MAP kinase kinase kinase-2 (MEKK2) regulates hypertrophic remodeling of the right ventricle in hypoxia-induced pulmonary hypertension


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MAP kinase kinase kinase-2 (MEKK2) regulates hypertrophic remodeling of the right ventricle in hypoxia-induced pulmonary hypertension. Am J Physiol Heart Circ Physiol 304:H269–H281, 2013. First published November 2, 2012; doi:10.1152/ajpheart.00158.2012.—Pulmonary hypertension (PH) results in pressure overload of the right ventricle (RV) of the heart, initiating pathological RV remodeling and ultimately leading to right heart failure. Substantial research indicates that signaling through the MAPK superfamily mediates pathological cardiac remodeling. These considerations led us to test the hypothesis that the regulatory protein MAPKKK-2 (MEKK2) contributes to RV hypertrophy in hypoxia-induced PH. Transgenic mice with global knockout of MEKK2 (MEKK2/−/− mice) and age-matched wild-type (WT) mice were exposed to chronic hypobaric hypoxia (10% O2, 6 wk) and compared with animals under normoxia. Exposure to chronic hypoxia induced PH in WT and MEKK2/−/− mice. In response to PH, WT mice showed RV hypertrophy, demonstrated as increased ratio of RV weight to body weight, increased RV wall thickness at diastole, and increased cardiac myocyte size compared with normoxic control animals. In contrast, each of these measures of RV hypertrophy seen in WT mice after chronic hypoxia was attenuated in MEKK2/−/− mice. Furthermore, chronic hypoxia elicited altered programs of hypertrophic and inflammatory gene expression consistent with pathological RV remodeling in WT mice; MEKK2 deletion selectively inhibited inflammatory gene expression compared with WT mice. The actions of MEKK2 were mediated in part through regulation of the abundance and phosphorylation of its effector, ERK5. In conclusion, signaling by MEKK2 contributes to RV hypertrophy and altered myocardial inflammatory gene expression in response to hypoxia-induced PH. Therapies targeting MEKK2 may protect the myocardium from hypertrophy and pathological remodeling in human PH.

Right ventricular (RV) hypertrophy (RVH) occurs in response to pulmonary hypertension (PH) resulting from clinically important pediatric and adult lung disorders (1). Originating from the hypoxic pulmonary environment, these lung pathologies reflect self-perpetuating processes of hypoxic vasoconstriction and pathological vascular remodeling leading to pressure overload and subsequent decompensation and failure of the RV. Although the lung vascular pathology is clearly the initiating disease mechanism, the cardiac consequences ultimately dominate the clinical outcome (10, 14, 27).

The mechanisms that control RVH and myocardial remodeling in PH are not well understood, and cardiac-directed therapies are not available. Emerging evidence indicates that the RV differs functionally, anatomically, and biochemically from the more-studied left ventricle (LV) (58). Collaborative studies from our group have shown alterations in RV myofilamental Ca2+ sensitivity and protein phosphorylation in hypoxia-induced PH distinct from previous findings in the pressure-overloaded LV (59).

Processes involving major cell systems of the heart should be considered as contributing mechanisms underlying these findings. First, hypertrophy of the cardiac myocyte, accompanied by characteristic signatures of myocyte gene expression, occurs as a direct consequence of the increased hemodynamic load. Although historically viewed as compensatory, cardiac hypertrophy in response to cardiovascular disease or injury is an indicator of ongoing pathology (34, 52). Cardioprotective therapies, including β-blockers, angiotensin-converting enzyme inhibitors, cardiac resynchronization, and LV assist devices, cause reverse remodeling of hypertrophy and pathological gene expression in systemic heart failure (16, 40). Similar results have been reported with iloprost vasodilator therapy to reduce RV pressure overload in experimental PH in rats (50). Second, inflammatory and fibrotic activation of infiltrating immune cells and resident cardiac cells have been suggested as major determinants of pathological myocardial remodeling in a variety of disease states. Extensive studies have documented inflammatory remodeling of the pulmonary vasculature in hypoxia-induced experimental PH (39, 44, 55). Inflammatory fibrotic cardiac remodeling has been suggested as the basis for the worsened prognosis for the progression of PH in patients with systemic scleroderma with interstitial lung disease (37).

The above description of macroscopic remodeling in the hypertrophic myocardium has stimulated intensive research into intracellular signaling events regulating the phenotypes of cardiac cells. In this regard, attention has focused on the superfamily of MAPKs as important mediators of hypertrophic stimuli in the heart (60). Canonical MAPK signaling is mediated through three-kinase cascades of related protein isoforms, beginning with an upstream MAPKKK (MEKK) and leading sequentially to MEK and to effector MAPK. MAPK pathways have been implicated in a wide array of cellular responses to environmental stimuli (18, 31). Extensive research has documented roles for the subdivisions of ERK, JNK, and p38 MAPK in specific aspects of cardiac remodeling (42, 47, 57, 60).

Despite these efforts, the mechanisms of pathological cardiac remodeling and failure are incompletely understood. In particular, the remodeling processes unique to the progression of RV dysfunction in PH are only beginning to be explored. Recent studies have demonstrated the novel MAPK pathway initiated by MEKK2 as a contributor to cardiac hypertrophy,
MEKK2 IN RIGHT VENTRICULAR REMODELING

MEKK2 was used in an experimental model of PH induced by chronic hypoxia. An integrated assessment of MEKK2-mediated RV remodeling in PH was performed in terms of quantitative parameters of myocyte hypertrophy and inflammatory remodeling. This approach provides insights into the potential benefit of therapies targeting MEKK2 in human PH.

MATERIALS AND METHODS

Experimental protocols. All procedures with experimental animals were performed according to approved protocols under the supervision of the Institutional Animal Care and Use Committee of the University of Colorado-Denver (Aurora, CO).

Transgenic mice. Transgenic mice with targeted disruption of MEKK2 (MEKK2−/− mice) by insertion of the neomycin resistance gene into the MEKK2 coding sequence were generated as previously described (17). This transgenic strain propagates successfully as a homozygous knockout, although reduced litter sizes were observed compared with wild-type (WT) mice. Strain-matched Sv129ImJ mice with an intact MEKK2 gene (WT mice) were used as controls. Mice were provided water and chow ad libitum with a daily cycle of 14-h light to 10-h darkness. Genotypes were verified by PCR analysis of buccal swabs for the WT MEKK2 gene or the inserted neomycin resistance gene using the following primers: MEKK2 forward 5′-ATGCTCAGCTCTCAATAGTA-3′ and reverse 5′-ATGCCGGAAC-TATTCAAGCAT-3′; and neomycin resistance, forward 5′-AATCG-GCTGCTCTGATGCCGC-3′ and reverse 5′-AAGGCGATGCCGT-GCGAATCG-3′.

Exposure to chronic hypoxia. Male mice (12–18 wk old at the beginning of the study) were used for the experiments. Mice were housed for 6 wk in chambers equipped for continuous hypobaric hypoxia (0.5 atm, Po2: 10%, equivalent to ~5,380-m altitude). Chambers were vented to room atmosphere weekly for cage maintenance. Age-matched control mice were housed at room atmosphere (Denver, CO). No morbidity or mortality of the mice was observed during the study period. There were no significant differences in body weight among the experimental groups in these conditions (body weight: WT normoxic, 31.4 ± 4.4 g; WT hypoxic, 26.9 ± 3.4 g; MEKK2−/− normoxic, 29.1 ± 4.4 g; and MEKK2−/− hypoxic, 28.6 ± 6.7 g; n = 7 mice/group).

Echocardiography. Echocardiography was performed in the University of Colorado-Denver Small Animal Hemodynamic and Imaging Facility on a Vevo 770 high-resolution ultrasonic imaging system with a 35-MHz probe dedicated for mouse echocardiography (2). Mice were lightly sedated with 1–1.5% isoflurane to maintain heart rates above 500 beats/min. Heart rates in the experimental groups were as follows: WT normoxic, 509 ± 37 beats/min; WT hypoxic, 544 ± 30 beats/min; MEKK2−/− normoxic, 558 ± 63 beats/min, and MEKK2−/− hypoxic, 542 ± 21 beats/min [all P = not significant (NS)].

Echocardiograms were obtained at the conclusion of the study period under hypoxia or normoxia, as appropriate, by aerosolizing the isoflurane in normal breathing air or breathing air compounded with 10% O2, respectively. The chest was depilated before standard echocardiograms were obtained. Two-dimensional guided M-mode images were acquired at the level of the papillary muscle to measure LV end-diastolic and end-systolic dimensions as well as free wall, anterior, septal, and posterior wall thicknesses. RV measurements [RV free wall thickness, RV fractional shortening, and pulmonary artery (PA) blood flow] were made from modified parasternal long-axis views. Pulsed-wave Doppler measurements of blood flow across the PA and tricuspid valves were obtained as further indicators of RV function and pulmonary hemodynamic status. All measurements were made in the expiratory phase of the respiratory cycle. These echo measurements were used to calculate LV and RV dimensions, cardiac function (fractional shortening and cardiac output), and pulmonary hemodynamics.
**RV pressure determination.** RV pressure measurements were performed essentially as previously described (13, 35). Briefly, mice were deeply anesthetized with ketamine-xylazine. The RV was directly catheterized with a fluid-filled catheter interfaced with a pressure transducer connected to a dedicated computer. Trains of 10 successive pressure waveforms were analyzed and averaged to determine RV systolic pressure.

**Tissue harvest.** Animals were deeply anesthetized with tribromoethanol. Blood was obtained by cardiac puncture or by abdominal incision and cannulation of the inferior vena cava. For RNA determinations, the heart was excised, the atria and blood vessels were removed, and the heart was immersed in cardioplegia solution and dissected into the RV free wall, LV free wall, and septum. In some experiments, these components were blotted and weighed. Tissue pieces were either stored in RNALater at −80°C for RNA isolation or snap frozen in liquid nitrogen and stored at −80°C for immunoblot analysis. For histochemistry, hearts were excised intact and transversely perfused via the aorta with cardioplegia solution to render the heart quiescent followed by paraformaldehyde (4%) in PBS. For immunohistochemical analysis of the lung, the circulation was flushed with PBS through the RV in situ, and the lungs were fixed in parafomaldehyde (4%) in PBS. Tissues were paraffin embedded and cut into 5-μm sections.

**Immunohistochemistry of smooth muscle α-actin.** Neomuscularization of lung microvessels in response to hypoxia was quantitated by immunohistochemical staining of lung sections with antibody to smooth muscle α-actin. Formalin-fixed and paraffin-embedded sections were deparaffinized in graded ethanol solutions and incubated with Na-citrate buffer (pH 5.5) in a pressure cooker to unmask antigens. Immunostaining was performed with mouse anti-human smooth muscle α-actin primary antibody (clone 1A4, DAKO, 1:50) followed by peroxidase-conjugated horse anti-mouse IgG (Vector Labs, 1:200) and developed with diaminobenzidine diluent (Vector Labs). Peripheral lung vessels of 20198 5

**Gene Accession Numbers**

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**Analysis of cardiac gene expression.** Total RNA from the indicated cardiac regions was individually isolated from 6–7 animals/experimental group with TRIzol reagent (Invitrogen) according to the manufacturer’s specifications. First-strand cDNA synthesis was performed using Superscript II reverse transcriptase and random hexamer primers (25). Target gene expression was quantitated by real-time PCR on an iCycler MyiQ with SYBR green supermix (Bio-Rad). Proband abundance was determined relative to the abundance of the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (15). The sequences of gene-specific primer sets used in this study are shown in Table 1.

**Immunoblot analysis.** RV tissues were immersed and rapidly homogenized (Fisher PowerGen 125) in 0.5 ml RIPA buffer [150 mM NaCl, 50 mM Tris-HCl (pH 8), 2 mM EDTA, 1% (wt/vol) Nonidet P-40 (Igepal CA630, Sigma), 0.5% (wt/vol) Na-deoxycholate, and 0.1% (wt/vol) SDS] containing protease inhibitors (P8340, Sigma) and phosphatase inhibitors (P5726 and P0044 Sigma), each at 1% (vol/vol). Homogenates were centrifuged for 10 min at 10,000 g at 4°C. Supernatants were collected for immunoblot analysis. Protein concentrations were determined by BCA assay (Pierce).

**Immunoblot procedures** were performed essentially as previously described (38). Tissue lysates (30 μg/lane) were mixed with 6× Laemmli sample buffer, electrophoresed on precast SDS gradient gels (8–15%, Bio-Rad), and electrophoretically transferred to nitrocellulose membranes. Membranes were probed with antibodies to phosphoERK5 (44612G, Invitrogen), ERK5 (3372S, Cell Signaling Technology), or MEKK3 (ab33918, Abcam). Anti-β-actin (A5441, Sigma) was used to verify equivalent sample loading between lanes. Antibody concentrations used were as previously described (38). Blots were developed with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence, visualized by autoradiography, and quantitated by imaging densitometry (Bio-Rad).

**Statistics.** In vivo exposure to chronic hypoxia or normoxia was performed on four separate cohorts of four to seven animals per experimental group. Experimental determinations are reported from one or more such cohorts. Data are reported as means ± SE. Pairwise statistical differences were evaluated by an unpaired two-tailed t-test. Multiple group comparisons were performed by one-way ANOVA with Bonferroni’s post hoc test. P values of <0.05 was used to determine statistical significance.

**RESULTS**

**Chronic hypoxia induces sequelae of PH in WT and MEKK2−/−/129SvImJ mice.** Prolonged exposure of experimental animals to hypobaric hypoxia initiates a series of physiological responses,
including the development of PH, and provides an established model that recapitulates important features of human clinical disease (8, 56). Elevated pulmonary vascular resistance and blood pressure result initially from reflex vasoconstriction, augmented by subsequent medial smooth muscle hypertrophy of the pulmonary vasculature. Net erythropoiesis also occurs to compensate for hypoxia and hypoxemia and may contribute in part to the resulting PH. These responses to hypoxia were comparable in WT and MEKK2\(^{-/-}\) mice, as shown in Fig. 1. The results shown in Fig. 1A demonstrate that pulmonary blood pressure, measured as RV systolic pressure through cardiac catheterization, was elevated in both WT and knockout mice. The data shown in Fig. 1B demonstrate that WT and MEKK2\(^{-/-}\) mice underwent erythropoiesis in response to chronic hypoxia, measured as hematocrit. This result was corroborated by measurement of serum hemoglobin concentration (data not shown). In the mouse, medial remodeling is visualized as neomuscularization of the parenchymal microvasculature with immunostaining for smooth muscle \(\alpha\)-actin. Figure 1C, which shows representative immunohistochemical images, and Fig. 1D, which shows cumulative quantitation, provide evidence documenting the predicted increase in muscularized vessels in WT mice exposed to hypoxia. MEKK2\(^{-/-}\) mice exhibited a significant increase in actin-positive vessels, although this response was attenuated compared with WT mice.

These results demonstrate that mice with MEKK2 disruption respond to hypobaric hypoxia comparably to WT mice with

![Image](http://ajpheart.physiology.org/)

**Fig. 1. Parameters of pulmonary hypertension (PH) in chronically hypoxic mice.** Experimental animals were exposed to chronic hypoxic or normal atmosphere as described in MATERIALS AND METHODS. End-point measurements were performed. A: right ventricular (RV) systolic pressure. RV systolic pressure was measured by direct cardiac catheterization as described in MATERIALS AND METHODS. \(n = 11–13\) animals in the normoxic group and 7–8 animals in the hypoxic group. B: erythropoiesis. Hematocrit was measured as described in MATERIALS AND METHODS. \(n = 11–12\) animals in the normoxic group and 4–5 animals in the hypoxic group. C: immunohistochemical staining of parenchymal lung vascular muscularization. Lung tissues were immunostained for smooth muscle \(\alpha\)-actin (SMA) as described in MATERIALS AND METHODS. Immunoreactive microvessels (\(\leq 50\mu m\) diameter) in lung parenchyma are indicated by arrows. Scale bar = 100 \(\mu m\). MEKK2\(^{-/-}\), MEKK2 knockout (KO). D: quantitation of parenchymal lung neomuscularization. The SMA-immunoreactive vessels shown in C were determined as described in MATERIALS AND METHODS. \(n = 7\) animals/group. \(*P < 0.05\) strain-matched hypoxic vs. normoxic animals; \(+P < 0.05\) for equivalent experimental treatment between WT and MEKK2\(^{-/-}\) strains.
predicted consequences of PH, pulmonary vascular remodeling, and hematopoiesis.

RVH induced by PH is attenuated in MEKK2−/− mice. Pressure overload of the RV of the heart in response to PH sets in motion a complex series of events described collectively as myocardial remodeling. Importantly, RVH, reflecting increased RV wall thickness and mass, occurs to compensate for elevated wall stress. The ratios of RV mass relative to the sum of the (LV + septum), or RV weight relative to total body weight, were therefore compared in animals exposed to chronic hypoxia or normoxia to quantitate selective RVH in WT and MEKK2−/− mice. As predicted, the results shown in Fig. 2A demonstrate that WT mice exhibited a selective increase in RV mass relative to the (LV + septum). In contrast, RVH was blunted in MEKK2−/− mice such that the RV-to-(LV + septum) mass ratio was not significantly different from normoxic control mice. The complementary analysis shown in Fig. 2B shows the ratio of RV or LV mass relative to body weight in animals subjected to prolonged hypoxia. After hypoxic exposure, WT mice demonstrated significantly increased RV-to-body weight ratios compared with MEKK2−/− mice, whereas LV-to-body weight ratios were equal for both strains. These data demonstrate that WT mice underwent significant macroscopic RVH after chronic hypoxia, whereas MEKK2 disruption attenuated this response.

Echocardiographic analysis of RV function and morphometry. Echocardiography was used to noninvasively assess the effects of MEKK2 deletion on RV morphometry and performance in vivo in response to hypoxia-induced PH. The results are shown in Fig. 3. One of the characteristic echocardiographic findings in hypoxia-induced PH is the acceleration (shortening) of the PA pressure pulse wave and the appearance of a characteristic E-A notch in hypertensive animals, as shown in the representative traces in Fig. 3A. Figure 3B shows the effect of PH on the PA pressure waveform: a shortened time to peak acceleration, increased acceleration rate, and decreased duration of PA ejection time. These parameters were significantly altered, and in the predicted direction, in WT and MEKK2−/− mice, consistent with the PH-induced elevation of RV systolic pressure shown in Fig. 1. A modest reduction of the PA acceleration rate in MEKK2−/− mice in response to hypoxia was noted. We attribute the difference in statistical significance between these parameters to the enhanced sensitivity of PA acceleration rates as a clinical indicator of PH (4). Figure 3C shows representative cardiac M-mode and B-mode echocardiographic images illustrating the morphometric parameters used for echo quantitation. The consequent effects on RV morphometry and function are shown in Fig. 3D. PH increased RV wall thickness in WT mice, as expected for RVH. Increased wall thickness was also observed in MEKK2−/− animals, but the effect was significantly reduced compared with WT animals. In addition, echocardiographic measurements showed increased intraventricular septum thickness in WT mice exposed to chronic hypoxia versus normoxia (0.98 ± 0.06 vs. 0.85 ± 0.08 mm, P < 0.05) but not in MEKK2−/− mice (0.79 ± 0.14 vs. 0.90 ± 0.18 mm, P = NS). PH had no effect on LV wall thickness in either strain (data not shown). Moreover, RV function, measured as RV fractional shortening and cardiac output, was not altered during chronic hypoxia in both WT and MEKK2−/− mice. We note that the component values of RV chamber dimensions from which the fractional shortening values were calculated were also unchanged between experimental groups (chamber dimensions: diastolic, WT normoxic 1.25 ± 0.18 mm, WT hypoxic 1.25 ± 0.23 mm, MEKK2−/− normoxic 1.43 ± 0.10 mm, and MEKK2−/− hypoxic 1.23 ± 0.27 mm, all P = NS; and systolic, WT normoxic 0.62 ± 0.10 mm, WT hypoxic 0.71 ± 0.15 mm, MEKK2−/− normoxic 0.71 ± 0.16 mm, and MEKK2−/− hypoxic 0.75 ± 0.24 mm, all P = NS). These in vivo data reinforce the conclusion that MEKK2−/− animals experienced comparable hypoxia-induced PH, but attenuated RVH, compared with WT animals.

Cardiac myocyte hypertrophy induced by PH is attenuated in MEKK2−/− mice. The anatomic and morphometric measures of RVH described above are predicted to reflect net myofibrillar accumulation and increased cell size at the level of the cardiac myocyte. This hypothesis was tested by histochemical measurements of myocyte cross-sectional area, as shown in Fig. 4. Myocyte perimeter was visualized by histochemical staining with fluorophore-conjugated wheat germ agglutinin, and nuclei were visualized with DAPI. The representative images shown in Fig. 4 demonstrate the increased myocyte size in WT mice exposed to chronic hypoxia compared with MEKK2−/−
A  PA Doppler

B  PA Hemodynamics

C  Cardiac Echo Images

D  RV Function
mice. Myocyte cross-sectional area was determined in cell profiles containing identified nuclei by computer-assisted morphimetry, as shown in Fig. 4B. These composite data demonstrate the significant increase in RV cardiac myocyte size in WT mice exposed to hypoxia in vivo, whereas in MEKK2−/− mice, RV myocyte size after hypoxic exposure was not different from normoxic control mice. Taken together, the anatomic, echocardiographic, and cell morphometric data confirm that MEKK2 disruption attenuates RVH in response to PH.

**Myocyte hypertrophic gene expression induced by PH.** In addition to increased cell size, cardiac myocytes respond to altered demand with complex changes in the expression of key gene products. The specific patterns of gene expression appear to reflect the nature of the input stimulus (e.g., physiological vs. pathological) and to underlie specific states of myocyte contractile performance. Notably, previous studies have identified a suite of mRNAs associated with pathological hypertrophy. These genes include isoform switching from α- to β-myosin heavy chain (MHC) isoforms, reexpression of skeletal α-actin normally expressed in utero, downregulation of sarco(endo)plasmic reticulum Ca2+/H+ ATPase (SERCA), and upregulation of genes for atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). This hypertrophic gene program has been validated in experimental studies in rodents and in clinical studies of human heart failure (32, 36, 46). Accordingly, the influence of MEKK2 disruption on the expression of myocyte hypertrophic genes was investigated in our experimental protocol of hypoxia-induced PH using real-time PCR analysis, as shown in Fig. 5. These data demonstrate that exposure of WT mice to chronic hypoxia results in the recapitulation of prominent elements of the pathological hypertrophic gene program, including isoform switching between MHC isoforms (decreased α-MHC and increased β-MHC) and increased expression of skeletal muscle α-actin, ANP, and BNP. SERCA2 expression was also reduced but did not reach statistical significance. MEKK2 deletion modestly enhanced hypertrophic gene expression in normoxic controls (increased skeletal muscle α-actin and BNP). Chronic hypoxic exposure further activated hypertrophic gene expression in MEKK2−/− animals, in parallel with WT animals, with MHC isoform switching, increased expression of fetal skeletal α-actin and natriuretic peptide mRNAs, and downregulation of SERCA2. These results indicate that MEKK2 regulates myocyte hypertrophic growth independent of pathological gene expression.

**MEKK2 disruption attenuates inflammatory gene expression induced by PH.** Converging lines of investigation from animal models and human patients have emphasized the importance of inflammation in pulmonary vascular and cardiac responses to PH (6, 54). Moreover, the studies described above point to potential roles for MEKK2 signaling in response to hypoxia and inflammatory stimuli (see the Introduction). It was therefore important to investigate the effect of MEKK2 disruption...
Hypoxia elicits a constellation of anatomic and hemodynamic responses in hypoxia-induced PH. To this end, expression of cardiac inflammatory mRNAs was quantitated using real-time PCR. The cytokine targets that were chosen for study were previously shown to be increased in the remodeling pulmonary vasculature of neonatal calves during hypoxia-induced PH (7) and in vascular adventitial fibroblasts isolated from hypertensive animals (33). The data are shown in Fig. 6. In WT mice, chronic hypoxia robustly induced the expression of important proinflammatory mRNAs in the RV, including IL-1β, S100A4, monocyte chemoattractant protein (MCP)-1, stromal cell-derived factor (SDF)-1, and C-X-C chemokine receptor type 4 (CXCR-4). In contrast, hypoxia-induced expression of each of these mRNAs was reduced in MEKK2−/− mice. IL-6 tended to increase with hypoxic exposure in both WT and MEKK2−/− strains but did not reach statistical significance. These results clearly demonstrate that MEKK2 disruption attenuates RV inflammatory activation in concert with reduced RVH in response to hypoxia-induced PH.

**MEKK2 regulates MAPK signaling in the hypertensive RV.** To define the mechanisms of MEKK2 signaling in RVH, the phosphorylation and abundance of downstream MAPK targets were evaluated by immunoblot analysis. First, the results shown in Fig. 7A demonstrate that MEKK2 abundance was unchanged by chronic hypoxia versus normoxia in WT mice and, as expected, was absent in mice with MEKK2 disruption. In addition, we found that the related protein MEKK3 was expressed in the RVs of both WT and MEKK2−/− animals under normoxia and chronic hypoxia-induced PH. As shown in Fig. 7B, the RVs of WT normoxic mice demonstrated constitutive ERK5 phosphorylation. Exposure to 5-wk hypoxia led to maintained ERK5 phosphorylation as well as increased total ERK5 abundance. In comparison, the RVs from normotensive MEKK2−/− mice showed downregulation of ERK5 abundance and phosphorylation. Exposure to chronic hypoxia caused an upregulation of ERK5 abundance, yet ERK5 phosphorylation remained attenuated in knockout animals compared with WT animals. On the other hand, neither JNK abundance nor phosphorylation were sensitive to MEKK2 disruption or chronic hypoxia under these experimental conditions, as also shown in Fig. 7B. The results of phospho-ERK5 and total ERK5 densitometry are shown in Fig. 7C. We also observed no change in the abundance and phosphorylation status of ERK1/2 and p38 MAPK in separate immunoblot experiments (data not shown). These results suggest, first, that MEKK2 signaling is constitutively activated in the normal RV and that loss of MEKK2 input downregulates both the expression and phosphorylation of the target ERK5. Second, ERK5 expression and phosphorylation in the RV are upregulated by chronic hypoxia-induced PH. This activation depends in part, but not solely, on input from MEKK2. These results point to contributions from the MEKK2 signaling pathway in both RV homeostasis and hypertrophic remodeling. Finally, MEKK2 appears to signal selectively through ERK5 MAPK in the mouse RV.

**DISCUSSION**

Chronic hypoxic exposure in experimental animal species elicits a constellation of anatomic and hemodynamic re-
responses, resulting in PH that recapitulates important aspects of human disease. Although this remodeling is typically more modest in the mouse, our data clearly demonstrate the predicted responses to chronic hypoxia, including overt PH, pulmonary vascular remodeling, and elevated erythropoiesis. Moreover, RV pressure overload resulting from hypoxic PH manifested as RVH and altered programs of hypertrophic and inflammatory gene expression. Our data show that hypertrophy is specific to the RV in this model, arguing that hemodynamic pressure overload is a necessary condition independent of global hypoxia or polycythemia, which are also experienced by the LV. Mice with global knockout of MEKK2 develop PH in response to the hypoxic environment. However, MEKK2 disruption results in a striking and selective attenuation of RVH and a concomitant reduction in the expression of inflammatory genes in response to PH. To our knowledge, this is the first study to identify MEKK2 as an important regulator of hypertrophic remodeling in the RV.

RVH occurs in response to increased wall stress caused by PH. Because cardiac myocytes in the postnatal animal are largely incapable of proliferation, the increased wall thickness reflects a physical increase in individual myocyte size, resulting from the ordered deposition of additional sarcomeres (3). In this regard, we also noted that chronically hypoxic MEKK2−/− mice exhibited reduced neomuscularization of pulmonary microvessels compared with WT mice, despite an elevation of RV systolic pressure in response to hypoxia. This result may reflect a similar role of MEKK2 in vascular hypertrophy as that observed for cardiac hypertrophy. MEKK2−/− mice exhibited attenuated PA acceleration rates in hypoxia-induced PH, further suggesting a role for MEKK2 signaling in hypertensive remodeling in the pulmonary vasculature.

Alterations in the cardiac myocyte gene program have been well characterized in the pathological hypertrophy that results from cardiac injury or disease. These alterations reflect a reversion of the myocyte toward a fetal pattern of gene expression, including an elevated expression of β-MHC relative to α-MHC, reexpression of skeletal muscle α-actin, reduced expression of SERCA, and reexpression of ANP and BNP in the ventricles (expression in the adult is normally restricted to the atria). Myocyte reprogramming collectively may serve a cardioprotective effect by decreasing myocardial O2 consumption, protecting against Ca2+ overload, and decreasing plasma volume (46). These alterations were observed in both WT and...
MEKK2−/− mice exposed to chronic hypoxia. Thus, MEKK2 deletion does not affect the development of PH or the ability of the cardiac myocyte to sense elevated pressure and respond with altered gene expression but, rather, selectively limits myocyte hypertrophic growth. Dissociation between myocyte gene expression and hypertrophic growth was previously observed in a study (24) where a dominant negative mutation of the Fos transcription factor selectively blocked pathological gene expression but not cardiac myocyte hypertrophy in vitro.

We further observed RV activation of inflammatory genes in WT mice with hypoxic-induced PH. These results underscore the close, and complex, relationship between hypertrophic remodeling and inflammatory activation of the RV in hypoxia-induced PH. Inflammatory activation in response to hypoxia or cardiac insult may initially be an adaptive response. However, dysregulated or excessive inflammation may exacerbate pathological cardiac remodeling and dysfunction (26). Inflammatory cytokines may act directly on cardiac myocytes to elicit contractile dysfunction or, importantly, to promote myocyte hypertrophy (65). Perhaps the most striking finding of our study is that MEKK2 disruption attenuated cardiac myocyte hypertrophy and the production of myocardial inflammatory cytokine mRNAs in concert. The mRNAs identified as targets for MEKK2 regulation include IL-1β, a master cytokine for inflammatory activation; S100A4, an endogenous ligand for receptor for advanced glycation end-product signaling, MCP-1, a key cytokine for the recruitment and activation of circulating monocytes, and the chemokine SDF-1 and its cognate receptor CXCR-4, which are also involved in the recruitment of circulating immune and progenitor cells. It is intriguing to note that resident cardiac mast cells have been proposed as important sources of cardiac cytokines and to play important roles in hypertrophic remodeling (5). In this context, the attenuated inflammatory activation in MEKK2−/− is consistent with the observation of defective activation of MEKK2-null mast cells previously reported (62).

A previous report (41) has demonstrated a role for ERK5, an important downstream effector of MEKK2, in the control of

**Fig. 7.** Immunoblot analysis of MEKK and MAPK abundance and phosphorylation in the RV. Homogenates of RV tissue from the specified experimental animals were immunoblotted with the indicated antibodies as described in MATERIALS AND METHODS. Immunoblot analysis for β-actin was used to measure equivalent protein loading between lanes. A: MEKK2 and MEKK3. Note that lane designations for MEKK3 are identical to those for MEKK2. B: phosphorylated (p)ERK5, ERK5, pJNK, and JNK. C: quantitation of ERK5 abundance and phosphorylation status. Immunoblots from replicate experimental animals were determined by imaging densitometry as described in MATERIALS AND METHODS. n = 4–6 animals/experimental group. *P < 0.05, strain-matched hypoxic vs. normoxic control animals; †P < 0.05 vs. equivalent experimental treatment compared between WT and MEKK2−/− strains.
myocyte hypertrophy in vitro and in vivo through overexpression of activated MEK5 constructs. We extended these findings to show that MEKK2 disruption attenuates ERK5 abundance and activation, consistent with the observed attenuation of RVH in response to PH in vivo. We further observed that constitutive ERK5 abundance and activity depend on MEKK2, suggesting an unappreciated role in normal RV homeostasis. At the same time, it should be noted that chronic hypertension upregulates ERK5 abundance and activation in the absence of MEKK2, suggesting additional compensatory mechanisms. In this regard, we observed expression of closely related MEK3 in the RV that may in part provide functional redundancy in the absence of MEKK2. Finally, MEKK2 appears to signal selectively through ERK5, as opposed to JNK, ERK1/2, or p38 MAPK, in these conditions of chronic pressure overload. Clearly, our experiments only examined steady-state activation after prolonged hypoxia-induced PH, and additional MAPKs may also contribute to the initiation of RVH. These results provide an impetus for further mechanistic studies on coordinate signaling by MEKK2-MEK3 and downstream MAPKs in RVH.

These actions of MEKK2 can be considered in the context of hypertrophic remodeling and the transition to RV failure. Our results show that MEKK2 contributes importantly to RVH and the accompanying inflammatory activation in pressure overload. Yet RV function, as determined echocardiographically, was preserved, likely reflecting the ability of the mouse heart to compensate for the moderate degree of PH in this model. It will be of interest to test the effect of MEKK2 deletion in more severe models of RV (or LV) injury. We predict that MEKK2 plays a beneficial and adaptive role when RVH is sufficient to compensate for the imposed cardiac load, whereas excessive MEKK2 activity will be detrimental in the setting of the transition to decompensated RV failure. This balance between beneficial actions of MEKK2-ERK5 signaling in adaptive cardiac hypertrophy versus adverse actions under excessive stimulation is illustrated by a recent study (29) on LV pressure-overloaded models showing that cardiac-specific ERK5 deletion prevents compensatory LV hypertrophy but simultaneously increases myocyte apoptosis; in contrast, a report by Nicol et al. (41) showed that constitutive activation of ERK5 by mutated MEK5 increased myocyte hypertrophy and sudden cardiac death. Thus, therapies that attenuate MEKK2 are predicted to show selective benefits on decompensated RV failure resulting from severe PH.

These results point to directions for further research. This in vivo model with global MEKK2 disruption provides an integrated perspective on the role of MEKK2 in cardiopulmonary remodelling. It will be important to define the roles of MEKK2 in the pulmonary vasculature as well as the RV to translate our findings into effective therapies. In the context of RV remodeling, it will be important to examine the effects of MEKK2 deletion on the phenotypes of cardiac myocytes and fibroblasts in vitro. In addition, the understanding of MEKK2 intracellular signaling, particularly in relation to myocardial remodeling, is at an early stage. Intracellular partner proteins including Lad, 14-3-3 proteins, and heat shock protein 90 have been identified in other model systems, and ERK5 and JNK MAPKs are established as important downstream effectors (63). However, a defining feature of the MAPKKKs is their ability to respond to and integrate diverse stimuli. The specific mechanisms whereby MEKK2 responds to mechanical stretch or hypoxia leading to coordinated cardiac hypertrophy and inflammation remain to be determined. The methodological and conceptual framework developed in this study can be used for the future evaluation of therapeutic agents targeting MEKK2 signaling in the development and progression of PH and RV failure.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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