Platelet-Derived RANTES Mediates Hypercholesterolemia-Induced Superoxide Production and Endothelial Dysfunction


Background—Hypercholesterolemia (HC) is known to elicit oxidative stress and impair endothelium-dependent vasodilation (EDV) in arterioles and large arteries. Although RANTES, a chemokine that promotes the recruitment of leukocytes, has been implicated in atherosclerosis, its role in HC has not been previously evaluated.

Methods and Results—Wire myography, a cytochrome C reduction assay, and RT-PCR were used to assess the HC-induced responses of aortic rings from wild-type (WT), RANTES-deficient (RANTES−/−), bone marrow chimeras (WT→WT, RANTES−/−→WT, and WT→RANTES−/−), and WT mice receiving antiplatelet serum (APS) to induce thrombocytopenia. HC led to superoxide (O2•−) production, Nox-2 expression, and EDV dysfunction in WT mice with a corresponding increase in plasma RANTES concentration. The HC-induced responses were absent in RANTES−/−, RANTES−/−→WT chimeras, and APS-treated WT mice. Exposure of WT aortic rings to RANTES elicited EDV impairment and O2•− production, which were blocked by incubation with heparin or metRANTES. Aortic rings from CD44-deficient mice exhibited responses similar to WT after RANTES incubation, suggesting that CD44 does not act as an auxiliary receptor in RANTES-mediated responses with HC.

Conclusions—These findings are consistent with a mechanism whereby HC promotes platelet release of RANTES, inducing a glycosaminoglycan- and CCR-dependent enhancement of O2•− production with impairment of EDV.

Key Words: ●●●

Hypercholesterolemia (HC) is a significant risk factor for the development of cardiovascular disease (CVD), a leading cause of morbidity and mortality worldwide. Several mechanisms have been proposed to explain the deleterious effects of HC on the vasculature, including oxidative stress, activation of the renin–angiotensin system, platelet hyperactivity, and immune cell activation. Like immune cells and platelets, endothelial cells are also activated in response to HC. This endothelial cell dysfunction appears to contribute to both the early vasomotor dysfunction and later atherosclerotic lesion development that are associated with HC. Although there is significant evidence implicating immune cells in both the early and late endothelium-dependent vascular pathology in HC, less is known about the role of platelets in this disease process and whether platelets act in concert with immune cells to mediate HC-induced endothelial cell dysfunction. There are several lines of evidence that suggest that platelets may contribute to the vascular disease associated with HC, including: (1) circulating platelets are rendered hyperactive by HC and exhibit an increased expression of P-selectin, (2) blockade of platelet adhesion, by targeting either platelet-associated GPIIb-IIIa or P-selectin in apoE−/− mice reduces leukocyte accumulation and plaque development in large arteries, (3) inhibition of platelet cyclooxygenase activity attenuates lesion development in apoE−/− mice, and (4) HC-induced arteriolar dysfunction is blunted in thrombocytopenic mice and in mice with genetic deficiency of platelet-associated P-selectin.

Platelets produce and secrete on activation a variety of substances that could account for their ability to mediate vascular pathology. RANTES (Regulated on Activation Normal T-cell Expressed and Secreted) is a chemokine secreted by activated platelets that promotes the recruitment of leukocytes to sites of inflammation and vessel injury. Several recent studies have implicated this chemokine in the development of atherosclerosis, providing evidence that (1) RANTES secreted by thrombin-stimulated platelets is immobilized on the surface of microvascular and aortic endothelium where it mediates monocyte recruitment, (2) a reduction of platelet-derived RANTES attenuates HC-induced recruitment of monocytes to the endothelium of apoE−/− mice, and (3) antagonism of RANTES receptors limits plaque formation in HC mice. While there is compelling evidence that invokes a role for platelet-derived RANTES in the development of atherosclerotic lesions during HC, the potential contribution of this chemokine to the early vasomotor dysfunction and NADPH oxidase-mediated oxidative stress induced by HC has not been previously addressed.
The overall objective of this study was to determine whether platelet-derived RANTES contributes to the endothelial dysfunction and enhanced superoxide production that are elicited in large arteries by hypercholesterolemia. Because RANTES is a highly cationic molecule that has been shown to exert its actions on immune cells and endothelial cells via interactions with cell surface glycosaminoglycans (GAGs), we also assessed the potential contribution of GAGs to the RANTES-mediated vascular responses. Inasmuch as previous work on HC-induced oxidative stress and vasomotor dysfunction invokes a role for immune cells and cytokines (IFN-γ) derived there-from, the possibility that HC-induced RANTES release is modulated by CD4+ T-cells and IFN-γ was also evaluated. Our findings are consistent with a mechanism whereby hypercholesterolemia causes platelets to release RANTES, which induces a GAG-dependent enhancement of O₂⁻ production from Nox-2 and the subsequent impairment of endothelium-dependent vasodilation.

Materials and Methods

Animals and Experimental Groups
Wild-type (WT), CD4+ T-cell deficient (CD4+/-), homozygous for the Cd4tm1Mak mutation and interferon (IFN)-γ-deficient (IFN-γ−/−); homozygous for the Ifngtm1sf mutation, and CD44-deficient (CD44−/−) mice, all on a C57BL/6J background, were obtained from Jackson Laboratories (Bar Harbor, Me). RANTES-deficient (RANTES−/−) mice (on C57BL/6J background) were obtained from S. Sarawar at the Torrey Pines Institute for Molecular Studies, San Diego. A breeding colony for the RANTES mice was established at the animal-resource facility of LSU Health Sciences Center-Shreveport. RANTES−/− mice were also used for the generation of bone marrow chimeras as described further in the supplemental material (available online at http://atvb.ahajournals.org). Briefly, irradiated WT mice received RANTES−/− bone marrow, such that only circulating blood cells were deficient in RANTES (RANTES−/−→WT), or irradiated RANTES−/− mice received WT bone marrow such that only the vessel wall lacked RANTES (WT→RANTES−/−). In addition, CD4+ T-cells isolated from WT-HC mice were adoptively transferred into either IFN-γ−/− HC (CD4+ T-cells→IFN-γ−/−HC or RANTES−/−HC (CD4+ T-cells→RANTES−/−HC) mice, as detailed in the supplemental methods file. The mice had ad libitum access to a standard diet and water until reaching the desired age (5 to 6 weeks). The mice were placed on either a normal diet (ND) or cholesterol-enriched (HC) diet (Teklad 90221 with 1.25% cholesterol, 0.125% choline chloride, 15.8% fat; Harlan Teklad) for 2 weeks (n=5 per group).

Experimental Protocol
Aortas were obtained from all groups for measurement of endothelium-dependent vasodilatation, or endothelium-independent contraction (phenylephrine) using wire myography on 2-mm aortic rings as described previously. Superoxide production by aortic tissue was determined using real-time PCR (RT-PCR). Details of these methodologies can be found in the supplemental file. Serum from CD4+ T-cells/IFN-γ-/- mice, WT-ND, WT-HC, CD4+/-HC, and IFN-γ-/-HC mice was taken for use in an ELISA assay (RD Systems Minneapolis) to examine the effects of CD4+ T-cells and CD4+ T-cell-derived IFN-γ on plasma RANTES concentration.

RANTES Incubation Experiments
In 3 series of experiments, aortic rings dissected from WT or mutant (RANTES−/- or CD44−/-) mice were incubated with recombinant RANTES (R&D Systems Minneapolis) at a concentration of 10 ng/mL for a 4-hour period. In some experiments, the rings were incubated with RANTES for 15 or 60 minutes. Aortic rings from WT mice, placed on either ND or HC, were incubated with heparin (50 ng/mL) for 4 hours. Another group of aortic rings obtained from WT mice was incubated with recombinant RANTES (10 ng/mL) and either heparin (50 ng/mL) or the RANTES receptor antagonist metRANTES (1 μg/mL) for a 4-hour period. In a final series of experiments, aortic rings from WT mice were incubated with either recombinant RANTES (10 ng/mL) with catalase, or catalase alone for a 4-hour period.

Statistical Analysis
All values are reported as mean±SE. ANOVA (Scheffe) was used for statistical comparison of the experimental groups with statistical significance set at P<0.05.

A detailed materials & methods section can be found in the supplemental materials (available online at http://atvb.ahajournals.org).

Results
Serum cholesterol concentration in WT and mutant mice placed on HC (189.45±17.53 mg/dL) was significantly (P<0.05) higher than that detected in their ND counterparts (54.55±10.09 mg/dL). No significant differences in serum cholesterol concentration were noted between any of the mutant or chimera mice groups placed on the ND or HC chow when compared to their same diet counterparts (data not shown).

The comparison of dose-response curves for endothelium-independent contraction (PE dose-response curve) and dilation (SNP dose-response curve) from aortic rings of WT or mutant mice placed on ND or HC revealed no statistically significant differences (data not shown). Endothelium-dependent vasodilation (acetylcholine dose-response curve) was significantly attenuated in the aortic rings of WT-HC mice when compared to the WT-ND controls (Figure 1A). This HC-induced endothelium-dependent vasomotor impairment in WT-HC mice was accompanied by an increased vascular superoxide production (Figure 1B). The HC-induced vasomotor dysfunction and increased O₂⁻ production were not detected in the RANTES−/− mice (Figure 1A and 1B). To determine whether the RANTES mediating the HC-induced vascular responses was derived from circulating blood cells versus non–bone marrow–derived cells within or outside the vessel wall, vasomotor dysfunction and O₂⁻ production were evaluated in aortic tissue derived from RANTES−/− bone marrow chimeras (Figure 1A and 1B). RANTES−/−→WT-HC mice exhibited endothelium-dependent vasodilation and superoxide production responses similar to those noted in WT→WT-ND controls. This attenuation of HC-induced endothelial dysfunction and increased vascular O₂⁻ production was not observed in the WT→RANTES−/−HC or WT→WT-HC chimeras, suggesting that RANTES derived from circulating blood cells, not from the vessel wall, mediate the HC-induced oxidative stress and endothelium-dependent vasomotor dysfunction.

The results summarized in Figure 2 demonstrate that 4-hour incubation of aortic rings from WT-ND mice with 10 ng/mL RANTES elicits an impaired acetylcholine-induced vasodilatory response and increased O₂⁻ production comparable to that observed in aortic tissue from WT-HC mice.
Shorter incubation times (15 or 60 minutes) did not elicit the responses noted with 4-hour incubation. The RANTES-mediated responses were not altered by coincubation with catalase. Similar responses were induced by RANTES incubation of aortic rings derived from normocholesterolemic RANTES−/− and RANTES−/−/− mice. These findings demonstrate that RANTES can directly act on aortic tissue to increase superoxide production and impair the vasomotor response, and indicates that prolonged RANTES deficiency (either in all cells or only in circulating blood cells) does not alter the responsiveness of aortic tissue to the chemokine.

Figure 1. Acetylcholine-induced (10−4 mol/L) dilation of (A) and superoxide production (B) by aortic rings from wild-type (WT) and RANTES-deficient (RANTES−/−) mice, as well as wild-type to wild-type (WT→WT), RANTES−/− to WT (RANTES−/−→WT), and WT to RANTES−/− (WT→RANTES−/−) bone marrow chimeras. Mice in each group were placed on either a normal (ND) or cholesterol-enriched (HC) diet. *P<0.05 vs WT-ND; #P<0.05 vs WT-HC; †P<0.05 vs WT→WT-ND; ‡P<0.05 vs WT→WT-HC. n=5 mice per group.

Figure 2. Acetylcholine-induced (10−4 mol/L) dilation (A) of and superoxide production (B) by aortic rings from wild-type (WT) mice on a normal diet (ND), wild-type (WT) mice on a cholesterol-enriched (HC) diet, RANTES-deficient (RANTES−/−) mice on ND, and RANTES−/− to WT (RANTES−/−→WT) bone marrow chimeras on ND. In one series of experiments, WT, RANTES−/−, and RANTES−/−→WT groups were incubated with 10 ng/mL RANTES for 4 hours. *P<0.05 vs WT-ND; #P<0.05 vs WT-HC. n=5 mice per group.
Figure 3 illustrates that WT mice rendered thrombocytopenic (blood platelet count reduced by 93%) with rabbit antimouse platelet antiserum (APS) do not exhibit the impaired vasomotor function and increased superoxide production normally induced by HC, suggesting that CD4+ T-cells are an unlikely source of the chemokine in this model.

Figure 4 summarizes the changes in acetylcholine-induced vasodilation and superoxide production in response to HC or RANTES incubation in the presence or absence of heparin. Previous work by others indicates that RANTES can use cell surface GAGs to mediate cell activation signals and that heparin can block these GAG-mediated signals.27,30 We noted that heparin incubation ablates the impaired vasomotor response as well as the increased superoxide production induced by either HC or RANTES incubation with ND control vessels. In addition, we noted that WT-ND rings incubated with the combination of RANTES and met-RANTES (a RANTES receptor antagonist) exhibited blunted EDV impairment and superoxide production, compared to incubation with RANTES alone. RANTES-induced O2− superoxide production and EDV dysfunction were not attenuated in rings from CD44−/− mice. Collectively, these findings indicate that RANTES-mediated EDV dysfunction and increased superoxide formation is dependent on both activation of the CCR5 receptor and an interaction with endothelial GAGs (which presumably elevates local RANTES concentration for interaction with the CCR5 receptors). The RANTES-mediated responses do not appear to involve the proteoglycan CD44, as previously proposed for other disease models.28

We have previously shown that the HC model used in this study elicits an increased expression of Nox-2 mRNA in mouse aortic tissue and that Nox-2 is the major source of the enhanced superoxide production in these vessels.14 The Table summarizes the changes in Nox-2 mRNA in aortic tissue derived from the different experimental groups of the present study. Nox-1 and Nox-4 mRNA was detected in all samples, however its expression did not differ between groups (data not shown). Whereas HC elicited an increased Nox-2 expression in WT mice, neither RANTES−/− mice, RANTES−/− → WT chimeras, nor thrombocytopenic (APS-treated) WT-HC mice exhibited a significant change in Nox-2 mRNA in response to HC. Aortic rings from WT mice incubated with RANTES for 4 hours also exhibited an increased expression of Nox-2 mRNA that was attenuated by the addition of heparin or the CCR5 receptor antagonist metRANTES. In addition, aortic rings derived from CD44-deficient mice exhibited no change in RANTES-induced Nox2 mRNA expression, when compared to WT controls. These findings suggest that RANTES elicits vascular Nox-2 mRNA expression via a mechanism that involves GAG binding and CCR5 activation, and is not dependent on the proteoglycan CD44.

Figure 5 summarizes the changes in plasma RANTES concentration that are elicited by HC in different experimental groups. WT-HC mice exhibited a large increase in plasma RANTES concentration, which was not observed when the mice were rendered thrombocytopenic with APS. Because previous work from our laboratory implicates CD4+ T-cells...
and T-cell-derived IFN-γ in the vasomotor dysfunction and increased superoxide production elicited by HC, we also evaluated the influence of genetic deletion of these factors on the HC-induced elevation in plasma RANTES. HC mice deficient in either CD4+ T-cells or IFN-γ also exhibited an attenuation of HC-induced RANTES secretion. However, the adoptive transfer of WT-HC CD4+ T-cells into IFN-γ-deficient HC mice restored the HC-induced elevation in plasma RANTES concentration, suggesting a role for CD4+ T-cell-mediated IFN-γ signaling in the platelet-dependent changes in plasma RANTES.

**Discussion**

RANTES (CCL5), a member of the CC-chemokine family, has been implicated in a variety of chronic inflammatory diseases, including atherosclerosis. It is generally held that the primary biological action of RANTES is to recruit and activate leukocytes at sites of inflammation. Although this chemokine is also known to elicit different biological responses in immune cells, such as proliferation and apoptosis, its actions on other cell populations remain poorly defined. The results of this study provide evidence for novel biological actions of RANTES that may explain the impaired vascular function that is associated with different risk factors for cardiovascular disease. We demonstrate for the first time that RANTES is a major determinant of the oxidative stress and vasomotor dysfunction that is induced in large arteries by hypercholesterolemia. In addition, we show that exogenous RANTES acts directly on arterial tissue to increase the expression of Nox-2 (NADPH oxidase) mRNA, enhance superoxide production, and inhibit vessel reactivity to the endothelium-dependent vasodilator acetylcholine. These findings, coupled to our observation that HC is associated with a significantly elevated RANTES concentration in plasma, support a role for RANTES as a mediator of ROS production and endothelial cell dysfunction induced by hypercholesterolemia.

RANTES is produced by a variety of cell populations, including platelets, T-lymphocytes, endothelial cells, mast cells, and vascular smooth muscle. By comparing the

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**Table. gp91phox (Nox-2) mRNA Expression in Aortic Tissue Determined by RT-PCR**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Relative gp91phox mRNA Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-ND</td>
<td>1.00±0.08</td>
</tr>
<tr>
<td>WT-HC</td>
<td>2.82±0.74*</td>
</tr>
<tr>
<td>RANTES–/-ND</td>
<td>1.01±0.11#</td>
</tr>
<tr>
<td>RANTES–/-HC</td>
<td>0.93±0.19#</td>
</tr>
<tr>
<td>WT→WT-ND</td>
<td>1.03±0.08#</td>
</tr>
<tr>
<td>WT→WT-HC</td>
<td>3.09±0.54*</td>
</tr>
<tr>
<td>RANTES–/-→WT-ND</td>
<td>0.88±0.14#</td>
</tr>
<tr>
<td>RANTES–/-→WT-HC</td>
<td>1.01±0.26#</td>
</tr>
<tr>
<td>WT-ND (APS)</td>
<td>1.17±0.21#</td>
</tr>
<tr>
<td>WT-ND (APS)</td>
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</tr>
<tr>
<td>WT-RANTES incubation</td>
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</tr>
<tr>
<td>WT-RANTES/heparin incubation</td>
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</tr>
<tr>
<td>WT-RANTES/metRANTES incubation</td>
<td>1.06±0.32#</td>
</tr>
<tr>
<td>CD44–/- RANTES incubation</td>
<td>2.03±0.28#</td>
</tr>
</tbody>
</table>

Each group was placed on either a normal diet (ND) or high-cholesterol diet for 2 weeks (HC). gp91phox levels were normalized to GAPDH and expressed relative to the gp91phox mRNA expression of the WT-ND group. n=5 mice per group.

*P<0.05 vs WT-ND; #P<0.05 vs WT-HC.
The molecular mechanisms that underlie the ability of RANTES to mediate the impaired vascular function in HC remain poorly defined. However, our results, coupled with previous reports demonstrating a critical role for vessel wall Nox-2 in the HC-induced superoxide production and vasomotor dysfunction, suggest that RANTES is likely to be mediating its effects via NADPH oxidase upregulation/activation.14,40 HC-induced upregulation of Nox-2 mRNA was prevented by RANTES deficiency, in RANTES−/−→WT chimeras, and in thrombocytopenic WT mice, suggesting that platelet-derived RANTES regulates Nox-2 expression in vascular endothelium. Collectively, our findings are consistent with a mechanism wherein RANTES acts on endothelial cells to increase Nox-2 levels, which results in accelerated rates of superoxide production. The impaired endothelium-dependent vasodilation likely reflects an effect of the enhanced superoxide flux to reduce the bioavailability of nitric oxide, which is known to mediate acetylcholine-induced endothelium-dependent vasodilation. The proposed action of RANTES on superoxide production via NADPH oxidase during HC is consistent with the results of a recent report that describes an enhanced superoxide production and impaired endothelium-dependent vasodilation in rat aorta incubated with the CX3C chemokine fractalkine.41 The fractalkine-mediated EDV response was prevented by coincubation of the aortic ring with tiron, and the enhanced superoxide production was significantly attenuated by treatment with the nonspecific NADPH oxidase inhibitor diphenyliodonium, but not the xanthine oxidase inhibitor allopurinol. These observations with fractalkine and our findings with RANTES suggest that chemokines may play a more important role in the vasoregulatory dysfunction associated with inflammation than previously considered.

An interesting and potentially important observation in this study is the ability of heparin to reduce superoxide production and restore endothelium-dependent vasodilation in aortic rings derived from HC mice and to exert the same protective effects in rings incubated with RANTES. Although endothelial cell express G protein–coupled receptors for RANTES (CCR1, CCR3, CCR5), the density of these receptors is low compared to other cell populations, such as T-lymphocytes.42 Nonetheless, this positively charged chemokine binds avidly to vascular endothelium by interacting with cell surface GAGs,30 which is believed to confer selectivity to the chemokine in mediating tissue-specific leukocyte recruitment. RANTES binding studies to isolated cell systems have revealed that the GAG-RANTES interactions can promote cell signaling in two ways: (1) it can create a high local concentration of the chemokine in the vicinity of its receptors, which facilitates cell signaling when receptor density is low, as with endothelial cells,43 and (2) it can activate cells via a receptor-independent mechanism wherein RANTES binds to GAG chains of the proteoglycan CD44 leading to activation of a MAPK pathway.33 Because the affinity interactions between GAGs and RANTES can be ablated by heparin,27 our results with heparin suggest that the HC-induced RANTES-mediated increase in Nox-2 expression and superoxide production, and impaired vasomotor function likely involves one of the aforementioned GAG-dependent signal-
ing pathways. Because vascular rings derived from CD44-null mice exhibited responses to RANTES that were similar to those elicited in rings from WT mice, and in view of the observed inhibitory actions of met-RANTES in our model, it appears likely that endothelial GAGs are acting to facilitate the interaction of RANTES with CCR5 on endothelial cells, rather than acting via a CD44-dependent pathway. Such a mechanism appears tenable because G protein–coupled signaling pathways have been implicated in the activation of NADPH oxidase.44

Recent work from our laboratory has implicated CD4+ T-lymphocytes and CD4+ T-cell–derived IFN-γ in the oxidative stress and endothelium-dependent vasomotor dysfunction induced by HC in mice.14 These observations, coupled to the results of the present study that implicate platelet-derived RANTES in the same responses to HC, raise the possibility that CD4+ T-cells and IFN-γ may mediate the release of RANTES from platelets during HC. Our measurements of plasma RANTES concentration in hypercholesterolemic mice deficient in either CD4+ T-cells or IFN-γ support this possibility, as well as our observation that the HC-induced elevation of plasma RANTES is restored by adoptive transfer of CD4+ T-cells into IFN-γ–deficient mice. Such a link between platelet release of RANTES and IFN-γ is also consistent with reports demonstrating a significantly elevated plasma IFN-γ concentration in WT-HC mice45 and the presence of IFN-γ receptors on platelets.46 Alternatively, it is conceivable that IFN-γ does not act directly on platelets to elicit the release of RANTES, but exerts its effects through an intermediate blood cell, which in turn directly or indirectly activates platelets to release RANTES. Additional work is needed to determine whether and how the release of platelet-derived RANTES is modulated by immune cells in the setting of HC.

In conclusion, our findings have revealed a novel role for RANTES in the NADPH oxidase–mediated oxidative stress and impaired vasomotor function associated with HC. The data are consistent with circulating blood cells, most likely platelets, as a major source of the RANTES that mediates the HC-induced vascular responses. Inverse cell–mediated activation of platelets and GAG-dependent CCR5 receptor activation on endothelial cells are also implicated in these responses. The data supports RANTES as a potential therapeutic target for the vascular pathology induced by HC.

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Disclosures
None.

References
Platelet-Derived RANTES Mediates Hypercholesterolemia-Induced Superoxide Production and Endothelial Dysfunction
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