Divergent contractile and structural responses of the murine PKC-ε null pulmonary circulation to chronic hypoxia


Loss of PKC-ε limits the magnitude of acute hypoxic pulmonary vasoconstriction (HPV) in the mouse. Therefore, we hypothesized that loss of PKC-ε would decrease the contractile and/or structural response of the murine pulmonary circulation to chronic hypoxia (Hx). However, the pattern of lung vascular responses to chronic Hx may or may not be predicted by the acute HPV response. Adult PKC-ε wild-type (PKC-ε+/+), heterozygous null, and homozygous null (PKC-ε−/−) mice were exposed to normoxia or Hx for 5 wk. PKC-ε−/− mice actually had a greater increase in right ventricular (RV) systolic pressure, RV mass, and hematocrit in response to chronic Hx than PKC-ε+/+ mice. In contrast to the augmented PA pressure and RV hypertrophy, pulmonary vascular remodeling was increased less than expected (i.e., equal to PKC-ε+/+ mice) in both the proximal and distal PKC-ε−/− pulmonary vasculature. The contribution of increased vascular tone to this pulmonary hypertension (PHTN) was assessed by measuring the acute vasodilator response to nitric oxide (NO). Acute inhalation of NO reversed the increased PA pressure in hypoxic PKC-ε−/− mice, implying that the exaggerated PHTN may be due to a relative deficiency in nitric oxide synthase (NOS). Despite the higher PA pressure, chronic Hx stimulated less of an increase in lung endothelial (e) and inducible (i) NOS expression in PKC-ε−/− mice than in PKC-ε+/+ mice. In contrast, expression of nNOS in PKC-ε−/− mice decreased in response to chronic Hx, while lung levels in PKC-ε+/− mice remained unchanged. In summary, loss of PKC-ε results in increased vascular tone, but not pulmonary vascular remodeling in response to chronic Hx. Blunting of Hx-induced eNOS and iNOS expression may contribute to the increased vascular tone. PKC-ε appears to be an important signaling intermediate in the hypoxic regulation of each NOS isoform.

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Hx is a more complex process than acute HPV and likely involves additional pathways and intermediates.

Decreased expression/activity of NOS contributes to many models of vascular injury and dysfunction (26). Although we did not find that basal differences in NOS could account for the blunted HPV found in PKC-ε−/− mice exposed to acute Hx (28), abnormalities in NOS expression/activity could still be important in adaptive responses to chronic Hx in this model (23). All three NOS isoforms [endothelial (eNOS), inducible (iNOS), and neuronal (nNOS)] are expressed in rodent lung (11, 24). Lung eNOS and iNOS expression increase in response to chronic hypoxia (23). All three NOS isoforms [endothelial (eNOS), inducible (iNOS), and neuronal (nNOS)] are expressed in rodent lung (11, 24). Lung eNOS and iNOS expression increase in response to chronic Hx in mice and rats (24). NO, derived from eNOS, and to a lesser extent iNOS, is thought to be the major mediator of vasodilation in the murine pulmonary circulation (11).

We used a knockout mouse approach to test the hypothesis that the absence of PKC-ε would decrease the contractile and/or structural response of the murine pulmonary circulation to chronic Hx. Gray and colleagues (13) recently completed an extensive cardiac assessment of the PKC-ε+/+ and PKC-ε−/− mice used in the current study. Their analysis included direct inspection, echocardiography, and left heart catheterization. They found no differences in cardiac structure or function at baseline or after acute ischemia reperfusion. These findings suggested that right ventricular systolic pressure (RVSP) measurements made here would provide a useful, albeit indirect, index of PA pressure. Interestingly, we found that chronically hypoxic PKC-ε−/− mice actually have increased PHTN, RV hypertrophy (RVH), and polycythemia without further pulmonary vascular remodeling compared with PKC-ε+/+ mice. PKC-ε−/− mice had an intermediate RVSP and RVH response. However, reactive polycythemia was no different in these mice than PKC-ε+/+ controls. Thus the remodeling in the PKC-ε−/− and PKC-ε+/+ pulmonary circulation was less than expected, considering the pressure elevation and extent of RVH. An acute vasodilator response to inhaled NO was then measured in additional chronically hypoxic PKC-ε+/+ and PKC-ε−/− mice to determine the contribution of pulmonary vasoconstriction vs. structural remodeling to the augmented RVSP response seen under chronically hypoxic conditions. An enhanced vasodilator response to inhaled NO in the more hypertensive PKC-ε−/− pulmonary circulation would strongly suggest that an increase in pulmonary vascular resistance was responsible for the elevated RVSP detected in chronically hypoxic PKC-ε+/+ mice. Because differences were observed, a comparison of NOS expression and activity was undertaken. Collectively, the findings from these studies indicate roles for PKC-ε in pulmonary vascular reactivity, remodeling, and induction of NOS isoforms during chronic Hx.

MATERIALS AND METHODS

Materials. All chemicals used were from Sigma Chemicals (St. Louis, MO) unless otherwise specified.

Animals. Adult female C57BL6 × SV129 PKC-ε wild-type (PKC-ε+/+), heterozygous null (PKC-ε+/-), and homozygous null (PKC-ε−/−) mice were obtained as described (18, 21). F1 hybrid mice were generated at the Gallo Research Center (Emeryville, CA; sea level altitude) by crossing PKC-ε+/+ SV129 females with wild-type C57BL6 males. Resulting F1 generation PKC-ε−/− mice were then bred to generate PKC-ε+/-, PKC-ε+/+, and PKC-ε−/- F2 offspring that were then sent to Denver (altitude 5,280 ft) at 11–13 wk of age for experiments. These mice were allowed to acclimate to this altitude for 5 wk before study. Animal protocols were approved by the University of Colorado Health Sciences Center Institutional Animal Care and Use Committee.

PKC-ε expression in mouse whole lung homogenates. To examine differences in PKC-ε expression in PKC-ε+/+, PKC-ε+/−, and PKC-ε−/− mice, quick-frozen lung tissue was weighed and homogenized on ice (PowerGen 700, Pittsburgh, PA) in 10 volumes of homogenization buffer [10 mM Tris pH 7.4 containing 0.15 M NaCl, 40 mM NaF, 100 μM Na orthovanadate, 1.0% Triton X-100, 1.0% Nonidet P-40, and a 1.0% protease inhibitor cocktail including 1.04 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.8 μM aprotinin, 21 μM leupeptin, 15 μM pepstatin A, 36 μM bestatin, and 14 μM E-64]. The homogenates were centrifuged at 1,500 g for 20 min at 4°C (Savant SpeedFuge, Holbrook, NY), and supernatant was retained. Whole lung homogenate (50 μg) was loaded per lane of an 8% reduced SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and processed as previously described (27). Representative mouse lung lysates subjected to Western blotting are shown in Fig. 1. The relative amount of PKC-ε protein in lungs from PKC-ε+/+ mice was found to be 52 ± 7.5% of PKC-ε−/− levels [n = 7 mice; current data pooled with that previously reported (27)].

Hypoxic exposure and hemodynamic measurement. Animals were exposed to either normoxia (Nx) (Denver altitude) or Hx (18,000 ft, hypobaric chamber) for 5 wk. Exposures were continuous with interruptions of <1 h every 3–4 days for animal maintenance. At the end of the treatment period, mice were anesthetized by intramuscular injection with Ketamine-Rompun (100 and 15 mg/kg; Fort Dodge and Miles Laboratories, respectively) and weighed. After calibration of the pressure transducer (Statham), closed-chest measurements of RVSP (an indirect index of PA pressure) and heart rate were performed and recorded on spontaneously breathing mice as previously described under normoxic conditions (27). Gray et al. (13) recently completed an extensive cardiac assessment of PKC-ε+/+ and PKC-ε−/− mice including direct inspection, echocardiography and left heart catheterization, and they found no differences in cardiac structure or function at baseline or after acute ischemia reperfusion. This work suggests that in these mice RVSP measurements should yield useful, albeit indirect, data on PA pressure changes.

Blood and tissue harvesting. Blood samples were routinely taken from the RV via percutaneous stick at the end of the hemodynamic
measurements via a separate syringe and 25-gauge needle. The hematocrit was measured with a capillary tube and standard techniques. Collected blood samples were allowed to coagulate for ~1 h, and serum was collected after centrifugation. After baseline hemodynamic measurements, the chest was opened, and 100 units of heparin were injected into the RV. Lungs were isolated, inflated, and fixed as previously described with modifications (11). The pulmonary circulation was gently perfused at a pressure of 40 cmH2O with PBS to remove blood. The left bronchus was ligated, and the left lung was removed and quick-frozen in liquid nitrogen. A slit was made in the trachea, and a 16-gauge tubing adapter was inserted and tied in place. A syringe filled with 1% warmed agarose (GIBCO, Grand Island, NY) and 1% paraformaldehyde in PBS was attached to the adapter. An inline pressure transducer (Statham) with Biopac recorder was used to monitor inflation pressure in the airway in selected studies (n = 5 mice). The right lung was routinely inflated over ~70 s to a highly reproducible visual endpoint (tip of the right lower lobe was completely inflated) and an airway pressure of 30.5 ± 3.9 cmH2O. In additional control studies, local tissue pressure in the peripheral lung before or after inflation was measured in separate mouse lungs by pleural puncture with a 25-gauge needle attached to the same pressure transducer. Baseline pressure was 2.1 ± 0.5 cmH2O with the technique described (n = 4 mice). A blinded lung pathologist (C. D. Cool) examined sections from the six experimental groups and judged the quality and consistency of inflation and fixation to be excellent across all groups. The trachea was tied off, and the lung was removed and placed in cold 2% paraformaldehyde/PBS for 2 h, cut, and placed in cassettes in 10% buffered formalin for up to 24 h before being embedded in paraffin. In selected experiments the heart was removed and fixed in 10% buffered formalin (VWR, West Chester, PA) before being embedded in paraffin.

Measurement of RVH. Immediately following removal of inflated lungs, hearts were routinely removed and weighed. The atria were trimmed away and the right ventricular free wall (RV) was dissected from the left ventricle (LV) and septum (S). Both ventricular sections were weighed wet and then reweighed after being air-dried for 7 days. A blinded pathologist (C. D. Cool) reviewed cross sections of hearts from all experimental groups and found the quality of fixation to be excellent.

Tissue staining. Sections (4 μm) of embedded mouse lung and heart tissues were deparaffinized at 60°C for 45 min and then rehydrated. Tissues were placed in 0.5% hydrogen peroxide (Fisher Scientific, Fairlawn, NJ) for 5 min, washed, and blocked in PBS containing 1% horse serum, 0.6% Triton 100 and avidin (Vector Lab, Burlingame, CA) for 2 h. Hematoxylin and eosin (H&E) staining was performed on lung and heart sections from PKC-ε-KO mice, no other agents were tested. The NO concentration was monitored by continuous gas analysis (Pulmonox II; Pulmonox Medical, Tofield, Alberta, Canada). Because of the effectiveness of inhaled NO in the PKC-ε−/− mice, no other agents were tested.

NO isoform expression in mouse whole lung homogenates and tissue sections. Quick-frozen lung tissue was weighed and homogenized on ice and processed as described for measurement of PKC-ε expression. Whole lung homogenate (50–80 μg) was loaded per lane of an 8% reduced SDS-polyacrylamide gel, and positive controls for iNOS [lipopolysaccharide (LPS)/PMN-activated RAW 264.7 murine macrophage cell lysates; BD Transduction Laboratories, San Diego, CA] and nNOS (mouse brain extract; Santa Cruz Biotechnology, Santa Cruz, CA) were used and transferred to a nitrocellulose membrane as previously described (27). Molecular weight protein markers (Bio-Rad, Hercules, CA) were also loaded into the first lane of each gel. Anti-eNOS and phospho-specific (p)-eNOS antibodies were purchased from BD Transduction Laboratories, and anti-nNOS antibody from Zymed (South San Francisco, CA). Using LPS/PMN-activated RAW 264.7 murine macrophage cell lysates as a positive control, we compared anti-iNOS antibodies from BD Transduction Laboratories (polyclonal and monoclonal antibodies), Affinity Bio Reagents, Biomol, and Santa Cruz Biotechnology under optimal conditions per manufacturer instructions for sensitivity and specificity by Western blot. The polyclonal iNOS antibody from BD Transduction Laboratories was found to be the most sensitive and specific and was used for all experiments.

After a 1-h incubation at room temperature (RT, 37°C) in 2% BSA/Triis-buffered saline (TBS)/0.05% Tween 20 [for eNOS and (Ser-1177 and Thr-495) p-eNOS detection] or ON incubation at 4°C in 5% dry milk/TBS/0.05% Tween 20 (for nNOS and iNOS detection) to block nonspecific binding, the nitrocellulose was probed with eNOS (1:1,000), p-eNOS (1:1,000), nNOS (1:1,000), or iNOS (1:1,000) antibodies ON at 4°C. Blots were incubated for 1 h at RT with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG at a
Comparisons were performed by two-way analysis of variance (ANOVA) followed by the Scheffé’s multiple-comparison tests for individual comparisons within and between groups of data points. Data were considered significantly different if \( P < 0.05 \).

**RESULTS**

Effect of chronic hypoxia on RVSP, heart rate, RVH, and hematocrit in PKC-ε\(^{+/+}\), PKC-ε\(^{-/-}\), and PKC-ε\(^{-/+}\) mice. To test the effect of reduced PKC-ε on the murine pulmonary hypertensive response to chronic hypoxia, we exposed adult PKC-ε\(^{+/+}\), PKC-ε\(^{-/-}\), and PKC-ε\(^{-/+}\) mice to N(5,200 ft, Denver altitude) or chronic Hx (18,000 ft) for 5 wk, and RVSP (an indirect measure of PA pressure) and heart rate were measured \((n = 9–13\) mice/group). An extensive cardiac assessment of PKC-ε\(^{+/+}\) and PKC-ε\(^{-/-}\) mice including direct inspection, echocardiography, and left heart catheterization was recently completed by Gray et al. (13). They found no differences in cardiac structure or function at baseline or after acute ischemia reperfusion. Baseline pressures were similar in PKC-ε\(^{+/+}\), PKC-ε\(^{-/-}\), and PKC-ε\(^{-/+}\) mice (Fig. 2). However, following chronic Hx, PKC-ε\(^{-/-}\) mice had augmented RVSP compared with PKC-ε\(^{-/+}\) mice. PKC-ε\(^{-/+}\) mice also tended to have higher RVSP than PKC-ε\(^{-/-}\) mice. The actual magnitude of the increase over baseline pressure rose progressively as a function of genotype \((10.8 \pm 1.3, 15.3 \pm 1.5, \text{and } 28.2 \pm 2.0 \text{ mmHg})\), respectively, for PKC-ε\(^{-/-}\), PKC-ε\(^{-/+}\), and PKC-ε\(^{-/+}\) mice. Heart rates were similar in PKC-ε\(^{+/+}\), PKC-ε\(^{-/-}\), and PKC-ε\(^{-/+}\) mice at baseline and following chronic Hx [Nx: PKC-ε\(^{+/+}\) = 359 ± 27, PKC-ε\(^{-/+}\) = 415 ± 16, and PKC-ε\(^{-/-}\) = 370 ± 24 bpm; Hx: PKC-ε\(^{-/+}\) = 370 ± 27, PKC-ε\(^{-/-}\) = 339 ± 18, PKC-ε\(^{-/+}\) = 377 ± 22 bpm; \( P = \text{not significant (NS)}; n = 11–13\) mice/group]. Another indirect index of PA pressure, RV mass, was similar at baseline for PKC-ε\(^{+/+}\), PKC-ε\(^{-/-}\), and PKC-ε\(^{-/+}\) mice (Fig. 3). PKC-ε\(^{-/-}\) mice also tended to have a lesser extent, PKC-ε\(^{-/-}\) mice had a greater increase in the RV/LV+S ratio (a measure of RVH) following chronic Hx.
compared with PKC-ε+/+ mice. The pattern of RVH closely paralleled the RVSP responses seen in the PKC-ε+/+, PKC-ε+/−, and PKC-ε−/− mice. An analysis of hypoxia-induced change in RVSP vs. hypoxia-induced change in RV/LV+S across the three genotypes revealed a strong correlation between increased RVSP and RV mass (y = 0.0103x + 0.0107, R² = 0.9351). A close relationship between increased mean PA pressure (measured by catheterization) and RVH has previously been demonstrated in a rat model of hypoxic PHN (33). Other studies in mice have also shown a strong correlation between elevated RVSP and RVH (19). Cross-sectional images of PKC-ε+/+ and PKC-ε−/− H&E-stained hearts demonstrated the range of RV hypertrophic response observed (Fig. 4). The intermediate response of PKC-ε−/− hearts is not shown.

Baseline hematocrits were similar in all three groups. No differences in O₂ saturation were detected between genotypes at baseline. PKC-ε+/+ mice had a modest polycythemic response to chronic Hx, PKC-ε−/−, but not PKC-ε+/−, mice had a greater polycythemic response to chronic Hx than PKC-ε+/+ mice. Thus PKC-ε+/− mice exhibited a gene dosing effect for increased RVSP and RVH, but not polycythemia. (Nx: PKC-ε+/+ = 43.6 ± 7.3%, PKC-ε−/− = 43.4 ± 1.9%, PKC-ε+/− = 39.5 ± 5.3%; Hx: PKC-ε+/+ = 57.0 ± 2.9%, PKC-ε−/− = 57.9 ± 5.0%, PKC-ε+/− = 70.3 ± 3.1% n = 3 mice/group). No difference in magnitude of desaturation was observed following chronic Hx in PKC-ε+/− vs. PKC-ε−/− mice.

Effect of chronic Hx on pulmonary vascular structure in PKC-ε+/+, PKC-ε+/−, and PKC-ε−/− mice. To determine the effect of reduced PKC-ε on pulmonary vascular structure at baseline and in response to chronic Hx, morphometric analysis of both proximal (50–125 μm) and distal (10–50 μm) vessels was performed on PKC-ε+/+, PKC-ε+/−, and PKC-ε−/− mouse lung sections following double staining for endothelial (factor VIII) and smooth muscle (α-SM actin) cells. Measurements of medial wall thickness of proximal vessels in PKC-ε+/+, PKC-ε+/−, and PKC-ε−/− mouse lungs were not dependent on vessel density calculations (Table 1). At baseline no differences in wall thickness across the three genotypes were detected. A subtle increase in the percent medial wall thickness was found in PKC-ε+/+, PKC-ε−/−, and PKC-ε−/− mice exposed to chronic Hx. The signal was of similar magnitude across all three genotypes and, when pooled, was significantly greater than the aggregate normoxic response. Thus despite the increased RVSP and RVH response, PKC-ε−/− and PKC-ε+/− mice did not have increased pulmonary vascular remodeling of their proximal vessels compared with PKC-ε+/+ mice. In a separate review, a blinded pulmonary vascular pathologist detected only a subtle difference in proximal vessels between Nx- and Hx-treated groups upon review of H&E-stained slides (C. D. Cool). No further differences between genotypes within these treatment groups were found. PKC-ε−/− and PKC-ε+/− mice also did not have increased vascular remodeling in their distal vessels (i.e., muscularization) compared with PKC-ε+/+ mice following chronic Hx (Fig. 5).

Table 1. Medial wall thickness of proximal vessels from PKC-ε+/+, PKC-ε+/−, and PKC-ε−/− mice at baseline and in response to chronic hypoxia

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Normoxia, %</th>
<th>Hypoxia, %</th>
</tr>
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<tbody>
<tr>
<td>PKC-ε+/+</td>
<td>9.4 ± 0.6</td>
<td>11.1 ± 0.5</td>
</tr>
<tr>
<td>PKC-ε−/−</td>
<td>9.0 ± 0.7</td>
<td>10.1 ± 0.6</td>
</tr>
<tr>
<td>PKC-ε+/−</td>
<td>9.7 ± 0.2</td>
<td>10.8 ± 0.9</td>
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Values are means ± SE. *P < 0.05 pooled hypoxic response of all 3 genotypes compared with normoxic values; n = 4–7 mice. The signal was of similar magnitude across all 3 genotypes and when pooled was significantly greater than the aggregate normoxic response. A minimum of 10 vessels in the 50 to 125-μm-size range were analyzed in a blinded manner per animal. Percent medial thickness was calculated as described in MATERIALS AND METHODS.
muscularization (PM + FM divided by total vessels \( \times 100 \)) were also not dependent on vessel density calculations. Vessel density (total 10- to 50-\( \mu \)m-diameter vessels divided by unit
area of lung examined) was similar in PKC-\( \varepsilon^{-/-} \), PKC-\( \varepsilon^{+/+} \), and PKC-\( \varepsilon^{-/-} \) mice at baseline (Nx: PKC-\( \varepsilon^{+/+} \) 13.3 \( \pm \) 1.3, PKC-\( \varepsilon^{-/-} \) 12.6 \( \pm \) 2.1, PKC-\( \varepsilon^{-/-} \) 16.3 \( \pm \) 2.4 vessels/\( \mu \)m\(^2\); \( P \) = not significant; \( n = 6–9 \) mice/group). After chronic Hx, the calculated density of 10- to 50-\( \mu \)m-diameter vessels in PKC-\( \varepsilon^{-/-} \) lungs was lower than was found in normoxic PKC-
\( \varepsilon^{-/-} \) lungs (Hx: PKC-\( \varepsilon^{+/+} \) 14.0 \( \pm \) 2.7, PKC-\( \varepsilon^{-/-} \) 16.9 \( \pm \) 1.7, PKC-\( \varepsilon^{-/-} \) \( * \) 10.4 \( \pm \) 0.9 vessels/\( \mu \)m\(^2\); \( * P < 0.05 \) vs. Nx PKC-
\( \varepsilon^{-/-} \); \( n = 6–9 \) mice/group).

Effect of acute inhaled NO on pulmonary hypertensive response of PKC-\( \varepsilon^{+/+} \) and PKC-\( \varepsilon^{-/-} \) mice to chronic Hx. To evaluate the contribution of sustained vasoconstriction to the increased RVSP in PKC-\( \varepsilon^{-/-} \) mice, NO was given (25 ppm \( \times \) 5 min) following either Nx or chronic Hx, and the effect on RVSP was measured. The decrease in RVSP in PKC-\( \varepsilon^{+/+} \) and PKC-\( \varepsilon^{-/-} \) mice under Nx conditions was slight (1–2 mmHg, data not shown) and consistent with previous reports (9). After chronic Hx, only a small increase in the vasodilatory response to NO was seen in PKC-\( \varepsilon^{-/-} \) mice. In contrast, chronically Hx PKC-\( \varepsilon^{-/-} \) mice had a more substantial vasodilatory response to inhaled NO showing reversibility of the exaggerated PHTN (Fig. 6).

Expression of NOS isoforms in PKC-\( \varepsilon \) wild-type and null mice at baseline and following chronic Hx. The selective reversal of PHTN observed following NO treatment suggested that eNOS or one of the other NOS isoforms may be downregulated in PKC-\( \varepsilon^{-/-} \) mice at baseline or following chronic Hx. Therefore, we compared the expression of eNOS, nNOS, and iNOS in whole lung homogenates from PKC-\( \varepsilon^{+/+} \) and PKC-\( \varepsilon^{-/-} \) mice under baseline Nx conditions and following chronic Hx. A 140-kDa eNOS band of similar intensity was detected in PKC-\( \varepsilon^{+/+} \) and PKC-\( \varepsilon^{-/-} \) mice at baseline (Fig. 7A). Expression of eNOS following chronic Hx was increased in PKC-\( \varepsilon^{+/+} \) and PKC-\( \varepsilon^{-/-} \) mice, but this response was greater in PKC-\( \varepsilon^{-/+} \) mice. At baseline, expression of nNOS in PKC-
\( \varepsilon^{+/+} \) lungs was higher compared with PKC-\( \varepsilon^{-/-} \) lungs. After chronic Hx, nNOS expression selectively decreased in PKC-
\( \varepsilon^{-/+} \) mouse lungs (Fig. 7B). A faint 170-kDa nNOS band was detected in both groups of lungs under Nx conditions, as well as in a positive control (mouse brain extract, not shown).

Expression of the iNOS protein could not be detected by Western blotting techniques in PKC-\( \varepsilon^{+/+} \) and PKC-\( \varepsilon^{-/-} \) lung homogenates at baseline or following chronic Hx despite increased sample loading, prescreening several available antibodies for sensitivity and specificity, and increasing the amounts of primary and secondary antibody used beyond manufacturer recommendations. This is consistent with previous studies that did not find iNOS protein expression at baseline in mouse lung homogenates and only saw upregulation of the iNOS protein following treatment with LPS (2, 14, 36). A 130-kDa band consistent with the molecular mass of the iNOS protein was detected in the positive control lanes containing 1 and 10 \( \mu \)g of an LPS/PMA-activated RAW 264.7 murine macrophage cell lysate (Fig. 8A). Expression of iNOS was therefore only qualitatively assessed by immunostaining (Fig. 8, B–G). A blinded observer scored (0–4+) stained sections for signal in proximal vessels, large airways, distal vessels, and alveolar walls. Variable staining of proximal vessels and large airways was observed in both PKC-\( \varepsilon^{+/+} \) and PKC-\( \varepsilon^{-/-} \) lungs (2+). No signal (0+) was appreciated in distal vessels or alveolar walls of PKC-\( \varepsilon^{+/+} \) or PKC-\( \varepsilon^{-/-} \) lungs. After chronic Hx, iNOS expression in PKC-\( \varepsilon^{+/+} \) lungs was increased in both proximal vessels and large airways (4+) but remained of variable intensity. Hx-induced iNOS expression in proximal vessels and large airways of PKC-\( \varepsilon^{-/-} \) lungs was less than that observed in PKC-\( \varepsilon^{-/+} \) mice (2–3+). In contrast to PKC-\( \varepsilon^{-/+} \) mice, no signal was detected in the distal vessels of hypoxia-exposed PKC-\( \varepsilon^{-/-} \) mice. These qualitative differences were appreciable when a more dilute concentration of primary antibody was applied (1:1,000).

Effect of chronic Hx on NOS activity in PKC-\( \varepsilon^{+/+} \) and PKC-\( \varepsilon^{-/-} \) mice. To relate the changes found in eNOS protein expression with eNOS activity, we assessed p-eNOS expression (Ser-1177 and Thr-495) in PKC-\( \varepsilon^{+/+} \) and PKC-\( \varepsilon^{-/-} \) lungs at baseline and following chronic Hx. eNOS activity is modulated in part by balancing the phosphorylation of Ser-1177 (activation) and Thr-495 (inhibition) residues by multiple
PKC-ε KNOCKOUT MICE AND CHRONIC HYPOXIA

**DISCUSSION**

This study demonstrates that the adaptive response of the pulmonary circulation to chronic Hx is dependent on PKC-ε although the resulting pulmonary vascular phenotype is different than we originally anticipated. We found that chronically hypoxic PKC-ε-/- and, to a lesser extent, PKC-ε+/+ mice have increased PHTN and RVH compared with PKC-ε+/+ mice. The PKC-ε-/- mice also develop a higher hematocrit than PKC-ε+/+ mice after 5 wk of Hx; however, the PKC-ε-/- mice do not. For this and other reasons discussed here, we think differences in polycythemia contribute only modestly to the exaggerated PHTN. Interestingly, the PHTN changes were not accompanied by exaggerated pulmonary vascular remodeling, suggesting that vasoconstriction is a major contributor to the increased pressure. We hypothesized and found that the increased RVSP detected in chronically hypoxic PKC-ε-/- mice could be reversed with acutely inhaled NO. Although direct PA pressure measurements were not performed in this study, the differential effect of inhaled NO observed here combined with the recent noninvasive and invasive cardiac assessment published by Gray et al. (13), our heart rate data, and the tight correlation we observed between RVSP and RVH all suggest that the RVSP measurements reported here are likely reflective of PA pressure changes. Whether the vasodilatory effect of NO is specific vs. other vasodilators is not yet known. Thus our studies suggest that vasoconstriction is a major contributor to the increased PHTN seen in the PKC-ε-/- and PKC-ε+/+ mice. In light of the pressure elevation and extent of RVH, the absence of PKC-ε actually appears to provide relative protection from increased pulmonary vascular remodeling.

The reversibility with NO suggested that inadequate baseline and/or Hx-induced expression/activity of one or more of the NOS isoforms might be contributing to the divergent responses seen here. Decreased NOS expression/activity has been found in models of systemic vascular injury (12, 24). Fagan et al. (11) have shown that NO derived from eNOS and to a lesser extent iNOS is the major mediator of vasodilation in the murine pulmonary circulation. Ping et al. (32) have shown that the eNOS and iNOS isoforms are physically associated with PKC-ε in the heart, suggesting a novel role in the modulation of these two NOS isoforms in myocytes. We found that baseline eNOS and iNOS protein expression by Western blotting and immunostaining, respectively, was similar in PKC-ε-/- vs. PKC-ε+/+ lungs. We also found a trend toward lower nNOS expression in PKC-ε-/- vs. PKC-ε+/+ lungs at baseline. However, this isoform of NOS does not have a known role in pulmonary vascular biology and probably is more important in airway function. Our findings in PKC-ε+/+ mice following chronic Hx are consistent with previous reports that expression...
of eNOS increases with chronic hypoxic PHTN in rodents (24). After 35 days of Hx, eNOS expression was induced to a greater extent in PKC-ε+/+ than PKC-ε−/− mice. We also found, by immunostaining, a trend that iNOS expression was also increased more in PKC-ε+/+ than PKC-ε−/− lungs. The results support a role for PKC-ε in the regulation of Hx-induced expression of eNOS rather than in the direct regulation of enzymatic activity by phosphorylation of the enzyme. PKC-ε+/+ and PKC-ε−/− lungs showed similar p-eNOS expression of the Thr-495 residue under normoxic and chronic hypoxic conditions, suggesting eNOS activity was not decreased relative to expression by this mechanism. These findings imply that PKC-ε is important in the regulation of Hx-induced expression of eNOS rather than enzymatic activity. Li et al. (25) showed that various PKC inhibitors prevent phorbol ester-induced enhancement of eNOS expression; furthermore, this enhancement seemed to be a transcriptional event. This observation suggested PKC is important in the induction of eNOS expression but did not implicate a specific isozyme, nor was it necessarily relevant to hypoxic responses.

The regulation of eNOS activity is complex and involves several kinases including PKC, PKA, Akt, and AMP-activated protein kinase as well as two phosphatases including PP1 and PP2A (29). PKC signaling has been implicated in eNOS protein phosphorylation at Thr-495 as well as promoting dephosphorylation of the protein at Ser-1177. These events result in decreased eNOS activity in bovine aortic as well as human umbilical vein endothelial cells by inhibiting the interaction between eNOS and calmodulin (28, 29). However, the specific PKC isozyme involved in this regulation of eNOS activity has not been determined, as only general PKC activation by phorbol 12,13-dibutyrate treatment has been observed. We thought it was possible PKC-ε was involved here; however, our results do not support this.

NOx measurements are similar to the results of p-eNOS (Ser-1177 and Thr-495 residues) blotting, which suggests that eNOS protein expression (but not activity per unit protein) was increased following Hx. The NOx measurements in PKC-ε+/+ mice are also consistent with similar studies that showed that rats breathing 10% O2 chronically did not have an increase in NO plasma metabolite levels, suggesting that Hx inhibited NO production despite increases in eNOS isoform expression (35). Limiting factors here are the sampling site (final RV stick after initial hemodynamic measurements with potential for mixing with arterial blood from the LV), the single 35-day time point, and the recent studies showing NOx measurements, regardless of site, may not be that informative (17). Although serum NOx concentrations have been shown to correlate with erythrocyte NOx concentrations, a recent study suggests that steady-state
NOx concentrations reflect renal excretion of NOx metabolites rather than NOx formation by endothelial cells in vivo (17).

The immunostaining data also support a role for PKC-ε in the regulation of Hx-induced expression of iNOS. Interestingly, macrophages from PKC-ε−/− mice have an attenuated response to LPS and interferon with decreased generation of NO (1). Macrophages from these PKC-ε−/− mice fail to induce iNOS expression, suggesting that PKC-ε is important in cytokine-induced expression of iNOS. Our observation of a blunted increase in iNOS expression in PKC-ε−/− mice exposed to chronic Hx is also consistent with a previous report in RAW 264.7 murine macrophages suggesting that PKC-ε is sufficient to induce iNOS synthesis via a mechanism that involves activation of response elements in the iNOS promoter region (7).

Reactive polycythemia is probably also contributing modestly to the increased PA pressure detected in PKC-ε−/− mice. Polycythemia has been shown to increase blood viscosity and pulmonary vascular resistance (16). Reactive polycythemia has only a small effect on PA pressure and takes several weeks to detect. Walker et al. (37) showed that administration of human recombinant erythropoietin (Epo) for 2 wk to rats to increase hematocrit from 45 to 66% made no difference in mean PA pressure. At 4 mo of age, mice that chronically overexpress Epo (10-fold over control) and have severely elevated hematocrit (86 vs. 45 for controls) have only modestly elevated PA pressures (22 vs. 15 for controls) (15). Finally, Petit et al. (31) have shown that potentiation of hematocrit in chronically hypoxic rats from 63 to 72% with rEpo (a scenario similar in some ways to what we are observing here) failed to further enhance PHTN. Polycythemia could alter eNOS expression or activity, thereby altering vascular reactivity. There are conflicting reports that polycythemia increases eNOS expression and NO production (16, 37). Walker et al. (37) showed that in rats administered rEpo, hematocrit levels were found to be increased compared with control; however, the increase in shear stress associated with this polycythemia did not increase eNOS expression or NO metabolites. The Epo-overexpression mouse with severely elevated hematocrit has increased eNOS expression (15, 16). If anything, these studies reinforce the importance of the blunted NOS response we observed.

Despite the increased RVSP and RVH response, PKC-ε−/− and PKC-ε+/− mice did not have increased pulmonary medial remodeling. Medial wall thickness of proximal vessels was subtly increased to the same extent in all three genotypes following chronic Hx. The distal vasculature also did not show increased pulmonary remodeling. Neither measurement was dependent on vessel density calculations. The extent of remodeling we observed was less than the maximal levels reported for other genetically altered mice, suggesting that a ceiling for extent of structural change had not yet been reached (19). Collectively, these findings demonstrate that the contractile and structural responses of the murine lung circulation to chronic Hx can be uncoupled from one another and from RVH. Chronically hypoxic PKC-ε−/− mice have a deficiency in Hx-induced eNOS expression in the lung that probably contributes to the increased PHTN detected. PKC-ε is likely an important intermediate in the regulation of Hx-induced eNOS as well as iNOS expression. Loss of PKC-ε provides relative protection from exaggerated pulmonary vascular, but not RV, remodeling in response to chronic Hx. These findings suggest that PKC-ε is an important determinant of susceptibility to chronic hypoxic PHTN.

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