MicroRNA-146a and microRNA-146b expression and anti-inflammatory function in human airway smooth muscle

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Asthma is characterized by inflammation, airway remodeling, and airway hyperresponsiveness. Airway smooth muscle cells function as immunomodulatory and contractile cells in the lung and contribute to the pathogenesis of asthma (59). A small subset of patients with severe asthma do not respond adequately to current therapies, exhibit corticosteroid insensitivity, and account for a large and disproportionate amount of all asthma-related health care costs (1, 10, 23). This subset of patients with poorly controlled asthma highlights the need for investigating novel anti-inflammatory drug targets.

MicroRNAs (miRNAs or miRs) are posttranscriptional regulators of gene expression that have been linked to a number of pathologies, including chronic obstructive pulmonary disease, pulmonary arterial hypertension, and idiopathic pulmonary fibrosis (14, 49, 60). MicroRNAs are differentially expressed in asthmatic human airway smooth muscle cells (hASMCs) and exhibit dynamic changes in expression in the lungs of mice during the development of allergic airway inflammation in models of asthma (11, 16, 28, 39, 45, 55). Members of the miR-146 family, consisting of miR-146a and miR-146b, are elevated in a murine model of asthma (16), miR-146a and miR-146b are negative regulators of inflammatory gene expression in numerous cell types, including monocytes (54), fibroblasts (49), endothelial (9), airway smooth muscle (34), and epithelial cells (46). Validated inflammation-related targets of the miR-146 family include cyclooxygenase-2 (COX-2) (49), human antigen R (HuR) (9), IL-1 receptor-associated kinase-1 (IRAK1) (34, 54), TNF receptor-associated factor 6 (TRAF6) (34, 54), IL-1β (41), IL-6 (34, 34, 46). Of these targets, only IRAK1, TRAF6, IL-6, and IL-8 have been validated in hASMCs (34). Thus, we sought to further characterize miR-146a and miR-146b expression and function in hASMCs from patients with and without asthma. We chose to investigate whether miR-146a and miR-146b regulate the expression of COX-2 and IL-1β due to their relevance to inflammation and also to determine novel cell-specific functions for the miRNAs in airway smooth muscle by investigating miR-146a regulation of HuR in these cells. The studies are stimulated by the important role of hASMCs in asthma and because expression and functional results in this cell type are limited to one study (34).

Larnier-Svenssson et al. and Perry and colleagues characterized miR-146 expression and function in hASMCs and human lung alveolar epithelial cells (34, 46). miR-146a expression in hASMCs was inducible by treatment with IL-1β. Treatment of human retinal pigment epithelial cells with a multicytokine cocktail consisting of IL-1β, TNF-α, and IFNγ induced significantly higher levels of miR-146a and miR-146b than treatment with IL-1β alone. A similar synergistic effect has been
reported in studies of hASMCs treated with the same cytokine cocktail (22, 51). Based on these studies, we chose to investigate miR-146a and miR-146b expression and function in hASMCs that were treated with the multicytokine cocktail or “cytomix” consisting of IL-1β, TNF-α, and IFNγ that is thought to mimic the inflammatory milieu that is present in late-stage inflammation in asthmatic airways (5, 57). Before this study, only miR-146a negative regulation of IRAK1, TRAF6, IL-6, and IL-8 had been investigated in airway smooth muscle (34). We demonstrate that miR-146a and miR-146b also negatively regulate COX-2 and IL-1β in hASMCs, that miR-146a negatively regulates HuR, and that both miR-146a and miR-146b are inducible in hASMCs treated with cytokym.

METHODS AND MATERIALS

Cell culture. Primary nonasthmatic and asthmatic hASMCs were isolated from nontransplantable donor lungs or resected lung tissue by enzymatic digestion at the University of Chicago or University of Manitoba, respectively. All tissue procurement and cell culture studies were conducted following protocols approved by the Human Research Ethics Board (University of Manitoba) and Institutional Review Boards for Protection of Human Subjects of the University of Chicago and the University of South Alabama. Cells were cultured in 5% CO₂ at 37°C in DMEM supplemented with 5% FBS, 0.5 μg/ml basic fibroblast growth factor, 2 μg/ml epidermal growth factor, 50 units/ml penicillin, and 100 μg/ml streptomycin. Cells were grown in culture in 50:50 DMEM-Ham’s F-12 supplemented with 0.25 mg/ml insulin, 0.11 mg/ml transferrin, and 0.1 μg/ml selenium. Nonasthmatic and asthmatic hASMCs were assayed at passage 6–8. Cells were treated with cytokym (10 ng/ml IL-1β, 10 ng/ml TNF-α, 10 ng/ml IFNγ), individual cytokines (10 ng/ml), or left untreated for 20 h as previously described (22, 51). Cells were not tested for the presence of mycoplasma. Culture media and supplements were obtained from Life Technologies (Grand Island, NY), Cell Generation (Fort Collins, CO), or Sigma-Aldrich (St. Louis, MO).

RNA isolation and quantitative RT-PCR. Total RNA was extracted using miRNeasy (Qiagen, Valencia, CA). RNA quality and concentration were assessed by spectrophotometry. miR-146a-5p and miR-146b-5p were assayed using Taqman miRNA assays (Life Technologies) according to the manufacturer’s protocol with an input of 40 ng total RNA and quantified using miRNA standard curves. miRNA concentrations were determined by spectrophotometry. miR-146a-5p and miR-146b-5p were assayed using Taqman miRNA assays (Life Technologies) according to the manufacturer’s protocol with an input of 40 ng total RNA and quantified using miRNA standard curves. miRNA concentrations were determined by spectrophotometry. miR-146a and miR-146b expression in asthmatic and nonasthmatic hASMCs. To determine the role of miR-146a and miR-146b in regulating COX-2 and IL-1β expression in hASMCs treated with cytokym, we first had to determine if miRNA expression was induced under these proinflammatory conditions. Asthmatic and nonasthmatic hASMCs were left untreated, treated with cytokym (10 ng/ml each of IL-1β, TNF-α, and IFNγ), or treated with the individual cytokines for 20 h. miR-146a and miR-146b abundance was measured using qRT-PCR. Treatment with cytokym induced expression of both both miR-146a (Fig. 1A) and miR-146b (Fig. 1B). miR-146a expression was higher in asthmatic than in nonasthmatic hASMCs treated with cytokym (Fig. 1A). Compared with cytokym, individual cytokines produced little or no upregulation of miR-146a or miR-146b. Only TNF-α increased miR-146a expression significantly in nonasthmatic hASMCs (Fig. 1A). These results demonstrated that miR-146a and miR-146b are inducible and that simultaneous stimulation with a mixture of proinflammatory cytokines is required for maximum expression. These results are consistent with previous reports of potentiation of COX-2 and IL-1β expression in hASMCs with cytokym compared with individual cytokines (22, 43, 51). miR-146a and/or miR-146b may act as negative regulators of the proinflammatory function of hASMCs under these conditions (9). To test whether changes in miR-146a or miR-146b are linked with changes in COX-2 and IL-1β expression we used miRNA mimics and inhibitors to determine effects on proinflammatory function of hASMCs.

miR-146a negatively regulates COX-2 expression in hASMCs. COX-2 is a validated target of miR-146a in fibroblasts (49). However, miR-146b is also predicted to target COX-2 due to sequence homology with miR-146a (54), but this has not been validated experimentally. To determine if miR-146a and miR-146b can both silence COX-2 expression in hASMCs, we transfected cells with specific duplex miRNA mimics and hairpin inhibitors. Successful transfection of miRNA mimics and inhibitors was validated by qRT-PCR and transfection of fluorescently labeled oligonucleotides, which successfully
miR-146a/b expression and function in airway smooth muscle

miR-146a/b expression and function in airway smooth muscle (hASMCs). miR-146a (A) and miR-146b (B) abundance in nonasthmatic (open bars) and asthmatic (solid bars) hASMCs treated with cytomix (10 ng/ml each of IL-1β, TNF-α, and IFN-γ), individual cocktail components, or left untreated for 20 h. Data (mean ± SE) obtained from 5 nonasthmatic and 7 asthmatic donors; n = 10–14 cultures. *P < 0.01 vs. nonasthmatic or asthmatic 20 h untreated control; **P < 0.05 vs. nonasthmatic 20 h untreated control; ***P < 0.05 vs. asthmatic 20 h treated with cytokin. P values for comparisons were calculated using 1-way ANOVA with Bonferroni’s test. P values for comparisons of treatments within either nonasthmatic or asthmatic groups were calculated using 1-way ANOVA with Dunnett’s test.

miR-146a negatively regulates IL-1β expression in hASMCs. miR-146a was previously shown to reduce IL-1β expression in mouse macrophages (41). To determine if miR-146a and miR-146b negatively regulate IL-1β expression in hASMCs, we used a gain-of-function and loss-of-function approach as above. Transfection of 30 nM miR-146a or 30 nM miR-146b mimics both reduced IL-1β mRNA (Fig. 4A) and pro-IL-1β protein (Fig. 4B) abundance in hASMCs treated with cytomix. Inhibition of miR-146a but not miR-146b using 30 nM of the inhibitor modestly increased pro-IL-1β protein abundance (Fig. 5). Transfecting cells with 90 nM of the miR-146a inhibitor did not have an additive effect on the enhancement of pro-IL-1β expression (data not shown). These results indicate that both miRNAs are capable of repressing IL-1β expression at pharmacological levels, but only miR-146a acts as an endogenous negative regulator of IL-1β expression in hASMCs.

**DISCUSSION**

Members of the miR-146 family are anti-inflammatory miRNAs that are induced in response to proinflammatory conditions (54). Here we report the novel finding that miR-
miR-146a and miR-146b expression is greatly enhanced in hASMCs treated with cytomix compared with treatment with the individual cytokines. Furthermore, we report that miR-146b is inducible in hASMCs treated with cytomix, whereas, previously, Larner-Svensson and colleagues observed no induction in hASMCs treated with IL-1β alone (34). Interestingly, we observed that miR-146a expression is greater in asthmatic hASMCs treated with cytomix, a novel finding that is supported by observations made by others of enhanced expression of cytokine-responsive genes in asthmatic hASMCs (4, 8, 27, 45). We demonstrate that miR-146a and miR-146b mimics are capable of negatively regulating the expression of COX-2 and IL-1β in hASMCs and thus are functioning as anti-inflammatory miRNAs as originally hypothesized by Taganov and colleagues (54). Furthermore, we demonstrate that, despite not being predicted to directly targeting the 3′-untranslated region of IL-1β mRNA, both miR-146a and miR-146b are capable of reducing steady-state levels of IL-1β mRNA in hASMCs. In support of this observation, we also observed that miR-146a repressed the expression of HuR in hASMCs, which may contribute to the reduction of IL-1β mRNA levels observed in this study and previous observations of miR-146a regulation of IL-6 and IL-8 by Larner-Svensson and colleagues (34). Interestingly, our loss-of-function studies indicate that only miR-146a is an endogenous negative regulator of COX-2 and IL-1β in hASMCs indicating that it is the functionally dominant miR-146 family member in hASMCs under these conditions, which is reflected by its slightly higher abundance under these conditions.

In this study, we sought to further characterize the expression and function of miR-146a and miR-146b in hASMCs. Larner-Svensson and colleagues (34) observed induction of miR-146a expression but not of miR-146b in hASMCs treated with IL-1β. We found that cytomix induced miR-146b expres-
miR-146a/b expression and function in airway smooth muscle

We observed significant increases in miR-146a expression when the cells were treated with TNF-α or cytomix. Interestingly, Larner-Svensson and colleagues observed an increase in miR-146a expression when hASMCs were treated with IL-1β (34) but our results indicated a trend toward an increase that was not statistically significant. Our results do not challenge those of Lindsay and colleagues but rather may be due to a difference in experimental design that requires corrections for multiple comparisons. These results highlight the large differences in miRNA expression that are observed when cells are treated with a multicytokine cocktail vs. individual cytokines as previously observed by Kutty and colleagues in epithelial cells (31). Furthermore, differences in expression between nonasthmatic and asthmatic cells were only evident when the cells were treated with the multicytokine cocktail that mimics the inflammatory milieu. Interestingly, no differences in basal miR-146a or miR-146b expression were observed between asthmatic and nonasthmatic cells at 0 or 20 h. These results are supported by a study by Williams and colleagues (58) who found no differences in miR-146a or miR-146b expression in samples from patients with mild asthma compared with control patients.

COX-2 was previously demonstrated to be a target of miR-146a (49), and we have confirmed this in hASMCs. Furthermore, Larner-Svensson and colleagues observed an increase in miR-146a expression when hASMCs were treated with IL-1β alone. We previously demonstrated that cytokyme has a synergistic effect on protein-coding gene expression compared with the individual cytokine components and such an effect may be occurring for expression of miRNA genes as well (22, 43, 51). Cytokine activates NF-κB, ERK1/2, JNK1/2, p38 MAPK, and JAK/STAT signal transduction pathways in hASMCs (22, 51, 52), ERK1/2 and JNK1/2 regulate miR-146a and miR-146b processing, and NF-κB is a transcriptional regulator of miR-146a expression (34, 47, 54). Interestingly, p38 MAPK has not been linked to the regulation of miR-146a and miR-146b expression despite recent evidence that the pathway regulates p68 nuclear translocation and increases the miRNA processing activity of the DROSHA microprocessor complex (24). This raises the question of whether there are subtle differences in miRNA processing depending on cell type and the pathways being activated at any given time. Therefore, we investigated expression and mRNA targeting of miR146a and miR146b in hASMCs under conditions that mimic the proinflammatory state in the airways of humans with asthma.

Fig. 4. miR-146 mimics decrease IL-1β mRNA and pro-IL-1β protein abundance in hASMCs following cytomix treatment. hASMCs were transfected with 30 nM control (open bars), miR-146a (solid bars), or miR-146b (hatched bars) mimics and then treated with cytomix (10 ng/ml each of IL-1β, TNF-α, and IFNγ) for 20 h. IL-1β mRNA (A) and protein (B) abundance was assayed and normalized relative to cytomix-treated control at 20 h. Data (mean ± SE) obtained from 3 to 5 donors; n = 5–11 mRNA cultures, n = 7–15 protein cultures. *P < 0.01 vs. control mimic treated with cytomix. All P values calculated using 1-way ANOVA with Dunnett’s test.

Fig. 5. miR-146a inhibition increases pro-IL-1β protein abundance in hASMCs following cytomix treatment. hASMCs were transfected with 30 nM control (open bars), 30 nM miR-146a (solid bars), or 30 nM miR-146b (hatched bars) inhibitors and then treated with cytomix (10 ng/ml each of IL-1β, TNF-α, and IFNγ) for 20 h. IL-1β protein abundance was assayed by Western blotting and normalized relative to cytomix-treated control at 20 h. Data (mean ± SE) obtained from 4 donors; n = 8–16 cultures. *P < 0.01 vs. control mimic treated with cytomix. All P values calculated using 1-way ANOVA with Dunnett’s test.
more, these results indicate that miR-146a contributes to the maintenance of steady-state COX-2 expression that we previously observed in hASMCs treated with cytomix (51). Additionally, we have demonstrated that miR-146b is also capable of repressing COX-2 in hASMCs when the miRNA is present at pharmacological levels. However, miRNA inhibition experiments show that only miR-146a is an endogenous negative regulator. This negative regulatory role is modest as demonstrated by the modest increases in COX-2 protein abundance seen after inhibition of miR-146a, but a modest increase is reasonable considering that miRNAs normally function as minor regulators of individual gene expression that are capable of regulating a large number of genes (Fig. 3). miRNA-mediated reductions in COX-2 expression correlated with a reduction in PGE2 secretion. B2-Adrenoceptor heterologous desensitization has been observed in some patients with asthma, and in vitro this has been linked to cytokine-mediated induction of COX-2 and autocrine PGE2 signaling in hASMCs (2, 20, 33, 42, 50). These results suggest targeted delivery of miR-146a and miR-146b in the airways of patients with asthma that are hyporesponsive to β2-adrenoceptor agonists may help increase patient responsiveness by reducing COX-2 expression.

The miR-146a- and miR-146b-mediated reductions in PGE2 secretion observed in this study were greater in magnitude than the reduction in relative COX-2 protein abundance. This discrepancy may be due to miR-146 repression of additional enzymes involved in PGE2 synthesis. Microsomal PGE2 synthase-1 and cytosolic phospholipase A2 are induced in parallel with COX-2 to promote PGE2 biosynthesis when hASMCs are stimulated with IL-1β (43, 44). Recently, miR-146a was shown to target prostaglandin E synthase-2 in murine bone marrow-derived mesenchymal stem cells, but prostaglandin E synthase-2 mRNA is not a predicted target for miR-146a in humans (3, 40). Human miR-146a and miR-146b are predicted to target microsomal prostaglandin E synthase-1 (3). Whether miR-146a and miR-146b are repressing microsomal prostaglandin E synthase-1 expression in hASMCs is unknown and will need to be investigated in the future.

miR-146a and miR-146b are important negative regulators of the innate immune response (37). This immunoregulatory function has been attributed to repression of IRAK1 and TRAF6 (54). However, Larner-Svensson and colleagues (34) presented evidence that, although miR-146a silences IRAK1 and TRAF6 expression in hASMCs, a reduction in the transcriptional activation of IL-6 and IL-8 was not responsible for the decrease in cytokine secretion. Our results support an alternate mechanism where stability of cytokine transcripts is reduced by increased miR-146a and reduced expression of the mRNA-binding protein HuR. miR-146a represses expression of HuR protein in hASMCs (Fig. 6) as previously described in endothelial cells (9). Thus our results taken together with Larner-Svenssson et al. (34) demonstrate that members of the miR-146 family negatively regulate cytokine-responsive gene expression in hASMCs by multiple mechanisms, including direct targeting of mRNAs and repression of HuR expression. In addition, miR-146a might inhibit translation of cytokine mRNAs. This notion is supported by recent studies showing that miR-146a promotes cytosolic localization of RNA-binding motif (RBM)-4 (6, 7). RBM4 inhibits the translation of bound transcripts by a mechanism that may require colocalization of argonaute-2, and such a mechanism may be partly responsible for the effects of miR-146a in hASMCs (6, 7, 34, 35).

The ability of mimics for miR-146a or miR-146b to act as anti-inflammatory agents may be beneficial as new add-on therapy with inhaled glucocorticoids (9, 34, 46, 54). Numerous additional inflammation-related targets have been identified for the miR-146 family that support the mimics as potential anti-inflammatory agents, including C-X-C chemokine receptor type 4 (32), IRAK2 (25), Kruppel-like factor 4 (53), Rho-

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Fig. 6. miR-146a represses human antigen R (HuR) expression in hASMCs. hASMCs were transfected with 30 nM control (open bars), 30 nM miR-146a (solid bars) mimics (A), or inhibitors (B) and then treated with cytomix (10 ng/ml each of IL-1β, TNF-α, and IFNγ) or left untreated for 20 h. HuR protein abundance was assayed and normalized relative to control. Data (mean ± SE) obtained from 4 donors; n = 8–16. *P < 0.001 vs. control mimic treated with cytomix. **P < 0.001 vs. control inhibitor 20 h untreated. ***P < 0.001 vs. control mimic treated with cytomix. #P < 0.05 vs. control inhibitor 20 h untreated. ##P < 0.01 vs. control inhibitor treated with cytomix. All P values calculated using 1-way ANOVA with Bonferroni’s test.
associated protein kinase-1 (36), and signal transducer and activator of transcription 1 (38). A number of these targets are involved in signal transduction, and, as a result, the miR-146 family has the potential to indirectly regulate the expression of a number of cytokine-responsive genes, including IL-1β (41), IL-6 (34), IL-8 (34, 46), MAPK phosphatase-1 (6), and regulated on activation, normal T cell expressed and secreted (46). Elevated miR-146a and miR-146b would likely repress Toll-like receptor (TLR)-4 and IL-1 receptor signal transduction in patients with inflammatory lung disease. TLR-4 activation on structural cells in the airways of mice is required for the development of allergic airway inflammation in response to sensitization and challenge with house dust mite extract, an allergen for which a high percentage of patients with asthma exhibit atopy (21, 56). Mattes and colleagues have demonstrated that allergic airway inflammation is attenuated in mice lacking TLR-4 following sensitization and challenge with house dust mite extract (39). Targeted delivery of miR-146a and miR-146b to the airways would mimic the effect of TLR-4 knockout that was observed in mice by antagonizing TLR-4 signaling (54).

In conclusion, miR-146a and miR-146b expression is induced in asthmatic and nonasthmatic hASMCs treated with a mixture of cytokines. miR-146a expression was higher in asthmatic hASMCs that were treated with cytokinin. miR-146a but not miR-146b may be a modest endogenous negative regulator of COX-2 and IL-1β gene expression in hASMCs. Furthermore, miR-146a negatively regulates HuR expression in hASMCs. Future studies will need to investigate miR-146a and miR-146b function in mouse models of asthma to determine if the miRNAs should be targeted in asthmatic patients.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

B.S.C. and W.T.G. conception and design of research; B.S.C. performed experiments; B.S.C. analyzed data; B.S.C. and W.T.G. interpreted results of experiments; B.S.C. prepared figures; B.S.C. drafted manuscript; B.S.C., B.C.M., P.C.K., A.J.H., J.S., and W.T.G. edited and revised manuscript; B.S.C., B.C.M., P.C.K., A.J.H., J.S., and W.T.G. approved final version of manuscript.

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