Endothelial Microparticle Formation by Angiotensin II Is Mediated via Ang II Receptor Type I/NADPH Oxidase/Rho Kinase Pathways Targeted to Lipid Rafts

Dylan Burger, Augusto C. Montezano, Nobuhiro Nishigaki, Ying He, Anthony Carter, Rhian M. Touyz

Objective—Circulating microparticles are increased in cardiovascular disease and may themselves promote oxidative stress and inflammation. Molecular mechanisms underlying their formation and signaling are unclear. We investigated the role of reactive oxygen species (ROS), Rho kinase, and lipid rafts in microparticle formation and examined their functional significance in endothelial cells (ECs).

Methods and Results—Microparticle formation from angiotensin II (Ang II)–stimulated ECs and apolipoprotein E−/− mice was assessed by annexin V or by CD144 staining and electron microscopy. Ang II promoted microparticle formation and increased EC O2•− generation and Rho kinase activity. Ang II–stimulated effects were inhibited by irbesartan (Ang II receptor type I blocker) and fasudil (Rho kinase inhibitor). Methyl-β-cyclodextrin and nystatin, which disrupt lipid rafts/caveolae, blocked microparticle release. Functional responses, assessed in microparticle-stimulated ECs, revealed increased O2•− production, enhanced vascular cell adhesion molecule/platelet-EC adhesion molecule expression, and augmented macrophage adhesion. Inhibition of epidermal growth factor receptor blocked the prooxidative and proinflammatory effects of microparticles. In vitro observations were confirmed in apolipoprotein E−/− mice, which displayed vascular inflammation and high levels of circulating endothelial microparticles, effects that were reduced by apocynin.

Conclusion—We demonstrated direct actions of Ang II on endothelial microparticle release, mediated through NADPH oxidase, ROS, and Rho kinase targeted to lipid rafts. Microparticles themselves stimulated endothelial ROS formation and inflammatory responses. Our findings suggest a feedforward system whereby Ang II promotes EC injury through its own endothelial-derived microparticles. (Arterioscler Thromb Vasc Biol. 2011;31:1898-1907.)

Key Words: angiotensin II ■ endothelium ■ reactive oxygen species ■ signal transduction ■ microparticles

Microparticles are submicron (0.1 to 1.0 μm) fragments that arise from plasma membrane blebbing and subsequently shed from activated or apoptotic cells.1 Microparticles are present in plasma of healthy subjects, and their levels are elevated in disease states such as in hypertension,2 atherosclerosis,1 hyperlipidemia,3 and chronic kidney failure.4–6 Although it is believed that all cells are capable of producing microparticles, most studies have focused on microparticles derived from cells of the vasculature (platelets, endothelial cells [EC], leukocytes). Platelet-derived microparticles promote coagulation,7–9 and leukocyte-derived microparticles promote oxidative stress and apoptosis in ECs.10–13 Circulating endothelial microparticles are increased in conditions of vascular disease, and an elevation in plasma endothelial microparticle levels has been considered as a biomarker of vascular damage.4,14 Microparticles have been reported to impair angiogenesis, promote oxidative stress, and impair vasorelaxation, and they themselves produce reactive oxygen species (ROS).15,16 Thus, endothelial microparticles may be more than just biomarkers but circulating bioactive effectors of vascular damage. However, mechanisms whereby microparticles regulate vascular cell function and molecular processes of activation remain elusive.

Plasma microparticle levels are elevated in conditions where angiotensin II (Ang II) is implicated. For example, patients with hypertension, diabetes, or hyperlipidemia exhibit elevated plasma platelet and endothelial-derived microparticles.17 Treatment with Ang II receptor type 1 (AT1) blockers and lipid-lowering agents, such as simvastatin, decreases plasma microparticle levels.3,17–19 As yet, it is unclear whether there is a direct effect of Ang II on microparticle formation or whether these reductions in plasma microparticle levels occur as an indirect consequence of improved vascular health. Although exact processes facilitating microparticle generation await clarification, lipid-rich domains, including lipid rafts/caveolae, have been implicated in platelet and monocyte microparticle formation,20,21 and it has been suggested that reorganization of cellular cytoskeletal...
organization through pathways involving Rho/Rho kinase and calpain may be involved.22,23

In the present study, we investigated molecular mechanisms underlying EC microparticle formation, focusing on the putative role of ROS and Rho kinase, through cholesterol-rich microdomains, in Ang II–stimulated cells, and we evaluated whether microparticles themselves influence EC function. In vitro studies using cultured mouse aortic ECs demonstrated that Ang II increases endothelial microparticle release via NADPH oxidase-derived ROS generation through processes involving Rho kinase targeted to lipid rafts. Microparticles stimulated EC ROS formation, induced expression of proinflammatory mediators, and increased macrophage adhesion. Using an in vivo model of Ang II–infused apolipoprotein E (ApoE)−/− hyperlipidemic mice, a model of endothelial dysfunction, we extended our in vitro studies to demonstrate that Ang II infusion increases plasma endothelial microparticle levels in a redox-sensitive, blood pressure–independent manner.

Methods
See supplemental material, available online at http://atvb.ahajournals.org, for a detailed Methods section.

Cell Culture and Animals
This study was approved by the University of Ottawa Animal Ethics Committee and performed according to the recommendations of the Canadian Council for Animal Care.

Cell Culture
For studies on acute in vitro effects of Ang II on microparticle formation, mouse aortic ECs were isolated and cultured as described.24

In Vivo Studies
Ang II–treated C57BL6 wild-type (WT) and ApoE−/− mice (males, 6 months, Jackson Laboratory) were studied. Ang II–infused ApoE−/− mice were used as a model of vascular damage, oxidative stress, and inflammation.25 Some mice were cotreated with the putative role of ROS and Ang II, through cholesterol-rich microdomains, in Ang II–stimulated cells, and we evaluated whether microparticles themselves influence EC function. In vitro studies using cultured mouse aortic ECs demonstrated that Ang II increases endothelial microparticle release via NADPH oxidase-derived ROS generation through processes involving Rho kinase targeted to lipid rafts. Microparticles stimulated EC ROS formation, induced expression of proinflammatory mediators, and increased macrophage adhesion. Using an in vivo model of Ang II–infused apolipoprotein E (ApoE)−/− hyperlipidemic mice, a model of endothelial dysfunction, we extended our in vitro studies to demonstrate that Ang II infusion increases plasma endothelial microparticle levels in a redox-sensitive, blood pressure–independent manner.

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Treatment of ECs With Endothelial Microparticles
Microparticles were isolated from cultured mouse aortic ECs in basal conditions as described above and quantified by flow cytometry (annexin V positivity). ECs were treated with microparticles (105 microparticles/mL).

Electron Microscopy
Microparticles, sorted by flow cytometry, were examined by electron microscopy.

Cholesterol Depletion
Cholesterol depletion and repletion experiments were performed using methyl−β−cyclodextrin (MBC) and nystatin.30

Western Blotting
Protein expression was examined in cultured ECs, mesenteric arteries of mice, and microparticles isolated from the medium of cultured ECs. Membranes were incubated with the following antibodies: anti-flotillin-2 (1:4000), anti–caveolin-1 (1:2000), anti–endothelial nitric oxide synthase (1:2000) anti–glyceroldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000), anti–proliferating cell nuclear antigen (1:2000), anti–heparin-binding epidermal growth factor (EGF)–like growth factor (1:1000), anti–platelet-EC adhesion molecule (PECAM) (1:2000), anti–vascular cell adhesion molecule (VCAM-1) (1:2000), or anti–vascular-endothelial cadherin (VE-cadherin) (1:2000). Washed membranes were incubated with horse-radish peroxidase–conjugated second antibodies (1:2000) and probed for immunoreactive proteins by chemiluminescence. Blots were analyzed densitometrically.

Measurement of Rho Kinase Activity
Rho kinase activity was assessed in ECs using the ROCK Activity Assay Kit (Cell Biolabs Inc).

Measurement of Superoxide Production and NADPH Oxidase Activity
Superoxide (O_2−) production was measured in ECs by dihydroethidium–high-performance liquid chromatography as described by Laurindo et al.31 NADPH oxidase–derived O_2− generation was measured in ECs by lucigenin chemiluminescence.32

Measurement of Macrophage Adhesion
Growth-arrested ECs were stimulated with microparticles (10^5 microparticles/mL, 8 hours). Murine J774A.1 macrophages (American Type Culture Collection) were labeled with calcine-acetoxyethyl ester (1 μmol/L, Molecular Probes). Fluorescently labeled J774A.1 cells (10^5 cells/mL) were added to microparticle-stimulated and unstimulated ECs and allowed to adhere (30 minutes, 37°C). Nonadherent cells were removed by washing in Hanks’ solution. Adherence was measured quantitatively by lysing cells in 0.1 mol/L NaOH and measuring fluorescence (Flowstar Galaxy, BMG, excitation 485 nm, emission 520 nm).

Microparticle Labeling and Immunohistochemistry
To evaluate physical interaction between microparticles and ECs, microparticles were loaded with carboxyfluorescein diacetate succinimidy ester (CFSE) (10 μmol/L, Molecular Probes), a nonfluorescent cell permeable compound that fluoresces when exposed to intracellular esterases.33 ECs were exposed to CFSE-
labeled microparticles (24 hours), after which cells were fixed in 20% acetone/80% methanol and incubated with anticaveolin-1 antibody, followed by labeling with a rhodamine-conjugated secondary antibody. Nuclei were stained with Hoechst 33342 (1 μg/mL), and cells were visualized with Zeiss Axioskop 2 MOT (magnification ×63).

Statistical Analysis
Data are presented as means±SEMs. Groups were compared using the Student t test, 1-way ANOVA, or 2-way ANOVA with a Newman-Keuls post test as appropriate. P<0.05 was significant.

Results

Microparticle Characterization
Microparticles were isolated from EC culture media (in vitro studies) and from plasma (in vivo studies) and identified by 3 methods: (1) size (0.1 to 1.0 μm), (2) positive staining for annexin V (annexin V−ve), and (3) morphologically by electron microscopy. Endothelial microparticles were further defined as staining positive for CD144. Cells were sorted by flow cytometry, and microparticles were identified as those events measuring 0.1 to 1.0 μm (Figure 1). Figure 1B demonstrates annexin V−ve events and Figure 1D CD144+ events, confirmed to be microparticles, as evidenced by characteristic size and morphology by electron microscopy (Figure 1E and 1F).

In Vitro Studies

Microparticle Formation Derived From Mouse Aortic ECs Is Increased by Ang II Through Redox-Sensitive Processes
The effects of Ang II on microparticle formation by mouse aortic ECs were examined in vitro. Ang II increased endothelial microparticle formation, as evidenced by an increase in annexin V−ve events at 16 and 24 hours (Figure 2A). This was significantly reduced in cells pretreated with irbesartan (Ang II type 1 [AT1] receptor inhibitor) and apocynin (Figure 2B). To confirm that Ang II induces EC NADPH oxidase-derived ROS generation, activation of NADPH oxidase and production of O2•− were measured in cultured ECs. As shown in Supplemental Figure IIA and IB, Ang II significantly increased NADPH oxidase activation and O2•− formation.

Role of Rho Kinase in Ang II–Mediated Microparticle Formation
To examine putative mechanisms responsible for cytoskeletal reorganization governing Ang II–mediated microparticle formation, we examined the role of Rho kinase 2 and calpain, which are important in cytoskeletal regulation and previously implicated in microparticle production. Ang II induced a rapid increase in Rho kinase 2 activation in ECs, as measured by cytosol-to-membrane translocation (Supplemental Figure IIA) and by phosphorylation of the downstream target MYPT1 (Supplemental Figure IIB). The effects of Ang II on Rho kinase activity were inhibited with fasudil (Rho kinase inhibitor) and apocynin (Supplemental Figure IIC). Fasudil blocked Ang II–stimulated endothelial microparticle formation (Figure 2C). Ang II–mediated increases in Rho kinase activity were blocked by MBC, suggesting that Rho kinase activation is associated with cholesterol-rich microdomains. Ang II did not significantly influence calpain activation, as measured by spectrin cleavage (Supplemental Figure IIIA and IIIB).

Role of Lipid-Rich Microdomains in Endothelial Microparticle Formation
Cholesterol-rich microdomains were disrupted in ECs by cholesterol depletion using MBC and nystatin. Under basal conditions, neither MBC nor nystatin had any effect on
microparticle formation (Figure 3A and 3B). Both agents decreased Ang II–mediated increase in annexin V+ve events (Figure 3A and 3B), suggesting a role for cholesterol-rich EC microdomains in microparticle formation.

To explore whether microparticles possess EC-derived lipid-rich domains, microparticles were probed for specific markers of lipid rafts/caveolae. As shown in Figure 3C, microparticles express vascular-endothelial cadherin (VE-cadherin) and endothelial nitric oxide synthase (EC markers), flotillin-2 and caveolin-1 (lipid raft/caveolae markers), and heparin-binding EGF-like growth factor (a protein that localizes to lipid rafts34). Proliferating cell nuclear antigen was not detected in microparticles but was abundantly expressed in ECs. GAPDH, a cytosolic protein, was detectable in microparticles. No expression of EGF was detected in either endothelial microparticles or ECs.

**Functional Effects of Endothelial Microparticles**

To evaluate whether endothelial microparticles are biologically active and whether they have functional significance, we investigated EC effects of microparticles on ROS production, proinflammatory responses, and macrophage adhesion. Mouse aortic ECs were treated with 105 microparticles/mL medium (a physiologically relevant dose that has previously been described to impair vasorelaxation in aortic rings and promote oxidative stress in cultured ECs16), and effects on O2− production and EC inflammation were evaluated. Microparticles significantly increased EC O2− production (Figure 4A and 4B), expression of VCAM-1 and PECAM (Figure 4C), and adhesion of macrophages (Figure 4D).

To examine molecular mechanisms governing the biological effects of microparticles, we examined whether microparticles interact physically with ECs. Microparticles were labeled with CFSE and incubated in the presence of ECs. After 24 hours, labeled microparticles were visible on the surface of ECs as punctate, CFSE-positive particles of 100 to 1000 nm, suggesting a surface interaction (Figure 5A to 5E). On the basis of this and the presence of heparin-binding EGF-like growth factor in endothelial microparticles, we hypothesized that activation of the EGF receptor (EGFR) may play a role in the biological effects of endothelial microparticles. Microparticle-stimulated increases in O2− production and VCAM/PECAM expression were not seen in ECs pretreated with the EGFR inhibitor gefitinib (Figure 5F and 5G).

**In Vivo Studies**

**Plasma Biochemistry and Lipid Profile**

Plasma biochemistry was similar in all treatment groups (Supplemental Table). Plasma cholesterol and triglyceride levels were increased in ApoE−/− mice and in ApoE−/− mice treated with Ang II compared with control counterparts (P<0.05). Apocynin had no effect on plasma biochemistry or lipid status. Blood urea nitrogen and creatinine levels were increased following Ang II treatment (P<0.05), whereas apocynin had no effect.

**Ang II Increases Blood Pressure and Promotes Left Ventricular Hypertrophy in ApoE−/− Mice, and Apocynin Has No Effect**

There was no significant difference between WT and ApoE−/− mice with respect to left ventricular mass (Supplemental Table) or systolic blood pressure (Figure 6A) for the duration of the study. Ang II treatment was associated with a significant increase in blood pressure, evident at 2 to 4 weeks posttreatment (Figure 6A), and left ventricular mass, evident at 4 weeks posttreatment (Supplemental Table). Apocynin had no effect on blood pressure or ventricular hypertrophy alone or in combination with Ang II.

**Vascular Expression of Proinflammatory Mediators Is Increased in Ang II–Infused ApoE−/− Mice**

Expression of adhesion molecules VCAM-1 and PECAM was significantly increased in mesenteric arteries from Ang II–infused ApoE−/− mice (Figure 6B). Apocynin significantly reduced expression of PECAM and VCAM-1.
Ang II Increases Plasma Microparticles in ApoE<sup>−/−</sup> Mice

Levels of endothelial (CD144<sup>−/−</sup>) and total (annexin V<sup>−/−</sup>) microparticles were measured in platelet-poor plasma. ApoE<sup>−/−</sup> mice exhibited a significant increase in circulating annexin V<sup>−/−</sup> particles compared with C57BL6 mice (Figure 6C). Ang II increased CD144<sup>−/−</sup> and annexin V<sup>−/−</sup> events in C57BL6 and ApoE<sup>−/−</sup> mice (Figure 6C and 6D). Under basal conditions, apocynin did not significantly alter annexin V<sup>−/−</sup> or CD144<sup>−/−</sup> events in ApoE<sup>−/−</sup> mice. However, although apocynin had no effect on Ang II–mediated increase in blood pressure, it significantly reduced Ang II–mediated increase in CD144<sup>−/−</sup> and annexin V<sup>−/−</sup> events.

Discussion

Major findings from the present study demonstrate that (1) Ang II is a potent stimulus for EC microparticle formation (in vivo and in vitro), an effect mediated by Ang II receptor type 1 that signals through NADPH oxidase and Rho kinase; (2) EC-derived microparticles are enriched in lipid rafts/caveolae, which themselves contribute to generation of microparticles; (3) microparticles are functionally active in that they stimulate ROS production and proinflammatory responses in ECs in an EGFR-dependent manner; and (4) in ApoE<sup>−/−</sup> mice, a model of significant endothelial dysfunction, Ang II increases endothelial microparticle formation, an effect that is redox-sensitive and blood pressure–independent. These in vitro and in vivo findings identify AT<sub>R</sub>, NADPH oxidase, and Rho kinase pathways, targeted to lipid-rich microdomains, in the generation of EC microparticles, which themselves influence EC function. Our study suggests a positive feedforward system whereby Ang II promotes EC injury through its own endothelial-derived microparticles. Such a phenomenon may contribute to endothelial dysfunction and vascular injury in cardiovascular diseases, particularly in conditions associated with activation of the renin angiotensin system.

Many factors have been implicated as inducers of EC vesiculation, membrane blebbing, and consequent microparticle release. In human umbilical vein ECs, ionomycin, complement complex C5b-9, and captopril (topoisomerase inhibitor) but not thrombin, phorbol myristate acetate, tissue plasminogen activator, or tumor necrosis factor-α stimulate microparticle release. Others showed that thrombin and collagen stimulate platelet microparticle formation. Physical factors, such as shear stress, and remodeling of membrane phospholipids, which causes exposure of phosphatidylserine on the outer membrane leaflet, facilitate EC vesiculation and microparticle release. Whether Ang II influences microparticle formation is unclear. Simak et al failed to demonstrate any effect of Ang II, whereas others reported that activation of the renin-angiotensin system is associated with increased microparticle release. Using cell culture and whole animal studies, we showed that Ang II has a direct stimulatory action on ECs to form microparticles. Microparticles were characterized by annexin V or CD144 staining, size <1.0 μm, and morphology as determined by electron microscopy. In cells exposed to irbesartan, an AT<sub>R</sub> blocker, Ang II–stimulated microparticle generation was inhibited, indicating the role of AT<sub>R</sub> in this process. Our data are supported by clinical findings where elevated plasma levels of monocyte-derived microparticles were significantly reduced by losartan in patients with diabetes and hypertension. However, in that study, losartan also reduced blood pressure, rendering it difficult to dissociate blood pressure effects from direct Ang II effects on microparticles.

Although there is growing evidence that plasma levels of microparticles are increased in chronic diseases, processes...
regulating microparticle formation remain elusive, and few studies have examined the intrinsic mechanisms, leading to the generation and release of microparticles from ECs, particularly in vivo. A study based on gene profiling identified a pathway of EC vesiculation induced by thrombin, involving activation of Rho kinase 2 and nuclear factor-κB by caspase 2. Other signaling molecules implicated in EC microparticle formation include p38 mitogen-activated protein kinase, interleukin-1, and interleukin-1 receptor antagonist. Here we demonstrate that Ang II, through AT1R, triggers microparticle generation through redox-sensitive, Rho kinase-dependent signaling pathways because apocynin and fasudil (inhibitors of NADPH oxidase and Rho kinase, respectively) blocked the actions of Ang II. To confirm that Ang II influences these systems in ECs, we show that activity of NADPH oxidase, O2•− generation and Rho kinase translocation are stimulated by Ang II. NADPH oxidase is the major source of Ang II–mediated O2•− production in the vasculature and has been widely implicated in the deleterious effects of Ang II in ECs. Among the many systems influenced by NADPH oxidase-derived O2•− is RhoA/Rho kinase, which itself can influence NADPH oxidase activity.

Rho kinase is a multifunctional kinase that regulates cytoskeletal organization; it is important in cell contraction, migration, and growth and consequently in the control of vascular function and structure. Here, we demonstrated that Rho kinase may also be important in EC microparticle formation, because Rho kinase inhibition blocked Ang II–induced release of microparticles. Rho kinase activation appears to be downstream of O2•− production as treatment with apocynin blocked Ang II–mediated increases in Rho kinase activity. Processes underlying these Rho kinase-dependent actions may involve cytoskeletal rearrangement and membrane blebbing, processes classically regulated by Rho signaling and previously demonstrated to be important in thrombin-stimulated microparticle generation. Myosin light chain kinase has been identified as a central component of AT1R-dependent membrane blebbing in HEK293 cells. As Ang II treatment was associated with increased phosphorylation of MYPT1, an upstream regulator of myosin light chain kinase, it is possible that a similar mechanism regulates the downstream effects of Rho kinase on endothelial microparticle formation. This seems to be a highly controlled process, because calpain activity, which is important in cytoskeletal rearrangement and microparticle formation under certain conditions, was not influenced by Ang II in our studies, as assessed by spectrin cleavage.

Molecular profiling of endothelial microparticles showed enrichment in proteins associated with lipid rafts/caveolae, including caveolin-1, flotillin-2, and endothelial nitric oxide synthase, suggesting the presence of lipid raft/caveolae in microparticles. On the basis of these observations, we...
hypothesized that endothelial microparticle formation by Ang II involves signaling through lipid rafts/caveolae, microdomains enriched in signaling molecules, including NADPH oxidase subunits, Rho kinase, and AT1R. Depletion of membrane cholesterol by MBC and nystatin attenuated Rho kinase activation and endothelial microparticle generation, indicating the pivotal role of lipid rafts/caveolae in microparticle formation by Ang II.

To evaluate the biological significance of our findings, we sought to determine whether endothelial-derived microparticles themselves influence EC signaling and function. Exposure of ECs to microparticles resulted in a significant increase in endothelial NADPH oxidase activity, enhanced \( \text{O}_2^- \) generation, increased expression of proinflammatory mediators, and increased macrophage adhesion. These findings indicate a prooxidative, proinflammatory action of microparticles. Our findings are consistent with previous reports, which suggested that leukocyte-derived microparticles reduce NO bioavailability and increase oxidative stress in microvascular ECs.50,51 Because microparticles typically derive from stressed/activated cells, endothelial damage may promote further damage via release of prooxidative, proinflammatory...
microparticles in a feed-forward manner. In addition, we report that EGFR may mediate these microparticle effects. NADPH oxidase has been implicated in O₂⁻ production and EC inflammation and represents a mechanism by which surface interaction between microparticles and ECs is translated into a biological effect.44,52,53 Terrisse et al identified both a surface interaction and internalization of microparticles by ECs and implicated the internalization of microparticles in their prooxidative and proinflammatory actions.54 Thus, although EGFR inhibition was associated with attenuation of the biological effects of microparticles in our studies, it is likely that EGFR represents 1 of several molecular mechanisms whereby microparticles mediate their functional actions. These mechanisms may include the transfer of mRNA, transcription factors, or protein to the target cell; free radical production by microparticles themselves; or other cell/microparticle surface interactions.

To examine whether our in vitro findings have physiological/pathophysiological significance, we examined endothelial-derived microparticle release in ApoE⁻/⁻ mice challenged with Ang II. These mice were studied because they typically exhibit endothelial dysfunction and vascular inflammation.55–57 This was confirmed in our study because vascular expression of proinflammatory adhesion molecules VCAM-1 and PECAM was increased in Ang II–infused ApoE⁻/⁻ mice. These mice also had elevated plasma levels of endothelial-derived microparticles. To evaluate whether ROS play a role in microparticle release in vivo, as we demonstrated in vitro, mice were treated with apocynin to inhibit NADPH oxidase activity and to scavenge ROS. Apocynin reduced levels of circulating microparticles in Ang II–infused ApoE⁻/⁻ mice, without a significant effect on blood pressure. The effects of NADPH oxidase inhibition on blood pressure is controversial, as studies have found either partial attenuation²⁵,5⁸ or no

Figure 6. Effects of Ang II treatment on systolic blood pressure (A), vascular expression of adhesion molecules PECAM and VCAM-1 (B) and annexin V⁺/ve(C) or CD144⁺/ve(D) microparticle (MP) levels in WT (C57BL6) and ApoE⁻/⁻ mice infused with Ang II (1.44 mg/kg per day), treated with apocynin (Apo, 2.5 mg/day in food), or both. *P<0.05 vs C57BL6, †P<0.05 vs ApoE⁻/⁻ mice, ‡P<0.05 vs ApoE⁻/⁻ + Ang II. Data are mean±SEM, n=4 to 7.
effect despite adequate ROS inhibition.\textsuperscript{99,60} Our results are consistent with no effect of apocynin on blood pressure, despite reductions in vascular inflammation and plasma microparticle levels. These findings suggest that endothelial-derived microparticle release involves redox-sensitive, blood pressure–insensitive processes. To further support a blood pressure–independent effect, ApoE\textsuperscript{−/−} hyperlipidemic mice exhibited increased microparticle levels but had blood pressures similar to those of their WT counterparts. Furthermore, in vitro treatment of cultured ECs with Ang II resulted in a significant increase in microparticle formation. Our results differ from those of others implicating shear stress and blood pressure in microparticle formation.\textsuperscript{3,17,19} Reasons for these divergent findings may relate to differences in models studied. For example, we studied microparticless in Ang II–treated ApoE\textsuperscript{−/−} mice, whereas other studies examined microparticless in hypertensive patients treated with AT\textsubscript{1}R blockers, which reduced both blood pressure and circulating microparticle levels.\textsuperscript{3,18} It should be noted that plasma microparticle levels are determined by both microparticle formation and clearance. Thus, one cannot rule out the possibility that changes in plasma microparticle levels may also occur as a result of altered microparticle clearance. In fact, ApoE\textsuperscript{−/−} mice have been shown to have altered clearance of apoptotic bodies,\textsuperscript{61} and this may explain, in part, the differences in plasma microparticle levels between WT and ApoE\textsuperscript{−/−} mice. In summary, our data suggest that Ang II has a direct stimulatory effect on endothelial microparticle formation, involving a novel AT\textsubscript{1},R/NADPH oxidase/Rho kinase pathway targeted to lipid rafts/caveolae. Microparticles, in turn, trigger an EGFR-dependent prooxidative, proinflammatory response in ECs, indicating that they are functionally active. In a mouse model exhibiting endothelial dysfunction, Ang II–mediated microparticle release involves redox-dependent, blood pressure–independent mechanisms. These findings support a feedforward process whereby ECs promote their own dysfunction through microparticle-induced oxidative stress and inflammation. Our in vitro data, supported by in vivo findings, provide new insights into molecular mechanisms of microparticle formation and their potential role in progressive vascular dysfunction.

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Disclosures
None.

References


SUPPLEMENTAL DATA

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Dylan Burger, Augusto C Montezano, Nobuhiro Nishigaki, Ying He, Anthony Carter, Rhian M Touyz.

Kidney Research Centre, Ottawa Hospital Research Institute, University of Ottawa, Canada.

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**Correspondence:**
Rhian M. Touyz MD, PhD
Canada Research Chair in Hypertension
Kidney Research Centre,
OHRI/University of Ottawa,
451 Smyth Road
Ottawa, K1H 8M5, Ontario.
Phone: (613) 562-5800 ext 8241; Fax: (613) 562-5487
Email: rtouyz@uottawa.ca
Methods

Cell Culture

Mouse aortic endothelial cells (ECs) were isolated and cultured according to the methods described by Suh et al. (1). Cells were seeded in Dulbecco’s modified Eagle Media (DMEM, Gibco) containing 10% fetal calf serum (Gibco), 50 mg/L of endothelial cell growth supplement (Sigma), 10 U/mL heparin, 100 U/ml penicillin/streptomycin, and 1x minimal essential amino acids (Gibco) and passed upon reaching confluence. Cells from passages 4 or 5 were used for all experiments and treated when approximately 80% confluence was reached.

Animals

All experiments in this study were approved by the University of Ottawa Animal Ethics Committee and performed according to the recommendations of the Canadian Council for Animal Care.

Ang II-treated C57BL6 wild-type (WT) and apolipoprotein E deficient (ApoE⁻/⁻) mice (males, 6 months, Jackson Laboratory (Bar Harbor, ME)) were studied. Ang II-infused ApoE⁻/⁻ mice were chosen as a model of significant vascular damage, oxidative stress and inflammation. Additionally, some mice were co-treated with the NADPH oxidase inhibitor apocynin, a pro-drug which is converted by peroxidase-mediated oxidation into an active dimer which inhibits cytosolic subunit assembly of NADPH oxidase. Mice were fed a standard rodent chow (Teklad Global 18% protein diet, Harland) and received tap water ad libitum. Animals were divided into six treatment groups: 1) untreated C57BL6 mice (n=5), 2) Ang II-treated C57BL6 mice (1.44 mg/kg/day, osmotic mini pumps) (n=4) 3) ApoE⁻/⁻ mice treated with vehicle (saline) via
subcutaneous osmotic mini pumps (model 2004, Alzet Corporation) (n=8), 4) ApoE<sup>−/−</sup> mice treated with Ang II (n=7), 5) Apo E<sup>−/−</sup> mice receiving apocynin (2.5 mg/day in food) (n=8), and 6) ApoE<sup>−/−</sup> mice receiving Ang II+apocynin (n=8). Experimental treatments lasted 4 weeks.

**Blood Pressure and plasma analysis.**

Blood pressure was measured using a tail cuff plethysmography system (BP-2000, Visitech Systems). Animals were trained to the system for one week prior to experiments, and blood pressure was recorded weekly for the duration of the experiment.

To measure electrolyte levels blood samples were collected via cardiac puncture. Levels of electrolytes (Na+, K+, Mg2+, Ca2+), glucose, cholesterol, triglycerides, and creatinine were determined using an automated analyzer (Synchron CX5 PRO, Beckman).

**Microparticle Isolation:**

*Cell culture studies.* Media was collected from cultured MAECs and samples were subjected to centrifugation at 1500 g for 20 minutes at 20°C to obtain cell-free media. Microparticles were then pelleted from cell-free media by centrifugation at 18000 g for 20 minutes at 20 °C. For flow cytometry, the supernatant was then aspirated and the microparticle-containing pellet was re-suspended in Annexin V binding buffer containing (in mM) 10 HEPES, pH 7.4, 140 NaCl, 2.5 CaCl<sub>2</sub>. For Western blot analysis, microparticles were re-suspended in a lysis buffer which contained (in mM) 10 HEPES, pH 7.4, 50 NaF, 5 NaCl, 5 EDTA, 5 EGTA, 50 Na pyrophosphate, with 0.5% phenylmethylsulfonyl fluoride, 0.5% Triton-X100, 1 µg/mL leupeptin, and 1 µg/mL aprotinin.
In vivo studies. Microparticles were isolated from either ApoE−/− and WT mice according to the methods of Biro et al. with modification (2). Blood was collected from the hearts of mice by cardiac puncture using a 25-5/8 gauge needle and transferred into heparinized tubes. Blood was centrifuged at 1500 g for 20 minutes and 18000 g for 20 minutes as above.

Flow Cytometric Detection of Microparticles:

Isolated microparticles were quantified using a MoFlo Fluorescence Activated Cell Sorter with Summit software (Dako Canada Inc, Mississauga, ON). As microparticles express surface phosphatidylserine, which Annexin V binds with high affinity, an Alexa-647-labeled Annexin V (Biolegend, San Diego, CA) was used to identify events as microparticles. Isolated microparticles were re-suspended in Annexin V binding buffer and labeled with 0.5 µg/ml of Annexin V; this concentration of Annexin V was titrated to determine optimal labeling conditions. Additionally, as a negative control, a sub-population of microparticles was re-suspended in Annexin V binding buffer lacking calcium, which is necessary for Annexin V binding to phosphatidylserine.

Annexin V+ve microparticles were defined as particles of less than 1.0 µm and greater than 0.1 µm in size that exhibited significantly more fluorescence than their negative controls. The size of sorted microparticles was further confirmed by electron microscopy (see below). A total of 0.5 mL of each sample was analyzed over approximately 5 minutes and the number of annexin V+ve microparticles/mL of plasma or /10^6 cells was reported.

Electron Microscopy
Microparticles, previously sorted by flow cytometry, were examined by electron microscopy. Microparticles were pelleted by centrifugation at 18000g for 20 minutes at 20°C and the supernatant was aspirated. The microparticle pellet was fixed in 2.5% gluteraldehyde in 1x phosphate buffered saline (PBS, pH 7.4) overnight at 20°C. The pellet was then washed in 0.1 M Na cacodylate buffer, postfixed in 2% OsO₄, and dehydrated in graded ethanol. The sample was embedded in Spurrs Resin and 60 nm sections were prepared on copper grids. Samples were visualized on a Hitachi H7100 electron microscope and microparticles were visible as small (0.1-1.0 μm), rounded objects with clear, intact membranes.

**Cholesterol depletion**

Cholesterol depletion and repletion experiments were performed using methyl-beta cyclodextrin (MβCD) and nystatin. MβCD has previously been shown to deplete membrane cholesterol in a rapid, and reversible manner (3). Cholesterol depletion was performed by incubating MAECs in media containing MβCD (10 mM) at 37°C for 45 minutes. For cholesterol loading experiments, cells were incubated in media containing a mixture of MβCD (10 mM) and cholesterol (1 mM) at 37°C for 45 minutes. Nystatin-mediated cholesterol sequestration was achieved by treating with 25 μg/ml of nystatin in media for 10 minutes.

**Measurement of superoxide production by dihydroethidium**

Superoxide (O₂⁻) production was measured in cultured MAEC extracts using a modification of the protocol outlined by Laurindo et al. (4). MAECs were isolated as above and treated with angiotensin II (10 nM) for 30 minutes. Cells were then washed with PBS and incubated with dihydroethidium (50 uM in a buffer containing1.3 mM CaCl₂, 0.8 mM MgSO₄, 5.4 mM KCl, 0.4 mM KH₂PO₄, 4.3 mM NaHCO₃, 137 mM NaCl, 0.3 mM Na2HPO₄, 5.6 mM glucose,
100uM diethylenetriamenepta-acetic acid, pH 7.4) for 30 minutes. Cells were then washed in PBS, and harvested in 100% acetonitrile, and centrifuged at 12000g (10 min, 4°C). Supernatants were then vacuum dried with a low dry rate and the heater turned off. The resultant pellets were maintained at –20°C in the dark until analysis. For HPLC analysis, the pellets were resuspended in 120 µl of 20% methanol with 0.1% trifluoroacetic acid and 100 µl was injected into the system and separated under the following gradient: A (20% methanol with 0.1% trifluoroacetic acid) and B (100% methanol) at a flow rate of 0.5 ml/min. An Agilent Zorbax 300SB C18 with a 5 um internal diameter was used as column. The gradient started at 0% solution B and increased linearly during the first 10 minutes to 50% solution B. Solutions were then held at this proportion until 20 minutes, at which time a 5 minute wash using 100% solution B was done. The system was then re-equilibrated with 0% solution B from 25 to 35 minutes. Dihydroethidium (the probe, and internal standard) was monitored by ultraviolet absorption at 245 nm and yielded a peak at approximately 11.1 mins. Ethidium (representing non-specific reactive oxygen species) and 2-hydroxyethidium (representing O_2^-) were monitored by fluorescence detection with an excitation of 480 nm and an emission of 580 nm and yielded peaks at approximately 13.9 and 14.4 mins respectively.

**Measurement of NADPH oxidase activity and superoxide production by lucigenin**

Superoxide production was also measured in cultured mouse aortic endothelial cell extracts using a lucigenin-based assay as described previously (5). Briefly, cells were washed with cold PBS and lysed in a buffer containing (20 mmol/L of KH2PO4, 1 mmol/L of EGTA, 1 µg/mL of aprotinin, 1 µg/mL of leupeptin, 1 µg/mL of pepstatin, and 1 mmol/L of PMSF). Lucigenin-derived chemiluminescence was measured using a plate luminometer (AutoLumat LB 953,
Berthold) and the enzymatic activity was expressed as arbitrary units of luminescence per minute per milligram of protein.

Western Blot Analysis

Protein expression was examined in cultured ECs, the mesenteric arteries of mice, or in microparticles isolated from the media of cultured (unstimulated) ECs by Western blot analysis. Approximately 15 µg of protein was isolated and separated by electrophoresis on 10% polyacrylamide gels. Proteins were then transferred onto a polyvinylidene difluoride membrane. Nonspecific binding sites were then blocked with 5% skim milk in Tris-buffered physiological saline solution with Tween (TBS-T) (1 h, room temperature). Membranes were incubated with the following antibodies: anti-flotillin-2 (1:4000, BD Biosciences), anti-caveolin-1 (1:2000, Santa Cruz Biotechnology), anti-endothelial nitric oxide synthase (eNOS) (1:2000, Santa Cruz Biotechnology), anti-glyceraldehydes-3 phosphate dehydrogenase (GAPDH) (1:5000, Chemicon), anti proliferating cell nuclear antigen (PCNA) (1:2000, Santa Cruz Biotechnology), anti heparin-binding EGF-like growth factor (HB-EGF) (1:1000, Santa Cruz Biotechnology), anti platelet-endothelial cell adhesion molecule (PECAM) (1:2000 Santa Cruz Biotechnology), anti vascular cell adhesion molecule (VCAM-1) (1:2000 Santa Cruz Biotechnology), or anti vascular-endothelial cadherin (VE-Cadherin) (1:2000, Santa Cruz Biotechnology). Washed membranes were incubated with the appropriate horseradish peroxidase-conjugated second antibodies (1: 2000). Washed membranes were incubated with the appropriate horseradish peroxidase-conjugated second antibodies (1: 2000) in TBS-T-milk (room temperature, 1 h). Membranes were then probed for immunoreactive proteins by chemiluminescence. Blots were analyzed densitometrically using ImageJ software (NIH).
**Measurement of Rho kinase activity**

Activation of Rho kinase was determined by assessing translocation from cytosol to membrane as described previously. Briefly, ECs were homogenized and fractionated to obtain cytosol and membrane fractions through centrifugation at 50,000g (1 hour, 4°C). The membrane containing pellet was resuspended in lysis buffer and protein analysis was performed on both membrane and cytosolic fractions by Western blot analysis using anti-RhoA antibody. Rho kinase activity was assessed in ECs using the ROCK Activity Assay Kit (Cell Biolabs Inc.) according to manufacturers instructions. This kit measures the inhibitory phosphorylation of myosin phosphatase target subunit 1 at Thr\(^{696}\) (MYPT1) an upstream regulator of myosin light chain phosphorylation.

**Statistical analysis**

Data are presented as means ± SEMs. Groups were compared using a Student’s t-test, 1, or 2-way ANOVA with a Bonferroni’s post-test as appropriate. A \(P<0.05\) was considered statistically significant.
References

   V, Droogmans G, Nilius B. Characterisation of explanted endothelial cells from mouse


4. Laurindo FR, Fernandes DC, Santos CX. Assessment of superoxide production and
   NADPH oxidase activity by HPLC analysis of dihydroethidium oxidation products.

5. Callera GE, Touyz RM, Tostes RC, Yogi A, He Y, Malkinson S, Schiffrin EL.
   Aldosterone activates vascular p38MAP kinase and NADPH oxidase via c-Src.
Figure legends

**Supplemental figure I.** Ang II (10 nm, 4 hours) significantly increased activation of NADPH oxidase, as assessed by enhanced lucigenin chemiluminescence (figure IA) and generation of •O₂⁻ production as measured by dihydroethidium/HPLC (figure IB). *P<0.05 vs. untreated control. Data are mean±SEM, n=3-8.

**Supplemental figure II.** Rho kinase in endothelial microparticle formation. Endothelial cells were cultured and treated with Ang II (10 nM, 30 mins) with or without membrane cholesterol depletion using MBC (10 mM, 45 mins). Rho kinase activity was assessed by measuring translocation to the membrane (figure IIA) or phosphorylation of MYPT1 (figure IIB). Ang II increased Rho kinase translocation and MYPT1 phosphorylation. This increase was absent in MBC-treated cells suggesting a role for lipid rafts in Rho kinase activation. Pretreatment with apocynin (Apo, 10 µM, 30 mins prior) or fasudil (10 µM, 30 mins prior) also reduced Rho kinase activity (figure IIB). Data are mean±SEM, n=3-6

**Supplemental Figure III.** Effects of Ang II on calpain activity. Ang II (10 nM) had no effect on calpain activity, measured by spectrin cleavage at 30 mins (figure IIA) or at 4 hours (figure IIB. Inserts: representative immunoblots. Data are mean±SEM, n=3
Table: Plasma electrolyte levels after 4 weeks treatment

<table>
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<tr>
<th></th>
<th>C57BL6</th>
<th>ApoE&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>ApoE&lt;sup&gt;−/−&lt;/sup&gt; + Ang II</th>
<th>ApoE&lt;sup&gt;−/−&lt;/sup&gt; + Apocynin</th>
<th>ApoE&lt;sup&gt;−/−&lt;/sup&gt; + Ang II + Apocynin</th>
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<tbody>
<tr>
<td>LVW/BW</td>
<td>2.34 ± 0.12</td>
<td>2.97 ± 0.23</td>
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<td>2.95 ± 0.26</td>
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<td>Na&lt;sup&gt;+&lt;/sup&gt;</td>
<td>151.08 ± 0.44</td>
<td>147.22 ± 1.16</td>
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<td>148.20 ± 0.59</td>
<td>146.90 ± 0.91 *</td>
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<tr>
<td>K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>4.17 ± 0.13</td>
<td>4.42 ± 0.24</td>
<td>4.52 ± 0.10</td>
<td>4.77 ± 0.17</td>
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<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>0.92 ± 0.02</td>
<td>0.95 ± 0.04</td>
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<td>0.98 ± 0.02</td>
<td>1.01 ± 0.05</td>
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<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>2.25 ± 0.02</td>
<td>2.14 ± 0.02</td>
<td>2.16 ± 0.04</td>
<td>2.08 ± 0.05 *</td>
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<tr>
<td>Cholesterol</td>
<td>3.15 ± 0.10</td>
<td>11.17 ± 1.11 *</td>
<td>12.41 ± 0.96 *</td>
<td>11.52 ± 0.63</td>
<td>10.97 ± 0.96 *</td>
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<td>Glucose</td>
<td>17.92 ± 1.10</td>
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<td>16.70 ± 1.12</td>
<td>15.36 ± 1.06</td>
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<tr>
<td>Triglycerides</td>
<td>1.20 ± 0.19</td>
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<td>4.00 ± 0.91 *</td>
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<tr>
<td>BUN</td>
<td>8.06 ± 0.42</td>
<td>7.48 ± 0.28</td>
<td>12.40 ± 0.83 *</td>
<td>8.60 ± 0.51</td>
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<tr>
<td>Creatinine</td>
<td>24.00 ± 0.55</td>
<td>22.00 ± 2.14</td>
<td>31.50 ± 2.10 †</td>
<td>24.20 ± 1.16</td>
<td>26.75 ± 1.75</td>
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</table>

BUN: Blood-Urea Nitrogen, LVW/BW: Left Ventricular Weight/Body Weight (mg/g)

Data are mean ± SEM, *P<0.05 vs. C57BL6, †P<0.05 vs. untreated ApoE<sup>−/−</sup> mice.
Supplemental II

A

Membrane ROCK2 (~180 kDa)

Cytosolic ROCK2 (~180 kDa)

<table>
<thead>
<tr>
<th>ROCK translocation (% Control)</th>
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<tbody>
<tr>
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B

<table>
<thead>
<tr>
<th>MYPT Tyr696 Phosphorylation (AU)</th>
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<tr>
<td>Control</td>
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Downloaded from http://atvb.ahajournals.org/ at University of Pennsylvania on August 16, 2011
Supplemental III

A

Cleaved αII-Spectrin (~140 kDa)

αII-Spectrin (240 kDa)

B

Cleaved αII-Spectrin (~140 kDa)

αII-Spectrin (240 kDa)

Control

Ang II

Cleaved Spectrin / Spectrin (arbitrary units)

Control

Ang II

Cleaved Spectrin / Spectrin (arbitrary units)