Rap1b in Smooth Muscle and Endothelium Is Required for Maintenance of Vascular Tone and Normal Blood Pressure

Sribalaji Lakshmikanthan, Bartosz J. Zieba, Zhi-Dong Ge, Ko Momotani, Xiaodong Zheng, Hayley Lund, Mykhaylo V. Artamonov, Jason E. Maas, Aniko Szabo, David X. Zhang, John A. Auchampach, David L. Mattson, Avril V. Somlyo, Magdalena Chrzanowska-Wodnicka

Objective—Small GTPase Ras-related protein 1 (Rap1b) controls several basic cellular phenomena, and its deletion in mice leads to several cardiovascular defects, including impaired adhesion of blood cells and defective angiogenesis. We found that Rap1b−/− mice develop cardiac hypertrophy and hypertension. Therefore, we examined the function of Rap1b in regulation of blood pressure.

Approach and Results—Rap1b−/− mice developed cardiac hypertrophy and elevated blood pressure, but maintained a normal heart rate. Correcting elevated blood pressure with losartan, an angiotensin II type 1 receptor antagonist, alleviated cardiac hypertrophy in Rap1b−/− mice, suggesting a possibility that cardiac hypertrophy develops secondary to hypertension. The indices of renal function and plasma renin activity were normal in Rap1b−/− mice. Ex vivo, we examined whether the effect of Rap1b deletion on smooth muscle–mediated vessel contraction and endothelium-dependent vessel dilation, 2 major mechanisms controlling basal vascular tone, was the basis for the hypertension. We found increased contractility on stimulation with a thromboxane analog or angiotensin II or phenylephrine along with increased inhibitory phosphorylation of myosin phosphatase under basal conditions consistent with elevated basal tone and the observed hypertension. Cyclic adenosine monophosphate–dependent tension was elevated arteriolar resistance because of increased second messengers, cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate, reduces Ca2+ sensitivity and induces SM relaxation (Ca2+ desensitization).

Increased SM contractility is counterbalanced by relaxing factors such as prostacyclin, endothelium-derived hyperpolarizing factor, and nitric oxide (NO), that are released from endothelium on increased flow shear stress. Decreased NO production, a hallmark of endothelial dysfunction, leads to hypertension.

Rap-related protein 1 (Rap1) is a ubiquitously expressed small GTPase that integrates signals from multiple receptors to control several basic cellular phenomena including adhesion, polarity, and migration. Two Rap1 isoforms exist, Rap1a and Rap1b; deletion of both isoforms leads to embryonic lethality. Therefore, we examined the function of Rap1b in regulation of blood pressure.

Conclusions—This is the first report to indicate that Rap1b in both smooth muscle and endothelium plays a key role in maintaining blood pressure by controlling normal vascular tone.

Key Words: relaxation • signal transduction • vasodilation
addition to described defects, our observation of frequent increased neonatal lethality and sudden death in male Rap1b−/− mice suggested severe underlying defects in cardiovascular function. In this article, we report that Rap1b−/− mice are hypertensive and develop cardiac hypertrophy. We hypothesize that vascular Rap1 deficiency in blood vessels increases vascular tone, resulting in hypertension. We show that Rap1b in SM and endothelium contributes to elevated vascular tone. Mechanistically, in SM, Rap1b counteracted RhoA-dependent Ca2+ sensitization.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Global Rap1b Deficiency Leads to Hypertension and Cardiac Hypertrophy

We observed that, in addition to previously described phenotypes,12,15,17 Rap1b−/− mice have an increased heart-to-body weight ratio because of grossly enlarged heart weight (Figure 1A and 1B; Figure 1A in the online-only Data Supplement) and increased dimensions (Figure 1C; Table), resulting partly from enlarged cardiomyocytes (Figure 1D). This remodeling response was accompanied by increased cardiac fibrosis (Figure 1E) with evidence of emerging diastolic dysfunction (increased mitral E-wave deceleration times; Table) and macrophage infiltration (Figure 1F), all consistent with pathological hypertrophy. Because such changes often are maladaptive and secondary to hypertension,1 we performed telemetric blood pressure measurements. We found that Rap1b−/− mice had significantly elevated blood pressure (135±6 versus 108±1 mm Hg; wild type [WT]; n≥4; Figure 2A–2C), but normal heart rate (514±15 versus 511±9 beats per minute; WT; n=4; Table). To test whether cardiac hypertrophy is secondary to elevated blood pressure in Rap1b−/− mice, we examined the effect of blood pressure–controlling medication on the development of cardiac hypertrophy in Rap1b−/− mice. To control hypertension, Rap1b−/− mice were chronically treated with losartan,18 a competitive inhibitor of angiotensin II type 1 receptor (AT,R). AT,R inhibitors have been demonstrated to prevent pressure overload–induced left ventricular hypertrophy in mouse models.19 After 8 to 12 weeks of treatment, blood pressure, heart, and body weights of treated animals were measured and compared with untreated controls. Systolic blood pressure in 8- to 12-week-old Rap1b−/− untreated mice (Figure 2D) was elevated compared with untreated WT mice, but lower than that in 6- to 10-month-old Rap1b−/− mice (Figure 2A). Importantly, correcting elevated blood pressure with losartan treatment (Figure 2D) led to decreased heart-to-body weight ratio and alleviated cardiac hypertrophy in Rap1b−/− mice (Figure 2E), suggesting that cardiac hypertrophy may be secondary to hypertension in Rap1b−/− mice. However, AT,R antagonist may have a direct effect on cardiomyocytes.20 We, therefore, attempted to correct elevated blood pressure with an antihypertensive drug hydralazine21 and examined the effect of this treatment on cardiac hypertrophy. We found that hydralazine treatment corrected elevated blood pressure in Rap1b−/− mice.
Therefore, it is possible that Rap1 deficiency in cardiomyocytes directly contributes to cardiac hypertrophy.

**Increased Basal and Agonist-Induced Contraction and Suppressed Relaxation in Rap1b−/− SM**

Indices of renal function and activation of the renin–angiotensin–aldosterone system were assessed to determine the contribution of these systems to the observed phenotype. We observed that plasma renin activity, plasma creatinine concentration, and urine albumin/creatinine excretion ratio (Figure 3) were within the normal range of values reported for mice,25,26 and renal histology, as evaluated by Masson trichrome and H&E staining (Figure 4), was not different between WT and Rap1b−/− mice. Therefore, we concluded that defects in renal function or in the activation of the circulating renin–angiotensin system are not causes of elevated blood pressure in Rap1b−/− mice and placed our efforts into the elucidation of altered vascular contractility responses.

To determine whether Rap1 deletion leads to increased vascular tone, we measured contraction of endothelium-denuded aortic segments isolated from Rap1b−/− and control mice. Although aorta has limited contribution to the overall blood pressure level, ex vivo analysis of aortic SM contractility is an accepted model to study underlying mechanisms of SM contraction and was used by Furchgott27 in the landmark discovery of endothelium-derived relaxing factor. SM contractility is regulated by a Ca2+-dependent component and a Ca2+-independent component, the latter modulated by small GTPase RhoA. For various agonists, the amount of force to calcium signal differs, and one agonist that promotes contractility via the RhoA

<table>
<thead>
<tr>
<th>Table. Measurements Acquired From the Echocardiography of Rap1b−/− and Wild-Type Mice</th>
<th>Rap1b−/+ (n=5)</th>
<th>Rap1b−/− (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AWD, mm</td>
<td>0.88±0.06</td>
<td>1.00±0.08</td>
</tr>
<tr>
<td>AWS, mm</td>
<td>1.25±0.09</td>
<td>1.38±0.13</td>
</tr>
<tr>
<td>PWd, mm</td>
<td>0.93±0.06</td>
<td>1.25±0.08*</td>
</tr>
<tr>
<td>PWs, mm</td>
<td>1.25±0.08</td>
<td>1.55±0.07*</td>
</tr>
<tr>
<td>LVIDd, mm</td>
<td>3.89±0.12</td>
<td>4.13±0.19</td>
</tr>
<tr>
<td>LVIDs, mm</td>
<td>2.84±0.16</td>
<td>3.06±0.22</td>
</tr>
<tr>
<td>RWTd†</td>
<td>0.47±0.03</td>
<td>0.56±0.05</td>
</tr>
<tr>
<td>% PW thickening‡</td>
<td>35±3</td>
<td>27±5</td>
</tr>
<tr>
<td>Peak E wave, cm/s</td>
<td>72±9</td>
<td>88±8</td>
</tr>
<tr>
<td>Peak A wave, cm/s</td>
<td>54±6</td>
<td>55±4</td>
</tr>
<tr>
<td>E wave deceleration time, ms</td>
<td>11±1</td>
<td>26±3*</td>
</tr>
<tr>
<td>LV fractional shortening,§ %</td>
<td>27±2</td>
<td>27±4</td>
</tr>
<tr>
<td>LV mass, mg</td>
<td>106±5</td>
<td>156±13*</td>
</tr>
</tbody>
</table>

Measurements were made under light isoflurane anesthesia (1–1.5%). AWD indicates anterior wall thickness during diastole; AWS, anterior wall thickness during systole; LVIDd, left ventricular (LV) internal diameter during diastole; LVIDs, LV internal diameter systole; PWd, posterior wall thickness during diastole; PWs, posterior wall thickness during systole; PWd, posterior wall thickness during systole; and RWTd, relative wall thickness.

*P<0.05 vs wild-type group.
†RWTd=(A+PWd/PWd/LVIDd).
‡%PW thickening=(PWs−PWd)/PWd*100.
§LV fractional shortening=([LVIDd−LVIDs]/LVIDd)×100.
||LV mass=1.05×([LVIDd+PWd+AWd]/[LVIDd]^3).
component is prostanoid thromboxane A2, a potent vasoconstrictor implicated in hypertension and acting via thromboxane prostanoid receptor.28 Thromboxane-induced activation of RhoA and its target Rho kinase leads to increased inhibitory phosphorylation of myosin light chain phosphatase, increasing RLC20 phosphorylation (Ca2+ sensitization).2–4 Because our previous work suggested that Rap1 may regulate contractility by regulating RhoA,29 we examined SM contractility in response to thromboxane analog, 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin F2α (U46619), which activates Rap1.29 We found a significant increase in contractile force in Rap1b−/− segments (Figure 5A and 5B; Table I in the online-only Data Supplement).

To examine the underlying mechanism of increased contraction in Rap1b−/− denuded aortic segments, we examined the effect of Rap1 deficiency on expression and activity of the contractile apparatus components.

Because hypertension is known to cause vascular remodeling, we investigated aortic morphology to determine whether an increased thickness in the medium layer may contribute to the enhanced contractile responses. We found no gross differences in diameter and wall thickness between WT and Rap1b−/− vessels when examined under a dissecting microscope. We also found no changes in the expression of actin or myosin (Figure 6A) in aortic strips of the same length and width from these mice. Lastly, we measured maximum force induced by high K+-induced contraction in strips that were cut at identical widths, and therefore force is only a function of the cross-sectional area of the strip. We found no difference in the maximum high K+-induced force in WT and Rap1b−/− aortae (Figure 5C), consistent with identical cross-sectional areas. Therefore, we conclude that these vessels contain the same amount of SM, and there is no hypertrophy in the vessel wall.

Importantly, however, the average basal level of phosphorylation of MYPT1/Thr853 (Rho kinase site) was significantly elevated by 2-fold (Figure 6B), which, with unchanged K+-induced maximum contraction (Figure 5C), suggested selective effect of Rap1b deletion on RhoA/Rho kinase signaling, and not changes in calcium homeostasis. Stimulation with the thromboxane analog, U46619, led to increased phosphorylation of RLC20 and MYPT1 at Thr853 during 5 minutes of treatment in WT aortae and a significantly higher increase in Rap1b−/− aortae, indicative of increased RhoA activity (Figure 6C). Note that the U46619-induced increases in RLC20 and MYPT1/Thr853 phosphorylation are each normalized to a paired nonstimulated muscle for comparison of the magnitudes of U46619-induced changes and thus do not reflect the increased basal phosphorylation shown in Figure 6B.

To examine whether SM contractility is altered in response to other agonists associated with hypertension, we examined...
contractile responses to angiotensin II. Angiotensin II is known to activate Rap1,20,30 and in SM it also activates the RhoA-mediated component.31,32 We found that the contractile responses were significantly elevated in Rap1b−/− aortae in response to angiotensin II (Figure 5D), whereas the expression of AT1R was unchanged (Figure 5E). Furthermore, because sympathetic system plays a critical role in maintaining basal blood pressure, we examined the contractile response to phenylephrine, an adrenergic receptor agonist, and found that it was also increased in Rap1b−/− aortae (Figure 5F). We, therefore, conclude that Rap1b modulation of agonist-induced SM contraction is not selective to U46619. These data show that Rap1b suppresses agonist-induced force and suggest that Rap1b is required to suppress RhoA activity, and that elevation of RhoA activity leading to inhibitory phosphorylation...
of myosin light chain phosphatase and increased RLC20 phosphorylation may contribute to increased basal tone and hypertension in Rap1b−/− mice.

SM tone is modulated under physiological conditions by cAMP33 and perturbed in disease states, such as obstructive pulmonary diseases and asthma.44 We have recently demonstrated that elevation of cAMP with 8-pCPT-2′-O-Me-cAMP (007), a cAMP analog that selectively activates Epac, a Rap1 GTP exchange factor, induces SM relaxation by downregulation of RhoA activity.29 To directly test whether Rap1 is involved in this mechanism of cAMP-dependent Ca2+ desensitization and SM relaxation, we measured in WT and Rap1b−/− aortae the U46619 dose–force relationship in the presence and absence of 007. Although 007 significantly increased U46619 EC50 values (Table I in the online-only Data Supplement) and suppressed force curves in both WT (Figure 5A, open versus filled squares) and Rap1b−/− aortae (Figure 5A, open versus filled circles), it was unable to reduce the force in the presence of Rap1b−/− segments to the level of WT controls (Figure 5A, open circles are upward shifted compared with open squares; the effect of 007 on contraction is blunted), consistent with a role for Epac in the Rap1b-mediated suppression of RhoA-mediated force (Figure 6A; Table I in the online-only Data Supplement).

Endothelial Dysfunction and Decreased Endothelial NO Synthase Activity in Rap1b−/− Mice

In response to turbulent or laminar blood flow, humoral factors, and increased SM contraction, endothelial cells (ECs) secrete vasodilator substances that relax SM to maintain normal blood pressure. Of these, NO is particularly important for relaxation of large vessels, including the aorta. Because Rap1b−/− mice showed increased vasoconstriction (Figure 5) and developed hypertension (Figure 2), we examined the effect of Rap1b deficiency on vessel dilation, a defect in NO-dependent vasodilation would likely lead to increased vascular tone and hypertension.35 Acetylcholine is a potent vasodilator acting via G-protein–coupled muscarinic receptors, and Rap1 has been previously implicated in signaling pathways downstream from M(1) subtype of muscarinic acetylcholine receptor.35 Acetylcholine-induced, NO-dependent dilation was significantly reduced in Rap1b−/− aortic segments preconstricted with U46619 (Figure 7A; black line, Rap1b−/−; grey line, WT; Table II in the online-only Data Supplement) or with methoxamine (data not shown). To distinguish whether Rap1 regulates NO release from ECs or NO sensitivity of SM, we blocked NO synthase (eNOS). Dose-dependent relaxation of wild-type (WT; grey line; n=11), Rap1b−/− (black line; n=10), and Tie2-CreEKO, Rap1b−/− (Rap1b−/−ECKO; black dashed line; n=6) aortic rings in response to acetylcholine (Ach; A) or sodium nitroprusside (SNP), an NO donor, in the presence of l-NG-nitro arginine methyl ester (n=8, 6, and 4, respectively; B). Relaxation is expressed as a fold maximal dilation relative to U46619 constriction. Plotted are calculated means±SEM; lines are fit plots. C, Immunoblot and quantification of vascular endothelial growth factor (VEGF)–induced phosphorylation of eNOS/Ser1177, normalized to actin in human umbilical vein endothelial cells transfected with control, scrambled siRNA, or Rap1b small interfering RNA. Fold change vs unstimulated control is shown (n=3).

Rap1b−/− aortae (Figure 7A and 7B, black lines). These results suggest that a defect in EC is responsible for decreased NO bioavailability. In blood vessels, NO is produced by endothelial NO synthase (eNOS), the activity of which is regulated by Ca2+/calmodulin, binding of regulatory cofactors, and post-translational modifications including phosphorylation of Ser1177, which sensitizes eNOS to Ca2+, stimulating NO production.36–38 To determine whether decreased eNOS activity is responsible for decreased NO production in Rap1b-deficient ECs, we examined agonist-induced phosphorylation of eNOS at Ser1177.19 Treatment of WT ECs with VEGF led to a significant induction of eNOS/Ser1177 phosphorylation, an effect that was significantly decreased in Rap1b-deficient ECs, indicating decreased eNOS activity in these cells (Figure 7C).

Together, these findings underscore the importance of Rap1 in both cellular compartments, EC and SM, in vascular tone control.

Discussion

The major finding of this study is that Rap1b plays a critical role in the regulation of vascular tone and blood pressure.
by controlling responses of endothelium and SM, 2 inter-
dependent cell types coordinating the relaxation–contraction
mechanism in blood vessels. In SM, denuded of endo-
thelium, we show that Rap1b regulates basal tone and mediates
3',5'-cAMP-induced desensitization of contraction. Rap1b sup-
presses RhoA-mediated RLC20 phosphorylation, Ca^2+ sensi-
tization, and relaxation, signaling that is further enhanced on
3',5'-cAMP-dependent activation of Rap1 by Epac (Figure 6D).
Ex vivo, we show that Rap1b is essential for regulation of
vasodilation in EC-autonomous manner, because Rap1b
deficiency leads to a significant decrease in NO-dependent
vasodilation, a novel and physiologically important function
of Rap1b. In vitro, we show that underlying the defect in
NO-dependent vasodilation is decreased eNOS activation in
Rap1b-deficient ECs. Together, Rap1b function in both cell
types is important for regulation of vascular tone. The sig-
nificance and novelty of our findings is underscored by the
phenotype of Rap1b-deficient mice, which includes endothe-
lial dysfunction and hypertension.

Regulation of vascular tone and blood pressure by Rap1b
may be one of its physiologically most critical functions in
the cardiovascular system in adult mice, compared with rela-
tively mild defects reported so far in other systems.12–15,40,41
Furthermore, it may help explain the lethality during embry-
onic development that we had previously described,12 which
includes hemorrhage on the side of the head after embryonic
day E12.5. Although no specific primary vascular defects have
been observed in Rap1b−/− embryos, abnormal vascular tone
may constitute a hemodynamic or physiological abnormal-
ity underlying the observed embryo lethality.11 Although our
study points to key functions of Rap1b in SM and endothe-
lium in control of blood pressure, blood pressure is regulated
by several mechanisms.42 Here we show that Rap1b in kidney
does not have a major function in that process, because we
have observed normal gross kidney histology and no major
changes in indices of renal function or plasma renin activity
in Rap1b−/− mice. However, several additional studies would
need to be performed to fully understand the role of Rap1b in
kidney function43 and in regulation of tissue renin–angiotensin
system, which also contributes to blood pressure regulation.
Likewise, it is conceivable that Rap1b in the sympathetic nerv-
ous system contributes to the regulation of blood pressure.
Although future studies should explore the contribution of
Rap1b in the sympathetic nervous system to the regulation of
blood pressure, our current findings demonstrate that Rap1b
functions in vessels are critical for vascular tone.

The phenotype of Rap1b-deficient mice is consistent with
Rap1’s role in hypertension and cardiac disease. We have not
found any reports of changes in Rap1 expression in hyper-
tension; interestingly, though, a genome-wide expression
profiling study reported upregulated expression of a negative
regulator of Rap1 activity, Rap1GAP, in lungs of patients with
idiopathic pulmonary hypertension,44 a change that would be
expected to lead to decreased Rap1 activity and increased air-
way resistance. Our findings implicating Rap1b in regulation
of vascular tone provide justification for additional examina-
tion of Rap1b activity in samples from hypertensive patients.
Additional studies are also needed to fully elucidate molecu-
lar mechanisms of Rap1b-mediated downregulation of RhoA
activity in SM. In other cell types, 2 different RhoGAPs have
been implicated in playing this role: ARAP3, which is involved
in regulation of neurite outgrowth,45,46 male germ cell devel-
opment,47 and monocyte migration48; and ArhGAP29,49 which
promotes EC spreading and barrier function.50 Identification
of the molecular connection between Rap1 and RhoA in SM
is our future area of investigation.

Several studies have linked increased Epac activity with
cardiac hypertrophy51; Epac expression correlates with patho-
lological cardiac hypertrophy,52 and, conversely, silencing Epac
expression blocks the hypertrophic response.53 However, pro-
hypertrophic Epac signaling is not mediated by Rap1, but
rather by Epac’s other effectors, Rac1 and H-Ras.54 In this
study, we report that correction of elevated blood pressure
with AT1R antagonist, losartan, ameliorates cardiac hypertro-
phy phenotype in Rap1b−/− mice. Although this novel finding
is consistent with hypertrophy being secondary to elevated
vascular tone and blood pressure, both AT1R and Rap1 are
expressed in cardiomyocytes, and the effect of losartan could
be cardiomyocyte-autonomous.50 Nonetheless, Rap1 may be
involved in other functions in cardiomyocytes; for example,
Rap1 signaling has been implicated in cardioprotection from
myocyte death induced by chronic adrenergic stimulation dur-
ing congestive heart failure.55 Therefore, Rap1 function in the
heart deserves a separate investigation.

In conclusion, through distinct signaling mechanisms, the
role of Rap1 in the endothelium and in SM is mutually rein-
forcing, having key mechanoeffecter functions required for
maintenance of normal vascular tone.

Acknowledgments
We thank B. Fleming and G. Slocum for histological processing and
expert technical assistance; A. Katch for statistical analysis of data; J.
Idsvoog for animal care assistance; and T. Johnson for proofreading
the article.

Sources of Funding
This work was supported by American Heart Association grant
(0950118G; to M. Chrzanowska-Wodnicka) and the National
Institutes of Health grants (HL11583 to M. Chrzanowska-Wodnicka;
HL29587 and DK96859 to D.L. Mattson; HL07707 and HL089471
to J.A. Auchampach; DK088905 and GM86457 to A.V. Somlyo; and
HL096647 to D.X. Zhang).

Disclosures
None.

References
1. Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL
Jr, Jones DW, Materson B, Oparil S, Wright JT Jr, Roccella EJ. National
Heart, Lung, and Blood Institute Joint National Committee on Prevention,
Detection, Evaluation, and Treatment of High Blood Pressure; National
Heart, Lung, and Blood Institute Joint National Committee on Prevention,
Detection, Evaluation, and Treatment of High Blood Pressure Education
Program Coordination Committee. The Seventh Report of the Joint National
Committee on Prevention, Detection, Evaluation, and Treatment of High
2. Matsuji T, Amano M, Yamamoto T, Chihara K, Nakafuku M, Ito M,
Nakano T, Okawa K, Iwamatsu A, Kaibuchi K. Rho-associated kinase, a
novel serine/threonine kinase, as a putative target for small GTP binding
3. Uehata T, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa
H, Yamagami K, Imai J, Maekawa M, Naramura S. Calcium sensitization


Increased vascular tone is a common observation in arterial hypertension, a major health problem and a risk factor for several cardiovascular diseases. Two of the major mechanisms controlling basal vascular tone are vascular smooth muscle–mediated vessel contraction and endothelium–dependent vessel vasodilation mediated by relaxing substances released in response to elevated shear flow of blood. Here, we demonstrate that a small GTPase Ras-related protein 1 (Rap1b) critically regulates both of these processes. Using Rap1b-deficient mice, we show that Rap1b regulates basal smooth muscle tone and mediates cyclic adenosine monophosphate–induced relaxation. In endothelium, Rap1b is essential for regulation of vasodilation, because Rap1b deficiency leads to a significant decrease in nitric oxide–dependent vasodilation. The significance of our findings is underscored by the phenotype of Rap1b-knockout mice, which includes hypertension and endothelial dysfunction. This report reveals a novel, physiologically important function of Rap1b and contributes to our understanding of the mechanisms of blood pressure regulation.
Rap1b in Smooth Muscle and Endothelium Is Required for Maintenance of Vascular Tone and Normal Blood Pressure

Sribalaji Lakshmikanthan, Bartosz J. Zieba, Zhi-Dong Ge, Ko Momotani, Xiaodong Zheng, Hayley Lund, Mykhaylo V. Artamonov, Jason E. Maas, Aniko Szabo, David X. Zhang, John A. Auchampach, David L. Mattson, Avril V. Somlyo and Magdalena Chrzanowska-Wodnicka

Arterioscler Thromb Vasc Biol. 2014;34:1486-1494; originally published online May 1, 2014; doi: 10.1161/ATVBAHA.114.303678

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/34/7/1486

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2014/05/02/ATVBAHA.114.303678.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Materials and Methods

Chemicals and antibodies. Unless indicated, chemicals were purchased from Sigma Aldrich. RNAiMAX was from Life Technologies. Monoclonal antibody to rat F4/80 was from Abcam and purified rat IgG2b control antibody was from Accurate Chemical. Antibodies to Rap1b, total MYPT1, phospho-MYPT1 (Thr853), total RMLC\textsubscript{20}, phospho-eNOS (Ser1177) were from Cell Signaling Technologies. Rabbit polyclonal anti-Angiotensin II Type-1 Receptor (AT\textsubscript{1}R) was from Alomone Labs. Antibodies to actin (goat polyclonal) were from Santa Cruz Biotechnology. All HRP conjugated secondary antibodies were from Jackson ImmunoResearch. Losartan was from AK Scientific, Inc. or LKT Laboratories, Inc. Recombinant Human VEGF 165 was from R&D Systems.

Genetic mouse models. All mouse procedures were performed according to approved Medical College of Wisconsin Institutional Animal Use and Care Committee protocols. Generation of Rap1b\textsuperscript{-/-} mice \textsuperscript{1} and endothelial-specific Rap1b KO mice (EC-Rap1b KO; Tie2-Cre\textsuperscript{+/0} Rap1b\textsuperscript{f/f}) \textsuperscript{2} has been previously described. Because estrogen is known to have protective effects on regulation of blood pressure and, in particular release of NO \textsuperscript{3}, male mice were used for blood pressure measurements, vasodilation and vasoconstriction studies. Unless indicated otherwise, both male and female mice, 2-4 months old, were used for all other experiments.

Heart isolation and histology. Isoflurane anesthetized mice were weighed and hearts were perfused with PBS. Dissected hearts were thoroughly rinsed in PBS, blotted quickly and weighed. Hearts were cut in a cross section just below mid level. Both halves of the heart were formalin fixed and embedded in paraffin. Sections (4 \textmu m) were prepared. The sections were stained with hematoxylin and eosin for examination of gross appearance and Masson's Trichrome or periodic acid-Schiff counterstained with hematoxylin (PAS-H) was employed to facilitate quantification of fibrosis and cardiomyocyte size, respectively.

Cardiac fibrosis. Masson's Trichrome-stained heart sections were scanned by Nikon cool scan at 4000dpi. The left ventricular muscle area was quantified by setting a threshold (138-255/HSI; MetaMorph 6.1) for purple color. Resetting the threshold (138-210) for blue color quantified the collagen content in the sclerotic areas of the left ventricle. Cardiac fibrosis was determined by calculating the percentage of Masson's Trichrome-blue stained area of interstitial fibrosis per total area of left ventricular cardiac tissue.

Cardiomyocyte hypertrophy. A cross-sectional area of 100-120 cardiomyocytes per PAS-H-stained section was measured in 8-10 randomly selected fields using Nikon E-600 with 20X objective. Cells with nearly circular profiles and centered nuclei in the left ventricular (LV) free wall were selected for analysis. MetaMorph 6.1 was used to apply shape factor to pick the circular profiled cardiomyocytes.
Cardiac inflammation. Monocytes/macrophages infiltrating cardiac tissue were detected by F4/80 immuno-localization on heart sections using F4/80 rat monoclonal antibody followed by Biotin-conjugated goat anti-rat IgG. ABC Elite kit (Vector) was used to visualize the immunoreaction. Positively stained cells per cross section were manually counted in Rap1b−/− and control samples.

Echocardiography. Echocardiography measurements were performed as previously described 4. WT and Rap1b−/− mice anesthetized with 1% isoflurane were analyzed using a VisualSonics Vevo 770 high-frequency ultrasound rodent imaging system equipped with a 30 MHz mouse probe designed for cardiac imaging (VS-RMV-707). Parasternal short-axis, long-axis, and apical views were used to obtain M-mode recordings for analysis of the anterior wall thickness (AW), posterior wall thickness (PW) and left ventricular internal diameter (LVID) at end-diastole (d) and end-systole (s) at the mid-papillary level. Apical views were used to measure the velocity of blood inflow through the mitral valve (peak E and A waves, E wave deceleration time) by pulsed Doppler. Calculations were as follows: relative wall thickness during diastole (RWTd) = (AWd+PWd)/LVIDd; % posterior wall thickening = (PWs-PWd)/PWd*100; % fractional shortening = (LVIDd-LVIDs)/LVIDd*100; left ventricular mass = 1.05*[LVIDd+PWd+AWd]3/[LVIDd]3).

Blood pressure and heart rate measurements. Blood pressure and heart rate measurements were performed using telemetry and/or tail cuff, as previously described 5, 6. For telemetric blood pressure measurement, mice were preanesthetized with isoflurane and then deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.). An implantable telemetric device (TA11PA-C20 BP; Data Sciences International) was implanted in the carotid artery. Both antibiotic (30 mg/kg cefazolin) and analgesic (0.1 mg/kg Buprenex, s.c. or 5 mg/kg Carprofen, s.c.) were administered post surgically, and the mice were allowed to fully awaken from anesthesia on a temperature-controlled pad. The mice were allowed to recover for three days following surgery. Blood pressure and heart rate measurements were obtained on post surgical days 4–6. For tail cuff blood pressure measurements, systolic blood pressure was determined bi-weekly in conscious 8- to 12-wk old animals using IITC Life Sciences Blood Pressure System. Mice were acclimated to the blood pressure chamber three times a week starting at 6 weeks of age. Recordings were taken after 30 minutes of acclimation. Three to six successful measurements were averaged as a single data point. For some groups of animals, we validated this method by comparing the values obtained with those from telemetry, performed in the same animals.

Anti-hypertensive drug treatments. Breeding pairs and resulting offspring were given plain water or losartan (10 mg/kg/day) or hydralazine (1mg/kg/day) in their drinking water. Weaned male mice were maintained on this drug treatment for another 8-12 weeks, until blood pressure measurement was completed, at which time the animals were sacrificed and heart and body weights were measured.

Analysis of kidney function. Mice were housed in metabolic cages for three days and urine was collected every 24 hours. Urine volume, urine microalbumin and creatinine
content were measured as previously described. The ratio of albumin to creatinine concentration in the sample was plotted as an indicator of kidney damage. For measurement of plasma creatinine and renin activity, mice were deeply anesthetized with isoflurane (initially in an induction chamber and maintained with a nose cone). The mice were placed on their backs and a midline abdominal incision was made. A 1 cc syringe with a 23-gauge needle was used to draw blood from the heart. The needle hub was filled with 1000 cc heparin/ml to prevent clotting. The needle was inserted through the diaphragm into the base of the heart, and blood was drawn. The blood was spun at 1000g for 5 min to separate the cells and plasma. The plasma aliquots were maintained at -80 °C until analyzed. Plasma creatinine and renin activity were determined in plasma aliquots, as previously described. Kidneys were harvested, weighed, fixed in 10% formalin and processed for pathology. Sections (4 µm) were prepared and stained with hematoxylin and eosin for examination of gross appearance and Masson's Trichrome was employed to facilitate quantification of protein cast, renal necrosis, renal fibrosis and glomerular alterations and hypercellularity.

**Tissue preparation and force measurements.** Preparation of tissue and contraction of aortic segments was performed essentially as previously described. Briefly, abdominal aortae were removed, cleaned of connective tissue and 3 helical strips 2 mm long and 400 µm wide were cut from the distal end of each aorta, mounted for tension recording on a "bubble plate", and equilibrated for 30 min at 22°C prior to stimulation. Cumulative additions of U46619 were carried out with and without the addition of 100 µM 8-pCPT-2'-O-Me-cAMP (007) added 5 min prior to the first stimulus. RLC and MYPT1/Thr853 phosphorylation on frozen, freeze-substituted samples have been previously described.

**Endothelium-dependent relaxation of aortic rings in response to acetylcholine.** Vasodilation was measured as previously described. Briefly, the thoracic aorta from control and Rap1-mutant mice was dissected and placed in Krebs physiological saline aerated with 21%O2/5% CO2. 1.5 mm segments were mounted on parallel pins in conventional myographs and maintained at optimal tension in Krebs buffer for 60 min at 37°C. The rings were preconstricted with 10 nM U46619, exposed to increasing concentration of acetylcholine in the absence or presence of 100 µM L-NAME and changes in vascular tension were recorded.

**Cell culture and analysis of eNOS phosphorylation.** HUVECs, passage 3 or fewer, were transfected with 50 nM scrambled siRNA or human Rap1b siGENOME siRNA pool (Dharmacon) using OptiMem medium and RNA iMAX reagent (Life Technologies) for 6 hours and cultured for additional 24 hours in Vasculife –EnGS complete medium. Cells were then serum-starved for 4 hours in Vasculife –EnGS basal medium and stimulated with 40 ng/ml VEGF for 0 or 5 min. Following stimulation, HUVECs were rapidly washed once with ice cold PBS and lysed with lysis buffer (0.001M EDTA, 0.15M NaCl, 1% NP-40, 5% glycerol in 0.025M Tris-pH 7.4, 1X protease/phosphatase inhibitor cocktail). Cells lysates were spun at 13K rpm for 5 minutes at 4°C and supernatants were stored at -80°C. Total lysates were resolved on 4-12% gradient gel and blotted using specific antibodies, as indicated. Band intensity was determined by densitometry of X-ray film,
values obtained for phosphoproteins were normalized to actin in the same sample and phosphorylation fold induction over the basal phosphorylation level was determined.

**Statistical analysis.** Mean values and standard error of mean (s.e.m.) were obtained from 3 or more independent experiments, as indicated. Statistical significance of group differences was determined using unpaired 2-tailed Mann-Whitney test or 2-tailed Student’s t-test. In cases where data did not follow Gaussian distribution, values were log2-transformed before statistical analysis. For analysis of aortic ring contraction a log-logistic dose response curve was fitted to the data from each strip, and the estimates of EC$_{50}$ (the concentration at which the response is 50% of its maximal value), Hill's slope coefficient, and the maximum response were obtained. Each parameter was analyzed over the experiments using a linear mixed effects model with fixed effects of genotype, inhibitor, and their interaction and random animal effects. For analysis of vasodilation, a Gompertz dose response curve with Ach/SNP concentration on the log10 scale was fitted to the data from each strip, and the estimates of EC$_{50}$, the slope coefficient, and the maximum response were obtained. Each parameter was analyzed over the experiments using a linear mixed effects model with a fixed genotype effect and random animal effects.$^{11}$

**References**


**SUPPLEMENTAL MATERIAL**

Supplemental material contains supplemental tables, supplemental reference and a supplemental figure with a legend.

**SUPPLEMENTAL TABLES**

**Supplemental Table I.** Statistical analysis of absolute force calculations for aortic segments from *Rap1b<sup>-/-</sup>* mice. A log-logistic dose response curve was fitted to the data from each strip, and the estimates of EC<sub>50</sub> (the concentration at which the response is 50% of its maximal value), Hill's slope coefficient, and the maximum response ("top") were obtained. Each parameter was analyzed over the experiments using a linear mixed effects model with fixed effects of genotype, compound 007 ("007"), and their interaction and random animal effects. There is strong evidence for the activity of 007, especially in increasing the EC<sub>50</sub>; and evidence of reduced EC<sub>50</sub> in *Rap1b<sup>-/-</sup>* animals. There is also evidence for increase in maximal force ("top") in *Rap1b<sup>-/-</sup>* animals. Among chimeric animals the effect size is similar, but the variability is larger, so statistical significance is not reached. There is no evidence of interaction between genotype and the presence of 007 for any of the parameters or experiments.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Comparison</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>wild type, no 007</td>
<td>208</td>
<td>52.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rap1b KO vs wild type, no 007</td>
<td>-25.62</td>
<td>73.4</td>
<td>0.7283</td>
</tr>
<tr>
<td></td>
<td>007 vs no 007</td>
<td>68.22</td>
<td>32.2</td>
<td>0.0389</td>
</tr>
<tr>
<td></td>
<td>interaction</td>
<td>-21.94</td>
<td>44.9</td>
<td>0.6267</td>
</tr>
<tr>
<td>slope</td>
<td>wild type, no 007</td>
<td>1.51</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rap1b KO vs wild type, no 007</td>
<td>0.0064</td>
<td>0.1</td>
<td>0.9495</td>
</tr>
<tr>
<td></td>
<td>007 vs no 007</td>
<td>0.15</td>
<td>0.08</td>
<td>0.0546</td>
</tr>
<tr>
<td></td>
<td>interaction</td>
<td>-0.059</td>
<td>0.1</td>
<td>0.5866</td>
</tr>
<tr>
<td>top</td>
<td>wild type, no 007</td>
<td>0.50</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rap1b KO vs wild type, no 007</td>
<td>0.21</td>
<td>0.1</td>
<td>0.0497</td>
</tr>
<tr>
<td></td>
<td>007 vs no 007</td>
<td>-0.054</td>
<td>0.09</td>
<td>0.5738</td>
</tr>
<tr>
<td></td>
<td>interaction</td>
<td>0.038</td>
<td>0.1</td>
<td>0.7725</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Rap1b KO vs wild type, with 007</td>
<td>-47.57</td>
<td>73.4</td>
<td>0.5195</td>
</tr>
</tbody>
</table>
Supplemental Table II Statistical analysis of vasodilation of aortic segments from Rap1b<sup>−/−</sup> mice. A Gompertz dose response curve with Ach/SNP concentration on the log<sub>10</sub> scale was fitted to the data from each strip, and the estimates of EC<sub>50</sub>, the slope coefficient, and the maximum response were obtained. Each parameter was analyzed over the experiments using a linear mixed effects model with a fixed genotype effect and random animal effects<sup>1</sup>. Note that in this model lack of responsiveness can present as zero or negative maximal response, in which case the other parameters are difficult to estimate or interpret (as in the chimera Ach data). Overall, there is evidence of impaired responsiveness to acetylcholine (Ach, A) and sodium nitroprusside (SNP, B) in Rap1b knockouts.

(A) Ach response in Rap1b<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Comparison</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>wild type</td>
<td>-7.21</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rap1b KO vs wild type</td>
<td>1.01</td>
<td>0.5</td>
<td>0.0635</td>
</tr>
<tr>
<td></td>
<td>Rap1b chimera vs wild type</td>
<td>0.40</td>
<td>0.6</td>
<td>0.4957</td>
</tr>
<tr>
<td>slope</td>
<td>wild type</td>
<td>0.97</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rap1b KO vs wild type</td>
<td>1.02</td>
<td>0.5</td>
<td>0.0633</td>
</tr>
<tr>
<td></td>
<td>Rap1b chimera vs wild type</td>
<td>0.96</td>
<td>0.6</td>
<td>0.1288</td>
</tr>
<tr>
<td>top</td>
<td>wild type</td>
<td>0.63</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rap1b KO vs wild type</td>
<td>-0.16</td>
<td>0.08</td>
<td>0.0473</td>
</tr>
<tr>
<td></td>
<td>Rap1b chimera vs wild type</td>
<td>-0.19</td>
<td>0.10</td>
<td>0.0699</td>
</tr>
</tbody>
</table>

(B) SNP response in Rap1b<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Comparison</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>wild type</td>
<td>-8.18</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rap1b KO vs wild type</td>
<td>0.33</td>
<td>0.2</td>
<td>0.1518</td>
</tr>
<tr>
<td></td>
<td>Rap1b chimera vs wild type</td>
<td>0.56</td>
<td>0.2</td>
<td>0.0436</td>
</tr>
<tr>
<td>slope</td>
<td>wild type</td>
<td>1.06</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rap1b KO vs wild type</td>
<td>-0.066</td>
<td>0.1</td>
<td>0.5788</td>
</tr>
<tr>
<td></td>
<td>Rap1b chimera vs wild type</td>
<td>0.29</td>
<td>0.1</td>
<td>0.0496</td>
</tr>
<tr>
<td>top</td>
<td>wild type</td>
<td>0.85</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rap1b KO vs wild type</td>
<td>-0.079</td>
<td>0.06</td>
<td>0.2432</td>
</tr>
<tr>
<td></td>
<td>Rap1b chimera vs wild type</td>
<td>-0.034</td>
<td>0.07</td>
<td>0.6398</td>
</tr>
</tbody>
</table>
SUPPLEMENTAL REFERENCES

Supplemental Figure I. Cardiac hypertrophy in Rap1b−/− hearts. (A) Absolute and mean values of heart (left panel) and (B) body (right panel) weight of WT (Rap1b+/+) and Rap1b−/− mice; age as indicated. (n≥10 per genotype in each age group). Values are means ± s.e.m.