Response of Intra-acinar Pulmonary Microvessels to Hypoxia, Hypercapnic Acidosis, and Isocapnic Acidosis

Kazuhiro Yamaguchi, Koichi Suzuki, Katsuhiko Naoki, Kazumi Nishio, Nagato Sato, Kei Takeshita, Hiroyasu Kudo, Takuya Aoki, Yukio Suzuki, Atsushi Miyata, Harukuni Tsumura

Abstract—To elucidate the differential reactivity of pulmonary microvessels in the acini to hypoxia, excessive CO₂, and increased H⁺, we investigated changes in the diameter of precapillary arterioles, postcapillary venules, and capillaries in isolated rat lungs on exposure to normocapnic hypoxia (2% O₂), normoxic hypercapnia (15% CO₂), and isocapnic acidosis (0.01 mol/L HCl). Microvascular diameters were precisely examined using a real-time confocal laser scanning luminescence microscope coupled to a high-sensitivity camera with an image intensifier. Measurements were made under conditions with and without indomethacin or N^ω-nitro-L-arginine methyl ester to assess the importance of vasoactive substances produced by cyclooxygenase (COX) or NO synthase (NOS) as it relates to the reactivity of pulmonary microvessels to physiological stimuli. We found that acute hypoxia contracted precapillary arterioles that had diameters of 20 to 30 μm but did not constrict postcapillary venules of similar size. COX- and NOS-related vasoactive substances did not modulate hypoxia-elicited arteriolar constriction. Hypercapnia induced a distinct venular dilatation closely associated with vasodilators produced by COX but not by NOS. Arterioles were appreciably constricted in isocapnic acidosis when NOS, but not COX, was suppressed, whereas venules showed no constrictive response even when both enzymes were inhibited. Capillaries were neither constricted nor dilated under any experimental conditions. These findings suggest that reactivity to hypoxia, CO₂, and H⁺ is not qualitatively similar among intra-acinar microvessels, in which COX- and NOS-associated vasoactive substances function differently. (Circ Res. 1998;82:722-728.)

Key Words: acinus ■ hypoxia ■ acidosis ■ cyclooxygenase ■ nitric oxide synthase

In the normal lung, vascular reactivity of pulmonary vessels responding to O₂, CO₂, and hydrogen ion (H⁺) is considered exceedingly important in regulating the distribution of pulmonary blood flow, allowing the lung to maintain a reasonable match between ventilation and blood flow. Alveolar hypoxia and increased H⁺ concentration induced either by alveolar hypercapnia (hypercapnic acidosis) or by isocapnic acidosis (ie, metabolic acidosis) have been shown to evoke active vasoconstriction in the pulmonary circulation.¹⁻⁷ However, the CO₂ molecule, per se, has been presumed to induce pulmonary vasodilation, as when Viles and Shepherd¹² found the pressor response of isolated feline lungs to hypercapnic acidosis to be less than that to isocapnic acidosis at the same pH level. Thus, pulmonary vascular response to hypercapnic acidosis may be the net result of vasoconstriction due to increased H⁺ concentration and vasodilation due to increased CO₂ tension. Direct evidence, however, for CO₂ molecule-elicited vasodilatation of pulmonary vessels has not been obtained conclusively. Although several groups of investigators¹⁻⁷ have attempted to produce definite evidence of active vasoconstriction of pulmonary vessels mediated by alveolar hypoxia or by alveolar hypercapnia, the effects of alveolar hypercapnia on pulmonary vessels are less consistent than those of alveolar hypoxia. Additionally, we would place great emphasis on the fact that inquiry has been largely absent in determining whether intra-acinar microvessels, including precapillary arterioles and postcapillary venules, having diameters of 20 to 30 μm can be actively constricted in response to various physiological stimuli.

Vasoactive PGs and NO have recently emerged as important substances modifying pulmonary vessel response to hypoxic stimulation.¹⁸⁻²⁰ However, their significance in HPV in the acini has not been critically evaluated. Furthermore, analysis of the importance of PGs and/or NO in H⁺- and CO₂-elicited vascular response is very limited for the pulmonary circulation and totally lacking for the intra-acinar microcirculation. In view of these facts, we also intend to shed light on the possible roles of vasodilating PGs, such as prostacyclin (PGI₂) and NO, both of which are produced mainly in vascular endothelial cells, in modifying the contraction and/or dilatation of intra-acinar microvessels in intact rat lungs.
when exposed to alveolar hypoxia, hypercapnic acidosis, and isocapnic acidosis.

Materials and Methods
Preparation of Isolated Perfused Rat Lungs
We used isolated perfused lungs that were prepared from male pathogen-free Sprague-Dawley rats (8 weeks old) weighing 250 to 300 g (n=112). The isolated perfused lung preparation has been described in detail elsewhere.22,23 Briefly, after heparin was administered into the left ventricle, catheters were inserted into the pulmonary artery and left atrium and were secured with strings. A ligature was placed around the aorta to prevent loss of the perfusate into the systemic circulation. The isolated lung was then fixed on a microscopic stage with animals in the supine position and perfused at a constant recirculating flow rate of 0.2 mL/s with a roller pump (RotoR 1500N, Taitec). Kresbs-Henseleit solution with 3% bovine serum albumin was used as the perfusate, in which the Ht was adjusted to 0.05 by adding fresh blood obtained from donor rats. The trachea was ligated at the end-inspiratory position, and gas exchange was maintained with an ECMO (Merasilox-S, Senko) inserted between the isolated lung and the roller pump. A gas mixture containing 21% O2 and 5% CO2, at 8 mL/s, was used as the gas blowing onto the lung surface to maintain a temperature of 37°C and to avoid desiccation of the lung surface. Ppa was monitored continuously at 16 mL/s to the lung surface to maintain a temperature of 37°C and to avoid desiccation of the lung surface. Ppa was continuously monitored with a pressure transducer (TP-400T, Nihon Kohden) connected to a small cannula whose tip was located in the catheter that was inserted into the main pulmonary artery.

Experimental Protocols
After stable Ppa was attained, the gas flowing into the ECMO and the gas blown onto the lung surface were simultaneously switched from the control gas (21% O2 and 5% CO2 in N2) to the following mixtures: (1) hypoxic-normocapnic gas composed of 2% O2 and 5% CO2 in N2 for HPV to be elicited (perfusate PO2 was reduced to 6.45 kPa [48 mm Hg], P CO2 to 5.1±0.3 kPa [38±2 mm Hg], and pH to 7.4±0.1). A warmed, humidified gas mixture containing the same composition of gases as used for the ECMO was supplied continuously at 16 mL/s to the lung surface to maintain a temperature of 37±0.5°C and to avoid desiccation of the lung surface. Ppa was continuously monitored with a pressure transducer (TP-400T, Nihon Kohden) connected to a small cannula whose tip was located in the catheter that was inserted into the main pulmonary artery.

Selected Abbreviations and Acronyms

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>CCD</td>
<td>charge-coupled device</td>
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<tr>
<td>COX</td>
<td>cyclooxygenase</td>
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<tr>
<td>ECMO</td>
<td>extracorporeal membrane oxygenator</td>
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<td>HPAEC</td>
<td>human pulmonary artery endothelial cell</td>
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<td>HPV</td>
<td>hypoxic pulmonary vasoconstriction</td>
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<td>Ht</td>
<td>hematocrit</td>
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<td>6-keto-PGF2α</td>
<td>6-ketoprostaglandin F2α</td>
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<td>L-NAME</td>
<td>N N-nitro-l-arginine methyl ester</td>
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<tr>
<td>NOS</td>
<td>NO synthase</td>
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<tr>
<td>PG</td>
<td>prostaglandin</td>
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<td>Ppa</td>
<td>pulmonary arterial pressure</td>
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Experimental condition were used to measure the extent of change in overall vascular resistance occurring in the pulmonary circulation, including intra-acinar microvessels and extra-acinar vessels of a larger size.

To elucidate the importance of the response of vasoactive PGs and NO in the pulmonary circulation to alveolar hypoxia, hypercapnic acidosis, or isocapnic acidosis, each animal group was subdivided into three categories based on the agents used: (1) medication-free group (N group: measurements were made without administration of any agent); (2) indomethacin group (I group: indomethacin [Sigma Chemical Co] was used to restrain a constitutive form of COX [COX-1], and the perfusate concentration of indomethacin was adjusted to 20 μmol/L); (3) L-NAME group (L group: a constitutive form of NOS [endothelial NOS] was inhibited with L-NAME [Sigma], whose concentration in the perfusate was maintained at 100 μmol/L).

Measurements of Pulmonary Microvascular Diameters
To obtain images suitable for precisely estimating the events occurring in pulmonary microvessels, we used a confocal luminescence microscope recently developed in our laboratory.24 The reflected light or fluorescent emission from the sample was imaged onto a high-sensitivity CCD camera with an image intensifier (EktaPro Intensified Image VSG, Kodak), which can detect even very low fluorescence signals. By incorporating an excitation wavelength of 488 nm emitted from a low-power air-cooled argon ion laser (532-BSA04; output power, 10 mW; Omnichrome) with appropriate filters, our confocal system enabled us to obtain apparently instantaneous images at 1000 frames/s. The final magnifying power of our system reached ×968, with a ×40 objective, on the video screen. The resulting field of view was 105×105 μm, corresponding roughly to a diameter of a single pulmonary microvessel running beside the terminal bronchiolo.25 We registered confocal images at a rate of 250 frames/s by means of a high-speed video analysis system (EktaPro 1000 Processor, Kodak) connected to the image-intensified CCD camera (EktaPro Intensified Image VSG, Kodak).

To determine microvessel diameter and architecture, we added 200 μL of 5% FITC-dextran with a molecular weight of 145 000 (Sigma) to the reservoir. Defining the edge of the microvessel of interest as the portion exhibiting a steep change of fluorescence signal, we calculated vessel diameters by processing a confocal video image with a computer-assisted digital image-analyzing system (Quanta 840A Image 1.58, Apple). To discriminate between precapillary arterioles and postcapillary venules, we added a small quantity of erythrocytes stained with FITC (Sigma) to the perfusion medium and measured their flow direction with the confocal luminescence microscope.

Measurements of PGI2 and NO-Related Metabolites in the Perfusate
The perfusate sample (2 mL) collected in the tube containing indomethacin and EDTA was centrifuged and acidified with acetic acid and frozen at −80°C until extraction. At extraction, 1 mL of acidified fluid was extracted into 4-mL ethyl acetate by shaking for 1 hour. The ethyl acetate layer was transferred to a second tube, evaporated to dryness under pure N2 gas, and stored frozen. At assay, the dried extract was reconstituted in the assay buffer. Immunoreactive 6-keto-PGF1α, a stable metabolite of PGI2, was determined by ELISA (EIA kit, Cayman Chemical).

As a measure of NO production in the lung, we examined the total concentration of end products of NO metabolism, NO2− and NO3−, in the perfusate.26 The NO2−/NO3− level was spectrophotometrically determined by modifying the method of Green et al.27 Five milliliters of the perfusate was transferred into a vessel containing 5 mL of 5% ammonium chloride and 25 mL of distilled water. The prepared sample was passed through a column packed with copper-plated cadmium, allowing the complete reduction of NO3− to NO2−. Ten milliliters of the effluent was mixed with the reagent containing 1

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of HCl via exposure to the control gas, we measured pH<sub>i</sub> changes caused by isocapnic acidosis again for 15 minutes. pH<sub>o</sub> during hypercapnia was maintained at 7.05±0.03. In the absence of medications, the effect of indomethacin on vasoactive PG genesis and that of L-NAME on NO production before and after a given stimulation were judged by virtue of the paired t test or the Wilcoxon test. The differences in transitional changes in pH during hypercapnic acidosis and isocapnic acidosis were examined on the basis of the Hotelling T statistic and the unpaired t test. Values are presented as mean±SD, and P<.05 was taken to be statistically significant.

**Results**

**Basic Hemodynamic Characteristics in the Pulmonary Circulation**

Baseline Ppa before stimulation with hypoxia, hypercapnia, or isocapnic acidosis was made by the paired t test or the Wilcoxon test. Baseline capillary diameters were statistically the same under all experimental conditions.

**Microvascular Diameter Changes at Hypoxia, Hypercapnic Acidosis, and Isocapnic Acidosis in the Medication-Free Group**

Introduction of hypoxia enhanced the Ppa by 4.1 mm Hg in association with a 2.7-μm reduction in arteriolar diameter.
TABLE 2. Effects of Indomethacin and L-NAME on Mean Ppa Incrementation

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<tr>
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<td>N Group</td>
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<tr>
<td>Hypoxia</td>
<td>4.1 ± 2.0 (8)</td>
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<tr>
<td>Hypercapnia</td>
<td>0.7 ± 0.6 (16)</td>
</tr>
<tr>
<td>Isocapnic acidosis</td>
<td>1.0 ± 0.4 (7)</td>
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*Significantly (P < .05) greater than values in the N group.

Addition of indomethacin did not alter the overall pressor response (Table 2) or the constrictive level of arterioles during hypercapnic acidosis but converted the venular state from dilation to constriction (Fig 1). Capillary diameter at hypercapnic acidosis was not modified by indomethacin.

The increment of Ppa and diameter changes in microvessels at isocapnic acidosis in the presence of indomethacin did not differ from the changes obtained in the agent’s absence (Table 2 and Fig 1).

Microvascular Diameters at Hypoxia, Hypercapnic Acidosis, and Isocapnic Acidosis in the L-NAME–Treated Group

Although increments of perfusate concentrations of NO-related products before and after hypoxic stimulation attained 2.8 μmol/L in the absence of L-NAME, they were only 0.4 μmol/L when L-NAME was administered (Table 3). Qualitatively, the same trend was observed under the conditions of hypercapnia and of isocapnic acidosis (Table 3).

Inhibition of NOS by L-NAME markedly enhanced hypoxia-induced overall pressor response of the pulmonary circulation (Table 2). The presence of L-NAME, however, exerted little influence on hypoxia-evoked diameter change in any microvessels in the acini (Fig 1).

L-NAME administration did not modify the Ppa increment (Table 2) or microvascular response to hypercapnic acidosis (Fig 1). Hypercapnic acidosis concomitant with L-NAME did not elicit arteriolar constriction and caused postcapillary venules to remain dilated by 2.1 μm (Fig 1). Capillary diameter was not altered by the presence of L-NAME at hypercapnia.

Although the extent of increase in overall Ppa at isocapnic acidosis was not modified by L-NAME administration (Table 2), the intra-acinar arterioles distinctly gained contractility in response to isocapnic acidosis; ie, the average arteriolar diameter was reduced by 1.4 μm (Fig 1). However, neither arteriolar constriction but caused a distinct venular dilatation (Fig 1). The venular diameter was enlarged by 2 μm, ie, an 8% increase in the venular size. Hypercapnic acidosis did not influence capillary diameters.

Isocapnic acidosis increased the Ppa by 1 mm Hg (Table 2) in association with no alteration of microvascular diameters (Fig 1).
venular nor capillary reactivity to isocapnic acidosis was changed by the presence of L-NAME.

**pH**<sub>i</sub> **During Hypercapnia and Isocapnic Acidosis**

pH<sub>i</sub> of HPAECs before the imposition of hypercapnic acidosis averaged 7.16±0.05 and was promptly changed immediately after the initiation of hypercapnia, attaining the plateau value of 6.83±0.01 within 1 minute (Fig 2). Baseline pH<sub>i</sub> before the introduction of HCl was 7.19±0.02, which was not different from the value obtained before hypercapnia exposure. Alterations of pH<sub>i</sub> during isocapnic acidosis were more gradual than those during hypercapnic acidosis and reached a nadir of 6.86±0.01 approximately 3 minutes later (Fig 2). The nadir level of pH<sub>i</sub> in isocapnic acidosis was comparable to that in hypercapnic acidosis. Thereafter, pH<sub>i</sub> during isocapnic acidosis slowly increased at a rate of 0.016/min and reached 7.05 fifteen minutes later. The pH<sub>i</sub> values obtained between 7 and 15 minutes after HCl administration were always more alkalotic than those observed after hypercapnia exposure.

### Discussion

**Critique of Methods**

To appraise the reactivity of intra-acinar microvessels to various stimuli, including hypoxia, hypercapnia, and isocapnic acidosis, we developed a real-time confocal laser scanning luminescence optical microscope, which allows precise discrimination of individual microvessels from neighbors despite close microvessel piling. Our novel method, however, has potential limitations since it was restricted to the in situ condition; ie, we used the isolated lung perfused at a constant flow rate with a buffer containing a small quantity of whole blood (Ht, 0.05). Hemodynamic aspects in a lung isolated from an animal may be rather different from those in the living animal. Furthermore, low erythrocyte densities in the circulating medium may blunt the vascular reactivity to various physiological stimuli, such as hypoxia. Shirai and colleagues have previously reported that hypoxia evokes a vasoconstriction of feline small pulmonary veins under in vivo conditions, contradicting our findings (Fig 1). In addition to the difference in animal species and venular size analyzed, one other important distinction between the present study and their studies is the method of lung preparation; the extent of microvascular constriction, especially that of the venule, may be underestimated in the isolated lung perfused with a low Ht medium.

**Hypoxia-Induced Constriction of Intra-acinar Microvessels**

Although previous histological and radiological studies have indicated that in intact lungs hypoxia causes vasoconstriction in pulmonary arteries (with the diameter ranging from 100 to 1000 μm), the question of whether much smaller intra-acinar arterioles are actually constricted when exposed to hypoxia has not been conclusively answered. Applying a monochromatic videomicroscope, Hillier et al demonstrated that canine pulmonary arterioles with a diameter of <70 μm were constricted during exposure to hypoxic gas; these findings were qualitatively consistent with those observed in the present study (Fig 1). Although the issue of whether
intra-acinar arterioles have a significant amount of cells capable of vasoconstriction has been largely controversial,24,25 our experimental results appear to support the findings of Davies et al.,26 who showed that intra-acinar arterioles with a diameter of >10 \( \mu m \) have appreciable quantities of cells with contractile properties.

The overall extent of the hypoxia-induced pressor response was significantly enhanced by inhibiting NOS but not by inhibiting COX (Table 2), suggesting that NO would function as an important modulator coping with excessive vasoconstriction of intact pulmonary vessels during hypoxic stimulation. These findings are in accordance with those reported by several groups of investigators.9–15,26 Our results additionally suggest that vasodilating PGs have little impact in opposition to the overall HPV occurring in intact rat lungs. These findings are consistent with those of other investigators,16–18 who have demonstrated that COX-related PGs exert no conspicuous influence on Ppa changes elicited by hypoxia in the intact lung. However, the importance of endogenous PGs in attenuating overall HPV was found in endotoxin-injured rat lungs27 as well as in canine lungs during isoflurane anesthesia.28

Although NOS inhibition not only enhanced Ppa changes (Table 2) responding to hypoxia, it did not alter the diameter of intra-acinar microvessels (Fig 1), indicating that NO would play no significant role in modulating hypoxia-induced vasoconstriction of microvessels. Assuming that Ppa changes reflect the sum of pressor response yielded by all of the intra- and extra-acinar vasculature, our experimental findings suggest that NO is important in preventing excessive vasoconstriction of relatively large resistive vessels located mainly outside the acini during hypoxia exposure.

We found no effects of COX inhibition on either the extent of vasoconstriction within the acinar microvessels (Fig 1) or the overall pressor changes during hypoxic stimulation (Table 2), indicating that COX-related vasoactive substances play little role in modifying the reactivity of intra-acinar or extra-acinar vessels to hypoxia, at least in intact rat lungs. Intra-acinar Microvessel Vasodilatation Induced by CO\(_2\) Molecule

Brimioulle et al.,4 found that the overall HPV in the canine lung is much greater in isocapnic acidosis than in hypercapnic acidosis. Baudouin and Evans5 have demonstrated that hypercapnic acidosis attenuates HPV in the isolated rat lung. However, the importance of endogenous PGs in modulating HPV in the canine pulmonary circulation, the most crucial issue in these studies is that transitional changes in pHi corresponding to changes in pH\(_o\) were not studied under conditions in which acid or base was added to the surrounding medium. For instance, Raffenstein and McMurtry35 and Farrukh et al.36 implicitly assumed that neither extracellular acidification nor alkalinization significantly alters pH\(_i\), at least within 10 minutes, leading them to assume that the response of pulmonary circulation to acid or base might be principally mediated by changes in pH\(_i\). We found that pericellular acidification by HCl lowered the pH within HPAECs at a rate slower than that caused by hypercapnic acidosis but led it to the nadir level within 3 minutes (Fig 2). These findings may allow us to conclude that the pressor response within 10 minutes after acute acidification by HCl should be taken to be elicited by both pH\(_i\) and pH\(_o\) changes, as in the case of hypercapnic acidosis. Although we investigated pH\(_i\) changes solely in HPAECs harvested from human large pulmonary arteries, these observations may qualitatively be generalized to endothelial cells and smooth muscle cells of the rat pulmonary microvasculature.
Neither isocapnic acidosis nor hypercapnic acidosis, in the absence of medication, enhanced the constriction of precapillary arterioles, whereas H⁺-elicited arteriolar constriction was evidently observed at isocapnic acidosis with NOS inhibition (Fig 1). Isocapnic acidosis with COX inhibition and hypercapnic acidosis with suppression of either COX or NOS did not restore arteriolar constriction (Fig 1). These findings suggest that arteriolar constriction on H⁺ stimulation is suppressed both by H⁺-dependent activation of NOS and by COX-evoked arteriolar dilatation, the latter of which appears to be unrelated to vasodilating substances yielded by COX or NOS. Although NOS inhibition augmented arteriolar constriction responding to H⁺, it did not increase overall pressor changes during isocapnic acidosis (Table 2), indicating that NO may not play a role in modifying vascular reactivity to excessive H⁺ in extra-arterial vessels of a larger size.

In conclusion, microvascular tones in the acini are actively but differently modulated by O₂, CO₂, and/or H⁺ in association with or independent of COX- or NOS-related vasoactive substances. The arteriolar constriction caused by hypoxia is only negligibly modulated by COX- and NOS-related metabolites, whereas venular dilatation at hypercapnia is importantly mediated by vasodilating substances produced through the CO₂-dependent activation of COX. Although arteriolar constriction is potentially induced by increased concentrations of extracellular and/or intracellular H⁺, it is generally hidden by NO-related products yielded through H⁺-associated augmentation in NOS activity.

References
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