Effect of CO₂ on Intracellular pH and Contraction of Retinal Capillary Pericytes

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**Purpose.** To test the potential participation of pericytes in the regulation of capillary blood flow according to metabolic needs.

**Methods.** The authors measured the change in extracellular pH (pHₒ), intracellular pH (pHᵢ), and contractile tone of cultured bovine retinal pericytes when the ambient carbon dioxide (CO₂) level was changed.

**Results.** Raising the partial pressure of CO₂ (Pco₂) from 5% to 10% or 20% acidified the medium, decreased the pHᵢ, and relaxed the pericytes. Lowering the Pco₂ from 5% to 0% or 2% alkalized the medium, raised the pHᵢ, and contracted the pericytes. The reactions of pericytes in both cases were dose dependent. When the pHᵢ was kept at ~7.4 as the Pco₂ was changed by adjusting the ratio of NaHCO₃ and NaCl in the medium (or by using HEPES buffer when Pco₂ was zero), both elevating and lowering the Pco₂ caused intracellular acidification but did not change the contractile tone of the retinal pericytes significantly.

**Conclusions.** The effect of CO₂ on the pHᵢ and the contractility of pericytes is mediated primarily by its influence on the pHₒ. Elevation of CO₂ causes pericytes to relax, whereas dispersion of CO₂ causes pericytes to contract under the condition that allows pHₒ to be affected by Pco₂. Therefore, pericytes may contribute to the regulation of local blood flow by their response to the change in local metabolic conditions. Invest Ophthalmol Vis Sci. 1997;38:643-651.

Pericytes, which enclose the capillaries, are abundant in the retina and optic nerve. They are contractile cells that respond to some of the same hormonal stimuli that affect vascular smooth muscle cells. Pericytes resemble vascular smooth muscle cells in their content of contractile proteins, membrane properties, receptors, and responsive intracellular second-messenger systems.

We hypothesize that pericytes may help regulate the local blood flow by responding to the chemical indicators of local metabolic conditions, in addition to the regional and global influence by neurotransmitters, hormones, and hormone-driven release of endothelium-derived agents. Specifically, we wonder whether pericytes might respond to an accumulation of carbon dioxide (CO₂) in the tissue, a sign that blood flow is inadequate. Capillaries in the central nervous system have been observed to dilate in response to CO₂, but the dilation could be caused either by a change in the intraluminal pressure caused by arterial changes upstream or by the relaxation of pericytes that reside in the wall of capillaries. Moreover, pericyte relaxation could be a direct response to CO₂ or an indirect response mediated by endothelial cells.

Therefore, we studied the direct effect of CO₂ on pericytes in pure culture, isolated from bovine retinal capillaries. We investigated how CO₂ affected the intracellular pH and the contractility of pericytes.

**MATERIALS AND METHODS**

**Pericyte Culture**

In keeping with the method of D’Amore, bovine retinas were removed from commercially obtained calf eyes (Vision Tech, Dallas, TX) and were transferred to a 100 mm presterilized petri dish filled with 10 ml phosphate-buffered saline (PBS). Choroid and retinal...
pigment epithelium were removed carefully. Retinas were rinsed thoroughly, minced, and suspended in PBS containing 0.2% collagenase and 0.2% bovine serum albumen. After a 1-hour digestion, the minced retinas were filtered through a spectrmesh (111 μm). Cells recovered from the filtrate by centrifugation were plated in 75 mm² (T75) flasks filled with Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum. After approximately 3 weeks, the cells grew into a near-confluent stage and were transferred onto either rectangular slides or silicone-coated dishes. In our experiments, only the cells from first or second passages were used.

Pericytes in culture were identified carefully and were characterized using anti-ganglioside antibody SG5, the most specific identifying marker for the pericytes. Cultures contained no endothelial cells, which engulf acetylate-low-density lipoprotein (Ac-LDL) marked with the fluorescent probe l,l’-dioctylfluorescein acetoxymethyl ester (BCECF-AM) which equilibrates the pHi with the pH in the medium.

Intracellular pH (pHi) Measurement

The pHi of the cultured pericytes was measured with a spectrofluorometer (model RC-M; Photon Technology International, South Brunswick, NJ). Pericytes grown on a slide were washed with a standard bicarbonate-buffered physiologic solution, pH 7.3 to 7.5. The autofluorescence of each slide at excitation wavelengths 440 nm and 490 nm was measured and then recorded at emission wavelength 530 nm in the spectrofluorometer. Pericytes were loaded with 5 μM 2',7'-bis-(2-carboxymethyl)-5(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) for 30 minutes at 37°C. After loading, the slide was inserted at a 45° angle into a polystyrene cuvette filled with the bicarbonate-buffered solution. The cuvette was placed in the thermostatic sample compartment of the spectrofluorometer in an orientation so that the incident light would excite the side of the slide with cells, and the reflected light was directed away from the photon detector. The slide was washed with the warm bicarbonate buffer for 30 minutes to eliminate the extracellular BCECF-AM.

During the experiments, pericytes were illuminated alternately at 440 nm and 490 nm. The light emitted at 530 nm was measured. After subtraction of the previously measured autofluorescence from the raw fluorescence signals, the ratio of the fluorescence excited at 440 nm to that excited at 490 nm (f440–f490) was calculated and converted to the corresponding levels of pHi from a ratio-pHi look-up table. (The ratio-pHi look-up table had been obtained by determining the f440–f490 in pericytes at pH 6.5, 6.8, 7.0, 7.5, 8.0, and 8.5. The pHj was set by incubating a test slide of cultured pericytes at 37°C in a solution that contained 145 mM KCl and 5 mM N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] (HEPES) and 5 mM HEPES sodium salt. The solution was adjusted to the desired pH with Tris plus 10 μM nigericin, a K⁺/H⁺ exchanger that equilibrates the pH, with the pH in the medium.

Contraction Measurement

As described by Harris et al,25 the bottoms of the 60 mm glass culture dishes were coated with silicone (dimethylpolysiloxane, 60,000 cp viscosity; Sigma, St. Louis, MO). The silicone was flamed to cause the formation of cross-linking on its surface. A thin elastic layer was formed on the bottom of each dish. The dishes were sterilized by ultraviolet radiation overnight.

Pericytes from the primary culture were seeded onto the silicone-treated Pyrex dishes maintained by the usual growth medium. After approximately 1 week, almost all the cells contracted and caused the wrinkles to be formed on the thin silicone layer. The experiments were conducted at this stage.

All experiments were performed at 37°C on an inverted microscope. The Pyrex dish with the pericytes grown on its silicone-coated bottom was placed in a specially designed chamber, within which the temperature and the gas environment were controlled. Two tubes were connected to the different sides of the dish. The change of solutions was accomplished by injection through one tube and suction by vacuum from the other tube. Pictures of the cells were taken at the desired times. The total length of the wrinkles (lₜ) was determined as the sum of lengths of all wrinkles under the pericytes in the selected field. An increase in lₜ represents the contraction of pericytes, and a decrease in lₜ represents cell relaxation. In some cases, cells detached from the silicone surface because of excessive contraction with loss of all the wrinkles. These cells were not used for evaluation of contraction. When the pericytes were incubated in the standard bicarbonate buffer gassed with 5% CO₂, 20% O₂, and 75% N₂, the count of lₜ was designated as lₜ, the baseline constrictive state of the chosen pericytes. At designated times under the experimental conditions, the count of lₜ was designated as l. The ratio of l to lₜ (l/ lₜ) was taken to be a measure of change in the contractile state of the pericytes. In some of the experiments, the pericytes had to be prerelaxed with sodium nitroprusside (SNP, 3 X 10⁻⁶ M) for 20 minutes before the initiation of the experiments (SNP was maintained in the medium throughout the experiments). In these experiments, the count of lₜ at 20 minutes after prerelaxation was used to represent the baseline contractile state and was designated as lₜ. The ratio of l to lₜ (l/lₜ) represents the change in the con-
TABLE 1. Compositions of the First Set of the Bicarbonate-Buffered Solutions*

<table>
<thead>
<tr>
<th>Pco2 (%)</th>
<th>PN2 (%)</th>
<th>pH ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (control)</td>
<td>5</td>
<td>75</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>78</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>70</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>60</td>
</tr>
</tbody>
</table>

* All the solutions contained NaHCO₃ 25 mM, NaCl 115 mM, KCl 5 mM, CaCl₂ 2 mM, MgCl₂ 1 mM, and glucose 10 mM. P = 20%.

Effect of CO2 on the Contraction of Pericytes in the Standard Bicarbonate Buffer

When Pco2 was increased to 10% or 20% in the standard bicarbonate buffer, the pericytes relaxed in a dose-dependent manner, as judged by the U/L ratio. At 20 minutes, 20% Pco2 caused ~24% relaxation when compared to the contractile state before the rise in Pco2; 10% Pco2 caused a statistically significant 8% relaxation only at 1 minute (Fig. 3).

When we tested the effect of decrease in Pco2 on the contractile tone of pericytes, we often observed that pericytes detached from the silicone surface with loss of underlying wrinkles. This appears to have been caused by excessive contraction of the pericytes. To prevent paradoxical loss of wrinkles with strong contraction, we partially prerelaxed the pericytes with SNP, a source of nitric oxide. The presence of SNP greatly reduced the occurrence of cell detachments and made it easier to document the contraction of pericytes quantitatively.

Sodium nitroprusside (3 × 10^-6 M) relaxed pericytes by ~28% after 20 minutes, and the relaxation reached a steady state (n = 45). Under the condition of partial prerelaxation, we observed that 0% and 2% Pco2 induced marked contraction (increase in wrinkles) as much as >40% compared to the state before Pco2 was lowered. The maximum effect of 0% Pco2 appeared faster (<10 minutes) than that of 2% Pco2 (~15 minutes) (Fig. 4).

Effect of CO2 on the pHj of Pericytes While pHo Was Maintained Constant

To explore the role of pHo on CO2-induced pHj change, we kept pHo constant between 7.3 and 7.5 by...
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**FIGURE 4.** Effect of lowering PCO₂ on the pericytes relaxed with sodium nitroprusside, shown as \( l/l \) (mean ± SE) over time. 0 minute indicates when PCO₂ was raised after cell prerelaxation. \(*P < 0.05; **P < 0.01; ***P < 0.001; n = 5\) for all groups; unpaired Student’s *t*-test, comparing the value of \( l/l \) for each PCO₂ to that of the control (PCO₂ = 5%) at the indicated time.

Adjusting the ratio of [NaHCO₃] and [NaCl] in the bicarbonate buffer as the PCO₂ was altered. When PCO₂ was 0%, HEPES buffer was used instead of the bicarbonate buffer (Table 2).

Although pHₒ was fixed, the movement of CO₂ and HCO₃ into and out of the cell would affect the pHᵢ, moderated by the intracellular buffering systems. Our results show that when pHₒ was kept constant, both 0% PCO₂ and 2% PCO₂ induced a gradual decrease in pHᵢ. At 20 minutes after the switching of the solutions, pHᵢ dropped 0.32 ± 0.09 U in HEPES buffer (PCO₂ = 0) and dropped 0.15 ± 0.03 U when PCO₂ was 2% (Fig. 5, above).

When PCO₂ was raised to 10% and 20% with pHₒ kept constant, a transient decrease in pHᵢ was induced, and the pHᵢ recovered to the original level within 20 minutes. At 10% PCO₂, the largest fall in pHᵢ was 0.27 ± 0.04 U, at 98 seconds. The 20% PCO₂ caused the largest fall in pHᵢ, 0.22 ± 0.04 U, at 82 seconds after the solutions were switched (Fig. 5, below). There was no significant difference between the effects of 10% and 20% PCO₂.

Effect of CO₂ on the Contraction of Pericytes While pHₒ Was Maintained Constant

When lowering PCO₂ in the buffer with constant pHₒ, the pericytes showed a tendency to relax over time. However, the relaxing effect by either 0% PCO₂ or 2% PCO₂ did not achieve statistical significance (Fig. 6, above). Raising PCO₂ to 10% did not have a significant effect on the contractile tone of the pericytes; 20% PCO₂ induced a small, transient, uncertain \((P = 0.074)\) relaxation at 1 minute (Fig. 6, below).

**Summary of Results**

To compare the qualitative effects of CO₂ on the pHᵢ and on the contractile state of the pericytes and to contrast the various experimental conditions, these results have been summarized in Table 3.

**DISCUSSION**

**Physiologic Implications**

Pericytes have a number of trophic functions, but they are also contractile. They may fine tune local tissue perfusion by changing capillary diameter and, thus, supplement the control exerted by the arterioles. We think the contractile tone of the retinal capillaries is modulated at least partially by local metabolic needs (metabolic autoregulation).

Our current study reveals that PCO₂, an important reflection of the balance between local metabolic activity and local blood perfusion, affects the pHᵢ and the contractile state of the retinal pericytes. Acute changes in the level of CO₂ induce contraction or relaxation of pericytes in the direction that would promote homeostasis. We did not exclude the possible...
Mechanism of the Effect of CO₂ on pHᵢ of the Retinal Pericytes

When P₉₀ is raised without restraining the pHᵢ, two expected immediate effects on the cell would lower the pHᵢ. First, CO₂ acidifies the external medium. The elevated extracellular concentration of H⁺ would cause external H⁺ to leak into the cytoplasm of the pericytes because H⁺ permeability is relatively large in most vertebrate cells. Second, CO₂, a nonionic molecule, can enter the cell easily and acidify the cytoplasm through the formation of carbonic acid in the presence of carbonic anhydrase.³⁹

In our experiments, with the elevation of P₉₀, the pHᵢ decreased immediately and reached the lowest point after 2 minutes (Fig. 2, below). However, the subsequent recovery of pHᵢ toward the baseline indicates that H⁺ was extruded from the cytoplasm of the pericytes. There are two primary known acid extrusion mechanisms in vertebrate cells: the Na⁺⁻H⁺ exchanger, which exchanges extracellular Na⁺ for intracellular H⁺; and the Na⁺-dependent HCO₃⁻-Cl⁻ antiport, which exchanges extracellular NaHCO₃ for intracellular HCl.⁸⁹⁻⁹¹ The former is primarily responsible for acid efflux after an acute severe acid load (pHᵢ < 6.5), whereas the latter is responsible under milder physiological changes (pHᵢ > 6.5).³¹ Because the pHᵢ never dropped below 6.5 in our experiments, it is most likely that the Na⁺⁻dependent HCO₃⁻⁻Cl⁻ antiport played a more important role in the recovery of pHᵢ.

When P₉₀ is lowered in the standard bicarbonate-buffered solution, opposite results are produced. First, the extracellular medium becomes alkalized, which, in turn, should produce a H⁺ efflux from the cytoplasm. Second, intracellular CO₂ diffuses out of the cells, which should produce a chain reaction that consumes intracellular protons and bicarbonate to create water and additional CO₂ to diffuse out of the cell. Both these effects lead to alkalization of the cytoplasm, as we observed (Fig. 2, above). However, the pHᵢ failed to recover, even after pHᵢ reached ~7.5. The results suggest that the Na⁺-independent HCO₃⁻⁻Cl⁻ antiport, which acidifies the cytoplasm after cell alkalization and is activated when pHᵢ increase above 7.1 in other cells,³⁰⁻³¹ is ineffective under these experimental conditions in pericytes.

We further explored the effect of CO₂ on the pHᵢ of the pericytes in a second set of experiments in which we kept pH₀ constant. The purpose of these experiments was to determine the contribution of pH₀

### Table 3. Summary of CO₂ Effects on Pericytes

<table>
<thead>
<tr>
<th>P₉₀</th>
<th>pHᵢ</th>
<th>Contractile Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH₀ unadjusted (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Increase</td>
<td>Contraction</td>
</tr>
<tr>
<td>2</td>
<td>Gradual increase</td>
<td>Contraction</td>
</tr>
<tr>
<td>10</td>
<td>Decrease followed by recovery</td>
<td>Relaxation</td>
</tr>
<tr>
<td>20</td>
<td>Decrease followed by recovery</td>
<td>Relaxation</td>
</tr>
<tr>
<td>pH₀ constant (7.4) (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Gradual decrease</td>
<td>Insignificant</td>
</tr>
<tr>
<td>2</td>
<td>Gradual decrease</td>
<td>Insignificant</td>
</tr>
<tr>
<td>10</td>
<td>Transient decrease</td>
<td>Insignificant</td>
</tr>
<tr>
<td>20</td>
<td>Transient decrease</td>
<td>Insignificant</td>
</tr>
</tbody>
</table>
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to the initial effect of CO₂ on pH, and on the subsequent restoration of pH toward the baseline.

In these experiments, when PCO₂ was elevated, we observed a transient decrease in pH, and a much faster recovery to its normal level (Fig. 5, below). The transient decrease in pH in this second experimental setting was caused solely by the entry of CO₂ into the cytoplasm without a contribution from a change in extracellular concentration of H⁺. The faster recovery of the pH, presumably results at least in part from a reduced load of H⁺ influx into the cytoplasm. Additionally, the higher concentration of extracellular HCO₃⁻ ([HCO₃⁻]₀) and the lower concentration of extracellular Cl⁻ ([Cl⁻]₀) (Table 2) favor the activation of Na⁺-dependent HCO₃⁻/Cl⁻ antiport, which also would hasten recovery.

When we lowered PCO₂ while maintaining pH₀, at a constant level, alkalization of the cytoplasm did not occur. The gradual decrease in pH, may have been caused by the high [Cl⁻], and the artificially low or absent [HCO₃⁻]₀ in the medium (Fig. 5, above, Table 2). These conditions would stimulate the Na⁺-independent HCO₃⁻/Cl⁻ antiport because it exchanges extracellular Cl⁻ for intracellular HCO₃⁻.29 Thus, this antiport seems to be present in pericytes and to be active under the conditions in this second set of experiments. The lack of recovery from the progressive acidification suggests that the operation of Na⁺-dependent HCO₃⁻/Cl⁻ antiport was hindered by the lack of HCO₃⁻ in the extracellular medium and provides further evidence that the Na⁺-H⁺ exchanger was not sufficiently active to accomplish the required acid extrusion.

The above results lead to two conclusions: The immediate CO₂-induced change in pH, is influenced heavily by its effect on pH₀. The Na⁺-dependent HCO₃⁻/Cl⁻ antiport may be the more active H⁺ extrusion system in pericytes within the PCO₂ range used in the experiments.

Mechanism of the Effect of CO₂ on the Contraction of the Retinal Pericytes

CO₂ is a potent vasodilator.3,35 Hypocapnia dilates brain capillaries and hypocapnia constricts them, although the mechanisms remain unclear. In general, the contractile state of the cells can be affected by the change of either their intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) or the sensitivity of the myofilament to Ca²⁺. Therefore, we must consider how changes in local PCO₂ might affect these two factors, either as a direct effect of the CO₂ molecule or as an indirect effect mediated by a change of intracellular pH and of extracellular pH.

Intracellular pH has several relationships with [Ca²⁺]ᵢ in vascular cells. Evidence has shown that a decrease in pHᵢ can inhibit Ca²⁺ release from the sarcoplasmic reticulum,29,34 and it can inhibit uptake of Ca²⁺ by the sarcoplasmic reticulum.35 It also decreases the sensitivity of myofilament to Ca²⁺.29,36,37 Inversely, an increase in pHᵢ results in a release of Ca²⁺ from sarcoplasmic reticulum34,35,38-40 and an inhibition in the Ca²⁺-ATPase activity.35

Similarly, the extracellular pH also influences [Ca²⁺]. An increase in pH₀ induces a Ca²⁺ influx that is independent of the change it causes in pHᵢ.35 A decrease in pH₀ can relax the cells by an inhibition in the Ca²⁺ influx or some other unknown mechanisms.41

In our experiments, changing PCO₂ without adjusting [HCO₃⁻]₀ changed both pHᵢ and pH₀ (Fig. 1, Fig. 2). Increasing PCO₂ acidified both the medium and the cytoplasm, and it relaxed the pericytes. We noticed that after PCO₂ was raised to 20% for 5 minutes, the pH₀ started to recover but the pericytes continued to relax (Fig. 2, below; Fig. 3). This observation suggests that the relaxation was not caused by pHᵢ solely. Either pH₀ or some other effect of CO₂ must be involved. Lowering PCO₂ increased pH₀ and pHᵢ. It also caused the prerelaxed pericytes to contract markedly (Fig. 2, above, Fig. 4).

When we changed PCO₂ but adjusted [HCO₃⁻]₀ to keep pH₀ constant, neither raising PCO₂ nor lowering PCO₂ caused a significant effect on the contractile tone of the pericytes (Fig. 5). Our results indicate that the effect of CO₂ on the contractility of pericytes is mediated primarily by its influence on pH₀.

Although the exact mechanisms are not elucidated, the experiments reported here suggest that pH₀ plays a major role in affecting the contractile state of the pericytes and that altered PCO₂ triggers compensatory changes that slowly alter the intracellular environment and the contractile state.

SUMMARY

Under conditions that allow the pH₀ to be affected by PCO₂, an elevation of CO₂ causes a decrease in pHᵢ and a relaxation of pericytes, whereas a reduction of CO₂ alkalizes the cytoplasm and contracts the pericytes. CO₂ may affect pHᵢ by diffusing across the cell membrane to change the production of carbonic acid, a reaction catalyzed by carbonic anhydrase, and by changing pH₀ to alter the H⁺ exchange across the cell membrane. The Na⁺-dependent HCO₃⁻/Cl⁻ antiport may be the more active H⁺ extrusion system for pericytes to recover from CO₂-induced cell acidification. The effect of CO₂ (within the range of 0% to 20% PCO₂) on the contractile tone of the retinal pericytes is attributed primarily to its effect on pH₀. An additional direct effect of CO₂ on the contractility of the pericytes is not ruled out. Either indirect or direct effects may change [Ca²⁺], or the sensitivity of the myofilament to Ca²⁺. In any event, retinal pericytes likely partici-
pate in the local regulation of the retinal microcirculation through their responses to local PCO₂ and other metabolic regulating factors.

**Key Words**
carbon dioxide, contraction, extracellular pH, intracellular pH, metabolic autoregulation of blood flow, pericyte, retinal capillaries

**References**


