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Genetic modifiers of hypertension in soluble guanylate cyclase α1–deficient mice

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Nitric oxide (NO) plays an essential role in regulating hypertension and blood flow by inducing relaxation of vascular smooth muscle. Male mice deficient in a NO receptor component, the α1 subunit of soluble guanylate cyclase (sGCα1), are prone to hypertension in some, but not all, mouse strains, suggesting that additional genetic factors contribute to the onset of hypertension. Using linkage analyses, we discovered a quantitative trait locus (QTL) on chromosome 1 that was linked to mean arterial pressure (MAP) in the context of sGCα1 deficiency. This region is syntenic with previously identified blood pressure–related QTLs in the human and rat genome and contains the genes coding for renin. Hypertension was associated with increased activity of the renin-angiotensin-aldosterone system (RAAS). Further, we found that RAAS inhibition normalized MAP and improved endothelium-dependent vasorelaxation in sGCα1–deficient mice. These data identify the RAAS as a blood pressure–modifying mechanism in a setting of impaired NO/cGMP signaling.

Introduction
Systemic arterial hypertension is one of the most widespread public health problems in the developed world and the most prevalent modifiable risk factor for cardiovascular disease (CVD) in both women and men (1). The pathogenesis of essential hypertension is multifactorial, and in the vast majority of cases the etiology of hypertension is unknown. Although major advances in the treatment of hypertension have decreased CVD-related deaths over the last decade (2), many of the molecular mechanisms underlying the development of hypertension remain elusive. Genome-wide association studies (GWAS) suggest that there is a substantial heritable component to blood pressure (3, 4). Although GWAS have identified several loci associated with blood pressure in human beings, including loci containing genes that either regulate cGMP levels (4–7) or the renin-angiotensin-aldosterone system (RAAS) (8), many of the genetic factors determining blood pressure and how these factors interact remain to be identified.

Renal abnormalities, such as decreased urinary sodium excretion in response to increasing renal perfusion pressure, and increased activity of the RAAS are generally considered to be a major contributor to the development of high blood pressure (9). However, other studies support the idea that hypertension can arise from primary vascular abnormalities (10, 11). The ability of NO to relax vascular smooth muscle and its essential role in the regulation of blood flow are well characterized (12, 13). Ample evidence suggests that altered NO signaling is involved in the pathogenesis of hypertension (14).

One of the primary receptors for NO is soluble guanylate cyclase (sGC), a heme-containing enzyme that generates cGMP. The impact of NO on the cardiovascular system is mediated, at least in part, by cGMP-dependent mechanisms (15). sGC is a heterodimeric enzyme, consisting of α and β subunits. Two isoforms of each subunit have been identified (α1, α2, β1, β2), but only sGCα1β1 and sGCα2β1 appear to function in vivo (16). Although the sGCα1β1 heterodimer is the most abundant sGC isoform in the cardiovascular (17), low levels of cGMP, generated by sGCα1β1, are sufficient to mediate many of NO’s cardiovascular effects (15, 18–20).

The role of sGCα1β1 in regulating blood pressure in humans and the importance of sGC as a potential therapeutic target for hypertension were recently highlighted in a GWAS identifying a locus that influences blood pressure and that contains the genes encoding the sGCα1 and sGCβ1 subunits (7). We previously reported that male but not female mice deficient for sGCα1 on a 129Sv6 (S6) background (sGCα1−/−) develop hypertension (18). These effects appear to be modulated by genetic factors, because male sGCα1−/− mice on the C57BL/6 (B6) background (sGCα1−/−B6) do not develop hypertension. The underlying pathophysiological mechanisms responsible for the differential impact on blood pressure of impaired NO/cGMP signaling in these inbred strains of mice remained unknown. Therefore, we sought to identify genetic determinants critical to the development of hypertension in sGCα1−/− mice using an unbiased linkage analysis approach. Moreover, we characterized the role of a candidate gene (renin), identified in the linkage analysis, in the hypertension associated with deficient NO/cGMP signaling.

Results
Identification of a blood pressure quantitative trait locus on chromosome 1, and renin as a candidate modifier gene for blood pressure in sGCα1−/− mice. As previously described (18, 21), mean arterial blood pres-
sure (MAP) was higher in male sGCα1−/− mice than in sGCα1−/−B6 mice (161 ± 2 and 112 ± 3 mmHg, respectively; Figure 1). This difference in blood pressure between inbred sGCα1−/− strains confirms a strong genetic influence on the regulation of blood pressure in sGCα1−/− mice. To exploit this difference for identification of modifier genes of blood pressure, we performed a reciprocal F1 intercross between sGCα1−/−B6 and sGCα1−/−S6 mice. sGCα1−/−F2 progeny exhibited a large variance in MAP, ranging from values observed in sGCα1−/−B6 to values observed in sGCα1−/−S6 mice, demonstrating that the hypertension associated with sGCα1 deficiency is preserved in a subset of mice in the F2 cross (Figure 1). Using the phenotypic variance in genetically homogeneous parental and F1 mice as an estimate of the trait’s variation from environmental influences and the phenotypic variance in F2 mice as an estimate of the trait’s variation from both genetic and environmental influences, we estimated the broad heritability of the trait to be greater than 0.5.

A linkage analysis in a first cohort of 92 F2 mice, using 120 genome-wide SNPs, identified a quantitative trait locus (QTL) on chromosome 1 that influences blood pressure (lod score, 3.0; Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI60119DS1). Genotyping of a second independent cohort of 96 additional F2 mice yielded a collective lod score of 5.3 at the chromosome 1 locus (genome-wide corrected, P = 0.006; Supplemental Figures 2 and 3). To refine the linkage peak through additional recombinants and higher marker density, the original 188 mice and a third independent cohort of 96 F2 mice were genotyped for 28 SNPs across the QTL on chromosome 1. Linkage at this locus was highly significant, with a final lod score of 6.3 (genome-wide corrected, P = 0.0006) (Figure 2). We designated this locus Hsgcq (hypertension associated with sGCα1-deficiency QTL). Two other loci of interest were identified, on chromosomes 4 and 9, with maximal lod scores of 3.6 and 3.9, respectively. Here, we focused on the locus (Hsgcq) with the highest lod score.

Hsgcq was localized to a position close to Mm37-1-130689858 (Figure 2). The 1.5 lod confidence interval spanned a region on chromosome 1 between Mm37-1-119319789 and Mm37-1-158911588. One of the genes in this region is renin 1 (position on chromosome 1: 135,247,143–135,256,900), a candidate modifier gene of hypertension (22). Mice on the B6 background have one renin gene (renin 1c), whereas mice on the S6 background have two renin genes, designated renin 1d and renin 2 (23–25). In addition, when comparing the genomic DNA sequence of renin 1c and renin 1d (~10,000 bp), we identified 201 nucleotide substitutions and 126 nucleotide insertions or deletions, resulting in a sequence variability of 3.3%. We identified 21 nucleotides that were different between renin 1c and renin 1d cDNA (1,592 bp for both; Supplemental Figure 4). On the protein level, we identified 5 amino acid differences (of 402 amino acids): R58W, I68T, V160S, D315E, and 1...
sGCα1−/− mice and suggest that the effect size of Hsgcq on blood pressure is approximately 11 mmHg.

The strain-specific hypertension in sGCα1−/− mice is associated with increased activity of the RAAS. To test whether the genetic background affected the RAAS, we measured plasma Ang II and plasma aldosterone levels in male WTS6, sGCα1−/−B6, WT S6, and sGCα1−/−S6 mice. Plasma Ang II levels were similar in WT and sGCα1−/− mice on either the B6 or S6 background but were higher in S6 mice than in B6 mice (Figure 5A), confirming the existence of strain-specific differences in the RAAS. Plasma aldosterone levels did not differ in WT S6 and sGCα1−/−B6 mice (Figure 5B). However, plasma aldosterone levels were greater in sGCα1−/−S6 than in WT S6 mice (Figure 5B). Expression levels of aldosterone synthase (cytochrome P450, family 11, subfamily b, polypeptide 2, or CYP11B2) in adrenal glands did not differ between sGCα1−/−B6 and WT S6 mice but were higher in sGCα1−/−S6 mice than in WT S6 mice (Supplemental Figure 6). Consistent with the observed hyperaldosteronism, plasma K+ levels were lower and Na+ levels were higher in sGCα1−/−S6 mice than in WT S6 mice (Table 1).

To further determine the contribution of the increased activity of the RAAS to the elevated blood pressure associated with sGCα2 deficiency, we treated male WT S6 and sGCα1−/−S6 mice with the clinically used renin inhibitor aliskiren, which is available for oral administration. Aliskiren did not alter blood pressure in male WT S6 mice but normalized blood pressure in male sGCα1−/−S6 mice (Figure 6A). Similarly, the aldosterone antagonist spironolactone did not alter blood pressure in male WT S6 mice but normalized blood pressure in male sGCα1−/−S6 mice (Figure 6B). No change in blood pressure was detected in either WT S6 or sGCα1−/−B6 mice treated with the same dose of spironolactone (Supplemental Figure 7). Furthermore, the Ang II type 1 receptor (AT1) antagonist losartan decreased blood pressure more in male sGCα1−/−S6 mice than in WT S6 mice (Supplemental Figure 8). Together, these data suggest that increased activity of the RAAS contributes to the hypertension associated with sGCα2 deficiency in S6 mice.

Normal glomerular filtration rate but decreased K+-excretion in sGCα1−/− mice. To test whether increased RAAS activity resulted in hypertension via an effect on renal function, we performed renal clearance studies in male WT S6 and sGCα1−/−S6 mice. While absolute (E Na) and fractional (FE Na) urinary sodium excretion were similar in sGCα1−/−S6 mice and in WT S6 mice, absolute (E K) and fractional (FE K) urinary potassium excretion were lower in sGCα1−/−S6 mice than in WT S6 mice. However, urinary volume and glomerular filtration rate (GFR), both corrected for body weight, were similar in WT S6 and sGCα1−/−S6 mice (Table 1). The lower body weight in sGCα1−/−S6 mice compared with WT S6 mice may be secondary to the mild gastrointestinal dysfunction observed in sGCα1−/−S6 mice (26). Together with the observation that blood urea nitrogen (BUN) levels were not higher in sGCα1−/−S6 mice than in WT S6 mice, these findings argue against a central role for renal dysfunction in the hypertension observed in sGCα1−/−S6 mice.

Activity of the RAAS modulates endothelium-dependent vascular reactivity. We previously reported that vascular smooth muscle relaxation in response to endothelium-derived NO was severely attenuated in aortic and femoral rings isolated from sGCα1−/−S6 mice (19). In an independent study, the ability of acetylcholine to induce vasorelaxation in isolated carotid arteries was attenuated in sGCα1−/−B6 mice (27). In the current study, we tested the hypothesis that differences in vascular reactivity between sGCα1−/− mice on the B6 and S6 backgrounds contribute to the observed strain-related difference in blood pressure.
in blood pressure. We compared vascular relaxation in response to acetylcholine in isolated pre-constricted aortic rings from male WT and sGCα1–/– mice on both the B6 and S6 backgrounds. Acetylcholine-induced vascular relaxation was impaired to a greater extent in sGCα1–/–S6 than in sGCα1–/–B6 mice (Figure 7). A similar result was obtained in mesenteric arteries (Supplemental Figure 9). These findings suggest that the decreased sensitivity of the vasculature to endothelium-dependent relaxation may contribute to the strain-specific hypertension in sGCα1–/– mice.

To investigate whether increased RAAS signaling in sGCα1–/–S6 mice reduces the ability of the vasculature to vasorelax, we studied the responsiveness to acetylcholine of aortic rings isolated from mice treated for 3 weeks with the angiotensin-converting enzyme (ACE) inhibitor enalapril. Importantly, after prolonged inhibition of RAAS signaling with enalapril, the ability of acetylcholine to relax aortic rings isolated from sGCα1–/–S6 mice no longer differed from that observed in aortic rings isolated from sGCα1–/–B6 mice (Figure 7). Treatment with enalapril also tended to improve the ability of acetylcholine to induce vasorelaxation in sGCα1–/–B6 mice (P = 0.069 vs. sGCα1–/–B6 mice that did not receive enalapril). Taken together, these results suggest that activity of the RAAS has a direct impact on endothelium-dependent vasorelaxation. Increased RAAS activity may therefore be, at least in part, responsible for the greater impairment of vascular relaxation in sGCα1–/–S6 than in sGCα1–/–B6 mice, thereby contributing to the hypertension observed in sGCα1–/–S6 mice.

Discussion

Animal and epidemiological studies have demonstrated that genetic factors contribute to the pathogenesis of essential hypertension. Recent advances in performing and interpreting GWAS and candidate gene–association studies have allowed the identification of common genetic variants that influence blood pressure (3, 5–8, 28). Genetic variation in both cGMP signaling, including in the genes encoding the sGCαβ1 isoform (7), and RAAS signaling pathways have been implicated in the regulation of blood pressure in both human (5–8) and animal (29–32) models of hypertension. Here, we used both a nonbiased genetic approach (linkage analysis) and a targeted approach (investigating a specific molecular pathway, i.e., RAAS) to identify renin as a candidate modifier gene for blood pressure in a setting of impaired NO/cGMP signaling. In addition, our findings suggest that a vascular, rather than a renal, effect of increased RAAS activity underlies the hypertension observed in sGCα1–/–S6 mice.

Variation in the genetic makeup of laboratory mouse strains affects the cardiovascular phenotype of those strains (33, 34). For example, ACE2 deficiency was associated with a modest increase in blood pressure in mice on the B6 background but had no effect on blood pressure in S6 mice (35). Inhibition of NO synthase had

| Renal function is similar in WT and sGCα1–/– mice |

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Values are means ± SEM. UV, urinary volume; Ua, urinary sodium concentration; U0, urinary potassium concentration; Pu, plasma sodium concentration; Pua, plasma potassium concentration. Absolute P values from Student’s t test are shown.
a more profound effect on renal blood flow in S6 mice than in B6 mice (36), blood pressure was consistently higher in S6 than in B6 mice (36), and S6 mice were found to be more susceptible to the development of hypertension and kidney injury after subtotal nephrectomy than were B6 mice (37). Several reports demonstrated that linkage studies in rodents represent a feasible and powerful approach to identify genetic loci and/or candidate modifier genes involved in the etiology of hypertension (31, 37–40). In the current study, we sought to identify genetic variants that modify blood pressure in sGCα1−/− mice by taking advantage of the observation that male sGCα1−/−S6 but not sGCα1−/−B6 mice develop hypertension (18, 21). Using a linkage analysis approach in 284 sGCα1−/−F2 mice, we identified a genomic locus (Hsgcq) on chromosome 1 (covering ~40 Mb) that was associated with hypertension in sGCα1−/− mice. This QTL (located on Mus musculus 1-E4) is syntenic to known hypertension QTLs in rats (Rattus norvegicus 13q13) and humans (Homo sapiens 1q32; Supplemental Figure 10). Importantly, variants of one of the genes located in this region, renin, were previously identified to be associated with blood pressure in rats (41, 42) and humans (43, 44). Additional research will be required to identify other blood pressure-modifying genes that contribute to the hypertension in sGCα1−/−S6 mice (e.g., in the other loci identified in our linkage analysis [on chromosomes 4 and 9]).

In the Hsgcq QTL identified in our linkage study, renin represents a strong biological candidate modifier gene of blood pressure. Renin is a key enzyme in the RAAS that is responsible for the control of fluid homeostasis and blood pressure. It is well established that inappropriate activation of the RAAS is an important contributor to the pathogenesis of hypertension (45). The strain-specific hypertension in sGCα1−/− mice is associated with greater impairment of vascular reactivity in sGCα1−/−S6 mice than in sGCα1−/−B6 mice. Acetylcholine-induced relaxation was studied in phenylephrine-precontracted aortic rings from male wild-type (black symbols, solid line) and sGCα1-deficient mice (sGCα1−/−) on a B6 (circles) or S6 (squares) genetic background. sGCα1−/− mice were either pretreated (gray symbols, dashed line) or not (white symbols, dotted line) with the ACE inhibitor (ACEI) enalapril. Acetylcholine-induced vascular relaxation was impaired to a greater extent in sGCα1−/−S6 than in sGCα1−/−B6 mice. In vivo pretreatment with enalapril restored vascular reactivity in sGCα1−/−S6 to levels observed in sGCα1−/−B6 mice. Overexpression of murine renin 2 in rats resulted in hypertension (46) that was attributed to RAAS-dependent blunting of NO/sGC-mediated vasorelaxation (47). In addition to the previously reported duplication of the renin gene in S6 mice, multiple genetic differences were identified between renin 1c and renin 1d, including 5 missense mutations. Whether the duplication and/or the renin 1 missense mutations contribute to the strain-specific and RAAS-dependent hypertension in sGCα1−/−S6 mice remains to be determined. It is well known that the mechanism of activation of pro-renin is species-specific (48) and that the active site of human renin is unique (49). Based on the extensive differences in genomic, CDNA, and amino acid sequence between the murine and human renin 1 genes (see Supplemental Figure 11 for the amino acid alignment between human renin 1 and mouse renin 1c), it is...
unlikely that identification of the causative mutation (or mutations) in the murine renin genes would be directly translatable to the human renin gene. However, the finding that the combination of altered RAAS signaling (regardless of the causative mutation) and impaired NO/cGMP signaling results in hypertension in mice is translatable to the human condition. Both increased RAAS activity (45) and impaired NO/cGMP signaling (associated with, for example, endothelial dysfunction [ref. 50] and oxidation of sGC [ref. 51]) can increase blood pressure in humans. Genetic variants in the renin gene (43, 44) and in a locus encoding the sGCα1 and sGCβ1 subunits (7) were found to be associated with blood pressure, highlighting the relevance of renin and sGC in regulating blood pressure in humans.

To confirm that renin is a modifier for blood pressure in a setting of impaired NO/cGMP signaling, we compared the activity of the RAAS in S6 and B6 mice. The observation that plasma Ang II levels were higher in S6 mice than in B6 mice confirms the existence of strain-specific differences in activity of the RAAS. It is likely that the observation that Ang II levels were higher in S6 than in B6 mice, regardless of their sGCα1 genotype, is a consequence of the difference in renin genotype, and that renin acts as a modifier of blood pressure in both sGCα1−/− and WT mice. Although blood pressures were higher in WT56 and sGCα1−/− mice (expressing renin 1d and renin 2) than in WT583 and sGCα1−/−B6 mice (expressing renin 1c), respectively, our results suggest that the blood pressure-modifying effect of renin is more apparent when NO/cGMP signaling is impaired (e.g., in sGCα1−/− mice) than when NO/cGMP signaling is intact (e.g., in WT mice).

Plasma aldosterone and adrenal expression of the aldosterone synthase CYP11B2 were higher in hypertensive sGCα1−/− mice than in normotensive WT583 mice. This was an unexpected finding, since an increase in blood pressure would be expected to lead to a homeostatic reduction in RAAS activity due to the renal baroreceptor mechanism (52). It is important to note that a higher adrenal expression level of CYP11B2 in sGCα1−/− than in WT583 mice does not provide additional evidence that renin is a modifier gene for blood pressure in sGCα1−/− mice, nor does it indicate that Cyp11b2 (located on chromosome 15qD3) is a modifier gene. Instead, higher adrenal CYP11B2 expression levels in sGCα1−/− mice than in WT583 mice suggest that NO/cGMP signaling modulates RAAS activity at the level of Cyp11b2 gene expression. Furthermore, increased adrenal CYP11B2 expression levels likely contribute to the elevated plasma aldosterone levels observed in sGCα1−/− mice.

Blocking the RAAS, by inhibiting renin activity with aliskiren or aldosterone receptor signaling with spironolactone, at doses that did not affect blood pressure in WT mice and sGCα1−/− mice, normalized blood pressure in sGCα1−/− mice. Furthermore, acute administration of the AT1 antagonist losartan decreased blood pressure to a greater extent in sGCα1−/− mice than in WT583 mice. The greater sensitivity of sGCα1−/− mice to the blood pressure-lowering effects of aliskiren, spironolactone, and losartan suggests that increased activity of the RAAS contributes to the hypertension associated with sGCα1 deficiency in S6 mice. It is conceivable that both genetics (renin genotype) and interactions between the NO/cGMP signaling and the RAAS (altered gene expression of Cyp11b2) contribute to the hypertension observed in sGCα1−/− mice. Additional characterization of the RAAS, both renal and extrarenal, is required to fundamentally understand the role of altered RAAS signaling in the hypertension associated with impaired NO/cGMP signaling.

Many studies have established an important role for the kidney in the development of high blood pressure (9). We did not find evidence of overt renal dysfunction in hypertensive sGCα1−/− mice. BUN levels were not increased in sGCα1−/− mice, and GFR was similar in WT583 and sGCα1−/− mice. The mechanisms underlying the lower absolute and fractional urinary potassium excretion in sGCα1−/− mice than in WT583 mice remain to be determined. As would be expected, higher plasma aldosterone levels in sGCα1−/− than in WT583 mice were associated with lower plasma K+ levels and higher plasma Na+ levels in the former. It is possible that kidneys in sGCα1−/− mice adapt to decreased K+ levels by increasing reabsorption and/or reducing secretion of K+ in an attempt to maintain homeostasis. Alternatively, the lower K+ excretion in sGCα1−/− than in WT583 mice may be due to reduced K+ channel activity in the kidneys of sGCα1−/− mice. The latter hypothesis stems from the observation that NO and cGMP can stimulate the activity of apical small-conductance K+ (SK) and basolateral K+ channels, respectively (53, 54). Finally, we cannot exclude the possibility that reduced K+ levels and excretion are attributable to gastrointestinal malabsorption, caused by abnormal gastric motility previously observed in sGCα1−/− mice (26).

In the absence of apparent renal dysfunction, hypertension was previously reported to arise from primary vascular abnormalities (10). In cGMP-dependent protein kinase 1α mutant mice, for example, hypertension was observed in the setting of normal renal function and normal renal salt handing, but decreased vascular relaxation in response to a nitrovasodilator (11). We previously reported that both endothelium-dependent and endothelium-independent vascular relaxation are attenuated in aortic and femoral artery rings isolated from sGCα1−/− mice (19) and in cardiac arteries isolated from sGCα1−/−B6 mice (27). These findings highlighted the importance of sGCα1β2-derived cGMP in the relaxation of vascular smooth muscle by NO. In the current study, we observed that the ability of acetylcholine to induce vasorelaxation is impaired to a greater extent in sGCα1−/− mice than in sGCα1−/−B6 mice, both in the aorta and in mesenteric arteries, suggesting that decreased vascular reactivity may contribute to the strain-specific hypertension in sGCα1−/− mice. Similarly, greater impairment of endothelium-dependent relaxation in WT583 mice than in WT583 mice may contribute to the higher blood pressure observed in WT583 mice than in WT583 mice (36).

Surprisingly, in vivo inhibition of RAAS signaling with the ACE inhibitor enalapril restored vascular reactivity in sGCα1−/− mice to the level observed in sGCα1−/−B6 mice that were either pretreated with enalaprtil or not pretreated with enalapril. Together, these results suggest that differences in RAAS activity can impact the ability of the vasculature to relax in response to acetylcholine and raise the possibility that Hsgcq influences blood pressure in the context of sGCα-deficiency by directly modulating endothelium-dependent vasorelaxation. To unequivocally demonstrate that Hsgcq modulates the ability of acetylcholine to induce vascular relaxation, vascular reactivity needs to be assessed in arteries isolated from sGCα1−/− mice.

Multiple studies have demonstrated that extrarenal (including vascular) RAAS signaling can modulate blood pressure (55, 56). Renin (57), aldosterone (58), and aldosterone receptors (59) were previously shown to be expressed in vascular tissue (60). In addition, aldosterone itself was reported to have a direct vasoconstrictive effect (61, 62). However, the exact function of the vascular RAAS remains elusive (60). Our model of RAAS-dependent hyper-
tension associated with sGCα1 deficiency may allow for further elucidation of the role of the vascular RAAS in regulation of vascular tone. One potential mechanism by which increased RAAS signaling may affect vascular function is an aldosterone-mediated increase in oxidative stress (55). The resulting oxidative modification of the Cys122 residue on sGCβ1 would convert sGCαβ1 in sGCα1−/− mice to an NO-insensitive state (55). Activity of sGCαβ1 was previously shown to contribute to the vasorelaxing effect of NO (15, 18, 19). Decreased activity of sGCαβ1 would further disrupt normal vasodilatory signaling in vascular smooth muscle. However, whether increased activity of the RAAS associated with sGCα1 deficiency results in subsequent oxidation and inactivation of sGCαβ1 remains to be determined. Additional studies are required to test whether NO-independent sGC activators that can activate oxidized sGC (63, 64) are able to rescue the vascular dysfunction seen in sGCα1−/− mice.

We previously reported that hypertension in sGCα1−/− mice was male specific and testosterone dependent (18). It is conceivable that androgens modulate RAAS activity, thereby affecting blood pressure in a sex-specific manner and contributing to the hypertension observed in sGCα1−/− mice (65). For example, renin 2 was reported to be an androgen-responsive gene (66, 67). Moreover, overexpression of murine renin 2 in rats resulted in testosterone-dependent hypertension (68). Furthermore, the RAAS has been suggested to have a greater impact on blood pressure regulation in males than in females: knocking out the gene encoding ACE1 decreased blood pressure in male but not female mice (69), and in a large population study, genetic linkage of the ACE locus with hypertension was found in men but not in women (70). Precisely how testosterone interacts with the RAAS to modulate blood pressure in sGCα1−/− mice remains to be investigated.

Associations between common gene variants and hypertension offer mechanistic insights into the regulation of blood pressure and may help in the development of novel intervention strategies to prevent and treat CVD. Improved knowledge of interactions between signaling pathways previously identified to regulate blood pressure will impact our understanding of the etiology of essential hypertension. For example, the observation that increased activity of the RAAS contributes to the pathogenesis of hypertension associated with impaired NO/cGMP signaling may have clinical implications. Both impaired NO/cGMP signaling (14, 15, 18) and increased activity of the RAAS (9) were reported to be involved in the development of hypertension. Therefore, a combination of pharmacological inhibition of the RAAS and pharmacological stimulation of sGC may offer novel strategies to treat hypertension (71, 72), especially in settings where use of classical NO donor compounds is problematic because of the development of tolerance (73).

In conclusion, the identification of a locus on mouse chromosome 1 that contains the renin gene(s) and that modifies blood pressure in sGCα1−/− mice, together with the observation that the strain-specific hypertension in sGCα1−/− mice is associated with increased activity of the RAAS, strongly suggests that activity of the RAAS modulates the susceptibility to hypertension in a setting of impaired NO/cGMP signaling. The finding that NO/cGMP signaling and RAAS signaling (two established pathways in the regulation of blood pressure in humans) interact, has great potential to advance our understanding of the etiology of human hypertension. Understanding the interaction of pathways that modulate blood pressure is particularly critical, given the multigenic pathogenesis of most forms of hypertension, and may help define genetically distinct subgroups of men and women with essential hypertension. Further elucidation of how interaction of NO/cGMP signaling and the RAAS affects cardiovascular function in mice is likely to impact the clinical development of sGC-activating compounds for treatment of CVDs (51) and may promote the search for new sGC isofrom–specific agonists.

Methods

Generation and blood pressure measurement of F2 offspring from an sGCα1−/− × sGCα1−/− intercross. Only male mice were studied. Generation of sGCα1−/− (generated on the S6 background) and sGCα2−/− mice (backcrossed 8 generations with B6 mice from The Jackson Laboratory) was described previously (18, 21). WTα and WTβ6 mice were purchased from The Jackson Laboratory and Taconic, respectively. Male sGCα1−/− and female sGCα1−/− mice (4 breeding pairs) were crossed to yield F1 offspring. The F1 offspring were subsequently intercrossed (16 breeding pairs) to yield F2 offspring. MAP was measured invasively in 284 male F2 offspring at 4 months of age, as previously described (18, 21). Briefly, mice were anesthetized by ip. injection with ketamine (100 mg/kg), fentanyl (50 mg/kg), and pancuronium (2 mg/kg); intubated; and mechanically ventilated (FiO2 of 1, 10 uLg, 120 breaths per minute). A fluid-filled catheter was inserted into the left carotid artery for infusion of saline solution (2 ml/h) and for measurement of MAP. The reproducibility of the invasive hemodynamic technique described here was validated previously by measuring blood pressure in WTα and sGCα1−/− mice using radiotelemetry (in unrestrained, unanesthetized mice), invasive hemodynamics (in anesthetized mice), and tail-cuff (in restrained, unanesthetized mice) (18).

Genotyping. Genomic DNA was isolated from ear tissue of 284 sGCα1−/2 mice using a commercially available kit (DNeasy, QIAGEN). For genome scans, mice were genotyped using 120 B6-S6 informative SNPs covering the murine autosomes (average inter-marker spacing, 22.2 Mb; largest inter-marker gap, 54.6 Mb). All SNPs were assayed using Sequenom MassARRAY iPLEX GOLD chemistry. Oligonucleotides were synthesized and subjected to quality control analysis by mass spectrometry at Integrated DNA Technologies. All SNPs were genotyped in multiplexed pools of up to 36 assays, designed by AssayDesigner v.3.1 software, starting with 10 ng DNA per pool. Seven nanoliters of reaction were loaded onto each position of a 384-well SpectroCHIP preloaded with 7 nl matrix (3-hydroxypicolinic acid). SpectroCHIPS were analyzed in automated mode by a MassArray MALDI-TOF Compact System 2 with a solid phase laser mass spectrometer (Bruker Daltonics). Variants were called by real-time SpectroCALLER algorithm and analyzed by SpectroTYPE r.4.0 software, and clusters were manually reviewed for validation of genotype calls. Poorly genotyped markers were removed prior to analysis. Subsequently, all 284 sGCα1−/− mice were genotyped using 28 SNPs spaced approximately 4 Mb apart and spanning a region on chromosome 1 extending from 30 Mb to 173 Mb. Reported genetic map positions for the markers were retrieved from the SNP database (build 36.1) of the National Center for Biotechnology Information (NCBI).

Linkage and sequence analysis. As the phenotype appears normally distributed, parametric linkage analysis was performed using MAPMAKER/QTL (74) to identify QTLs associated with blood pressure. For an F2 intercross, a lod of approximately 2.8 is considered “suggestive” (expected to be seen by chance once per genome scan), a lod of approximately 4.3 is considered “significant” (with a genome-wide corrected P = 0.05, a score expected to be seen by chance only once per 20 genome scans), and a lod of approximately 6.1 is considered “highly significant” (genome-wide corrected P = 0.001) (75). A region within 1.5 lod score units of the maximum lod was used to represent the 95% interval for a strong QTL (76). The genome-wide scans were plotted with the use of the J/qtl mapping pro-


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Measurement of renin genotype. Total RNA was extracted from whole kidneys or adrenal glands harvested from 8- to 16-week-old mice using TRZol reagent (Invitrogen), and cDNA was synthesized using Moloney’s murine leukemia virus reverse transcriptase (Promega). Renin 1c, renin 1d, renin 2, CYP11B2, and 18S RNA transcript levels were measured by real-time PCR using the SYBR or TaqMan universal PCR master mix (Applied Biosystems) in a Mastercycler ep realplex 2 (Eppendorf). Primers were designed for renin 1 (detecting renin 1c and renin 1d; 5'-CTCTCTGGGCACTCTTGTTGC-3′ and 5'-GGAGGGTAAAGATTGGTCAAAGGA-3′), CYP11B2 (5'-TTGCAACAGGTGGAAGTTATG-3′ and 5'-GTCGGTGAGACGC-CATTCT-3′), and 18S RNA (5'-CGGCTACCATCCTCAGGAA-3′ and 5'-GCTGGAATTCGCGGCTG-3′) for normalization. For renin 2, TaqMan primer sets were used (Applied Biosystems). Changes in the relative gene expression normalized to levels of 18S rRNA were determined using the relative cycle threshold method.

Renin genotyping. Genomic DNA isolated from ear tissue of sGCα1−/− mice was amplified using primers yielding a 543-bp fragment specific for renin 1c and renin 1d (5'-CGACCCGAGATTATCAG-3′ and 5'-ACTCCGACACGCTTCTTCCTACAT-3′); primers amplifying a 1,100-bp fragment specific for renin 2 (5'-GCCAAAGGAAAGACTACAT-3′ and 5'-CGGCCCAAGCGCAAATCCA-3′); and primers amplifying a 695-bp fragment of intergenic DNA in the renin 1c locus but not in the renin 1d−/− renin 2 locus (5'-CAATACGTGTTTGGTTGATCTC-3′ and 5'-AGGTAACGCTGTTAAGTGTTGAG-3′). Combination of these 3 PCR reactions allows identification of mice homozygous for renin 1c, homozygous for renin 1d and renin 2, or heterozygous (renin 1c/renin 1d and renin 2).

Measurements of plasma aldosterone, plasma Ang II, and blood Na+, K+, and ENa, EK, FENa, and FEK. Blood pressure, urine volume, GFR, ENa, EK, FENa, and FEK were calculated by standard methods.

Measurement of vascular reactivity in isolated aortic and mesenteric rings. Male WT and sGCα1−/− mice were euthanized with pentobarbital (200 mg/kg, i.p.), Krebs-Henseleit physiological salt solution was prepared as previously described (84). Two milliliters of ice-cold Krebs solution were injected retrograde into the left ventricle, and the thoracic aorta or a mesenteric artery was dissected free of connective and adipose tissue and placed in ice-cold Krebs-Henseleit physiological salt solution pre-equilibrated with 95% O2/5% CO2 for 15 minutes. Four rings 2–4 mm in length per mouse were taken from the aorta or mesenteric artery. Aortic rings were mounted between two tungsten wire hooks and suspended vertically in 10-ml organ baths of the myograph (TSE Systems) containing physiological salt solution and maintained at 37°C, pH 7.4, in a mixture of 95% O2/5% CO2. The rings were equilibrated for 90 minutes at a resting force of 1.0 g, with replacement of the bathing solution at 15 minute intervals. Mesenteric arteries were mounted onto glass cannulas in a pressure myograph (DMT), maintained at 85 mmHg, and perfused as described previously (85). The viability of the vessel was confirmed by measurement of stable and reproducible responses to the addition of phenylephrine (10−6 mol/l). Cumulative concentration-tension response curves to sequential addition of acetylcholine (10−9 to 10−5 mol/l) were obtained. The integrity of the endothelium was confirmed by a reproducible and stable relaxation of phenylephrine-precontracted rings by acetylcholine. The results of the 4 rings studied per mouse were averaged to yield an n = 1 per mouse.

Statistics. All continuous measurements are expressed as mean ± SEM. Statistical comparisons of MAP between multiple strains of mice (sGCα1−/−, sGCα1−/−, sGCα1−/− mice or mice with different renin allele combinations, including mice homozygous for the B6 renin locus or the S6 renin locus, or mice heterozygous for the renin locus) were performed by 1-way ANOVA with Bonferroni post-hoc testing. A 2-way ANOVA with Bonferroni post-hoc testing was used when comparing gene expression, plasma Ang II levels, plasma aldosterone levels, and MAP in multiple groups (WTα1−/−, WTα1−/−, sGCα1−/−, and sGCα1−/− mice, as well as WT and sGCα1−/− mice treated with vehicle or a RAAS inhibitor). When comparing two groups (renal clearance studies, MAP), the independent Student’s t test was used. The vascular reactivity in aortic rings and mesenteric arteries and the acute effect of losartan on MAP were analyzed by repeated-measures ANOVA. In all cases, a P value less than 0.05 was considered statistically significant.

Study approval. Housing and procedures involving experimental animals (mice) were approved by the Subcommittee on Research Animal Care of Massachusetts General Hospital, the Institutional Animal Care and Use Committee, Yale University, and the Animal Ethical Committee of Ghent University.

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Renal clearance experiments. GFR was measured by inulin clearance as previously described (83). Briefly, male WTα1−/− and sGCα1−/− mice were anesthetized by i.p. injection of thiobutabarbitual sodium (Inactin, Sigma-Aldrich, 100–120 mg/kg body weight), and the right jugular vein was catheterized for fluid infusion. The left carotid artery was cannulated for continuous measurement of MAP and blood sampling, and the bladder was cannulated for urine collection. Tritiated inulin (10 μCi/ml; NEN) in Ringer solution (in mmol/l: 111 NaCl, 30 NaHCO3, and 4.7 KCl) was intravenously infused at 3 μl/min for assessment of GFR. After surgery and a 60-minute equilibration period, two 30-minute urine samples were collected. Blood samples (30 μl) were taken after each clearance period. Plasma and urinary 1H-inulin concentration was measured for calculation of GFR. Urine Na+ and K+ concentrations were measured by flame photometry (type 480 Flame Photometer, Corning Medical and Scientific). Blood pressure, urine volume, GFR, ENa, EK, FENa, and FEK were calculated by standard methods.
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