Role of Blood Cell–Associated AT1 Receptors in the Microvascular Responses to Hypercholesterolemia


Objective—Hypercholesterolemia elicits a proinflammatory and prothrombogenic phenotype in the microvasculature that is characterized by activation and adhesion of blood cells. The angiotensin II receptor-1 antagonist Losartan prevents the induction of these responses. The objective of this study was to determine the relative contributions of blood cell-associated versus endothelium-associated AT1a-R to these hypercholesterolemia-induced microvascular alterations.

Methods and Results—Leukocyte adhesion and emigration and platelet adhesion were quantified by intravital microscopy in postcapillary venules. C57Bl/6 mice were placed on a normal (ND) or high-cholesterol (HCD) diet for 2 weeks. AT1a-R bone marrow chimeras that express AT1a-R on the vessel wall but not blood cells and AT1a-R knockouts were placed on HCD. Venular shear rate was comparable in all groups. Platelet and leukocyte adhesion and leukocyte emigration were significantly increased in HCD mice versus ND. Leukocyte recruitment was significantly reduced in the HCD–AT1a-R bone marrow chimera group, whereas platelet adhesion remained at HCD levels. However, in HCD-AT1a-R knockout mice, platelet and leukocyte adhesion were reduced to ND levels.

Conclusions—These data indicate that the platelet-vessel wall adhesion elicited by hypercholesterolemia is mediated by AT1a-R engagement on the endothelial cell rather than the platelet, whereas leukocyte recruitment is mediated by blood cell-associated AT1a-R. (Arterioscler Thromb Vasc Biol. 2006;26:0-0.)

Key Words: angiotensin II type 1 receptor ■ microcirculation ■ hypercholesterolemia ■ platelets ■ leukocytes
mediating the proinflammatory and prothrombogenic responses elicited by hypercholesterolemia and to define the specific cell populations that contribute to these AT1a-R-dependent adhesion responses. The latter objective was achieved by comparing hypercholesterolemia-induced leukocyte and platelet adhesion among WT mice, AT1a-R<sup>−/−</sup> mice, and AT1a-R<sup>+/−</sup> chimeras (mice characterized by AT1a-R deficiency on circulating blood cells but not on vascular endothelium). The findings from this study strongly implicate a role for the AT1a-R on circulating leukocytes in mediating the adhesion of these cells, whereas endothelial cell-associated AT1a-R mediates the platelet adhesion observed during hypercholesterolemia.

**Methods**

**Animals**

Male WT C57Bl/6J mice, AT1a-R<sup>−/−</sup> mice (Jackson Laboratories), and chimeras produced by the transfer of bone marrow from WT mice into WT recipients (WtCh) or AT1a-R<sup>−/−</sup> into WT recipients (AT1aCh) were placed on either a normal (ND) or high-cholesterol diet (HCD; Teklad 90221 containing 1.25% cholesterol, 15.8% fat, and 0.125% choline chloride, Harlan Teklad) for 2 weeks. In all of the groups, n=3 to 6 per group.

**Production of Bone Marrow Chimeras**

Bone marrow cells were isolated from the femurs and tibias of WT or AT1a-R<sup>−/−</sup> donor mice and resuspended at 4×10<sup>7</sup> cells/mL in PBS. Recipient (CD45 congenic WT) mice were irradiated with 2 doses of 500 to 525 Rads, 3 hours apart, after which 8×10<sup>6</sup> donor bone marrow cells in 200 μL of PBS were injected into the femoral vein. The chimeras were kept in autoclaved cages, with 0.2% water was used. Flow cytometry was used to verify chimera reconstitution (usually requiring 6 to 8 weeks) by staining for CD45.1 and CD45.2 expression on circulating leukocytes with an fluorocsein isothiocyanate-labeled anti-CD45.1 antibody and a biotinylated anti-CD45.2 antibody with a streptavidin-PerCP secondary antibody (PharMingen). This procedure normally yields >90% penetrance of the transferred marrow at ≥6 weeks after transplant. This bone marrow transfer protocol allowed for the creation of mice wherein normal levels of AT1a were expressed by all of the cells (HCD-WTCh group) or the genetic deficiency of AT1a-R was confined to the circulating blood cells (HCD-AT1aCh group).

**Surgical Protocol**

Mice were anesthetized with ketamine hydrochloride (150 mg/kg body weight, IP) and xylazine (7.5 mg/kg body weight, IP). The right jugular vein was cannulated for administration of heparinized saline and platelets, and the left carotid artery was cannulated for systemic arterial pressure measurement. Core body temperature was maintained at 35±0.5°C. Animal procedures were approved by the Louisiana State University Health Sciences Center Institutional Animal Care and Use Committee and were in accordance with the guidelines of the American Physiological Society.

**Platelets**

Platelets were collected, isolated, and labeled as previously described. 15

**Intravital Microscopy**

The cremaster muscle was prepared for intravital microscopy as described previously. 16 Postcapillary venules (20 to 40 μm diameter) with wall shear rates of ≥500 s<sup>−1</sup> were studied. The total number of adherent leukocytes and platelets were quantified during playback of videotaped images. Platelets (number per millimeter square) were considered adherent if they arrested for ≥2 s. A leukocyte was

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**Table:**

<table>
<thead>
<tr>
<th>Groups</th>
<th>SCC, mg/dL</th>
<th>WSR, s&lt;sup&gt;−1&lt;/sup&gt;</th>
<th>MAP, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND-WT</td>
<td>65±9.8</td>
<td>685±24.9</td>
<td>57±2.0</td>
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<tr>
<td>HCD-WT</td>
<td>169±21.3*</td>
<td>695±145.4</td>
<td>57±3.6</td>
</tr>
<tr>
<td>HCD-AT1a-R&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>156±24.9*</td>
<td>574±33.9</td>
<td>30±2.6†</td>
</tr>
<tr>
<td>HCD-WTCh</td>
<td>150±23.2*</td>
<td>593±46.7</td>
<td>60±3.6</td>
</tr>
<tr>
<td>HCD-AT1aCh</td>
<td>180±7.3*</td>
<td>743±75.4</td>
<td>55±1.5</td>
</tr>
</tbody>
</table>

SCC indicates serum cholesterol concentration; WSR, wall shear rate; MAP, mean arterial pressure.

*P<0.005 vs ND-WT; †P<0.0001 vs all other groups.

considered adherent if it remained stationary for ≥30 s (number per millimeter square). Platelet and leukocyte adhesion was measured throughout the observation periods. Leukocyte emigration was measured online at the end of each observation period and expressed as the number of interstitial leukocytes per high-powered field of view adjacent to the observed segment (number per field).

**Experimental Protocol**

Venules were selected for observation after a 30-minute stabilization period. Platelets (10<sup>10</sup> in a volume of 120 μL) were infused via the jugular vein over 5 minutes and allowed to circulate for an additional 5 minutes. Mice in the ND-WT, HCD-WT, HCD-AT1a-R<sup>−/−</sup>, HCD-WTCh, and HCD-AT1aCh groups received platelets from matching donors. In other groups, HCD-WT mice received platelets from either hypercholesterolemic AT1aCh mice (HCD-AT1aCh) or hypercholesterolemic AT1aCh mice treated with Losartan (HCD-AT1aCh-Los). Five-minute recordings of the leukocytes (light microscopy) followed by 1-minute 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. The mean value of each variable within a single venule was calculated, and comparisons were made between the experimental groups.

**Serum Cholesterol Levels**

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

**Statistical Analysis**

All of the values are reported as mean±SEM. ANOVA with Fisher post-hoc test was used for statistical comparison of experimental groups, with statistical significance set at P<0.05.

**Results**

**Serum Cholesterol, Wall Shear Rate, and Mean Arterial Pressure**

A significant (2- to 3-fold) increase in total serum cholesterol was detected in all of the mice placed on a HCD (HCD-WT, HCD-WTCh, HCD-AT1a-R<sup>−/−</sup>, and HCD-AT1aCh), when compared with the normal diet group (Table). Although HCD-AT1a-R<sup>−/−</sup> mice exhibited a lower arterial blood pressure than the other groups, venular wall shear rate, a factor that can influence the adhesion of blood cells in venules, was not significantly different between any of the groups studied (Table).

**Role of Blood Cell-Associated and Endothelial Cell-Associated AT1a-R in Hypercholesterolemia-Induced Leukocyte Recruitment**

The number of adherent leukocytes in postcapillary venules of HCD-WT and HCD-WTCh mice was significantly higher...
than that observed in ND-WT mice (Figure 1). The hypercholesterolemia-induced increase in adherent leukocytes was significantly blunted in HCD-AT1a-R⁻/⁻ mice and in HCD-AT1aCh mice (Figure 1). Leukocyte emigration was also increased in the HCD-WT and HCD-WTCh mice compared with the ND-WT group (Figure 2). Much like the leukocyte adherence responses, a profound reduction in leukocyte emigration was noted in both HCD-AT1a-R⁻/⁻ and HCD-AT1aCh mice.

Regarding the role of blood cell-associated AT1a-R in hypercholesterolemia-induced platelet adhesion, platelets derived from HCD-WT mice administered into HCD-WT-recipient mice exhibited an elevated level of adhesion in postcapillary venules when compared with ND-WT platelet adhesion in matched recipient (ND-WT) mice (Figure 3). The number of adherent platelets in the WT chimeras was comparable to HCD-WT mice. This response to hypercholesterolemia was completely abolished in HCD-AT1a-R⁻/⁻ mice that received platelets from HCD-AT1-R⁻/⁻ donor mice. However, the attenuation of the hypercholesterolemia-induced platelet adhesion response noted in AT1a-R knockouts was not observed when platelets from AT1aCh mice were administered into AT1aCh recipients. To additionally explore the contribution of platelet-associated AT1a-R to the platelet-vessel wall adhesion response to hypercholesterolemia, HCD-AT1aCh platelets were monitored in HCD-WT recipients (Figure 4). In these mice, a normal response to hypercholesterolemia was noted. In a previous report,¹³ we demonstrated that the AT1-R antagonist Losartan effectively blocks the platelet adhesion response to hypercholesterolemia, largely via an action on the endothelial cell. Conversely, platelets from hypercholesterolemic mice treated with Losartan exhibited an exaggerated adhesion response when administered to HCD-WT mice. However, we were unable to completely rule out a nonspecific action of the drug. Therefore, we examined the adhesion response of platelets derived from Losartan-treated HCD-AT1aCh mice in HCD-WT recipients (Figure 4). In these studies, no attenuation or exaggeration of platelet adhesion in response to hypercholesterolemia was noted.

**Discussion**

Angiotensin receptor activation has been implicated in the altered vascular responses to hypercholesterolemia. Previous reports have described a role for the AT1-R in mediating the development of atherosclerotic lesions.³⁻⁶,¹⁶ the impaired endothelium-dependent vasodilation manifested in large ar-
teries and arterioles, the oxidative stress experienced by all segments of the vascular tree, and the blood cell-endothelial cell interactions observed in postcapillary venules. Although a recent report from our laboratory has invoked a role for AT1-R in the inflammatory and thrombogenic responses during hypercholesterolemia, concerns regarding potential nonspecific actions of Losartan on circulating blood cells suggest that alternative strategies are needed to definitively implicate these receptors in the pathogenesis of hypercholesterolemia. Furthermore, treatment with AT1-R antagonists like Losartan does not allow for a clear distinction of the specific cell types in which AT1-R activation occurs.

In this study, we used AT1a-R−/− mice and AT1a-R−/− bone marrow chimeras to define the role of the AT1a-R in mediating the proinflammatory and prothrombogenic responses observed in the microvasculature during hypercholesterolemia and to define the specific cell populations that are primarily responsible for these AT1a-R-dependent adhesion responses.

Hypercholesterolemia has been shown to promote the adhesion and emigration of leukocytes in postcapillary venules of different tissues, including skeletal muscle, intestine, liver, and brain. Neutrophils are the dominant leukocyte population recruited into venules in the first 2 weeks of hypercholesterolemia. The leukocyte adhesion elicited in this model appears to be linked to the oxidative stress experienced by endothelial cells. The inflammatory cell recruitment is additionally exacerbated by an enhanced production of cytokines (interferon γ) by T lymphocytes in response to an elevated blood cholesterol concentration. The results of the present study strongly support the view that AT1a-R activation is a major factor in inducing the inflammatory phenotype caused by hypercholesterolemia. A comparison of our leukocyte adhesion data from the AT1a-R−/− mice and AT1a-R−/− chimeras reveals that leukocyte-associated AT1a receptors are largely responsible for mediating the inflammatory phenotype induced by hypercholesterolemia. This conclusion is based on the observation that the AT1a-R−/− chimeras, which have leukocytes that are devoid of AT1a-R but still express AT1a-R on endothelial cells, exhibited an attenuated leukocyte adhesion response. The reduction in leukocyte emigration may be a direct result of the decreased leukocyte adhesion, because firm adhesion is a prerequisite for emigration. These results are consistent with 2 distinct roles for the AT1-R on leukocytes. First, engagement of AT1-R on neutrophils leads to activation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase and mitogen-activated protein kinase, which both promote the activation of redox-sensitive transcription factors, such as nuclear factor κB, thereby inducing an inflammatory phenotype. This possibility is supported by data that invokes a role for neutrophil NADPH oxidase in the leukocyte adhesion elicited by hypercholesterolemia. Second, engagement of the AT1-R on circulating T lymphocytes may elicit the enhanced production of interferon γ, which has been shown to drive the neutrophil adhesion and oxidative stress responses to hypercholesterolemia.

There is a growing body of evidence that inflammatory conditions are associated with a corresponding accumulation of adherent platelets. This phenomenon of simultaneous inflammatory and thrombogenic responses in venules has been demonstrated in animal models of ischemia reperfusion, endotoxemia, sickle cell disease, and hypercholesterolemia. In all of these experimental models, the adhesion molecule P-selectin has been identified as a critical determinant of the platelet-vessel wall interactions in the inflamed venules. The results of the present study also support a role for the AT1a-R in the prothrombogenic response elicited in venules by hypercholesterolemia. Furthermore, a comparison of the platelet adhesion data derived from the AT1a-R−/− and AT1a-R−/− bone marrow chimeras indicate that, unlike with leukocytes, it is the endothelial cell AT1a-R, rather than the platelet or leukocyte receptor, that mediates the hypercholesterolemia-induced platelet-vessel wall interactions. The absence of a contribution of platelet-associated AT1a-R is additionally supported by our observation that AT1a-R−/− platelets bind to a similar extent in venules of WT hypercholesterolemic mice as WT platelets in the same recipients. The role of endothelial cell AT1a-R in mediating platelet adhesion may result from an increased P-selectin expression on endothelial cells that is induced by AT1a-R activation. However, this possibility is inconsistent with our previous observation that Losartan treatment does not alter the increased P-selectin expression on endothelial cells that is induced by hypercholesterolemia. Another potential reason is that the hypercholesterolemia-induced platelet recruitment is linked to an AT1a-R-mediated oxidative stress in endothelial cells. This possibility is supported by our previous report describing an inhibition of the hypercholesterolemia-induced oxidative stress in postcapillary venules of Losartan-treated mice. The fact that the attenuation of leukocyte adhesion observed in AT1a-R−/− chimera mice was not accompanied by a similar reduction in platelet recruitment, despite our previous findings that platelets primarily

**Figure 4.** The adhesion of platelets derived from hypercholesterolemic (HCD) WT (n=5), angiotensin II receptor type 1 bone marrow chimeras which lacked AT1 on the blood cells (AT1aCh; n=5), and AT1aCh mice treated with the AT1-R antagonist Losartan (AT1aCh-Los; n=4) was determined in HCD-WT recipient mice. *P<0.005 vs ND-WT.
adhere to the hypercholesterolemic vessel wall by attaching to adherent leukocytes, is an interesting observation. This suggests that the platelet recruitment that occurred in the absence of leukocyte adhesion was a result of direct contact between platelets and the vascular endothelium. Whether this enhancement of platelet-endothelial interactions in the AT1a-R/H11002 chimeras is attributable to more angiotensin II being available to bind and activate the endothelium because the leukocytes and platelets do not express AT1a-R, remains unclear.

We have found previously that platelets from Losartan-treated animals exhibit exaggerated adhesion in hypercholesterolemic recipients when compared with nontreated platelets.13 Although it has been discovered that Losartan possesses antiplatelet properties that are independent of AT1a-R,21 our observations suggested that Losartan may actually enhance platelet activity in vivo. Therefore, we investigated whether this potentially deleterious effect of Losartan was specific to the AT1a-R. Because Losartan did not alter platelet adhesion in the absence of AT1a-R on platelets, our findings indicate that the exaggerated adhesion observed previously for Losartan-treated HCD-WT platelets may be invoked by an AT1a-receptor-mediated pathway during hypercholesterolemia and the proposed mechanisms through which this occurs (based on our previous findings and those of others). Elevated cholesterol levels are associated with increased expression of AT1-R and activation of NADPH oxidase in both the vessel wall and leukocytes. This leads to leukocyte and platelet recruitment in postcapillary venules. The administration of an AT1-R antagonist during hypercholesterolemia reduces the oxidative stress and attenuates the inflammatory and thrombogenic phenotypes by acting on both leukocytes and endothelial cells. Thus, it is plausible that AT1a-R on the leukocyte mediates the hypercholesterolemia-induced leukocyte-endothelial interactions through the activation of NADPH oxidase-mediated inflammatory pathways, whereas occupation of AT1a-R on the vascular endothelium initiates platelet recruitment, possibly through reactive oxygen species-sensitive adhesion molecule upregulation.

Collectively, the results of this study indicate that AT1a-R is a major contributor to the leukocyte and platelet adhesion responses to hypercholesterolemia, with the leukocyte-associated AT1a-R participating in the inflammatory response and endothelial cell AT1a-R mediating the thrombogenic response (Figure 5). In light of previous in vitro27 and in vivo18 findings, it is plausible that AT1a-R engagement on the leukocyte and endothelial cell initiates NADPH oxidase activation in both cell types during hypercholesterolemia. In the endothelium, this could lead to the activation of redox-sensitive transcription factors, such as nuclear factor κB activation, thereby inducing adhesion molecule expression,32,33 which may support the platelet adhesion. The exact mechanism through which AT1a-R and NADPH oxidase activation generates the adhesive phenotype in neutrophils remains unclear, because several studies have demonstrated a lack of effect of Losartan on adhesion molecule expression on leukocytes. Nonetheless, the current findings suggest that cell-specific targeting of AT1-R may be a useful therapeutic strategy for thrombogenic and inflammatory diseases.

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**References**


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