Midkine (MK) is expressed in a precise temporal-spatial pattern during lung morphogenesis; however, its role in pulmonary homeostasis is unknown. Increased MK staining and mRNA expression were observed in the lungs of hypoxia-susceptible CAST/eiJ mice during hypoxia. MK expression was induced by hypoxia in cell lines in vitro. Because the transcription factor hypoxia-inducible factor-1α (HIF-1α) modulates cellular responses to hypoxia, we tested whether increased expression of MK in the lung was mediated by HIF-1α. HIF-1α enhanced the transcription of MK, acting on HIF-1α regulatory elements located in the MK gene promoter. Site-directed mutagenesis of the 3′ HIF response element in the MK promoter blocked the stimulatory effects of HIF-1α. To directly assess the role of MK on lung morphogenesis, transgenic mice were generated in which MK was expressed in the respiratory epithelial cells of the developing lung. MK increased muscularization of small pulmonary arteries, increasing α-smooth muscle actin and caldesmon staining and the expression of myocardin. MK directly enhanced the expression of myocardin and the smooth muscle-specific genes α-smooth muscle actin, calponin, and SM-22 in vascular smooth muscle precursor cells. Expression of MK in the respiratory epithelium is regulated by hypoxia and HIF-1α. These data provide a model wherein the respiratory epithelium responds to hypoxia via HIF-1α-dependent regulation of MK, enhancing myocardin expression to influence pulmonary vascular gene expression.
The binding of HIF-1α/HIF-1β to HREs assists in the recruitment of coactivator molecules that form transcription initiation complexes to enhance the expression of genes that mediate cellular and physiologic responses to hypoxia (16).

Epithelial-mesenchymal interactions, mediated by autocrine-paracrine signals, play a critical role in directing morphogenesis by providing signals that induce genetic programs in pulmonary cells (17). The process of alveolarization occurs primarily during the postnatal period and is completed by 3–4 weeks of age in the mouse. During this period alveoli are increasing in number and alveolar walls thin to generate an efficient gas exchange compartment. Also required in this process is the formation of the pulmonary vasculature. Muscularization of pulmonary arteries develops late in gestation and is mediated by autocrine-paracrine signaling, which directs smooth muscle cell-specific gene expression (18). Smooth muscle differentiation is directed by a serum response factor (SRF) coactivator, myocardin, (19) which forms protein-DNA complexes consisting of dimerized SRF bound to CArG box sequences (20). Although epithelial-mesenchymal interactions are known to be critical to lung morphogenesis, the molecular mechanisms by which epithelial and mesenchymal cells communicate during lung formation or a response to hypoxia remain poorly understood.

In the present study we demonstrate that MK expression was induced by HIF-1α during hypoxia. Furthermore, increased expression of MK in the respiratory epithelium increased the expression of myocardin, smooth muscle cell markers and increased the muscularization of small pulmonary arteries. These data provide a mechanism by which hypoxia influences gene expression in the respiratory epithelium to generate midkine, a paracrine signal, that influences the formation of the pulmonary vasculature.

**EXPERIMENTAL PROCEDURES**

**Mouse Models and Animal Husbandry—**CAST/eiJ mice were obtained from The Jackson Laboratories (Bar Harbor, ME) and housed in a temperature-controlled room (22–25 °C) under a 12-h light/12-h dark cycle. Animal husbandry followed protocols approved by the Institutional Animal Care and Use Committee at Cincinnati Children’s Hospital Research Foundation.

Human surfactant protein C (SP-C) is selectively expressed in endodermally derived epithelial cells in the embryonic lung and is expressed by subsets of alveolar and bronchiolar epithelial cells after birth (21). The SP-C-CAT gene (22) was inserted into a plasmid with a rSV promoter upstream of the reverse tetracycline transactivator (rtTA) gene (23). The (tetO)5-CMV-MK-3'/5' or 5'-11/3' gene was generated by inserting a 696-bp mouse MK cDNA downstream of a minimal CMV promoter containing seven concatenated tetracycline receptor binding sites. Transgenic mice were generated by pronuclear injection using standard techniques, and genotypes were determined by PCR. (tetO)5-CMV-MK-1/3' or 3'/5' mice were identified by amplification of a 559-bp sequence of the transgene by using the following primers: forward (5'-GGT CTT TGA CTT GGT CTT GGA GG-3') and reverse (5'-GGT CTT TGA CTT GGT CTT GGA GG-3'). PCR parameters included heating at 95 °C for 5 min followed by 30 cycles at 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 45 s followed by a 7-min extension at 72 °C. SP-C-rtTA-1/3' mice were identified by PCR amplification as previously described (23). Double transgenic mice (SP-C-rtTA-1/3', (tetO)5-CMV-MK-1/3' or 5'/3') were created by crossing the SP-C-rtTA-1/3' transgenic mice with (tetO)5-CMV-MK-1/3'. Transgenic MK expression was induced by feeding animals doxycycline (5 mg/kg, Harlan Teklad, Madison, WI) and DNase I (24) at room temperature for 15 min.

**In Vitro Exposure to Hypoxia—**Human placental adenocarcinoma (JEG-3), mouse fetal lung mesenchyme (MFLM-4) cell lines were maintained in minimal essential medium, Dulbecco’s modified Eagle’s medium, and RPMI, respectively, containing 10% fetal calf serum, 2 mM glutamine, and antibiotics. Cells were cultured in 5% CO2, 95% air (nmonic conditions) at 37 °C. At 80–90% confluence, cells were split and plated in 35-mm dishes and allowed to grow in normoxia for 18 h. Oxygen was then placed into an incubator in which the atmosphere was changed (Thermo Forma Series II Water Jacketed CO2 Incubator, model 3100, Marietta, OH). Cells were exposed to a mixture of 5% O2, 90% N2, and 5% CO2 for 4 h. Following the manufacturer’s instructions, cells were rapidly lysed, and total RNA was isolated using the Absolutely RNA® RT-PCR Miniprep kit (Stratagene, La Jolla, CA).

**In Vitro Addition of MK Protein—**MFLM-4 cells were maintained as outlined above. At 80–90% confluence, cells were split and plated in 35-mm dishes. After 6 h, 10 μg/ml recombinant human MK (rhMK 258-MD, R&D Systems, Minneapolis, MN) in Dulbecco’s modified Eagle’s medium containing 3% fetal calf serum was added. Cells were lysed, and RNA was isolated as outlined previously after 24 h in culture.

**Quantitative Real-Time Analysis—**Total RNA was isolated from wild-type and doxycycline-induced MK transgenic mice with (5'-AGA CAA CAA GCA GTC GTG G3') and reverse (5'-AAATG CGT CGG CCT AAA TAA G3') primers by using TRIzol® reagent (Invitrogen) and from cells grown in culture by using the Absolutely RNA® RT-PCR Miniprep kit (Stratagene) following the manufacturer’s instructions. 2-μg aliquots of total RNA were treated with 1 μl each of RNasin (Promega, Madison, WI) and DNase I (Invitrogen) at room temperature for 15 min. The DNase I was heat-denatured at 90 °C for 10 min, and reverse transcription was performed with SuperScript™II RT (Invitrogen) according to the manufacturer’s protocol. PCR was performed using 2-μl aliquots of the generated cDNA using Taq polymerase (Roche Applied Science). Products were electrophoresed on a 1.5% agarose gel with appropriate molecular weight standards. Primers used for the PCR reactions included MK primers (forward 5'-GGT CTT TGA CTT GGT CTT GGA GG-3' and reverse 5'-GGT CTT TGA CTT GGT CTT GGA GG-3'). Primers used for the PCR reactions included MK primers (forward 5'-GGT CTT TGA CTT GGT CTT GGA GG-3' and reverse 5'-GGT CTT TGA CTT GGT CTT GGA GG-3'). Primer PCR parameters included an initial heating at 95 °C for 5 min. Amplification was performed by 35 cycles at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s. HIF-1α was amplified by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. Both were followed by a 7-min extension at 72 °C. All other primer pairs were utilized as outlined (25–28). 1 μl of cDNA was isolated from mouse lungs and reverse-transcribed to cDNA. Oligonucleotide SYBR green primer pairs for myocardin (28) and β-actin (29) were used (5'-TGCT TGT TGG TAT AGG AAC ATG TA-3' and reverse 5'-AAA TGT CCT CTC ATC ATG CTC G3'). Quantitative Fluorescent amplification of cDNA was performed in the Smart Cycler® processing block, model SC1000-1 (Cepheid, Sunnyvale, CA) and by using the LightCycler-DNA Master SYBR Green I kit (Roche Applied Science). The relative abundance of mRNA was determined from standard curves generated from the amplification from serially diluted standard pools of cDNA and normalized to β-actin mRNA.

**Plasmid Construction and Mutagenesis—**The entire 2.5-kilobase (p2.5MK-luc) and a 5'-truncated 1.7-kilobase (p1.7MK-luc) mouse MK expression construct was directionally cloned into the pCRII-Blunt vector (Invitrogen) and verified by sequencing as previously described (8). Site-directed mutagenesis of the two putative HREs in the p2.5MK-luc reporter construct was performed by using the QuikChange™ site-directed mutagenesis kit (Stratagene). Synthetic oligonucleotides containing the desired mutation (RGGT → CCTG) were extended during the mutagenesis reaction to generate sense and antisense primers that contained the desired 5'-HRE, p2.5MKΔ5 HRE-luc, and a mutant 3' HRE, p2.5MKΔ3 HRE-luc. All constructs were verified by sequencing.

**Transfection and Reporter Gene Assays—**Assays of reporter gene constructs were performed in triplicate using transient transfection of JEG-3 as previously outlined (8). Plasmid concentrations included 500 ng/pCMV-HIF-1α, 500 ng/pCMV-HIF-1p, and 200 ng/pCDNA control vector to bring the total amount of DNA in each transfection to 1.08 μg. β-Galactosidase assays were performed as previously described (29). Reporter gene constructs were normalised for transfection efficiency based on the β-galactosidase activity.
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Morphometric Analysis—Mice were killed as previously described (23). At the time of necropsy, hearts were removed and dissected. The right ventricle (RV) was separated from the left ventricle and septum (LV+S) and weighed on an analytical balance. Ratios of RV weight/LV+S weight and RV weight/body weight are represented. Lungs were inflation fixed, washed in phosphate-buffered saline, dehydrated in a series of alcohols, and embedded in paraffin (30). To assess muscularizaton of the peripheral pulmonary vessels, lung sections were immuno-histochromically stained with an α-SMA monoclonal antibody as described above. In each randomized 60× field, vessels (15–50-μm external diameter) were classified as fully muscularized (actin staining >75% of the circumference), partially muscularized (actin staining 25–75% of the circumference), or non-muscularized (actin staining <25% of the circumference) as previously described (31). The percentage of peripheral pulmonary blood vessels that were fully or partially muscularized was calculated, and t tests were performed to demonstrate significant differences between double transgenic and non-transgenic littermates at the p < 0.05 level.

In Vivo Exposure to Hypoxia—Double transgenic and non-transgenic mice were placed in a normobaric chamber and exposed to FiO₂ of 0.10 for 5 weeks. Normobaric hypoxia was achieved by displacement with N₂ regulated by a Pro-Ox Model 110 unit (Biospherix Ltd., Redfield, NY). The chamber was changed regularly with activated carbon (Fisher), Drierite (Hammond Drierite Co., Ltd., Xenia, OH), and Baralyme® (Allied Healthcare Products, Inc., St. Louis, MO) to prevent the toxic accumulation of ammonia, excess humidity, and carbon dioxide. Once a week, mice were exposed to room air for less than 20 min to obtain body weights, clean cages, and/or replenish food and water.

Pulmonary Arteriograms, Histology, and Arterial Density Counts—Adult mice were sacrificed with a sodium pentobarbital (26%) euthanasia solution (Fort Dodge Animal Health, Fort Dodge, IA), and lungs were infused with a heated solution of gelatin and barium through the pulmonary artery as previously described (32). Pulmonary arterial architecture was imaged by x-ray radiography. The left lungs were subsequently embedded in paraffin and sectioned as previously described. To determine arterial density, barium-filled pulmonary arteries were counted by a blinded observer in randomly selected high-powered (60×) fields of distal lung. Fields containing large airways and/or large vessels were excluded. All vessels were counted, and five high-powered fields were counted per animal.

Western Blot Analysis—Western blot analysis was performed with the following primary antibodies: a rabbit polyclonal antibody to Tie-2 (Santa Cruz Biotechnology, Santa Cruz, CA), lung homogenates (25 μg) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blocked overnight at 4°C in 5% nonfat dried milk. Immunodetection was performed by incubating the membranes with the primary antibody diluted in blocking buffer for 1 h at room temperature. After washing, a secondary horseradish peroxidase-conjugated antibody (Santa Cruz) was diluted in blocking buffer at room temperature. After washing, a secondary horseradish peroxidase-conjugated antibody (Santa Cruz) was diluted in blocking buffer at room temperature, membranes were incubated with the secondary antibody for 1 h, washed, and developed using ECL Plus detection (Amersham Biosciences) was performed, and autoradiographs were taken.

RESULTS

Increased MK Expression during Hypoxia in Vivo and in Vitro—CAST/eiJ mice, an inbred strain, develop vascular remodeling and cor pulmonale during hypoxia (FiO₂ of 0.10), whereas FVB/N mice are resistant to hypoxia. MK was not induced in the lungs of adult FVB/N mice during hypoxia (Fig. 1, A and B). Although MK was barely detectable in the lungs of adult CAST/eiJ mice housed in normoxic conditions, intense staining of MK was observed in alveolar epithelial cells and pulmonary vasculature of adult CAST/eiJ mice exposed to hypoxia (FiO₂ of 0.10) for 4 weeks (Fig. 1, C and D). MK staining was observed in the peripheral pulmonary epithelium and smooth muscle of the pulmonary vasculature (Fig. 1, E and F). Intensity of staining with MK increased during exposure to hypoxia (Fig. 1, G and L). MK staining was induced in the epithelial cells lining proximal bronchioles after 1 week in hypoxia (Fig. 1, G and L). When hypoxic exposure was lengthened to 2 weeks MK staining was observed in the alveolar regions of the lung parenchyma (Fig. 1L). After exposure to hypoxia for 3 and 4 weeks MK staining was observed throughout the lung and was detected in epithelial cells of the conducting and peripheral airways and in the smooth muscle cells of the peripheral pulmonary blood vessels (Fig. 1, K and L). MK mRNA was detected in the lungs of CAST/eiJ mice after hypoxic exposure for 1 week, and expression steadily increased as the hypoxic exposure time was lengthened to 2 weeks (Fig. D), 3 weeks (K), and 4 weeks (L). MK staining increased in the respiratory epithelium and in the pulmonary vasculature. Images are at 40× (A–D, G–L) and 100× (E–F). Bar = 100 μm.

FIG. 1. MK staining in FVB/N and CAST/eiJ mice during hypoxia. MK staining was not detected in the lungs of adult FVB/N mice after exposure to normoxia (A) or hypoxia (FiO₂ of 0.10, B) for 5 weeks. Compared with normoxic conditions (C), MK staining was markedly increased in adult CAST/eiJ mice exposed to hypoxia for 4 weeks (D). Staining for α-SMA (F) revealed that MK was localized to the peripheral epithelium and vascular smooth muscle in the lungs of CAST/eiJ mice exposed to hypoxia for 4 weeks. MK was not observed in the lungs of CAST/eiJ mice exposed to hypoxia for 1 day (G) or 3 days (H), but staining was detected in bronchiolar epithelial cells after 1 week in hypoxia (L). As the hypoxic exposure time was lengthened to 2 weeks (J), 3 weeks (K), and 4 weeks (L), MK staining increased in the respiratory epithelium and in the pulmonary vasculature. Images are at 40× (A–D, G–L) and 100× (E–F). Bar = 100 μm.

The effect of hypoxia on MK expression was assessed in various cell lines including human placental adenocarcinoma (JEG-3), mouse fetal lung mesenchyme (MFMIL-4), and human pulmonary adenocarcinoma (H-441). HIF-1α and MK mRNA expression were increased by hypoxia in all of the cell lines tested (Fig. 3).

HIF-1α Regulates MK Transcription in Vitro—Because HIF-1α is a known transcriptional mediator of response to hypoxia, effects of HIF-1α on MK transcription were assessed. JEG-3 cells were co-transfected with a luciferase-reporter plasmid containing 2.5 kilobases of the 5′-region of the mouse MK gene (p2.5MK-luc) and an expression vector containing HIF-1α cDNA. HIF-1α activated the MK promoter (Fig. 4) and acted in an additive manner when co-transfected with TTF-1 (Fig. 4B). There are two potential HREs in the 2.5MK-luc plasmid, located at −1701 to −1697 (5′-HRE) and +48 to +52 (3′-HRE). Deletion of the 5′-HRE in the promoter (p1.7MK-luc) did not inhibit HIF-1α responses (Fig. 4C). To identify HREs that mediate HIF-1α-induced MK transcription, site-directed mutagenesis was performed on the HREs located at −1701 to
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Fig. 2. MK mRNA expression in CAST/eiJ mice during hypoxia. CAST/eiJ mice were exposed to hypoxia (FiO2 of 0.10) for 0, 1, 2, 3, or 4 weeks, and whole lung RNA was isolated and reverse-transcribed to cDNA. MK mRNA was detected by RT-PCR, and relative expression levels were compared with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Fig. 3. Hypoxia induces MK in vitro. JEG-3, MFLM-4, and H-441 cells were placed in hypoxia (FiO2 of 0.05) or normoxia (FiO2 of 0.20) for 4 h, and RNA was isolated. HIF-1α and MK mRNAs were detected by RT-PCR analysis. Student’s t tests were performed, and fold increases in MK expression were normalized to normoxic culture levels (p < 0.05). Images are representative of four separate in vitro experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

-1697 and +48 to +52, generating p2.5MK(Δ5′ HRE)-luc and p2.5MKΔ3′ HRE)-luc, respectively (Fig. 4D). Mutation of the 5′-HRE did not significantly inhibit transactivation by HIF-1α. In contrast, HIF-1α did not induce MK transcription when the 3′ HRE was mutated (Fig. 4D), demonstrating that the proximal HRE regulates HIF-1α-induced expression of MK.

Inducible Expression of MK in the Murine Lung—To assess the effects of chronic MK expression during lung morphogenesis, mouse MK was expressed in the respiratory epithelium of transgenic mice using the human surfactant protein C promoter (21). The human surfactant protein C promoter mediates respiratory epithelial cell-specific expression of the rtTA, which binds doxycycline and induces MK expression in vitro.

Pulmonary arteriograms—Pulmonary arteriograms were performed using barium infusion of the lungs through the pulmonary artery of double transgenic mice and non-transgenic littermates. There were no observable differences in the alveolar architecture. The numbers of pulmonary arteries that ranged in size from the main pulmonary artery to small arterioles, no differences in lung morphology were observed in MK-expressing mice after exposure to hypoxia. In the double transgenic mice exposed to hypoxia, MK staining in the respiratory epithelium was observed, and staining for α-SMA and caldesmon was increased (data not shown). These data demonstrate that expression of MK in the respiratory epithelium of the developing lung causes thickening of the muscular media of pulmonary arteries.

Respiratory Epithelial Cell-specific Expression of MK Does Not Induce Blood Vessel Formation—Pulmonary arteriograms were performed using barium infusion of the lungs through the pulmonary artery of double transgenic mice and non-transgenic littermates. There were no observable differences in the alveolar architecture. The numbers of pulmonary arteries that ranged in size from the main pulmonary artery to small arterioles, no differences in lung morphology were observed in MK-expressing mice after exposure to hypoxia. In the double transgenic mice exposed to hypoxia, MK staining in the respiratory epithelium was observed, and staining for α-SMA and caldesmon was increased (data not shown). These data demonstrate that expression of MK in the respiratory epithelium of the developing lung causes thickening of the muscular media of pulmonary arteries.

MK Induces Myocardin Expression in Vitro and in Vivo—Myocardin is a SAP (SAP-A/B, acinus, Pias) domain-containing protein (19). Myocardin forms a stable ternary complex with SRF and CArG box DNA and activates most smooth muscle cell differentiation markers (20). To test whether MK induces smooth muscle gene expression via myocardin, myocardin expression was assessed in a cell line derived from fetal lung mesenchyme (MFLM-4) (24) grown in culture containing MK. MK markedly increased myocardin expression in MFLM-4 cells in vitro and enhanced the expression of the smooth muscle cell-specific markers α-SMA, SM-22, and calponin (Fig. 9A).

Myocardin mRNA was assessed after MK was induced in the SP-C-rtTA, (tetO)5-CMV-MK transgenic mice. Myocardin expression was significantly increased in double transgenic mice exposed to doxycycline from conception until PN14 (Fig. 9B). Likewise, myocardin mRNA and MK increased coordinately.
during hypoxic exposure (FiO2 of 0.10) of CAST/ei mice (Fig. 9C and Fig. 2).

**DISCUSSION**

**MK Is Induced during Hypoxia Both in Vivo and In Vitro**—In the present study chronic hypoxia induced the expression of MK in association with increased muscularization of pulmonary arteries and increased expression of α-SMA in the CAST/eiJ mice. Adaptation to hypoxia is a topic of considerable clinical relevance. Persistent pulmonary hypertension of the newborn (PPHN) is a relatively common disorder associated with labile hypoxia (33, 34). PPHN is associated with severe
pulmonary vasoconstriction, hypertrophy/hyperplasia of vascular smooth muscle in peripheral pulmonary arteries, and increased pulmonary vascular resistance. Chronic hypoxia contributes to the morbidity and mortality of infants with bronchopulmonary dysplasia, causing pulmonary hypertension (35). Pulmonary vascular remodeling occurs in the perinatal and postnatal periods (36) during the transition from high pulmonary vascular resistance in utero to a state of low pulmonary vascular resistance and high pulmonary blood flow characteristic of the postnatal infant. The findings that MK directly enhances pulmonary vascular muscularization in the postnatal lung and that hypoxia induces MK expression in cell lines in vitro and in vivo support a model by which oxygen sensing in the respiratory epithelium generates a paracrine signal, MK, which selectively causes pulmonary vascular remodeling.

Chronic hypoxia markedly increased MK expression in hypoxia-susceptible CAST/eiJ mice in vivo and in cell lines in vitro. Pulmonary vascular remodeling did not occur after postnatal exposure of FVB/N mice to hypoxia. In contrast, lungs of CAST/eiJ mice are highly susceptible to hypoxia, and chronic exposure to hypoxia induces pulmonary vascular remodeling and RVH. MK was not induced when adult FVB/N mice were exposed to hypoxia. In contrast, MK was present at low levels in the lungs of CAST/eiJ mice exposed to normoxia. After hypoxic exposure for 4 weeks, MK staining in bronchiolar and respiratory epithelial cells was markedly increased in the lungs of CAST/eiJ mice and was associated with pulmonary vascular remodeling and RVH. Likewise, hypoxia induced MK expression in several cell types in vitro. Although the mechanism whereby the oxygen-sensitive CAST/eiJ mice respond to hypoxia remains to be clarified, it is possible that CAST/eiJ mice either lack the ability to employ homeostatic mechanisms triggered by hypoxia or that pathways influencing the expression of MK during hypoxia are activated. Because the expression of MK is regulated by the respiratory epithelium-specific transcription factor, TTF-1 (8), the finding that MK expression is increased in the respiratory epithelium of CAST/eiJ mice during hypoxia suggests that MK acts in a paracrine manner on smooth muscle precursors in the peripheral pulmonary vasculature.

**HIF-1α Regulates MK Expression during Hypoxemia**—Hypoxia enhances the activity of HIF-1α, which induces the transcription of downstream genes (13). Although binding of HIF-1α/β heterodimers to HREs activate target genes, the precise genes and pathways induced during hypoxia and their role in the pathogenesis of hypoxia-induced pulmonary disease remain to be discerned (16, 37). Because the MK gene promoter contains two potential HREs and MK expression was induced in CAST/eiJ mice and cell lines exposed to hypoxia, the ability of HIF-1α...
MK Causes Pulmonary Vascular Remodeling—The effect of increased expression of MK was assessed in the murine lung by generating transgenic mice that express MK in the respiratory epithelium via a doxycycline-inducible transgenic system. Alveolarization and cellular proliferation were not perturbed, vascular remodeling characterized by increased muscularization of small pulmonary arteries as indicated by increased expression of α-SMA, caldesmon, and calponin, was evident. Because pulmonary vascular remodeling was not induced when animals were given doxycycline after PN21, the effects of MK on vascular remodeling in the FVB/N mice functioned in a developmentally specific manner. MK is expressed

in the respiratory epithelium of the lung and is detected in vascular tissue during alveolarization (8) but is not detected later in development. Furthermore, colocalization with epithelial markers in the mature lung and direct regulation by the

HIF-1α Regulates Midkine Expression during Hypoxemia

**Table 1**

| Condition | Control | SP-C-rtTA<sup>Cre<sup>Cre</sup>, (tetO)-CMV-MK<sup>tg</sup>/α<sup>tg</sup> | SP-C-rtTA<sup>Cre<sup>Cre</sup>, (tetO)-CMV-MK<sup>tg</sup>/α<sup>tg</sup> | % Muscularization | % Muscularization |
|-----------|---------|--------------------------|--------------------------|----------------|----------------|
| Normoxic  | RV/LV + S | 0.39 ± 0.03 | 0.42 ± 0.04 | 40.2 ± 6.03 | 77.5 ± 2.93<sup>a</sup> |
| Hypoxic   | RV/LV + S | 0.43 ± 0.03 | 0.49 ± 0.03<sup>a</sup> | 45.8 ± 3.22 | 75.8 ± 3.31<sup>a</sup> |

<sup>a</sup> p < 0.05 versus non-transgenic littermates.
epithelial cell-specific transcription factor, TTF-1, suggest that MK serves as a paracrine signal that originates in the epithelium and targets pulmonary vascular cells or their precursors. Because staining for smooth muscle markers was not increased in the bronchial smooth muscle of transgenic mice expressing MK, the increased muscularization of pulmonary arteries observed in this transgenic model support the concept that MK has a selective effect on vascular smooth muscle.

**MK Enhances Smooth Muscle Gene Expression**—Vascular smooth muscle hypertrophy and/or hyperplasia are associated with clinical conditions such as primary pulmonary hypertension (38, 12), bronchopulmonary dysplasia (35), and cor pulmonale-associated severe pulmonary diseases including emphysema, cystic fibrosis, and chronic obstructive pulmonary disease (39). Staining of smooth muscle-specific proteins has been useful in identifying changes in vascular smooth muscle cells (40). Muscularization of pulmonary arteries can be assessed by staining for α-SMA (41), caldesmon (42), calponin, and SM-22 (43). In the present study, immunohistochemical staining for α-SMA and caldesmon identified the sites of increased muscularization of small pulmonary arteries induced by the chronic expression of MK. Similarly, increased MK, α-SMA, and caldesmon immunostaining in the pulmonary arteries of CAST/eiJ mice during hypoxia suggests a role for MK in vascular remodeling and pulmonary hypertension in this hypoxia-sensitive strain. It remains unclear whether the enhanced expression of smooth muscle markers induced by MK indicates hyperplasia, hypertrophy, or changes in cell migration and differentiation.

The signaling pathways mediating the effects of MK are only partially understood at present. Recently, MK has been shown to bind to a member of the low density lipoprotein receptor family, low density lipoprotein receptor-related protein-6 (LRP-6) (44), which functions together with Frizzled as a Wnt receptor (45). LRP is highly expressed in vascular smooth muscle cells of both normal and atherosclerotic vessels (46). In these studies MK was translocated to the nucleus of mouse embryonic fibroblasts (47). MK was not internalized in LRP-deficient cells, whereas transfection with an LRP expression vector restored MK internalization and nuclear translocation. Internalized MK in the cytoplasm of mouse embryonic fibroblast cells was associated with nucleolin, a nucleocytoplasmic shuttle protein. Studies involving mouse embryonic fibroblast cells that express nuclear localization sequence-deficient nucleolin demonstrate that functional nucleolin is vital for the nuclear targeting of MK (47). Mutation of the phosphorylation sites of TTF-1 results in decreased expression of LRP as compared with non-mutant control mice (48). The findings that TTF-1 regulates both MK (8) and LRP (48) support the possibility that various components of the MK pathway are controlled directly or indirectly by TTF-1.

**MK Induces Myocardin Expression**—In the present study MK induced the expression of myocardin, a potent smooth muscle gene regulator (19) in MFLM-4 cells. MFLM-4 cells were originally isolated from mouse fetal lung mesenchyme, which serves as a source for pulmonary endothelial cells, smooth muscle cells, and fibroblasts (24). MK enhanced the expression of myocardin and its known targets, α-SMA, SM-22, and calponin in vitro. Likewise, myocardin mRNA was increased in the lungs of transgenic mice expressing MK and in CAST/eiJ mice exposed to hypoxia. Smooth muscle genes are regulated by SRF, a ubiquitous MADS (MCM1, Agamous, Deficiens, SRF) box transcription factor that binds as a homodimer to cis-acting CArG box DNA sequences (49). Most smooth muscle promoters contain essential CArG boxes in their control regions (20), and myocardin is a cofactor that activates these genes via protein/protein interactions with SRF. The myogenic capacity attributed to myocardin is similar to MyoD, which can convert non-muscle cells into skeletal muscle (50). Consistent with SRF-dependent transactivation, mutations in the SRF binding regions of myocardin block its myogenic activity, and SRF-dependent reporter genes are not activated by myocardin in SRF-deficient embryonic stem cells (51). Thus, the present data support a model in which MK acts as a paracrine signal in a regulatory pathway that directs pulmonary vascular remodeling via myocardin-dependent regulation of smooth muscle-specific genes including α-SMA, calponin, and SM-22.

**Conclusions**—MK expression was markedly increased in the lungs of hypoxia-sensitive CAST/eiJ mice during exposure to hypoxia. HIF-1α directly activated MK expression mediated by a HIF response element located in the MK gene promoter. Increased expression of MK in murine respiratory epithelial cells during postnatal lung morphogenesis caused remodeling of the peripheral pulmonary vasculature characterized by increased arterial smooth muscle staining of α-SMA and caldesmon. MK production in the respiratory epithelium is regulated by hypoxia via HIF-1α and serves as a paracrine signal that selectively enhances muscularization of small pulmonary arteries, a process that is likely mediated by increased expression of myocardin. Taken together these data suggest that MK is likely to play a role in pathological remodeling of the pulmonary vascular bed in various pulmonary diseases.
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