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Prostaglandin (PG)E₂ exhibits anti-fibrotic activity in vocal fold fibroblasts

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Abstract

Objectives/Hypothesis—Prostaglandin (PG)E₂ has been implicated in a variety of disease processes. It has been described as antifibrotic in the lower airway, yet scar-inducing in the skin. We seek to describe the effects of PGE₂ on vocal fold fibroblasts and its interactions with TGF-β1. In addition, we describe a novel organotypic model, a critical step in the development of therapeutic trials.

Study Design—In vitro, ex vivo

Level of Evidence—N/A

Methods—Collagen secretion by human vocal fold fibroblasts (HVFF) was assayed in response to TGF-β1, PGE₂, and specific EP receptor agonists. Basal HVFF migratory rate was also quantified in response to PGE₂. TGF-β1 induced COX-2 mRNA expression/PGE₂ secretion. Excised vocal folds were subjected to exogenous IL-1β; PGE₂ secretion into the supernatant was then assayed.

Results—TGF-β1-induced collagen secretion was blunted in a dose-dependent manner in response to PGE₂. This effect appears to be mediated primarily through the EP1 and EP2 receptors. TGF-β1 induced COX-2 mRNA expression and PGE₂ secretion. In our organ culture model, IL-1β stimulated PGE₂ secretion in a dose-dependent manner.

Conclusions—PGE₂ is anti-fibrotic; this finding suggests that the upper airway response to this inflammatory mediator differs significantly from the lower airway. These data have important clinical implications for a variety of pathological processes. Furthermore, exogenous TGF-β1 elicits induction of COX-2, suggesting inherent complexity regarding these processes and PGE₂, signaling specifically. In addition, our organ culture model may prove useful as a means to quantify biological phenomena in the vocal folds.

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Conflicts of interest: NONE

Keywords

Vocal fold; inflammation; cyclooxygenase; prostaglandin

INTRODUCTION

The complexity of interactions between multiple cell types, matrix, and soluble mediators of wound healing following injury are substantial. As evidenced by data regarding the pathological processes involved in pulmonary fibrosis, the events following injury are also organ-specific. These observations not only confound our collective insight regarding a generic tissue response following injury, but also impede the translational generalizability of data from other systems to the vocal folds, an inherently unique organ from a biomechanical perspective. Furthermore, relatively small regions of fibrosis appear to have disproportionately detrimental effects on phonatory physiology. In general, little is known regarding the events following injury in the vocal folds, beyond global histological outcomes. To address these pervasive issues which likely contribute to the lack of standardized, efficacious therapies for vocal fold fibrosis, we have attempted to continue to describe specific pathways as a means to potentially provide targets for therapeutic interventions with the ultimate goal of directing wound healing toward a more regenerative, less fibrotic outcome.

Specifically, our laboratory has long posited that interventions which target the inflammatory or early events following injury have the potential to coordinate a more favorable wound outcome. Generally, the response following injury involves a series of coordinated, overlapping phases including inflammation, proliferation, and remodeling, in adults, yielding an overproduction of poorly organized collagen fibers.¹ In contrast, wounding during the first and second trimester of fetal gestation heal quickly and without scar.¹⁻⁴ The hallmark of scarless fetal healing appears to be the absence of a robust inflammatory response.⁵⁻¹¹ The development of anti-fibrotic therapeutics based on the inherent biochemical switches responsible for the transition from scarless to adult healing along with their signaling pathways should prove to be clinically valuable.

Cyclooxygenase (COX)-2, the rate-limiting enzyme in prostaglandin synthesis via the conversion of arachadonic acid, is induced during the acute response to injury¹² and may be implicated in this transition as prostaglandins, and particularly prostaglandin (PG)E₂, are multifunctional; active in both the acute inflammatory response and the subsequent fibroblastic events including alteration of cytoskeletal dynamics as well as fibroblast migration, contraction, proliferation, and extracellular matrix metabolism.¹³⁻¹⁵ In most cases, PGE₂ is considered a potent proinflammatory mediator implicated in the pathogenesis of several processes including periodontitis, UVB-mediated cutaneous inflammation, rheumatoid arthritis, cancer growth, and keloid formation.¹⁶⁻¹⁹ Interestingly, in most cases, inhibition of COX-2 is effective at diminishing disease progression. Furthermore, topical inhibition of COX-2 has been shown to be anti-fibrotic in full thickness skin wounds.²⁰ In contrast, however, the lung may serve as a privileged site for PGE₂²¹ whereby increased PGE₂ concentrations may be therapeutic in the context of pulmonary fibrosis.²²

The vocal folds provide an interesting model for investigating these phenomena given their proximity to the lungs. Both COX-2 and PGE₂ have been shown to be upregulated following acute vocal fold injury,²³⁻²⁴ and our laboratory previously characterized COX-2 signaling in human vocal fold fibroblasts.²⁵ We now seek to further elucidate the regulatory role of PGE₂ on these cells with a specific interest in the interaction between transforming growth factor (TGF)- β 1, a putative profibrotic member of the TGF superfamily which

implicated in a variety of fibroproliferative processes including vocal fold fibrosis,²⁶⁻²⁹ and PGE₂. In addition, acknowledging that current models of investigation in the vocal folds are relatively limited, we sought to develop an *ex vivo*, organ culture system to quantify the tissue response to exogenous mediators of healing. Ultimately, this model may prove to be a critical link between *in vitro* data and *in vivo* trials.

MATERIALS AND METHODS

Cell Model and Reagents

The HVOX human vocal fold fibroblast cell line developed by our laboratory was employed for each *in vitro* experiment described.²⁹ Interleukin-1 β and PGE₂ were purchased from Sigma-Aldrich (St. Louis, MO) and Cayman Chemical (Ann Arbor, MI), respectively. EP receptor agonists, 17-phenyl trinor Prostaglandin E₂ (EP1), butaprost (EP2), sulprostone (EP3), and 11-deoxy Prostaglandin E₁ (EP4) were purchased from Cayman Chemical (Ann Arbor, MI). TGF- β 1 was purchased from R&D Systems (Minneapolis, MN).

Fibroblast Collagen Secretion

Total secreted collagen in the supernatant media was quantified using the Sircol Soluble Collagen Assay (Biocolor, Ltd, Ireland). Near-confluent cells grown in 6-well plates were treated for 24 hours and the post-culture medium (supernatant) was collected. We first sought to describe the effects of PGE₂ on TGF- β 1-mediated collagen secretion. For these experiments, physiological concentrations of both PGE₂ (1.0 and 10.0uM) and TGF- β 1 (10ng/mL) were applied to the HVOX cultures, concurrently. Secondly, we sought to determine the relevant EP receptors mediating this effect. As such, we concurrently treated HVOX with TGF- β 1 (10ng/mL) and the individual EP receptor agonists described below. Each agonist was employed at a concentration of 10uM, as suggested by the manufacturer. Sircol Dye Reagent was added to 200 μ L of supernatant media and vortexed every 5 minutes for 30 minutes, then centrifuged at 12,000rpm for 10 minutes. The unbound dye in the supernatant was then removed by inversion and the pellet incubated for 10 minutes with 1mL of alkali reagent to dissolve bound dye. The samples were then read at 550nm using a multi-well plate reader. Absorbance values were extrapolated to collagen concentration via standard curve. All data were standardized to total protein content using the bicinchoninic acid assay.

Fibroblast Migration

HVOX migratory rate was assessed using an *in vitro* scratch assay previously described by our laboratory and others.²⁹ Confluent HVOX in pre-marked 24-well plates were serum-starved overnight. The monolayers were interrupted by scratching along the longitudinal axis of each well using a 200 μ L pipette tip, washed twice with PBS, and then incubated for eight hours in serum-free medium containing 1.0 and 10.0uM PGE₂. Digital images were captured of each well immediately following the scratch procedure and again, eight hours later, using the markings on the plates as landmarks to ensure identically positioned photographs were obtained at both time points. The difference in the relative area (pixels) of the scratch over time was assessed using ImageJ (National Institutes of Health). The following equation was employed to calculate HVOX migration over the period of observation: $initial\ area - final\ area = \Delta area / 8\ hours$.

Polymerase Chain Reaction

In order to determine the effects of exogenous TGF- β 1 on COX-2 mRNA expression, we isolated total RNA from HVOX following 24 hours of treatment with TGF- β 1 (0.1, 1, and 10ng/mL). The OneStep RT-PCR Kit (Qiagen Inc., Santa Clara, CA) was then employed to

reverse transcribe and amplify the RNA, following the manufacturer's protocol. Briefly, 13.0 μ L of OneStep PCR Mix was added to 10ng RNA with 2.0 μ L of COX-2 primer, incubated at 37° for one hour, heated to 95° for six minutes, and then subjected to 28 cycles of 30 seconds at 37°, 30 seconds at 60°, and one minute at 72°. PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide.

PGE₂ Immunoassay

PGE₂ synthesis was assayed via a commercially-available ELISA kit (R&D Systems, Minneapolis, MN). Concentrations were standardized to total cellular protein.

Vocal Fold Organ Culture

We then sought to provide preliminary data regarding the viability of ex vivo methods to quantify biological phenomena in the vocal folds, primarily the secretion of mediators of wound healing. As such, adult, female New Zealand rabbits employed by other investigators were sacrificed and subjected to total laryngectomy. The membranous vocal folds were then dissected from the anterior commissure to the vocal process in the anterior-posterior dimension and from the inferior border of the fold to the superior surface within the ventricle under magnification. These specimens included epithelium, lamina propria, and vocalis muscle. The specimens were weighed, washed in phosphate buffered saline (PBS) three times, and placed in serum-free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% antibiotic/antimycotic cocktail for 4 hours. The media was then changed to include supplementation with various concentrations of IL-1 β and left at 37°C for 24 hours. The media was then harvested, diluted appropriately, and subjected to immunoassay for PGE₂. Immunoassay data were standardized to tissue weight. Data are presented as pg/mL/gram of tissue.

Statistical Analyses

One way Analyses of Variance were performed using SPSS (v12.0). Pending a significant main effect at $p=0.05$, post hoc comparisons were employed using the Tukey method.

RESULTS

PGE₂ limited TGF- β 1-induced HVOX collagen synthesis

As shown in Figure 1A, exogenous TGF- β 1 elicited a significant increase collagen secretion by HVOX ($p<0.05$), consistent with our previous findings.²⁹ PGE₂ abrogated this effect at concentrations of 1.0 and 10.0 μ M ($p<0.05$). This effect was amplified at increased concentrations; 10 μ M of PGE₂ decreased TGF- β 1 mediated collagen synthesis to below baseline levels. We then employed specific agonists of the four cell surface prostaglandin receptors to further investigate the mechanism of this effect. At baseline, treatment with each EP receptor agonists decreased collagen secretion slightly, and in a consistent manner. This effect did not achieve statistical significance (Figure 2). Treatment with each agonist decreased TGF- β 1-induced collagen secretion, but in a more heterogeneous fashion. This effect, however, only achieved significance when HVOX were treated with agonists of EP1 and EP2 ($p=0.003$ and 0.001 , respectively).

PGE₂ decreased the basal migratory rate of HVOX

A dose-dependent effect in basal migratory rate was observed (Figure 1B); 1 μ M PGE₂ decrease migratory rate by approximately 20%. This decrease did not achieve statistical significance. However, at 10.0 μ M, PGE₂ decreased HVOX basal migratory rate by nearly 80% ($p=0.038$).

TGF- β 1 induced COX-2 mRNA expression and PGE₂ synthesis in HVOX

Given that PGE₂ appears to have some inherent anti-fibrotic characteristics, we sought to determine if TGF- β 1 induced COX-2 expression and PGE₂ synthesis, potentially, as a component of an autocrine anti-fibrotic feedback loop. As shown in Figure 3A, COX-2 mRNA expression increased in a dose-dependent fashion following 24 hours of exposure to TGF- β 1. PGE₂ also increased over 24 hours of TGF- β 1 exposure. However, only the highest concentration (10.0ng) of TGF- β 1 induced a statistically-significant increase in PGE₂ synthesis ($p=0.039$).

IL-1 β induced PGE₂ synthesis in an ex vivo organ culture model

Acknowledging that current models of vocal fold wound healing are limited, we sought to describe physiological phenomena in an ex vivo model. Specifically, we sought to determine if an inflammatory tissue phenotype could be elicited, in this case, characterized by increased PGE₂ synthesis, by treating freshly-excised vocal fold tissue with exogenous IL-1 β . Using this model, PGE₂ synthesis into the supernatant increased by more than 60% ($p=0.038$) and approximately 100% ($p=0.004$) in response to 1.0 and 10.0ng/mL of IL-1 β , respectively (Figure 4).

DISCUSSION

Treatment for vocal fold fibrosis remains suboptimal. Investigation regarding the sequence of events following vocal fold injury is likely to provide potential targets for intervention. We hypothesize that readily-available pharmacological agents targeting well-described pathways have been largely overlooked. One therapeutic option is targeting signaling that crosses the boundary between inflammation and fibrosis. In that regard, COX-2 and its downstream product PGE₂ appear to be ideal. Our laboratory previously reported that PGE₂ secretion can be maintained given that it is fibroblast-secreted.²⁵ This finding is particularly relevant given that the effects are likely to be relatively long-term, compared to other shorter lived cytokines and chemokines. We previously showed that PGE₂ had a bimodal expression pattern with peak expression in the acute inflammatory phase and then increased concentrations approximately 1 to 2 weeks following injury.³⁰ We hypothesized that this temporal pattern related primarily to the complexity of PGE₂ as a regulator of both inflammation as well as the proliferative phase of healing, and its interactions with mesenchymal cells recruited into the wound bed.

Given the relationship between inflammation and fibrosis, many hypothesize that increased PGE₂ is related to an aberrant inflammatory response resulting in hypertrophic scar or keloid formation. Furthermore, preliminary data suggested that topical inhibition of COX-2 limited dermal fibrosis.²⁰ However, this response appears to be organ-specific as increased bleomycin-induced pulmonary fibrosis was observed in COX-2 deficient mice.³¹ In the current study, we attempted to characterize the regulatory effects of exogenous PGE₂ on vocal fold fibroblasts in an attempt to reconcile some of these issues and potentially describe a reasonable pharmacological target for manipulation.

The development of fibrosis appears to be driven by cytokines like TGF- β 1, which is a master regulator of wound healing. TGF- β 1 is a multifunctional cytokine implicated in the pathogenesis of a variety of fibrotic diseases.³²⁻³³ Our laboratory and others have previously shown that TGF- β 1 is associated with type I collagen synthesis related to fibrosis, among other cell activities.²⁹ However, targeted anti-TGF- β 1 therapeutics have largely failed. Recent data suggest that TGF- β 1 may, in fact, induce an anti-fibrotic feedback loop via the regulation of COX-2, further complicating the putative role of the COX-2/PGE₂ pathway as either a cornerstone of fibrosis or a potent antifibrotic agent. Our data suggest that PGE₂

does, in fact, limit TGF- β 1-induced collagen secretion and this phenomenon is largely mediated via the EP1 and EP2 receptors in HVFF. Furthermore, PGE₂ limits basal migratory rates of HVFF migration, further contributing to its putative antifibrotic properties. In addition, TGF- β 1 induced HVFF COX-2 mRNA and PGE₂ secretion into the supernatant, suggesting the induction of a putative anti-fibrotic mechanism inherent to the fibrotic phenotype. These data also suggest some clinical value to altering this pathway. Pharmacological therapies have already been developed; we simply must determine the relevant temporal variables to optimize therapies related to altered PGE₂ signaling.

Although traditional cell culture methods are critical to describing fundamental cell processes, the complexity of the wound healing processes are largely overlooked and oversimplified. Furthermore, anatomic factors related to both the size and location of the vocal folds often limit investigation regarding vocal fold dynamics. We, therefore, sought to compliment our in vitro data with ex vivo, organ culture data. This model, although not recapitulating the complexity of in vivo processes, attempts to close the gap between the quasi-artificial in vitro environment and the overly complex in vivo milieu. Our data suggest that freshly excised vocal folds do respond to exogenous mediators of healing. In this particular case, IL-1 β induced significantly increased PGE₂ secretion from the tissue. Clearly, further investigation is warranted to validate such techniques including immunolocalization to determine the cell source of these secreted mediators. Regardless, we are optimistic that this organotypic model is likely to yield critical information regarding the development of therapeutic trials.

In summary, these data implicate the intricate balance of positive and negative signaling pathways in the regulation of basal physiological activities. Increased insight regarding this balance and the sequelae associated with an imbalance of these processes is critical to understanding pathological process, and the development of novel therapies. Our longstanding hypothesis is that modulating or attenuating the acute inflammatory response following vocal fold injury is likely to yield a less fibrotic outcome to the wound healing process. We also hypothesize that the COX-2 pathway provides an ideal opportunity for pharmacological intervention as it appears to be relatively ubiquitous during the wound healing process. However, the complexity of this particular signaling pathway is significant and as our data suggest, there is likely critical temporal variable that must be considered moving forward.

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Figure 1. Collagen secretion by HVOX in response to treatment with exogenous PGE₂ +/- TGF-β1 (A; * <0.05 from baseline, † <0.05 from TGF-β1 treatment) and relative changes in basal migratory rate in response to PGE₂ (B; * <0.05).

Figure 2. Collagen secretion by HVOX in response to treatment with exogenous PGE₂ +/- specific EP receptor agonists (*<0.05 from baseline, #<0.05 from TGF- β 1 treatment).

Figure 3. COX-2 mRNA expression (A) and PGE₂ secretion (B) in response to exogenous TGF- β 1 (* <0.05).

Figure 4. PGE₂ secretion from excised rabbit vocal folds treated with IL-1 β (A; * <0.05 , raw data provided, B).