Effect of Phalloidin on Structure and Permeability of Rete Capillaries in the Normal and Hypoxic State

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The effects of $10^{-4}$ M phalloidin on reperfusion-injured blood capillary structure and permeability were studied in the countercurrent perfused rete mirabile of the eel swim bladder. In the normal rete, the addition of phalloidin to the perfusion medium did not induce morphological or functional changes. When flow was arrested for 30 minutes, during which time the capillaries were exposed to inhibitors of ATP generation, and flow was then resumed with an oxygenated medium, cell membrane blebs and vacuolization, mitochondrial swelling, pericyte shrinkage, and interstitial space edema were observed. The permeability coefficients for labeled albumin, sucrose, and sodium increased to three to four times baseline values, whereas the permeability to water was not significantly modified. When the same protocol was repeated with phalloidin present in the medium throughout the experiment, the structural integrity of the endothelial cells was completely preserved and pericyte shrinkage was abolished, but interstitial space edema still occurred. The permeability to albumin, sucrose, and sodium increased only to 1.5 times baseline values, a significantly decreased increment in comparison with the experiments performed without phalloidin. We concluded that although phalloidin does not improve the capillary barrier of the normal rete, it provides protection against the structural and functional damage induced by hypoxia and reperfusion. (Circulation Research 1989;65:591-599)

It has been suggested that the cytoskeleton of the endothelial cell could be involved in the regulation of microvascular permeability. Cytochalasin B, which disassembles filamentous or F-actin, increases the permeability of endothelial cell monolayers and promotes pulmonary edema, whereas phalloidin, which stabilizes F-actin, decreases monolayer permeability. In addition, other experimental evidence has indicated an association between the permeability effects of various inflammatory agents and their actions on F-actin. The observations of apparent actin-associated permeability change have been made primarily on cultured monolayers, derived from endothelial cells harvested from macrovessels. We inferred that changes in the quantity and disposition of cytoskeletal actin fibers might also affect the way materials traverse capillary endothelium in situ. We also reasoned that a substance affecting actin might modify the manner in which capillary permeability is changed by an extraneous influence in vivo and that this might then introduce a new set of working tools to this area of biology.

We therefore designed and carried out experiments to examine the effects of phalloidin in a capillary organ, the rete mirabile of the eel swim bladder. This preparation has the advantage that permeability can be recorded easily during countercurrent perfusion. We first tested the effects of phalloidin in the normal rete. We then induced substantial morphological and functional damage with stagnant hypoxia and reperfusion, and repeated these experiments in the presence of phalloidin. The phalloidin was found to exert a substantial protective effect on the structure and permeability of the rete, such that reperfusion injury was much decreased.

**Materials and Methods**

Large female eels, caught in the St. Lawrence River, were kept without food in oxygenated tap...
In Vitro Metabolic Studies

The swim bladder artery was cannulated, and the retia were rapidly flushed clear of blood with a Krebs-Ringer bicarbonate buffer (pH 7.4) containing albumin (4 g/100 ml) and glucose (5 mM) and equilibrated with a 95% O2-5% CO2 gas mixture. Six retia were removed from three eels and dissected free of their capsular tissue and vascular poles. The remaining capillary tissue was cut into small clusters of filaments and distributed into vials containing 1 ml perfusion medium either without phalloidin or with phalloidin (10^-6 M). Approximately 40 mg wet weight of capillary tissue was added to each vial. Glucose has previously been observed to be the major energy substrate of rete capillaries, and the activity of their glycolytic pathway has been found to be very high. Two-hour incubations were carried out at 37° C, with a gas phase consisting initially of a mixture of 95% O2-5% CO2. Glucose uptake and lactate output were measured by enzymatic procedures. Glucose conversion into CO2 was calculated from the ratio of U^14C activity in CO2, trapped in a scoop filled with phenethylamine, to the specific activity of glucose ^14C (ul) in the medium at the start of the incubation.

Permeability Studies

The rete was countercurrent perfused through its arterial and venous inputs, and samples were simultaneously collected from the arterial and venous outputs. The perfusions were carried out at room temperature, with a constant flow in each direction of 0.5 ml/min at a constant pressure head of 45 cm H2O. The wet weights of the perfused retia averaged 250 mg. The following labeled materials were added at time zero to the medium at the arterial input: human [125I]albumin (Frosst, Kirkland, Quebec; more than 95% of the labeled iodine precipitated with 10% trichloroacetic acid), [14C]sucrose (ul) (New England Nuclear, Boston, Massachusetts; 2-10 mCi/mmol), titrated water (New England Nuclear; biological quality, 0.1 mCi/g), and ^22Na (New England Nuclear; as sodium chloride, 99.9% radionuclide purity). No radioactive tracers were added to the medium at the venous input. The radioactivity of the tracers at the arterial input and at the arterial and venous outputs was measured on a 10% trichloroacetic acid precipitate, washed with excess potassium iodide for [125I]albumin and on protein-free supernatant for the other tracers, by use of Packard gamma scintillation or liquid scintillation spectrometers (Packard Instrument, Downers Grove, Illinois). All values were corrected for background and crossover.

The permeability surface product (PS) of the arterial endothelium–interstitial space–venous endothelial barrier, where P is the permeability coefficient and S is the surface area, can be measured for each tracer when steady-state concentrations are attained. At equilibrium, the sum of the tracer concentrations at the two outputs was within 2% of the arterial input concentration. At steady state, PS=F (V_a/A_o), where F is the unidirectional flow and V_a and A_o are simultaneously measured concentrations at venous and arterial outputs. Therefore, the permeability coefficient P is proportional to the ratio V_a/A_o. It has been estimated that the total surface area-to-volume ratio of the eel rete is of the order of 1 cm^2/mm^3 and, by approximation, 1 cm^2/mg wet wt. With this value, permeability P can be estimated for each tracer from steady-state concentrations. The surface area S was derived, in each case, from the wet weight of the rete at the end of the experiment. Various degrees of edema were observed at this time in the stagnant hypoxia and reperfusion experiments, and with the edema, permeability values would be underestimated. On the other hand, the PS values, which measure the diffusion capacity of the retia, showed a greater variability than usual because the individual weights of the retia varied more widely than in any previous group. Therefore, we expressed our findings as permeability estimates. The dimensions of relative change with time, nevertheless, will be expected to be correct.

The first set of experiments was designed to study the effects of phalloidin on the permeability of the normal rete. Five retia were perfused with Krebs-Ringer bicarbonate buffer (pH 7.4) containing albumin (4 g/100 ml) and glucose (5 mM) for a control period of 1 hour. The perfusion was then switched to a medium of the same composition containing 10^-6 M phalloidin for 2 additional hours. In a second set of experiments, we tested the protective effects of phalloidin on the permeability of the rete compromised by hypoxia and reperfusion. Eight retia were first perfused for 30 minutes with the control medium. They then were perfused for 10 minutes with a medium without glucose but supplemented with potassium cyanide (1 mM) and sodium iodacetate (1 mM) and with a Po2 of 10 mm Hg, and were clamped at both poles for 30 minutes. We have shown that this procedure combines the effects of arrest of both energy generation and flow. Finally, the retia were reperfused with oxygenated control medium for 1 hour. The protocol was then repeated with six other retia in similar fashion, except that the perfusion medium throughout the experiment contained 10^-4 M phalloidin.

Morphological Studies

At the end of each experiment, fragments of the perfused rete were fixed by immersion in a 1% glutaraldehyde solution, postfixed with 1% osmium tetroxide, and embedded in Epon. The other retes, which were not perfused, was removed during the initial preparative procedures and similarly pre-

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In Vitro Metabolic Studies were analyzed per eel for both control and experimental retia subjected to hypoxia and reperfusion in the absence or in the presence of phalloidin. In both sets of experiments, control retia were also examined. The surfaces occupied by each intercapillary space and the pericytes in that space were measured by direct planimetry on electron micrographs. The difference between the two values was also calculated to provide a value for the interstitial space. Twenty micrographs were analyzed per eel for both control and experimental rete. The micrographs were recorded at x4,400 and enlarged to a final magnification of x11,000. A modular system for quantitative digital image analysis (Videoplan 3, Carl Zeiss, Don Mills, Ontario) was used for the evaluations.

Results

In Vitro Metabolic Studies

Table 1 gives the values of the in vitro glucose utilization by normal capillary tissue and shows the effect of 10^-6 M phalloidin on glucose utilization. Neither glucose uptake nor glucose conversion into lactate and CO₂ was significantly modified by the addition of phalloidin to the medium during a 2-hour incubation at 37°C. Values for medium with phalloidin are not significantly different from control values (evaluated with Student's t test for paired experiments).

The effect of phalloidin on the rete permeability changes induced by stagnant hypoxia and reperfusion is shown in Figure 2. Without phalloidin (left panel), reperfusion of the rete after stasis and hypoxia induced a significant increase in the V_o/A_o values for labeled albumin, sucrose, and sodium within 5–10 minutes; the values then continued to rise with reperfusion until they reached plateau levels at 30–60 minutes. With 10^-6 M phalloidin (right panel), a significant rise in V_o/A_o values for the same tracers was also observed during reperfusion after stasis and hypoxia, but the effect was delayed and much reduced in magnitude in comparison with the values obtained in the absence of phalloidin. In neither set of experiments was any change in the V_o/A_o concentration ratio for tritiated water observed during reperfusion.

Table 3 shows the permeability values measured during control periods and during the plateau periods of reperfusion in the absence and in the presence of phalloidin. In the absence of phalloidin, the coefficient of permeability P for the tracers albumin, sucrose, and sodium increased, on average, during reperfusion to values 2.5 to 4 times initial control values (p<0.05; Student's t test for paired samples). In the presence of phalloidin, the rise was only to values approximately 1.5 times the control values (p<0.05; Student's t test for paired samples). Figure 3 shows a graphic representation of the proportional increase in the permeability, expressed as a multiple of baseline. The phalloidin is seen to have significantly reduced the permeability-increasing effects of hypoxia and reperfusion (p<0.05; Student's t test for unpaired samples).

Morphological Studies

The normal microvascular rete (Figure 4, top panel) consists of arterial endothelial cells, which are high and continuous; venous endothelial cells, which are thin and fenestrated; and numerous per-
cytes, all embedded in continuous basal laminae. The cells are closely apposed, and the interstitial space, filled with collagen fibers, is so reduced that it is confined to the area where capillaries do not come into contact with one another. The structure of the reticulum tissue remained entirely normal after continuous perfusion with phalloidin 10^-6 M (Figure 4, bottom panel). When the reticulum was subjected to stagnant hypoxia and reperfusion (Figure 5, top panel), extensive damage was found in the membranes and organelles of the endothelial cells; the tight junctions and fenestrations, however, remained intact. Many pericytes were separated from the venous endothelium in areas of edema. Morphometric analysis of the intercapillary space (Table 4) shows expansion of the interstitial space and, unexpectedly, shrinkage of the pericytes. The addition of 10^-6 M phalloidin to the medium abolished the endothelial lesions induced by hypoxia and reperfusion (Figure 5, bottom panel). Pericyte shrinkage was not found, but a characteristic additional change in pericytes was observed: the filling of pericyte cytoplasm by a network of actin filaments. The change was seen in more than half of the pericytes. When it occurred, cytoplasmic structures (endoplasmic reticulum and mitochondria) and the nucleus were displaced to the side of the pericyte. After phalloidin, close contacts appeared to persist between the plasma membranes of the pericytes and their neighboring endothelial cells, but despite this condition interstitial edema accumulated, albeit to a significantly smaller degree (Table 4) than when phalloidin was absent.

Discussion

Phalloidin has the capacity to bind to F-actin in a variety of cells; it either modifies cell architecture to induce toxic effects, or stabilizes it to protect against denaturation by chemical or physical treatments. There have been recent speculations that the cytoskeleton of endothelial cells may play a role in the regulation of diffusion permeability across the capillary vascular barrier. We have reported that inhibition of energy metabolism and arrest of flow, followed by reperfusion, induces substantial structural and functional changes in the rete. Other in vitro studies have suggested that reduced ATP generation results in restructuring and loss of actin filaments in endothelial cells. Therefore, normal and reperfused rete preparations were used in this study, with the idea that phalloidin might maintain the microvascular barrier through stabilization of cellular cytoskeletal microfilaments. There was an underlying premise that phalloidin, which is excluded...
from all normal cells except hepatocytes, would enter damaged cells.

As phalloidin also could have exerted cytotoxic effects, we first studied its action on the energy metabolism of rete microvascular tissue. At the end of a 2-hour incubation in vitro at 37°C, there were no significant differences in glycolytic or oxidative pathways of glucose utilization between control and phalloidin-treated samples; from Table 1, the total expected yield of ATP averaged 45 μmol/g/hr for each preparation.

During continuous perfusion of the normal rete, 10⁻⁶ M phalloidin was introduced after 60 minutes and continued for 2 subsequent hours. There were no detectable changes in the permeability coefficients of the tracers with respect to the initial control period (Figure 1 and Table 2). The morphology of the endothelial cells and pericytes was indistinguishable from that observed in the contralateral rete removed at the beginning of the experiment (Figure 4). These results would have been predicted if the phalloidin did not gain access to the interior of the cells. Alternatively, if phalloidin did enter the endothelial cells, it could be argued that the phalloidin that did reach the cytoskeleton could not improve an already optimally tight system. Our previous studies have shown the barrier of the rete to be generally poorly permeable; this helps to explain the way the rete functions with the eel swim bladder in vivo, that is, to multiply the partial pressure of oxygen along the arterial capillary, via a Root effect.

When the rete was perfused with a normal oxygenated medium for assessment of its baseline permeability and then exposed to stagnant and cytotoxic hypoxia followed by reperfusion with a control medium, important morphological and functional lesions were observed. Cell membranes in the endothelium were swollen and vacuolated, with protrusions and debris in the lumen. There were, however, no visible disruptions of tight junctions and fenestrations. The interstitial spaces showed patches of edema, especially evident where shrunken pericytes on the venous side separated from their adjoining endothelial cells (Figure 5, top panel, and Table 4). The permeability to albumin, sucrose, and sodium was increased threefold to fourfold (Figure 3 and Table 3). The time course of these tracer concentrations at the outputs of the rete indicated that the permeability-increasing effect evolved largely during the reperfusion (Figure 2, left panel). The observation is compatible with, but not proof of, a role for free oxygen radicals, generated during reoxygenation of the hypoxic rete, in the induction of the capillary injury. The lack of change in the permeability to water during reperfusion would be expected if water readily diffuses across the whole surface area of the endothelium, as opposed to more limited pathways for albumin, sucrose, and sodium. What the paths of transport for these solutes are cannot be derived from the results of our

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<th>Table 3. Effect of Phalloidin on the Rete Permeability Changes Induced by Stagnant Hypoxia and Reperfusion</th>
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Results are expressed as mean±SEM. Each tracer was tested in eight paired experiments without phalloidin and six paired experiments with phalloidin. Medium before and after stagnant hypoxia was a Krebs-Ringer bicarbonate buffer with purified bovine albumin (4 g/100 ml) and glucose (5 mM). Stagnant hypoxia was induced by clamping the rete for 30 minutes in a Krebs-Ringer bicarbonate buffer with purified bovine albumin (4 g/100 ml), no glucose, potassium cyanide (1 mM), and iodoacetate (1 mM), equilibrated with 95% N₂, 5% CO₂. Pₚ, permeability coefficient at 25°C.

*p<0.05 vs. control (Student's t test for paired experiments).
FIGURE 4. Top panel: Electron micrograph of arterial (AC) and venous (VC) capillaries of a normal rete. Intercellular junctions (J) are tight in both endothelia. Interstitial space (IS) contains pericytes (P) and collagen fibers. Arrows indicate zones of close contact between endothelial cells and pericytes (magnification, ×17,600). Bottom panel: Electron micrograph of arterial and venous capillaries of a rete perfused with phalloidin 10^{-6} M. Morphological features are identical to those found with an ordinary control rete. f, fenestration (magnification, ×23,320).
**Figure 5.** Top panel: Electron micrograph of arterial (AC) and venous (VC) capillaries of a rete subjected to stagnant hypoxia and reperfusion. Endothelial cells are vacuolated, and several membrane-bound vacuoles protrude into the lumen (arrows). Interstitial space (IS) is edematous. One pericyte (P) appears detached from its adjacent endothelial cells (magnification, ×18,900). Bottom panel: Electron micrograph of rete capillaries after stagnant hypoxia and reperfusion, where the medium contained phalloidin 10⁻⁶ M throughout the experiment. Morphology of tissue resembles that of a normal control rete. Filament-like structures fill the major part of a pericyte. Inset: Arrows indicate close contacts between plasma membranes of a pericyte filled with filaments and an adjoining endothelial cell. J, intercellular junction (magnification, ×18,900).
widening of the interendothelial cell junctions, which was not displaced from the underlying endothelium; they appeared packed with cross sections of filaments that confined the nucleus and organelles to a small area of the cytoplasm, near the plasma membrane (Figure 5, bottom panel). Functionally, the permeative effect of hypoxia and reperfusion on albumin, sucrose, and sodium was still present and measurable against control values. The change, although modest, was not spurious: we have, indeed, shown that the pericyte preparation does not become spontaneously leaky over a 2-hour period of perfusion. 6

When the same experiments were repeated with 10^-6 M phalloidin present at all times, striking protective effects were observed. Morphologically, the capillary endothelial cells looked normal in all aspects, but edema nevertheless accumulated between the capillaries to a somewhat lesser degree. The pericytes did not shrink (Table 4), and they protected the structure and function of the rete capillaries and in the interstitium, but not within the tight junctions. 19

The mechanisms whereby phalloidin was able to protect the structure and function of the rete capillaries against injury due to hypoxia and reperfusion are conjectural. One must presume that phalloidin gained access to the cells, possibly through changes in plasma membrane selectivity induced by hypoxia. There appear to have been two major effects: 1) the rapid stabilization of F-actin in the meshwork underlying the surface endothelial cell membrane has prevented its vacuolization, presumably by supporting it, so that surface flow stress does not damage the membrane in the presence of hypoxia; and 2) stabilization of the microfilament system appears to have maintained junctional integrity, to some extent, just as it does in epithelia. 20 It has been proposed that the disposition and number of actin filaments in endothelial cells are sensitive to energy metabolism, and that loss of F-actin is induced by a severe reduction in ATP generation. 14,15 If this also occurred in the rete, it would indicate that the interaction of phalloidin and low ATP levels is at a common effector, the microfilament, and that there is a protective effect of the former against the latter. It would also indicate that the role of the cytoskeleton in the regulation of microvascular permeability needs both emphasis and further exploration, especially under abnormal circumstances. At the same time, the increase in filaments in the pericytes also suggests that these cells provide a backup bulkwork for and reinforce interendothelial cell junctions, and that they may have contributed to the moderation of the permeability-increasing effects of hypoxia. 21

In conclusion, phalloidin does not modify the structure and permeability of normal capillaries of the rete. However, when capillaries are injured by hypoxia and reperfusion, phalloidin provides a major protection against both morphological and functional alterations.

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References


KEY WORDS: rete mirabile • capillary permeability • ischemia • reperfusion • phalloidin • cytoskeleton • actin microfilaments
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