, smaller amounts of COX-1 were detected in alveolar MØ compared with those from normal peritoneal MØ (Figure 2A). COX-1 was not detected in alveolar MØ examined by immunofluorescent confocal microscopy, but was seen in peritoneal MØ (Figures 1 and 2). Unexpectedly, NE-dissociated COX-1 was induced in alveolar MØ by intranasal administration of HK-BCG or HK-*P. acnes* (Figures 1A and 1B).

These studies demonstrate that *in vivo* phagocytosis of HK-BCG by alveolar MØ induces the NE-dissociated forms of both

Figure 1. Cyclooxygenase (COX)-1 and COX-2 in alveolar macrophages (MØ) after *in vivo* treatment with heat-killed *Mycobacterium bovis* BCG (HK-BCG) and other microbes. Alveolar MØ were isolated from groups of mice 24 hours after intranasal administration of 0.5 mg HK-BCG, -*P. acnes*, -*E. coli*, or -*L. monocytogenes*. (A) COX-1, COX-2, and β-actin levels were determined by Western blot as indicated in MATERIALS AND METHODS. β-actin bands show equivalent loading of samples. Results are representative of three separate experiments. (B) Subcellular localization of COX-1 and COX-2. Cells were examined by confocal microscopy after immunofluorescent staining with anti-COX-1 or anti-COX-2 antibody (green) and PI (red) for the nucleus and HK microbes. Results are representative of three separate experiments. Bar = 10 µm.

Figure 2. COX-1 and COX-2 in alveolar MØ treated *in vitro* with HK-BCG and other microbes. (A) Constitutive COX-1 detection. COX-1 and β -actin levels in normal alveolar MØ (AM) were determined by Western blot at 1.5, 12.5, and 25 μ g protein per lane. Normal peritoneal MØ (PM) were used as comparison controls. (B) Induced COX-1 and COX-2 detection. Normal alveolar MØ (AM) were treated with 20 μ g/ml HK-BCG, *-P. acnes*, *-E. coli*, or *-L. monocytogenes* for 24 hours. COX-1, COX-2, and β -actin levels were determined by Western blot as indicated in MATERIALS AND METHODS. β -actin bands show equivalent loading of samples (1.5 μ g protein/lane). Results are representative of three separate experiments. (C) Subcellular localization of COX-1 and COX-2. Cells were examined by confocal microscopy after immunofluorescent staining with anti-COX-1 or anti-COX-2 antibody (green) and PI (red) for the nucleus. HK microbes were detected by PI staining. Results are representative of three separate experiments. Bar = 10 μ m.

COX-2 and COX-1. The formation of NE-dissociated COX is dependent on specific microbe properties, since *E. coli* and *L. monocytogenes* did not induce the inactive COX isoforms.

COX Expression In Vitro

We further examined the effects of *in vitro* exposure to HK-*P. acnes*, *E. coli*, and *L. monocytogenes* compared with the effects of HK-BCG on normal alveolar MØ. All of these microbes induced similar amounts of COX-2 protein in alveolar MØ (Figure 2A), which by confocal microscopy was NE-associated (Figure 2B). Treatment with lower (1 μ g/ml) or higher (100 μ g/ml) doses of HK-BCG further confirmed the induction of

NE-associated COX-2 (data not shown). COX-2 levels induced by 20 μ g/ml of HK-BCG were comparable to those induced by 100 μ g/ml of HK-BCG (data not shown). In contrast to COX-2, however, COX-1 was not induced *in vitro* by HK-BCG or HK-*P. acnes* (Figures 2B and 2C).

PGE₂ Release by Alveolar MØ with NE-Dissociated COX-1 and COX-2

Assays of cellular supernatants demonstrated that normal alveolar MØ produced less PGE₂ than normal peritoneal MØ (Figure 3). This constitutive production of PGE₂ is probably dependent on the constitutive presence of COX-1 (21). When

Figure 3. PGE₂ release by alveolar MØ. For *in vitro* HK-BCG treatment, normal alveolar (AM) and peritoneal MØ (PM) as comparison controls were incubated with 20 µg/ml HK-BCG for 24 hours. For *in vivo* HK-BCG treatment, mice received 0.5 mg intranasal HK-BCG and alveolar MØ were harvested 24 hours after administration. (A) MØ suspension (10⁶/ml) was cultured in RPMI 1640 for 2 hours after which PGE₂ was assayed by ELISA. Values are mean ± SD, *n* = 3. **P* < 0.05 compared with control alveolar MØ (*in vitro*). ***P* < 0.0005 compared with control alveolar MØ (*in vitro*). (B) Cells were treated with EDAC to cross-link carboxyl groups of PGE₂ to amines in adjacent proteins. After immunofluorescent staining with anti-PGE₂ antibody, cells were examined by confocal microscopy. Results are representative of 10 separate experiments. Bar = 10 µm.

alveolar MØ were treated *in vitro* with HK-BCG, PGE₂ production increased (Figure 3), consistent with the localization of COX-2 at the NE as seen in Figure 2C. In contrast, for alveolar MØ treated with HK-BCG *in vivo*, intracellular PGE₂ and cellular PGE₂ production were unchanged from the saline control (Figure 3).

NE-Dissociated COX-1 and COX-2 Formation Persists for 28 Days *In Vivo*

Previously we found that splenic MØ expressed the inactive form of COX-2 1 day after intraperitoneal administration of HK-BCG, and then switched to active COX-2 after an additional 6 to 13 days (20). We also noted that intracellular BCG is preferentially associated with inactive COX-2; conversely active COX-2 was less likely to be associated with intracellular BCG. Therefore, after intranasal administration of HK-BCG we

monitored the COX-2 localization and presence of intracellular BCG in alveolar MØ for up to 28 days.

PI-stained intracellular BCG were detected in 79%, 72%, and 68% of alveolar MØ at Days 7, 14, and 28, respectively, after intranasal HK-BCG administration (Figure 4). The MØ with intracellular BCG exclusively expressed the inactive forms of COX-1 and COX-2. In sharp contrast, intracellular HK-BCG were observed in only 5%, 1%, and less than 0.5% of peritoneal MØ isolated 7, 14, or 28 days, respectively, after mice were given intraperitoneal HK-BCG (Figure 4). Only the inactive form of COX-2 was observed in the presence of intracellular HK-BCG in these peritoneal MØ (Figure 4). Unlike splenic MØ at 7 to 14 days, in the experiments described here neither alveolar nor peritoneal MØ exhibited the active form of COX-2 even after 28 days (data not shown). Moreover, MØ from these two tissues did not undergo apoptosis after phagocytosis of HK-BCG (data not shown).

Figure 4. COX-1 and COX-2 expression in alveolar MØ after intranasal HK-BCG administration. Alveolar MØ (AM) were harvested at indicated periods from mice treated intranasally with 0.5 mg HK-BCG. As comparison controls, peritoneal MØ (PM) were obtained from mice given 1 mg HK-BCG intraperitoneally. To evaluate the presence of COX isoforms and intracellular HK-BCG, MØ were examined by confocal microscopy as indicated in Figures 1 and 2. Results are representative of two separate experiments. Bar = 20 µm.

HK-BCG Promotes Production of TNF- α and Nitric Oxide in the Lungs

To confirm the immunomodulatory activity of HK-BCG and HK-*P. acnes* *in vivo*, we measured TNF- α in lung lavage. TNF- α is a critical contributor to the granulomatous inflammation of tuberculosis and sarcoidosis (34, 35). As shown in Figure 5A, TNF- α production increased on Day 1 after intranasal HK-BCG, and an even larger increase was seen after treatment with HK-*P. acnes*. NO, which is released by activated M ϕ and measured as nitrite (36), was also increased after the treatments (Figure 5B). In contrast, IL-10 was produced constitutively and IL-10 levels in the lung lavage were not increased by treatment with either microbe (Figure 5C). In alveolar M ϕ , cell surface receptors, including the TLRs, play differential roles in activation by these two bacteria (37, 38), which may explain the differential responses in production of COX-2 and inflammatory mediators.

As seen in Figure 5D, TNF- α and nitrite levels in the lungs were significantly higher than those in controls even 28 days after HK-BCG administration. PGE₂ levels (Day 0, 120 $\pm$ 42; Day 1, 138 $\pm$ 46; Day 7, 141 $\pm$ 54; Day 14, 54 $\pm$ 43; Day 28, 43 $\pm$ 33 pg/ml [mean $\pm$ SE, *n* = 4]) did not increase during these 28 days after intranasal HK-BCG). In contrast, TNF- α and NO were not detected in the lung lavage 7 days after HK-*E. coli* or *L. monocytogenes* administration, although comparable levels of the mediators were detected on Day 1 (data not shown).

Other HK-Mycobacteria and HK-BCG Components

To further explore the production of COX-2 in alveolar M ϕ , we determined whether other HK-mycobacteria and fractionated components of HK-BCG induced NE-dissociated COX-2 after intranasal administration. As summarized in Table 1, NE-dissociated COX-2 was induced not only by HK-*M. tuberculosis* and *M. butyricum*, but also by a sonicated lysate of HK-BCG, as well as components of HK-BCG obtained by treatment with chloroform/methanol or 1 M hydrochloric acid. However, HK-BCG pre-treated with 1 M sodium hydroxide did not induce either NE-associated or -dissociated COX-2. These data suggest that mycobacterial proteins and/or acid-resistant carbohydrates

are needed for the *in vivo* induction of NE-dissociated COX-2. In this regard purified protein derivative (PPD), a HK-mixture of secretory mycobacterial proteins, which induced M ϕ COX-2 *in vitro*, did not induce COX-2 *in vivo*.

DISCUSSION

Our previous studies clearly demonstrated that the route of administration of mycobacteria determines whether activated M ϕ express catalytically active or inactive COX-2. The type of COX-2 expressed depended on the tissue type, phagocytosis of the mycobacteria *in vivo*, and the nature of M ϕ precursors (20, 21, 23). The data presented here indicate that alveolar M ϕ activated by *in vivo* phagocytosis of mycobacteria express COX-2 that is NE-dissociated and catalytically inactive, failing to increase release of PGE₂. Moreover, this type of M ϕ activation remains dominant for at least 28 days after intranasal administration of HK-BCG. Even at this late time point, we were unable to detect either M ϕ expressing the NE-associated form of COX-2, or increased PGE₂ release. Our results clearly demonstrate that a single administration of mycobacteria induces a persistent state of M ϕ activation, pulmonary inflammation and anti-microbial activities (e.g., suppressed PGE₂ release, with elevation of TNF- α and NO) that continue for 28 days, implying granulomatous inflammation. Based on this and our previous studies (7, 20, 21, 23), Figure 6 summarizes HK-BCG-induced COX-2 expression among alveolar, splenic, and peritoneal M ϕ .

The respiratory system is exposed to inhaled particulates including various microbes that must be cleared constantly from the airways. Alveolar M ϕ contribute to this clearance largely by phagocytosis of microbes and particulate matter. Our studies of these M ϕ indicate that the induction of a catalytically inactive form of COX-2 by potential pathogens administered *in vivo* is microbe dependent. However, organisms that produce Th1-mediated granulomatous inflammation, *M. tuberculosis*, *M. butyricum*, and *P. acnes*, as well as *M. bovis* BCG (2, 33, 39), all induce NE-dissociated COX-2 in alveolar M ϕ . In sharp contrast, *in vivo* administration of HK-microbes of *E. coli* or *L.*

Figure 5. TNF- α , IL-10, and NO levels in whole lung lavage. TNF- α /IL-10 and nitrite were measured by ELISA and Griess assay, respectively, in the supernatant obtained from a 1-ml whole lung lavage from mice given 0.5 mg HK-BCG or HK-*P. acnes* intranasally. (A) TNF- α , (B) nitrite, and (C) IL-10 in lavage from mice 1 day after administration. Values are mean $\pm$ SD; *n* = 3. **P* < 0.0005 compared with saline control group. ***P* < 0.0005 compared with BCG group. (D) Time-course of TNF- α and nitrite in lavage for mice treated with HK-BCG. Values are mean; *n* = 4. **P* < 0.0005 compared with saline control group. ***P* < 0.005 compared with saline control group. Each result represents mice from two experiments.

Figure 6. HK-BCG induces tissue-dependent COX-2⁺ MØ formation. Results obtained from this and previous studies (7, 20, 21, 23) are summarized. HK-BCG at 0.5 mg or 1 mg doses was given C57Bl/6 mice intranasally or intraperitoneally, respectively. Alveolar MØ (AM, this study), peritoneal MØ (PM, this study), and splenic MØ (SM, 7, 20, 21, 23) were isolated and the % MØ with NE-associated (active) (—) and NE-dissociated (inactive) forms of COX-2 (---) were determined by immunohistology as indicated in MATERIALS AND METHODS.

monocytogenes induces NE-associated COX-2 or no COX-2, respectively.

Phagocytosis and internalization of HK-BCG by alveolar MØ *in vivo* is associated with formation of NE-dissociated COX-2, which is detected even 28 days after HK-BCG administration. Mycobacteria and *P. acnes* have complex cell wall structures resistant to host anti-microbial activity and lysosomal digestion, compared with those of *E. coli* or *L. monocytogenes*. The altered response of alveolar MØ may also relate to the fact that their clearance pathway to the draining lymph node is different from that of peritoneal MØ (40, 41); peritoneal MØ phagocytosing HK-BCG are cleared within 7 days. We determined that the clearance of these peritoneal MØ is not associated with apoptosis (data not shown).

TABLE 1. SUMMARY OF THE EFFECTS OF HK-MICROBES AND FRACTIONATED HK-BCG COMPONENTS ON THE EXPRESSION OF COX-2

	<i>In Vitro</i> Induced COX-2	<i>In Vivo</i> Induced COX-2
HK- <i>M. tuberculosis</i>	NE-associated	NE-dissociated
HK- <i>M. butyricum</i>	NE-associated	NE-dissociated
HK- <i>M. bovis</i> BCG	NE-associated	NE-dissociated
HK-BCG pre-treated with:		
Chloroform/methanol (3:1)	NE-associated	NE-dissociated
1 M hydrochloric acid	NE-associated	NE-dissociated
1 M sodium hydroxide	Not observed	Not observed
PPD	NE-associated	Not observed
Sonicated, filtered HK-BCG lysate	NE-associated	NE-dissociated

Definition of abbreviations: BCG, bacillus Calmette-Guérin; COX, cyclooxygenase; HK, heat-killed; NE, neutrophil elastase; PPD, purified protein derivative.

Our previous studies demonstrated that NE-dissociated COX-2 is isolated in the microsomal fraction but is not co-localized with phagosomal HK-BCG or lysosome-associated membrane protein 1 (21), and that an endoplasmic reticulum protein, calreticulin, was not co-localized with NE-dissociated COX-2 (unpublished observation). It is of particular significance that the BCG lysate still induces NE-dissociated COX-2 (Table 1), demonstrating that the soluble molecules are sufficient to induce this altered MØ response, and particulate forms of mycobacteria are not required. Our results (Table 1) suggest that mycobacterial proteins and/or carbohydrates play a key role in the induction of inactive COX-2. In this regard, despite induction of COX-2 *in vitro*, PPD did not induce COX-2 *in vivo* (Table 1). Further studies are needed to identify the mycobacterial molecules which activate alveolar MØ with expression of inactive forms of COX-2.

This study further confirms that *in vivo* factors associated with HK-BCG administration are obligatory for establishing the catalytically inactive form of COX-2 and consequently the regulation of prostaglandin production. Our previous studies with peritoneal MØ in culture (21), however, do not support the hypothesis that extracellular factors produced in response to intraperitoneal HK-BCG administration in the peritoneal cavity or present in sera regulate the subcellular localization of COX-2. Thus, it appears that other endogenous factors present *in vivo* but not *in vitro* are important for the induction of catalytically inactive COX-2. From our results, these factors are organism dependent and appear to be effective for at least 28 days after intranasal administration of HK-BCG. We are not aware of previous reports indicating that mycobacteria localize in the peritoneal region of infected MØ.

COX-1 is the constitutive isoform of COX, but may be inducible in rare cases. For example, developmental induction of COX-1 in ovine pulmonary tissue, especially pulmonary artery, has been reported (42). Our results indicate that normal alveolar MØ exhibit a relatively small amount of COX-1 compared with peritoneal MØ, and that COX-1 is induced by mycobacteria *in vivo* but not *in vitro*. Similar differential COX-1 expression between the two MØ was shown in normal rats (43). Alveolar MØ in alveolar space are exposed to high oxygen tension and have direct contact with alveolar epithelial cells that constitutively produce surfactant and soluble mediators including IL-10 and PGE₂. Although no significant increases in these mediators were noted in the lavage fluid assays in this study, it is possible that increases in these mediators might have occurred, but binding to cells or tissues may have prevented their detection in lavage supernatants. This unique environment may down-regulate constitutive COX-1 expression. It is unknown whether the mechanism for induction of COX-1 after *in vivo* phagocytosis of mycobacteria is similar to that for induction of NE-dissociated COX-2 under the same conditions.

Recent studies with mice deficient in COX-1 and/or COX-2 indicate that COX-1 deficiency is detrimental, whereas COX-2 deficiency is beneficial to the host during pulmonary infection with influenza virus (44) and in airway allergic responses (45). Experiments using specific COX inhibitors and genetic knock-down approaches in selenium-supplemented MØ indicate that COX-1, but not COX-2, is responsible for the increased synthesis of 15-deoxy- $\Delta^{12,14}$ -PGJ₂, an endogenous anti-inflammatory eicosanoid (46). This eicosanoid (prostaglandin) stimulates MØ through peroxisome proliferator-activated receptor (PPAR γ) activation, and down-regulates MØ anti-microbial activities including IL-12 production, oxidative burst, and NO release (47). Unlike peritoneal MØ, however, alveolar MØ isolated from normal mice express PPAR γ constitutively (47). Furthermore, PPAR γ is also detected in alveolar MØ from

healthy humans, but is deficient in alveolar MØ from patients with pulmonary sarcoidosis, a granulomatous disease with Th1 inflammation (48). Therefore, it remains to be studied whether PPAR γ activity in alveolar MØ is reduced in response to mycobacteria or *P. acnes* in a COX-dependent manner.

PGE₂, constitutively produced mainly by airway epithelial cells and fibroblasts, influences physiologic functions including increases in surfactant secretion and wound closure (49, 50). Our preliminary immunohistochemical analysis of lung sections indicated that expression of constitutive NE-associated COX-1 and the absence of COX-2 in type II pneumocytes were unchanged by the intranasal administration of HK-BCG (data not shown). This is consistent with our observation that PGE₂ release into lung lavage is not increased by exposure to mycobacteria.

In conclusion, we have observed a relatively slower clearance of mycobacterial components by airway alveolar MØ compared with that for peritoneal MØ. After the phagocytosis of mycobacteria, alveolar MØ produce an inactive form of COX-2. This results in “hyperactivated” and persistent inflammation with production of the inflammatory mediators, TNF- α and NO, apparently without a contribution from PGE₂ production. Taken together, our series of studies (7, 20, 21, 23) has demonstrated that the kinetics and mechanisms of alveolar MØ activation by mycobacteria are distinct from those of peritoneal and splenic MØ, suggesting tissue-dependent development of chronic inflammation. Additional work is needed to elucidate whether the mechanism of NE-dissociated COX-2⁺ alveolar MØ formation is dependent on stages of MØ maturation at persistent mycobacterial pulmonary inflammation. Finally, heat-resistant, filterable molecules, which may be common among mycobacteria and *P. acnes*, are essential for the expression of the NE-dissociated (inactive) COX-2 and for the up-regulation of host immune responses. Whether this is a direct consequence of suppressed production of PGE₂ remains to be established.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors thank Dr. Ikuro Honda, Japan BCG Laboratory, Tokyo, for providing HK-BCG and PPD for this study and Dr. C. Kathleen Dorey, Florida Atlantic University, for critical review of the manuscript.

References

- Ehlers S, Benini J, Held HD, Roeck C, Alber G, Uhlig S. Alphabeta T cell receptor-positive cells and interferon-gamma, but not inducible nitric oxide synthase, are critical for granuloma necrosis in a mouse model of mycobacteria-induced pulmonary immunopathology. *J Exp Med* 2001;194:1847–1859.
- Fulton SA, Martin TD, Redline RW, Henry Boom W. Pulmonary immune responses during primary *Mycobacterium bovis*-Calmette-Guerin bacillus infection in C57Bl/6 mice. *Am J Respir Cell Mol Biol* 2000;22:333–343.
- Erb KJ, Holloway JW, Sobeck A, Moll H, Le Gros G. Infection of mice with *Mycobacterium bovis*-Bacillus Calmette-Guerin (BCG) suppresses allergen-induced airway eosinophilia. *J Exp Med* 1998;187:561–569.
- Sakuma T, Suenaga T, Yoshida I, Azuma M. Mechanisms of enhanced resistance of *Mycobacterium bovis* BCG-treated mice to ectromelia virus infection. *Infect Immun* 1983;42:567–573.
- Davies M, Sabbadini E. Effect of different doses of BCG on an allogeneic cell-mediated antitumor response in mice. *J Natl Cancer Inst* 1977;59:263–265.
- Engleman EG, Sonnenfeld G, Dauphinee M, Greenspan JS, Talal N, McDevitt HO, Merigan TC. Treatment of NZB/NZW F1 hybrid mice with *Mycobacterium bovis* strain BCG or type II interferon preparations accelerates autoimmune disease. *Arthritis Rheum* 1981;24:1396–1402.
- Shibata Y, Henriksen RA, Honda I, Nakamura RM, Myrvik QN. Splenic PGE₂-releasing macrophages regulate Th1 and Th2 immune responses in mice treated with heat-killed BCG. *J Leukoc Biol* 2005;78:1281–1290.
- Sheibanie AF, Tadmori I, Jing H, Vassiliou E, Ganea D. Prostaglandin E₂ induces IL-23 production in bone marrow-derived dendritic cells. *FASEB J* 2004;18:1318–1320.
- Garg A, Barnes PF, Roy S, Quiroga MF, Wu S, Garcia VE, Krutzik SR, Weis SE, Vankayalapati R. Mannose-capped lipoarabinomannan- and prostaglandin E₂-dependent expansion of regulatory T cells in human *Mycobacterium tuberculosis* infection. *Eur J Immunol* 2008;38:459–469.
- Cross ML, Slobbe LJ, Buchan GS, Griffin JF. In vitro responses of cervine macrophages to bacterial stimulants. *Vet Immunol Immunopathol* 1996;53:249–256.
- Keller R, Keist R, van der Meide PH. Modulation of tumoricidal activity, induced in bone-marrow-derived mononuclear phagocytes by interferon gamma or *Corynebacterium parvum*, by interferon beta, tumor necrosis factor, prostaglandin E₂, and transforming growth factor beta. *Int J Cancer* 1991;49:796–800.
- Rangel Moreno J, Estrada Garcia I, De La Luz Garcia Hernandez M, Aguilar Leon D, Marquez R, Hernandez Pando R. The role of prostaglandin E₂ in the immunopathogenesis of experimental pulmonary tuberculosis. *Immunology* 2002;106:257–266.
- van der Pouw Kraan TC, Boeije LC, Smeenk RJ, Wijdenes J, Aarden LA. Prostaglandin-E₂ is a potent inhibitor of human interleukin 12 production. *J Exp Med* 1995;181:775–779.
- Harbrecht BG, Kim YM, Wirant EA, Simmons RL, Billiar TR. Timing of prostaglandin exposure is critical for the inhibition of LPS- or IFN-gamma-induced macrophage NO synthesis by PGE₂. *J Leukoc Biol* 1997;61:712–720.
- Pillinger MH, Philips MR, Feoktistov A, Weissmann G. Crosstalk in signal transduction via EP receptors: prostaglandin E1 inhibits chemoattractant-induced mitogen-activated protein kinase activity in human neutrophils. *Adv Prostaglandin Thromboxane Leukot Res* 1995;23:311–316.
- Shinomiya S, Naraba H, Ueno A, Utsunomiya I, Maruyama T, Ohuchida S, Ushikubi F, Yuki K, Narumiya S, Sugimoto Y, *et al.* Regulation of TNF α and interleukin-10 production by prostaglandins I(2) and E(2): studies with prostaglandin receptor-deficient mice and prostaglandin E-receptor subtype-selective synthetic agonists. *Biochem Pharmacol* 2001;61:1153–1160.
- Pavlovic S, Du B, Sakamoto K, Khan KM, Natarajan C, Breyer RM, Dannenberg AJ, Falcone DJ. Targeting prostaglandin E2 receptors as an alternative strategy to block cyclooxygenase-2-dependent extracellular matrix-induced matrix metalloproteinase-9 expression by macrophages. *J Biol Chem* 2006;281:3321–3328.
- Otto JC, Smith WL. The orientation of prostaglandin endoperoxide synthases-1 and -2 in the endoplasmic reticulum. *J Biol Chem* 1994;269:19868–19875.
- Morita I, Schindler M, Regier MK, Otto JC, Hori T, DeWitt DL, Smith WL. Different intracellular locations for prostaglandin endoperoxide H synthase-1 and -2. *J Biol Chem* 1995;270:10902–10908.
- Yamashita M, Tsuji S, Nishiyama A, Myrvik QN, Henriksen RA, Shibata Y. Differential subcellular localization of COX-2 in macrophages phagocytosing heat-killed *Mycobacterium bovis* BCG. *Am J Physiol Cell Physiol* 2007;293:C184–C190.
- Yamashita M, Shinohara T, Tsuji S, Myrvik QN, Nishiyama A, Henriksen RA, Shibata Y. Catalytically inactive cyclooxygenase 2 and absence of prostaglandin E₂ biosynthesis in murine peritoneal macrophages following in vivo phagocytosis of heat-killed *Mycobacterium bovis* bacillus Calmette-Guerin. *J Immunol* 2007;179:7072–7078.
- Spencer AG, Thuresson E, Otto JC, Song I, Smith T, DeWitt DL, Garavito RM, Smith WL. The membrane binding domains of prostaglandin endoperoxide H synthases 1 and 2: peptide mapping and mutational analysis. *J Biol Chem* 1999;274:32936–32942.
- Shibata Y, Gabbard J, Yamashita M, Tsuji S, Smith M, Nishiyama A, Henriksen RA, Myrvik QN. Heat-killed BCG induces biphasic cyclooxygenase 2+ splenic macrophage formation—role of IL-10 and bone marrow precursors. *J Leukoc Biol* 2006;80:590–598.
- Shibata Y, Bautista AP, Pennington SN, Humes JL, Volkman A. Eicosanoid production by peritoneal and splenic macrophages in mice depleted of bone marrow by 89Sr. *Am J Pathol* 1987;127:75–82.
- Shibata Y, Honda I, Justice JP, Van Scott MR, Nakamura RM, Myrvik QN. Th1 adjuvant N-acetyl-D-glucosamine polymer up-regulates Th1 immunity but down-regulates Th2 immunity against a mycobacterial protein (MPB-59) in interleukin-10-knockout and wild-type mice. *Infect Immun* 2001;69:6123–6130.

26. Melancon-Kaplan J, Hunter SW, McNeil M, Stewart C, Modlin RL, Rea TH, Convit J, Salgame P, Mehra V, Bloom BR, *et al*. Immunological significance of *Mycobacterium leprae* cell walls. *Proc Natl Acad Sci USA* 1988;85:1917–1921.
27. Wang L, Slayden RA, Barry CE III, Liu J. Cell wall structure of a mutant of *Mycobacterium smegmatis* defective in the biosynthesis of mycolic acids. *J Biol Chem* 2000;275:7224–7229.
28. Lizon C, Fritsch P. Chemical toxicity of some actinides and lanthanides towards alveolar macrophages: an in vitro study. *Int J Radiat Biol* 1999;75:1459–1471.
29. Ding AH, Nathan CF, Stuehr DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages: comparison of activating cytokines and evidence for independent production. *J Immunol* 1988;141:2407–2412.
30. D'Avila H, Melo RC, Parreira GG, Werneck-Barroso E, Castro-Faria-Neto HC, Bozza PT. *Mycobacterium bovis* Bacillus Calmette-Guerin induces TLR2-mediated formation of lipid bodies: intracellular domains for eicosanoid synthesis in vivo. *J Immunol* 2006;176:3087–3097.
31. Billiau A, Matthys P. Modes of action of Freund's adjuvants in experimental models of autoimmune diseases. *J Leukoc Biol* 2001;70:849–860.
32. Chillingworth NL, Donaldson LF. Characterisation of a Freund's complete adjuvant-induced model of chronic arthritis in mice. *J Neurosci Methods* 2003;128:45–52.
33. McCaskill JG, Chason KD, Hua X, Neuringer IP, Ghio AJ, Funkhouser WK, Tilley SL. Pulmonary immune responses to *Propionibacterium acnes* in C57BL/6 and BALB/c mice. *Am J Respir Cell Mol Biol* 2006;35:347–356.
34. Bean AG, Roach DR, Briscoe H, France MP, Korner H, Sedgwick JD, Britton WJ. Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol *Mycobacterium tuberculosis* infection, which is not compensated for by lymphotoxin. *J Immunol* 1999;162:3504–3511.
35. Medica I, Kastrian A, Maver A, Peterlin B. Role of genetic polymorphisms in ACE and TNF-alpha gene in sarcoidosis: a meta-analysis. *J Hum Genet* 2007;52:836–847.
36. Nathan CF. Secretory products of macrophages. *J Clin Invest* 1987;79:319–326.
37. Yadav M, Schorey JS. The beta-glucan receptor dectin-1 functions together with TLR2 to mediate macrophage activation by mycobacteria. *Blood* 2006;108:3168–3175.
38. Kalis C, Gumenscheimer M, Freudenberg N, Tchaptchet S, Fejer G, Heit A, Akira S, Galanos C, Freudenberg MA. Requirement for TLR9 in the immunomodulatory activity of *Propionibacterium acnes*. *J Immunol* 2005;174:4295–4300.
39. Nishiwaki T, Yoneyama H, Eishi Y, Matsuo N, Tatsumi K, Kimura H, Kuriyama T, Matsushima K. Indigenous pulmonary *Propionibacterium acnes* primes the host in the development of sarcoid-like pulmonary granulomatosis in mice. *Am J Pathol* 2004;165:631–639.
40. Bellingan GJ, Caldwell H, Howie SE, Dransfield I, Haslett C. In vivo fate of the inflammatory macrophage during the resolution of inflammation: inflammatory macrophages do not die locally, but emigrate to the draining lymph nodes. *J Immunol* 1996;157:2577–2585.
41. Jakubzick C, Tacke F, Llodra J, van Rooijen N, Randolph GJ. Modulation of dendritic cell trafficking to and from the airways. *J Immunol* 2006;176:3578–3584.
42. Brannon TS, North AJ, Wells LB, Shaul PW. Prostacyclin synthesis in ovine pulmonary artery is developmentally regulated by changes in cyclooxygenase-1 gene expression. *J Clin Invest* 1994;93:2230–2235.
43. Wilborn J, DeWitt DL, Peters-Golden M. Expression and role cyclooxygenase isoforms in alveolar and peritoneal macrophages. *Am J Physiol Lung Cell Mol Physiol* 1995;268:L294–L301.
44. Carey MA, Bradbury JA, Seubert JM, Langenbach R, Zeldin DC, Germolec DR. Contrasting effects of cyclooxygenase-1 (COX-1) and COX-2 deficiency on the host response to influenza A viral infection. *J Immunol* 2005;175:6878–6884.
45. Carey MA, Germolec DR, Bradbury JA, Gooch RA, Moorman MP, Flake GP, Langenbach R, Zeldin DC. Accentuated T helper type 2 airway response after allergen challenge in cyclooxygenase-1–/– but not cyclooxygenase-2–/– mice. *Am J Respir Crit Care Med* 2003;167:1509–1515.
46. Vunta H, Davis F, Palempalli UD, Bhat D, Arner RJ, Thompson JT, Peterson DG, Reddy CC, Prabhu KS. The anti-inflammatory effects of selenium are mediated through 15-deoxy-Delta12,14-prostaglandin J2 in macrophages. *J Biol Chem* 2007;282:17964–17973.
47. Reddy RC, Keshamouni VG, Jaigirdar SH, Zeng X, Leff T, Thannickal VJ, Standiford TJ. Deactivation of murine alveolar macrophages by peroxisome proliferator-activated receptor-gamma ligands. *Am J Physiol Lung Cell Mol Physiol* 2004;286:L613–L619.
48. Culver DA, Barna BP, Raychaudhuri B, Bonfield TL, Abraham S, Malur A, Farver CF, Kavuru MS, Thomassen MJ. Peroxisome proliferator-activated receptor gamma activity is deficient in alveolar macrophages in pulmonary sarcoidosis. *Am J Respir Cell Mol Biol* 2004;30:1–5.
49. Morsy MA, Isohama Y, Miyata T. Prostaglandin E(2) increases surfactant secretion via the EP(1) receptor in rat alveolar type II cells. *Eur J Pharmacol* 2001;426:21–24.
50. Savla U, Appel HJ, Sporn PH, Waters CM. Prostaglandin E(2) regulates wound closure in airway epithelium. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L421–L431.