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## Roles of platelet and endothelial cell COX-1 in hypercholesterolemia-induced microvascular dysfunction

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**Taylor A, Wood KC, Wallace JL, Specian RD, Granger DN.** Roles of platelet and endothelial cell COX-1 in hypercholesterolemia-induced microvascular dysfunction. *Am J Physiol Heart Circ Physiol* 293: H3636–H3642, 2007. First published October 12, 2007; doi:10.1152/ajpheart.01105.2006.—Aspirin is a common preventative therapy in patients at risk for cardiovascular diseases, yet little is known about how aspirin protects the vasculature in hypercholesterolemia. The present study determines whether aspirin, nitric oxide-releasing aspirin (NCX-4016), a selective cyclooxygenase (COX)-1 inhibitor (SC560), or genetic deficiency of COX-1 prevents the inflammatory and prothrombotic phenotype assumed by hypercholesterolemic (HC) venules. Aspirin or NCX-4016 (60 mg/kg) was administered orally for the last week of a 2-wk HC diet. COX-1-deficient (COX-1<sup>-/-</sup>) and wild-type (WT) mice were transplanted with WT (WT/COX-1<sup>-/-</sup>) or COX-1<sup>-/-</sup> (COX-1<sup>-/-</sup>/WT) bone marrow, respectively. HC-induced adhesion of platelets and leukocytes in murine intestinal venules, observed with intravital fluorescence microscopy, was greatly attenuated in aspirin-treated mice. Adhesion of aspirin-treated platelets in HC venules was comparable to untreated platelets, whereas adhesion of SC560-treated platelets was significantly attenuated. HC-induced leukocyte and platelet adhesion in COX-1<sup>-/-</sup>/WT chimeras was comparable to that in SC560-treated mice, whereas the largest reductions in blood cell adhesion were in WT/COX-1<sup>-/-</sup> chimeras. NCX-4016 treatment of platelet recipients or donors attenuated leukocyte and platelet adhesion independent of platelet COX-1 inhibition. Platelet- and endothelial cell-associated COX-1 promote microvascular inflammation and thrombogenesis during hypercholesterolemia, yet nitric oxide-releasing aspirin directly inhibits platelets independent of COX-1.

aspirin; NCX-4016; platelets; leukocytes; cyclooxygenase

ASPIRIN THERAPY IS WIDELY used in patients at risk of developing atherosclerosis and thrombosis. The therapeutic efficacy of aspirin in cardiovascular diseases (CVD) is attributed to its platelet-inhibitory function, which results from irreversible inhibition of cyclooxygenase (COX) activity and thromboxane (Tx) generation (2, 33). Although platelets are a rich source of the constitutive isoform of COX (COX-1), endothelial cells also express COX-1 in addition to the inducible isoform, COX-2. This likely accounts for evidence implicating endothelial cell COX-1 inhibition in the beneficial effects of aspirin (but not COX-2 inhibitors) on the inflammation associated with atherosclerotic lesions in apolipoprotein E-deficient and low-density lipoprotein receptor-deficient mice (8, 25). While the anti-inflammatory action of aspirin in this setting may also result from platelet inhibition (8), nonsteroidal anti-inflammatory drugs (NSAIDs) and their metabolites (i.e., salicylate) can

directly interfere with adhesion of inflammatory cells to vascular endothelium, both in vitro (5, 11) and in vivo (1, 6). Hypercholesterolemia, diabetes, hypertension, and other risk factors for CVD promote inflammatory and thrombotic responses in lesion-prone arteries. Similar responses are detected in microvasculature before the development of atherosclerotic lesions and may render tissues even more vulnerable to ischemic tissue injury (30). Nonetheless, it remains unclear whether COX-1 inhibition by aspirin or other agents can attenuate these deleterious adhesion-dependent microvascular responses to CVD risk factors, and which cell type (platelets, endothelial cell, or leukocytes) is the primary target of aspirin.

A major limitation to aspirin therapy is gastrointestinal bleeding from reduced production of COX-1-derived cytoprotective prostaglandins. A new series of nitric oxide (NO)-releasing NSAIDs have been developed to reduce aspirin-mediated gastrointestinal damage while maintaining aspirin's desired anti-inflammatory and anti-thrombotic properties. NO-releasing aspirin 2-(acetyloxy)benzoic acid 3-(nitrooxymethyl) phenyl ester (NCX-4016) inhibits thrombus formation in vivo through a mechanism that appears to be largely dependent on NO release and partially related to a weak inhibitory effect on COX-1-dependent TxA<sub>2</sub> synthesis (34–36). This NO-releasing NSAID also markedly suppresses formyl-methionine-leucine-phenylalanine-induced leukocyte adhesion in rat mesenteric venules (37), an effect that likely results from NO inhibition of leukocyte-endothelial cell adhesion (16). However, whether NO-releasing aspirin provides additional benefit to plain aspirin in modulating the microvascular responses to CVD risk factors, such as hypercholesterolemia, has not been assessed to date.

The overall objective of this study was to determine whether chronic aspirin therapy protects against hypercholesterolemia-induced microvascular dysfunction or injury. Specific questions addressed were whether 1) treatment with aspirin or the selective COX-1 inhibitor 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole (SC560) prevents hypercholesterolemia-associated inflammatory and prothrombotic venular responses; 2) platelets, leukocytes, and/or vascular endothelial cells are primarily responsible for the COX-1-dependent responses to hypercholesterolemia; and 3) if NO-releasing aspirin provides additional benefit to plain aspirin in modulating the microvascular responses to hypercholesterolemia.

### METHODS

**Animals.** Male wild-type (WT) C57BL/6J, CD45 congenic mice (B6.SJL-Ptprap3b/BoyJ) (which express CD45.1) were obtained

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from the Jackson Laboratory (Bar Harbor, ME), while COX-1-deficient (COX-1<sup>-/-</sup>) mice on a C57BL/6 background (B6;129P2-*Ptgs1<sup>tm1Unc</sup>*) (6–8 wk of age) were obtained from Taconic (Germantown, NY). The mice were housed in a climate-controlled room, exposed to 12:12-h light-dark cycles, with free access to normal rodent chow and drinking water. All experimental procedures were reviewed and approved by the Louisiana State University Health Sciences Center-Shreveport Institutional Animal Care and Use Committee and performed according to the criteria outlined in the National Institutes of Health guidelines.

**Treatments.** WT and COX-1<sup>-/-</sup> mice ( $n = 5-9$ ) were placed on either normal diet (ND) or cholesterol-enriched diet [hypercholesterolemic (HC)] (Teklad 90221) containing 1.25% cholesterol, 15.8% fat, and 0.125% choline chloride for 2 wk. For the last 7 days of the 2-wk HC diet, acetylsalicylic acid (aspirin, 60 mg/kg, Sigma, St. Louis, MO) was administered in drinking water. In other experiments, an equimolar dose of NO-releasing aspirin (NCX-4016; a generous gift from Dr. J. L. Wallace, Calgary, Ontario) dissolved in 1% carboxymethyl cellulose (CMC) (Sigma Chemicals) or the vehicle alone was administered. In separate experiments, some mice were placed on the HC diet and received 2 wk of daily oral treatment (either alone or in combination with aspirin, as described above) with the selective COX-1 inhibitor, SC560 (15 mg/kg, Sigma Chemical).

**Measurement of COX activity.** To determine the oral dose of aspirin required to inhibit platelet COX activity in vivo, generation of the stable product of platelet-derived TxB<sub>2</sub> was measured in platelets harvested from mice orally treated with different doses of aspirin (5, 10, 15, 20, 40, and 60 mg/kg) for 7 days. Blood was collected, and washed platelets were prepared as described below. Platelets ( $100 \times 10^6$ ) were stimulated with calcium A23187 ionophore (1  $\mu$ M, Sigma) for 10 min at 37°C. Cells were centrifuged (10,000 rpm) for 10 min, and supernatants were collected and frozen (-80°C) for TxB<sub>2</sub> determination using a commercial enzyme immunoassay kit (Cayman, Arbor, MI).

**Platelet isolation.** Platelets were isolated and labeled with the fluorochrome carboxyfluorescein diacetate succinimidyl ester (CFSE, 90  $\mu$ M; Molecular Probes, Eugene, OR), as previously described (32). The platelet isolation procedure yielded negligible contamination with other cellular components and has no significant effect on activation state or viability (28, 32). Platelets ( $100 \times 10^6$ ) were infused into recipient mice via the jugular vein over 5 min, yielding ~5% of the total murine platelet count. Platelets were allowed to circulate for 5 min before the observation and recording period.

**Surgical procedure and intravital fluorescence microscopy.** Mice were anesthetized with ketamine hydrochloride (150 mg/kg ip) and xylazine (7.5 mg/kg ip). The right carotid artery was cannulated for blood pressure measurement, while the right jugular vein was cannulated for infusion of CFSE-labeled platelets and/or rhodamine 6G (0.02%, Sigma), which was used for in vivo labeling of leukocytes. A midline laparotomy was performed, the animal was placed in a supine position, and a loop of the small bowel was exteriorized and superfused with warm bicarbonate buffer solution (pH 7.4). The preparation was allowed to stabilize for 10 min before infusion with CFSE-labeled platelets and/or rhodamine 6G.

**Production of bone marrow chimeras.** Bone marrow (BM) transfer (groups indicated by donor/recipient) was employed to create COX-1<sup>-/-</sup> chimeric mice, wherein the genetic deficiency of COX-1 is confined to either circulating blood cells (COX-1<sup>-/-</sup>/WT chimeras) or non-blood tissue (WT/COX-1<sup>-/-</sup> chimeras), with WT/WT marrow transfer used to produce control chimeras. Briefly, BM was isolated from the femurs and tibias of donor (e.g., congenic WT mice expressing CD45.1 or COX-1<sup>-/-</sup> mice expressing CD45.2 leukocyte antigens) mice and administered ( $8 \times 10^6$  cells in 200  $\mu$ l of PBS) via the femoral vein into recipient (COX-1<sup>-/-</sup> or CD45 congenic WT) mice previously irradiated with two doses of 500–525 rad, 3 h apart. Chimeras were kept in autoclaved cages, with 0.2% neomycin drinking water for 2 wk, followed by normal drinking water. Flow cytometry

was used to verify chimera reconstitution (at 6–8 wk) by staining for leukocyte CD45.1 (CD45 congenic mice) vs. CD45.2 (expressed by C57Bl/6 mice or knockout mice on a C57Bl/6 background) expression using a FITC-conjugated anti-CD45.1 antibody and a biotinylated anti-CD45.2 antibody with streptavidin-peridinin chlorophyll protein as secondary antibody (BD Pharmingen, San Diego, CA). This procedure yields  $\geq 90\%$  penetrance of the transferred BM at  $\geq 6$  wk after transplant.

**Image analysis.** Leukocyte and platelet interactions with the venular wall were examined in three to five postcapillary venules (25–40  $\mu$ m) per animal with a  $\times 40$  objective. Firmly adherent platelets and leukocytes remained stationary  $\geq 30$  s along 200- $\mu$ m vessel length. Adhesion data are expressed as the number of cells/mm<sup>2</sup> venular surface, assuming cylindrical vessel shape (19). Estimates of venular wall shear rates were calculated using Poiseuille's law for a Newtonian fluid: pseudoshear rate =  $(V_{pit}/D) \times 8$ , where  $V_{pit}$  is velocity of flowing platelets, and  $D$  is venular diameter (3). Differential circulating blood and platelet counts were performed on all animals.

**Serum cholesterol levels.** Frozen (-80°C) serum samples from each mouse were spectrophotometrically assayed (Sigma) for total cholesterol levels.

**Histological examination.** Each mouse stomach was flushed of food and fixed in 4% paraformaldehyde-2.5% glutaraldehyde in 0.1 M phosphate buffer overnight at 4°C. Samples were washed in phosphate buffer, dehydrated with 95% ethanol, and embedded in JB-4 (Polysciences, Warrington, PA). Sections (1–2  $\mu$ m) were cut on glass knives and stained with hematoxylin and eosin. Micrographs were recorded on a Nikon Diaphot microscope with a SenSys digital camera (Photometrics, Huntington, CA). All micrographs were processed identically in Adobe Photoshop.

**Statistical analysis.** Data were analyzed by standard statistical analysis, i.e., one-way ANOVA with Scheffé's (post hoc test). All values are reported as means  $\pm$  SE, from 5–9 mice per group, and statistical significance was set at  $P < 0.05$ .

## RESULTS

All HC groups exhibited increased total serum cholesterol concentration compared with their ND counterparts. Aspirin, NCX-4016, or SC560 treatment did not alter serum cholesterol levels (Table 1). Blood leukocyte and platelet counts were similar between groups (Table 1).

**Aspirin inhibits HC-induced platelet and leukocyte adhesion.** Our first objective was to determine whether aspirin altered the proinflammatory and prothrombotic phenotype that is assumed by HC venules. The dose of aspirin (60 mg·kg<sup>-1</sup>·day<sup>-1</sup>  $\times$  7 days) used to study aspirin effects on platelet and leukocyte adhesion produced complete inhibition of platelet COX-1 activity, as reflected by an absence of TxB<sub>2</sub> generation by aspirin-treated platelets (Fig. 1A, *inset*). HC mice receiving HC platelets (but not treated with aspirin) had significantly increased numbers of adherent platelets ( $358.2 \pm 51.7/\text{mm}^2$ ), platelet-free ( $268.5 \pm 17.9/\text{mm}^2$ ) adherent leukocytes, and platelet-bearing ( $215.5 \pm 25.3/\text{mm}^2$ ) adherent leukocytes (Fig. 1), compared with mice placed on a ND ( $11.7 \pm 6.2$ ,  $4.1 \pm 4.1$ , and  $4.5 \pm 4.5/\text{mm}^2$ , respectively). Aspirin treatment of either ND platelet donors or recipient mice did not alter platelet or leukocyte adhesion. Untreated HC recipients of aspirin-treated HC platelets exhibited leukocyte and platelet adhesion responses similar to untreated HC recipients of untreated HC platelets. However, aspirin-treated HC recipients of untreated HC platelets exhibited significantly reduced (76%) platelet adhesion, as well as reduced adhesion of platelet-free (50%) and platelet-bearing (66%) leukocytes, compared with untreated HC recipients of untreated HC platelets.

Table 1. Total serum cholesterol level, blood pressure, and leukocyte and platelet counts in recipient mice

Group	Total Serum Cholesterol, mg/dl	Blood Pressure, mmHg	Leukocytes, $\times 10^3/\mu\text{l}$	Platelets, $\times 10^6/\mu\text{l}$
<i>Platelet donor recipient</i>				
ND $\rightarrow$ ND	97.8 $\pm$ 2.9	51.2 $\pm$ 3.3	1.5 $\pm$ 0.5	1.0 $\pm$ 0.04
ND + ASA $\rightarrow$ ND	95.9 $\pm$ 6.8	56.9 $\pm$ 2.2	3.7 $\pm$ 1.1	1.4 $\pm$ 0.20
ND $\rightarrow$ ND + ASA	131.8 $\pm$ 19.5	60.5 $\pm$ 3.7	4.9 $\pm$ 1.8	1.3 $\pm$ 0.10
HC $\rightarrow$ HC	230.9 $\pm$ 16.0*	48.6 $\pm$ 3.1	3.3 $\pm$ 0.3	0.9 $\pm$ 0.07
HC + ASA $\rightarrow$ HC	202.5 $\pm$ 15.6*	51.7 $\pm$ 4.6	1.7 $\pm$ 0.3	1.1 $\pm$ 0.04
HC $\rightarrow$ HC + ASA	206.0 $\pm$ 18.7*	50.6 $\pm$ 3.4	2.4 $\pm$ 0.5	0.9 $\pm$ 0.09
HC + NCX-4016 $\rightarrow$ HC	174.9 $\pm$ 12.8*	54.3 $\pm$ 4.9	3.6 $\pm$ 0.5	1.2 $\pm$ 0.10
HC $\rightarrow$ HC + NCX-4016	174.1 $\pm$ 12.8*	47.6 $\pm$ 0.97	3.3 $\pm$ 0.5	1.2 $\pm$ 0.06
HC $\rightarrow$ HC + SC560	208.1 $\pm$ 12.4*	52.6 $\pm$ 1.8	3.9 $\pm$ 0.5	1.3 $\pm$ 0.10
HC $\rightarrow$ HC + SC560 + ASA	189.0 $\pm$ 14.1*	48.5 $\pm$ 2.6	3.8 $\pm$ 0.5	1.1 $\pm$ 0.05
<i>HC chimeras: marrow donor/recipient</i>				
WT $\rightarrow$ WT/WT	215.6 $\pm$ 20.9*	59.1 $\pm$ 5.7	3.1 $\pm$ 0.5	0.8 $\pm$ 0.07
COX-1 <sup>-/-</sup> $\rightarrow$ COX-1 <sup>-/-</sup> /WT	166.6 $\pm$ 17.1*	68.2 $\pm$ 17.9	6.2 $\pm$ 1.1	0.7 $\pm$ 0.08
WT $\rightarrow$ WT/COX-1 <sup>-/-</sup>	169.2 $\pm$ 34.6*	88.4 $\pm$ 5.6*	1.5 $\pm$ 0.4	1.0 $\pm$ 0.12
COX-1 <sup>-/-</sup> $\rightarrow$ WT	163.6 $\pm$ 14.2*	56.4 $\pm$ 3.5	3.7 $\pm$ 1.1	1.1 $\pm$ 0.08

Values are mean  $\pm$  SE;  $n = 5-7$  animals/group. ND, normal diet; ASA, aspirin; HC, hypercholesterolemic mice; NCX-4016, nitric oxide-releasing aspirin 2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester; SC560; 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole; WT, wild type; COX-1, cyclooxygenase-1. " $\rightarrow$ " Designates platelet transfer from donor to recipient; "/" designates marrow transfer from donor to recipient. \* $P < 0.05$  vs. WT animals on normocholesterolemic diet. WT/COX-1<sup>-/-</sup> has significantly higher blood pressure vs. all groups.

*COX-1 modulates HC-induced platelet and leukocyte adhesion.* The anti-inflammatory and anti-thrombogenic actions of aspirin have been attributed to its ability to irreversibly inhibit COX-1. While the aspirin dose used in our studies appeared sufficient to completely inhibit platelet COX-1 activity, platelets derived from aspirin-treated mice did not exhibit an altered adhesion response in untreated HC recipients, suggesting that the high dose of aspirin required to completely inhibit mouse platelet COX-1 may exert nonspecific effects on blood cell-vessel wall interactions, via an action on other cell types. Hence, additional experiments were performed using platelet donor mice treated with the selective COX-1 inhibitor SC560 for 2 wk, either alone or in combination with aspirin for the last 7 days of HC. The dose of SC560 (15 mg $\cdot$ kg<sup>-1</sup> $\cdot$ day<sup>-1</sup>) used to study platelet and leukocyte adhesion also resulted in complete inhibition of platelet COX-1 activity (Fig. 2A, inset). Untreated HC recipients receiving SC560-treated HC platelets exhibited a dramatic reduction in adhesion of platelets (85%) and leukocytes (63%) compared with untreated HC recipients receiving untreated HC platelets. The reduction in leukocyte adhesion was primarily due to the fall in platelet-bearing leukocytes (78%). Further reductions in platelet and leukocyte adhesion were not observed in platelet donor mice treated with both SC560 and aspirin.

The cellular origin (blood cell vs. vessel wall) of the SC560-implicated COX-1 involved in HC-induced blood cell-vessel wall interactions was addressed in BM chimeras (Fig. 3). The control chimeras (WT/WT) exhibited leukocyte and platelet adhesion responses that were comparable to WT HC counterparts, suggesting that the BM transplant procedure did not alter the inflammatory phenotype induced by HC. A role for circulating cell (platelet) COX-1 was addressed by transplanting COX-1<sup>-/-</sup> BM into WT mice (COX-1<sup>-/-</sup>/WT) that were subsequently placed on a HC diet. Adhesion of COX-1<sup>-/-</sup> platelets in these HC chimeras was reduced 67% ( $P < 0.05$ ) compared with adhesion of WT platelets in WT/WT chimeras. Although total adherent leukocytes (platelet free + platelet bearing) were reduced by 38% in the COX-1<sup>-/-</sup>/WT chimeras,

this reduction resulted entirely from a decrease (62%) in platelet-bearing adherent leukocytes. Virtually identical responses for both platelet and leukocyte adhesion were noted when COX-1<sup>-/-</sup> platelets were administered to HC WT mice, suggesting that platelet-derived COX-1 products (rather than leukocyte-associated COX-1) are important mediators of platelet adhesion to leukocytes during HC. However, vessel wall-associated COX-1 appears to be more important for modulating the HC-induced platelet and leukocyte adhesion responses, inasmuch as WT/COX-1<sup>-/-</sup> chimeras (receiving donor-matched WT platelets) demonstrated a virtual absence of either platelet or leukocyte adhesion.

*NO-releasing aspirin (NCX-4016) attenuates HC-induced platelet and leukocyte adhesion via a COX-1-independent mechanism.* Mice treated orally with 60 mg $\cdot$ kg<sup>-1</sup> $\cdot$ day<sup>-1</sup> aspirin for 7 days developed gastric erosions, as determined by gross inspection and histology. However, mice treated orally with equimolar NCX-4016 demonstrated no evidence of gastric injury, which is consistent with previous reports using NO-releasing aspirin (35, 37). Furthermore, unlike aspirin, NCX-4016 treatment did not significantly alter platelet COX-1 activity (Fig. 4A, inset). WT mice treated with 1% CMC alone did not induce an inflammatory response, indicating that the vehicle has no effect on cellular adhesion. Despite an absence of effect on COX-1 activity, NCX-4016 does appear to afford significant protection against the deleterious microvascular responses elicited by hypercholesterolemia in a manner that differs from aspirin (Fig. 4). Untreated HC mice receiving platelets from NCX-4016-treated donors exhibited a 44% reduction in platelet adhesion, in contrast to the absence of effect noted with aspirin-treated platelets in the same model. NCX-4016-treated platelets elicited a comparable (41%) reduction in the total number of adherent leukocytes in HC recipients, with 35 and 48% reductions in platelet-free and platelet-bearing leukocytes, respectively. However, NCX-4016-treated HC mice receiving untreated platelets only exhibited reductions in platelet (70%) and leukocyte adhesion (total, platelet free, and

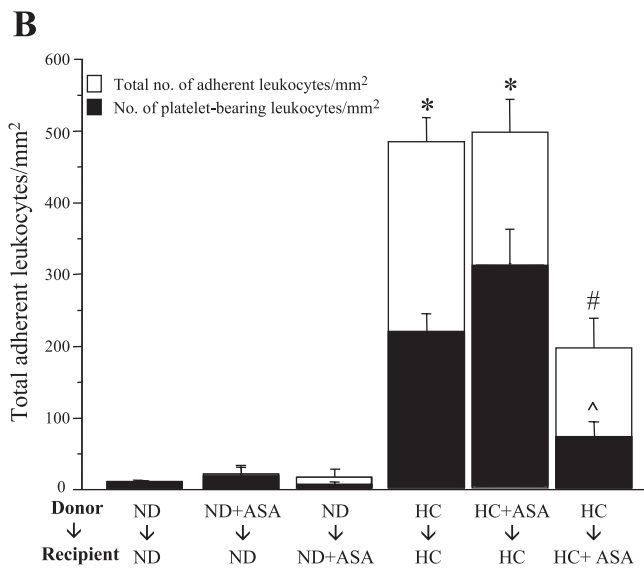
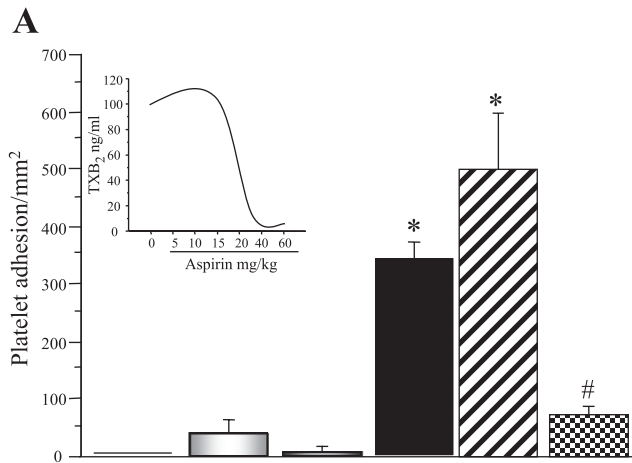


Fig. 1. Effect of aspirin (ASA) on platelet (A) and leukocyte (B) adhesion in postcapillary venules of hypercholesterolemic (HC) mice. The dose-response relationship between platelet cyclooxygenase (COX)-1 activity [measured as the generation of thromboxane (Tx) B<sub>2</sub>] and the oral daily dose of ASA for 7 days is demonstrated in the *inset*. ND, normal diet; HC, high-cholesterol diet. ND → ND, ND platelets in ND recipient; ND+ASA → ND, ASA-treated ND platelets in ND recipient; ND → ND+ASA, ND platelets in ASA-treated ND recipient; HC → HC, HC platelets in HC recipient; HC+ASA → HC, ASA-treated HC platelets in HC recipient; HC → HC+ASA, HC platelets in ASA-treated HC recipient. \* $P < 0.001$  vs. ND → ND; # $P < 0.001$  vs. HC → HC; ^ $P < 0.004$  vs. platelet-bearing leukocytes in HC → HC ( $n = 5-9$ /group).

platelet bearing) that were comparable to the responses noted in aspirin-treated recipients.

## DISCUSSION

Hypercholesterolemia elicits a systemic inflammatory response that is characterized by endothelial cell activation and accumulation of inflammatory cells and platelets in lesion-prone arteries and multiple microvascular beds. The inflammatory and thrombogenic phenotype that is assumed by the microcirculation during hypercholesterolemia precedes large-vessel disease, suggesting that hypercholesterolemia-induced microvascular alterations may contribute to the initiation and/or progression of the large-vessel disease (30). Widely used prophylactic therapies that target the deleterious vascular

consequences of hypercholesterolemia include statins to lower blood cholesterol and aspirin to avert thrombotic events that can accompany the development/disruption of atherosclerotic plaques in large arteries. While there is considerable evidence that statins also attenuate the inflammatory and thrombogenic responses of the microvasculature to hypercholesterolemia, the literature is silent regarding the effects of prophylactic aspirin therapy on the microcirculation during hypercholesterolemia.

Our results demonstrate that prophylactic aspirin therapy effectively reduces hypercholesterolemia-induced leukocyte and platelet adhesion in postcapillary venules. Aspirin's effects on leukocyte-endothelial cell adhesion are controversial, with reports of aspirin-inducing (1, 17), as well as attenuating (5, 6, 11), leukocyte adhesion. The findings of our study suggest that aspirin has beneficial effects via an action on the endothelium. Our study also reveals a role for endothelial cell COX-1 in

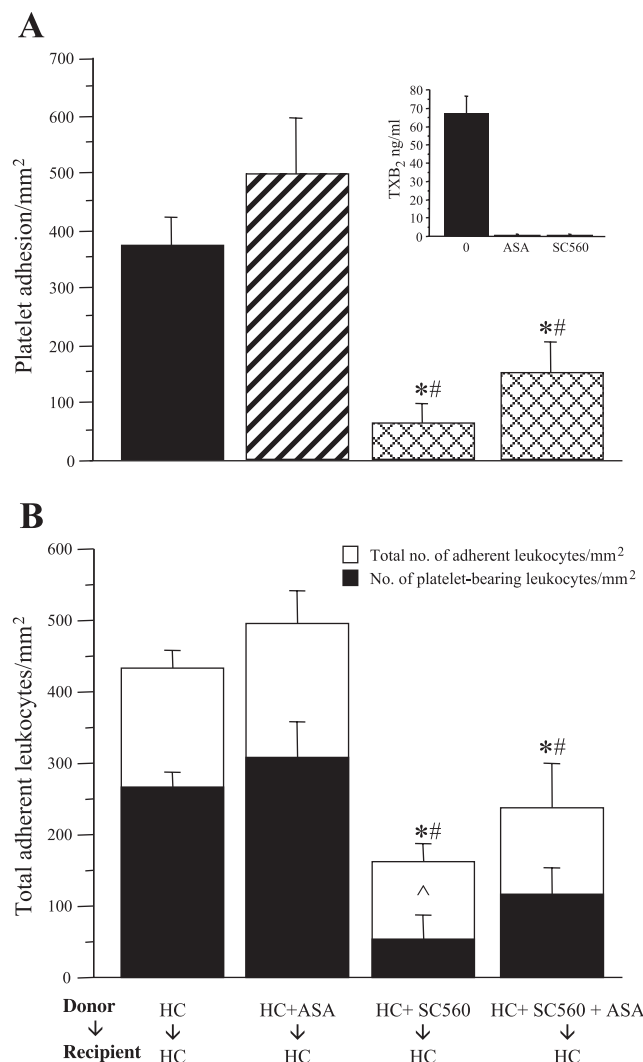


Fig. 2. Effects of the specific COX-1 inhibitor (SC560) on platelet (A) and leukocyte (B) adhesion in postcapillary venules of HC mice. Inhibitory effects of 60 mg/kg ASA and SC560 on platelet COX-1 activity are compared in the *inset*. HC+ASA → HC, ASA-treated HC platelets in HC recipient; SC560 → HC, SC560-treated HC platelets in HC recipient; HC+SC560 + ASA → HC, SC560 + ASA-treated HC platelets in HC recipient. \* $P < 0.001$  vs. HC → HC; # $P < 0.05$  vs. HC+ASA → HC; ^ $P < 0.01$  vs. platelet-bearing leukocytes in HC → HC ( $n = 5-9$ /group).

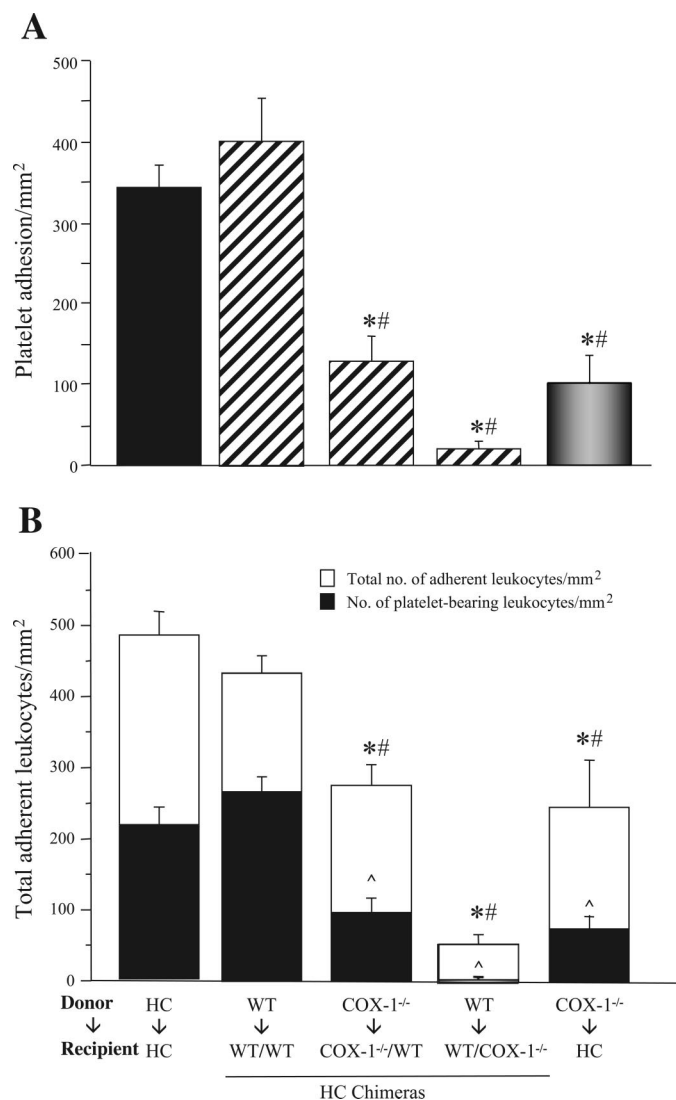


Fig. 3. Effects of COX-1 deficiency in blood cells or vessel wall on platelet (A) and leukocyte (B) adhesion in postcapillary venules of HC mice. Wild type (WT) → WT/WT, WT platelets in WT/WT chimeras; COX-1<sup>-/-</sup> → COX-1<sup>-/-</sup>/WT, COX-1<sup>-/-</sup> platelets in COX-1<sup>-/-</sup>/WT chimeras; WT → WT/COX-1<sup>-/-</sup>, WT platelets in WT/COX-1<sup>-/-</sup> chimeras; COX-1<sup>-/-</sup> → WT, COX-1<sup>-/-</sup> platelets in WT recipients. \**P* < 0.001 vs. WT HC → HC; #*P* < 0.05 vs. WT/WT chimeras (HC); ^*P* < 0.05 vs. platelet-bearing leukocytes in WT/WT chimeras (HC), (*n* = 5–9/group).

mediating cell-cell adhesion during hypercholesterolemia. These conclusions are based on the following observations: 1) aspirin treatment and WT/COX-1<sup>-/-</sup> BM chimeras exhibit qualitatively similar blood cell adhesion responses during hypercholesterolemia; 2) aspirin's anti-adhesive actions were noted only when vessels (rather than transferred platelets) were exposed to aspirin; and 3) the most dramatic reductions in HC-induced blood cell adhesion occurred when the vessel wall (rather than blood cells) was genetically deficient in COX-1.

The importance of COX-1 in the inflammation associated with hypercholesterolemia has been demonstrated in chronic models of atherosclerosis, wherein the expression of both COX-1 and -2 is accompanied by an enhanced platelet adhesion in the lesion area (4, 22). The results of the present study also support a role for COX-1 in the early microvascular

alterations elicited by hypercholesterolemia. However, our findings in aspirin-treated mice and in COX-1 BM chimeras reveal that both endothelial and blood cell (platelets) COX-1 are important in modulating the inflammatory responses elicited in venules by hypercholesterolemia. While the precise mechanism by which COX-1 modulates this inflammatory phenotype remains unclear, our finding underscores the early and important role of this enzyme in modulating the cell-cell interactions elicited in the microvasculature by hypercholesterolemia.

The molecular basis for the anti-inflammatory effects of aspirin on the endothelial cell appears to involve both a platelet-independent mechanism, as well as COX-1 inhibition. Endothelial cells are known to express both the constitutive (COX-1) and inducible (COX-2 isoforms) of COX, with the latter isoform found in leukocytes as well. Although aspirin is

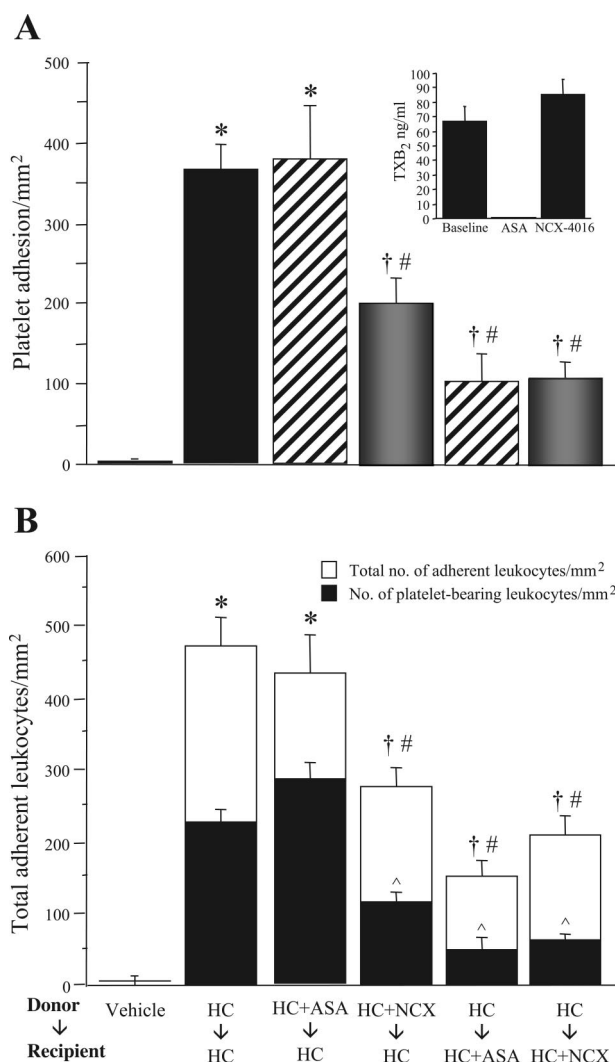


Fig. 4. Effects of nitric oxide-releasing ASA (NCX-4016) on platelet (A) and leukocyte (B) adhesion in postcapillary venules of HC mice. Vehicle refers to 1% carboxymethyl cellulase alone. HC+AS → HC, ASA-treated HC platelets in HC recipient; HC+NCX → HC, NCX-treated HC platelets in HC recipient; HC → HC+ASA, HC platelets in ASA-treated HC recipient; HC → HC+NCX, HC platelets in NCX-treated HC recipient. \**P* < 0.001 vs. ND → ND; †*P* < 0.05 vs. HC → HC; #*P* < 0.05 vs. HC+ASA → HC; ^*P* < 0.001 vs. platelet-bearing leukocytes in HC+ASA → HC (*n* = 5–9/group).

a highly specific inhibitor for the COX-1 isoform, at high doses it can also inhibit COX-2 activity (23). Furthermore, it has been reported that the elevated levels of COX-1 and -2-derived prostanoids (prostaglandin and  $\text{TxA}_2$ ) detected in atherogenic models are attenuated following aspirin treatment (4, 27). Therefore, it is possible that the blunted inflammatory and thrombogenic phenotype associated with aspirin treatment of HC mice reflect an inhibition of both COX isoforms. Since aspirin has also been shown to accelerate the production of COX-2-derived anti-inflammatory mediators, such as lipoxins (7, 26, 38), which has been linked to endothelial NO synthase (26), the NSAID may also be exerting its COX-1- and platelet-independent effects through such a pathway. Additional studies are needed to more clearly define these alternate pathways of aspirin action in the microvasculature of HC mice.

Although COX-1 activity of platelets from aspirin-treated mice was almost completely inhibited, hypercholesterolemia-induced adhesion of aspirin-treated platelets to the vessel wall was unaffected, a response that contrasts with the well-known inhibitory effect of aspirin on platelet aggregation (homotypic adhesion) (10, 33). However, the significant reduction in hypercholesterolemia-induced adhesion of platelets treated with SC560, a specific COX-1 inhibitor, or genetically deficient in COX-1 (monitored in COX-1<sup>-/-</sup>/WT chimeras), suggests that selective inhibition of platelet COX-1 activity will block platelet-vessel wall interactions. In light of our results with COX-1<sup>-/-</sup> and SC560-treated WT HC platelets, and the divergent responses of aspirin-treated platelets, it is possible that the beneficial effects of platelet COX-1 inhibition by aspirin are offset by COX-1-independent effects of aspirin that promote platelet adhesion.

Our findings also suggest that leukocyte-associated COX-1 does not contribute to the platelet adhesion responses, since we noted the adhesion of COX-1<sup>-/-</sup> platelets in HC WT mice (only platelets are COX-1 deficient) was nearly identical to the adhesion observed when COX-1<sup>-/-</sup> platelets were observed in COX-1<sup>-/-</sup>/WT HC chimeras (both platelets and leukocytes are COX-1 deficient). In both of these groups, platelet adhesion was significantly lower than that observed in WT HC mice receiving matched platelets. Since nearly identical leukocyte adhesion responses to hypercholesterolemia were also noted in these two experimental groups (i.e., reduced vs. WT HC), it also appears unlikely that leukocyte-associated COX-1 contributes to the hypercholesterolemia-induced leukocyte adhesion.

Our results invoke a major role for platelet-associated COX-1 activity in mediating cell-cell interactions in venules of HC mice. In the presence of either SC560-treated (but not aspirin) or COX-1<sup>-/-</sup> platelets, hypercholesterolemia-induced leukocyte adhesion was reduced, especially by platelet-bearing leukocytes, suggesting that platelet-derived COX-1 products promote these hypercholesterolemia-induced blood cell interactions, possibly through transcellular production of mediators or activation of signaling pathways that induce adhesion molecule expression and/or render platelet-bearing leukocytes more responsive to activation signals (14, 29). Our inability to demonstrate an influence of aspirin-treated platelets on platelet-leukocyte and leukocyte-vessel wall adhesion in HC venules is consistent with aspirin's reported failure to inhibit increased expression of P-selectin on platelets and platelet-leukocyte aggregation associated with several CVD, such as

ischemic heart disease (31), intracoronary stenting (21), and atherosclerosis (15).

The coupling of NO to aspirin has resulted in a unique set of compounds (e.g., NCX-4016) that retain aspirin's anti-platelet properties but are devoid of its gastric-damaging effects (9, 12, 38). NO-releasing aspirin has been shown to inhibit thrombus formation in rats and mice (24, 35) and to attenuate formyl-methionine-leucine-phenylalanine-stimulated and colitis-induced leukocyte adhesion in mesenteric rat venules (36, 37). The failure of NCX-4016 (60 mg/kg) to inhibit COX-1 activity in our study agrees with reports of higher doses (compared with aspirin) of NO-aspirin derivatives (NCX-4016 and NCX-4215) being required to inhibit platelet COX-1 activity (34, 35). Nonetheless, NCX-4016-treated HC mice exhibited blunted platelet and leukocyte adhesion responses comparable to their aspirin-treated counterparts. However, unlike aspirin-treated platelets, NCX-4016-treated platelets attenuated adhesion of platelets and leukocytes, especially platelet-bearing leukocytes. In the absence of platelet COX-1 inhibition, these NCX-4016 effects likely result from NO release, since other NO-releasing compounds inhibit leukocyte- and platelet-vessel wall interactions, as well as platelet-leukocyte aggregation in different models of inflammation (13, 16). The reduced NO bioavailability that accompanies hypercholesterolemia and promotes cell-cell adhesion may be reversed with NO-releasing aspirin, which can scavenge excess superoxide generated during hypercholesterolemia and/or improve diminished cellular (platelet, leukocyte, and endothelial cell) cGMP, restoring a noninflammatory and nonthrombogenic phenotype to the microcirculation. Another plausible explanation for our observations is that NO may reduce cell adhesion by altering adhesion molecule. Several reports in the literature have described the ability of NO to downregulate adhesion molecule expression on either endothelial cells (e.g., ICAM-1), or on circulating blood cells (leukocytes or platelets), either by affecting transcription or by inhibiting translocation of preformed pools of adhesion molecules in endothelial cells (e.g., P-selectin) (18, 20).

In conclusion, these results indicate that aspirin treatment largely prevents the inflammatory and thrombogenic responses of the microvasculature to hypercholesterolemia. The beneficial effects of aspirin appear to be largely mediated through COX-1 inhibition in the vessel wall, with no apparent involvement of platelet COX-1 products. Similar to aspirin, strategies that selectively inhibit (or genetically eliminate) COX-1 activity reduce hypercholesterolemia-induced microvascular responses; however, they primarily do so by inhibiting heterotypic platelet adhesive interactions. The gastroprotective NO-releasing aspirins may hold promise as prophylactic therapy for the microvascular dysfunction, systemic inflammation, and consequent large-vessel disease associated with hypercholesterolemia.

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