Hypoxic pulmonary vasoconstriction and pulmonary artery tissue cytokine expression are mediated by protein kinase C

Ben M. Tsai, Meijing Wang, Jeffrey M. Pitcher, Kirstan K. Meldrum, and Daniel R. Meldrum.

Hypoxic pulmonary vasoconstriction and pulmonary artery tissue cytokine expression are mediated by protein kinase C. Am J Physiol Lung Cell Mol Physiol 287: L1215–L1219, 2004. First published August 20, 2004; doi:10.1152/ajplung.00179.2004.—Pulmonary arteries exhibit a marked vasoconstriction when exposed to hypoxic conditions. Although this may be an adaptive response to match lung ventilation with perfusion, the potential consequences of sustained pulmonary vasoconstriction include pulmonary hypertension and right heart failure. Concomitant production of proinflammatory mediators during hypoxia may exacerbate acute increases in pulmonary vascular resistance. We hypothesized that acute hypoxia causes pulmonary arterial contraction and increases the pulmonary artery tissue expression of proinflammatory cytokines via a protein kinase C (PKC)-mediated mechanism. To study this, isometric force displacement was measured in isolated rat pulmonary artery rings during hypoxia in the presence and absence of the PKC inhibitors calphostin C or chelerythrine. In separate experiments, pulmonary artery rings were treated with the PKC activator thymeleatoxin for 60 min. After hypoxia, with or without PKC inhibition, or PKC activation alone, pulmonary artery rings were subjected to mRNA analysis for TNF-α and IL-1β via RT-PCR. Our results showed that, in isolated pulmonary arteries, hypoxia caused a biphasic contraction and increased expression of TNF-α and IL-1β mRNA. Both effects were inhibited by PKC inhibition. PKC activation resulted in pulmonary artery contraction and increased the pulmonary artery expression of TNF-α and IL-1β mRNA. These findings suggest that hypoxia induces the expression of inflammatory cytokines and causes vasoconstriction via a PKC-dependent mechanism. We conclude that PKC may have a central role in modulating hypoxic pulmonary vasoconstriction, and further elucidation of its involvement may lead to therapeutic application.

Address for reprint requests and other correspondence: D. R. Meldrum, 545 Barnhill Dr., Emerson Hall 215, Indianapolis, IN 46202 (E-mail: dmeldrum@iupui.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
experiments, phenylephrine-precontracted PA rings (n = 5) were incubated in the presence of the PKC activator thymeleatoxin (THX, 1 μmol/l) for 60 min. THX has been demonstrated to be an effective activator of multiple PKC isozymes (25, 26).

RT-PCR. Semiquantitative RT-PCR was used to assess TNF-α and IL-1β gene expression in PA rings. After tissue homogenization of PA rings, total RNA was extracted from each PA segment using RNA STAT-60 (TEL-TEST, Friendswood, TX). Total RNA (0.1 μg) was subjected to cDNA synthesis with a cloned avian myeloblastosis virus first-strand cDNA synthesis kit (Maxim Biotech, South San Francisco, CA). cDNA from each sample was used for PCR of cytokines using message screen rat PCR kits (Maxim Biotech). PCR products were separated by electrophoresis on 1% agarose gel stained with ethidium bromide. Gels were photographed with a Polaroid Gelcam (Polaroid, Waltham, MA) under ultraviolet illumination (Spectronics, Westbury, NY). Gel photographs were scanned with an Epson Perfection 3200 scanner (Epson America, Long Beach, CA) onto an iMac PowerPC G4 computer and analyzed with ImageJ software (NIH).

Chemicals and reagents. All chemical reagents were obtained from Sigma (St. Louis, MO) unless otherwise specified. All drug concentrations are expressed as final molar concentration in the organ bath. All reagents were dissolved in deionized distilled water. KH solution is a physiologically balanced salt solution containing (in mmol/l): 127 NaCl, 4.7 KCl, 17 NaHCO3, 1.17 MgSO4, 1.18 KH2PO4, 2.5 CaCl2, and 5.5 D-glucose. Final pH of all solutions was 7.35–7.45.

Experimental protocol and groups. Before starting experimental protocols, we stretched PA rings to a predetermined optimal passive tension of 750 mg and allowed them to equilibrate for 60 min, during which time KH solution was changed every 15 min. We then checked viability of each PA ring by measuring contractile response to 80 mmol/l KCl. This dosage of KCl was determined to produce maximal contractile response in previous experiments. After washout of KCl, endothelial integrity of each PA ring was assessed with dilution to acetylcholine (1 μmol/l) after phenylephrine (1 μmol/l) preconstriction. Rings demonstrating <200 mg contraction to phenylephrine were discarded. In endothelium-intact PA, rings demonstrating <50% vasorelaxation to acetylcholine were discarded. After washout of acetylcholine, PA rings were precontracted with phenylephrine, and hypoxia was induced by changing the gas to 95% N2/5% CO2. Experiments were terminated after 60 min of hypoxia, and rings were immediately flash-frozen in liquid nitrogen before mRNA analysis. This protocol was performed in the presence or absence of the PKC inhibitors chelerythrine (1 μmol/l) or calphostin C (1 μmol/l). PKC inhibitors were added 30 min before the onset of hypoxia. In separate experiments, phenylephrine-precontracted PA rings (n = 5) were incubated in the presence of the PKC activator thymeleatoxin (THX, 1 μmol/l) for 60 min. THX has been demonstrated to be an effective activator of multiple PKC isozymes (25, 26).

RESULTS

Hypoxic pulmonary vasoconstriction. To measure the effect of hypoxia on PA, we gassed phenylephrine-precontracted PA with 95% N2/5% CO2 for 60 min. This produced a PO2 of 30–35 mmHg. Hypoxia caused a biphasic PA vasoconstriction (Fig. 1). The initial contraction occurred ~1–3 min following the onset of hypoxia. This was followed by a vasodilation and then a delayed and sustained vasoconstriction ~15–20 min after the onset of hypoxia. Maximum contractions were measured as the differences between the highest and lowest force displacements during hypoxia (Fig. 1) and expressed as a percentage of the precontractile force (phenylephrine, 1 μmol/l). The maximum early contraction of endothelium-intact control PA (n = 12) was 40.09 ± 8.12%, and the maximum delayed contraction was 94.07 ± 5.94%.

Role of PKC on hypoxic pulmonary vasoconstriction. To measure the effect of PKC inhibition on hypoxic pulmonary vasoconstriction (HPV), selective PKC inhibitors chelerythrine (1 μmol/l) or calphostin C (1 μmol/l) were added to the organ baths 30 min before phenylephrine preconstriction. PKC inhibition (n = 7/group) significantly attenuated delayed, but not early, hypoxic contraction (Figs. 2A and 3). The maximum early contraction was 28.13 ± 3.86% with chelerythrine (P = 0.269 vs. hypoxia alone) and 54.85 ± 10.04% with calphostin C (P = 0.274 vs. hypoxia alone), whereas the maximum delayed contraction was 61.55 ± 3.91% with chelerythrine...
PKC MEDIATES HPV AND TISSUE CYTOKINE EXPRESSION

HPV has been extensively studied, yet the exact mechanisms are still unclear. We and others (3, 10, 28) have reported that in isolated rat PA, a biphasic pattern of contraction occurs in response to acute hypoxia. The role of PKC on HPV in isolated perfused lung has been demonstrated in previous studies (1, 32); however, the involvement of PKC in the isolated PA is less clear. Moreover, the role of PKC on local proinflammatory cytokine expression during acute hypoxia has not previously been shown. In this study, we have demonstrated that, in isolated PA 1) PKC mediates delayed hypoxic contraction, 2) hypoxia upregulates PA tissue expression of TNF-α and IL-1β, and 3) the upregulation of TNF-α and IL-1β expression during hypoxia is dependent on PKC activation.

(P < 0.001 vs. hypoxia alone) and 68.04 ± 5.87% with calphostin C (P ≤ 0.05 vs. hypoxia alone).

**PKC activation.** To investigate the effect of PKC activation on isolated PA, we incubated phenylephrine-precontracted PA with the PKC activator THX (1 μmol/l) for 60 min. THX caused a transient relaxation followed by a gradual sustained vasoconstriction. The force displacement curve of THX is superimposed on the hypoxia alone curve (Fig. 2B) to demonstrate its temporal and quantitative similarities. THX-induced contraction (90.70 ± 23.13%) after 60 min was similar in magnitude (Fig. 3) to the delayed contraction induced by hypoxia (94.07 ± 5.94%).

**Proinflammatory cytokine expression during hypoxia.** After 60 min of hypoxia, PA rings were flash frozen in liquid nitrogen and later subjected to mRNA analysis via RT-PCR for the proinflammatory cytokines TNF-α and IL-1β. Hypoxia increased the expression of TNF-α (Fig. 4) and IL-1β (Fig. 5) compared with matched normoxic controls. In the presence of chelerythrine, the hypoxic expression of TNF-α and IL-1β was attenuated. Furthermore, PA rings treated with THX demonstrated increased expression of TNF-α and IL-1β (Fig. 6).

![Fig. 2. Role of PKC in hypoxic pulmonary vasoconstriction. A: in the presence of the PKC inhibitor chelerythrine (1 μmol/l, n = 7), delayed hypoxic contraction was significantly attenuated compared with hypoxia alone (n = 12). ‡P < 0.001 vs. hypoxia alone. B: when the PKC activator thymeleatoxin (THX, 1 μmol/l, n = 5) was added to a PE-precontracted pulmonary artery, a transient relaxation was followed by a gradual sustained contraction over 60 min. This curve was similar in magnitude and timing to the delayed contraction induced by hypoxia alone.](http://ajplung.physiology.org/)

![Fig. 3. Maximum contractile force with PKC inhibition or activation. In a comparison of maximum contractile force, PKC inhibition (chelerythrine or calphostin C, 1 μmol/l) significantly attenuated delayed hypoxic contraction (61.55 ± 3.91% with chelerythrine and 68.04 ± 5.87% with calphostin C vs. 94.07 ± 5.94% with hypoxia alone). PKC activation (THX, 1 μmol/l) resulted in a maximum contraction (90.70 ± 23.13%), similar to the delayed contraction induced by hypoxia. *P < 0.05 and ‡P < 0.001 vs. hypoxia alone.](http://ajplung.physiology.org/)

![Fig. 4. Hypoxia upregulates expression of TNF-α via a PKC-dependent mechanism. Representative gel photographs (2 lanes per group shown) depicting increased expression of TNF-α mRNA in pulmonary arteries exposed to 60 min of hypoxia compared with matched controls (60 min of normoxia). In the presence of the PKC inhibitor chelerythrine (1 μmol/l), TNF-α is not upregulated by hypoxia.](http://ajplung.physiology.org/)

**DISCUSSION**

HPV has been extensively studied, yet the exact mechanisms are still unclear. We and others (3, 10, 28) have reported that in isolated rat PA, a biphasic pattern of contraction occurs in response to acute hypoxia. The role of PKC on HPV in isolated perfused lung has been demonstrated in previous studies (1, 32); however, the involvement of PKC in the isolated PA is less clear. Moreover, the role of PKC on local proinflammatory cytokine expression during acute hypoxia has not previously been shown. In this study, we have demonstrated that, in isolated PA 1) PKC mediates delayed hypoxic contraction, 2) hypoxia upregulates PA tissue expression of TNF-α and IL-1β, and 3) the upregulation of TNF-α and IL-1β expression during hypoxia is dependent on PKC activation.
We hypothesized that the signaling pathway of HPV involves common intracellular enzymes such as PKC. Indeed, several investigators have demonstrated in isolated lung that delayed contraction is mediated by PKC activation (7, 10, 32). Our results of PKC-dependent delayed hypoxic contraction in isolated PA are consistent with previous studies using isolated perfused lung. Furthermore, we and others (19) demonstrated that PKC activation results in a slow, sustained contraction in isolated arteries. The magnitude and timing of this contraction appear similar to that of the delayed contraction induced by hypoxia (Figs. 2A and 3). Although the exact mechanisms of HPV remain unknown, previous studies (2, 6, 10–12, 21, 23, 27) have hypothesized two signaling processes: 1) inhibition or secretion of an endogenous mediator that results in vasocostriction and 2) opening or closing of calcium or potassium channels in the pulmonary vascular smooth muscle cell that leads to contraction. There is abundant evidence to support both hypotheses, and the precise mechanism of HPV may actually be multifactorial. Furthermore, each phase of the biphasic hypoxic contractions observed in the isolated PA may have different mechanisms, and there is conflicting evidence regarding the mechanisms responsible for each phase. In the current study, we postulated that the PKC plays a central role in HPV irrespective of extracellular mediators.

PKC activation causes vascular smooth muscle contraction by increasing the influx of calcium, and hypoxic contraction via PKC activation may likewise involve calcium influx. An increase in cytosolic calcium concentration appears to be a key event in HPV (2, 9, 21, 31). Calcium accumulation occurs by release from intracellular stores such as the sarcoplasmic reticulum or influx through voltage-dependent channels. Investigators have shown that blocking calcium channels (2, 10, 22), depletion of intracellular calcium stores (6, 8, 9, 22), and removing extracellular calcium (10, 13) inhibit hypoxic contraction, whereas calcium agonists potentiate HPV (24). Thus we speculate that the downstream events in PKC activation during hypoxia involve calcium influx and subsequent smooth muscle contraction.

There is evidence that hypoxemia alone, in the absence of blood loss or tissue injury, has been shown to induce the systemic release of proinflammatory cytokines by macrophages (5). In this study, hypoxia upregulated the expression of TNF-α and IL-1β from the PA itself, and this response was inhibited by chelerythrine. Pharmacological activation of PKC also upregulated PA expression of TNF-α and IL-1β. PKC isoforms have previously been shown to mediate TNF-α and IL-1β production from macrophages (16), but this is the first demonstration of PKC-mediated cytokine expression in PA. Although it is not clear what role proinflammatory cytokine production from the PA plays, or the magnitude of cytokine production from the PA, these findings suggest that the local production of cytokines may effect direct changes in vascular reactivity. Indeed, previous studies have shown that inflammatory cytokines potentiate HPV (33). This could be compared with the relatively recent recognition that production of inflammatory mediators from cardiomyocytes may contribute to myocardial dysfunction following ischemia-reperfusion or other acute insults (14).

The upregulation of cytokine expression from PA may occur at the pretranscriptional level. In this regard, nuclear factor (NF)-κB, a transcription factor involved in the transcription of proinflammatory molecules, may be involved in the signaling pathway of HPV. This was suggested by a study showing that NF-κB inhibitors blocked HPV (29). PKC may also be involved in the expression of NF-κB-dependent genes because Page et al. (20) demonstrated that activation of PKC-Δ in airway epithelial cells induces NF-κB nuclear translocation and binding to DNA. Thus the activation of inflammation as well as the physiological response of the vasculature to acute hypoxia may be dependent on PKC activation.

In summary, we have demonstrated for the first time that acute hypoxia upregulates the PA tissue expression of proinflammatory cytokines. This was correlated with the well-recognized physiological observation that hypoxia causes PA contraction, and both processes were attenuated by PKC inhibition. These findings suggest a putative therapeutic role for PKC inhibition in the treatment of HPV.

Fig. 5. Hypoxia upregulates expression of IL-1β via a PKC-dependent mechanism. Representative gel photographs (2 lanes per group shown) depicting increased expression of IL-1β mRNA in pulmonary arteries exposed to 60 min of hypoxia compared with matched controls (60 min of normoxia). In the presence of the PKC inhibitor chelerythrine (1 μmol/L), IL-1β is not upregulated by hypoxia.

Fig. 6. PKC activation upregulates expression of TNF-α and IL-1β. Representative gel photographs (2 lanes per group shown) depicting increased expression of TNF-α (top) and IL-1β (bottom) mRNA in pulmonary arteries exposed to 60 min of the PKC activator THX (1 μmol/M) compared with controls (60 min without PKC activator).
REFERENCES


