Arginase II Knockout Mouse Displays a Hypertensive Phenotype Despite a Decreased Vasoconstrictory Profile

Ngan N. Huynh, Karen L. Andrews, Geoffrey A. Head, Sacha M.L. Khong, Dmitry N. Mayorov, Andrew J. Murphy, Gavin Lambert, Helen Kiriazis, Qi Xu, Xiao-Jun Du, Jaye P.F. Chin-Dusting

Abstract—Arginase upregulation is associated with aging and cardiovascular diseases. In this study we report on the cardiovascular phenotype of the arginase II knockout (KO) mouse. We demonstrate that vascular sensitivity and reactivity altered over time in these animals such that no influence on responses to vasoconstrictor activity was observed in 7-week-old KO mice, but dampened responses to norepinephrine and phenylephrine were observed by 10 and 15 weeks with Rho kinase influencing these effects in the 15-week-old animals. Despite these dampened vasoconstrictory responses, KO mice demonstrated increased mean arterial pressure from 8 weeks old. This hypertensive phenotype was associated with an increase in left ventricular weight, left ventricular systolic pressure, and diminished diastolic function. KO mice also show enhanced plasma norepinephrine turnover, suggesting an increased sympathetic outflow. In conclusion, our data suggest that global loss of arginase II activity results in hypertension. We suggest that this strain of mouse warrants further investigation as a potentially novel model of hypertension. (Hypertension. 2009;54:294-301.)

Key Words: arginase □ NO □ hypertension □ vasculature □ L-arginine

Arginase, a key hepatic urea cycle enzyme, hydrolyzes L-arginine to urea and L-ornithine and is responsible for the disposal of excess nitrogen resulting from amino acid and nucleotide metabolism. This enzyme, once thought to be largely confined to the liver, is now known to exist extracellularly in ≥2 isoforms, arginase I and II. Each isoform is encoded by 2 separate genes, which differ in intracellular and tissue expression, gene transcription, and transduction regulators, as well as metabolism. Arginase I is a cytosolic enzyme abundantly expressed in the liver, whereas mitochondrial arginase II is ubiquitously expressed in extrahepatic tissues, including the vasculature and the kidney. Together with the production of urea, arginase is also responsible for the biosynthesis of polyamines and the amino acids ornithine, proline, and glutamate. Vascular endothelial and smooth muscle cells express both arginase I and II, although the distribution appears vessel and species dependent. Importantly, arginase II appears to be the predominant isoenzyme in human endothelial cells.

Because arginase and NO synthase share L-arginine as their precursor substrate, arginase has been postulated to inhibit NO synthesis by competing with endothelial NO synthase (eNOS) for L-arginine. However, on closer examination, a much higher L-arginine affinity of eNOS (Michaelis constant = ~2 to 20 μmol/L) than that of arginase (Michaelis constant = ~1 to 5 mmol/L) makes this competition kinetically unlikely; however, inducible NO synthase–mediated S-nitrosylation of arginase can decrease the Michaelis constant of arginase to a level where it can compete with eNOS. Furthermore, studies examining arginine metabolism in activated macrophages show that the majority of L-arginine is consumed for the production of urea rather than NO, such that when arginase is inhibited or the culture medium is supplemented with L-arginine, increased NO synthesis is observed. In endothelial cells, inhibition of arginase stimulates NO production, whereas overexpression of arginase I or II decreases intracellular L-arginine concentrations and suppresses NO synthesis. Lastly, it has been shown recently that arginase II is found in the mitochondria where it regulates eNOS.

NO is an important vasodilator and antiproliferative and antiatherogenic compound. In various models of hypertension, increased arginase expression or activity is associated with increased vascular proliferation and decreased NO bioavailability, which results in reduced vascular compliance and heightened vascular reactivity, respectively. Although blood pressure (BP) is primarily regulated by the tone of resistance vessels, increased arginase and decreased NO bioavailability are found in both conduit and resistance vessels in hypertensive animals.

The relative contribution of either arginase I or II to whole body cardiovascular function remains unknown. Three types of arginase murine models have been developed: an arginase I, an arginase II, and a double arginase I and II knockout (KO) mouse. Although a comparison of vascular responses...
between arginase I and arginase II KOs was desirable, both the arginase I and double arginase I and II KO mice develop a severe form of hyperammonemia and die within 10 to 14 days after birth. In the present article we report on the cardiovascular phenotype of the arginase II KO mouse.

Materials and Methods
An expanded Materials and Methods section can be found in an online data supplement available at http://hyper.ahajournals.org.

Animals and Treatments
The study protocol was approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee. Studies were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Two pairs of the arginase II KO breeders with C57BL/6 background were a generous gift from Prof William O’Brien for establishment of a colony at the Baker IDI Heart and Diabetes Institute Animal Services Facility. Animals were studied at 7, 10, and/or 15 weeks.

BP Telemetry
Telemetry devices (TA11PA-C10, Data Sciences International) were implanted in control and arginase II KO mice at 7 weeks of age under open-circuit halothane gas anesthesia to monitor mean arterial pressure (MAP), heart rate (HR), and locomotor activity, as described previously.17

Assessment of Baroreflex by Spectral Analysis and Sequences
Cardiovascular variability and baroreflex sensitivity were measured noninvasively by power spectral analysis, as described previously.18

Cardiac Function and In Vivo Vascular Reactivity
Fifteen-week-old age-matched control and arginase II KO mice were anesthetized with a mixture of ketamine, xylazine, and atropine (100:0.1:0.1 mg/kg of body weight, respectively, IP) and cardiac function measured by cardiac catheterization, as described previously.19

Plasma Catecholamines
Norepinephrine (NE), dihydroxyphenylalanine (catecholamine precursor), dihydroxyphenylglycol (NE metabolite), and epinephrine levels were measured by high-performance liquid chromatography, as described previously.20

Plasma and Kidney Urea Levels
Plasma and kidney urea levels were measured using a QuantiChrom Urea Assay Kit (DIUR-500).

Isolated Aortic Rings
Vascular reactivity studies were performed on aorta isolated and mounted in Mulvany myographs (model 610 mol/L, JP Trading), as described previously.21

Imaging of NO Production
Using 4-amino-5-methylamino-2’7’-difluorofluorescein diacetate, real-time fluorescence imaging of isolated segments of aorta was performed as described previously.22

Nitrate/Nitrite Assay
Plasma NO metabolites (nitrate/nitrite) were measured using a Griess Reaction kit (Cayman Chemicals).

Results

Hypertensive Phenotype in Arginase II KO Mice
From 8 weeks of age and until ≥15 weeks of age, MAP, recorded by telemetry in conscious mice, was significantly greater in the arginase II KO mice compared with control mice (Figure S1). Furthermore, the increase in MAP at night (compared with day) was greater in the arginase II KO (16.5 mm Hg) than in control mice (10.7 mm Hg; P<0.001). HR and locomotor activity were higher in the arginase II KO mice at night but not during the day. The hypertensive phenotype was confirmed in animals under anesthesia recorded by micromanometry showing higher systolic, diastolic, and pulse pressures with no discernible difference in HR in arginase II KO mice (Table). Dietary L-arginine supplementation (25 g/L in their drinking water) for 4 weeks resulted in increased plasma L-arginine concentrations in both control (28±6 versus 66±28 μmol/L; P<0.05) and KO (39±6 versus 72±22 μmol/L; P<0.05) mice and increased MAP in the arginase II KO mice while having no effect in controls.

Short-Term Cardiovascular Variability and Baroreflex Sensitivity
Spectral analysis of 24-hour BP was used to determine whether there were specific changes within the frequency bands, low frequency, mid frequency, and high frequency, as a measure of the modulation of vascular reactivity, sympathetic activity, and parasympathetic activity, respectively. There were no significant differences in total BP and HR variability or variability in the frequency bands assessed by spectral analysis in arginase II KO compared with control mice (Table S1). However, the gain of the baroreceptor HR reflex calculated from the cross-spectral transfer function between MAP and HR in the midfrequency was higher in arginase II KO compared with control mice (Table S1).

Cardiac Functional and Morphological Changes
Although atrial and right ventricular weights were comparable, left ventricular weight was ∼34% greater in the arginase II KO mice when normalized to tibial length (Table), in keeping with hypertensive cardiac hypertrophy. Left ventricular systolic pressure was significantly greater in arginase II KO mice. Although rate of cardiac contraction was maintained, arginase II KO mice displayed increment in the left ventricular end diastolic pressure, increased 7, and significant reduction in the rate of cardiac relaxation (Table), indicating significant diastolic dysfunction.

Plasma Catecholamines and Cardiovascular Stress Response
Plasma levels of the NE precursor, dihydroxyphenylalanine, and the intraneuronal NE metabolite, dihydroxyphenylglycol, were significantly elevated in arginase II KO mice, indicating enhanced NE turnover (Figure 1A). However, plasma NE and epinephrine levels were not different between control and arginase II KO mice. The animals’ response to an arousal feeding stimulus (Figure S2A to S2B) or when they were stressed for 5 minutes either by being placed on a shaker (Figure S2C to S2D) or in a restrainer (Figure 1B to 1C) resulted in increases in pressure and HR in both strains. For
the restraint stress (Figure 1D to 1E) and the response to feeding (Figure S2E to S2F) but not the shaker stress (Figure S2G to S2H), the magnitude of increase in MAP but not HR was significantly greater in the arginase II KO mice.

Plasma and Kidney Urea Levels
Plasma urea levels were not different between the 2 strains (control versus arginase II KO mice; 21.3 ± 1.3 versus 19.9 ± 1.3 mmol/L; n = 10; P > 0.05). Similarly, kidney urea levels were unaltered by the arginase II deletion (control versus arginase II KO mice; 17.9 ± 2.4 versus 16.9 ± 2.8 mmol/L; n = 8; P > 0.05).

Vascular Responses In Vitro and In Vivo
Maximal contractile responses to high K⁺ levels significantly increased with age (from 7 to 15 weeks of age) in both control and arginase II KO mice. Although these responses were not different between groups in the 7-week- and 15-week-old mice, the 10-week-old arginase II KO mice exhibited diminished sensitivity compared with control mice (Figure S3).

Responses to NE were similar in aorta isolated from control and arginase II KO mice at 7 weeks of age (Figure 2A) but significantly blunted in aorta from arginase II KO animals at 10 and 15 weeks of age (Figure 2B and 2C). Similar responses were observed with the vasoconstrictors PE (15 weeks of age: E_max control versus arginase II KO mice, 0.44 ± 0.12 versus 0.14 ± 0.03; n = 9; P < 0.05) and endothelin I (15 weeks of age: E_max control versus arginase II KO mice, 0.35 ± 0.06 versus 0.14 ± 0.04; n = 8 to 9; P < 0.05). The contractile responses to NE were enhanced with the addition of the NO synthase inhibitors N-nitro-L-arginine (NOLA) or N²-nitro-L-arginine methyl ester, in both control and arginase II KO mice (Figure 2A through 2C). However, the magnitude of augmentation was not different between the 2 strains regardless of age. Responses to acetylcholine (ACh) were not different between groups in the 7- or 15-week-old mice but trended strongly toward an increased response in arginase II KO in the 10-week-old mice (Figure 2D through 2F). Responses to the vasodilator ACh were abolished in the presence of NOLA or N-G-nitro-L-arginine methyl ester and were not different in control and arginase II KO mice. Similarly, responses to isoprenaline, which were partially blocked in the presence of NOLA, were not different between control and arginase II KO mice (data not shown). Responses to sodium nitroprusside in both control and arginase II KO mice were not different between groups (Figure S4). Dietary l-arginine supplementation had no significant effect on vascular reactivity in response to ACh or NE (Table S2). The attenuated aortic constrictor responses to PE in arginase II KO mice were also observed in vivo, and dietary l-arginine supplementation had no significant effect (Figure S5).

Vascular and Plasma NO Levels
Real-time, fluorescence imaging (using 4-amino-5-methylamino-2′,7′-dichlorofluorescein diacetate to measure NO production) of isolated segments of aorta from 10- and 15-week-old control and arginase II KO mice showed no differences in fluorescence between groups at any time point (for 240 seconds, see Figure 3A). Furthermore, as indices of NO production, plasma NO metabolites were not significantly different be-

### Table. Heart and Lung Weights and Cardiac Function in Arginase II Knockout and Control Mice (n = 6 to 9 per group) at 15 Weeks of Age

<table>
<thead>
<tr>
<th>Strain</th>
<th>Control</th>
<th>Arginase II KO</th>
<th>Control + L-Arginine Supplement</th>
<th>Arginase II KO + L-Arginine Supplement</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>34.3 ± 0.8</td>
<td>29.0 ± 0.4*</td>
<td>31.8 ± 1.2</td>
<td>29.4 ± 0.6</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>337 ± 19</td>
<td>336 ± 22</td>
<td>323 ± 13</td>
<td>365 ± 14</td>
</tr>
<tr>
<td>SAP, mm Hg</td>
<td>118 ± 5</td>
<td>163 ± 11*</td>
<td>117 ± 2</td>
<td>185 ± 4*</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>90 ± 4</td>
<td>116 ± 8*</td>
<td>92 ± 2</td>
<td>134 ± 3†‡</td>
</tr>
<tr>
<td>DAP, mm Hg</td>
<td>75 ± 3</td>
<td>92 ± 7*</td>
<td>79 ± 1</td>
<td>109 ± 1†‡</td>
</tr>
<tr>
<td>PP, mm Hg</td>
<td>43 ± 2</td>
<td>71 ± 6*</td>
<td>37 ± 2</td>
<td>76 ± 3†</td>
</tr>
<tr>
<td>LVSP, mm Hg</td>
<td>8 ± 1</td>
<td>11 ± 1</td>
<td>6 ± 1</td>
<td>11 ± 1†</td>
</tr>
<tr>
<td>LV weight, mg/mm</td>
<td>1.2</td>
<td>29.4</td>
<td>29.8</td>
<td>31.8</td>
</tr>
<tr>
<td>PP weight, mg/mm</td>
<td>0.4*</td>
<td>31.8</td>
<td>32.0</td>
<td>33.5</td>
</tr>
<tr>
<td>RV weight, mg/mm</td>
<td>0.4 17.6</td>
<td>0.6 18.2</td>
<td>0.6 18.2</td>
<td>0.6 18.2</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>8* 9</td>
<td>12 2</td>
<td>16 2</td>
<td>20 3</td>
</tr>
<tr>
<td>dP/dt max, mm Hg/s</td>
<td>967 ± 368</td>
<td>10069 ± 675</td>
<td>7514 ± 368*</td>
<td>10420 ± 277†</td>
</tr>
<tr>
<td>t, ms</td>
<td>14 ± 1</td>
<td>22 ± 3*</td>
<td>16 ± 2</td>
<td>24 ± 3†</td>
</tr>
<tr>
<td>Lung weight, mg</td>
<td>152 ± 5</td>
<td>147 ± 4</td>
<td>156 ± 10</td>
<td>143 ± 3</td>
</tr>
<tr>
<td>Tibial length, mm</td>
<td>18.0 ± 0.4</td>
<td>17.6 ± 0.6</td>
<td>18.2 ± 0.2</td>
<td>18.1 ± 0.2</td>
</tr>
<tr>
<td>Atria weight, mg/mm</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>RV weight, mg/mm</td>
<td>5.2 ± 0.3</td>
<td>4.7 ± 0.7</td>
<td>4.2 ± 0.1</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td>LV weight, mg/mm</td>
<td>5.8 ± 0.3</td>
<td>7.8 ± 0.6*</td>
<td>5.1 ± 0.3</td>
<td>6.2 ± 0.4</td>
</tr>
</tbody>
</table>

LV indicates left ventricle; RV, right ventricle (normalized for tibial length); LVEDP, left ventricular end diastolic pressure; LVSP, left ventricular systolic pressure; dP/dt max, rate of cardiac contraction; dP/dt min, rate of cardiac relaxation. Data are presented as mean ± SEM.

*P < 0.05 vs control (C57BL/6).
†P < 0.05 vs control + l-arginine supplement.
‡P < 0.05 vs arginase II KO using 1-way ANOVA with Bonferroni posthoc analysis.
Rho Kinase Contribution

In light of the finding that the dampened constrictory effects observed in the arginase II KO animals were not attributable to alterations in NO production in the 15-week-old mice, we investigated the contribution of Rho kinase, an important regulator in calcium sensitization and vascular contraction. The addition of the Rho kinase inhibitor Y-27632 or HA-1077 attenuated the responses to PE and NE in both control and arginase II KO mice (Figure 4A through 4C). The relative contributions of Rho kinase in the aorta of arginase II KO mice were significantly (≥60%) less than that observed in aorta from control mice (see difference in area under the curve analysis before the addition of inhibitor in Figure 4D through 4F).

Discussion

Primary hypertension is a major risk factor for cardiovascular disease, the etiology of which is not well understood. Several reports suggest that changes in blood vessel structure and function precede BP elevation and that limited NO bioavailability can cause hypertension. Arginase I and/or II in the vasculature have been reported to promote vascular proliferation, increase arterial stiffness, and reduce NO bioavailability, leading to adverse cardiovascular outcomes. Thus, it was our a priori anticipation that the arginase II KO mouse would exhibit low BP.

Instead, our findings clearly demonstrate for the first time a hypertensive phenotype in these mice when BP was assessed in the conscious animal by telemetry or under anesthesia by arterial catheterization. Despite recent findings that suggest that arginase II inhibition may be suitable therapy for heart failure, we also demonstrated cardiac diastolic dysfunction and left ventricular hypertrophy in the arginase II KO mice as early as 15 weeks of age, suggesting that a lack of arginase II is not sufficient to preserve cardiac function in the face of sustained pressure overload. In comparison with the eNOS KO mice, where the MAP of 12- to 16-week-old mice was 15 mm Hg higher than the C57BL/6 strain, the arginase II KO mice demonstrate a ~25 mm Hg greater BP than age-matched C57BL/6 mice, suggesting a more fundamental role for arginase II than for eNOS in BP regulation. Indeed, with the exception of the angiotensin II receptor IA KO mouse, which also exhibits an ~25-mm Hg drop in BP, few other single gene deletion models have reported such a profound increase in BP.

In light of the recent published findings of Lim et al., who observed a dampened vasoconstrictory profile in these mice that could be attributed to increased NO bioavailability, ie, findings that needed reconciliation with our data of a hypertensive phenotype, we re-examined the vascular profile of these mice. Similar to Lim et al., we observed a dampened vasoconstrictory profile to NE, PE, and endothelin I but only in 10- and 15-week-old mice. Again, comparable to Lim et al. (who studied only 10-week-old mice) we observed a trend (P=0.07) toward an increased response to ACh, and we concur with their conclusion that there may be increased NO production in the aorta of the 10-week-old arginase II KO mouse. However, this finding appears to be age specific in that no differences between strains in the dilatory response to the endothelium-dependent agonist ACh nor to the partial endothelium dependent agonist isoprenaline, responses to both of which were confirmed to be NO synthase dependent, were observed in the 7- and/or 15-week-old animals. Furthermore, although NO synthase inhibition significantly augmented the responses to NE, the magnitude of augmentation was not different between the 2 strains, suggesting that basal NO release was not different in the arginase II KO mouse. Indeed, unlike the findings by Lim et al., using fluorescence imaging of NO release, there were no significant differences in responses to ACh-induced NO production between 10- or 15-week-old control and KO mice. Because NO bioavailability is usually reduced during hypertension, L-arginine supplementation has successfully been used in the past to overcome reduced NO bioavailabil-

Figure 1. A. Plasma catecholamine levels (DHPG indicates dihydroxyphenylglycol; DOPA: dihydroxyphenylalanine; Epi, epinephrine) and effect of aversive restraint stress on (B) MAP and (D) HR in arginase II KO mice. Change in (C) MAP and (E) HR from rest to 5-minute stress period are shown. Data are presented as mean±SEM. *P<0.05 using unpaired t test between control and arginase II KO mice at either 7 or 15 weeks of age but were significantly increased with dietary L-arginine supplementation (Figure 3B).
Dietary supplementation of \( \text{l-arginine} \) resulted in increased plasma \( \text{l-arginine} \) concentration levels and had no significant effects on MAP in controls; however, MAP in the arginase II KO mice was unexpectedly increased.

Given the previous finding by Ming et al\(^4\) that the addition of thrombin to human endothelial cells stimulates arginase II via a Rho A/Rho kinase–dependent mechanism and that Rho kinase is involved with regulating vascular contraction via calcium sensitization, vascular smooth cell proliferation and migration, remodeling of actin cytoskeleton, and downregulation of eNOS,\(^29\) we examined its role in the context of its contribution to the constrictory responses in the 15-week-old animals. Although increased Rho-kinase expression and/or activity has been reported in hypertension,\(^30\) this appears to vary dependent on the vessel bed. In the present study on the mouse aorta, the vascular reactivity findings do not explain the hypertensive phenotype of the arginase II KO mouse was robust and consistent from 8 to 15 weeks of age. Regardless of age and the effect on vascular reactivity, however, the hypertensive phenotype of the arginase II KO mouse was robust and consistent from 8 to 15 weeks of age. The vascular reactivity findings do not explain the hypertensive phenotype observed, although a similar contrafinding of a diminished vasconstrictory profile in a hypertensive animal has been reported previously,\(^31\) where the authors postulated that the reduced cardiovascular sensitivity may result in vessel rigidity, thus reducing vascular compliance, resulting in high BP. In the present study, the hypertensive phenotype was observed as early as at adolescent age (8 weeks old; the

Figure 2. Full concentration response curves to (A through C) NE and (D through F) ACh before and after the addition of NOLA (100 \( \mu \text{mol/L} \)) or N\(^3\)-nitro-L-arginine methyl ester (10 \( \mu \text{mol/L} \)) and to (G and H) single-nucleotide polymorphism were obtained in control (CON) and arginase II KO (KO) mice at 7, 10, and 15 weeks of age. All of the data are presented as mean±SEM. EC\(_{50}\) and E\(_{\text{max}}\) responses were compared using 1-way ANOVA with a Bonferroni posthoc test, where \(* P<0.05\), \(** P<0.01\), and \(*** P<0.001\).

<table>
<thead>
<tr>
<th>Condition</th>
<th>EC(_{50}) (( \mu \text{mol/L} ))</th>
<th>E(_{\text{max}}) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>1.8 ± 0.2</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>ArgII KO</td>
<td>2.5 ± 0.3</td>
<td>95 ± 3</td>
</tr>
</tbody>
</table>

Plasma NOx (\( \mu \text{M} \))

<table>
<thead>
<tr>
<th>Condition</th>
<th>Plasma NOx (( \mu \text{M} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>ArgII KO</td>
<td>15 ± 2</td>
</tr>
</tbody>
</table>

Figure 3. A, NO release, measured by 4-amino-5-methylamino-2′,7′-dichlorofluorescein diacetate fluorescence, in response to ACh (10 \( \mu \text{mol/L} \)) in segments of aorta from 10- and 15-week-old control (CON) and arginase II knockout (ArgII KO) aorta 240 seconds after stimulation (\(P>0.05\); 1-way ANOVA). B, Plasma nitrites and nitrates (NO\(_x\)) in control (CON) and arginase II KO (ArgII KO) mice at 7 or 15 weeks of age and with \( l \)-arginine dietary supplementation (\(+\) L-arg). \(P<0.05\) vs CON mice without \( l \)-arginine supplementation using 1-way ANOVA with a Bonferroni posthoc test. All of the data are presented as mean±SEM and represent \( n=4 \) to 10.
earliest it was recorded via telemetry in this study) and was apparent until at least mature adult age (15 weeks old), suggesting that the abnormality resided in organs responsible for the long-term regulation of BP, namely, the brain, with later influence of vasoconstriction resulting from structural and functional changes in the vasculature leading to deterioration of vital organ (eg, kidney, brain, and heart) function.

In the nucleus tractus solitarius and hypothalamus, coexpression of arginase II and neuronal NO synthase has been shown.32 Centrally, some studies have suggested that NO can enhance reflex bradycardia and depressor response33,34; others believe that increased NO can set BP at a higher level.35 Given that arginase II may reciprocally regulate NO bioavailability, it is also possible that a lack of arginase II may result in a central resetting of BP. Increased sympathetic activation is classically present in hypertension and arousal or stress.36 In this study, the application of restraint stress or during an arousal feeding stimulus led to a significantly greater pressor response in the arginase II KO mice, but there was no difference in the pressor response to a mechanically induced response (oscillating table response). These findings suggest that sympathetic function was augmented in the arginase II–deficient mice. The mechanism underlying increased sympathetic activity during hypertension is not well established and can arise from changes in the periphery or centrally within the brain. Our findings show greater levels of the plasma NE precursor and the metabolite, dihydroxyphenylalanine and dihydroxyphenylglycol, respectively, in the arginase II KO mice, suggesting that sympathetic outflow may be increased. It is recognized, however, that measuring plasma catecholamines is not the best approach to estimate central sympathetic outflow because of limitations associated with blood sampling and the choice of anesthesia.37 Mice are nocturnal animals and are more active during the night; as such, BP in both strains was increased during the night, however, the BP differences between day and night in the arginase II KO mice were significantly higher compared with the control mice. This finding further supports the view of an increased sympathetic nervous system contribution to hypertension, because the circadian changes in BP are largely attributable to changes in sympathetic nervous system activity. Intriguingly, we demonstrated an increase in baroreflex gain that is usually seen with exercise training,38 whereas impairment of baroreflex function is observed in hypertension.39

Asides from the brain, the kidney is also thought to be a major organ responsible for the long-term regulation of BP.
Arginase II is highly expressed in both the cortex and medulla of the kidney in many species, including mice. Interestingly, decreased arginase II expression in the kidney has been reported to be an underlying cause of salt-sensitive hypertension. When these rats were fed for 6 weeks with 5% urea, hypertension was reduced by 20 mm Hg, suggesting that urea deficit, because of the lack of arginase II, may be responsible for the increase in BP. Because urea inhibits sodium reabsorption in the proximal tubule, as well as the Na-K-2Cl pump found in the thick ascending limb, the lack of urea would consequently increase sodium reabsorption, shifting the pressure-natriuresis curve to the right, thus increasing BP. However, the arginase II KO mice in the current study exhibited no differences in either plasma or kidney urea levels.

A notable finding of the current study is that changes in vascular sensitivity and reactivity over time were clearly apparent. Because the increase in BP was observed from week 8 of age, but no vascular differences were observed in week 7 (in the current study) and were only seen in week 10 (supported by Lim et al) and maintained albeit via a potentially different signaling pathway in week 15, the etiology of the hypertension would appear not to be because of vascular modifications but rather other causes, eg, an increase in sympathetic drive.

In conclusion, the phenotype of the arginase II KO mouse suggests that global loss of arginase II activity appears to result in hypertension. Because of the as-yet-undefined nature of the role of arginase II in the promotion of this hypertensive phenotype, we suggest that data from isolated vessels from the strain of mouse in the context of vascular responses be interpreted with caution and that it warrants further investigation as a potential model of resistant hypertension.

Perspectives

Despite recent findings that increased arginase activity and expression lead to adverse cardiovascular outcomes, the present study clearly demonstrates that, in arginase II KO mice, a global arginase II ablation results in a hypertensive phenotype (=25 mm Hg greater). These mice have cardiac diastolic dysfunction and left ventricular hypertrophy but a decreased vasoconstrictory profile, the mechanism of which appears age dependent, minimally involving NO but where a loss of Rho kinase activity is apparent. KO mice also showed enhanced plasma NE turnover, suggesting an increased sympathetic outflow. These findings suggest a fundamental role for arginase II in BP regulation through a currently undefined mechanism.

Sources of Funding

This work was supported by grants from the National Health and Medical Research Council of Australia.

Disclosures

None.

References


Arginase II Knockout Mouse Displays a Hypertensive Phenotype Despite a Decreased Vasoconstrictory Profile

Ngan N. Huynh, Karen L. Andrews, Geoffrey A. Head, Sacha M.L. Khong, Dmitry N. Mayorov, Andrew J. Murphy, Gavin Lambert, Helen Kiriazis, Qi Xu, Xiao-Jun Du and Jaye P.F. Chin-Dusting

Hypertension. 2009;54:294-301; originally published online June 22, 2009;
doi: 10.1161/HYPERTENSIONAHA.108.121731

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/54/2/294

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2009/06/08/HYPERTENSIONAHA.108.121731.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension 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 Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
Online Supplement

The arginase II knockout mouse displays a hypertensive phenotype despite a decreased vasoconstrictory profile.

Ngan N Huynh, Karen L Andrews, Geoffrey A Head, Sacha ML Khong, Dmitry N Mayorov, Andrew J Murphy, Gavin Lambert, Helen Kiriazis, Qi Xu, Xiao-Jun Du, Jaye PF Chin-Dusting*

Baker IDI Heart and Diabetes Institute, 75 Commercial Rd, Melbourne, Australia 3004.

Running title: Hypertension of the arginase II KO mouse

*Author for Correspondence:

Jaye PF Chin-Dusting
Head, Vascular Pharmacology
Baker IDI Heart and Diabetes Institute
PO Box 6492 St Kilda Rd Central
Victoria 8008 Australia
Ph: +61 3 8532 1505
Ph: +61 3 8532 1160
e-mail: jaye.chin-dusting@bakeridi.edu.au
Expanded Materials and Methods

The study protocol was approved by the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee. Studies were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Animals and treatments
Two pairs of the arginase II knockout (arginase II KO) breeders with C57BL/6 background were a generous gift from Prof William O’Brien1 for establishment of a colony at the Animal Services Facility at the Baker IDI Heart and Diabetes Institute. Prior to experimentation genotyping was performed by standard PCR on DNA extracted from tail clippings to ensure a homozygous colony. These mice have been reported to have low arginase activity in the kidney, where arginase II is ubiquitously expressed.1 Age-matched male C57BL/6 mice were used as controls. All animals were housed under pathogen-free environments in a temperature and humidity controlled facility on a 12:12 hour light–dark cycle, fed standard chow (containing 0.4% NaCl, Specialty Feeds, Glenn Forrest, Australia) and provided with water ad libitum. Animals were studied at 7, 10 and/or 15 weeks as stated. Mice were anaesthetized with 80% CO2 and 20% O2 and exsanguinated by cardiac puncture. Blood was collected into heparinized saline lined tubes and the thoracic aorta was excised and placed into ice-cold Krebs modified buffer (composition in mmol/L; NaCl 119, KCl, 4.7, MgSO4·7H2O 1.17, NaHCO3 25, KH2PO4 1.18, CaCl2, 2.5 glucose 11 and EDTA 0.03). Blood was centrifuged at 10,000 g for 10 mins at 4°C and the plasma was then aliquoted and stored at −20°C for subsequent analysis. In a subset of mice an L-arginine (L-arginine USP, Musashi Pty.Ltd, Notting Hill, Australia) supplement was added to the drinking water (25g/L) of 11-week old age-matched C57Bl/6 and arginase II KO mice for 4 weeks and mice allowed access ad libitum. At 15-weeks of age the mice were either utilized for in vitro study or cardiac catheterization.

Blood pressure telemetry
Telemetry devices (TA11PA-C10, Data Sciences International, MN USA) were implanted in control and arginase II KO mice at 7 weeks of age (the minimum age/weight recommended by the manufacturers) under open circuit halothane gas anaesthesia to monitor mean arterial pressure (MAP), heart rate (HR) and locomotor activity as previously described.2 Briefly, the catheter tip was inserted into the aortic arch via the left carotid artery and the transmitter body placed under the skin along the flank. Following recovery, animals were individually housed and radiotelemetry signals collected as previously described3. Blood pressure was recorded continuously over the weekend every week between 8 and 15 weeks of age.

Beat-to-beat values were filtered to remove values greater than 2.5 times the running standard deviation and then MAP and HR values were separated into light and dark periods to provide a single value for day and night for each animal
each week. To examine cardiovascular stress responses using blood pressure telemetry, animals were restrained without physical pressure (in a plexiglass restrainer) for 5 min on 2 consecutive days mid-week at 8, 11 and 15 weeks of age as previously described\(^4\). Beat-to-beat data for feeding, restraint and shaker stress was binned into 30-sec intervals 5 min before, during and after stress stimuli. In addition, the average change during stress stimuli was subtracted from baseline resting period in MAP and HR to calculate the magnitude of cardiovascular responses.

**Assessment of baroreflex by spectral analysis and sequences**

Cardiovascular variability and baroreflex sensitivity was measured non-invasively by power spectral analysis to determine if there were specific changes within the frequency bands, low frequency, midfrequency and high frequency as a measure of vascular reactivity, sympathetic activity and parasympathetic activity, respectively as previously described\(^5\). An hour of beat-to-beat recording of MAP and HR data at 11 am (inactive period) and 11 pm (active period) were analyzed at week 8, 11 and 15 in CON and KO mice. The auto- and cross-power spectra were calculated for multiple overlapping (by 50%) segments of MAP and HR using Fast Fourier Transform\(^5\). The average value of the transfer gain in the frequency band between 0.3 and 0.5 Hz was used as the estimate of the baroreflex sensitivity\(^6\). Other frequency bands included in the analysis were low frequency (0.08 - 0.3 Hz) and high frequency (0.5 - 1 Hz) and the total power was calculated between 0 and 1 Hz\(^7\).

**Cardiac function and in vivo vascular reactivity**

15-week old age-matched control and arginase II KO mice were anaesthetized with a mixture of ketamine, xylazine and atropine (100/10/1.2 mg/kg body weight respectively, i.p.) and cardiac function measured by cardiac catheterization as previously described\(^8\). The measurements were made from 8-10 consecutive beats and averaged. In addition, another catheter (30G) was inserted into the jugular vein for delivery of phenylephrine (0.25 – 8 µg/kg) where responses to phenylephrine were analysed as change in MAP above baseline values. At the end of the experiments animals were killed with a bolus injection of the anaesthetic mixture and the tibia, lung and heart excised. Tibia length and organ weights were recorded. The heart was dissected into the left ventricle (LV), right ventricle (RV) and atria and weighed and normalised to tibia length.

**Plasma and Kidney Urea Levels**

Plasma and kidney urea levels were measured using a QuantiChrom Urea Assay Kit (DIUR-500). The assay utilises a modified-Jung method,\(^9\) which uses a chromogenic reagent to form a coloured complex specifically with urea, the colour intensity of which is then measured at 520 nm.
**Isolated aortic rings**

Vascular reactivity studies were performed on aorta isolated from mice at 7, 10 and 15 weeks of age. The thoracic aorta was cleaned and mounted in a Mulvany myograph (model 610M, JP Trading, Denmark) as previously described\(^\text{10}\). Responses to KPSS (composition in mmol/L; KCl 123, MgSO\(_4\)\(\cdot\)7\(\text{H}_2\text{O}\) 1.17, NaHCO\(_3\) 25, KH\(_2\)PO\(_4\) 1.18, CaCl\(_2\) 2.5 glucose 6.05 and EDTA 0.03) were obtained in all vessels and endothelium integrity was assessed by a response to 1 \(\mu\)mol/L of acetylcholine (ACh). Full concentration response curves (CRCs) were constructed to norepinephrine (NE; 0.1 nmol/L – 1 \(\mu\)mol/L), phenylephrine (PE; 0.1 nmol/L – 1 \(\mu\)mol/L) and endothelin-1 (1 nmol/L – 1 \(\mu\)mol/L), acetylcholine (ACh; 1 nmol/L – 100 \(\mu\)mol/L), sodium nitroprusside (SNP; 0.1 nmol/L – 10 \(\mu\)mol/L) and isoproterenol (0.1 nmol/L – 100 \(\mu\)mol/L). Some of these agonists were conducted in endothelium intact or denuded vessels or repeated after incubation of an inhibitor for 30 min. The inhibitors used include the NOS inhibitor, \(N^\omega\)-nitro-L-arginine (NOLA, 10 \(\mu\)mol/L) and L- \(N^G\)-Nitroarginine methyl ester (hydrochloride) (L-NAME, 10 \(\mu\)mol/L), the \(\beta\)-blocker, propranolol (10 \(\mu\)mol/L), and the rho-kinase inhibitors, Y-27632 (1 \(\mu\)mol/L) and HA-1077 (10 \(\mu\)mol/L). Where a CRC was constructed to a vasodilator, vessels were precontracted with NE or PE producing 70% maximal NE or PE response. No more than two CRCs were performed in any one aortic ring. Curves were fitted to responses to the vasodilators, ACh and SNP, using the Hill Equation in GraphPad Prism to obtain the \(E_{\text{max}}\) and –logEC\(_{50}\) value. To assess the relative contribution of rho-kinase, the difference between the area under the curve (AUC) of responses to constrictor agonist before and after Y-27632 or HA-1077 was calculated. AUC was analysed in GraphPad Prism with the baseline set at \(y = 0\).

**Imaging of NO production**

Using DAF-FM, real time, fluorescence imaging of isolated segments of aorta from 10 and 15 week old control and arginase II knockout mice was performed as previously described\(^\text{11}\). Briefly, rings of aorta (5mm) were incubated in 1 mL of Kreb’s containing DAF-FM DA (10 \(\mu\)mol/L) for 30 min, followed by a washout period of 30 min. The ring was sliced longitudinally to expose the luminal surface and the adventitial surface mounted on glass slides with silicon grease. The tissue was immersed in 100\(\mu\)L of Kreb’s buffer and visualised under an Olympus Fluorescent microscope and baseline rates of fluorescence changes assessed for 5 min. Kreb’s containing acetylcholine (1 \(\mu\)mol/L) was perfused over the tissue and fluorescence changes assessed at 0, 10, 20, 40, 60, 120, 180, 240, and 300 seconds either in the absence or presence of L-NAME (100 \(\mu\)mol/L). L-NAME significantly attenuated the response to ACh, validating a measurement of NO (data not shown).
Nitrate/Nitrite assay

Plasma NO metabolites (nitrate/nitrite) were measured using a Griess Reaction kit (Cayman Chemicals). Plasma from whole blood collected in heparinised saline-lined tubes was centrifuged at 14,000 g for 20 min in Nanosep® Centrifugal Device spin column (10K molecular weight cut off, Pall Life Sciences, Lane Cove, NSW, Australia) to filter all haemolysed sample prior to performing a Griess reaction (Griess Reaction kit, Cayman Chemicals, MI, USA, sourced from Sapphire Biosciences P/L, Redfern, NSW, Australia) to measure NO metabolites, nitrates (NO3-) and nitrites (NO2). The Nanosep® devices were pre-rinsed twice (centrifuged at 14,000 g for 5 min each time) with deionised water to remove traces of sodium azide found in the filtering membrane. Briefly, the kit is a two step colorimetric assay performed in 96 well flat bottom plates (Linbro®, ICN Biomedicals Incorporated, Ohio, USA); the first step involves the conversion of nitrate to nitrite with the addition of nitrate reductase and the second step requires the addition of Griess reagents 1 (Sulfanilamide) and 2 [N – (1-Napthyl) ethylenediamine], resulting in the conversion of the nitrite into a purple azo compound. Standard curves for both nitrates and nitrites were performed using nitrate and nitrite standards provided in the kit. All samples were performed in duplicate with a plasma volume of 40 µl, total sample volume was adjusted to 80 µl of assay buffer provided in the kit. Absorbance was measured at 540 nm on the Victor V3 Multiplate Reader (Perkin Elmer), replicate absorbances were averaged and the concentration calculated from the standard curve. Nitrate and nitrite results were combined to provide a total concentration in µmol/L for NO metabolites (NOx).

Statistical analysis

All data was presented as mean±SEM and considered statistically significant if \(P<0.05\). Blood pressure telemetry was analysed using a split-plot repeated measures ANOVA (Microsoft Excel). Plasma catecholamines, cardiac size and change in stress responses were compared using an unpaired student t-test in GraphPad Prism (v4.0). In vivo responses to PE, functional cardiac measurements and NO measurements were compared using a one-way ANOVA with a Bonferroni post-hoc analysis. For isolated aortic rings a comparison of either the \(-\log EC_{50}\) or the \(E_{\text{max}}\) between control and arginase II KO for the contractile and dilatory responses an unpaired t-test was used if one curve was constructed from each animal. Alternatively, a one-way ANOVA was used to compare either the \(-\log EC_{50}\) or the \(E_{\text{max}}\) if two curves for each animal were performed.
References


### Table S1. Average spectral power in low mid and high frequency bands for mean arterial pressure and heart rate in arginase II KO and control (CON) mice.

<table>
<thead>
<tr>
<th>Strain</th>
<th>CON</th>
<th>arginase II KO</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean Arterial Pressure (mmHg)²</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Frequency Power</td>
<td>1.69 ± 0.18</td>
<td>1.80 ± 0.31</td>
<td>NS</td>
</tr>
<tr>
<td>Mid Frequency Power</td>
<td>0.40 ± 0.06</td>
<td>0.35 ± 0.06</td>
<td>NS</td>
</tr>
<tr>
<td>High Frequency Power</td>
<td>0.50 ± 0.12</td>
<td>0.56 ± 0.10</td>
<td>NS</td>
</tr>
<tr>
<td>Total Power of variance</td>
<td>4.98 ± 0.60</td>
<td>7.14 ± 2.23</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Heart Rate (beats/min)²</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Frequency Power</td>
<td>457 ± 134</td>
<td>622 ± 127</td>
<td>NS</td>
</tr>
<tr>
<td>Mid Frequency Power</td>
<td>147 ± 47</td>
<td>230 ± 59</td>
<td>NS</td>
</tr>
<tr>
<td>High Frequency Power</td>
<td>329 ± 164</td>
<td>394 ± 110</td>
<td>NS</td>
</tr>
<tr>
<td>Total Power of variance</td>
<td>2925 ± 1380</td>
<td>6244 ± 2633</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Baroreflex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gain</td>
<td>12.3 ± 0.9</td>
<td>18.5 ± 1.7</td>
<td>*</td>
</tr>
<tr>
<td>Coherence</td>
<td>0.51 ± 0.02</td>
<td>0.51 ± 0.03</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data is mean±SEM, indicating between animal variance. Probabilities are NS not significant *P<0.01, for arginase II KO compared to CON, Low frequency 0.08-0.3 Hz, Mid frequency 0.3-0.5 Hz, High frequency 0.5-1 Hz, Total, 0 - 1 Hz.
Table S2 Effect of dietary L-arginine supplementation on vascular reactivity. EC$_{50}$ and E$_{\text{max}}$ values for concentration response curves to acetylcholine (ACh) and norepinephrine (NE) in 15 week old control (CON) and arginase II KO (KO) mice with and without (control) dietary L-arginine supplementation (4 weeks in drinking water prior to experimentation).

<table>
<thead>
<tr>
<th>Agonist (EC$_{50}$)</th>
<th>Control CON (n=6)</th>
<th>KO (n=7)</th>
<th>With L-arginine Supplementation CON (n=9)</th>
<th>KO (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh</td>
<td>7.3 ± 0.2</td>
<td>7.1 ± 0.2</td>
<td>7.0 ± 0.1</td>
<td>7.1 ± 0.2</td>
</tr>
<tr>
<td>NE</td>
<td>8.0 ± 0.1</td>
<td>7.8 ± 0.1</td>
<td>8.0 ± 0.1</td>
<td>7.7 ± 0.1</td>
</tr>
<tr>
<td>Agonist (E$_{\text{max}}$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ach (%)</td>
<td>86 ± 4</td>
<td>88 ± 2</td>
<td>86 ± 3</td>
<td>83 ± 3</td>
</tr>
<tr>
<td>NE (g)</td>
<td>0.58 ± 0.11</td>
<td>0.29 ± 0.06*</td>
<td>0.62 ± 0.05</td>
<td>0.30 ± 0.06*</td>
</tr>
</tbody>
</table>

All data are presented as mean±SEM. *P<0.05 vs CON
Figure S1

Averaged day and night recordings showing mean arterial pressure (MAP), heart rate (HR) and activity (arbitrary units) in arginase II KO mice (KO; n=7, open circles) and control (CON; n=7, closed circles) mice. Data are presented as mean±SEM. **P<0.01 and ***P<0.001 using a split-plot repeated measures ANOVA.
Figure S2

A

MAP (mmHg)

CON (n = 8)  
KO (n = 8)

0 5 10 15

0 5 10 15

Time (mins)

B

HR (bpm)

CON (n = 8)  
KO (n = 8)

0 5 10 15

0 5 10 15

Time (mins)

C

MAP (mmHg)

CON (n = 6)  
KO (n = 7)

0 5 10 15

0 5 10 15

Time (mins)

D

HR (bpm)

CON (n = 6)  
KO (n = 7)

0 5 10 15

0 5 10 15

Time (mins)

E

\[ \Delta \text{MAP (mmHg)} \]

CON  KO

F

\[ \Delta \text{HR (bpm)} \]

CON  KO

G

\[ \Delta \text{MAP (mmHg)} \]

CON  KO

H

\[ \Delta \text{HR (bpm)} \]

CON  KO
**Figure S2** Effect of either an arousal feeding stimulus (almond meal) on A, mean arterial pressure (MAP) and B, heart rate (HR) or shaker stress on C, MAP and D, HR responses. P1 denotes phase 1 and P2 denotes phase 2 of a biphasic MAP and HR response. The delta change from baseline (averaged of stress period minus average of rest period) during feeding stimulus for E, MAP but not F, HR was greater in the arginase II knockout (KO) mice when compared to control (CON) mice. Averaged change over 5 min period during shaker stress from baseline in G, MAP and H, HR. No differences were observed between strains if only the first 2 minutes (P1) were compared. Data are presented as mean±SEM. *P<0.05 using unpaired Student t-test.
Figure S3 Responses to aortic vasoconstrictor agonists *in vitro* to KPSS at 7, 10 and 15 weeks of age. All data are presented as mean±SEM. *P*<0.05 compared control (CON) with arginase II KO (KO). Data was analysed using a two-way ANOVA with a Bonferroni post-hoc test.
Figure S4

Full concentration response curves to SNP were obtained in control (CON) and arginase II KO (KO) mice at A, 7, B, 10 and C, 15 weeks of age. All data are presented as mean±SEM. EC$_{50}$ and E$_{max}$ responses were compared using a Student’s t-test.
Figure S5

*Figure S5* In vivo responses to phenylephrine (PE) (change in mean arterial pressure; MAP) were obtained in 15 week old control (CON) and arginase II KO (KO) mice with (+ L-arg) and without L-arginine supplementation in their drinking water (25g/L) for 4 weeks prior to experimentation. All data are presented as mean±SEM. Group sizes are indicated in each figure. *P<0.05, ***P<0.001 vs CON. Data was analysed using a two-way ANOVA with a Bonferroni post-hoc test.