Angiotensin II Type-1 Receptor Antagonism Attenuates the Inflammatory and Thrombogenic Responses to Hypercholesterolemia in Venules


Abstract—Hypercholesterolemia elicits an inflammatory response in the microvasculature that is accompanied by an increased expression of angiotensin II type-1 receptors (AT1-R) on platelets, leukocytes, and endothelial cells. AT1-R blockade attenuates inflammatory responses to angiotensin II (eg, adhesion molecule expression and reactive oxygen species production). We investigated whether AT1-R antagonism attenuates the platelet and leukocyte recruitment induced by acute hypercholesterolemia in postcapillary venules. Leukocyte and platelet adhesion and oxidative stress were quantified by intravital microscopy in cremaster muscle, and P-selectin and AT1-R expression was determined in mice placed on a normal diet (ND) or high-cholesterol diet (HCD) for 2 weeks. Platelet and leukocyte adhesion was significantly elevated by hypercholesterolemia. In HCD mice receiving losartan (HCD-Los) in drinking water, platelet and leukocyte recruitment was reduced to ND levels. Increased platelet adhesion was observed in HCD mice receiving platelets from HCD-Los mice, consistent with a direct beneficial action of losartan on the vessel wall. Hypercholesterolemia elicited an oxidative stress in venules and an increased expression of P-selectin and AT1-R. The oxidative stress and AT1-R upregulation were reduced by losartan, but the P-selectin response was not. We propose that AT1-R engagement contributes to the prothrombogenic and proinflammatory state induced in venules by hypercholesterolemia. (Hypertension. 2005;45:209-215.)

Key Words: receptors, angiotensin II — microcirculation — hypercholesterolemia — leukocytes

It is well established that angiotensin II (Ang II), the effector component of the renin-angiotensin system, is a potent vasoconstrictor that plays a key role in the maintenance of blood pressure and fluid homeostasis. However, it is becoming increasingly apparent that Ang II also possesses potent proinflammatory properties, such as enhancing reactive oxygen species (ROS) generation, increasing the expression of cell adhesion molecules (CAMs) and stimulating the release of cytokines and chemokines such as interleukin (IL)-6, IL-8, and monocyte chemotactic protein-1. Ang II stimulates superoxide release from NAD(P)H oxidase by engaging the high-affinity Ang II type-1 receptor (AT1-R), thereby initiating the phosphorylation of critical enzyme subunits. In vitro, Ang II elevates surface expression of CAMs such as vascular CAM-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin. In vivo, superfusion of postcapillary venules with Ang II results in leukocyte recruitment and ROS generation. These leukocyte adhesion responses appear to be mediated by a ROS-dependent mobilization of preformed P-selectin to the endothelial surface. Ang II can act as an immunomodulatory agent that engages the AT1-R to increase the number of Th1 cytokine (interferon-γ)—producing T cells and elevate CD40 expression on circulating monocytes. Ang II may also exert some of its proinflammatory effects through activation of platelets. Platelets express AT1-R, and AT1-R antagonists attenuate platelet adhesion and aggregation in vitro and exert an antithrombotic effect in vivo.

Inflammatory responses have been implicated in the development of many cardiovascular diseases (CVDs), such as myocardial infarction, stroke, and atherosclerosis. It is becoming increasingly accepted that Ang II, through its interaction with the AT1-R, is linked to CVD by promoting inflammation. For example, clinical trials with AT1-R blockers show some benefit in reducing ischemic events and mortality related to CVD beyond blood pressure lowering, and AT1-R antagonists inhibit LDL lipid peroxidation and development of atherosclerotic lesions in animal models of hypercholesterolemia. It is well established that hypercholesterolemia elicits systemic inflammatory responses, which are characterized by an oxidative stress, elevated cytokines, and activation of blood cells. In the macrovasculature, these responses are confined to large arteries and contribute to development of atherosclerosis. The...
alterations that occur in small vessels precede atherosclerotic lesion development and are manifested as endothelial dysfunction and the adhesion of leukocytes and platelets to the venular wall.28,29 Ang II has been implicated in the inflammatory responses induced by hypercholesterolemia, and recent studies have shown that AT1-R is upregulated on platelets, leukocytes, and endothelial cells during hypercholesterolemia.30–32 Furthermore, AT1-R antagonists blunt the atherogenic responses to elevated cholesterol levels and attenuate hypercholesterolemia-induced endothelial cell dysfunction.33,34 Nonetheless, it remains unclear whether and how the AT1-R contributes to the early microvascular alterations elicited by hypercholesterolemia.

The overall goal of this study was to determine whether AT1-R antagonism (with losartan) reduces the platelet and leukocyte recruitment induced by acute hypercholesterolemia in postcapillary venules and to determine the relative contributions of platelets and endothelial cells to the AT1-R-mediated responses. Oxidative stress and the expression of AT1-R and P-selectin on microvascular endothelial cells were also monitored in these studies.

Materials and Methods

Animals
At 6 to 8 weeks of age, male wild-type C57BL/6J mice (Jackson Laboratories) were placed on either a normal diet (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. Separate groups of mice were treated with the AT1-R antagonist losartan (25 mg/kg/day) in drinking water for 7 days, beginning at day 8 on HCD (HCD mice receiving losartan [HCD-Los] group). In all groups, n=5 to 8 per 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 described previously.35 Intravital Microscopy
The cremaster was prepared for intravital microscopy as described previously.36 Postcapillary venules (20 to 40 μm diameter) with a wall shear rate (WSR) of ≥500/s37 were studied. The number of adherent leukocytes and platelets were quantified during playback of videotaped images. Platelets (number per square millimeter) were considered salting if they arrested for ≥2 s and adherent if they remained stationary for ≥30 s. Total platelet adhesion was defined as the sum of saltation and adherence. A leukocyte was considered adherent if it remained stationary for ≥30 s (number per square millimeter) and was measured throughout the observation period. 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 venule (number per field).

Experimental Protocol
Venules were selected for observation after a 30-min stabilization period. Platelets (in 120 μL) were infused via the jugular vein over 5 minutes and allowed to circulate for an additional 5 minutes. Mice in the ND, HCD, and HCD-Los groups received platelets from matching donors. In the HCD/HCD-Los group, HCD mice received platelets from losartan-treated hypercholesterolemic (HCD-Los) mice. Five-minute recordings of the leukocytes (light microscopy) followed by 1-minute recordings of the platelets (fluorescent microscopy) were made in the first 100 μm of every 300-μm venule length, 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.

Dihydrorhodamine-123 Oxidation
Separate groups of ND, HCD, and HCD-Los mice were prepared for intravital microscopy. Fluorescence of the first 100 μm of every 300 μm was recorded along the length of the postcapillary venule before (background; Ibgd) and after 15 minutes of superfusion with dihydrorhodamine-123 (DHR; 1 mmol/L [a nonfluorescent dye that is oxidized to the fluorescent compound rhodamine-123]; Itr) as described previously.38 The ratio of Itr/Ibgd was calculated for each section, and the average ratio for each animal was determined.

In Vivo Measurements of P-Selectin and AT1-R Expression
The endothelial surface expression of P-selectin and AT1-R was measured in separate ND, HCD, and HCD-Los groups using the dual-radiolabeled antibody technique.39 AT1-R expression was measured in an additional group of HCD mice receiving 50 mg/kg per day losartan (HCD-High Los). The binding antibodies were RB40.34 rat anti-mouse P-selectin (10 μg/mouse; BD Pharmingen) and N-10 rabbit polyclonal AT1-R (20 μg/mouse; Santa Cruz Biotechnology), which were labeled with 125I (DuPont NEN) using the iodogen method. The nonbinding antibody used for the P-selectin and AT1-R studies was P23 murine anti-human P-selectin (Pharmacia-Upjohn) labeled with 111I (DuPont NEN). Receptor levels were expressed as nanograms of antibody per gram of tissue as described previously.39

Blood Leukocyte and Platelet Counts
At the end of each experiment, blood was drawn from the heart and 25 μL was mixed with 465 μL 3% acetic acid and 10 μL 1% crystal violet. Circulating blood leukocyte count was performed with the aid of a hemocytometer. Circulating platelet counts were achieved using the Unopette system (Becton Dickinson).

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

Statistical Analysis
All values are reported as mean±SEM. ANOVA with Scheffe post hoc test was used for statistical comparisons, with statistical significance set at P<0.05.

Results
Mice placed on a cholesterol-enriched diet (HCD, HCD-Los, and HCD/HCD-Los groups) exhibited a 2- to 3-fold elevation of total serum cholesterol levels when compared with the ND group (Table 1). Coadministration of the AT1-R antagonist losartan did not affect the magnitude of the hypercholesterolemia. Venular WSR, a factor that can influence the adhesion of blood cells in venules, revealed no significant difference between any groups (Table 1). The mean arterial pressure was also comparable between the different groups (Table 1).
Role of AT1-R in Hypercholesterolemia-Induced Leukocyte Recruitment

Mice on HCD exhibited a significant increase in the number of leukocytes adhering in postcapillary venules when compared with the control group (Figure 1). Leukocyte emigration was also elevated in the HCD versus ND mice (Figure 2). However, in those mice receiving losartan (HCD-Los) the leukocyte recruitment was reduced to normocholesterolemic levels (Figure 1). Leukocyte emigration showed a similar response pattern (Figure 2).

Role of AT1-R in Hypercholesterolemia-Induced Platelet Adhesion

The hypercholesterolemia-induced adhesion of leukocytes was accompanied by substantial platelet recruitment. Platelet saltation and adhesion were significantly elevated in the HCD group when compared with ND animals (Figure 3). Treatment with losartan completely prevented these adhesive interactions (transient and firm adhesion) as shown in the HCD-Los group (Figure 3).

<table>
<thead>
<tr>
<th>Groups</th>
<th>SCC, mg/dL</th>
<th>WSR, s⁻¹</th>
<th>MAP, mm Hg</th>
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</thead>
<tbody>
<tr>
<td>ND</td>
<td>77±5.3</td>
<td>803±100.1</td>
<td>56±1.5</td>
</tr>
<tr>
<td>HCD</td>
<td>236±17.6*</td>
<td>606±26.5</td>
<td>53±1.5</td>
</tr>
<tr>
<td>HCD-Los</td>
<td>228±6.8*</td>
<td>832±97.6</td>
<td>53±2.5</td>
</tr>
<tr>
<td>HCD/HCD-Los</td>
<td>241±7.9*</td>
<td>616±18.4</td>
<td>56±1.2</td>
</tr>
</tbody>
</table>

*P<0.0001 vs ND.
SCC indicates serum cholesterol concentration.

Contribution of Endothelial-Associated Versus Platelet-Associated AT1-R in Hypercholesterolemia-Induced Platelet Adhesion

Based on our results, we were left with the question whether the effects of losartan on platelet adhesion were attributable to an action on platelets, endothelial cells, or both. In the ND, HCD, and HCD-Los groups, platelet donor and recipient animals belonged to the same dietary and treatment groups. Therefore, to address the possibility that the endothelium is the cellular target of action of losartan in attenuating platelet–vessel wall adhesion, platelets isolated from HCD-Los mice were administered into untreated hypercholesterolemic mice (HCD/HCD-Los). The recruitment of losartan-treated platelets was not attenuated when administered to HCD mice (Figure 3). In fact, HCD-Los platelets adhered to a greater extent than untreated HCD platelets in untreated HCD recipients, suggesting that the antithrombogenic effect of losartan was exerted on the endothelium rather than the platelets.

Effect of Hypercholesterolemia and Losartan on Oxidant Production by Venules

DHR oxidation, a measure of oxidative stress, was significantly increased in hypercholesterolemic mice when compared with ND mice (3.39±0.288 versus 2.08±0.193, respectively; P<0.01). However, treatment of HCD mice with losartan significantly reduced the hypercholesterolemia-induced oxidant stress toward ND values (2.42±0.182; P<0.05 versus HCD).
Influence of Hypercholesterolemia and Losartan on Endothelial Expression of P-selectin and AT1-R

P-selectin was upregulated in the lung, small bowel (Table 2), colon, and cecum of hypercholesterolemic mice. Losartan did not change the expression of P-selectin. Hypercholesterolemia increased AT1-R expression in the cremaster (Figure 4A), lung, and small bowel (Figure 4B). Treatment with losartan for the second week of HCD blunted the increased AT1-R expression in these organs. The heart, liver, stomach, pancreas, and muscle exhibited similar but smaller changes (data not shown).

Discussion

Ang II is a major contributor to the pathogenesis of prevalent human diseases such as diabetes, hypertension, myocardial infarction, congestive heart failure, and stroke. Several experimental and clinical trials indicate that the role of Ang II in these diseases is mediated through activation of AT1-R because AT1-R blockers have proven to be effective therapeutic agents. Recent evidence also points to the possibility that Ang II (acting through AT1-R) may contribute to the inflammatory responses associated with hypercholesterolemia, a condition known to increase the density of AT1-R on endothelial cells and circulating blood cells. In the present study, we assessed the potential role of AT1-R in mediating the platelet–endothelial and leukocyte–endothelial cell adhesion elicited in the microvasculature by hypercholesterolemia. Our results indicate that AT1-R engagement contributes to the prothrombogenic and proinflammatory phenotype induced in postcapillary venules by hypercholesterolemia. The beneficial effect of AT1-R antagonism on platelet–vessel wall adhesion appears to reflect an action on the endothelial cell rather than the platelet.

Hypercholesterolemia elevates AT1-R expression on vascular cells and on circulating blood cells. Blockade of AT1-R attenuates the hypercholesterolemia-induced super-

![Figure 3. Effects of hypercholesterolemia on the recruitment of saltating (A), adherent (B), and total adherent (C) platelets in mice placed on an ND, HCD, HCD plus treatment of platelet donor and recipient mice with losartan (HCD-Los) and untreated HCD mice receiving platelets from HCD donor mice treated with losartan (HCD/HCD-Los). Platelet recruitment was significantly higher in the HCD group when compared with ND animals. However, the HCD/HCD-Los mice displayed exaggerated responses to hypercholesterolemia. P<0.05 vs ND; #P<0.05 vs HCD; †P<0.05 vs HCD-Los.

![Figure 4. AT1-R expression in the cremaster (A), lung, and small bowel (B) of mice placed on ND, HCD, or HCD treated with 25 mg/kg per day (HCD-Los) or 50 mg/kg per day losartan (HCD-High Los). AT1-R expression was elevated in HCD mice when compared with ND animals. These increases were blunted in the HCD-Los and HCD-high Los groups. P<0.05 vs ND; #P<0.05 vs HCD.

| TABLE 2. P-Selectin Expression (Nanograms of Antibody per Gram of Tissue) in the Lung and Small Bowel of ND, HCD, or HCD-Los Mice |
|-------------------------------|------------------|------------------|
| Groups                        | Lung             | Small Bowel      |
| ND                            | 2.2±0.88         | 2.3±0.25         |
| HCD                           | 10.6±1.13*       | 5.1±0.69*        |
| HCD-Los                       | 11.6±2.36†       | 5.2±0.35†        |

*P<0.05 vs ND; †P<0.005 vs ND.
oxide production in arteries and the accompanying endothelial cell dysfunction. Ang II mediates some of the long-term effects of hypercholesterolemia on large vessels, and it rapidly induces changes in the microvasculature. Topical application of Ang II elicits leukocyte–endothelial interactions in venules that are dependent on endothelial expression of P-selectin. The leukocyte adhesion appears to be initiated by the production of intracellular and extracellular ROS, which occurs via an AT1-R–mediated pathway. In light of our previous finding that postcapillary venules respond to hypercholesterolemia with endothelial cell dysfunction, characterized by an enhanced production of ROS and subsequent adhesion of neutrophils to the vessel wall, we assessed the role of AT1-R in the generation of leukocyte recruitment in the microvasculature of acutely hypercholesterolemic animals. Treatment of mice with the AT1-R antagonist losartan for the second half of a 2-week HCD regimen attenuated hypercholesterolemia-induced leukocyte adhesion and emigration in postcapillary venules. These data suggest that hypercholesterolemia may act via AT1-R on venular endothelial cells or leukocytes to induce adhesion molecule expression, thereby supporting leukocyte–endothelial interactions.

We demonstrated recently that adherent platelets are also recruited into postcapillary venules during hypercholesterolemia. This response is mediated by P-selectin on the platelets and venular endothelial cells. Because hypercholesterolemia is also associated with an increased expression of AT1-R on platelets, we investigated whether AT1-R contributes to the hypercholesterolemia-induced thrombogenic response in venules. When platelet donor and recipient mice were pretreated with losartan, the hypercholesterolemia-induced platelet adhesion response was completely abolished, implicating AT1-R in the prothrombogenic response. An interesting and potentially important observation is that when platelet donor mice (not recipients) were treated with losartan, no beneficial effect on platelet adhesion was noted. Instead, the platelet adhesion response to hypercholesterolemia was exaggerated, suggesting that engagement of the platelet AT1-R by the antagonist conversely activates these blood cells, whereas engagement of the endothelial cell receptor imparts an inhibitory state. The net effect when both cell types are exposed to an AT1-R antagonist during hypercholesterolemia is a dominant inhibitory action on platelet adhesion that is mediated through the endothelial cell.

The oxidative stress induced by hypercholesterolemia can lead to increased endothelial cell adhesion molecule expression and the subsequent recruitment of adherent blood cells. Such an oxidant-mediated process is consistent with our previous observation that superoxide generated from p47phox-containing NAD(P)H oxidase in the venular wall and circulating cells is critical for hypercholesterolemia-induced leukocyte adhesion. Interestingly, AT1-R activation enhances production of ROS in several cell types by initiating the phosphorylation of NAD(P)H oxidase subunits such as p47phox with subsequent activation of this enzyme. Here, we observed that inhibition of AT1-R signaling with losartan significantly reduced the oxidative stress observed in venules during hypercholesterolemia. Thus, it is conceivable that losartan is exerting its inhibitory effects on leukocyte and platelet recruitment in our model by blocking hypercholesterolemia-induced NAD(P)H oxidase activation. It is noteworthy that losartan appears to exert some of its protective actions via enhancement of NO levels. Hence, losartan may protect the microvasculature by reducing hypercholesterolemia-induced superoxide production, which would minimize the inactivation of NO, thereby preserving an anti-inflammatory and antithrombogenic environment in postcapillary venules.

It has been demonstrated that the leukocyte recruitment induced by hypercholesterolemia is mediated by P-selectin and that endothelium- and platelet-associated P-selectin mediates platelet adhesion. Furthermore endothelial P-selectin mediates Ang II–induced leukocyte recruitment, and our findings here suggest that losartan is exerting its inhibitory effect on the endothelium. Therefore, an important question is whether losartan interferes with upregulation of vascular P-selectin. Our results indicate that although P-selectin expression was increased by hypercholesterolemia in several vascular beds, this response was not altered by blocking AT1-R, suggesting that the protective effects of losartan involve an action on the vessel wall that is independent of P-selectin. This could involve the modulation of a different adhesion molecule (eg, ICAM-1) or may reflect an effect of losartan on the production of inflammatory mediators induced by hypercholesterolemia.

Because engagement of AT1-R induces inflammatory pathways that can further increase the expression of AT1-R, we examined whether just 2 weeks of high cholesterol feeding could induce AT1-R expression and whether this response was altered by losartan treatment. We found that AT1-R expression was significantly increased in an organ-specific manner in hypercholesterolemic mice and that the AT1-R upregulation was prevented by losartan. There are at least 2 possible explanations for the latter observation. First, losartan may bind to the same site on AT1-R as the antibody (N-10) used to quantify receptor expression, thereby resulting in an underestimation of AT1-R expression. Second, the prevention of Ang II–mediated signaling may prevent any positive feedback on AT1-R that would otherwise promote its upregulation. For example, cytokines (eg, IL-6) have been shown to induce AT1-R expression, whereas chronic inhibition of NO synthase upregulates AT1-R expression, suggesting that any actions of losartan to inhibit proinflammatory (cytokine) pathways while preserving anti-inflammatory (NO) pathways would result in an attenuation of the AT1-R upregulation associated with hypercholesterolemia.

Collectively, our results support a role for AT1-R in mediating the proinflammatory phenotype induced in postcapillary venules by hypercholesterolemia. In addition, losartan appears to exert its antithrombogenic effect primarily through engagement of endothelium-associated AT1-R, whereas the drug exerts a direct nonspecific effect.
on platelets that leads to an exaggerated platelet adhesion response in hypercholesterolemic venules. Although in many instances, the primary target of AT1-R antagonists is blood pressure, several clinical trials with these drugs have revealed unexpected benefits that are unrelated to antihypertensive effects, primarily through reducing ROS generation and elevating NO bioavailability. Our findings not only support a role for losartan in modulating the oxidative stress associated with hypercholesterolemia but also implicate novel avenues through which AT1-R blockers can affect disease induction or progression (ie, by targeting circulating inflammatory cells and vascular endothelial cells) and by preventing upregulation of AT1-R. Our novel findings on the actions of losartan on platelet adhesion also raise the appealing possibility that treatment with AT1-R blockers may reduce the incidence of thrombotic events in individuals who are at risk for CVD.

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References


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