Reperfusion-induced changes in capillary perfusion and filtration: effects of hypercholesterolemia

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Harris, Norman R. Reperfusion-induced changes in capillary perfusion and filtration: effects of hypercholesterolemia. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H669–H675, 1999.—Fluid filtration rate (J/S) and red blood cell velocity (V_{RBC}) in individual mesenteric capillaries of normocholesterolemic (NC) and hypercholesterolemic (HC) rats were measured before and after ischemia and reperfusion (I/R). In NC rats, a correlation was found between baseline J/S and the percent of the feeding arteriole length that was paired (<15 µm) with a postcapillary venule (A-V pairing), but not in the HC group. Additionally, in NC rats only, a correlation was found between baseline V_{RBC} and A-V pairing. In capillaries in which A-V pairing was substantial (>20%), V_{RBC} dropped after reperfusion in the HC group (54% of baseline; P < 0.05), but not in the NC group (79%). The decrease in V_{RBC} in HC rats could be attenuated by a P-selectin antibody (PB1.3). PB1.3 was also able to attenuate the increase in I/R-induced capillary J/S in HC rats (median increase = 1.26-fold vs. 1.53-fold without PB1.3). These data suggest a role for A-V pairing in capillary perfusion in NC rats and a potential role for P-selectin in I/R-induced microvascular dysfunction in HC rats.

Microvascular permeability; leukocyte-endothelial cell adhesion; acute inflammation; endothelial barrier dysfunction

Hypercholesterolemia increases mortality rates and is considered to be a major risk factor for cardiovascular disease (23). Whether inherited or diet induced, prolonged hypercholesterolemia can result in severe changes in arteries, such as atherosclerosis and reduced elasticity (12). However, even a single high-fat meal can also attenuate arterial vasoactivity (27). At the microvascular level, short-term feeding (~2 wk) of a high-cholesterol diet increases injury associated with ischemia-reperfusion (I/R). For example, Kurose et al. (17) found that I/R caused significantly more leukocyte adherence, leukocyte emigration, albumin leakage, and platelet aggregation in mesenteric venules of rats fed a high-cholesterol diet for 2 wk than those fed normal chow. Furthermore, they found that administration of monoclonal antibodies directed against CD11/CD18 and intercellular adhesion molecule-1 (molecules involved in firm leukocyte-endothelial cell adherence) were able to attenuate the increased venular albumin leakage.

Our laboratory has initiated a study into the effect of hypercholesterolemia on I/R-induced changes in capillary function. In a similar model to that of Kurose et al. (17), we previously reported that I/R-induced capillary filtration rate (J/S) was enhanced by a high-cholesterol diet and could be attenuated by administration of anti-neutrophil serum (10). These results, together with those of Kurose et al. (17), suggest that leukocyte adhesion may be involved in the enhanced microvascular permeability associated with I/R in hypercholesterolemia. In the present study, we continue this investigation by injecting a monoclonal antibody against P-selectin (involved in leukocyte rolling) to see whether this adhesion molecule is involved in I/R-induced increases in J/S in hypercholesterolemic rats.

Reduced capillary flow or “no-reflow” after reperfusion is a phenomenon that has received increasing attention in recent years. The mechanisms of no-reflow have been difficult to define, in part because of an incomplete knowledge of how capillary flow is normally regulated. One contributing factor in reduced capillary flow after reperfusion has been described by Zamboni et al. (30). In their study, they observed reperfusion-induced arteriolar constriction (which would tend to decrease capillary flow) when the observed arteriole was in close proximity (<15 µm) with a postcapillary venule. Their results suggest that mediators produced in postcapillary venules send a distance-limited signal to paired arterioles that are capable of controlling capillary perfusion. In the present study, we observe a fundamental difference in arterio-venular control of capillary flow between normo- and hypercholesterolemic rats that could be useful in explaining control of capillary perfusion before or after I/R. Our objectives of this study are to evaluate the role of P-selectin and arterio-venular pairing in the microvascular dysfunction associated with hypercholesterolemia.

METHODS

Animal preparation. Sixty-five male Sprague-Dawley rats were fed normal chow or a high-fat diet containing 1.2% cholesterol, 0.4% cholic acid, and 2.5% olive oil (Dyets, Bethlehem, PA). The rats were purchased in the 100- to 124-g weight range and were maintained on the appropriate diet for 2–3 wk before the experiments, which were performed in a similar manner to that described previously (10). At the beginning of each experiment, rats were anesthetized with an intraperitoneal injection of 135 mg/kg thiobutabarbital (Inactin; Research Biochemicals, Natick, MA). A thoracotomy was performed to facilitate breathing, the right carotid artery was cannulated for injection of a P-selectin monoclonal antibody (in selected experiments) and for administration of an overdose of pentobarbital sodium (160 mg/kg) at the conclusion of each experiment.

After a midline abdominal incision, a snare was created around the superior mesenteric artery using vinyl tubing (OD = 0.8 mm). The small intestine was then exteriorized, and the rat was turned on its right side on a Plexiglas board so that a selected section of mesentery could be draped over a

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KCl, 1.2 MgSO₄, 20 NaHCO₃, and 2.0 CaCl₂. After the board under study, was covered with gauze soaked in bicarbonate-exposed intestine, except for the selected mesenteric section glass coverslip glued on a hole centered in the board. The H670 HYPERCHOLESTEROLEMIA AND CAPILLARY FLUID FILTRATION will increase to equal arteriolar pressure after an occlusion. (Part)

intravascular pressure to be completed. (Capillary pressure allow enough time to refocus the objective after occlusion and position of the capillary segment containing the two cells. The calculation with an image processor during playback of the videotape in

Images were then directed through a time-date generator using a 100-W halogen light source, and bright-field images were acquired with a color camera (Sony DXC-107A). The images were then recorded to video tape with a video cassette recorder (JVC BR-S601MU), and the taped image was used for playback analysis with an image grabber (Imaging Technology Visionplus-AT, Bedford, MA) and image processor (Bioscan Optimas, Edmonds, WA). Capillary red blood cell velocity (V_RBC; mm/s) was measured with an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M, College Station, TX).

Measurement of capillary filtration. Capillary J_v/S was measured using a modified Landis technique (18) in which a selected capillary is occluded and filtration is monitored from the decreasing distance between two red blood cells (19). All selected capillaries had diameters ~10 µm and were “true” capillaries, i.e., divergent at their upstream end and convergent at their downstream end. Each selected capillary was occluded near its venous end with a micropipette drawn to a tip diameter of ~5 µm with a pipette puller (model 700C; David Kopf, Tujunga, CA). The micropipette tip was rounded with a microforge (model 51512; Stoelting, Wood Dale, IL) to minimize damage to the capillary during occlusion. Positioning of the micropipette was accomplished with the use of a micromanipulator (Narishige MO-302) mounted on the microscope. During capillary occlusion, individual red blood cells within the vessel gradually move closer together and toward the occlusion site as the intravascular fluid separating the cells filters across the endothelial barrier into the surrounding tissue. To measure J_v/S, two red blood cells ~80–100 µm apart were selected, and the distance between those cells was monitored for a period of 16 s. In general, a more rapid decrease in the distance between the two selected cells is indicative of a higher value of J_v/S. The capillary geometry was assumed to be a uniform circular cylinder with diameter D, and the decreasing distance between the two cells (l) was used to calculate J_v/S by dividing the volume filtered by a given time period t (to give the filtration rate J_v) and normalizing to the surface area (S) of the capillary segment containing the two cells. The calculation of J_v/S can be simplified and expressed as

J_v/S = -D(4l) × (dl/dt)

The change in l with respect to time (dl/dt) was measured with an image processor during playback of the videotape in seven 2-s periods beginning 2 s after the occlusion, and the average J_v/S of the seven periods was computed. The measurement was not made during the first 2 s of the occlusion, to allow enough time to refocus the objective after occlusion and enough time for a brief vascular expansion due to a higher intravascular pressure to be completed. (Capillary pressure will increase to equal arteriolar pressure after an occlusion.) The increase in diameter caused by higher pressure causes red blood cells within the vessel to move closer together independent of filtration, since a larger diameter requires less height for a given cylindrical volume. Figure 1 shows the increase in diameter after an occlusion in normal and high-cholesterol-diet rats; the diameter change appears to be effectively complete within the first 0.5 s in both groups. (The same was true after the I/R protocol.) The seven 2-s measurements required that the capillary be occluded for a total of 16 s, which was long enough to obtain an accurate estimate of J_v/S but brief enough to allow consistent capillary reflow when the pipette was lifted. J_v/S declines over the 16-s period, with the average over the entire 16-s period ~75% of the value obtained in the first 2-s sampling period (11). In each measurement, the downstream red blood cell was chosen so that it was at least 25–35 µm from the occlusion site where the pipette flattens the shape of the capillary and impedes cell movement. The second cell was chosen as far upstream on the video monitor as possible, typically including 80–100 µm of capillary length. Each capillary under study was occluded one time during baseline conditions and again after I/R. Because the pipette could have damaged the capillary at the occlusion site, the I/R occlusion was made ~5–10 µm upstream of the baseline occlusion so that any damage incurred by the pipette would not effect the subsequent measurement. Although increased J_v/S can reflect an increase in permeability, it can also reflect an increase in hydrostatic pressure or a decrease in capillary osmotic pressure. Starling’s equation describes the factors that determine the rate at which the fluid filters

\[ J_v/S = L_p[P_{c} - P_i - \sigma(H_c - H_i)] \]

where L_p is hydraulic conductivity, P_c is capillary hydrostatic pressure, P_i is interstitial pressure, H is the osmotic reflection coefficient, H_c is capillary osmotic pressure, and H_i is interstitial osmotic pressure. Because the mesentery was exteriorized and superfused with protein-free buffer, both P_i and H_i should be close to 0 mmHg. Additionally, the hydrostatic pressure in the occluded capillary is equal to the hydrostatic pressure in the arteriole (P_{art}) that feeds the capillary. Therefore, Starling’s equation is simplified to

\[ J_v/S = L_p[P_{art} - \sigma H_c] \]

Determination of P_{art} and protein concentration (to estimate H_c) were made so that any changes in endothelial permeability (L_p and \sigma) could be detected; either an increase in L_p or a decrease in \sigma results in increased J_v/S.

Measurement of arteriolar pressure. Because hydrostatic pressure can affect measurements of capillary fluid filtration,
arterial pressure was measured with a servo-null apparatus (model 5A; Instrumentation for Physiology and Medicine, San Diego, CA) using pipettes filled with 2 M NaCl and having a tip resistance of ~3–6 MΩ (11–10–5; Frederick Haer, Bowdoinham, ME). Positioning of the micropipette into the arteriole was facilitated with a micromanipulator. Pressure measurements were considered valid if there was a tight coupling with waveforms of carotid arterial pressure and if there was no change in measurement with a small increase in gain.

Measurements from blood samples. Blood samples (400 µl) were taken during the baseline period and after I/R via the carotid arterial cannula, adding ~5 µl of 1,000 U/ml heparin to prevent coagulation. The blood was spun in a centrifuge, and 50 µl of plasma were drawn off for cholesterol measurement (kit 352–20; Sigma); another 50 µl of plasma were drawn off and placed in a refractometer to measure plasma protein concentration (Cp). The value of Cp was calculated with the equation derived for rat plasma proteins (5); \( c = 2.01C_p + 0.34 \), where \( c \) is in millimeters mercury and \( C_p \) is in grams per deciliter.

Arterio-venular pairing. Arterioles and venules are often paired in a parallel countercurrent arrangement. Pairing was defined in this study as the percentage of the arteriole length that was within 15 µm of a postcapillary venule (30). The length of arteriole was measured from the branching capillary as far upstream as possible, until reaching the fat cells surrounding the major arcade vessels.

I/R protocol. A 10- to 15-min stabilization period preceded baseline measurements of arterio-venular (A-V) pairing, \( V_{RBC} \), and \( J/S \) (or \( P_{art} \) in separate experiments). Baseline measurements, mesenteric ischemia (capillary \( V_{RBC} \) reduced to an average of 15–20% of baseline) was produced by tightening the snare surrounding the superior mesenteric artery. The ischemic period lasted 10–15 min (10 min in experiments where each capillary under study was completely shut down during ischemia; 15 min otherwise). After 30 min of reperfusion, measurements of either 1) \( V_{RBC} \) and \( J/S \) or 2) \( P_{art} \) were repeated. The following three groups of animals underwent the I/R protocol: 1) rats on normal chow (normocholesterolemia), 2) rats on high-cholesterol chow (hypercholesterolemia), and 3) hypercholesterolemic (HC) rats injected with a P-selectin monoclonal antibody (PB1.3; Cytel, San Diego, CA) 5 min into the ischemic period.

Statistics. Analysis of fewer than three sets of data was performed with t-tests (parametric) or the Wilcoxon test (nonparametric). Bonferroni’s post hoc test was used to compare more than two groups when a significant F-test resulted from ANOVA. Linear regression was performed to determine whether relationships existed between various parameters. Each test was performed with Instat software (GraphPad Software, San Diego, CA) using a 95% confidence level to determine significant differences. Data from multiple capillaries were pooled before statistical tests except when analysis of individual capillaries was more appropriate. Error bars are presented as ± SE.

**RESULTS**

Table 1 lists several statistics of the three experimental groups, i.e., normocholesterolemic (NC), HC, and HC rats injected with the P-selectin antibody, PB1.3 (HC/PB1.3). The high-fat diet increased circulating cholesterol concentrations by a factor of about six to seven, which also increased the total plasma protein concentration. Carotid pressure, arteriolar pressure, duration of ischemia, and percent ischemia were similar between groups. We have reported previously (10) that the arteriolar pressure drop in normal and HC rats as well as the drop in plasma protein concentration are also present in time-matched sham experiments, indicating that these events are not due to mesenteric I/R.

Figure 2 gives \( J/S \) before ischemia and after 30 min of reperfusion in the three groups. Although no significant difference was observed between the increases in \( J/S \) between the NC and HC groups, we have previously (10) found that the two groups can be statistically distinguished using a longer period of ischemia (30 min) that resulted in a greater increase in \( J/S \). When PB1.3 was administered to the HC group, I/R did not produce a significant increase in \( J/S \). The lack of a significant increase in \( J/S \) in the HC/PB1.3 group is not merely due to fewer experiments (n = 12) compared with the NC and HC groups (19 each). After 12 experiments in the HC group, statistical significance had already been achieved (\( P = 0.007 \)), whereas the P value in the HC/PB1.3 group was 0.176 (power of statistical test = 0.30). A limited supply of PB1.3 prevented further experiments.

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Data are given as means ± SE with the number of animals included in parentheses. NC, normocholesterolemic; HC, hypercholesterolemic; HC/PB1.3, HC injected with a P-selectin antibody. I/R, ischemia-reperfusion. P < 0.05 vs. NC (Bonferroni’s post hoc test; *), HC (Bonferroni’s post hoc test; †), and baseline (paired t-test; ‡).
The relative increases in \( J_v/S \) (I/R divided by baseline) in the NC and HC groups appear to be nonparametric. Median relative increases were 1.14-fold (\( P < 0.05 \)) in the NC group, 1.53-fold (\( P < 0.001 \)) in the HC group, and 1.26-fold (not significant, \( P = 0.13 \)) in the HC/PB1.3 group.

In each of the three groups, separate experiments were performed to establish the effect of I/R on arteriolar pressure. In these experiments, \( P_{art} \) was obtained from a selected arteriole with a diameter of \( 15–20 \) \( \mu \)m. I/R had very little effect on \( P_{art} \) (see Table 1), as we have reported previously (10). The values of \( P_{art} \) and \( \pi_c \) (calculated from plasma protein concentration in Table 1) can be inserted into the simplified Starling’s equation (given in METHODS) to predict their influence on \( J_v/S \). In the HC group, for example, if \( \pi_c \) drops from 20.8 to 18.0 mmHg (5.4–4.9 g/dl protein concentration) and if \( P_c \) drops from 39 to 38 mmHg, the predicted relative increase in \( J_v/S \) in the absence of any change in permeability (\( L_0 \) or \( \sigma \)) would be \( L_0[38 - 0.9(18)] / L_0[39 - 0.9(20.8)] = 1.07 \), using a \( \sigma \) value of 0.9 (6, 21). Therefore, with \( P_{art} \) and \( C_p \) remaining near baseline values, the increase in \( J_v/S \) is primarily due to changes in capillary permeability.

In approximately half of the experiments in which capillaries were occluded, capillary \( V_{RBC} \) and A-V pairing were also measured. Several intriguing observations were made with these data. A significant (\( P = 0.0001 \)) correlation existed between A-V pairing and baseline \( J_v/S \) in the NC group, as shown in Fig. 3A. However, this relationship was completely absent (\( P = 0.18 \)) in the HC group (Fig. 3B). Capillary \( V_{RBC} \) had a similar relationship to A-V pairing as shown in Fig. 4, A and B, where the correlation between the two was only present in the NC group. These data suggest the possibility that A-V pairing is one factor that determines capillary perfusion and filtration.

When the data from all capillaries are included, there were no significant differences (between the three groups) in the extent of capillary reperfusion, i.e., restoration of \( V_{RBC} \) to baseline values. However, among the capillaries that branched from highly paired arterioles (>20% of their length), capillary \( V_{RBC} \) dropped more significantly in the HC group than in the NC group, as shown in Fig. 5. This trend was reversed with injection of PB1.3.

**DISCUSSION**

Reperfusion of ischemic tissue causes adverse effects in each of the three main segments of the microcirculation (arterioles, capillaries, and venules). Arterioles tend to lose their ability to dilate (22), capillaries increase their permeability to water (10) and often do not completely reperfuse (7, 14), and venules increase their adhesiveness for leukocytes and increase their permeability to protein (17). The present study contributes to the growing body of evidence suggesting that these responses are not isolated phenomena but are
coordinated in a communicated fashion between the three segments.

The microvascular dysfunction associated with I/R is more pronounced with the added risk factor of hypercholesterolemia (10, 17), possibly due to a basal state of dysfunction. For example, hypercholesterolemia significantly attenuates the arteriolar response to endothelium-dependent dilator agents (12) and appears to promote the adherence of venular endothelium to circulating leukocytes (1, 15, 25). Furthermore, capillary density decreases by a factor of two in rabbits with heritable hyperlipidemia (26). Although there may be several potential explanations for decreased capillary perfusion with hypercholesterolemia, the present study suggests that a contributing factor could be decreased venular input toward arteriolar dilation.

The idea of venules controlling arteriolar tone has become more prominent in recent years. Several investigators (3, 4, 13, 24) have suggested that vasoactive metabolites can diffuse from postcapillary venules into paired arterioles. Additionally, Collins et al. (2) have determined that venules exposed to ATP send a vasoactive signal to upstream arterioles through the capillaries, most likely by gap junctional communication conducted along the vascular wall. Finally, venules appear to constrict paired arterioles (which are <15 µm away) during reperfusion (30). The present study supports the concept of A-V communication by demonstrating a significant correlation between capillary perfusion and A-V pairing, a correlation that was absent in HC animals. Therefore, the degree to which A-V pairing influences capillary function may be dependent on two main factors, i.e., the proximity of communicating arterioles and venules and the presence of a condition (such as hypercholesterolemia) that may alter the concentrations or activity of signaling molecules.

A-V pairing also appeared to determine baseline capillary \( J_{V/S} \) in the present study. Filtration is dependent on two main factors, pressure and permeability. It is possible that the hydrostatic pressure in capillaries feeding off of highly paired arterioles is higher than from relatively unpaired arterioles, especially if venules normally release a vasodilator, such as nitric oxide (3, 4), which would decrease precapillary resistance. Arteriolar dilation that increases capillary \( V_{RBC} \) would also increase capillary shear rates. Considering that increased shear has been associated with increased endothelial permeability (28), our observed relationship between A-V pairing and baseline \( J_{V/S} \) could be a result of inseparable permeability and pressure effects.

We have recently demonstrated that increases in capillary permeability induced by the inflammatory mediator platelet-activating factor are dependent on A-V pairing (11) as well as on neutrophil adhesion (8, 9). Therefore, it is possible that the correlation between baseline \( J_{V/S} \) and A-V pairing is a phenomenon due to low-level, neutrophil-mediated inflammation causing an increase in capillary permeability that is independent of arteriolar dilation. However, if the latter explanation were true, we would have expected a similar correlation between baseline \( J_{V/S} \) and A-V pairing in HC rats, given their apparent increased inflammatory potential.

When A-V pairing exceeded 20%, injection of PB1.3 allowed better capillary reperfusion than in the untreated HC group. There are at least two possible explanations for this observation. Assuming that PB1.3 inhibits leukocyte-endothelial cell adhesion, a lower level of leukocyte adhesion may allow for less resistance to flow. However, if this were the case, one might expect to see a tendency for an increase in arteriolar pressure in the HC group after reperfusion (due to downstream obstruction) and an attenuation of this pressure increase in the HC/PB1.3 group. As shown in Table 1, no such tendencies were present. Another possibility relates back to the aforementioned study by Zamboni et al. (30) in which reperfusion-induced arte-
riolar constriction was limited to sites of a nearby postcapillary venule. Their study raises the possibility that leukocyte adhesion in venules stimulates the release of a vasoconstrictive agent that reaches paired arterioles, causing reduced capillary flow. Therefore, our injection of PB1.3 may have prevented the first step in the proposed mechanism, leukocyte adhesion, and thereby improved capillary perfusion. However, we cannot rule out the possibility that the protection given by PB1.3 could be independent of its ability to attenuate leukocyte adhesion. For example, platelets also express P-selectin, and PB1.3 could conceivably interfere with a platelet function (other than facilitating leukocyte adhesion) that affects capillary perfusion.

Another benefit of PB1.3 administration in our study was its ability to prevent as large of an increase in reperfusion-induced capillary permeability in the HC group. This finding was not unexpected, given the reported benefits of P-selectin blockade in preventing reperfusion-induced increases in capillary filtration coefficient (20) and venular protein leakage (16). Furthermore, at the level of single capillary studies, we have previously found PB1.3 to prevent increased capillary permeability induced by platelet-activating factor (8), which is a primary mediator of reperfusion injury. The mechanism of protection may likely involve the prevention of leukocyte adhesion. However, Weiser et al. (29) has proposed that the protection against I/R-induced vascular permeability given by PB1.3 may be due to an adhesion-independent mechanism, in light of the fact that PB1.3 did not significantly prevent leukocyte sequestration in their model.

The mechanism of increased capillary permeability after I/R is unclear. Our observations that increased J/s can be prevented by anti-neutrophil serum (10) and by PB1.3 suggest that the mechanism may involve neutrophil-endothelial cell adhesion. One possible mechanism of increased permeability is that neutrophil-endothelial cell interactions within venules initiates a signal delivered by paired arterioles to downstream capillaries. Such a scenario may not be easy to detect in our model due to conflicting effects of pairing on J/s. For example, arterioles tightly paired to venules may send a strong permeability-increasing signal to downstream capillaries (which would increase J/s); however, the same arterioles may receive a vasoconstrictive signal (which would decrease J/s) after I/R.

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REFERENCES


