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

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Am J Physiol Lung Cell Mol Physiol 307: L727–L734, 2014. First published September 12, 2014; doi:10.1152/ajplung.00174.2014.—MicroRNA (miR)-146a and miR-146b are negative regulators of inflammatory gene expression in lung fibroblasts, epithelial cells, monocytes, and endothelial cells. The abundance of cyclooxygenase-2 (COX-2) and IL-1β is negatively regulated by the miR-146 family, suggesting miR-146a and/or miR-146b might modulate inflammatory mediator expression in airway smooth muscle thereby contributing to pathogenesis of asthma. To test this idea we compared miR-146a and miR-146b expression in human airway smooth muscle cells (hASMCs) from nonasthmatic and asthmatic subjects treated with cytomix (IL-1α, TNF-α, and IFNγ) and examined the miRNAs’ effects on COX-2 and IL-1β expression. We found that cytokin treatment elevated miR-146a and miR-146b abundance. Induction with cytokint was greater than induction with individual cytokines, and asthmatic cells exhibited higher levels of miR-146a expression following cytokinin treatment than nonasthmatic cells. Transfection of miR-146a or miR-146b mimics reduced COX-2 and IL-1β expression. A miR-146a inhibitor increased COX-2 and IL-1β expression, but a miR-146b inhibitor was ineffective. Repression of COX-2 and IL-1β expression by miR-146a correlated with reduced abundance of the RNA-binding protein human antigen R. These results demonstrate that miR-146a and miR-146b expression is inducible in hASMCs by proinflammatory cytokines and that miR-146a expression is greater in asthmatic cells. Both miR-146a and miR-146b can negatively regulate COX-2 and IL-1β expression at pharmacological levels, but loss-of-function studies showed that only miR-146a is an endogenous negative regulator in hASMCs. The results suggest miR-146 mimics may be an attractive candidate for further preclinical studies as an anti-inflammatory treatment of asthma.

cyclooxygenase-2; human antigen R; interleukin-1β; miRNA-146; inflammation

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 ade-
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 cytokymix.

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 mg/l basic fibroblast growth factor, 2 µg/l epidermal growth factor, 50 units/ml penicillin, and 100 µg/ml streptomycin. Cells were grown in 50:50 DMEM-Ham’s F-12 supplemented with 0.25 mg/l insulin, 0.11 mg/l transferrin, and 0.1 µg/l selenium. Nonasthmatic and asthmatic hASMCs were assayed at passage 6–8. Cells were treated with cytokymix (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 expressions were synthesized by IDT (Coralville, IA) using sequences according to the miRBase (version 19) (18, 19, 29). Reverse transcription was performed using 1 µg total RNA and an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time amplification was performed using RT² SYBR green/fluorescent quantitative RT-PCR (qPCR) Mastermix (Qiagen) and previously published primers for COX-2 (51), 18S rRNA (51), and IL-1β (48).

Mimic and inhibitor miRNA transfection. Either miR-146a-5p or miR-146b-5p miRIDIAN duplex mimics, Caenorhabditis elegans (cel)-67 miRIDIAN negative control duplex mimics, miR-146a-5p or miR-146b-5p miRIDIAN hairpin inhibitors, or cel-67 miRIDIAN negative control inhibitors (Thermo Fisher Scientific, Waltham, MA) were transfected into hASMCs by reverse transfection at 30 nM using 1 µg total RNA and an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time amplification was performed using RT² SYBR green/fluorescent quantitative RT-PCR (qPCR) Mastermix (Qiagen) and previously published primers for COX-2 (51), 18S rRNA (51), and IL-1β (48).

Western blotting. Protein samples were prepared as previously described (51). Total protein for COX-2 (15 µg), pro-IL-1β (15 µg), and HuR (10 µg) was separated by SDS-PAGE on 10% bis-Tris polyacrylamide gels in NuPAGE MOPS buffer (Life Technologies) and transferred to nitrocellulose in NuPAGE transfer buffer containing 10% methanol. Equal loading was verified using Faststain (G-biosciences, St. Louis, MO). Blocking was performed using 50:50 phosphate-buffered saline/Odyssey blocking solution (LI-COR, Lincoln, NE). Anti-COX-2 (sc-1745; Santa Cruz, Dallas, TX) and anti-IL-1β (AF201NA; R&D Systems, Minneapolis, MN) antibodies were used as previously published (22, 51). Anti-HuR (sc-5261; Santa Cruz) antibody was used at a dilution of 1:500. Fluorescent secondary antibodies (IRDye 800CW; LI-COR) were detected with an Odyssey near-infrared scanner (LI-COR). Integrated intensities of protein bands were expressed relative to protein levels in control mimic or control inhibitor-treated cells as appropriate.

RESULTS

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 cytokymix, we first had to determine if miRNA expression was induced under these proinflammatory conditions. Asthmatic and nonasthmatic hASMCs were left untreated, treated with cytokymix (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 cytokymix induced expression of both miR-146a (Fig. 1A) and miR-146b (Fig. 1B). miR-146a expression was higher in asthmatic than in nonasthmatic hASMCs treated with cytokymix (Fig. 1A). Compared with cytokymix, 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 cytokymix 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

Fig. 1. MicroRNA (miR)-146a and miR-146b abundance in cytokine-treated human airway smooth muscle cells (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. nonasthmatic 20 h treated with cytomix. 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/b expression and function in airway smooth muscle

miR-146a/b expression and function in airway smooth muscle. 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.

miR-146a represses HuR expression in hASMCs. miR-146a is not predicted to directly target the 3′-untranslated region of the IL-1β transcript (3), which raises the question of what the mechanism of action is for repressing IL-1β expression. Early studies observed that miR-146a/b repressed the expression of IRAK1 and TRAF6 (3a, 54). Later, Cheng and colleagues demonstrated that miR-146a also directly represses the expression of HuR in endothelial cells (9). HuR is downstream of IRAK1 and TRAF6 and is a direct mRNA-binding protein that stabilizes many inflammation-related miRNAs (15). Because miR-146a downregulation of cytokine synthesis was found not to be mediated by repression of IRAK1 and TRAF6 in a previous study in hASMCs (34), we chose to investigate miR-146a targeting of HuR. We used a gain-of-function and loss-of-function approach as above. Transfection of 30 nM miR-146a mimic reduced HuR protein abundance (Fig. 6A) in untreated cells and in cells that were treated with cytomix. Inhibition of miR-146a using 30 nM of the inhibitor increased HuR protein abundance (Fig. 6B) in untreated cells and in cells treated with cytomix. Transfecting the cells with 90 nM of the miR-146a inhibitor did not have an additive effect on the enhancement of HuR expression (data not shown). We did not investigate whether miR-146b negatively regulated HuR expression in hASMCs because there is no evidence that it is an endogenous negative regulator of IL-1β expression in cells treated with cytomix (see Fig. 5). Our results indicate that miR-146a can negatively regulate expression of HuR in hASMCs, which could contribute to destabilization of cytokine-stimulated miRNAs and reduced pro-IL-1β abundance.

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/b 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-146b expression depending on cell type and the pathways being activated at any given time. Therefore, we investigated expression and processing of miR-146a and miR-146b in hASMCs under conditions that mimic the proinflammatory state in the airways of humans with asthma.

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. Further-

![Graph A](image1.png)

**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.

![Graph B](image2.png)

**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. β2-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-
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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