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P-selectin-mediated adhesion impairs endothelium-dependent arteriolar dilation in hypercholesterolemic mice

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IT IS WELL ESTABLISHED that the early stages of hypercholesterolemia induce endothelial dysfunction in microvessels well before the formation of atherosclerotic lesions in large arteries. The hypercholesterolemic microvasculature experiences an increased oxidative stress and diminished availability of nitric oxide (NO). This imbalance of superoxide and NO could induce a proinflammatory response in postcapillary venules, which is manifested as enhanced adhesion of leukocytes and platelets (12, 29, 33, 34). The hypercholesterolemia-enhanced expression of P-selectin on endothelial cells mediates the rolling of leukocytes on endothelial cells (11). Additionally, the increased expression of P-selectin on activated platelets mediates their interactions with both leukocytes and endothelial cells during hypercholesterolemia (34, 35).

Hypercholesterolemia results in an impairment of endothelium-dependent vasodilation in arteries and small arterioles (8, 14, 17, 30). The underlying mechanism associated with hypercholesterolemia-induced arteriolar dysfunction is known to be related to an oxidant-dependent pathway; that is, a hypercholesterolemia- and oxidant-induced decrease in arteriolar NO bioavailability leads to attenuated arteriolar dilation. Recently, we have shown that venular leukocyte adherence also could lead to arteriolar constriction by limiting NO bioavailability when arterioles were closely paired with inflamed venules (16). The possible role of platelet and/or leukocyte adhesion in venule-dependent arteriolar constriction has been studied in experimental models of ischemia-reperfusion (37), diabetes (23), and dextran sodium sulfate-induced colitis (13). However, there has been little investigation of the possible link between hypercholesterolemia-induced venular inflammation and arteriolar dysfunction.

In this study, we have tested the hypothesis that the dysfunction elicited by hypercholesterolemia in arterioles and venules might be tightly associated in a common mechanism; that is, venular adhesion of leukocytes and platelets may be responsible for arteriolar constriction. The major objectives of this study were to determine the following: 1) the extent to which P-selectin mediates hypercholesterolemia-induced venular leukocyte and platelet adhesion in the mouse intestinal submucosa, 2) whether the enhanced leukocyte and platelet adhesion contributes to impaired arteriolar dilation, 3) whether endothelial dysfunction is more severe when arterioles are more closely paired with the inflamed venules, and 4) whether superoxide participates in the venule-dependent arteriolar dysfunction. To accomplish these goals, venular leukocyte and platelet adherence and arteriolar responses to bradykinin (BK) were observed via intravital microscopy in hypercholesterolemic mice in which either P-selectin-mediated adhesion or superoxide was blocked.

MATERIALS AND METHODS

Animal preparation. Male C57BL/6J wild-type and B6.129S7-S (P-selectin-deficient) mice were purchased (from Jackson Labs; Bar Harbor, ME) in the 20- to 30-g weight range. At 6 wk of age the C57BL/6J wild-type mice were placed on either a normal or a high-cholesterol diet (1.25% cholesterol, 0.125% cholic acid, and 15.8% fat; Harlan Teklad; Madison, WI) for a period of 4 wk. P-selectin-deficient mice were also placed on the high-cholesterol diet for 4 wk. Mice were housed 3–5 per cage in a controlled environment (12 h/12 h, light/dark cycle). Animal procedures were approved by the University Health Sciences Center, Shreveport, Louisiana.

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Institutional Animal Care and Use Committee of Louisiana State University Health Sciences Center.

**Surgical procedure.** Mice were anesthetized with ketamine hydrochloride (150 mg/kg) and xylazine (10 mg/kg). The right carotid artery was cannulated to measure systemic blood pressure and to collect blood samples for platelet isolation from donor mice. The right jugular vein was cannulated for injection of fluorescently labeled platelets, rhodamine 6G, and in some mice, a monoclonal antibody against P-selectin (2 mg/kg, RB40.34, BD Pharmingen, San Diego, CA). The P-selectin antibody was injected 30 min before baseline measurements. A segment of the small intestine was exteriorized through a midline abdominal incision, and the spontaneously breathing mouse was placed on its right side on a Plexiglas board. Once the small intestine was exteriorized, two small incisions in the intestinal wall were made to perfuse the small intestine with bicarbonate-buffered saline (BBS) consisting of (in mM) 131 NaCl, 4.7 KCl, 1.2 MgSO4, 20 NaHCO3, and 3.5 CaCl2. A polyethylene tube (P-190, Becton Dickinson) was inserted through a hole incised in the mid small intestine to form an input port. The output port was located distally ~5 cm. The cannulated intestinal segment was covered with gauze soaked in warm BBS. After the board was mounted onto the stage of an inverted microscope, the intestine was continuously perfused with BBS bubbled with a 95% N2-5% CO2 gas mixture and warmed to 37°C. The perfusion flow rate was set to 0.5 ml/min.

**Fluorescent labeling of platelets.** Approximately 0.9 ml of blood from a donor mouse was collected via the right carotid artery into acid-citrate-dextrose buffer (Sigma; St. Louis, MO). The blood was centrifuged at 1,200 rpm for 8 min to obtain a layer of platelet-rich plasma (PRP). The PRP was drawn off and subsequently centrifuged at 1,200 rpm for an additional 3 min to pellet the remaining red blood cells and leukocytes. The PRP was centrifuged at 3,000 rpm, and the pellet was resuspended slowly in 0.5 ml phosphate-buffered saline (pH 7.37). Five microliters were withdrawn for platelet counting using a hemacytometer, and the remaining platelets were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; final concentration 10 μM) and incubated for 10 min at room temperature. The CFSE-labeled solution was centrifuged at 3,000 rpm for 10 min, and the pellet was resuspended in PBS. Approximately 1 × 106 cells were injected into the recipient mouse, comprising ~10% of the total circulating platelets. The platelet-donor mouse was matched with a recipient mouse with respect to strain (wild-type or P-selectin-deficient) and diet (normal or high cholesterol).

**Intravital microscopy.** The intestinal microvessels were observed through a 20× objective (Nikon Plan Apo, 0.4 numerical aperture) using a 100-W halogen (brightfield) and 100-W mercury (fluorescence) light sources, and images were captured with a color camera (CCD DXC-390, SONY). The fluorescence image was viewed using filter cubes for fluorescein and rhodamine wavelengths. Rhodamine 6G (0.2 ml of a 0.3 mg/ml solution) was infused slowly through the right jugular vein to visualize blood vessels and adherent leukocytes. Subsequently, labeled platelets were infused through the right jugular vein over a period of 5 min and allowed to circulate before the observation. The images were directed into a DVD recorder (DMR-E30, Panasonic), and the recorded images were used for playback analysis with an image grabber and image processor (Scion Image) for length and diameter measurements.

**Cholesterol measurements.** At the end of each experiment, a blood sample (~200 μl) was withdrawn from a cannulated jugular vein and gently mixed with ~5 μl of 1,000 U/ml heparin. Approximately 50 μl of the blood sample was used in a test panel of a cholesterol measurement kit (Polymer Technology System, Indianapolis, IN).

**Venular leukocyte rolling, leukocyte adherence, and platelet adherence.** The number of rolling leukocytes, adherent leukocytes, and adherent platelets were quantified during playback analysis of recorded images. The number of rolling leukocytes were defined as the number of leukocytes that passed a selected cross section of venule per minute. Leukocyte and platelet adherence were defined as the number of leukocytes and platelets that remained stationary on the venular wall for a period of at least 30 s and were expressed as the number of cells per square millimeter of venular surface.

**Experimental protocols.** Experimental groups were divided as follows: 1) wild-type mice with a normal cholesterol diet (NC), 2) wild-type mice with a high-cholesterol diet (HC), 3) wild-type mice with a high-cholesterol diet and injection of a monoclonal antibody against P-selectin (HC+Anti-P-sel), 4) P-selectin-deficient mice with a high-cholesterol diet (HC+P-selP−/−), and 5) wild-type mice with a high-cholesterol diet and intraluminal exposure to the superoxide dismutase mimetic Tempol (HC+Tempol).

After surgical preparation of the intestine, arterioles with or without closely paired venules in the submucosa were selected for intravital study. First-order arterioles come into the intestinal submucosa from the mesentery paired with a venule, and the pairing is maintained by most second-order arterioles, albeit with varying separation distances. Pairing becomes even more variable in branching third-order arterioles and is rarely found in fourth-order arterioles. The present study limits the second-order arterioles with or without a paired venule close enough to be in the microscopic field of view. To ensure that all groups were composed of vessels from similar branching sites, second-order arterioles were chosen at a site where the paired venule (if present in the field of view) was ~<100 μm in diameter. After 20 min of stabilization with BBS perfusion, baseline measurements of leukocyte rolling, leukocyte adherence, and platelet adherence were obtained from venules that were closely paired with the arterioles of interest. Arteriolar diameters also were measured, and in selected experiments, remeasured after 10 min intraluminal perfusion of 10−4 mM Tempol (Sigma). After baseline measurements, the endothelium-dependent vasodilator BK, dissolved in BBS, was perfused for 15 min before repeating measures of arteriolar diameter. In the groups of untreated NC and HC mice, dose-dependent increases in arteriolar diameters were measured at BK concentrations of 10−4, 10−3, and 10−2 mM. In experimental groups of HC+Anti-P-sel, HC+P-selP−/−, and HC+Tempol, BK responses were measured at 10−2 mM concentrations (including 10−4 mM Tempol in the latter group). After BK measurements, perfusate was changed to BBS to allow the arteriolar diameter to return to baseline values, and perfusion was continued for 10 min. The perfusate then was changed to a papaverine solution (1 mM, Sigma), and endothelium-independent maximal arteriolar dilations were obtained after 10 min. Arterioles that did not respond to papaverine were not included in the data analysis.

**Statistics.** GraphPad Instat (San Diego, CA) software was used for statistical analysis. Data were compared with standard t-tests or with ANOVA (using Bonferroni post hoc corrections) for multiple groups or repeated measures. Minitab software (Minitab, State College, PA) was used for regression analysis. Error bars are presented as means ± SE. Statistical significance was set at P < 0.05.

**RESULTS**

Table 1 shows statistical data from the five groups of mice. Systemic blood pressures and vascular diameters were similar in all groups, but cholesterol levels were elevated more than twofold in the groups given the high-cholesterol diet (to ~145–163 mg/dl compared with ~70 mg/dl in controls).

**Influence of high-cholesterol diet and arteriovenular distance on BK-induced vasodilation.** Second-order arterioles in the submucosa of NC and HC mice were exposed to BK concentrations ranging from 0 to 1 × 10−2 mM. BK-induced dilations were attenuated in the HC group by ~50% at all concentrations (P < 0.001; Fig. 1A). Figure 1B presents the individual BK-induced dilations (10−2 mM BK) as a function of distance between the arteriole and paired venule. The majority of second-order arterioles are paired with parallel, countercurrent venules, but if no venule was present in the

AJP-Heart Circ Physiol • VOL 292 • JANUARY 2007 • www.ajpheart.org
microscopic field of view (which encompassed a distance of ~150 μm), the data are presented at the far right of the x-axis and were not included in the following regression analysis (1 case in NC; 3 cases in HC). The BK-induced dilations in NC mice appeared to be independent of the distance between the arteriole and the paired venule (P = 0.55, r² = 0.03), whereas a significant relationship existed in the HC mice (P = 0.01, r² = 0.35). The regression equation for the HC data was dilation (%) = 12.2 + 0.0726 × pairing distance (μm), with dilation increasing by an approximate factor of 2 over the range of pairing distances. The HC data were further categorized as closely paired (<50 μm separation distance) or more distantly paired/unpaired (>50 μm) as shown in Fig. 1C. BK-induced dilations (10⁻² mM BK) were significantly attenuated (P < 0.001) compared with NC (31.1 ± 1.2% dilation) in both pairing subsets of HC data (Fig. 1C), but the attenuation in closely paired arterioles (to 13.7 ± 1.6% dilation) was significantly more severe (P < 0.01) compared with the more distantly paired arterioles (21.2 ± 1.4% dilation).

High cholesterol-induced venular leukocyte and platelet recruitment. NC mice demonstrated minimal leukocyte rolling, leukocyte adherence, and platelet adherence to the endothelium of postcapillary venules (Fig. 2). However, 4-wk HC diet provided to wild-type mice resulted in ~5- to 10-fold increases (P < 0.001) in adhesion as shown in Fig. 2, B–D. When HC mice were treated acutely with a P-selectin antibody (HC+Anti-P-sel), the number of rolling leukocytes, adherent leukocytes, and adherent platelets were reduced significantly (P < 0.001), to approximately the same number found in NC mice. Similar attenuations also were observed for P-selectin-deficient mice placed on the HC diet (HC+P-sel⁻/⁻), suggesting that P-selectin expressed on platelets or venular endothelium plays a crucial role in venular leukocyte and platelet recruitment.

Effect of P-selectin on HC-induced impairment of endothelium-dependent arteriolar dilation. To determine whether effective reductions in leukocyte and platelet recruitment in venules can restore impaired endothelium-dependent arteriolar dilation in HC mice, BK (10⁻² mM) responses were observed from both closely venule-paired and distantly venule-paired/unpaired arterioles (Fig. 3). In closely paired arterioles, BK-induced dilation was enhanced significantly in HC mice given the P-selectin antibody (P < 0.01) and in P-selectin-deficient HC mice (P < 0.001; Fig. 3A). Even though the same trends were present in the more distantly paired HC arterioles, no significant differences were observed. However, some of the small variability in the BK response could be reduced further by normalizing the BK-induced dilation to the maximal dilation observed with papaverine exposure (Fig. 3B). After this...
normalization, a small but significant (P < 0.05) improvement in BK-induced dilation was found in the more distantly paired HC arterioles of the P-selectin-deficient mice.

The BK-induced dilation in NC mice averaged 93.7 ± 1.2% of the papaverine response (Fig. 3B) compared with a dramatically lower 30.1 ± 3.2% in closely paired HC arterioles and 55.3 ± 6.9% in more distantly paired HC arterioles. In the closely paired HC arterioles, the normalized BK responses improved to 68.9 ± 5.3% and 76.4 ± 4.6% in the P-selectin antibody and deficient groups (Fig. 3B), to values that were similar to those in the more distantly paired HC arterioles (68.8 ± 3.1% and 76.9 ± 3.3%, respectively).

Most experimental groups demonstrated similar dilation in response to papaverine (Fig. 3C), suggesting that the endothelium-independent vasodilatory ability was intact in the early stage of hypercholesterolemia. However, the papaverine-induced dilation in closely paired arterioles of the HC group treated with the P-selectin antibody was significantly smaller (P < 0.05) than that of the untreated HC group. This observation could be consistent with a small degree of P-selectin-mediated leukocyte and platelet adhesion and was dependent on the distance to closely paired venules. We provide evidence that 1) 10⁻² mM BK-induced arteriolar dilation in the mouse submucosa is reduced in hypercholesterolemia, 2) the HC-induced attenuation in the BK response is more dramatic in arterioles that are closely paired with inflamed venules, 3) P-selectin blockade completely reverses HC-induced venular leukocyte and platelet recruitment, and 4) P-selectin and superoxide blockade substantially (and to a similar extent) improve the HC-attenuated dilation of closely paired arterioles.

**Effect of superoxide dismutase mimetic on HC-induced impairment of endothelium-dependent arteriolar dilation.** To determine whether superoxide might be involved in the HC-induced impairment of endothelium-dependent arteriolar dilation, BK responses were assessed in the presence of Tempol (Fig. 4). The Tempol treatment significantly increased the baseline diameters of HC arterioles (Fig. 4A), but not of NC arterioles, suggesting a basal superoxide-dependent vasoconstriction in HC. (The dilation in NC arterioles was near the approximate limit of resolution.) Moreover, Tempol was able to substantially restore the BK response (with and without papaverine normalization) in closely paired HC arterioles (Fig. 4, B and C) but did not have a statistically significant effect in more distantly paired arterioles. The BK-induced dilation in Tempol-treated HC mice averaged 67.1 ± 4.8% of the papaverine response in closely paired arterioles compared with a significantly lower 30.1 ± 3.2% in HC mice without Tempol (P < 0.001).

**DISCUSSION**

This study addresses the possibility of a link between two major endothelial dysfunctions in early hypercholesterolemia (before atherosclerosis): 1) P-selectin-dependent leukocyte and platelet adhesion and 2) attenuated endothelium-dependent arteriolar dilation. The novel finding of this study was that endothelium-dependent arteriolar dysfunction occurred primarily via a pathway associated with P-selectin-mediated leukocyte and platelet adhesion and was dependent on the distance to closely paired venules. We provide evidence that 1) 10⁻² mM BK-induced arteriolar dilation in the mouse submucosa is reduced in hypercholesterolemia, 2) the HC-induced attenuation in the BK response is more dramatic in arterioles that are closely paired with inflamed venules, 3) P-selectin blockade completely reverses HC-induced venular leukocyte and platelet recruitment, and 4) P-selectin and superoxide blockade substantially (and to a similar extent) improve the HC-attenuated dilation of closely paired arterioles.

P-selectin is stored in α-granules of platelets as well as in Weibel-Palade bodies of endothelial cells, and proinflammatory stimuli rapidly mediate the fusion of these granules with the plasma membrane and subsequent exposure of P-selectin to the cell surface (6, 31). The elevated P-selectin expression on platelets or endothelial cells is known to mediate the adhesion of platelets and leukocytes to endothelial cells (11) and mediate platelet-leukocyte interactions during hypercholesterolemia (34, 35). Our results obtained from intestinal venules also were consistent with those findings as evidenced by increased platelet and leukocyte adhesion in hypercholesterolemic mice, with the response inhibited by P-selectin blockade. Although there
have been many studies of hypercholesterolemia-induced blood cell-endothelial cell interactions or impaired vasodilation, relatively little attention has been devoted to the possible link between these two manifestations. The mechanism by which adhesion limits arteriolar vasodilation is still not clear; however, several possible explanations can be considered.

One possibility is that the presence of adherent leukocytes in venules may limit the concentration of NO in paired arterioles, as we have shown previously (16, 24), and may ultimately impair the endothelium-dependent vasodilation of nearby arterioles via a diffusion-limited mechanism. We have found that NO synthase inhibition induces specific constriction of venules near arteriolar smooth muscle to induce vasoconstriction. In a previous study of the mouse intestinal submucosa, we found a median arteriovenular pairing distance of 17 μm (13), which included several microns of arteriolar smooth muscle. Therefore, an extravasated leukocyte often would have to emigrate only a few microns from the venules to be in contact with the arteriolar smooth muscle. In our study, chronic blockade of P-selectin (the use of P-selectin-deficient mice) in HC mice improved the BK response slightly more than by using a P-selectin antibody in wild-type mice (Fig. 3), despite equivalent success in eliminating venular leukocyte and platelet recruitment (Fig. 2). This difference potentially could be attributed to the likelihood that acute administration of P-selectin antibody would not have eliminated the presence of interstitial leukocytes to the same extent as it did in P-selectin-deficient mice. This speculation is supported by the recent finding obtained from postcapillary venules of cremaster muscle in hypercholesterolemic mice, in which treatment with a P-selectin antibody did not significantly alter the number of emigrated leukocytes, in contrast to the almost complete attenuation observed in HC mice with a chronic P-selectin deficiency (bone marrow transfer into wild-type mice from P-selectin-deficient mice) (32). It also has been reported that P-selectin-deficient mice have reduced neutrophil clearance and peritoneal macrophage recruitment in comparison with those seen in wild-type mice in response to an inflammatory insult (15). Therefore, it is plausible that emigrated leukocytes surrounding postcapillary venules might lead to impaired arteriolar dilation in nearby arterioles by releasing mediators via a diffusion- or emigration-limited mechanism.

We also observed that the HC-induced attenuated dilation, and reversal by P-selectin blockade, were present (although to a lesser extent) even in distantly paired arterioles. This finding can be addressed by at least two potential explanations: 1) relevant P-selectin-dependent events occur in HC arterioles paired arteriolar pathways (22) and that arteriolar NO is decreased with higher levels of venular leukocyte adherence (16, 24). In arterioles that are closely paired with inflamed venules (having high leukocyte adherence), NO levels are significantly enhanced with Tempol (16).

Leukocyte-endothelial cell adhesion activates leukocyte NADPH oxidase, which in turn catalyzes the production of the superoxide radicals that rapidly react with NO (2). Substantial evidence suggests that a hypercholesterolemia-induced excess of superoxide could be responsible for impaired vasodilation of atherosclerotic large arteries (20). However, there has been less investigation into whether superoxide also contributes to attenuated dilation in small arterioles in the early stages of hypercholesterolemia. Superoxide radicals are also known to be produced by NADPH oxidase localized in the arteriolar endothelium, which could lead to increased degradation of NO (36). Taken together, it is conceivable that superoxide could be released either from activated leukocytes in adjacent venules or from arteriolar endothelial cells and may reduce arteriolar dilation by decreasing NO bioavailability when arterioles are in close proximity to the inflamed venules. The findings of this study support this scenario, in which the BK-induced dilation of closely paired arterioles was significantly improved in the presence of the superoxide dismutase mimetic Tempol.

Another possibility is that leukocytes extravasate through venular walls to the interstitium and would be intimately located near arteriolar smooth muscle to induce vasoconstriction. In a previous study of the mouse intestinal submucosa, we found a median arteriovenular pairing distance of 17 μm (13), which included several microns of arteriolar smooth muscle. Therefore, an extravasated leukocyte often would have to emigrate only a few microns from the venules to be in contact with the arteriolar smooth muscle. In our study, chronic blockade of P-selectin (the use of P-selectin-deficient mice) in HC mice improved the BK response slightly more than by using a P-selectin antibody in wild-type mice (Fig. 3), despite equivalent success in eliminating venular leukocyte and platelet recruitment (Fig. 2). This difference potentially could be attributed to the likelihood that acute administration of P-selectin antibody would not have eliminated the presence of interstitial leukocytes to the same extent as it did in P-selectin-deficient mice. This speculation is supported by the recent finding obtained from postcapillary venules of cremaster muscle in hypercholesterolemic mice, in which treatment with a P-selectin antibody did not significantly alter the number of emigrated leukocytes, in contrast to the almost complete attenuation observed in HC mice with a chronic P-selectin deficiency (bone marrow transfer into wild-type mice from P-selectin-deficient mice) (32). It also has been reported that P-selectin-deficient mice have reduced neutrophil clearance and peritoneal macrophage recruitment in comparison with those seen in wild-type mice in response to an inflammatory insult (15). Therefore, it is plausible that emigrated leukocytes surrounding postcapillary venules might lead to impaired arteriolar dilation in nearby arterioles by releasing mediators via a diffusion- or emigration-limited mechanism.

We also observed that the HC-induced attenuated dilation, and reversal by P-selectin blockade, were present (although to a lesser extent) even in distantly paired arterioles. This finding can be addressed by at least two potential explanations: 1) relevant P-selectin-dependent events occur in HC arterioles...
(not just in venules), or 2) HC-induced mediator diffusion and/or blood cell emigration from venules to arterioles may extend well beyond our close pairing definition of 50 μm. With respect to the latter, it is known that mediator signaling can be effective over much larger distances than 50 μm (10), and leukocyte emigration rates can exceed 10 μm in a single minute (5).

With respect to the former, there is a recent study reporting P-selectin-mediated leukocyte adhesion to arteriolar endothelium (under exposure to angiotensin II) in rat mesentery (1). However, we observed that leukocyte and platelet adhesion to submucosal arterioles was negligible in our model of early hypercholesterolemia. However, another P-selectin-mediated event that could be independent of close arteriovenular pairing is the formation of platelet-leukocyte aggregates in the systemic circulation. Platelet activation upregulates the affinity and avidity of leukocyte P-selectin glycoprotein ligand-1 (PSGL-1) and results in subsequent interaction between the two cell types (9, 32). Indeed, the increased formation of circulating platelet-leukocyte aggregates has been implicated in large artery diseases and proposed as an important clinical marker in cardiovascular diseases (26–28). The prevalence of these aggregates along the venular endothelium also should be noted: in a study by Tailor and Granger (35), HC-induced platelet adhesion in intestinal venules was highly dependent on leukocytes, with ~80% of the venular adherent platelets bound to adherent leukocytes. The binding of activated platelets to leukocytes stimulates increased release of superoxide (21) and cytokines (25) from leukocytes through a P-selectin-dependent mechanism. Therefore, in our study, it is possible that P-selectin blockade could inhibit the formation of both circulating and venule-bound platelet-leukocyte aggregates, thereby reducing superoxide production. Our finding that the improvements in the BK response with P-selectin blockade were comparable to those with Tempol treatment support a possibility that superoxide released from circulating and/or venule-bound platelet-leukocyte aggregates might contribute to the impaired arteriolar dilation.

Our data demonstrate substantial improvement in endothelium-dependent arteriolar dilation in HC mice with treatments targeting either P-selectin or superoxide; however, the BK responses were still not equal to those of the NC mice. This could suggest the involvement of additional vasoconstrictors released from activated leukocytes and/or platelets (whether adherent or not) that might act via an oxidant-independent mechanism. Activated leukocytes release proteases that facilitate the production of the vasoconstrictor endothelin-1 (ET-1); both chymase and cathepsin G each cleave the precursor Big ET-1 into ET-1 (18). Hypercholesterolemia has been implicated in the increased level of ET-1 and subsequent impaired endothelial function of coronary arteries (19). The chronic antagonism of endothelin receptors markedly improves coronary endothelial function in experimental hypercholesterolemia (4). Hypercholesterolemia also is known to increase the number of activated platelets in the circulation, which is positively correlated with the release of the vasoconstrictor thromboxane (TxA2) (7). Additionally, the interactions between activated platelets and leukocytes facilitate enhanced thromboxane release from platelets (9). The inhibition of TxA2 with a receptor antagonist has been shown to restore impaired acetylcholine-induced vasodilation in short-term HC-diet rats, supporting the possibility that HC-induced synthesis of TxA2 may also contribute to constriction and counteract the vasodilatory effect (3).

The findings of our study build on the recent report by Stokes et al. (32), who investigated the role of P-selectin-mediated events on acetylcholine-induced dilation of arterioles in the mouse cremaster. As in our study, the investigators found that hypercholesterolemia induced a P-selectin-dependent dysfunction in arteriolar dilation, which could be restored, in whole or in part, by treatments including anti-neutrophil serum, anti-platelet serum, and P-selectin deficiency. There-
fore, the mechanism of deficient dilation induced by hypercholesterolemia is found in both intestine (our study) and cremaster (32) and potentially exists in tissues throughout the body.

In conclusion, this study demonstrates that hypercholesterolemia-induced increases in adherent leukocytes and platelets may contribute to an impaired endothelium-dependent vasodilation of closely paired arterioles via distance-limited, P-selectin- and oxidant-dependent mechanisms. The results of our study indicate that the hypercholesterolemia-induced activation of platelets and/or leukocytes plays a crucial role in endothelial dysfunction in arterioles as well as venules (26–28).

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