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PLATELET-ASSOCIATED NAD(P)H OXIDASE CONTRIBUTES TO THE THROMBOGENIC PHENOTYPE INDUCED BY HYPERCHOLESTEROLEMIA

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Abstract

Elevated cholesterol levels promote pro-inflammatory and prothrombogenic responses in venules and impaired endothelium-dependent arteriolar dilation. Although NAD(P)H oxidase-derived superoxide has been implicated in the altered vascular responses to hypercholesterolemia, it remains unclear whether this oxidative pathway mediates the associated arteriolar dysfunction and platelet adhesion in venules. Platelet and leukocyte adhesion in cremasteric postcapillary venules, and arteriolar dilation responses to acetylcholine were monitored in wild-type (WT), Cu,Zn-superoxide dismutase transgenic (SOD-TgN) and NAD(P)H oxidase-knockout (gp91^{phox}^{-/-}) mice placed on normal (ND) or high cholesterol (HC) diet for 2 wk. HC elicited increased platelet and leukocyte adhesion in WT mice, versus ND. Cytosolic subunits of NAD(P)H oxidase (p47^{phox} and p67^{phox}) were expressed in platelets. This was not altered by hypercholesterolemia, however platelets and leukocytes from HC mice exhibited elevated generation of reactive oxygen species when compared to ND mice. Hypercholesterolemia-induced leukocyte recruitment was attenuated in SOD-TgN-HC and gp91^{phox}^{-/-}-HC mice. Recruitment of platelets derived from WT-HC mice in venules of SOD-TgN-HC or gp91^{phox}^{-/-}-HC recipients was comparable to ND levels. Adhesion of SOD-TgN-HC platelets paralleled the leukocyte response and was attenuated in SOD-TgN-HC recipients, but not in WT-HC recipients. However, gp91^{phox}^{-/-}-HC platelets exhibited low levels of adhesion comparable to WT-ND in both hypercholesterolemic gp91^{phox}^{-/-} and WT recipients. Arteriolar dysfunction was evident in WT-HC mice, compared to WT-ND. Overexpression of SOD or, to a lesser extent, gp91^{phox} deficiency, restored arteriolar vasorelaxation responses towards WT-ND levels. These findings reveal a novel role for platelet-associated NAD(P)H oxidase in producing the thrombogenic phenotype in hypercholesterolemia and demonstrate that NAD(P)H oxidase-derived superoxide mediates the HC-induced arteriolar dysfunction.

Keywords

Platelets; NAD(P)H oxidase; leukocytes; arteriolar dysfunction; superoxide; microcirculation

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Introduction

Hypercholesterolemia is a major risk factor for large vessel disease and it rapidly elicits endothelial activation throughout the microvasculature [1]. One of the earliest manifestations of the endothelial dysfunction noted in hypercholesterolemic humans [2] and animals [3,4] is impaired endothelium-dependent vasodilation. Oxidative stress has been implicated in this impaired vascular response, through both an enhanced production of reactive oxygen species (ROS) and a reduced bioavailability of nitric oxide [5]. Superoxide is the major ROS implicated in the arterial dysfunction during hypercholesterolemia, and xanthine oxidase has been proposed to be a major source of this superoxide [6,7]. NAD(P)H oxidase, another superoxide-producing enzyme that is expressed by many cell types found both in the vessel wall and in blood, has also been implicated in the pathogenesis of hypercholesterolemia [8,9]. While NAD(P)H oxidase expression/activity is increased in arteries of human subjects with coronary artery disease, and the enhanced presence of the enzyme is associated with impaired arterial dilation [10], there is little direct evidence that supports a role for NAD(P)H oxidase-derived superoxide in the impaired endothelium-dependent vasodilation responses elicited by hypercholesterolemia.

NAD(P)H oxidase-derived superoxide also appears to contribute to the recruitment of both leukocytes and platelets that is elicited in postcapillary venules by hypercholesterolemia [11, 12]. The hypercholesterolemia-induced leukocyte adhesion involves superoxide generated from NAD(P)H oxidase that is expressed in the vessel wall as well as circulating blood cells. The hypercholesterolemia-induced platelet accumulation in venules is a P-selectin-dependent process [4,13] that appears to result from an interaction between P-selectin on platelets and PSGL-1 on adherent leukocytes, with direct interactions between platelets and the vascular endothelium accounting for only a small portion of platelet recruitment [4,14]. Although NAD(P)H oxidase has been implicated in the recruitment of platelets during hypercholesterolemia, the relative importance of leukocyte-, endothelial- and platelet-associated forms of NAD(P)H oxidase in this process remains unclear. NAD(P)H oxidase is present in platelets and the activation of platelets is known to be associated with both the activation of a gp91^{phox}-dependent enzyme and increased expression of P-selectin on the cell surface [15]. A potential role for platelet-associated NAD(P)H oxidase in hypercholesterolemia is suggested by reports describing an enhanced production of superoxide, released through a DPI-sensitive pathway, in humans manifesting this risk factor [16].

Based on the growing body of evidence that NAD(P)H oxidase is a key enzyme in the generation of ROS that mediate the endothelial dysfunction elicited by several risk factors for cardiovascular disease, we applied the technique of intravital videomicroscopy to hypercholesterolemic mice that were either deficient in NAD(P)H oxidase (gp91^{phox}^{-/-}) or overexpress Cu,Zn-superoxide dismutase (SOD-TgN) to: 1) determine whether platelet-associated NAD(P)H oxidase is a critical factor that accounts for the thrombogenic phenotype that is assumed by postcapillary venules during hypercholesterolemia, and 2) assess the role of NAD(P)H oxidase-derived superoxide in the arteriolar dysfunction that occurs in the presence of elevated blood cholesterol levels.

Materials & Methods

Animals

Male wild-type C57Bl/6J (WT), B6.129S6-*Cybbtm1Din/J* (gp91^{phox}^{-/-}) and breeder stocks for C57BL/6-Tg(SOD1)3Cje/J (SOD TgN) mice on a C57BL/6 background were obtained from Jackson Laboratories, Bar Harbor, Maine. The SOD-TgN mice were identified by qualitative demonstration of Cu,Zn-SOD using nondenaturing gel electrophoresis followed by nitroblue tetrazolium staining. Non-transgenic littermates were used as controls (SOD-nonTgN). At 6-8

wk of age the mice were placed on either a normal diet (ND) or high cholesterol diet (HC) (Teklad 90221 containing 1.25% cholesterol, 15.8% fat and 0.125% choline chloride, Harlan Teklad) for two weeks (n=5-6/group).

Surgical Protocol

Mice were anesthetized with ketamine hydrochloride (150 mg/kg body weight, i.p.) and xylazine (7.5 mg/kg body weight, i.p.). Core body temperature was maintained at $35\pm0.5^{\circ}\text{C}$. Animal handling procedures were approved by the LSU Health Sciences Center Institutional Animal Care and Use Committee and were in accordance with guidelines of the American Physiological Society.

Platelet Isolation—Approximately 0.9 ml of blood was collected via the carotid artery from a donor animal and anticoagulated with 0.1 ml ACD buffer. Platelets were isolated from whole blood by a series of centrifugation steps, labeled with the fluorochrome carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene OR) as described previously [17], and resuspended in PBS at a concentration of 8.33×10^5 cells/ μl . This technique does not cause platelet activation as determined by P-selectin expression using flow-cytometry [13]. Before infusion, it was confirmed with the aid of a hemocytometer that there was no leukocyte contamination of the platelet suspension.

Intravital Microscopy

The right jugular vein of platelet recipient mice was cannulated for infusion of fluorescently labeled platelets. The left carotid artery was cannulated for systemic arterial pressure measurement. The cremaster muscle was prepared for intravital microscopy as described previously [11]. An upright multi-purpose microscope system (Zeiss, Thornwood, NY) with a 40X water immersion objective lens (Achromplan 40X/0.75 W) was used to observe the cremasteric microcirculation. The cremaster was either transilluminated (with a 12 V-100 W direct current-stabilized light source) for visualization of adherent leukocytes or epi-illuminated (HBO 50W mercury lamp) for visualization of the fluorescently-labeled platelets [18]. Postcapillary venules (20-40 μm diameter) were studied. Venular diameter (D_v) and centerline red blood cell velocity (V_{rbc}) were measured online using a video caliper and an optical Doppler velocimeter respectively (Microcirculation Research Institute, Texas A&M University). Venular blood flow (VBF_{mean}) was calculated as follows: $\text{VBF}_{\text{mean}} = V_{\text{rbc}}/1.6$. Venular wall shear rate (WSR) was calculated based on the Newtonian definition $\text{WSR} = 8 (\text{VBF}_{\text{mean}}/D_v)$ as previously reported [18,19]. In order to minimize inflammation-independent cell recruitment, a threshold WSR of $\geq 500/\text{s}$ was selected based on previous reports describing a propensity for leukocytes and platelets to adhere in venules at low WSRs [18]. The microscopic images were recorded on DVD and leukocyte and platelet parameters were analyzed off-line.

Experimental Protocol—At the end of a 30 min stabilization period, if more than one postcapillary venule met the criteria outlined above, the venule with the lowest number of adherent leukocytes was studied in order to minimize bias towards inflammation in HC mice, and to avoid assessing inflammation due to surgical manipulation. If several venules met these criteria, the venule with the most sections available for recording was chosen. 100×10^6 (120 μl) platelets were infused via the jugular vein over 5 min and allowed to circulate for a further 5 min (Groups expressed as “platelet donor $\rightarrow$ recipient”). Five minute recordings of the leukocytes (light microscopy) followed by 1 min recordings of the platelets (fluorescent microscopy) were made of the first 100 μm of every 300 μm along the length of the unstimulated vessel, beginning as near to the source of the venule as possible. A leukocyte was considered adherent if it remained stationary for ≥ 30 s ($\#/\text{mm}^2$) and was measured throughout the 5 min observation period. Leukocyte emigration was measured online at the end of each observation

period. Emigrated leukocytes were expressed as the number of interstitial leukocytes per field of view surrounding the venular segment under observation (#/field). Platelets were considered adherent if they remained stationary for ≥ 2 s, and adhesion was expressed as #/mm². The mean value of each variable within a single venule was calculated and comparisons were made between groups.

Endothelium-Dependent Arteriolar Vasodilation Responses

Once the venule data were collected, the bicarbonate-buffered saline (BBS) superfusion was increased to 2 ml/min and the animals were allowed to stabilize for 20-30 min. Arterioles with diameters between 15-40 μ m and WSR of ≥ 500 /s were chosen for study as described previously [4]. After baseline measurements of diameter and V_{rbc} were taken in a single section from each arteriole of interest, the preparation was superfused for 5 min with 10^{-5} M of the endothelium-dependent vasodilator, acetylcholine (ACh). Diameter and V_{rbc} were measured in the vessel regions as described above. The preparation was then superfused with BBS and allowed to return to baseline values. Any preparation that contained arterioles that were unresponsive to ACh was then superfused with the endothelium-independent vasodilator, papaverine (10^{-3} M) to determine if the lack of response was a feature of the smooth muscle rather than the endothelium. Data from arterioles that also failed to respond to papaverine were excluded from the study. Arteriolar vasorelaxation responses to ACh were expressed as the percentage diameter change versus baseline.

Serum Cholesterol Levels

Serum was frozen for subsequent measurement of cholesterol levels using a spectrophotometric assay (Sigma Chemicals Co., St. Louis, MO).

Platelet and Leukocyte ROS Generation

Platelets were isolated as above, resuspended in PBS with 5 mM glucose, 1 mM EDTA, and allowed to rest at 37°C for 30 min before labeling as previously described [20]. Red blood cells in separate whole blood samples were lysed, and leukocytes were resuspended in PBS-glucose-EDTA. The platelet and leukocyte samples were then incubated with 50 μ M dichlorohydrofluorescein diacetate (Invitrogen Corp., Carlsbad, CA) for 30 min at 37°C, washed with HEPES, and left unstimulated or were stimulated with thrombin or 1 mM PMA respectively, before dilution in HEPES and analysis using a FACSCALibur flow cytometer using CellQuest software (BD Biosciences, San Jose, CA).

Expression of NAD(P)H oxidase subunits in Platelets

Platelets were isolated from WT-ND and WT-HC mice as described above. The platelet pellet was resuspended in modified Tyrodes buffer, lysed and the debris removed by centrifugation as previously described [21]. The supernatant was frozen for subsequent measurement of p47^{phox} and p67^{phox} using western immunoblotting. Proteins from the platelet lysates (10×10^6 platelets per lane) were separated on a 10% SDS-polyacrylamide gel, and transferred onto a nitrocellulose membrane which was probed for p47^{phox} or p67^{phox} using goat anti-human polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The secondary antibody was a HRP-conjugated donkey anti-goat IgG antibody. NAD(P)H oxidase subunits were detected using ECL Western blotting detection reagents (Amersham, GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Leukocytes were used as a positive control.

Statistical Analysis

All values are reported as mean $\pm$ SEM. ANOVA with Bonferroni post-hoc test or t-tests were used for statistical comparison of experimental groups where appropriate, with statistical significance set at $P < 0.05$.

Results

Blood Cholesterol Concentration and Wall Shear Rate

Placement of WT and mutant mice on a cholesterol-enriched diet for two weeks led to a 2-3-fold increase in blood cholesterol concentration, with no statistical difference between the HC groups (Table 1). Wall shear rate was comparable between all groups examined, suggesting this was not a factor in producing the differences in blood cell recruitment. Furthermore, no differences in circulating leukocyte or platelet counts were noted between any of the experimental groups.

Leukocyte Recruitment in Postcapillary Venules

Hypercholesterolemia resulted in significant leukocyte adhesion (Figure 1) and emigration (Figure 2) in postcapillary venules when compared with normocholesterolemic controls. The SOD-nonTgN-HC controls behaved similar to WT-HC mice, with comparable levels of leukocyte recruitment in postcapillary venules. In contrast, overexpression of the superoxide scavenger SOD conferred protection against hypercholesterolemia-induced venular inflammation. Furthermore, mice deficient in the NAD(P)H oxidase subunit, gp91^{phox}, demonstrated an abrogation of hypercholesterolemia-induced leukocyte adhesion and emigration towards control levels observed in the WT-ND group (Figures 1 and 2), suggesting that NAD(P)H oxidase-derived superoxide contributes to the inflammatory phenotype in postcapillary venules.

Platelet Adhesion Responses to Hypercholesterolemia

We assessed platelet recruitment in mice receiving platelets from either matched or non-matched donors (platelet donor→recipient). In all cases where matched platelets were administered, platelet recruitment mirrored the leukocyte adhesion responses of the recipient animal. WT-HC mice exhibited a significant elevation in the adhesive interactions of matched platelets in postcapillary venules, when compared to their ND counterparts (Figure 3). The hypercholesterolemic SOD-nonTgN-HC mice also exhibited high levels of adhesion of platelets from matched donors. Platelets isolated from SOD-TgN-HC mice were recruited to a similar degree as platelets from their non-transgenic littermates when administered to SOD-nonTgN-HC recipients, reflecting endogenous leukocyte recruitment. Mice overexpressing Cu,Zn-SOD that received platelets from matched donors demonstrated a significant attenuation of platelet recruitment when compared with their non-transgenic counterparts. Platelets from the SOD-nonTgN-HC donors also failed to adhere in SOD-TgN-HC recipients.

In hypercholesterolemic gp91^{phox}^{-/-} mice receiving matched NAD(P)H oxidase-deficient platelets, the recruitment of platelets in postcapillary venules was comparable to levels observed in WT-ND mice, and significantly lower than WT-HC mice receiving WT-HC platelets (Figure 4). WT-HC platelets infused into gp91^{phox}^{-/-}-HC recipients also failed to adhere, rather the WT platelets behaved similar to knockout platelets, and followed the leukocyte adhesion responses. Interestingly, NAD(P)H oxidase-deficient platelets were not recruited in postcapillary venules of WT-HC recipients, despite high levels of leukocyte adhesion in these mice, suggesting that platelet-associated gp91^{phox} contributes to platelet recruitment.

Arteriolar Vasodilation Responses to Hypercholesterolemia

The vasodilation responses to acetylcholine were significantly impaired in hypercholesterolemic WT mice, when compared to WT mice fed a normal diet (Figure 5). The SOD-nonTgN-HC mice exhibited a low level of endothelium-dependent vasodilation that was comparable to the WT-HC group. However the overexpression of SOD (SOD-TgN-HC group)

led to an abrogation of the impaired arteriolar response to acetylcholine. Hypercholesterolemic mice deficient in NAD(P)H oxidase also showed an improvement of the vasodilatory response to acetylcholine, although this protection was slightly (but not significantly) less than that observed in the SOD-TgN-HC group.

Blood cell ROS generation and Platelet NAD(P)H oxidase subunit expression

Leukocyte ROS production was significantly elevated in WT-HC mice when compared to their WT-ND counterparts under basal conditions (Figure 6A). WT-HC exhibited increased levels of ROS when stimulated with PMA, whereas levels generated from the stimulated WT-ND leukocytes were only slightly elevated. Platelets from WT-HC also produced significantly higher baseline levels of ROS when compared with platelets from WT-ND controls (Figure 6B). Thrombin stimulation led to a small non-significant increase in the levels of ROS from WT-ND and, to a lesser extent, WT-HC platelets. Platelets from both WT-ND and WT-HC mice expressed p47^{phox} and p67^{phox}, two subunits of NAD(P)H oxidase, although there was no difference in expression between the two groups (Figure 6C). Although we tried to measure gp91^{phox} expression in these cells, we were unable to detect gp91^{phox} in either the platelets or the positive control (leukocytes) using commercially available antibodies (as has been previously found by Krotz et al. [21]).

Discussion

Hypercholesterolemia elicits an inflammatory response in the microvasculature of many organs, including skeletal muscle, intestine, brain, and mesentery [3,4,11,12,14]. This inflammatory response is characterized by endothelial dysfunction/activation, and manifests as impaired endothelium-dependent vasodilation in arterioles [3,4], and leukocyte and platelet recruitment in postcapillary venules [4,14,22]. In hypercholesterolemic humans and animals, oxidative stress has been implicated as one of the major underlying mechanisms that initiate these inflammatory and thrombogenic responses [5]. NAD(P)H oxidase-derived superoxide is generated at elevated levels in diseased large vessels in mice [23], and activation of this enzyme in diseased human arteries is associated with endothelial cell dysfunction [8,9]. Here we provide the first direct evidence that NAD(P)H oxidase mediates the arteriolar dysfunction that results from a moderate rise in blood cholesterol concentration. Previously we demonstrated that NAD(P)H oxidase-derived superoxide generated from blood cells and the vessel wall mediates leukocyte recruitment in postcapillary venules of hypercholesterolemic mice [11]. The present study extends these observations to demonstrate that platelet-associated NAD(P)H oxidase is a key factor that contributes to the prothrombogenic response that accompanies hypercholesterolemia.

Much of the focus on the role of NAD(P)H oxidase in the vascular complications associated with cardiovascular disease has centered on the importance of this enzyme in hypertension and diabetes [24-26]. Less is known about its role in creating the inflammatory and thrombogenic environment within the microvasculature during hypercholesterolemia. Previous findings in our laboratory have implicated superoxide, in particular superoxide derived from NAD(P)H oxidase, in the leukocyte recruitment observed in the cremaster muscle [11] and brain [12] of mice on a cholesterol-enriched diet, and this was confirmed here using Cu,Zn-SOD-overexpressing and gp91^{phox}-deficient mice. Since the thrombogenic phenotype induced by hypercholesterolemia appears to be dependent on leukocyte recruitment [4,14], it might be expected that platelet adhesion in our model of the cremaster microcirculation would also be mediated by NAD(P)H oxidase-derived superoxide. Indeed, using SOD-overexpressing mice, we demonstrated a role for superoxide in the platelet accumulation in postcapillary venules, and as previously reported for the brain microcirculation [12], a gp91^{phox}-containing NAD(P)H oxidase was shown to contribute to this response.

In previous experiments employing bone marrow chimeras we elucidated that NAD(P)H oxidases in unidentified circulating blood cells and in the vessel wall were important components of the leukocyte recruitment response in postcapillary venules during hypercholesterolemia [11]. While platelets were not considered as a possible source of the NAD(P)H oxidase that contributes to the inflammatory response, the recent surge of interest in platelet NAD(P)H oxidase and mounting evidence that platelets may initiate and/or perpetuate the hypercholesterolemia-induced inflammatory response in both the microvasculature [4] and in large vessels [27] suggests that platelet associated NAD(P)H warrants further attention. Further evidence supporting a potential role for platelet-associated NAD(P)H oxidase in hypercholesterolemia is provided by the observation that platelets isolated from hypercholesterolemic humans exhibit increased superoxide production via a DPI-sensitive pathway [16]. In fact we demonstrated that platelets from hypercholesterolemic mice generated higher levels of ROS under basal conditions when compared with normocholesterolemic counterparts.

Although the NAD(P)H oxidase enzyme has not been well characterized in platelets to date, several subunits of this enzyme (p22^{phox}, p47^{phox}, p67^{phox} and gp91^{phox}) have been identified in platelets [15,21,28]. It has also been shown that platelet activation leads to gp91^{phox}-dependent superoxide generation and P-selectin upregulation [15]. Previous findings in our laboratory have revealed a role for platelet-associated P-selectin not only in platelet recruitment but also in leukocyte adhesion in postcapillary venules of hypercholesterolemic mice [4]. However, it remains unclear whether platelet NAD(P)H oxidase-derived superoxide per se can lead to P-selectin upregulation thereby accounting for the P-selectin-dependent adhesion of platelets in venules during hypercholesterolemia. By monitoring the adhesion of platelets derived from gp91^{phox}-deficient mice in venules of wild-type hypercholesterolemic recipients, we obtained the first evidence that the thrombogenic events elicited in the microvasculature by hypercholesterolemia are mediated by a gp91^{phox}-containing enzyme in platelets. We also identified p47^{phox} and p67^{phox}, two subunits of NAD(P)H oxidase, in platelets isolated from WT mice, in agreement with others [21]. These subunits were not upregulated by hypercholesterolemia, although this is not necessarily surprising in a cell type that does not contain a nucleus. Rather it is plausible that these subunits are in the cytosol under normal conditions, and translocate to the membrane to form the active NAD(P)H oxidase enzyme during hypercholesterolemia. It is noteworthy that the platelet recruitment induced by hypercholesterolemia involves both leukocyte- and endothelium-dependent mechanisms [14], and that leukocyte recruitment is significantly blunted in hypercholesterolemic mice that are genetically deficient in platelet P-selectin [4]. This raises the possibility that platelet NAD(P)H oxidase may also contribute to the recruitment of leukocytes in venules of hypercholesterolemic mice. Unfortunately the ubiquitous expression of NAD(P)H oxidase in many blood cell populations makes it difficult to selectively deplete platelet, but not leukocyte, NAD(P)H oxidase in order to definitively address this possibility in vivo.

Our findings in hypercholesterolemic Cu,Zn-SOD transgenic mice indicate that while SOD overexpression exerts an anti-adhesion effect similar to that noted in gp91^{phox}-deficient mice (or gp91^{phox}-deficient platelets), overexpression of the superoxide scavenging enzyme must occur in cells other than the platelets in order to exert this protective action, i.e., donor platelets derived from SOD-TgN behaved similar to their wild-type counterparts while SOD-TgN recipients showed a marked attenuation of platelet adhesion irrespective of the donor source. This may suggest that the principal target cell (e.g., leukocyte and/or endothelial cell) of the platelet-derived superoxide must exhibit elevated SOD activity in order to protect itself against this pro-adhesive stimulus. Alternatively, the localization of Cu,Zn-SOD and NAD(P)H oxidase in different compartments (e.g, cytosol vs. cell membrane) may render the overexpressed Cu,ZnSOD ineffective in scavenging the superoxide generated by NAD(P)H oxidase.

It is well documented that enhanced superoxide generation is an important component of the impaired vascular reactivity to endothelium-dependent vasodilators and vasoconstrictors that accompanies different risk factors for cardiovascular disease [5]. We have previously shown that endothelium-dependent arteriolar dysfunction develops within two weeks of cholesterol feeding [4]. Here we reveal that superoxide contributes to this impairment of vasodilation. Xanthine oxidase has been widely implicated in the generation of this vascular phenotype in hypercholesterolemic humans [6,7]. However, there is mounting evidence that implicates NAD(P)H oxidase as a common underlying mechanism of the endothelial dysfunction that accompanies several pathophysiological conditions such as renovascular hypertension [29], angiotensin-induced hypertension [30,31], hyperhomocysteinemia [32] as well as aging [33], with relatively little direct evidence demonstrating the involvement of NAD(P)H oxidase in the altered vascular responses during hypercholesterolemia. Schneider et al. [34] have inferred that NAD(P)H oxidase does not participate in the impaired endothelium-dependent vasodilation observed in hypercholesterolemic humans because expression of a variant of the gene encoding p22^{phox}, a subunit of NAD(P)H oxidase that has been implicated in coronary artery disease, was not correlated with altered forearm blood flow responses to acetylcholine. Others have reported an association between the impaired vasorelaxation responses to acetylcholine and the elevated levels of NADH-dependent superoxide release in veins [8] and aortas [9] during hypercholesterolemia, but a direct link between the two responses was not demonstrated. We assessed arteriolar function in gp91^{phox}-deficient mice maintained on a cholesterol-enriched diet in order to specifically determine whether NAD(P)H oxidase contributes to this response. Our findings reveal that NAD(P)H oxidase was at least partially responsible for the impaired endothelium-dependent vasodilation resulting from acute hypercholesterolemia. In view of our previous observation that platelet-associated P-selectin mediates the arteriolar dysfunction during hypercholesterolemia [4], we can speculate that platelet-associated NAD(P)H oxidase may contribute to this arteriolar dysfunction by mediating the upregulation of P-selectin on hypercholesterolemic platelets. Inasmuch as overexpression of Cu,Zn-SOD was more effective than genetic ablation of gp91^{phox} (also known as Nox2) in attenuating the vasodilatory response, it is likely that another source of superoxide e.g. xanthine oxidase, participates in this dysfunction, and/or that an NAD(P)H oxidase enzyme that possesses a different form of Nox, e.g. Nox1 [35], is also involved. The protective effects of abrogating the formation of superoxide from NAD(P)H oxidase, or blocking the actions of superoxide (by promoting its conversion to hydrogen peroxide by Cu,Zn-SOD), on the arteriolar function may be due to an enhanced bioavailability of nitric oxide that results from lower levels of superoxide, which normally reacts with nitric oxide. This mechanism may also contribute to the reduction in the leukocyte and platelet recruitment observed in postcapillary venules of gp91^{phox}-deficient and Cu,Zn-SOD-TgN mice, since nitric oxide is known to possess anti-inflammatory and anti-thrombogenic properties, and nitric oxide bioavailability is decreased during hypercholesterolemia [5].

Our study supports indirect findings in large vessels that NAD(P)H oxidase-derived superoxide mediates impaired endothelium-dependent vasodilation during hypercholesterolemia, and provides the first evidence for a role for platelet NAD(P)H oxidase in the thrombogenic phenotype induced by elevated cholesterol levels. The exact mechanism through which NAD(P)H oxidase is activated in the microvasculature during hypercholesterolemia remains unclear. Based on a study in the aorta of hyperlipidemic rabbits where AT1-R blockade abrogated vascular superoxide generation from NADH oxidase [9], and previous findings in our model that AT1-R activation mediates the leukocyte adhesion and oxidative stress in postcapillary venules [36] it is conceivable that the vascular and perhaps leukocyte NAD(P)H oxidase is activated through an AT1-R receptor-dependent pathway. Although platelet superoxide release can be stimulated by angiotensin II acting through AT1-R activation [37], platelet AT1-R does not appear to participate in the thrombogenic responses in our model [38]. Consequently, the activation of platelet NAD(P)H oxidase may involve an alternative

pathway such as CD40L [39], which has also been implicated in hypercholesterolemia-induced inflammation [40,41]. Taken together with previous findings, our results suggest that NAD(P)H oxidase in both the vasculature and in circulating platelets is a key enzyme that leads to the induction of an inflammatory and prothrombogenic phenotype in the microvasculature during hypercholesterolemia.

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Figure 1.

Leukocyte adhesion in postcapillary venules of wild-type (WT), superoxide non-transgenic (SOD-nonTgN), SOD transgenic (SOD-TgN), and gp91^{phox}-knockout (gp91^{phox}-/-) mice maintained on a normal (ND) or high cholesterol diet (HC) for two weeks. *P<0.0001 versus WT-ND; #P<0.005 versus WT-HC; ^P<0.005 versus SOD-nonTgN-HC.

Figure 2.

The role of superoxide (using SOD-overexpressing mice (SOD-TgN), and corresponding non-transgenic controls (SOD-nonTgN)) and NAD(P)H oxidase (using gp91^{phox}-deficient (gp91^{phox}^{-/-}) mice) in hypercholesterolemia-induced leukocyte emigration into the tissue surrounding postcapillary venules. WT: wild-type mice; ND: normal diet; HC: high cholesterol diet. *P<0.0001 versus WT-ND; #P<0.005 versus WT-HC; ^P<0.005 versus SOD-nonTgN-HC.

Figure 3.

Contribution of superoxide to hypercholesterolemia-induced platelet recruitment in postcapillary venules. Platelets were isolated from wild-type (WT), superoxide non-transgenic (SOD-nonTgN), and SOD overexpressing (SOD-TgN) donors for observation in matching, or non-matching recipients maintained on a normal (ND) or cholesterol-enriched (HC) diet for two weeks (Platelet donor→Recipient). * $P < 0.0001$ versus WT-ND; # $P < 0.0001$ versus WT-HC; ^ $P < 0.0001$ versus SOD-nonTgN-HC→SOD-nonTgN-HC and SOD-TgN-HC→SOD-nonTgN-HC.

Figure 4.

Platelet recruitment in postcapillary venules of wild-type (WT), and gp91^{phox}-knockout (gp91^{phox-/-}) mice placed on a normal (ND) or high cholesterol (HC) diet for two weeks. Platelets were isolated from donors for observation in matching, or non-matching recipients (diet matched in all cases) (Platelet donor→Recipient). *P<0.0001 versus WT-ND; #P<0.0001 versus WT-HC.

Figure 5.

The involvement of NAD(P)H oxidase-derived superoxide in hypercholesterolemia (HC)-induced impairment of endothelium-dependent vasodilation in arterioles. In order to determine the role of superoxide, SOD non-transgenic (SOD-nonTgN), and SOD overexpressing (SOD-TgN) mice were used. The contribution of NAD(P)H oxidase was addressed by examining arteriolar responses in gp91^{phox}-deficient mice (gp91^{phox-/-}). Diameters of arterioles were measured at baseline and following superfusion of 10⁻⁵ M ACh; vasodilation in response to ACh is expressed as % diameter change versus baseline. ND: normal diet. *P<0.0005 versus WT-ND; #P<0.005 versus WT-HC.

Figure 6.

ROS generation from leukocytes (Panel A) and platelets (Panel B) from wild-type (WT) mice maintained on a normal diet (ND) or high cholesterol diet (HC) for two weeks. Flow cytometry was used to detect the oxidation of dichlorohydrofluorescein diacetate to its fluorescent form in cells under baseline or stimulated conditions (PMA for leukocytes, thrombin for platelets). Panel C: Western immunoblot showing expression of p47^{phox} and p67^{phox} in platelets isolated from WT-ND and WT-HC mice. * $P < 0.05$ versus corresponding WT-ND; # $P < 0.05$ versus unstimulated WT-HC.

Table 1

Serum cholesterol concentration, venular wall shear rate (WSR) and circulating leukocyte and platelet counts in wild-type (WT), and mutant mice maintained on a normal diet (ND) or high cholesterol (HC) diet for 2 wk

	Cholesterol (mg/dL)	WSR (s⁻¹)	Leukocytes (#/μL)	Platelets (# × 10⁶/μL)
WT ND	93±6.9	676±55.9	7019±736.7	1.0±0.11
WT HC	198±18.7	577±14.8	7594±1110.7	1.0±0.09
SOD-nonTgN-HC	175±37.4 *	558±20.0	11070±1856.5	1.4±0.12
SOD-TgN-HC	214±14.1	688±50.0	6100±566.4	1.2±0.11
gp91 ^{phox-/-} -HC	193±10.4 *	587±24.5	8775±1549.1	1.1±0.10

* P<0.001 vs. WT-ND group