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Article

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Cytosolic phospholipase A_2 in hypoxic pulmonary vasoconstriction

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Introduction

Hypoxic pulmonary vasoconstriction (HPV) is an intrinsic property of pulmonary vascular smooth muscle that produces vasoconstriction in poorly ventilated lung regions (1). This important mechanism acts to enhance the matching of ventilation with perfusion and maintain arterial oxygenation in lung disease. Despite intensive investigation, a comprehensive understanding of the cellular mechanisms that underlie HPV remains elusive.

In the lung, the metabolism of arachidonic acid (AA) by cyclooxygenases (COX), lipoxygenases (LOs), and cytochrome P450 (CYP450) enzymes generates a wide variety of eicosanoids, including prostaglandins, thromboxanes, leukotrienes, epoxyeicosatrienoic acids (EETs), and hydroxyeicosatetraenoic acids (HETEs), that importantly regulate vascular tone (for recent reviews see refs. 2, 3). Potential roles for eicosanoids in HPV have been studied extensively with conflicting results reported. For example, administration of exogenous AA has been shown to either attenuate (4) or enhance HPV (5). Moreover, studies of HPV using inhibitors of COX, LOs, and CYP450 enzymes are limited by the incomplete specificity of the inhibitors, as well as the metabolic diversion of AA to alternate pathways.

Unesterified AA is present at very low levels within cells, and its rate of formation generally controls the biosynthesis of bioactive eicosanoids (2). Release of AA from the *sn*-2 position of membrane phospholipids is mediated by phospholipase A_2 (PLA_2) enzymes. PLA_2 enzymes can be classified into three main types based on biological properties, including secretory PLA_2 ($sPLA_2$), cytosolic PLA_2 ($cPLA_2$), and intracellular Ca^{2+}-independent PLA_2 ($iPLA_2$). Here, we studied group IVA $cPLA_2$, because AA generated by this PLA_2 is tightly linked to the COX and LO pathways (6) and this PLA_2 is known to be expressed in murine lung (7).

To assess the contribution of $cPLA_2$ to HPV, we compared the increase in left lung pulmonary vascular resistance induced by selective left lung hypoxia in wild-type mice and $cPLA_2$-deficient mice. Here, we report that an absence of $cPLA_2$ activity prevents HPV in mice.

Methods

All animal experiments were conducted under protocols reviewed and approved by the Subcommittee on Research Animal Care of the Massachusetts General Hospital. Mice with a deletion of the *PLA2g4a* gene ($cPLA_{2\alpha}^{-/-}$) (7) and their wild-type littermates ($cPLA_{2\alpha}^{+/+}$) were maintained at the Massachusetts General Hospi-

tal Animal Resource Facility. C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). We tested mice of both sexes with an age range of 2–5 months, weighing 18–30 g. Animals in each experimental group were matched for sex and age.

Measurement of HPV in mice. We surgically prepared mice for hemodynamic study as described previously, and systemic artery pressure (SAP), pulmonary artery pressure (PAP), and left pulmonary artery blood flow (QLPA) were continuously recorded (8). To estimate the left lung pulmonary vascular resistance (LPVR), the inferior vena cava (IVC) was partially occluded with a circumferential 5-0 silk ligature to transiently reduce cardiac output until QLPA was reduced by approximately 50%. To calculate LPVR, the flow-pressure relationship was constructed by plotting approximately 50 consecutive digitized data points of linear parts of PAP and QLPA tracings during transient IVC occlusions. The best-fit line that describes the relationship between PAP and QLPA was obtained by linear regression analysis. The slope of this best-fit line represents incremental LPVR during the IVC occlusion.

Left lung alveolar hypoxia was induced by reversibly occluding the left main stem bronchus (LMBO) with a microvascular clip. Complete collapse of the left lung was visually observed within about a minute and confirmed by transient overinflation of the right lung. Transient IVC occlusion was repeated three times before and during LMBO in each animal, and the average of three slopes each was reported as LPVR at baseline and during LMBO. The percentage of increase in LPVR induced by LMBO (ΔLPVR) was obtained by calculating the percentage of change of the mean value of the slopes in each mouse (9).

Vessel morphometry. After breathing for 3 weeks at an inspired oxygen fraction (F_IO_2) of 0.21 (normoxia) or 0.10 (hypoxia), the airways and pulmonary vessels of $cPLA_{2\alpha}^{-/-}$ and $cPLA_{2\alpha}^{+/+}$ mice (n = five each group) were perfusion-fixed. Lung tissue was embedded in Historesin Plus (Leica Microsystems Inc., Deerfield, Illinois, USA), and 2-μm-thick sections were stained with 0.1% toluidine blue (10). Vessels (greater than 14 and less than 100 μm in external diameter [ED]) associated with alveolar ducts or in the alveolar wall were evaluated. The percentage of wall thickness (%WT) of each transversely cut vessel was calculated (as WT × 100/ED for partially muscular and 2 × WT × 100/ED for fully muscular vessels). The endothelial component of the vessel wall was excluded from the measurement of WT.

Measurement of PLA_2 activity in lung homogenates. Mice were sacrificed after breathing at a F_IO_2 of 0.10 or 0.21 for 3 weeks, and the right lung was homogenized in ice-cold buffer containing 10 mM HEPES, 1 mM EDTA, 0.34 M sucrose, 1 μg/ml aprotinin, 1 μM pepstatin A, 1 mM PMSF, and 100 μM leupeptin. Crude homogenates were centrifuged at 4°C for 5 minutes at 5,000 *g*, and PLA_2 activity in the supernatant was measured, as described previously (11), using 1-stearoyl-2-[^{14}C] arachidonyl-phosphatidylcholine (PC) as substrate for $cPLA_2$ and 1-stearoyl-2-[^{14}C] arachidonyl-phosphatidylethanolamine (PE) for both $sPLA_2$ and $cPLA_2$. To measure activity of $iPLA_2$, 30 μg of lung protein were incubated with 50 nmol 1-palmitoyl-2-[^{14}C] palmitoyl-phosphatidylcholine, 1 mM ATP, and 2 mM DTT in a reaction volume of 500 μl for 30 minutes at 37°C (12).

Identification of murine lung eicosanoids during HPV. At the end of HPV experiments, $cPLA_{2\alpha}^{-/-}$ and $cPLA_{2\alpha}^{+/+}$ mice were sacrificed (n = 5 each), and their hypoxic left lungs and oxygen-ventilated right lungs were rapidly excised and snap-frozen. The samples were prepared by solid-phase extraction, and liquid chromatography-tandem mass spectrometry (LC/MS/MS) was performed to characterize the eicosanoid profiles with an LCQ ion trap mass spectrometer system (Finnigan Corp., San Jose, California, USA) (13). Eicosanoids were identified by their respective MS/MS and retention times compared with those of synthetic standards. In some experiments, deuterium-labeled LTB_4-d_4 and thromboxane B_2-d_4 (Cayman Chemical, Ann Arbor, Michigan, USA) were used as internal standards. In addition, levels of prostaglandins E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$) and thromboxane B_2 (TXB_2) were analyzed by ELISA using commercially available ELISA kits (Neogen Corp., Lexington, Kentucky, USA).

Measurements of HPV in $cPLA_{2\alpha}^{-/-}$ mice. Measurements of LPVR were carried out in $cPLA_{2\alpha}^{-/-}$ mice (n = 10) and $cPLA_{2\alpha}^{+/+}$ mice (n = 11) before and 5 minutes after LMBO. After LPVR was measured, arterial blood was sampled by direct left ventricular puncture for blood gas analysis during LMBO.

Effects of $cPLA_2$ inhibitor on HPV. C57BL/6 mice were treated with the selective $cPLA_2$ inhibitor, arachidonyl trifluoromethyl ketone (ATK) (20 mg/kg dissolved in 100 μl of 2.5 vol% DMSO; n = 4) or were treated with vehicle (n = 3) by a single intravenous injection 30 minutes before measurement of ΔLPVR. The dose of ATK and timing of administration were chosen based on data published previously (14).

Effects of exogenous AA on HVP. The $cPLA_{2\alpha}^{-/-}$ mice (n = 6) and $cPLA_{2\alpha}^{+/+}$ mice (n = 3) received a continuous intravenous infusion of sodium salt of AA (1 μg/kg/min) for 60 minutes before measurements of ΔLPVR. We selected this dose of AA on the basis of results from pilot experiments.

Pulmonary vascular response to angiotensin II. Measurements of total pulmonary and systemic vascular resistances (TPVR and TSVR, respectively) were obtained in $cPLA_{2\alpha}^{-/-}$ mice (n = 6) and $cPLA_{2\alpha}^{+/+}$ mice (n = 6) before and during an intravenous infusion of increasing doses of angiotensin II (0.05, 0.5, and 5 μg/kg/min), as described previously (8). Cardiac output was estimated by measuring lower thoracic aortic flow (QLTAF), while SAP and PAP were continuously recorded. In additional mice, the effects of ATK pretreatment (20 mg/kg) on the pulmonary vasoconstriction induced by angiotensin II infusion were examined.

Measurements of effects of COX inhibition on HPV. ΔLPVR was measured 30 minutes after intravenous adminis-

Table 1
Hemodynamic measurements

			HR (beats per minute)		SAP (mmHg)		PAP (mmHg)		QLPA (μl/min/g)		LPVR (mmHg/ml/min/g)	
		n	Baseline	LMBO	Baseline	LMBO	Baseline	LMBO	Baseline	LMBO	Baseline	LMBO
Untreated	$cPLA_{2\alpha}^{+/+}$	11	591 ± 20	595 ± 26	76 ± 5	63 ± 3	16 ± 1	17 ± 1	70 ± 9	50 ± 7[C]	107 ± 13	216 ± 29[C]
	$cPLA_{2\alpha}^{-/-}$	10	567 ± 18	550 ± 20	77 ± 4	64 ± 3	15 ± 1	15 ± 1	80 ± 5	70 ± 5	97 ± 6	100 ± 10[B]
DMSO-treated	C57BL/6	5	462 ± 38	447 ± 24	66 ± 9	61 ± 10	18 ± 1	18 ± 1	83 ± 6	51 ± 4[C]	117 ± 28	179 ± 43[C]
ATK-treated	C57BL/6	3	475 ± 93	453 ± 80	69 ± 8	71 ± 6	15 ± 1	15 ± 1	107 ± 6	85 ± 15	67 ± 12	72 ± 13
Indomethacin	$cPLA_{2\alpha}^{+/+}$	4	389 ± 23[A]	431 ± 30	87 ± 15	92 ± 15	14 ± 1	16 ± 1	63 ± 8	49 ± 6	78 ± 25	142 ± 33[C]
-treated	$cPLA_{2\alpha}^{-/-}$	5	358 ± 23[A]	402 ± 51	80 ± 3	83 ± 4	16 ± 2	18 ± 2	80 ± 12	61 ± 13	68 ± 14	139 ± 30[C]
L-NAME–treated	$cPLA_{2\alpha}^{+/+}$	3	499 ± 46	474 ± 30	72 ± 8	68 ± 10	17 ± 2	20 ± 4	80 ± 18	44 ± 16	167 ± 68[A]	448 ± 45[C]
	$cPLA_{2\alpha}^{-/-}$	5	516 ± 36	503 ± 35	78 ± 10	69 ± 14	15 ± 1	16 ± 1	54 ± 3	29 ± 3[C]	110 ± 15	261 ± 38[C]
After 3 weeks	$cPLA_{2\alpha}^{+/+}$	6	462 ± 19	475 ± 26	60 ± 6	60 ± 8	20 ± 2[A]	22 ± 3	84 ± 9	49 ± 6[C]	122 ± 6	226 ± 18[C]
at F_IO_2 0.10	$cPLA_{2\alpha}^{-/-}$	10	443 ± 16	432 ± 18	65 ± 4	64 ± 3	23 ± 1[A]	23 ± 1	129 ± 10[A,B]	77 ± 7[C]	83 ± 8[B]	157 ± 15[B,C]

Hemodynamic measurements before LMBO (baseline) and 5 minutes after LMBO in $cPLA_{2\alpha}^{+/+}$ and $cPLA_{2\alpha}^{-/-}$ mice. All values at baseline before LMBO were compared between groups by ANOVA. [A]$P < 0.05$ vs. untreated mice of respective genotype. [B]$P < 0.05$ vs. $cPLA_{2\alpha}^{+/+}$ mice in the same treatment group. The effect of LMBO on each parameter was analyzed in each group by ANOVA with a post-hoc comparison. [C]$P < 0.05$ vs. baseline value of the same parameter in the same group. HR, heart rate.

tration of indomethacin (Sigma-Aldrich, St. Louis, Missouri, USA) at a dose of 5.0 mg/kg in $cPLA_{2\alpha}^{-/-}$ mice ($n = 5$) and $cPLA_{2\alpha}^{+/+}$ mice ($n = 4$). This dose was shown to completely inhibit COX activity in mice (15) and to enhance HPV in rabbits (16).

Measurements of effects of nitric oxide synthase inhibition on HPV. ΔLPVR was measured 30 minutes after intravenous administration of nitro-L-arginine methylester (L-NAME; Sigma-Aldrich) at a dose of 100 mg/kg in $cPLA_{2\alpha}^{-/-}$ mice ($n = 4$) and $cPLA_{2\alpha}^{+/+}$ mice ($n = 3$). This dose was chosen based on results from a previous study (17).

Measurements of effects of prolonged hypoxia on right ventricular hypertrophy, pulmonary vascular remodeling, hemoglobin concentration, and HPV. $cPLA_{2\alpha}^{-/-}$ and $cPLA_{2\alpha}^{+/+}$ mice were housed in specially constructed environmental chambers (18), wherein for 3 weeks they breathed at F_IO_2 0.10 (hypoxia) or 0.21 (normoxia). Thereafter, in $cPLA_{2\alpha}^{-/-}$ mice ($n = 15$) and $cPLA_{2\alpha}^{+/+}$ mice ($n = 12$), the ratio of the weight of the right ventricle (W_{RV}) to the sum of the weights of the left ventricle and septum (W_{LV+S}) (Fulton's ratio: W_{RV}/W_{LV+S}) was calculated, and hemoglobin (hb) levels were determined in whole blood. Lungs were perfusion fixed for histology as described above. At the end of the exposure period, additional $cPLA_{2\alpha}^{+/+}$ mice ($n = 5$) and $cPLA_{2\alpha}^{-/-}$ mice ($n = 10$) were removed from the chamber, anesthetized, and ventilated with 100% oxygen, and the increase of LPVR in response to LMBO was examined at thoracotomy.

Effects of an inhibitor of iPLA₂ on HPV. ΔLPVR was measured in $cPLA_{2\alpha}^{-/-}$ ($n = 3$) and $cPLA_{2\alpha}^{+/+}$ ($n = 3$) mice that were treated with the selective iPLA₂ inhibitor, bromoenol lactone (BEL); (Biomol Research Laboratories, Plymouth Meeting, Pennsylvania, USA; dissolved in 100 μl of 2.0 volume percent DMSO) by two intravenous injections of 0.1 mg/kg 30 minutes before and during LMBO. The dose of BEL was calculated to produce a plasma concentration of 10 μM, which was previously shown to abrogate hypoxia-induced increase in AA release from cardiac myocytes (19). Additional mice of each genotype were treated with vehicle alone ($n = 3$ for each genotype).

Statistical analysis. Differences between groups were determined using a two-way ANOVA. When significant differences were detected by ANOVA, a post hoc Scheffé test was employed (Statistica for Windows; StatSoft, Inc., Tulsa, Oklahoma, USA). A P value less than 0.05 indicated a significant difference. All data are expressed as SEM.

Results

Unilateral alveolar hypoxia and the contribution of cPLA₂. To assess the contribution of cPLA₂ to HPV, we examined changes of LPVR in response to LMBO in $cPLA_{2\alpha}^{+/+}$ and $cPLA_{2\alpha}^{-/-}$ mice. Before LMBO, hemodynamic parameters did not differ between the two genotypes (Table 1). LPVR was similar in $cPLA_{2\alpha}^{+/+}$ mice (107 ± 13

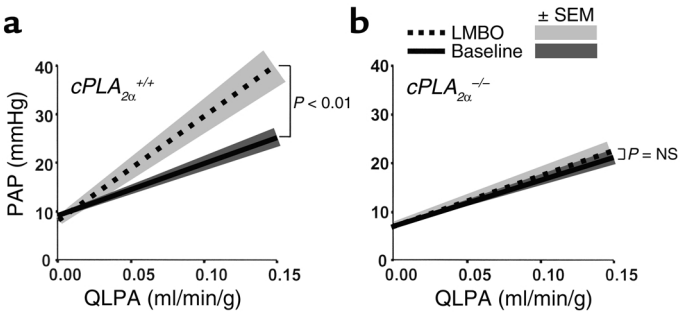

Figure 1
The left lung pulmonary blood flow-pressure relationship before (baseline) and after 5 minutes of LMBO in (**a**) $cPLA_{2\alpha}^{+/+}$ mice ($n = 11$) and (**b**) $cPLA_{2\alpha}^{-/-}$ mice ($n = 10$). The increase of the slope of the left lung pulmonary blood flow-pressure relationship after LMBO in $cPLA_{2\alpha}^{+/+}$ mice represents the LMBO-induced increased in LPVR ($P < 0.01$ vs. baseline). In contrast, in $cPLA_{2\alpha}^{-/-}$ mice, LMBO did not increase LPVR (**b**).

Table 2
Arterial blood gas analysis

	$cPLA_{2\alpha}^{+/+}$	$cPLA_{2\alpha}^{-/-}$
pH	7.38 ± 0.04	7.32 ± 0.04
$PaCO_2$ (mmHg)	36.3 ± 5.1	37.7 ± 2.9
PaO_2 (mmHg)	298 ± 46	159 ± 23[A]
HCO_3^- (mmol/l)	20.1 ± 1.6	18.7 ± 1.1
Hb (g/dl)	14.3 ± 1.4	13.4 ± 0.6

Arterial blood gas analyses at the end of hemodynamic studies in $cPLA_{2\alpha}^{+/+}$ mice (n = 6) and $cPLA_{2\alpha}^{-/-}$ mice (n = 8) breathing F_IO_2 = 1 during LMBO. [A]P < 0.05 vs. $cPLA_{2\alpha}^{+/+}$ mice.

mmHg/ml/min/g) and $cPLA_{2\alpha}^{-/-}$ mice (97 ± 6 mmHg/ml/min/g). In $cPLA_{2\alpha}^{+/+}$ mice, LMBO decreased QLPA without changing PAP, more than doubling LPVR (216 ± 29 mmHg/ml/min/g; P < 0.005; Table 1 and Figure 1a). In contrast, LMBO did not change LPVR in $cPLA_{2\alpha}^{-/-}$ mice (100 ± 10 mmHg/ml/min/g; Figure 1b), consistent with impaired HPV. In a subset of animals of each genotype (n = 3–5), QLTAF was measured as an estimate of cardiac output: LMBO did not alter QLTAF or TPVR in either genotype, demonstrating that the LMBO-induced changes in LPVR cannot be attributed to alterations in cardiac output.

To estimate the impact of impaired HPV on arterial oxygenation, we performed arterial blood gas analysis at the end of HPV measurements during LMBO while the right lung was ventilated at F_IO_2 = 1. Arterial partial pressure of oxygen (PaO_2) was significantly higher in $cPLA_{2\alpha}^{+/+}$ mice than in $cPLA_{2\alpha}^{-/-}$ mice (298 ± 46 and 159 ± 23 mmHg, respectively; P < 0.05; Table 2). There was no difference in blood pH, arterial partial pressure of carbon dioxide ($PaCO_2$), or HCO_3^- (Table 2). These observations are consistent with increased intrapulmonary shunting in $cPLA_{2\alpha}^{-/-}$ mice. Next, we tested whether or not pharmacological inhibition of $cPLA_2$ impaired HPV in wild-type mice. The LMBO-induced increase in LPVR was markedly attenuated when mice were studied 30 minutes after treatment with the $cPLA_2$ inhibitor, ATK, as compared with mice treated with vehicle alone (Figure 2). These results confirm that decreased $cPLA_2$ activity is associated with impaired HPV in mice.

Since $cPLA_2$ is believed to be a major enzymatic source of AA for eicosanoid biosynthesis (3, 20) in most mammalian cells, we examined whether administration of AA restores HPV in $cPLA_{2\alpha}^{-/-}$ mice. After continuous intravenous infusion of AA at 1 μg/kg/min for 60 minutes, LMBO increased LPVR in $cPLA_{2\alpha}^{-/-}$ mice (Figure 2). In contrast, when studied in $cPLA_{2\alpha}^{+/+}$ mice, the magnitude of the LMBO-induced increase of LPVR was not altered by exogenous AA. These results suggest that impaired HPV induced by $cPLA_2$ deficiency was attributable to decreased availability of AA in the hypoxic lung of $cPLA_{2\alpha}^{-/-}$ mice.

To examine the specificity of the effects of $cPLA_2$ deficiency on HPV, we examined whether $cPLA_2$ deficiency attenuates the normoxic pulmonary vasoconstrictor response to intravenous infusion of 0.05, 0.5, and 5 μg/kg/min of angiotensin II. TPVR increased from 74 ± 12 mmHg/ml/min/g at baseline to 177 ± 18 mmHg/ml/min/g at 5 μg/kg/min angiotensin II in $cPLA_{2\alpha}^{+/+}$ mice (P < 0.01) and from 69 ± 13 to 217 ± 39 mmHg/ml/min/g in $cPLA_{2\alpha}^{-/-}$ mice (P < 0.001). TSVR increased from 468 ± 66 mmHg/ml/min/g at baseline to 956 ± 216 mmHg/ml/min/g at 5 μg/kg/min angiotensin II in $cPLA_{2\alpha}^{+/+}$ mice (P < 0.01) and from 435 ± 47 to 899 ± 144 mmHg/ml/min/g in $cPLA_{2\alpha}^{-/-}$ mice (P < 0.001). At each angiotensin II infusion dose, there was no difference in either the TPVR or TSVR between $cPLA_{2\alpha}^{+/+}$ and $cPLA_{2\alpha}^{-/-}$ mice. Similarly, the pulmonary vasoconstrictor response to angiotensin II did not differ between ATK-treated and vehicle-treated wild-type mice (data not shown). Taken together, our results demonstrate that $cPLA_2$ deficiency attenuated pulmonary vasoconstriction to hypoxia, without causing nonspecific dysfunction of the vasomotor contractile apparatus.

Pulmonary eicosanoid profiles. To identify the major eicosanoids generated in the lungs of $cPLA_{2\alpha}^{-/-}$ and $cPLA_{2\alpha}^{+/+}$ mice, we carried out profiling of eicosanoids using both ELISA and LC/MS/MS analyses. In lungs obtained from both $cPLA_{2\alpha}^{+/+}$ and $cPLA_{2\alpha}^{-/-}$ mice, we identified PGE_2, TXB_2, 5S-HETE, 12S-HETE, and 15S-HETE by matching their respective mass spectra and retention times with those of authentic synthet-

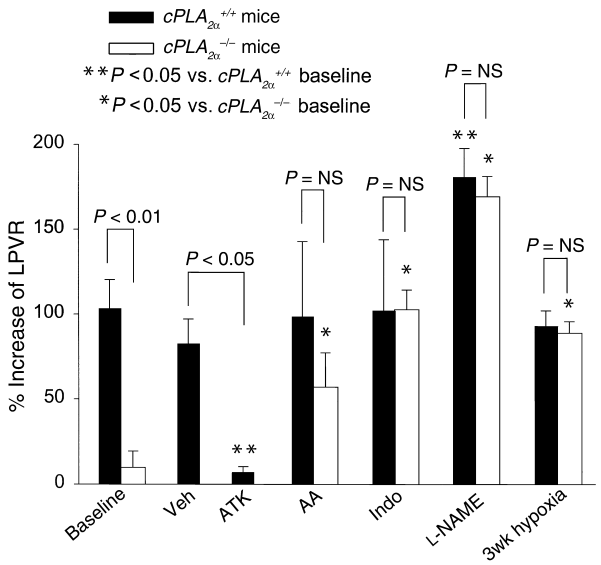

Figure 2
LMBO-induced increase in LPVR is shown for $cPLA_{2\alpha}^{+/+}$ (n = 11) and $cPLA_{2\alpha}^{-/-}$ (n = 10) mice at baseline and for wild-type C57BL/6 mice treated with vehicle (Veh; n = 5) or with ATK (n = 5). LMBO-induced increase in LPVR is also shown for mice during AA administration (n = 3 for $cPLA_{2\alpha}^{+/+}$ and n = 6 for $cPLA_{2\alpha}^{-/-}$), after administration of indomethacin (Indo; n = 4 for $cPLA_{2\alpha}^{+/+}$ and n = 5 for $cPLA_{2\alpha}^{-/-}$), after administration of L-NAME (n = 3 for $cPLA_{2\alpha}^{+/+}$ and n = 5 for $cPLA_{2\alpha}^{-/-}$), and after breathing at F_IO_2 0.10 for 3 weeks (3wk Hypoxia; n = 6 for $cPLA_{2\alpha}^{+/+}$ and n = 10 for $cPLA_{2\alpha}^{-/-}$). *P < 0.05 vs. $cPLA_{2\alpha}^{-/-}$ mice at baseline. **P < 0.05 vs. $cPLA_{2\alpha}^{+/+}$ mice at baseline.

ic standards (Figure 3, a and b). Leukotriene B_4 and EETs, as well as their respective omega-oxidation products, were not present in appreciable amounts in snap-frozen lungs from either genotype (Figure 3c) (2, 21). Moreover, 20-HETE was not detected in lungs of either genotype (22).

To examine the possibility that decreased levels of vasoconstrictor eicosanoids contribute to the impaired HPV in *cPLA*$_{2\alpha}^{-/-}$ mice, we quantitated vasoactive eicosanoids PGE_2, $PGF_{2\alpha}$, and TXB_2 by ELISA in *cPLA*$_{2\alpha}^{+/+}$ and *cPLA*$_{2\alpha}^{-/-}$ mice after LMBO. PGE_2 levels were higher in left than in right lungs in both genotypes ($P < 0.05$ for both; Figure 4a). $PGF_{2\alpha}$ and thromboxane levels were greater in left than in right lungs in *cPLA*$_{2\alpha}^{+/+}$ mice ($P < 0.05$ for both; Figure 4, b and c) but not in *cPLA*$_{2\alpha}^{-/-}$ mice. These observations suggest that COX products are increased in hypoxic left lungs of *cPLA*$_{2\alpha}^{+/+}$ mice. However, statistically significant differences were not found in the amounts of PGE_2, $PGF_{2\alpha}$, or TXB_2 when comparing corresponding lungs of *cPLA*$_{2\alpha}^{+/+}$ and *cPLA*$_{2\alpha}^{-/-}$ mice.

Both indomethacin and L-NAME restored HPV in cPLA$_{2\alpha}^{-/-}$ *mice*. To examine the possibility that HPV is impaired in *cPLA*$_{2\alpha}^{-/-}$ mice due to an altered vasoconstrictor/vasodilator balance, we studied the effects on HPV of inhibition of endothelium-dependent vasodilators, PGI_2, and nitric oxide (NO) by indomethacin and L-NAME, respectively. It has been reported that indomethacin and L-NAME enhance HPV at doses that do not alter the baseline pulmonary vascular resistance (16). Thirty minutes after the administration of indomethacin (5 mg/kg intravenously), the heart rate was decreased in both *cPLA*$_{2\alpha}^{+/+}$ and *cPLA*$_{2\alpha}^{-/-}$ mice as compared with untreated mice (591 ± 20 vs. 389 ± 23 and 567 ± 24 vs. 358 ± 23 beats per minute, respectively; $P < 0.001$ for each), but other hemodynamic parameters were unaffected (Table 1). The LMBO-induced increase in LPVR did not differ between indomethacin-treated and untreated *cPLA*$_{2\alpha}^{+/+}$ mice (ΔLPVR: 102% ± 42% vs. 103% ± 17%, respectively). However, in *cPLA*$_{2\alpha}^{-/-}$ mice, indomethacin treatment restored the ability of LMBO to increase the LPVR (103% ± 12%, $P < 0.01$, vs. untreated *cPLA*$_{2\alpha}^{-/-}$ mice) to the level measured in *cPLA*$_{2\alpha}^{+/+}$ mice (Figure 2).

In concordance with results from earlier studies (16, 17), 30 minutes after administration of L-NAME, baseline hemodynamic parameters, including PAP, QLPA, and SAP, were unchanged in both genotypes (Table 1). In *cPLA*$_{2\alpha}^{+/+}$ mice, L-NAME administration augmented the LMBO-induced increase in LPVR. In *cPLA*$_{2\alpha}^{-/-}$ mice, L-NAME administration restored the ability of LMBO to increase LPVR (Figure 2). Taken together, these results suggest that the pulmonary vasculature of *cPLA*$_{2\alpha}^{-/-}$ mice is capable of vasoconstriction in response to hypoxia when synthesis of an endothelium-dependent vasodilator is inhibited.

Chronic hypoxia in cPLA$_{2\alpha}^{-/-}$ *mice*. To learn if the attenuated vasoconstrictor response to acute hypoxia in *cPLA*$_{2\alpha}^{-/-}$ mice protects mice against the pulmonary vascular remodeling associated with prolonged exposure to hypoxia, we studied *cPLA*$_{2\alpha}^{-/-}$ and *cPLA*$_{2\alpha}^{+/+}$ mice that breathed at either F_IO_2 0.10 or 0.21 for 3 weeks. W_{RV}/W_{LV+S} was not different in *cPLA*$_{2\alpha}^{+/+}$ mice and *cPLA*$_{2\alpha}^{-/-}$ mice breathing air (25.2% ± 1.4% and 24.2% ± 1.5%, respectively). Although prolonged hypoxia increased W_{RV}/W_{LV+S} in both genotypes, RV hypertrophy was less prominent in *cPLA*$_{2\alpha}^{-/-}$ mice (30.3% ± 1.4 %) than in *cPLA*$_{2\alpha}^{+/+}$ mice (35.5% ± 1.4%; $P < 0.05$). The *cPLA*$_{2\alpha}^{+/+}$ mice and *cPLA*$_{2\alpha}^{-/-}$ mice had

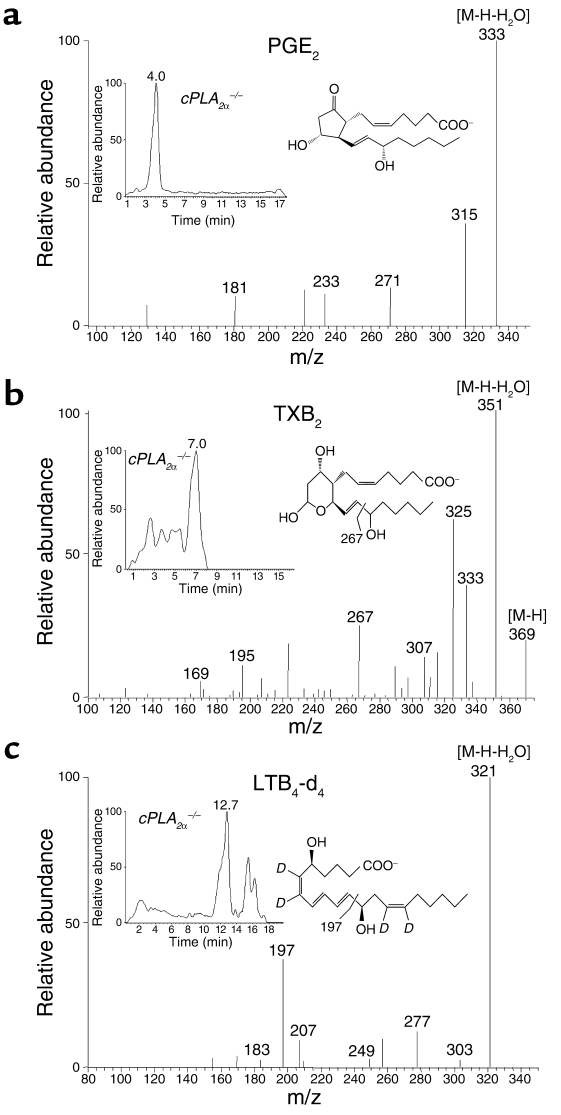

Figure 3
LC/MS analyses of eicosanoids from *cPLA*$_{2\alpha}^{-/-}$ mouse lungs. Results show representative LC/MS/MS analyses of lung extracts obtained from at least four separate experiments. LC retention times (RT), parent ions, and daughter ions for products extracted from murine lungs were diagnostic and matched those of authentic standards (see Methods for details). (**a**) LC/MS/MS spectrum (RT 4 minutes) of PGE_2. Inset: Chromatogram of ion mass/charge (m/z) 333. (**b**) LC/MS/MS spectrum (RT 7 minutes) of TXB_2. Inset: Chromatogram of ion m/z 369. (**c**) LC/MS/MS spectrum (RT 12.7 min) of LTB_4-d_4 used as internal standard. Inset: Chromatogram of ion m/z 339 showing the internal standard extracted from lung and the apparent absence of m/z 335 for endogenous LTB_4. *D*, deuterium.

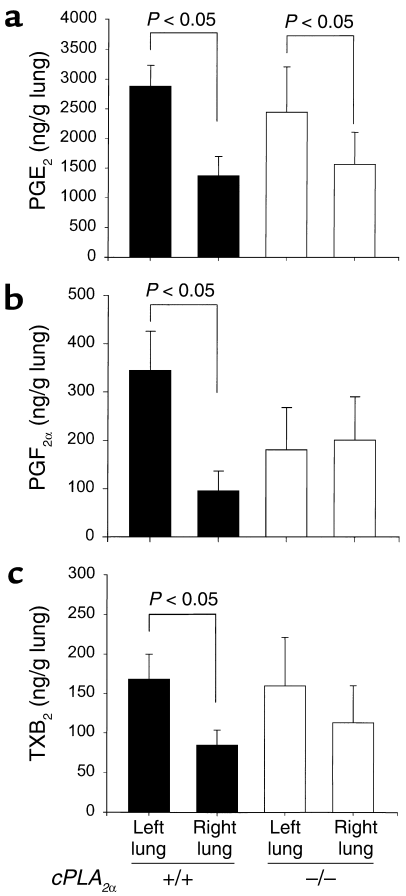

Figure 4
PGE_2 (**a**), $PGF_{2\alpha}$ (**b**), and TXB_2 (**c**) levels in right and left lung homogenates obtained from $cPLA_{2\alpha}^{+/+}$ and $cPLA_{2\alpha}^{-/-}$ mice after LMBO and measured by ELISA. Filled bars indicate $cPLA_{2\alpha}^{+/+}$ ($n = 5$) and open bars indicate $cPLA_{2\alpha}^{-/-}$ mice ($n = 5$). PGE_2 levels were higher in left than in right lungs in both genotypes ($P < 0.05$). $PGF_{2\alpha}$ and TXB_2 levels were higher in left than in right lungs in $cPLA_{2\alpha}^{+/+}$ mice ($P < 0.05$), but not in $cPLA_{2\alpha}^{-/-}$ mice. There was no difference in levels of PGE_2, $PGF_{2\alpha}$, and TXB_2 in either lung between the two genotypes.

similar hemoglobin levels under normoxic conditions (12.9 ± 0.5 and 12.9 ± 0.3 g/dl, respectively). Although chronic hypoxia increased the circulating hemoglobin concentration in both genotypes, $cPLA_{2\alpha}^{-/-}$ mice developed less polycythemia than $cPLA_{2\alpha}^{+/+}$ mice (16.5 ± 1.6 vs. 19.0 ± 0.3 g/dl; $P < 0.05$). Quantitative analysis revealed that the WT of pulmonary arterial vessels with external diameter between 14 and 100 μm was similar in $cPLA_{2\alpha}^{-/-}$ mice and $cPLA_{2\alpha}^{+/+}$ mice breathing at F_IO_2 0.21 (8% ± 2% vs. 8% ± 2%). Three weeks of hypoxia increased the WT of these pulmonary vessels similarly in both groups of mice (14% ± 2% vs. 14% ± 1%). Qualitative differences in the components of the vessel walls of the two genotypes were not evident after 3 weeks of hypoxia.

Baseline PAP, QLPA, and LPVR were measured at thoracotomy in mice during ventilation at F_IO_2 1. PAP was greater in both $cPLA_{2\alpha}^{+/+}$ and $cPLA_{2\alpha}^{-/-}$ mice that breathed at F_IO_2 0.10 for 3 weeks than in mice that breathed at F_IO_2 0.21 (Table 1). Breathing at F_IO_2 0.10 for 3 weeks did not increase the baseline LPVR significantly in mice of either genotype. Prolonged hypoxia did not alter the ability of LMBO to increase LPVR in $cPLA_{2\alpha}^{+/+}$ mice. In contrast, after 3 weeks of hypoxia, $cPLA_{2\alpha}^{-/-}$ mice showed a robust increase of LPVR in response to LMBO, demonstrating restored HPV in $cPLA_{2\alpha}^{-/-}$ mice (Figure 2). Of note, after breathing at F_IO_2 0.10 for 3 weeks, $cPLA_{2\alpha}^{-/-}$ mice had lower LPVR at baseline and during LMBO compared with $cPLA_{2\alpha}^{+/+}$ mice, apparently due to increased QLPA in $cPLA_{2\alpha}^{-/-}$ mice (Table 1).

Lung homogenates obtained from normoxic $cPLA_{2\alpha}^{+/+}$ mice de-esterified AA from PC, whereas lung homogenates from normoxic $cPLA_{2\alpha}^{-/-}$ mice metabolized PC only minimally ($P < 0.05$, Figure 5a). In contrast, lung homogenates from the normoxic $cPLA_{2\alpha}^{-/-}$ mouse contained PLA_2 activity directed against PE in assay conditions favoring the activity of $sPLA_2$. PE-metabolizing PLA_2 activity was greater in $cPLA_{2\alpha}^{+/+}$ mice ($P < 0.05$ vs. $cPLA_{2\alpha}^{-/-}$ mice; Figure 5b) most likely because $cPLA_2$ also acts on PE. Prolonged hypoxic exposure did not affect the activity of pulmonary $cPLA_2$ or $sPLA_2$ in either $cPLA_{2\alpha}^{+/+}$ mice or $cPLA_{2\alpha}^{-/-}$ mice (Figure 5, a and b). Lung homogenates obtained from normoxic $cPLA_{2\alpha}^{+/+}$ mice contained $iPLA_2$ activity, and it was not affected by breathing at F_IO_2 0.10 for 3 weeks (Figure 5c). The $iPLA_2$ activity in lung homogenates obtained from normoxic $cPLA_{2\alpha}^{-/-}$ mice was modestly greater than that in lung homogenates from $cPLA_{2\alpha}^{+/+}$ mice ($P = 0.055$), and it was decreased by breathing at F_IO_2 0.10 for 3 weeks ($P < 0.05$ vs. normoxic $cPLA_{2\alpha}^{-/-}$ mice; Figure 5c). Taken together, chronic hypoxia restored HPV in $cPLA_{2\alpha}^{-/-}$ mice without augmenting pulmonary PLA_2 activities.

Effects of inhibition of $iPLA_2$ on HPV. Since pulmonary $iPLA_2$ activity tended to be greater in normoxic $cPLA_{2\alpha}^{-/-}$ mice than in $cPLA_{2\alpha}^{+/+}$ mice, and $iPLA_2$ activity in $cPLA_{2\alpha}^{-/-}$ mice was decreased by prolonged breathing at F_IO_2 0.1, we considered the possibility that compensatory upregulation of $iPLA_2$ in $cPLA_{2\alpha}^{-/-}$ mice impaired HPV at baseline and reduction of $iPLA_2$ by chronic hypoxia restored HPV in $cPLA_{2\alpha}^{-/-}$ mice. To test this hypothesis, we examined effects of BEL, a selective $iPLA_2$ inhibitor, on HPV. Administration of BEL or vehicle did not affect baseline hemodynamics (data not shown). Administration of BEL did not affect HPV in $cPLA_{2\alpha}^{+/+}$ mice ($\Delta LPVR$ = 117% ± 20% and 102% ± 18% in BEL-treated and vehicle-treated $cPLA_{2\alpha}^{+/+}$ mice, respectively) or in $cPLA_{2\alpha}^{-/-}$ mice ($\Delta LPVR$ = 21% ± 10% and 28% ± 12% in BEL-treated and vehicle-treated $cPLA_{2\alpha}^{-/-}$ mice, respectively; both $P < 0.05$ vs. $cPLA_{2\alpha}^{+/+}$ mice with the same treatment). These results suggest that it is unlikely that a compensatory upregulation of $iPLA_2$ is responsible for impaired HPV in $cPLA_{2\alpha}^{-/-}$ mice.

Discussion

The present study demonstrates that $cPLA_2$ deficiency impairs HPV in mice resulting in a reduced ability to preserve systemic oxygenation after LMBO. Restoration

of HPV in *cPLA*$_{2\alpha}^{-/-}$ mice with exogenous AA suggests that AA availability itself and/or one or more downstream bioactive eicosanoids contribute to HPV in wild-type mice. Although we found that *cPLA*$_{2\alpha}^{-/-}$ mice are capable of producing eicosanoids (Figure 3) and could not demonstrate quantitative differences in the levels of AA-derived eicosanoids in lungs taken from *cPLA*$_{2\alpha}^{-/-}$ and *cPLA*$_{2\alpha}^{+/+}$ mice (Figure 4), it is possible that vasoactive eicosanoids present at low levels and/or generated from a minority of cells within the lungs of *cPLA*$_{2\alpha}^{+/+}$ mice (and hence not detected in whole-lung extracts using LC/MS/MS or ELISA) may be absent from those cells in *cPLA*$_{2\alpha}^{-/-}$ mice, thereby impairing HPV.

Alternatively, deficiency of AA production in *cPLA*$_{2\alpha}^{-/-}$ mice may modulate HPV. For example, a NADPH oxidase has been implicated in the sensing of pulmonary hypoxia (23, 24). Recently, Dana et al. used a cPLA$_2$-deficient cell line and showed that cPLA$_2$ and released AA are required for NADPH oxidase activation (25). It is possible that administration of AA restored HPV in *cPLA*$_{2\alpha}^{-/-}$ mice by activating NADPH oxidase-related events.

Impaired HPV in the *cPLA*$_{2\alpha}^{-/-}$ mice may be due to an alteration in the pulmonary vasoconstrictor/vasodilator balance, and administration of exogenous AA may have restored "balance." This hypothesis is supported by our observations that both indomethacin and L-NAME restored HPV in *cPLA*$_{2\alpha}^{-/-}$ mice using doses that do not alter baseline values of PAP and QLPA. Given the increased levels of COX products we detected in the left lungs after LMBO (Figure 4), the restoration of HPV by indomethacin and L-NAME is likely to be attributable to inhibition of endothelium-dependent production of vasodilator prostaglandins (i.e., PGE$_2$ and PGI$_2$) and NO, respectively, thereby shifting the "balance" in favor of vasoconstriction (16). These observations suggest that the pulmonary vasculature of *cPLA*$_{2\alpha}^{-/-}$ mice remains capable of constricting in response to hypoxia after major vasodilator metabolic pathways are blocked.

Because agents that attenuate HPV can prevent pulmonary hypertension and vascular remodeling in chronically hypoxic animals (26–28), we studied the effects of chronic hypoxia on *cPLA*$_{2\alpha}^{+/+}$ and *cPLA*$_{2\alpha}^{-/-}$ mice. Breathing at F_IO_2 0.10 for 3 weeks increased PAP similarly in both mouse genotypes. In addition, pulmonary vascular remodeling was evident in both *cPLA*$_{2\alpha}^{+/+}$ and *cPLA*$_{2\alpha}^{-/-}$ mice, and there was no difference in pulmonary vascular wall thickness between these genotypes. The observation that cPLA$_2$ deficiency partially protects against the development of RV hypertrophy in chronically hypoxic mice is potentially attributable to multiple factors. The initial absence of HPV, as well as the more modest increase in PVR after chronic hypoxia, likely decreased the stimulus for RV hypertrophy in *cPLA*$_{2\alpha}^{-/-}$ mice. In addition, cPLA$_2$ deficiency may have attenuated RV hypertrophy by reducing the degree of polycythemia (18).

Since *cPLA*$_{2\alpha}^{-/-}$ mice unexpectedly developed pulmonary vascular remodeling after prolonged breathing at F_IO_2 0.10, we investigated whether prolonged hypoxic exposure restored HPV in *cPLA*$_{2\alpha}^{-/-}$ mice. In wild-type mice, prolonged breathing at F_IO_2 0.10 did not alter the pulmonary vasoconstrictor response to LMBO. This finding in mice contrasts with previous reports suggesting that chronic hypoxia attenuates HPV in rats (29) and is possibly attributable to differences in the methods used to monitor HPV (30). In *cPLA*$_{2\alpha}^{-/-}$ mice, 3 weeks of breathing at F_IO_2 0.10 restored the ability of LMBO to increase LPVR to the levels measured in wild-type mice without augmenting pulmonary PLA$_2$ activities (Figure 5). It is possible that currently unappreciated compensatory mechanisms provided AA in the *cPLA*$_{2\alpha}^{-/-}$ mice after breathing at F_IO_2 0.10 for 3 weeks,

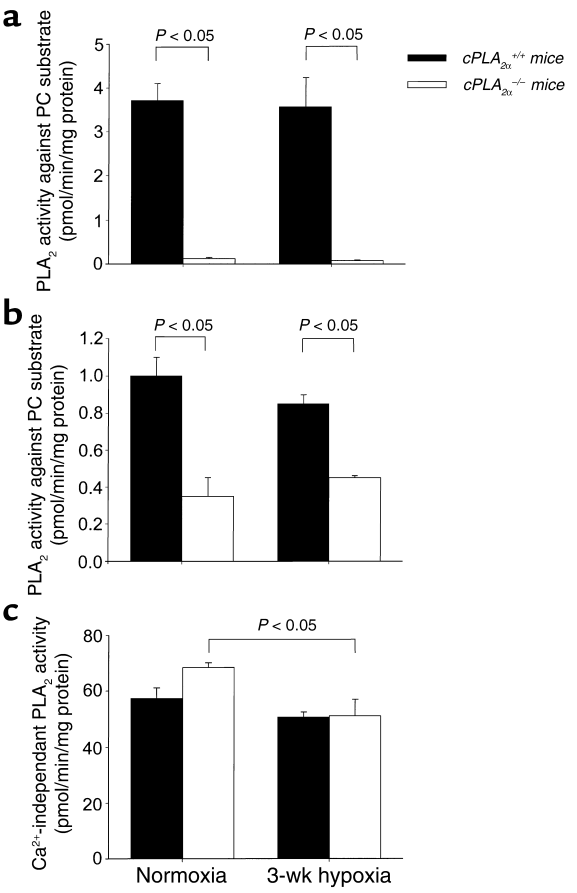

Figure 5
Calcium-dependent PLA$_2$ activity using PC substrate (**a**) and PE substrate (**b**) and iPLA$_2$ activity (**c**) in lung homogenates from the *cPLA*$_{2\alpha}^{+/+}$ and *cPLA*$_{2\alpha}^{-/-}$ mice at baseline and after 3 weeks of breathing at F_IO_2 0.10. Note that lung homogenates from the *cPLA*$_{2\alpha}^{+/+}$ have PC substrate-metabolizing activity (in assay conditions favoring the activity of cPLA$_2$), while homogenates from the *cPLA*$_{2\alpha}^{-/-}$ mice have essentially no phospholipase activity for the PC substrate at baseline or after 3 weeks of hypoxia. In contrast, the *cPLA*$_{2\alpha}^{-/-}$ mouse lung homogenate contained PE-metabolizing PLA$_2$ activity (in assay conditions favoring the activity of sPLA$_2$). Three weeks of breathing at F_IO_2 0.10 did not alter cPLA$_2$ or sPLA$_2$ activities in either the *cPLA*$_{2\alpha}^{+/+}$ or *cPLA*$_{2\alpha}^{-/-}$ mouse lungs. The iPLA$_2$ activity was modestly greater in *cPLA*$_{2\alpha}^{-/-}$ than in *cPLA*$_{2\alpha}^{+/+}$ mice at baseline ($P = 0.055$) and was decreased by breathing at F_IO_2 0.10 for 3 weeks in *cPLA*$_{2\alpha}^{-/-}$ mice ($P < 0.05$ vs. normoxic *cPLA*$_{2\alpha}^{-/-}$ mice).

thereby restoring HPV. For example, compensatory downregulation of AA turnover in cellular membranes could lead to increased levels of AA in the cellular compartment (31). Alternatively, chronic hypoxia may increase basal pulmonary vascular tone by modifying pulmonary arterial myocyte function, augmenting synthesis of other vasoactive factors or enhancing vasoresponsiveness to these mediators (32), and thereby restored HPV in $cPLA_2\alpha^{-/-}$ mice.

Recently, it has been suggested that inhibition of $cPLA_2$ may be beneficial in the treatment of acute lung injury. Nagase et al. reported that after administration of either lipopolysaccharide/zymosan or HCl, $cPLA_2\alpha^{-/-}$ mice developed less lung injury, as reflected by improved oxygenation and decreased edema formation and polymorphonuclear neutrophil sequestration (33). These results suggest that from the perspective of systemic oxygenation in lung injury the beneficial anti-inflammatory effects of decreased $cPLA_2$ activity may be greater than the detrimental effects of $cPLA_2$ deficiency on HPV and that inhibition of $cPLA_2$-initiated pathways could be a promising strategy in the management of acute lung injury (20, 33). Nonetheless, our findings suggest that pharmacological inhibition of $cPLA_2$ should be approached with caution since the loss of $cPLA_2$ activity may impair the ability of the lung to match ventilation and perfusion and thereby cause systemic hypoxemia.

In summary, our studies revealed that a deficiency of $cPLA_2$ activity, induced either by gene deletion or by inhibition of this enzyme, abolished HPV in mice. These results also demonstrate that $cPLA_2$ is not essential for HPV in conditions of enhanced pulmonary vasoconstrictor tone. These results underscore an important modulatory role of $cPLA_2$ in HPV.

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