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Increased Sensitivity to Endothelial Nitric Oxide (NO) Contributes to Arterial Normotension in Mice with Vascular Smooth Muscle-selective Deletion of the Atrial Natriuretic Peptide (ANP) Receptor*

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Atrial natriuretic peptide (ANP) plays a key regulatory role in arterial blood pressure homeostasis. We recently generated mice with selective deletion of the ANP receptor, guanylyl cyclase-A (GC-A), in vascular smooth muscle (SMC GC-A knockout (KO) mice) and reported that resting arterial blood pressure was completely normal in spite of clear abolition of the direct vasodilating effects of ANP (Holtwick, R., Gotthardt, M., Skryabin, B., Steinmetz, M., Potthast, R., Zetsche, B., Hammer, R. E., Herz, J., and Kuhn M. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 7142–7147). The purpose of this study was to clarify mechanisms compensating for the missing vasodilator responses to ANP. In particular, we analyzed the effect of the endothelial, cGMP-mediated vasodilators C-type natriuretic peptide and nitric oxide (NO). In isolated arteries from SMC GC-A KO mice, the vasorelaxing sensitivity to sodium nitroprusside and the endothelium-dependent vasodilator, acetylcholine, was significantly greater than in control mice. There was no difference in responses to C-type natriuretic peptide or to the activator of cGMP-dependent protein kinase I, 8-para-chlorophenylthio-cGMP. The aortic expression of soluble GC (sGC), but not of endothelial NO synthase or cGMP-dependent protein kinase I, was significantly increased in SMC GC-A KO mice. Chronic oral treatment with the NO synthase inhibitor N^{w}-nitro-L-arginine methyl ester increased arterial blood pressure, the effect being significantly enhanced in SMC GC-A KO mice. We conclude that SMC GC-A KO mice exhibit a higher vasodilating sensitivity to NO. This can be attributed to an enhanced expression of sGC, whereas the expression and/or activity levels of downstream cGMP-effector pathways are not involved. Increased vasodilating responsiveness to endothelial NO contributes to compensate for the missing vasodilating effect of ANP in SMC GC-A KO mice.

Cyclic GMP-dependent modulation of vascular tone is fundamental to the regulation of blood pressure. The levels of cGMP in vascular smooth muscle cells (SMC)[1] are regulated by the activities of three different guanylyl cyclases (GCs): soluble GC (sGC), the intracellular receptor for endothelial nitric oxide (NO) (1); particulate GC-A, a specific membrane receptor for the cardiac natriuretic peptides, atrial (ANP) and B-type (BNP) natriuretic peptides (2, 3); and particulate GC-B, a specific receptor for the endothelial C-type natriuretic peptide (CNP) (4, 5). Stimulation of either GC results in the conversion of GTP to the intracellular messenger cGMP. Subsequent increases in cellular cGMP modulate the activity of specific cGMP-effector molecules, *i.e.* cGMP-dependent protein kinase I (PKG I), ultimately leading to decreased cytosolic calcium levels and relaxation of vascular smooth muscle cells (6, 7). Thereby, local factors released by the vascular endothelium (NO and CNP) and circulating hormones (ANP and BNP) cooperate in the cGMP-mediated regulation of vascular tone. In addition, it has been suggested that the vasodilating effect of ANP involves endothelial GC-A and is partly mediated by the stimulation of the local release of NO and CNP (8–11).

In the past years, the development of several monogenetic mouse models contributed to elucidate the role of these factors and their respective receptor-GCs in the regulation of blood pressure. In particular, mice lacking endothelial NO synthase (eNOS) (12), ANP (13), or the ANP receptor, GC-A (14, 15), exhibit drastic arterial hypertension, experimental observations that emphasize the importance of cGMP-dependent vasodilation in cardiovascular homeostasis.

Because the soluble and particulate GC/cGMP systems have complementary roles in blood pressure homeostasis, an interaction between these pathways to regulate cGMP levels in vascular smooth muscle cells might represent an important physiological mechanism to control vascular tone. In this way, an excess or deficiency in one mediator could be compensated by the other, or conversely, the interaction may constitute a negative feedback system that prevents overactivation of cGMP signaling in SMC by NO and/or natriuretic peptides. Indeed, acute or chronic alterations of endothelial NO production in human and murine arteries resulted in reciprocal changes in the vasorelaxing responses to ANP (16). The authors suggested that the NO/sGC system modulates the sensi-

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[1] The abbreviations used are: SMC, smooth muscle cells; ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; CNP, C-type natriuretic peptide; NO, nitric oxide; NOS, NO synthase; eNOS, endothelial NO synthase; GC-A, guanylyl cyclase-A; sGC, soluble guanylyl cyclase; KO, knockout; PKG, cGMP-dependent protein kinase; 8p-CPT-cGMP, 8-para-chlorophenylthio-cGMP; SNP, sodium nitroprusside; L-NAME, N^{w}-nitro-L-arginine methyl ester; IBMX, 3-isobutyl-1-methylxanthine; SIN-1, 3-morpholinosydnonimine; bpm, beats per minute.

tivity and/or expression levels of GC-A in a cGMP-dependent manner (16). Vice versa, it is not clear whether changes in the vasodilating effects of ANP modulate the activity and/or vasodilating effects of the NO/sGC and CNP/GC-B systems.

In a recent study, we developed a new genetic mouse model in which the GC-A receptor is selectively deleted in vascular smooth muscle cells (SMC GC-A KO mice) (17). Intriguingly, despite the clear abolition of the direct vasorelaxing effects of ANP, the resting blood pressure of conscious SMC GC-A KO mice is completely normal. This was unexpected since the decisive role of the ANP/GC-A system in the chronic regulation of arterial blood pressure has been clearly shown by the hypertensive phenotype of mice with generalized GC-A gene deletion (14, 15). One possibility is that in the long term setting, other cGMP-dependent vasodilating systems, such as NO/sGC or CNP/GC-B, compensate for the missing vasodilating effects of ANP. To address this possibility, in the current study, we evaluated the responsiveness of SMC GC-A KO mice to different cGMP-mediated vasodilators. In addition, we took advantage of the selective inhibition of GC-A expression in vascular smooth muscle cells to dissect the specific endothelium-mediated vasodilating effects of ANP in intact vessel preparations.

EXPERIMENTAL PROCEDURES

Animals and Tissue Preparations—Mice with selective abolition of the ANP receptor, GC-A, in vascular smooth muscle (SMC GC-A KO mice) were generated as described (loxP/Cre recombination system) (17). Genotypings were by Southern blot (verification of the floxed GC-A and the Cre-deleted GC-A alleles) and PCR analyses (detection of the SM22-Cre transgene) of tail DNA. Studies were done with 4-month-old floxed GC-A mice (which retain normal GC-A expression levels) and SMC GC-A KO littermates (floxed GC-A mice harboring the SM22-Cre transgene) (17). All experimental protocols included in this manuscript were approved by the local animal care committee and conform with the *Guide for the Care and Use of Laboratory Animals* published by the U. S. National Institutes of Health (32). For the *in vitro* studies, mice were killed by cervical dislocation. The aorta was dissected free of surrounding tissue and used for organ chamber studies, Western blot analysis, and determination of cGMP content and sGC activity.

In Vitro Studies of Vascular Tone—Ring segments of the descending thoracic aorta (luminal diameter, Ø, 2000–2200 μm) were mounted in a myograph (model 410A; J.P. Trading, Aarhus, Denmark) for recording of isometric wall tension (17, 18). After a 15-min equilibration in temperated (37 °C), oxygenated (95% O_2, 5% CO_2) Krebs-Ringer bicarbonate buffer, rings were contracted with phenylephrine (10 μM; Sigma), and the relaxant response to cumulative concentrations of ANP, CNP (both obtained from Calbiochem-Novabiochem), sodium nitroprusside (SNP), acetylcholine (both from Sigma), or 8-para-chlorophenylthio-cGMP (8p-CPT-cGMP; from Biolog, Bremen, Germany) was tested. ANP was tested in the presence and absence of the NO synthase (NOS) inhibitor, N^{ω}-nitro-L-arginine methyl ester (100 μM L-NAME; Sigma).

Western Blot Analysis—To determine the expression levels of eNOS, sGC, and PKG I, frozen aortas were homogenized and analyzed by Western blot. Samples (20 μg of protein/lane) were separated on 8% SDS-polyacrylamide gels and then blotted onto nitrocellulose membrane. Membranes were first incubated with specific antisera against PKG I (19, 20) (diluted 1:3000), sGCα_1, or sGCβ_1 (21) (both diluted 1:1000) or eNOS (BD Bioscience; diluted 1:500) and then with a peroxidase-labeled anti-rabbit antibody in an ECL detection system (Amersham Biosciences) (19, 20). For quantitative analysis, the blots were scanned and quantified using Amersham Biosciences ImageQuant software. The antibody against PKG I was a generous gift from Dr. Suzanne Lohmann (19).

Determination of cGMP Content in Isolated Blood Vessels—Aortic rings were incubated in temperated (37 °C) Dulbecco's modified Eagle's medium (Invitrogen) containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 1 mM). After 15 min, rings were treated with either vehicle (Dulbecco's modified Eagle's medium) or the NO donor, 3-morpholinosydnonimine (SIN-1, 10 or 100 μM; Sigma) for an additional 5 min. Thereafter, rings were frozen in liquid nitrogen and homogenized, and cGMP was extracted with ice-cold 70% (v/v) ethanol. After centrifugation (13000 × *g*, 10 min, 4 °C), the supernatants were dried in a speed vacuum concentrator, resuspended in sodium acetate buffer (50 mM, pH 6.0), and acetylated, and then the cGMP contents were quantified by radioimmunoassay (17, 20). The pellets of the ethanol extracts were used for determination of protein content according to the method of Bradford (17, 20).

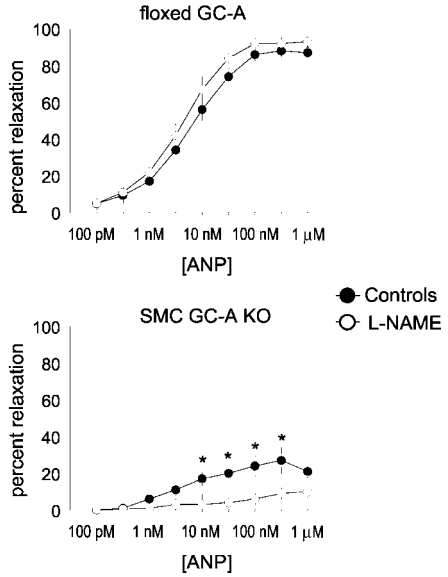

FIG. 1. **Relaxations of isolated aortas in response to ANP.** Aortic rings from floxed GC-A (*top*) and SMC GC-A KO mice (*bottom*) were contracted with phenylephrine (10 μM), and then cumulative concentrations of ANP were added. Experiments were performed in the presence (*white dots*) or absence (*black dots*, controls) of the NOS inhibitor L-NAME (100 μM). The vasorelaxing effects are presented as a percentage of the phenylephrine-induced contraction ($n = 6$ in each group; *, $p < 0.05$ *versus* rings in the presence of L-NAME).

Determination of Guanylyl Cyclase Activity—NO-dependent guanylyl cyclase activity in aortic homogenates was determined (17) according to Li *et al.* (22). Individual aortas were homogenized in 1 ml of ice-cold buffer containing 50 mM Tris·HCl (pH 7.6), 1 mM EDTA, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Extracts were centrifuged for 10 min at 500 × *g* (4 °C) (23). To initiate cyclase activity, supernatants (4 μg of protein) were incubated in assay buffer containing 50 mM Tris·HCl, pH 7.5, 4 mM $MgCl_2$, 2 mM IBMX, 1 mM GTP, 7.5 mM creatine phosphate, and 200 μg/ml creatine phosphokinase (185 units/mg) at 37 °C in the presence of the NO donor SIN-1 (10 μM). At 5 min of incubation, the reaction was stopped by addition of ice-cold 100% (v/v) ethanol (final concentration, 70%), and cGMP was extracted and measured as described above.

In Vivo Experiments—The animals were housed under a 12-h day/night cycle and fed a standard diet containing 0.6% NaCl (normal salt conditions). Floxed GC-A mice ($n = 10$) and SMC GC-A KO littermates ($n = 9$) were treated with the inhibitor of NO synthase, L-NAME (~100 mg/kg/day), via drinking water for 21 days according to published studies (24). Arterial blood pressure and heart rate were measured in conscious mice by tail-cuff plethysmography (Softron, Tokyo) as described previously (17, 25), before and during oral L-NAME treatment.

Data Analysis—Results are expressed as means ± S.E. (n = number of animals). The EC_{50} for agent-induced relaxation in aortic rings was calculated by nonlinear regression analysis of mean contraction (% of relaxation) *versus* log [agent] (GraphPad Prism 1.00; GraphPad, San Diego, CA). Statistical comparison of floxed GC-A mice (as controls) and SMC GC-A KO mice was performed by unpaired Student's t test. The serial changes in arterial blood pressure and heart rate before (control period) and after L-NAME treatment were analyzed by a repeated measures analysis of variance followed by Student-Newman-Keuls multiple comparisons test (GraphPad InStat software). p values of less than 0.05 were considered statistically significant.

RESULTS

Organ Chamber Studies—The contracting responses of aortic rings to phenylephrine (10 μM) were not different between genotypes. ANP (100 pM to 1 μM) induced the complete relaxation of preconstricted aortic rings obtained from floxed GC-A mice (controls, with normal GC-A expression levels) but had only marginal relaxing effects on arteries from SMC GC-A KO

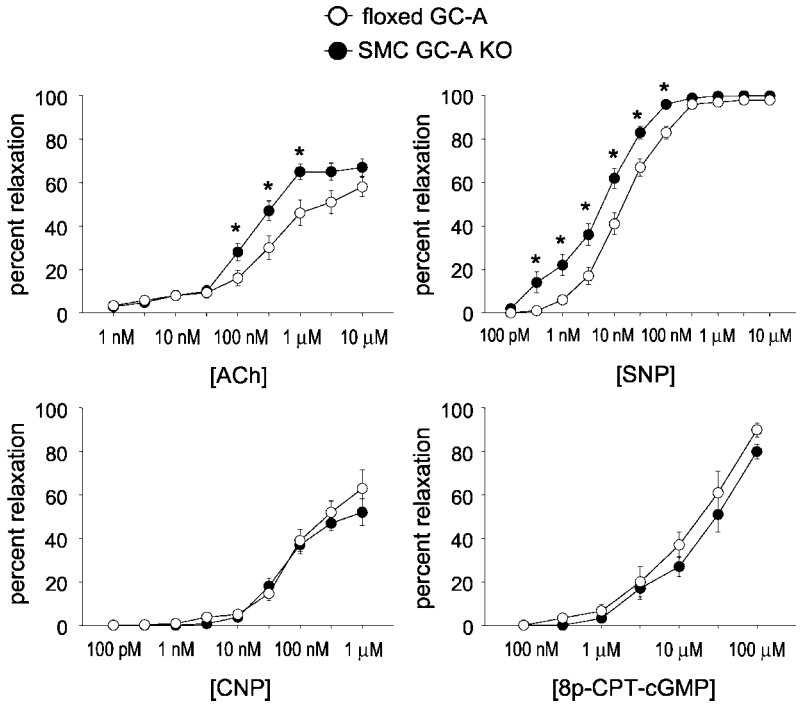

FIG. 2. **Relaxations of isolated aortas in response to acetylcholine (*ACh*), SNP, CNP, and 8p-CPT-cGMP.** Aortic rings from floxed GC-A and SMC GC-A KO mice were contracted with phenylephrine (10 μM), and then cumulative concentrations of test agents were added. The vasorelaxing effects are presented as a percentage of the phenylephrine-induced contraction ($n = 9$ in each group; *, $p < 0.05$ *versus* floxed GC-A).

mice (E_{max}, 88 ± 3% *versus* 26 ± 5%) (Fig. 1). Pretreatment of aortic rings with the NOS inhibitor L-NAME (100 μM) completely abolished the small relaxing responses to ANP observed in aortas from SMC GC-A KO mice, whereas relaxations in floxed GC-A mice were unaffected. The EC_{50} for ANP relaxation in floxed GC-A aortas was 6.9 ± 1.4 nM in the absence *versus* 4.8 ± 1.4 nM in the presence of L-NAME (no significant difference) (Fig. 1). L-NAME pretreatment totally prevented the relaxing responses to the endothelium-dependent vasodilator acetylcholine (1 nM to 10 μM) in both genotypes (E_{max} for acetylcholine, 7 ± 3% in the presence *versus* 66 ± 4% in the absence of L-NAME, $p < 0.05$).

Relaxations in response to acetylcholine (1 nM to 10 μM) and to the NO donor SNP (100 pM to 10 μM) were both significantly greater in aortas from SMC GC-A KO mice as compared with floxed GC-A mice (Fig. 2). The EC_{50} for acetylcholine relaxation in floxed GC-A mice was 346 ± 60 nM, and in SMC GC-A KO mice, it was 158 ± 21 nM ($p < 0.05$). The EC_{50} for SNP was 17.4 ± 3.8 in floxed GC-A and 6.5 ± 1.3 in SMC GC-A KO mice ($p < 0.05$). In contrast, CNP (100 pM to 1 μM) and the PKG-activator 8p-CPT-cGMP (100 nM to 100 μM) had similar effects in aortas from both genotypes (Fig. 2). The EC_{50} for CNP was 93.2 ± 19 nM in floxed GC-A and 136 ± 23 nM in SMC GC-A KO mice, and for 8p-CPT-cGMP, it was 33.7 ± 13 μM *versus* 32.6 ± 6.6 μM (no significant difference). The lower potency of CNP relative to that of ANP is similar to previous reports (25).

Western Blot Analysis—To investigate the mechanisms mediating the enhanced vasorelaxing responses to SNP and acetylcholine, downstream effectors were studied. As shown in Fig. 3, the expression levels of both sGC subunits α_1 and β_1 were significantly enhanced in aortas from SMC GC-A KO mice as compared with floxed GC-A mice. In contrast, the expression levels of eNOS and of a downstream target for cGMP, PKG I, were not different between genotypes (Fig. 3).

cGMP Content of Aortic Rings—The basal cGMP content in aortic rings was slightly greater in floxed GC-A as compared with SMC GC-A KO mice (235 ± 17 *versus* 179 ± 14 pmol/mg of protein, $n = 5$; $p < 0.05$). Incubation with SIN-1 increased cGMP content in aortic rings from floxed GC-A (at 100 μM SIN-1) and SMC GC-A KO mice (at 10 and 100 μM SIN-1) (Fig. 4). When compared with the respective basal cGMP content of each individual aorta, the increases of cGMP in response to SIN-1 were significantly greater in SMC GC-A KO (6.6 ± 0.7-fold increase at 100 μM SIN-1 *versus* untreated controls) as compared with floxed GC-A mice (3.4 ± 0.5-fold increase at 100 μM SIN-1; $n = 5$; $p < 0.05$) (Fig. 4).

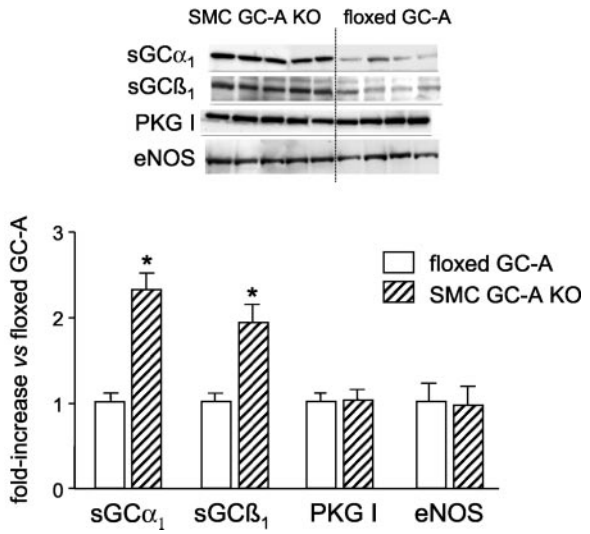

FIG. 3. **Aortic sGC, PKG I, and eNOS protein expression.** Aortic extracts from floxed GC-A and SMC GC-A KO mice were subjected to SDS-PAGE and Western blot analysis. *Top*, Western blots. *Bottom*, relative expression levels of sGC, PKG I, and eNOS (fold increase of floxed GC-A). sGCα_1 (80 kDa), sGCβ_1 (68 kDa), PKG I (78 kDa), and endothelial NOS (135 kDa) were detected using specific antisera and a peroxidase-labeled anti-rabbit antibody in an ECL detection system. The expression levels of sGC (both subunits) but not PKG I or eNOS were significantly enhanced in the aortas from SMC GC-A KO mice ($n = 9$ per genotype, *, $p < 0.05$ *versus* floxed GC-A).

sGC Activity—First, to test the linearity of the assay, SIN-1-stimulated sGC activity was compared in samples containing 1, 2, or 4 μg of protein extracted from wild-type mouse aortas. As shown in Fig. 5*A*, doubling the protein content of the samples (and thereby the amount of sGC protein) resulted in a

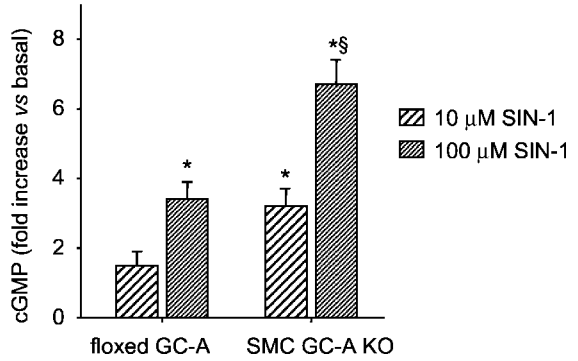

FIG. 4. **Effect of SIN-1 (10 or 100 μM) on cGMP content of aortic rings from floxed GC-A and SMC GC-A KO mice.** Rings were pretreated with the phosphodiesterase inhibitor IBMX (1 mM, 15 min) and then incubated with vehicle (Dulbecco's modified Eagle's medium) or SIN-1 for an additional 5 min in the presence of IBMX. Responses to SIN-1 are expressed as fold increase of cGMP content as compared with parallel vehicle-treated rings prepared from the same individual aortas (n = 5 per genotype, *, $p < 0.05$ *versus* basal; §, $p < 0.05$ *versus* floxed GC-A).

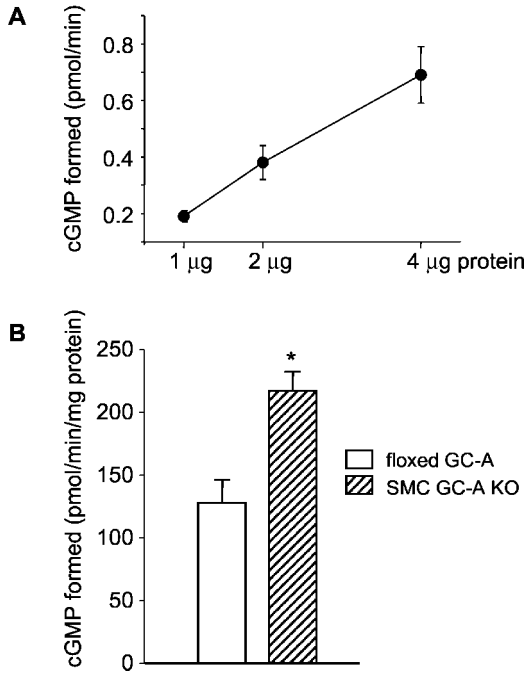

FIG. 5. **Determination of vascular sGC activity.** *A*, linearity of the sGC assay showing correlation between increasing amounts of aortic protein and SIN-1 (10 μM)-stimulated, NO-dependent sGC activity. Enzymatic activity is expressed as picomoles of cGMP formed per minute (n = 3). *B*, SIN-1 (10 μM)-stimulated activity of sGC in aortic homogenates obtained from floxed GC-A and SMC GC-A KO mice. Enzymatic activity is expressed as picomoles of cGMP formed per milligram of protein per minute (n = 5 mice per genotype, with sGC activity determinations performed in duplicate; *, $p < 0.05$ *versus* floxed GC-A).

proportional increase of SIN-1-stimulated cGMP formation (n = 3). Fig. 5*B* demonstrates that SIN-1 (10 μM)-stimulated sGC activity in protein extracts from SMC GC-A KO aortas was significantly higher than in floxed GC-A aortas by ~ 170% (n = 5; $p < 0.05$).

Effects of L-NAME on Blood Pressure—To ascertain whether increased sensitivity to endothelial NO affects chronic blood pressure levels, conscious homozygous floxed GC-A (n = 10) and SMC GC-A KO mice (n = 9) were treated with the NOS-inhibitor L-NAME. Systolic blood pressure was measured in awake mice by tail-cuff plethysmography. As reported previously (17), initial blood pressures and heart rates were not

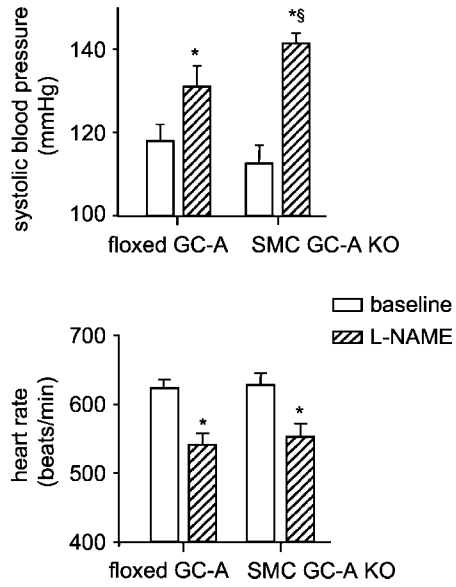

FIG. 6. **Systolic blood pressure (*top*) and heart rate (*bottom*) in floxed GC-A (n = 10) and SMC GC-A KO mice (n = 9) before and after 21 days of oral treatment with L-NAME (~100 mg/kg of body weight/day).** Measurements were obtained in awake mice using a tail-cuff method. Significant changes are indicated (*, $p < 0.05$ *versus* baseline; §, $p < 0.05$ *versus* floxed GC-A mice).

significantly different between floxed GC-A and SMC GC-A KO mice (Fig. 6). L-NAME (100 mg/kg of body weight/day, orally) provoked a significant rise in systolic blood pressure levels by 13 ± 3.7 mm Hg in floxed GC-A mice (from 118 ± 2.6 mm Hg at baseline to 131 ± 3.8* mm Hg after L-NAME) and by 28 ± 3.3 mm Hg in SMC GC-A KO mice (113 ± 2.7 mm Hg at baseline; 141 ± 1.6* mm Hg after L-NAME; *, $p < 0.05$ *versus* baseline). This was associated with a significant decrease in heart rate (floxed GC-A mice, 623 ± 12 bpm at baseline and 541 ± 17* bpm after L-NAME; SMC GC-A KO mice, 632 ± 18 bpm at baseline and 549 ± 21* bpm after L-NAME; *, $p < 0.05$ *versus* baseline). As shown in Fig. 6, the hypertensive but not the bradycardic response to L-NAME was significantly greater in SMC GC-A KO mice as compared with floxed GC-A mice.

DISCUSSION

In the present study, we demonstrate that mice with selective deletion of the ANP receptor in vascular smooth muscle exhibit an increased sensitivity to nitrovasodilators and endothelial NO but not to downstream targets of the cGMP pathway (PKG I) and other cGMP-mediated vasodilators, such as CNP. The results indicate a close interaction between the ANP/GC-A and NO/sGC pathways, which does not affect the CNP/GC-B pathway.

Using the Cre/loxP gene recombination strategy, we recently generated mice with selective deletion of the ANP receptor, guanylyl cyclase-A (GC-A), in vascular smooth muscle cells (SMC GC-A KO mice) and reported that chronic blood pressure was completely normal in spite of clear abolition of the direct vasodilating effects of ANP (17). This was unexpected because the decisive role of the ANP/GC-A system in the chronic regulation of arterial blood pressure has been clearly shown by the hypertensive phenotype of mice with generalized GC-A gene deletion (14, 15). We hypothesized that the chronic reduction of blood pressure by ANP might be mainly mediated by the other known endocrine actions of the hormone such as the inhibition of the sympathetic and renin-angiotensin-aldosteron systems as well as the stimulation of renal function (2, 3). Alternatively other vasodilating systems might be able to compensate for the missing vasodilating effects of ANP when all other cardiovascular actions of the peptide are preserved. To address this

possibility, in the current study, we compared the vascular expression levels of eNOS as well as the responsiveness of floxed GC-A mice (which exhibit normal GC-A expression levels) and SMC GC-A KO mice to different cGMP-mediated vasodilators. As shown, the vascular expression levels of eNOS were similar in both genotypes. Also, the vasorelaxing responses to CNP and to the membrane-permeable cGMP analog, 8p-CPT-cGMP, were identical in aortas of floxed GC-A and SMC GC-A KO mice. However, the latter exhibited an increased vasorelaxing sensitivity to the endothelium-dependent vasodilator, acetylcholine, and to the NO donor, SNP.

ANP may alter the NO-sGC-cGMP transduction cascade in several ways, affecting the release of endothelial NO (10) or the expression as well as the activity of sGC and targets further downstream. Since the vasorelaxing responses to CNP and to 8p-CPT-cGMP were identical in aortas with and without deletion of GC-A in smooth muscle cells, alterations of downstream effectors or modulators of cGMP, such as PKG I or phosphodiesterases (6, 7), are excluded as a cause for the increased NO sensitivity in SMC GC-A KO mice. Indeed, Western blot analysis showed that the vascular expression levels of PKG I, a common downstream target for all cGMP-dependent vasodilators (6, 7), are not different in floxed GC-A and SMC GC-A KO mice. In contrast, the vascular expression levels of the sGC α_1 and β_1 subunit proteins were significantly enhanced in aortas from SMC GC-A KO as compared with floxed GC-A mice. This was concordant with an increased effect of the NO donor, SIN-1, on both the cGMP content of aortic rings and the enzymatic activity of sGC in aortic homogenates from SMC GC-A KO mice. Thus, deletion of the GC-A gene in SMC leads to increased expression of sGC, which renders the arteries more susceptible to increases in cGMP and to vasodilation by NO. Notably, despite the 2-fold increase in vascular sGC expression levels, basal cGMP contents in aortas from SMC GC-A KO mice were slightly lower as compared with floxed GC-A littermates. However, basal cGMP levels in vascular tissues are regulated by the activity of both soluble and particulate guanylyl cyclases (GC-A and GC-B). Therefore, it is likely that the deletion of GC-A in smooth muscle cells accounts for the lower cGMP levels observed in unstimulated aortic rings from SMC GC-A KO mice under *in vitro* conditions. The increased protein expression of sGC was also observed in aortas obtained from younger, 6–8-week-old SMC GC-A KO mice (not shown), indicating that the compensatory changes in the NO/sGC/cGMP pathway are present already at early stages.

To determine whether the studies with isolated arteries reflected *in vivo* effects on blood pressure, adult (4-month-old) mice were subjected to chronic treatment with the NO synthase inhibitor L-NAME. As shown, L-NAME significantly increased the blood pressure levels of floxed GC-A and SMC GC-A KO littermates. Remarkably, the magnitude of blood pressure increases was significantly more pronounced in the latter group, suggesting that increased vasodilator responsiveness to endogenous, endothelial NO indeed contributes to the maintenance of physiological arterial blood pressure levels in mice with abolished vasodilator responses to ANP.

Our observations somehow differ from the results by Melo *et al.* (26) in ANP-deficient mice (ANP−/−). In this study, the hypertensive responses to L-NAME were not different in ANP−/− and wild-type mice, suggesting that the synthesis of or responsiveness to endothelial NO was not enhanced by chronic ANP deficiency. The following are possible explanations for the divergent results: absence of ANP (ANP−/−) may result in an increased expression of vascular SMC GC-A, and the consequent increase in basal activity of this receptor may prevent compensatory changes of the NO/sGC system; also, circulating BNP levels may still activate vascular GC-A receptors in ANP−/− mice.

Why did the deletion of SMC GC-A enhance the vasorelaxing responsiveness to NO and not to CNP? We cannot exclude that the CNP/GC-B system was up-regulated at the level of the local, endothelial synthesis and/or secretion of CNP (11). However, we and others have shown previously that the potency of CNP for vasodilation or reduction of blood pressure is much lower as compared with ANP (2, 25, 27). Even more, CNP-deficient mice do not exhibit arterial hypertension (28).[2] Taken together, these data might indicate that the CNP/GC-B system is not as crucial as the ANP/GC-A and NO/sGC systems in the regulation of vascular tone.

Within the vascular system, not only SMC but also endothelial cells are rich in GC-A and respond to ANP with increased production of cGMP (8, 9). In cultured endothelial cells, elevation of cGMP levels inhibits endothelin-1 (29) and stimulates NO (10) and CNP synthesis (11). Thus, it has been reported that ANP-induced vasodilation is partly mediated by the endothelial release of NO (30). By selective disruption of GC-A in smooth muscle cells (17), we generated an elegant mouse model allowing us to dissect the specific endothelial effects of ANP in intact vessel preparations. As shown, the vasorelaxing effect of ANP was almost completely abolished in SMC GC-A-deficient aortas. We observed a small relaxation at higher ANP concentrations, which was totally prevented by the inclusion of L-NAME in the organ chambers, indicating that these responses were mediated by the ANP/GC-A-stimulated release of endothelial NO. However, the NO-mediated vasorelaxing effect of ANP observed in SMC GC-A KO arteries was rather small as compared with ANP effects on arteries with intact SMC GC-A expression levels. Even more, inhibition of endothelial NO synthesis by L-NAME did not affect the vasorelaxing responses of the latter, floxed GC-A arteries to ANP. Taken together, these observations indicate that under normal conditions, the contribution of endothelial NO to the vasodilating effects of ANP is minor, if any. Thus, the role of the endothelium in the maintenance of blood pressure and volume homeostasis by ANP remains intriguing. Many studies have shown that ANP modulates endothelial permeability (31), an effect that might be essential for the physiological regulation of blood volume. In our future studies, we will take advantage of this new mouse model with SMC-specific deletion of GC-A to elucidate how the endothelial GC-A receptor contributes to the known actions of ANP on vascular permeability and, overall, to the regulatory effects of this peptide on blood volume homeostasis.

In summary, we demonstrated that isolated arteries from SMC GC-A KO mice exhibit a higher sensitivity to nitrovasodilators and endothelial NO as compared with arteries with normal GC-A expression levels. This effect can be attributed to an enhanced expression of sGC, whereas the expression and/or activity levels of eNOS and of downstream cGMP-effector pathways are not involved. Increased vasodilating responsiveness to endogenous endothelial NO contributes to compensate for the missing vasodilating effect of ANP in SMC GC-A KO mice. Our study adds an important piece of information to the local, reciprocal interactions between the ANP/GC-A and NO/sGC systems within the vascular wall. Given that blood pressure is elevated in mice with generalized deletion of ANP (13), GC-A (14), or eNOS (12), neither system is able to fully compensate for the complete loss of the other. However, their mutual interactions may moderate the hypertensive phenotype and prevent an otherwise lethal form of hypertension.

[2] Y. Ogawa, personal communication.

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