Pharmacologic Agents Elevating cAMP Prevent Arginase II Expression and Proliferation of Pulmonary Artery Smooth Muscle Cells

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Arginase II has been shown to be involved in the hypoxia-induced proliferation of human pulmonary artery smooth muscle cells (hPASMCs). The signal transduction pathways responsible for the induction of arginase II are poorly understood. Cyclic AMP is involved in many intracellular processes, and cAMP levels are regulated by a balance between production via adenylate cyclases and degradation via phosphodiesterases. The purpose of this study was to determine the effects of cAMP on hypoxia-induced arginase expression, activity, and proliferation in hPASMCs. We found that the cAMP analog 8-Bromo-cAMP (8-Br-cAMP), the adenylate cyclase activator forskolin, and the phosphodiesterase 3 inhibitor cilostamide prevented the hypoxic induction of arginase II mRNA and protein expression in hPASMCs. The inhibition of arginase II protein was found to be mediated by exchange protein directly activated by cAMP. Arginase activity was decreased by 8-Br-cAMP, as evidenced by significantly lower V_{max} for arginase in normoxia and hypoxia. The hypoxia-induced hPASMC proliferation was completely prevented by the addition of 8-Br-cAMP, forskolin, or cilostamide. These data are the first to describe the inhibitory effect of cAMP on arginase activity, expression, and resultant proliferation of hypoxic hPASMCs.

Keywords: pulmonary hypertension; L-arginine; vascular remodeling; exchange protein directly activated by cAMP (Epac); phosphodiesterase

Pulmonary artery hypertension (PAH) is a life-threatening complication of chronic hypoxic lung diseases. The hallmarks of PAH are elevated pulmonary vascular resistance and vascular remodeling (1, 2). The smooth muscle layer, in particular, plays an integral role in the pathogenesis of PAH, exhibiting smooth muscle extension into smaller, normally nonmuscular pulmonary arteries within the respiratory acinus (1, 3). In addition, pulmonary artery smooth muscle cells (PASMCs) markedly proliferate, resulting in decreased luminal diameters, and may lead to the obstruction of resistance level pulmonary arteries (1, 4).

Arginase, of which there are two described isoforms, has been shown to be important in the proliferation of different cell types (5). Arginase I is a cytosolic enzyme highly expressed in the liver, while arginase II is an extrahepatic mitochondrial protein (6). Both arginase isoforms are expressed in the lung (7). Arginase metabolizes the conversion of L-arginine to L-ornithine,

CLINICAL RELEVANCE

Pulmonary hypertension (PH), with characteristic findings of vascular remodeling and vascular smooth muscle cell proliferation, is a life-threatening disease involving the L-arginine and arachidonic acid pathways. Only supportive therapies are available with no known cure. The use of cAMP-elevating agents has been reported as a therapy for PH; therefore, understanding the mechanisms involved, including the interactions of these pathways, is critical for discovering novel therapeutic targets for PH.

and urea and is the first step in polyamine and proline synthesis. Polyamines and proline are critical for cell proliferation, differentiation, tissue repair, and growth (8, 9). We have previously shown that hypoxia-induced proliferation in human PASMCs (hPASMCs) depends on the induction of arginase II (10).

Cyclic nucleotides, such as cAMP, are critical intracellular messengers involved in many intracellular processes, including signal transduction. Pharmacologic agents that increase cAMP levels have been used to treat PAH due to their ability to inhibit growth of PASMCs and cause vasodilation (11). Cyclic AMP levels in the cell are regulated by a balance between its production through the adenylate cyclases (ACs) and degradation by the cyclic nucleotide phosphodiesterases (PDEs) (12, 13). In the pulmonary vasculature, PDE3 is implicated as one of the predominant isoforms for cAMP degradation (14, 15). Furthermore, cAMP analogs or cAMP-elevating agents have been shown to inhibit hPASMC proliferation (16, 17) and hypoxia-induced hPASMC proliferation (18). Therefore, in the present study, we tested the hypothesis that pharmacologic interventions that increase intracellular cAMP levels would prevent hypoxia-induced arginase II expression and the resultant arginase-dependent hPASMC proliferation. We used the cell-permeable cAMP analog 8-Br-cAMP, the adenylate cyclase activator forskolin, and the PDE3 inhibitor cilostamide to augment intracellular levels of cAMP. Furthermore, we sought to determine the mechanism by which cAMP exerts its effects on arginase expression and cell proliferation using cAMP pathway-selective analogs.

MATERIALS AND METHODS

Cell Culture

hPASMCs (Lonza, Basel, Switzerland) were grown in 21%O₂-5%CO₂-balance N₂ (normoxia) at 37°C in smooth muscle growth media (Lonza). The hPASMCs were used in experiments between passages 5 through 8, during which no cell morphology changes were noted. The cells were grown to approximately 80 to 90% confluence and washed, and fresh media was placed on the cells. The cAMP analog 8-Bromo-cAMP (8-Br-cAMP) (Enzo Life Sciences, Plymouth Meeting, PA) (5, 10, or 50 μ M), the AC activator forskolin (Enzo Life Sciences) (1, 10, or

(Received in original form January 16, 2011 and in final form March 13, 2012)

This work was supported by National Institutes of Health grants HL-075261 (L.D.N.) and 1K08HL105677-01A1 (B.C.) from the National Heart, Lung, and Blood Institute.

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Am J Respir Cell Mol Biol Vol 47, Iss. 2, pp 218-226, Aug 2012

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Originally Published in Press as DOI: 10.1165/rcmb.2011-0015OC on November 17, 2011

Internet address: www.atsjournals.org

100 μ M), or the PDE3 inhibitor cilostamide (Enzo Life Sciences) (3, 10, or 30 μ M) was added to the cells, and cells were incubated in normoxia or 1% O₂-5% CO₂-balance N₂ (hypoxia) for 24 or 48 hours.

The membrane-permeant, putative exchange protein directly activated by cAMP (Epac)-specific cAMP analog 8-(4-chlorophenylthio)-2'-O-Me-cAMP-AM (8-pCPT-AM; Biolog Life Science Institute, Bremen, Germany) (1 μ M) and/or the putative PKA-specific cAMP analog N⁶-Monobutyladenosine-3',5'-cAMP (6-MB-cAMP; Biolog) (50 μ M) were also used.

RNA Isolation, Reverse Transcription, and Quantitative Real-Time PCR

RNA was isolated from hPASMCs and reverse transcribed, and quantitative real-time PCR performed as previously described (10). Relative arginase I and II mRNA amounts were normalized to 18S mRNA expression using the $\Delta\Delta$ CT method (19). Samples were analyzed in duplicate.

Protein Isolation and Western Blot

After treatment, hPASMCs were harvested for total protein as previously described (10), and protein concentration was determined by the Bradford method (20). Protein was assayed for arginase I (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA) or arginase II (1:500) (Santa Cruz Biotechnology) expression by Western blot analysis as previously described (10). Protein bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) and quantified using densitometry (Sigma Gel; Jandel Scientific, San Rafael, CA). Expression of arginase I and II were normalized to β -actin (1:10,000) (Abcam, Cambridge, MA).

Arginase Activity

Arginase activity was measured in lysed hPASMCs as previously described (10). For one experiment, 16 10-cm-diameter cell culture plates were used to yield 200 μ g protein for each sample. The effect of cAMP on arginase activity in hPASMCs was assessed by adding vehicle or 8-Br-cAMP (50 μ M) to hPASMCs before incubation in normoxia or hypoxia for 48 hours. Arginase activity was calculated as the amount of urea produced (μ mol) over time normalized to protein (μ mol/[mg protein/min]). The reaction velocity was plotted against substrate concentration, and the $V_{\max}$ and K_m values were determined using the Michaelis-Menten equation (GraphPad Prism, San Diego, CA).

Proliferation Assay

Cell proliferation was determined as previously described (10). To determine the role of cAMP in hypoxia-induced hPASC proliferation, 8-Br-cAMP, forskolin, or cilostamide was added to the media, and cell proliferation was determined. 8-pCPT-AM and/or 6-MB-cAMP were also added to the media, and cell proliferation was determined.

Statistical Analysis

Values are given as means $\pm$ SEM. Unpaired *t* test or one-way or two-way ANOVA was used to compare the data between groups. Data were analyzed using one-way ANOVA with Bonferroni's *post hoc* testing for parametric data. Two-way ANOVA was used with treatment concentrations and oxygen exposure as independent variables. Individual differences were detected using modified *t* test if significance was determined by two-way ANOVA (GraphPad). Differences were considered significant when $P < 0.05$.

RESULTS

To determine the effects of pharmacologic manipulation of the cAMP pathway on hypoxia-induced arginase mRNA expression, increasing concentrations of the cAMP analog 8-Br-cAMP, the AC activator forskolin, or the PDE3 inhibitor cilostamide were added to the media of hPASMCs, grown to approximately 80 to

90% confluence, and incubated in normoxia or hypoxia for 24 or 48 hours. RNA was harvested, and arginase I and arginase II mRNA expression were assessed by quantitative real-time PCR. Data are shown as fold-change relative to normoxia-exposed hPASMC controls at each respective time-point. Overall, there was an approximately 3-fold increase ($P < 0.01$) in arginase II mRNA expression in hPASMCs exposed to hypoxia relative to those exposed to normoxia at 24 and 48 hours (Figures 1A, 1B, 2A, 2B, 3A, and 3B). All concentrations of 8-Br-cAMP tested prevented the hypoxia-induced increase in arginase II mRNA at 24 and 48 hours (Figures 1A and 1B). The highest concentration of forskolin (100 μ M) tested also prevented the hypoxia-induced increase in arginase II mRNA expression (Figures 2A and 2B). Cilostamide (30 μ M) inhibited the hypoxia-induced arginase II mRNA expression to levels equivalent to vehicle-treated, normoxia controls at 24 and 48 hours (Figures 3A and 3B). There were no differences at 24 or 48 hours in arginase I mRNA expression between hPASMCs exposed to normoxia or hypoxia, and the addition of 8-Br-cAMP, forskolin, or cilostamide had no effect on arginase I mRNA expression (data not shown).

To determine the effects of pharmacologic manipulation of the cAMP pathway on hypoxia-induced arginase protein expression, increasing concentrations of 8-Br-cAMP, forskolin, or cilostamide were added to the media of hPASMCs grown to approximately 80 to 90% confluence and incubated in normoxia or hypoxia for 24 or 48 hours. Protein was harvested, and arginase I and arginase II protein expression was measured using Western blot analysis and quantified by densitometry. Data are shown as fold-change relative to normoxia-exposed hPASMC controls at each respective time point. Overall, hPASMCs exposed to hypoxia had an approximately 2- to 3-fold ($P < 0.01$) increase in arginase II protein expression at 24 hours (Figures 1C, 1D, 2C, 2D, 3C, and 3D) and an approximately 3- to 4-fold ($P < 0.01$) increase at 48 hours (Figures 1E, 1F, 2E, 2F, 3E, and 3F) relative to those exposed to normoxia at the same time points. The addition of 8-Br-cAMP to the media prevented the hypoxia-induced increase in arginase II protein levels at all concentrations studied at 24 (Figure 1C and 1D) and 48 hours (Figure 1E and 1F) to levels equivalent to vehicle-treated, normoxia controls at each respective time point. Forskolin had little effect on arginase II protein levels at 24 hours (Figures 2C and 2D). However, forskolin caused a concentration-dependent inhibition of hypoxia-induced arginase II protein levels such that at the highest concentration (100 μ M), the hypoxia-induced arginase II protein levels were not different from vehicle-treated normoxia controls at 48 hours (Figures 2E and 2F). Cilostamide inhibited the hypoxia-induced arginase II protein expression to levels not different from vehicle-treated normoxia controls at 30 μ M concentrations at 24 (Figures 3C and 3D) and 48 hours (Figures 3E and 3F). There was no difference in arginase I protein expression between hPASMCs exposed to normoxia and hypoxia with and without 8-Br-cAMP, cilostamide, or forskolin at 24 or 48 hours (data not shown).

We determined the effects of pharmacologic manipulation of the cAMP pathway on arginase activity in hPASMCs. hPASMCs were treated with vehicle or 8-Br-cAMP and incubated in normoxia or hypoxia for 48 hours. The cell lysates were incubated with increasing concentrations of L-arginine, and urea production was measured. Michaelis-Menten kinetics for arginase were determined. Hypoxia significantly increased the $V_{\max}$ for arginase relative to normoxic vehicle-treated cells (Figures 4A and 4B) ($V_{\max}$ 0.50 ± 0.11 μ mol/[mg protein/min] [hypoxia] versus 0.21 ± 0.05 μ mol/[mg protein/min] [normoxia]; $P < 0.05$). The K_m for arginase was not different between the two groups (1.6 ± 0.2 mM, hypoxia versus 2.1 ± 0.8 mM, normoxia). The addition of 8-Br-cAMP (50 μ M) to hPASMCs had no significant

Figure 1. 8-Br-cAMP inhibits hypoxia-induced arginase II mRNA and protein expression in human pulmonary artery smooth muscle cells (hPASMCs). hPASMCs were incubated with vehicle or 8-Br-cAMP ($n = 5-9$) and exposed to 21% O₂ (normoxia) or 1% O₂ (hypoxia) for (A, C, D) 24 or (B, E, F) 48 hours. RNA and protein were harvested. Arginase II mRNA levels were analyzed by quantitative real-time PCR and normalized to 18S expression using the $\Delta\Delta CT$ method. Data are shown as fold change $\pm$ SEM with the addition of 8-Br-cAMP (A, B) relative to respective normoxia controls. Arginase II protein expression was analyzed by Western blot and normalized to β -actin. Representative Western blots are shown for arginase II and β -actin in hPASMCs exposed to normoxia or hypoxia at 24 (C) or 48 hours (E). Data are shown as fold change $\pm$ SEM relative to β -actin at 24 (D) and 48 hours (F). *Hypoxia different from normoxia controls at same time point: $P < 0.01$ (A, B); $P < 0.05$ (D, F). †Hypoxia 8-Br-cAMP-treated different from hypoxia, vehicle-treated: $P < 0.05$ (A, B); $P < 0.01$ (D, F).

effect on the $V_{\max}$ for arginase in normoxic hPASMCs; however, it completely inhibited the hypoxia-induced arginase activity ($P < 0.05$) (Figures 4A and 4B) to levels not different from the baseline arginase activity of normoxic vehicle-treated cells. The K_m for arginase was not different between the hypoxic vehicle-treated and hypoxic 8-Br-cAMP-treated hPASMCs (1.6 ± 0.2 mM [vehicle] versus 3 ± 0.8 mM [8-Br-cAMP]). When 8-Br-cAMP was added directly to the cell lysates of hypoxic vehicle-treated hPASMCs at the start of the arginase activity assay, there was no effect of 8-Br-cAMP on arginase activity (Figures 4C and 4D).

To determine the role of pharmacologic manipulation of the cAMP pathway on hypoxia-induced proliferation in hPASMCs, 8-Br-cAMP, forskolin, or cilostamide were used. hPASMCs were seeded on 6-well plates at a density of approximately 10,000 cells per well, and each of the pharmacologic agents or vehicle was added to the medium. The cells were incubated for 120 hours in normoxia or hypoxia, and viable cell numbers were determined

using trypan blue exclusion. Data are shown as a percentage of normoxia vehicle-treated controls. Overall, hypoxia significantly increased cell proliferation (Figure 5). In normoxia, 8-Br-cAMP significantly decreased hPASMC proliferation, and 8-Br-cAMP prevented hypoxia-induced hPASMC proliferation (Figure 5A). Indeed, the 10 and 50 μ M concentrations resulted in hPASMC proliferation at levels below that of normoxia vehicle-treated control (Figure 5A). Treatment of hPASMCs with forskolin decreased proliferation in normoxia (Figure 5B). In addition, forskolin completely prevented the hypoxia-induced hPASMC proliferation (Figure 5B). In normoxia, cilostamide (30 μ M) did not significantly decrease hPASMC proliferation (Figure 5C). However, cilostamide completely prevented the hypoxia-induced hPASMC proliferation, such that viable cell numbers in the hypoxic cilostamide-treated hPASMCs were not different from viable cell numbers in the normoxic vehicle-treated hPASMCs (Figure 5C).

Human PASMCs were treated with the pathway selective cAMP analogs 8-pCPT-AM (a membrane permeant),

Figure 2. Forskolin inhibits hypoxia-induced arginase II mRNA and protein expression in hPASMCs. hPASMCs were incubated with vehicle or forskolin ($n = 5-6$) and exposed to 21% O₂ (normoxia) or 1% O₂ (hypoxia) for (A, C, D) 24 or (B, E, F) 48 hours. RNA and protein were harvested. Arginase II mRNA levels were analyzed by quantitative real-time PCR and normalized to 18S expression using the $\Delta\Delta CT$ method. Data are shown as fold change $\pm$ SEM with addition of forskolin (A, B) relative to respective normoxia controls. Arginase II protein expression was analyzed by Western blot and normalized to β -actin. Representative Western blots are shown for arginase II and β -actin in hPASMCs exposed to normoxia or hypoxia at 24 (C) or 48 hours (E). Data are shown as fold change $\pm$ SEM relative to β -actin at 24 (D) and 48 hours (F). *Hypoxia different from normoxia controls at same time point: $P < 0.01$ (A, B); $P < 0.05$ (D, F). †Hypoxia, forskolin-treated different from hypoxia, vehicle-treated: $P < 0.01$.

Epac-selective cAMP analog, and 6-MB-cAMP (a PKA-selective cAMP analog) to determine the pathway by which cAMP alters protein expression. In these sets of experiments, 8-Br-cAMP, which acts through Epac and PKA, 8-pCPT-AM, 6-MB-cAMP, or a combination of 8-pCPT-AM + 6-MB-cAMP, were added to the media of hPASMCs grown to approximately 80 to 90% confluence and incubated in normoxia or hypoxia for 48 hours. Protein was harvested, and arginase II protein expression was measured using Western blot analysis and quantified by densitometry. Data are shown as mean density of arginase II normalized to β -actin. 8-Br-cAMP inhibited the hypoxia-induced arginase II protein expression, as shown previously (Figure 6). The pathway selective analog 6-MB-cAMP did not have a statistically significant effect on arginase II protein expression (Figure 6); however, the activation of the Epac pathway via 8-pCPT-AM significantly prevented the hypoxia-induced arginase II protein expression to the same extent as the 8-Br-cAMP-treated hypoxic hPASMCs. The activation of both pathways using the combination of

8-pCPT-AM + 6-MB-cAMP did not result in further inhibition of hypoxia-induced arginase II protein than 8-pCPT-AM alone.

To elucidate the pathway by which cAMP alters hypoxia-induced hPASMC proliferation, 8-Br-cAMP, 8-pCPT-AM, 6-MB-cAMP, or a combination of 8-pCPT-AM + 6-MB-cAMP were used. hPASMCs were seeded on 6-well plates at a density of approximately 10,000 cells per well, and each of the pharmacologic agents or vehicle was added to the medium. The cells were incubated for 120 hours in normoxia or hypoxia, and viable cell numbers were determined using trypan blue exclusion. Data are shown as a percentage of normoxia vehicle-treated controls. Similar to previous results, in normoxia 8-Br-cAMP significantly decreased hPASMC proliferation, and 8-Br-cAMP prevented hypoxia-induced hPASMC proliferation (Figure 7). Activation of the Epac pathway using 8-pCPT-AM significantly attenuated hPASMC proliferation in normoxia and hypoxia (Figure 7). The addition of the PKA-selective cAMP analog 6-MB-cAMP had similar effects, with

Figure 3. Cilostamide inhibits hypoxia-induced arginase II mRNA and protein expression in hPASCs. hPASCs were incubated with vehicle or cilostamide ($n = 4-6$) and exposed to 21% O₂ (normoxia) or 1% O₂ (hypoxia) for (A, C, D) 24 or (B, E, F) 48 hours. RNA and protein were harvested. Arginase II mRNA levels were analyzed by quantitative real-time PCR and normalized to 18S expression using the $\Delta\Delta C_T$ method. Data are shown as fold change $\pm$ SEM with the addition of cilostamide (A, B) relative to respective normoxia controls. Arginase II protein expression was analyzed by Western blot and normalized to β -actin. Representative Western blots are shown for arginase II and β -actin in hPASCs exposed to normoxia or hypoxia for 24 (C) or 48 hours (E). Data are shown as fold change $\pm$ SEM relative to β -actin at 24 (D) and 48 hours (F). *Hypoxia different from normoxia controls at same time point: $P < 0.01$ (A, B); $P < 0.05$ (D, F). †Hypoxia, cilostamide-treated different from hypoxia, vehicle-treated: $P < 0.01$.

attenuated cell proliferation in normoxia and hypoxia. Although the Epac-selective cAMP analog and the PKA-selective cAMP analog individually inhibited hPASC proliferation in normoxia and hypoxia, neither did so to the same extent as that caused by 8-Br-cAMP (Figure 7). The activation of the Epac and PKA pathways using a combination of 8-pCPT-AM + 6-MB-cAMP resulted in a further synergistic decrease in cell proliferation in normoxia and hypoxia but not to the same extent as that caused by 8-Br-cAMP (Figure 7).

DISCUSSION

The main objective of the present study was to determine the effects of pharmacological manipulation of the cAMP pathway on hypoxia-induced arginase II expression, arginase activity, and hypoxia-induced proliferation in hPASCs. The major findings of this study were: (1) consistent with our previous findings (10), hypoxia increased arginase activity, arginase II mRNA and

protein expression, and increased proliferation in hPASCs; (2) pharmacologic agents that raise intracellular cAMP levels prevented hypoxia-induced increases in arginase II mRNA and protein expression; (3) pharmacologic agents that raise cAMP levels prevented the hypoxia-induced increase in arginase activity; (4) cAMP inhibits hypoxia-induced arginase II protein expression via a PKA-independent pathway through Epac; (5) pharmacologic agents that raise intracellular cAMP levels prevented hypoxia-induced hPASC proliferation; and (6) the attenuation of hypoxia-induced arginase II protein expression via Epac-mediated signaling by cAMP does not solely account for the inhibition of hypoxia-induced hPASC proliferation. Taken together, these findings reveal that in hypoxic hPASCs, arginase II expression and activity are significantly inhibited by cAMP through Epac. Although the Epac-selective pathway only partially accounts for the antiproliferative effects of cAMP, these studies are the first to describe the inhibitory effect of cAMP on arginase II induction.

Figure 4. cAMP inhibits arginase activity in hypoxic hPASCs. hPASCs were incubated with vehicle or 8-Br-cAMP and exposed to 21% O₂ (normoxia) or 1% O₂ (hypoxia) for 48 hours ($n = 3$ for each group). Cell lysates were incubated with increasing concentrations of L-arginine, and urea production was measured. The reaction velocity was calculated as μ mol/(mg protein/min). (A) The data were fit using the Michaelis-Menten equation to determine $V_{\max}$ and K_m values. (B) Data are shown as a bar graph representing $V_{\max}$ for arginase. There was no significant difference in the $V_{\max}$ for arginase in the normoxic cells when treated with 8-Br-cAMP (0.21 $\pm$ 0.05 μ mol/[mg protein/min], vehicle versus 0.10 $\pm$ 0.01 μ mol/[mg protein/min], 8-Br-cAMP). Hypoxia significantly increased the $V_{\max}$ for arginase relative to normoxic, vehicle-treated cells (0.50 $\pm$ 0.11 μ mol/[mg protein/min], hypoxia versus 0.21 $\pm$ 0.05 μ mol/[mg protein/min], vehicle; $P < 0.05$), whereas the K_m for arginase was unchanged (1.6 $\pm$ 0.2 mM, hypoxia versus 2.1 $\pm$ 0.8 mM, vehicle). The addition of 8-Br-

cAMP to hypoxic hPASCs significantly decreased the $V_{\max}$ for arginase relative to hypoxia, vehicle-treated cells (0.21 $\pm$ 0.08 μ mol/[mg protein/min], 8-Br-cAMP versus 0.50 $\pm$ 0.11 μ mol/[mg protein/min], vehicle; $P < 0.05$), such that the $V_{\max}$ for arginase is not different from normoxia, vehicle-treated hPASCs. The K_m for arginase was not significantly different in hypoxic hPASCs (3.0 $\pm$ 0.8 mM, 8-Br-cAMP versus 1.6 $\pm$ 0.2 mM, vehicle). *Hypoxia-treated different from vehicle-treated: $P < 0.05$. †Hypoxia, 8-Br-cAMP-treated different from hypoxia, vehicle-treated: $P < 0.05$. 8-Br-cAMP was also added to the cell lysates after exposure to normoxia and hypoxia for 48 hours ($n = 1$ for each group). (C, D) There was no significant difference in arginase activity or $V_{\max}$ with the addition of 8-Br-cAMP in normoxia or hypoxia.

The effects of cAMP on arginase may be specific to the isoform induced, the stimuli, or the cell type. For example, in contrast to the inhibitory action of cAMP on arginase expression and activity in hypoxic hPASCs, forskolin enhanced the production of cAMP, increased basal arginase activity, and potentiated cytokine-induced arginase I expression in rat aortic smooth muscle cells (21). In the same study, inhibition of PKA, an enzyme downstream of cAMP, decreased basal arginase activity and expression and attenuated the increase in arginase activity and induction by IL-4 and IL-13 (21). Cyclic AMP analogs have been found to induce arginase activity in bone marrow-derived macrophages and RAW 264.7 cells (22–24). In fact, Sosroseno and colleagues (24) showed an increased arginase activity with cAMP-elevating agents (no effect was seen with cGMP-elevating agents) in nonstimulated RAW 264.7 cells and to a greater extent in cells stimulated with LPS. Erdelyi and colleagues (25) found that arginase activity due to arginase I mRNA and protein induction was slightly increased with cAMP-elevating agents and synergistically increased with cytokine stimulation. Arginase I expression was also induced by cAMP in hepatocytes (26). In Caco-2 tumor cells, arginase activity due to arginase II induction increased after treatment with cAMP-elevating agents (27). Thus, cAMP can induce or potentiate arginase activity due to cytokine

stimulation in some cell types. To our knowledge, we are the first to describe not only the effects of cAMP on arginase in hPASC but also the inhibitory action of cAMP-elevating agents on hypoxia-induced arginase expression. We also found that cAMP was not a direct inhibitor of arginase activity (Figure 4) but rather inhibits the hypoxic induction of arginase mRNA and protein in hPASCs.

The finding that cAMP inhibited hypoxia-induced arginase expression and activity in hPASCs was distinctive given its induction of arginase in other cell types. The mechanism(s) underlying the cAMP inhibition of arginase expression in hypoxic hPASCs appear to be mediated through a PKA-independent pathway. Two classic cAMP signaling pathways that have been studied are the cAMP-dependent protein kinase (PKA) and the Epac proteins, which may be responsible for downstream transcriptional and/or translational effects of cAMP. We have found that using a membrane-permeant, putative Epac-selective cAMP analog inhibited the hypoxia-induced arginase II protein expression in hPASCs, whereas using a putative PKA-selective cAMP analog alone had no effect on arginase II protein expression. Combining the PKA- and Epac-selective cAMP analogs did not further inhibit arginase II protein expression compared with an Epac-selective analog alone. Indeed, the combination of PKA- and Epac-selective cAMP analogs had the same effect on arginase as 8-Br-cAMP,

a membrane-permeable activator of PKA and of Epac. Thus, the mechanism responsible for the cAMP inhibitory effect of arginase expression in hypoxic hPASMCs is largely via the activation of Epac. In RAW-macrophages, the cAMP-dependent regulation of arginase expression has not been found to be associated with Epac (28) but rather is a result of PKA (24). However, the cAMP effects on arginase expression were clearly different in

Figure 5. cAMP-elevating agents prevent hypoxia-induced proliferation of hPASMCs. hPASMCs were incubated with vehicle, 8-Br-cAMP, forskolin, or cilostamide and exposed to 21% O₂ (normoxia) or 1% O₂ (hypoxia) for 120 hours ($n = 6$ for each group). Proliferation of hPASMCs was determined by trypan blue exclusion and cell counting using a hemacytometer. Initial seeding density was approximately 10,000 cells per well. Data are shown as % of normoxia, vehicle-treated controls $\pm$ SEM with the addition of 8-Br-cAMP (A), forskolin (B), or cilostamide (C). *Hypoxia, vehicle-treated different from normoxia, vehicle-treated controls: $P < 0.02$. †Hypoxia, cAMP-elevating agent different from hypoxia, vehicle-treated: $P < 0.02$. ‡cAMP-elevating agent different from normoxia, vehicle-treated controls: $P < 0.01$ (A); $P < 0.05$ (B).

RAW cells than in hPASMCs; the different pathway may account for the different mechanism of action.

Epac, of which there are two isoforms, are guanine nucleotide exchange factors for the Ras family of small GTPases, Rap1 and Rap2. Increasing evidence indicates that the Epac are involved in multiple biological actions of cAMP, including roles in cardiovascular physiology and pathophysiology (29). Although several studies have demonstrated that the Epac signaling results in Rap1 activation (30), more recent data suggest that some of the effects of cAMP on vascular smooth muscle cells may be Rap1 independent (31). Further studies are needed to fully elucidate the signaling mechanisms involved in the inhibition of hypoxia-induced arginase II expression as a result of activation of Epac by cAMP.

The growth inhibitory effect of cAMP is consistent with previous studies. Antiproliferative effects of cAMP have also been observed in vascular endothelial growth factor- or fibroblast growth factor-stimulated endothelial cells treated with 8-Br-cAMP or forskolin (32). Prostacyclin analogs, which increase cAMP production, inhibited DNA synthesis and proliferation in PDGF-BB-stimulated hPASMCs (16). In addition, beraprost sodium, a prostacyclin analog, and 8-Br-cAMP prevented the hypoxia-induced proliferation of hPASMCs (18). Interestingly, Millen and colleagues (33) found that hypoxia resulted in increased intracellular levels of cAMP. Elevated cAMP levels have been shown to arrest cells in the G1 phase of the cell cycle, at least in part via inhibition of G1-regulatory proteins Cyclin D₁ and Skp2 (34–36). Other researchers have found higher levels of cAMP response element-binding protein phosphorylation, p53, and p21 protein responsible for the cell cycle arrest associated with elevated cAMP levels (37). We have recently shown that hypoxia promotes hPASMC proliferation specifically through the induction of the arginase II isoform (10), and we are the first to describe that cAMP prevents hypoxia-induced hPASMC proliferation at least in part by inhibiting induction of arginase II. Although we found that cAMP prevented the hypoxia-induced arginase II expression via Epac-mediated signaling, the hypoxia-induced cell proliferation was not solely a result of its inhibitory effect on arginase. We found that the Epac-selective cAMP analog and the PKA-selective cAMP analog each attenuated hPASMC proliferation in normoxia and hypoxia to the same extent (Figure 7). The combination of both pathway-specific cAMP analogs resulted in a synergistic inhibitory effect on hPASMC proliferation but was less than using 8-Br-cAMP, which is a nonselective cAMP analog. Hower and colleagues recently showed no effect on vascular smooth muscle cell proliferation using a PKA-selective cAMP analog or an Epac-selective analog alone (31). The authors did find that, although the activation of PKA or Epac individually is insufficient, consistent with our findings, the simultaneous activation of both of the cAMP-dependent pathways resulted in growth arrest in

Figure 6. Exchange protein directly activated by cAMP (Epac)-selective cAMP analogs inhibit hypoxia-induced arginase II protein expression in hPASCs. hPASCs were incubated with vehicle, 8-Br-cAMP ($n = 5$), 8-pCPT-AM ($n = 5$), 6-MB-cAMP ($n = 5$), or 8-pCPT-AM+6-MB-cAMP ($n = 5$) and exposed to 21% O₂ (normoxia) or 1% O₂ (hypoxia) for 48 hours. (A) Arginase II protein expression was analyzed by Western blot and normalized to β -actin. Representative Western blots are shown for arginase II and β -actin in hPASCs exposed to normoxia or hypoxia at 48 hours. (B) Data are shown as mean density of arginase II normalized to β -actin $\pm$ SEM. *Hypoxia different from normoxia controls: $P < 0.05$. †Hypoxia, cAMP analog different from hypoxia, vehicle-treated: $P < 0.05$.

vascular smooth muscle cells (31). Taken together, these findings suggest that the cAMP attenuation of arginase II protein expression via an Epac-dependent pathway is another mechanism involved in the cAMP inhibition of cell proliferation. Furthermore, our findings suggest that, although a substantial portion of the cAMP effect on hypoxia-induced hPASC proliferation is via inhibition of arginase II, there are other anti-proliferative effects of cAMP that do not involve arginase II expression.

In conclusion, we found that clinically relevant pharmacologic agents that increase intracellular cAMP concentrations inhibit hypoxia-induced arginase II expression and activity and prevent the hypoxia-induced proliferation of hPASCs. Cyclic AMP acts through a PKA-independent pathway via Epac to alter protein expression of arginase II and inhibit the resultant proliferation of hPASCs. These studies continue to support the notion that using pharmacologic agents that elevate intracellular cAMP concentrations may be beneficial for preventing

Figure 7. Pathway-selective cAMP analogs partially inhibit hypoxia-induced proliferation of hPASCs. hPASCs were incubated with vehicle, 8-Br-cAMP, 8-pCPT-AM, 6-MB-cAMP, or 8-pCPT-AM+6-MB-cAMP and exposed to 21% O₂ (normoxia) or 1% O₂ (hypoxia) for 120 hours ($n = 9-15$ for each group). Proliferation of hPASCs was determined by trypan blue exclusion and cell counting using a hemacytometer. Initial seeding density was approximately 10,000 cells per well. Data are shown as % of normoxia, vehicle-treated controls $\pm$ SEM. Each of the cAMP-analog-treated cells in normoxia and hypoxia were different from

vehicle-treated controls in normoxia and hypoxia ($P < 0.01$). *Hypoxia, vehicle-treated different from normoxia, vehicle-treated controls: $P < 0.01$. †cAMP analog-treated different from hypoxia, 8-Br-cAMP-treated: $P < 0.01$. ‡Hypoxia, cAMP analog-treated different from hypoxia, 8-pCPT-AM+6-MB-cAMP-treated: $P < 0.05$.

the remodeling and smooth muscle cell proliferation seen in pulmonary hypertensive diseases associated with hypoxia.

Author disclosures are available with the text of this article at www.atsjournals.org.

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