

Attenuation of Monocrotaline-Induced Pulmonary Hypertension by Luminal Adeno-Associated Virus Serotype 9 Gene Transfer of Prostacyclin Synthase

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Abstract

Idiopathic pulmonary arterial hypertension (iPAH) is associated with high morbidity and mortality. We evaluated whether luminal delivery of the human prostacyclin synthase (*hPGIS*) cDNA with adeno-associated virus (AAV) vectors could attenuate PAH. AAV serotype 5 (AAV5) and AAV9 vectors containing the *hPGIS* cDNA under the control of a cytomegalovirus-enhanced chicken β -actin (CB) promoter or vehicle (saline) were instilled into lungs of rats. Two days later, rats were injected with monocrotaline (MCT, 60 mg/kg) or saline. Biochemical, hemodynamic, and morphologic assessments were performed when the rats developed symptoms (3–4 weeks) or at 6 weeks. Luminal (airway) administration of AAV5 and AAV9CBhPGIS vectors (MCT-AAV5 and MCT-AAV9 rats) significantly increased plasma levels of 6-keto-PGF_{1 α} as compared with MCT-controls, and closely resembled levels measured in rats not treated with MCT (saline–saline). Right ventricular (RV)/left ventricular (LV)+septum (S) ratios and RV systolic pressure (RVSP) were greater in MCT-control rats than in saline–saline rats, whereas the ratios and RVSP in MCT-AAV5CBhPGIS and MCT-AAV9CBhPGIS rats were similar to saline–saline rats. Thickening of the muscular media of small pulmonary arteries of MCT-control rats was detected in histological sections, whereas the thickness of the muscular media in MCT-AAV5CBhPGIS and MCT-AAV9CBhPGIS rats was similar to saline–saline controls. In experiments with different promoters, a trend toward increased levels of PGF_{1 α} expression was detected in lung homogenates, but not plasma, of MCT-treated rats transduced with an AAV9-*hPGIS* vector containing a CB promoter. This correlated with significant reductions in the RV/LV + S ratio and RVSP in MCT-AAV9CBhPGIS rats that resembled levels in saline–saline rats. No changes in levels of PGF_{1 α} , RV/LV + S, or RVSP were detected in rats transduced with AAV9-*hPGIS* vectors containing a modified CB promoter (CB7) or a distal epithelial cell-specific promoter (CC10). Thus, AAV9CBhPGIS vectors prevented development of MCT-induced PAH and associated pulmonary vascular remodeling.

Introduction

IDIOPATHIC PULMONARY ARTERIAL HYPERTENSION (iPAH) is a potentially fatal disease of the pulmonary vasculature characterized by pulmonary artery endothelial cell, smooth muscle cell, and adventitial cell proliferation leading to vascular luminal obliteration, increased pulmonary vascular pressures and resistance, and right-sided heart failure with resultant high morbidity and mortality (McLaughlin *et al.*, 2002). Current therapies, including prostacyclin and its analogs, endothelial receptor antagonists, and phosphodiesterase-5 inhibitors, have led to improvements in dyspnea, exercise

tolerance, and survival (Rubin *et al.*, 2002; Galie and Branzi, 2005; Galie *et al.*, 2005; McLaughlin *et al.*, 2005; Sitbon *et al.*, 2005; McLaughlin, 2013). However, far too many affected individuals still die prematurely.

Although familial disease has been linked to mutations in *BMPR2*, *Alk1*, *endoglin*, and *SMAD9* (Deng *et al.*, 2000; International PPH Consortium *et al.*, 2000; Shintani *et al.*, 2009), the etiology of the majority of cases of PAH is complex with multiple pathophysiological mechanisms. A variety of molecular approaches have been considered for therapy of PAH, including cell-based approaches and direct vector delivery to the lung or vasculature (Campbell *et al.*,

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1999, 2001; Nagaya *et al.*, 2000; Kido *et al.*, 2005; McMurtry *et al.*, 2005; Zhao *et al.*, 2005). Prostacyclin synthase (PGIS), the rate-limiting enzyme for prostacyclin production, is an attractive therapeutic transgene for molecular approaches, as prostacyclin infusions have been reasonably well tolerated clinically and improve exercise tolerance and survival in patients with PAH. Attenuation of chronic hypoxic PAH by overexpression of the rat *PGIS* cDNA under the control of the epithelial-specific human surfactant protein C (hSPC) promoter established the feasibility of paracrine delivery of prostacyclin to the pulmonary vasculature (Geraci *et al.*, 1999). Intratracheal and direct hepatic injections of the hemagglutinating virus of Japan (HVJ)-PGIS liposomes have confirmed this concept (Ono *et al.*, 2004). Delivery of adeno-associated virus serotype 1 (AAV1)- and AAV2-hPGIS vectors by intramuscular injection has been shown to attenuate monocrotaline (MCT)-induced and chronic hypoxic PAH in rodents (Ito *et al.*, 2007; Kawakami *et al.*, 2007; Kataoka *et al.*, 2013). Recently, attenuation of established MCT-induced PAH by aerosolized airway luminal delivery of AAV1-SERCA2 with a Penn Century Device has been reported (Hadri *et al.*, 2013).

In this study, we evaluated whether noninvasive luminal airway delivery of AAV5- and AAV9-hPGIS serotype vectors could attenuate MCT-induced PAH. We also tested the ability of constitutive and tissue-specific promoters to mediate efficient gene transfer and attenuate MCT-induced PAH.

Materials and Methods

Vector production

Several vector constructs were generated for this project (Fig. 1A). The *hPGIS* cDNA was originally cloned from human mRNA by Dr. Wanda O'Neal (University of North Carolina at Chapel Hill, NC) and cloned into an AAV2

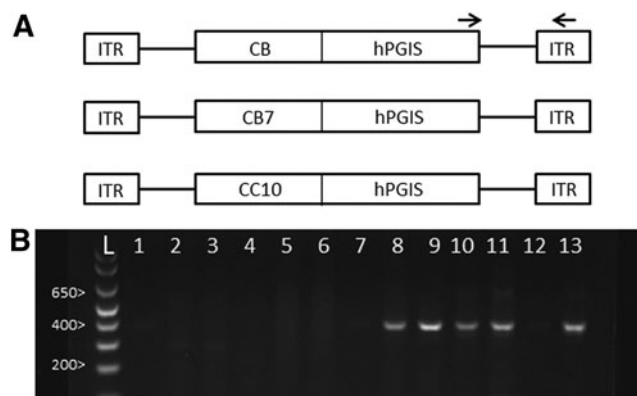


FIG. 1. (A) Schematic of AAV vectors. CB, cytomegalovirus-enhanced chicken β -actin promoter; CB7, modified CB promoter; CC10, clara cell secretory protein promoter. (B) PCR analysis of AAV-CBhPGIS vector DNA in rat lungs 4–6 weeks after AAV-CBhPGIS administration. Lanes 1 and 2, saline–saline; lanes 3 and 4, MCT-control; lanes 5–7, MCT-AAV-CBhPLAP; lanes 8–11, MCT-AAV-CBhPGIS; lane 12, negative control (water); lane 13, positive control (pAAVCBhPGIS plasmid); lane L, 1 kb ladder. Arrows in (A) depict the location of PCR primers selected for (B). AAV, adeno-associated virus; MCT, monocrotaline; PCR, polymerase chain reaction.

plasmid vector backbone (R. Jude Samulski, University of North Carolina at Chapel Hill, NC). This transgene was also cloned from human mRNA into the AAV backbone by Dr. Julie Johnston (Penn Vector Core, Philadelphia, PA). Three different promoters provided by the Penn Vector Core were used for our studies: the cytomegalovirus (CMV)-enhanced chicken β -actin (CB) promoter (Fisher *et al.*, 1996; Auricchio *et al.*, 2002), a modified CB (CB7) promoter (Penn Vector Core), and the CC10 promoter (Stripp *et al.*, 1992; Auricchio *et al.*, 2002). The CB7 promoter contains 282 bp of the core CB promoter as compared with 260 bp within the CB promoter, a second CMV enhancer element, and a large hybrid intron with a CB splice donor and rabbit β -globin splice acceptor. The AAV5 and AAV9 capsids were also provided by the Penn Vector Core. For this study, vectors produced with the AAV5 and AAV9 capsids contain an AAV2 vector genome and are referred to henceforth as AAV5 and AAV9 vectors.

The AAV9CC10hPGIS, AAV5CB7hPGIS, and initial lot of AAV9CBhPGIS vectors were produced locally and titered by dot blot assay as previously described (Grieger *et al.*, 2006). Clinical-grade AAV9CBhPGIS and AAV9-CB7hPGIS for experiments were obtained from the Penn Vector Core under the aegis of the NHLBI's Gene Therapy Resource Program.

Vector administration

All animal experiments and procedures were approved by the Institutional Animal Care and Use Committees of the University of Arkansas for Medical Sciences and the Central Arkansas Veterans Healthcare System. Rats (~150 g) were purchased from Charles River Laboratories. Experiments were conducted in two different strains of rats. The Fisher 344 rat strain was employed in our initial studies of AAV5 versus AAV9 serotypes (Figs. 2 and 3). However, subsequent studies with different promoters were conducted in Sprague Dawley rats because of the inability to obtain adequate numbers of age- and size-matched Fisher rats from vendors (Fig. 4). Rats were anesthetized with ketamine (80 mg/kg) and xylazine (15 mg/kg) by IP injection. AAV5 or AAV9hPGIS vector, irrelevant AAV9-human placental alkaline phosphatase (hPLAP) or AAV9-empty transgene vector (200 μ l; 2×10^{11} vector genomes), or vehicle (saline, 200 μ l) was instilled into the lungs of rats by orotracheal instillation (Johnson *et al.*, 2003). Forty-eight hours later, rats were injected with MCT (60 mg/kg IP) or an equal volume of saline. Rats were monitored daily until they developed symptoms of pulmonary hypertension (typically 3–4 weeks) such as weight loss and tachypnea, or until 6 weeks after vector administration. Hemodynamic, morphologic, and biochemical assessments were performed at this time.

Polymerase chain reaction detection of AAVCBhPGIS vector DNA

DNA was extracted from formalin-fixed paraffin wax-embedded lungs and/or from frozen lung samples taken from MCT-treated rats 4–6 weeks after AAV-CBhPGIS administration using the RecoverAll total RNA Isolation kit (Ambion). Polymerase chain reaction (PCR) was performed on 300 ng of the isolated DNA using primers that spanned the *hPGIS* cDNA and the 3' AAV inverted terminal repeat

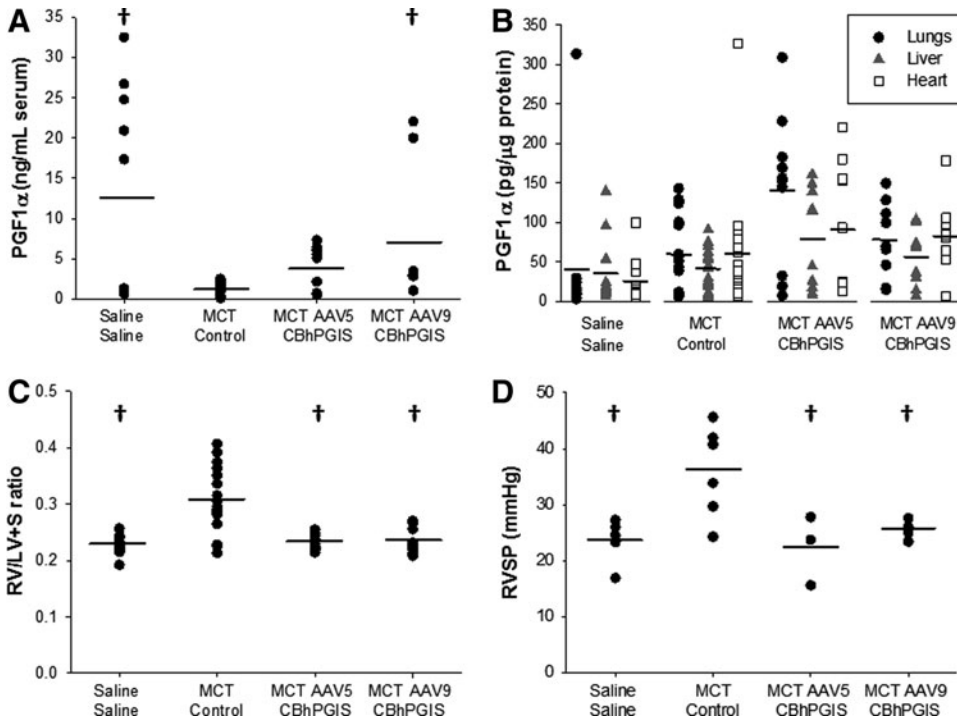


FIG. 2. Biochemical and functional assessment of AAV-mediated *hPGIS* expression in MCT-treated rats. (A) PGF1 α in plasma of MCT-treated rats ($n > 8$, $p = 0.018$). (B) PGF1 α in lung, liver, and heart tissue 4–6 weeks after AAV5 or AAV9-CBhPGIS administration to MCT-treated rats ($n > 9$). (C) RV/LV+S ratios 4–6 weeks after AAV5 or AAV9-CBhPGIS administration to MCT-treated rats ($n \geq 9$, $p \leq 0.001$). (D) RVSP measurements 4–6 weeks after delivery of AAV5 or AAV9-CBhPGIS to MCT-treated rats ($n \geq 6$, $p = 0.008$). †Significantly different from MCT-control. *hPGIS*, human prostacyclin synthase.

(forward primer, AGG TAC GGC TTC GGT CTG AT; reverse primer, AGT GGC CAA CTC CAT CAC TAG G) to amplify a 400 bp product. A negative control (water) and positive control (pAAV-CBhPGIS) were included in the PCR process. The PCR products were electrophoresed on a 1% agarose gel stained with ethidium bromide and visualized using UV light.

Measurement of *hPGIS* expression

To assess expression of the *PGIS* transgene, blood was collected by cardiac puncture at the time of euthanasia via CO₂ asphyxiation and centrifuged at 514 \times g for 5 min, and the supernatant collected. Levels of 6-keto PGF1 α , the nonenzymatic hydrolysis product of PGI₂, were measured by enzyme immunoassay kit (EIA Assay Design, Inc.) in the supernatant (plasma). For measurement of 6-keto PGF1 α in the tissues, organs were harvested and homogenized.

Measurement of right ventricular mass and right ventricular pressures

The right internal jugular vein of anesthetized rats was surgically exposed and cannulated with a 2F pressure transducer catheter (Millar Instruments) interfaced to a control unit (PC-2000) and a data acquisition system (Power Lab 8/30; AD Instruments). The catheter was advanced into the right ventricle and the pressures recorded. Right ventricular systolic pressure (RVSP) was measured in the vast majority, but not all rats, as either some MCT-treated rats had severe symptoms that prohibited collection of hemodynamic data or the measurements were technically inadequate for interpretation. After euthanasia, the hearts of all experimental animals were removed and blotted, and the RV was dissected free of the left ventricle + septum (LV + S) and each portion was weighed.

Histology

After euthanasia, the chest was opened and trachea exposed by sharp dissection. The trachea was cannulated and the lungs were inflation-fixed at 20 cm H₂O with buffered paraformaldehyde (4%). Lungs were removed and subsequently immersed in the paraformaldehyde solution. Specimens were then embedded in paraffin, sectioned (5 μ m) at the hilum, and stained with hematoxylin and eosin. Parallel sections were stained with an Elastic von Giessen stain for measurement of vascular media and outer-diameter wall thickness. Images were captured by visualization under a Zeiss Axioimager light microscope interfaced to a computer. Morphometric analysis was performed to assess vessel diameter.

Statistics

Data are presented as the mean \pm SE. The data were analyzed with the application of the one-way ANOVA method. A p -value < 0.05 was considered statistically significant. Corrections for multiple comparisons were performed using Dunn's method.

Results

Vector delivery

We performed PCR analysis of vector DNA in rat lung 4–6 weeks after administration of AAVCBhPGIS. The forward primer was selected from the *hPGIS* cDNA and the reverse primer was selected from the ITR to generate a 400 bp PCR product specific for AAV-*hPGIS* vector DNA. As shown in Fig. 1B, vector DNA was detected in the lungs of rats 4 weeks postdelivery of AAV9CBhPGIS vectors (lanes 8–11), but not in the lungs of rats that received luminal administration of an AAV9CBhPLAP vector (lanes 5–7) or lungs of rats that received vehicle (saline, lanes 1–4). These data

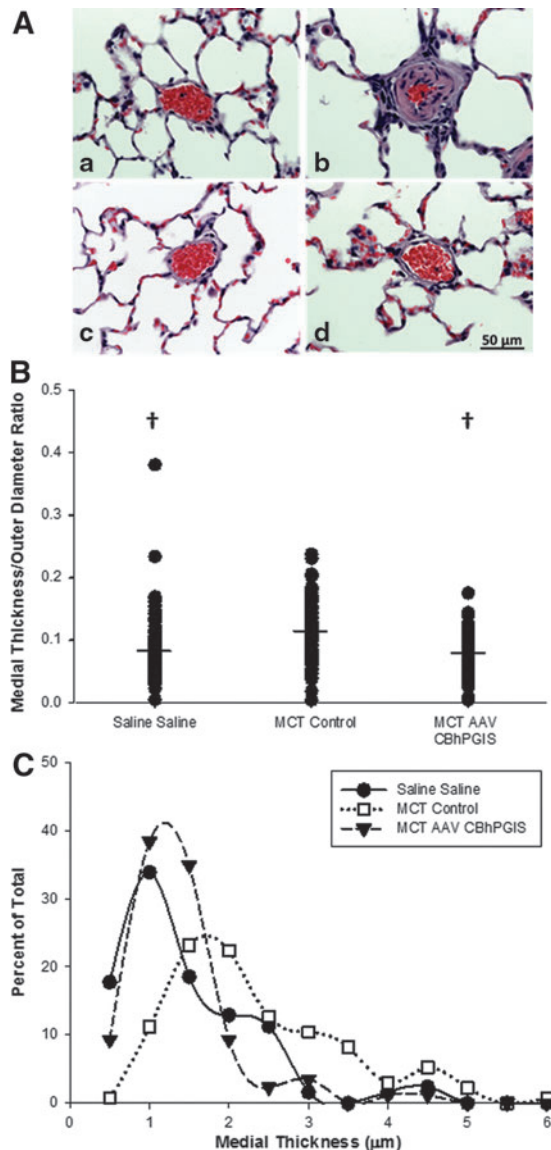


FIG. 3. Effect of AAV-hPGIS on pulmonary vascular remodeling. (A) Hematoxylin and eosin stains of (a) saline-saline control, (b) MCT-control, (c) MCT-AAV5-hPGIS, and (d) MCT-AAV9-hPGIS sections of rat lung. (B) Ratios of vascular medial (smooth muscle) thickness to outer diameter (total vessel wall thickness) of small pulmonary arteries ($n=4$ [AAV5=3, AAV9=1], $p \leq 0.001$). †Significantly different from MCT-control. (C) Distribution of vascular medial thickness in small pulmonary arteries of AAV and control rats.

confirmed the specificity of the PCR for the AAVhPGIS vectors and established delivery of the AAVCBhPGIS vector genome to the lung.

Expression of hPGIS

Intraluminal administration of AAV5 and AAV9CBhPGIS vectors increased serum PGF_{1 α} levels by ~3-fold and ~7-fold, respectively, as compared with MCT-control rats, which closely resembled levels measured in rats not treated with MCT (Fig. 2A). A trend toward an increase in the levels of PGF_{1 α} was detected in the lung and heart ho-

mogenates of MCT-AAV9CBhPGIS rats as compared with MCT-control or saline-saline rats (Fig. 2B), but did not reach significance. No differences in PGF_{1 α} levels were detected in liver homogenates from rats.

To assess the effects of hPGIS overexpression mediated by AAV5 and AAV9CBhPGIS vectors on function, we measured RV/LV+S ratios and RVSP. The RV/LV+S weight ratios were greater in MCT-control rats (0.306 ± 0.013) than in saline-saline control rats (0.228 ± 0.005), consistent with the development of PAH induced by MCT. The ratios in MCT rats transduced with AAV5 and AAV9CBhPGIS vectors (0.232 ± 0.005 and 0.235 ± 0.008 , respectively) were also different from MCT-control ratios, but similar to the ratios in saline-saline controls, consistent with prevention of MCT-induced cardiac remodeling (Fig. 2C). Similarly, the RVSP was nearly twofold greater in MCT-control rats (36.0 ± 3.3 mm Hg) than in saline-saline control rats (23.6 ± 1.8 mm Hg), whereas RVSP in MCT-AAV5 and MCT-AAV9CBhPGIS rats (22.4 ± 3.6 and 25.5 ± 0.9 mm Hg, respectively) was similar to measurements in saline-saline rats (Fig. 2D). Thus, AAV5 and AAV9CBhPGIS vectors attenuated the development of MCT-induced PAH.

Prevention of pulmonary vascular remodeling by AAV-mediated hPGIS expression

The effects of AAV-mediated expression of hPGIS on pulmonary vascular remodeling were measured (Fig. 3) in paraffin sections of rat lung. MCT-control rats exhibited a markedly thickened muscular media in the small pulmonary arteries, consistent with pulmonary hypertension, as compared with small pulmonary arteries in saline-saline rats (Fig. 3A). In contrast, the small pulmonary arteries of the MCT-AAV5CBhPGIS and MCT-AAV9CBhPGIS were similar to saline-saline control rats, consistent with prevention of MCT-induced PH.

Morphometric analysis of Elastic von Giessen-stained sections confirmed the thickening of pulmonary artery smooth muscle media in MCT-control rats as compared with MCT rats transduced with AAVCBhPGIS vectors (Fig. 3B). The diameters of the muscular medial thickness and the outer wall of small pulmonary arteries were measured, and the ratio of the media to total wall thickness was calculated. The ratios of medial thickness to outer wall diameter for the AAV vector-transduced groups were compared with the control groups (Fig. 3B). MCT-control rats had a greater ratio of medial thickness to outer vessel wall diameter than saline-saline control rats, whereas MCT rats transduced with AAV5 or AAV9CBhPGIS vectors had ratios that were not different from saline-saline controls. The distribution of medial wall thickness in PAs <50 μm in size is depicted in Fig. 3C. MCT-control rats had an increased medial thickness of small pulmonary arteries as compared with the saline-saline control group or the MCT-AAV9CBhPGIS rats ($n=4$ [3 AAV5 and 1 AAV9 specimen]). These data are consistent with the attenuation of pulmonary vascular remodeling in rats transduced with AAV5 or AAV9CBhPGIS vectors.

Promoter effects on hPGIS expression

Because of promising preliminary results with AAV9 vectors containing the CB7 promoter by others *in vivo* (Julie Johnston, PhD, Penn Vector Core, Philadelphia, PA,

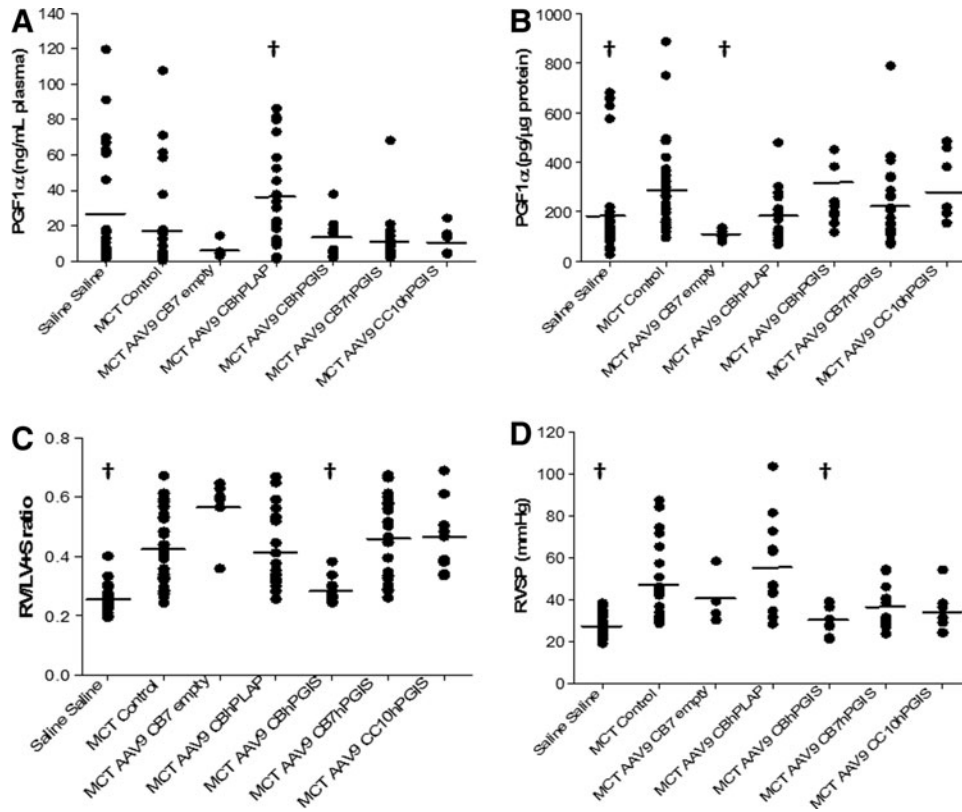


FIG. 4. Biochemical and functional assessment of AAV-hPGIS vectors containing different promoters. **(A)** PGF1 α in plasma 4–6 weeks after delivery of AAV9-hPGIS vectors with CB ($n=12$), CB7 ($n=23$), or CC10 ($n=8$) promoters to lungs of MCT-treated rats ($p=0.012$). **(B)** PGF1 α in lung homogenates 4–6 weeks after AAV9-hPGIS administration to MCT-treated rats [n is same as for **(A)**, $p\leq 0.001$]. **(C)** RV/LV+S ratios 4–6 weeks after administration of AAV9-hPGIS vectors to MCT-treated rats [n is same as for **(A)**, $p\leq 0.001$]. **(D)** RVSP measurements in MCT-treated rats after delivery of AAV9-hPGIS vectors containing CB ($n=9$), CB7 ($n=17$), or CC10 ($n=8$) promoters ($p\leq 0.001$). A smaller n for RVSP measurements reflects the inability to obtain hemodynamic data from some MCT-treated rats. †Significantly different from MCT–saline control. RV/LV+S, right ventricle/(left ventricle + septum).

personal communication), we began a series of experiments with clinical-grade AAV-CB7hPGIS vectors in the MCT rat model. Disappointing results led us to perform experiments with the clinical-grade AAV9CBhPGIS vectors and a locally produced vector containing the CC10 epithelial-specific promoter, AAV9CC10hPGIS. The summary data for these experiments are presented in Fig. 4. No significant increases in PGF1 α levels in plasma (Fig. 4A) or lung homogenates (Fig. 4B) were detected in MCT-AAV9CBhPGIS-transduced rats as the responses were highly variable. However, significant reductions in RV mass (RV/LV+S ratios) and RVSP as compared with MCT-control rats (Fig. 4C and D) were detected, consistent with attenuation of MCT-induced PAH. These hemodynamic findings were similar to the measurements in naïve rats (saline–saline) that did not receive vector or MCT. The attenuation in RV mass and pressures was restricted to rats treated with AAV9-hPGIS vectors containing the CB promoter and could not be reproduced with AAV5CBhPGIS vectors in these latter experiments (data not shown). No significant changes in levels of PGF1 α , RV/LV+S, or RVSP were detected in MCT rats transduced with AAV9-hPGIS vectors containing the CB7 or CC10 promoters as compared with MCT-controls. Surprisingly, PGF1 α was increased in rats treated with an AAV9CBhPLAP vector ($p=0.012$), possibly reflecting

some cross reactivity of the transgene, but no evidence of functional changes in RV mass or RVSP was detected. Thus, AAV9CBhPGIS vectors transduced MCT-treated rats and attenuated PAH, whereas AAV5-hPGIS vectors with CB7 or CC10 promoters did not.

Discussion

This study demonstrated that expression of *hPGIS* mediated by a single luminal (airway) instillation of AAV9-hPGIS vector containing a CB promoter prevented the development of MCT-induced PAH and associated pulmonary vascular remodeling. An AAV5-hPGIS vector with the same CB promoter also prevented the development of MCT-induced PAH. Measurements of the nonenzymatic hydrolysis product of prostacyclin, PGF1 α , were highly variable, limiting the ability of this measure to confirm *hPGIS* expression statistically, although some trends may be present. This is not an unexpected result as the assay for detection of 6-keto-PGF1 α cannot differentiate between the human and rat molecules.

One of the aims of the study was to determine which of the two selected AAV serotypes would be the best for gene delivery. A variety of serotypes have been considered for gene transfer to the lung, including AAV serotypes 1, 2, 5, 6, and 9 (Zabner *et al.*, 2000; Auricchio *et al.*, 2002;

Limberis and Wilson, 2006; Limberis *et al.*, 2009). Previous studies of AAV-hPGIS vectors in chronic hypoxic mice or MCT-treated rats relied extensively on the AAV1 and AAV2 serotype vectors delivered by intramuscular injection (Ito *et al.*, 2007; Kawakami *et al.*, 2007; Kataoka *et al.*, 2013). While AAV1 has been used for lung gene transfer, its utility in muscle gene transfer has been recognized in many fields of clinical gene transfer (Song *et al.*, 1998; Chao *et al.*, 2000; Flotte *et al.*, 2011). Noninvasive aerosol lung delivery of AAV2 vectors has been tested in human clinical trials of cystic fibrosis, but showed limited clinical efficacy (Moss *et al.*, 2004). Subsequently, AAV5, AAV6, and AAV9 pseudotyped vectors were developed as promising vectors for improved airway and distal lung delivery (Halbert *et al.*, 2001; Auricchio *et al.*, 2002; Limberis *et al.*, 2009). In preliminary studies, AAV5 vectors were superior to AAV1 vectors for mediating efficient hPLAP reporter gene transfer to the lungs of mice after luminal airway delivery (data not shown). In contrast, Flotte *et al.* (2010) reported greater efficiency of AAV1 vectors as compared with AAV5 vectors in a chimpanzee *in vivo* airway model that was predictive of findings in cultures of polarized human airway epithelia. Our study focused on delivery of AAV5 and AAV9 vectors to distal lung, with prior studies showing that AAV5 transduces both airway and alveolar epithelial cells, whereas AAV9 vectors primarily transduce alveolar (type II) epithelial cells (Auricchio *et al.*, 2002; Limberis and Wilson, 2006; Limberis *et al.*, 2009). Published data also suggested that AAV9 vectors could efficiently transfer a variety of transgenes under the control of various promoters after luminal airway instillation (Auricchio *et al.*, 2002; Limberis *et al.*, 2009). The possibility that the AAV9 capsid vector with AAV2 genome backbone could be readministered, even in the presence of circulating neutralizing antibodies, as early as 1 month after initial exposure, made it a promising gene transfer vector candidate for use in humans (Auricchio *et al.*, 2002; Limberis and Wilson, 2006; Limberis *et al.*, 2009). Accordingly, we selected AAV5 and AAV9 vectors for the current study.

One of the hallmarks of the prior *in vivo* gene transfer studies with AAV-hPGIS vectors was the consistent selection of the CB or CAG promoters to drive transcription of the hPGIS cDNA (Ito *et al.*, 2007; Kawakami *et al.*, 2007; Kataoka *et al.*, 2013). We tested the CB promoter against a modified version of the CB promoter known as CB7 (provided by the Penn Vector Core) and against the epithelial-specific promoter, CC10 (Stripp *et al.*, 1992; Auricchio *et al.*, 2002). The results of our study supported the selection of the constitutive CB promoter chosen by others, as AAV9-hPGIS vectors containing the CC10 and CB7 promoters did not attenuate MCT-induced PAH. These results were unexpected as the CC10 promoters had previously been shown to efficiently mediate gene transfer to murine lungs (Auricchio *et al.*, 2002). Because data from a personal communication (Julie Johnston, PhD) suggested that the CB7 promoter was a highly effective promoter, we devoted significant resources to testing this vector *in vivo*, but found that this promoter failed to mediate efficient transgene expression in rats. We were also unable to reproduce functional expression of AAV5CBhPGIS in Sprague Dawley rats (data not shown). We do not know whether the failure of these promoters and AAV serotype 5 to mediate gene expression in Sprague Dawley rats was a

species-specific phenomenon or whether unforeseen epigenetic factors played a role.

A limitation of our study was the MCT-induced PAH prevention model in which rats received noninvasive lung delivery of vector 2 days before MCT administration. Prior studies of intramuscular injection of AAV1 and AAV2-hPGIS vectors also used a prevention model approach (Ito *et al.*, 2007; Kawakami *et al.*, 2007; Kataoka *et al.*, 2013). In contrast, Hadri *et al.* (2013) administered an AAV1-SERCA2 vector by luminal airway aerosol administration with a Penn Century Device in both prevention and established MCT-PAH models with similar results. Unlike our study, Hadri and associates performed their studies in older male Sprague Dawley rats (350 g), whereas our studies were performed in younger females and males (150 g). Moreover, the severity of PAH was greater in the Hadri study with an RVSP of 50–60 mm Hg as compared with an RVSP of ~40 mm Hg in our study, with equivalent doses of MCT delivered by intraperitoneal injection. This difference in RVSP between the two studies may have reflected gender differences in MCT-induced PAH severity between male and female rats (Bal *et al.*, 2013). However, our findings that the RV/LV+S ratios and RVSP measured in MCT-injected male rats did not differ from the measurements in female rats (data not shown) fail to support the gender difference argument. Rather, our data likely demonstrated strain differences as the RV/LV+S ratios and RVSP in MCT-injected Fisher 344 rats (Fig. 2C and D) were lower than the same measurements in Sprague Dawley rats (Fig. 4C and D), which are consistent with data from Hadri *et al.* (2013) (Pan *et al.*, 1993). Despite these strain differences, the data presented from our study compare favorably to the data from the intramuscular injection of AAV1 and AAV2-hPGIS vectors and luminal airway aerosol administration of AAV-SERCA2 vectors in a prevention of MCT-induced PAH model (Ito *et al.*, 2007; Kawakami *et al.*, 2007; Hadri *et al.*, 2013; Kataoka *et al.*, 2013).

In conclusion, noninvasive luminal lung delivery of AAV5- and AAV9CBhPGIS gene transfer vectors significantly attenuated the severity of MCT-induced PAH and prevented cardiac and pulmonary vascular remodeling. Although the constitutive CB promoter was best for *in vivo* delivery to rat lung, we do not know if a CB promoter would be ideal for human studies. Overall, these data demonstrate a potential role for noninvasive luminal airway delivery of AAV-hPGIS vectors for paracrine delivery of prostacyclin to the pulmonary vasculature in human clinical trials of PAH.

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Disclaimer

The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of National Center for Toxicological Research or NIH.

Author Disclosure Statement

No competing financial interests exist.

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