GRK2 Targeted Knock-down Results in Spontaneous Hypertension, and Altered Vascular GPCR Signaling*

Received for publication, September 30, 2014, and in revised form, December 25, 2014. Published, JBC Papers in Press, January 5, 2015, DOI 10.1074/jbc.M114.615658

Elena Tutunea-Fatan†, Fabiana A. Caetano‡, Robert Gros§,∥, and Stephen S. G. Ferguson¶

From the † J. Allyn Taylor Centre for Cell Biology, ‡ Vascular Biology Group, Robarts Research Institute, and Departments of ¶ Medical Sciences, ‡ Physiology and Pharmacology, University of Western Ontario, London, Ontario N6A 5K8, Canada

Background: GRK2 contributes to the desensitization of GPCRs is involved in cardiovascular disease.

Results: Genetic knockdown of GRK2 in mice results in the development of hypertension and altered vascular signaling.

Conclusion: Reduced GRK2 expression leads to the development of hypertension.

Significance: Therapeutic strategies that target GRK2 activity, not expression, may be more effective for the treatment of hypertension.

Hypertension, elevated arterial pressure, occurs as the consequence of increased peripheral resistance. G protein-coupled receptors (GPCRs) contribute to the regulation of vasodilator and vasoconstrictor responses, and their activity is regulated by a family of GPCR kinases (GRKs). GRK2 expression is increased in hypertension and this facilitates the development of the hypertensive state by increasing the desensitization of GPCRs important for vasodilation. We demonstrate here, that genetic knockdown of GRK2 using a small hairpin (sh) RNA results in important for vasodilation. We demonstrate here, that genetic knockdown of GRK2 using a small hairpin (sh) RNA results in increased altered vascular reactivity and the development of hypertension between 8–12 weeks of age in shGRK2 mice due to enhanced signaling. Vascular smooth muscle cells (VSMCs) cultured from shGRK2 knockdown mice show increases in GPCR-mediated signaling, as the consequence of reduced GRK2-mediated desensitization. In addition, agonists and biased agonists exhibited age-dependent alterations in ERK1/2 and Akt signaling, as well as cell proliferation and migration responses in shGRK2 knockdown VSMCs when cultured from mice that are either 3 months or 6 months of age. Changes in angiotensin II-stimulated ERK1/2 phosphorylation are observed in VSMCs derived from 6-week-old shGRK2 mice prior to the development of the hypertensive phenotype. Thus, our findings indicate that the balance between mechanisms regulating vascular tone are shifted to favor vasoconstriction in the absence of GRK2 expression and that this leads to the age-dependent development of hypertension, as a consequence of global alterations in GPCR signaling. Consequently, therapeutic strategies that target GRK2 activity, not expression, may be more effective for the treatment of hypertension.

Vascular reactivity and blood pressure homeostasis is controlled via a complex network of cell signaling mechanisms (1). In the hypertensive state, elevated arterial pressure is a consequence of increased peripheral resistance, which is regulated by cellular mechanisms that control a balance between blood vessel vasoconstriction and vasodilation (1, 2). Among the network of receptors and signaling molecules regulating blood vessel reactivity, G protein-coupled receptors (GPCRs) are known to play a central role and represent an important target for the treatment of hypertension (1). In this context, it is evident that a better understanding of the mechanisms underlying GPCR regulation remains of paramount importance.

GPCRs function to modulate vascular tone by transducing hormonal signals into changes in intracellular second messenger levels, effector enzymes, and channel activity (3). A prevailing hypothesis in hypertension research is that reduced blood vessel dilation occurs as the consequence of the decreased production of second messengers in response to increased receptor desensitization (1). Of particular importance, GPCRs are phosphorylated by a family of G-protein-coupled receptor kinases (GRKs), which promote the binding of β-arrestins that function to uncouple GPCRs from heterotrimeric G proteins (4). β-Arrestin interactions with GPCRs also couple GPCRs to G protein-independent intracellular signaling cascades (5, 6).

From a mechanistic perspective, alterations in GRK2 expression have been detected in both human and animal models of hypertension (7–17). Specifically, GRK2 protein expression is elevated in lymphocytes from hypertensive patients and is correlated with reductions in the β-adrenergic receptor (βAR)-stimulated vasodilation (7). Moreover, increases in systolic

* This study was supported by grants from the Canadian Institutes of Health Research MOP 111093 (to S. S. G. F.) and MOP 82756 (to R. G.). This manuscript is dedicated to the memory of our great friend and colleague Dr. Hubert H. M. Van Tol who was responsible for generation of the shGRK2 construct. The authors would also like to thank Dr. Hubert H. M. Van Tol for his valuable input throughout the course of this study.

† Supported in part through funding provided by Canadian Institutes of Health Research MOP 111093 (to S. S. G. F.) and MOP 82756 (to R. G.).

‡ Supported by a New Investigator Award from the Heart and Stroke Foundation of Canada. To whom correspondence may be addressed: Robarts Research Institute, University of Western Ontario, 100 Perth Dr., London, Ontario N6A 5K8, Canada. Tel.: 519-663-5777, Ext. 24429; Fax: 519-931-5252; E-mail: rgros@robarts.ca.

§ Supported by a Tier I Canada Research Chair and Career Investigator Award from the Heart and Stroke Foundation of Ontario. To whom correspondence may be addressed: Robarts Research Institute, University of Western Ontario, 100 Perth Dr., London, Ontario N6A 5K8, Canada. Tel.: 519-931-5706; Fax: 519-931-5252; E-mail: ferguson@robarts.ca.

∥ Received for publication, September 30, 2014, and in revised form, December 25, 2014. Published, JBC Papers in Press, January 5, 2015, DOI 10.1074/jbc.M114.615658.

© 2015 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.
Altered Vascular Activity in GRK2 Knockdown Mice

blood pressure in humans correlate well with increases in GRK2 mRNA expression, but not with either GRK3 or GRK5 levels. Reductions in GRK2 expression may also protect against angiotensin II (Ang II)-induced hypertension by increasing nitric oxide bioavailability (11). Consequently, alterations in GRK2 expression are proposed to induce a shift in the balance between vasoconstrictor and vasodilator mechanisms suggesting GRK2 may function as a potential molecular mediator contributing to the induction of hypertension (1). Thus, inhibition of GRK2 may represent a viable therapeutic solution for the treatment of hypertension (18).

GRK2 is an essential gene, for which the genetic deletion is embryonically lethal, that plays a critical role in regulating both phosphorylation-dependent and -independent GPCR desensitization and endocytosis, as well as β-arrestin-biased cellular signaling (19–22). Because GRK2 knock-out embryos do not survive beyond embryonic day 15.5 (19), in collaboration with Dr. Hubert H. M. Van Tol, we utilized small hairpin interfering RNA (shRNA) to universally knockdown GRK2 protein expression in transgenic mice. We created a line of shGRK2 knockdown mice with reduced GRK2 in all tissues tested. These mice are intraterine growth retarded, are spontaneously hypertensive and have impaired vascular reactivity. In addition, vascular smooth muscle cells (VSMCs) cultured from shGRK2 knockdown mice demonstrate reduced Gαq- and Gα11/11 GPCR desensitization, altered extracellular regulated kinase 1/2 (ERK1/2) and Akt signaling with age, as well as age-dependent increases in cellular proliferation and migration in response to angiotensin receptor (ATR), but not βAR, activation. Our data indicate that a loss of GRK2 expression shifts the balance of GPCR-regulated vasodilator and vasoconstrictor mechanisms in favor of Gα11/11-signalizing that leads to the establishment of a hypertensive phenotype.

MATERIALS AND METHODS

Development and Characterization of Transgenic Mice—The shGRK2 transgenic mouse strain was generated by Dr. Hubert H. M. Van Tol using a U6 RNA polymerase III promoter to drive ubiquitous mGRK2-shRNA AATCTTTTGACTCTCTATAATTAT (shGRK2) expression. The shGRK2 sequence was subcloned into the pSilencer 2.0-U6 vector and a fragment containing both the U6 promoter and shGRK2 was microinjected into B6C3F1 mouse embryos transferred to pseudopregnant recipients. The integration of the genomic DNA was confirmed into B6C3F1 mouse embryos transferred to pseudopregnant recipients. The experimental protocol was approved by the Animal Use Subcommittee of Western University, according to the guidelines of the Canadian Council on Animal Care.

Assessment of Blood Pressure—Blood pressures and heart rates were recorded from conscious mice at 4–5, 8, 12–14, and 24–25 weeks old, using the non-invasive CODA tail-cuff blood pressure system (Kent Scientific, Torrington, CT), as described previously (23). To assess the possible influence of anxiety on blood pressure, non-invasive tail-cuff blood pressures and heart rates were obtained in 12-week-old anesthetized (ketamine/xylazine 100/10 mg/kg intraperitoneal) mice. Invasive arterial blood pressures and heart rates were recorded in 12-week-old anesthetized (ketamine/xylazine) mice after cannulating the right carotid artery with a Millar-tip transducer catheter (model SPR-261, 1.4F; Millar Instruments, Inc., Houston TX). To assess in vivo vasoconstrictor responsiveness, mice were injected with 1 mmol/liter phenylephrine intraperitoneal and blood pressures were recorded for an additional 10 min.

Indirect Calorimetry, Activity, and Inactivity—Assessment of metabolic and activity parameters were obtained using the Comprehensive Laboratory Animal Monitoring System metabolic chamber (Columbus Instruments, Columbus, OH) as described previously (23).

Vasomotor Studies—Vascular reactivity was assessed in second-order mesenteric arteries obtained from 12–14-week-old wild-type and shGRK2 mice were mounted on a pressure myograph (Living Systems Instrumentation, St. Albans, Vermont) as previously described in detail (24–25). Passive and active vessel diameter perfusion pressure relationships (20–120 mm Hg) were obtained. The set-point was defined as the lowest perfusion pressure at which significant myogenic constriction was first observed. Extent was defined as the magnitude of the percent myogenic tone at a given perfusion pressure, and stiffness was defined as the slope of the active diameter-pressure relationship. Contractile responses to phenylephrine (1 nmol/liter to 10 μmol/liter) or KCl (10 to 120 mmol/liter) were recorded in vessels pressurized to 60 mm Hg. To examine Ca2+ sensitivity, vessels were washed twice in Ca2+-free physiological salt solution (1 mmol/liter EGTA) and placed in Ca2+-free physiological salt solution (PSS) containing 120 mmol/liter KCl. Contractile responses to increasing extracellular CaCl2 (0.5 to 3.0 mmol/liter) were recorded. Vasodilatatory response to isoproterenol (1 nmol/liter to 10 μmol/liter) or sodium nitroprusside (0.1 nmol/liter to 10 μmol/liter) were performed in 60 mm Hg pressurized vessels pre-contracted with 3 μmol/liter of phenylephrine.

VSMC Cell Culture—Vascular smooth muscle cells (VSMCs) were isolated by enzymatic digestion from thoracic aortas of 12- and 24-week-old GRK2 shRNA transgenic and age-matched C57Bl/6 male mice and maintained in primary culture for a subsequent number of 7 passages as previously described (26). Cells were maintained in Dulbecco’s Modified Eagle Medium (Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (BioShop, Burlington, ON, Canada), 50 μg/ml platelet-derived growth factor (PDGF)-B (Sigma-Aldrich) in a humidified incubator with 5% CO2 at 37 °C.

Cyclic Adenosine Monophosphate (cAMP)-Glo Assay Analysis—The cAMP Glo assay (Promega, Madison, WI) was carried out according to the manufacturer’s protocol. VSMCs were seeded into a poly-d-lysine coated, white, clear-bottom 96-well plates (~10,000 cells per well) and incubated overnight. Cells were treated with increasing concentrations of agonists for 15 min and lysed with cAMP-Glo Lysis Buffer. Then, cAMP-Glo detection solution containing protein kinase A was added for 20 min, followed by the addition of Kinase-Glo
reagent. Luminescence was measured using a Victor plate reader (PerkinElmer Life Sciences, Billerica, MA).

Fura-2 Fluorescence Ca\(^{2+}\) Imaging—VSMCs grown on 25 mm round glass cover slips were loaded with 5 \(\mu\)M Fura-2 acetoxy-methyl ester (Invitrogen) at 37 °C for 20 min in Krebs Ringers buffer (pH 7.4). Cells were then washed and incubated for another 10 min in Krebs Ringers buffer. Fura-2 dual excitation and emission was accomplished using 340- and 380-nm excitation filter and a Photon Technology International DeltaRam V imaging system. The coefficients required for \([Ca^{2+}]\) calculations were calibrated by means of the method described in Ref. 27.

Western Blot Analysis—For preparation of the protein lysate, cells were treated with M-PER\textsuperscript{®} Mammalian Protein Extraction Reagent supplemented with HALT Protease Inhibitor Mixture and Phosphatase Inhibitor Mixture (Thermo Scientific, Rockford, IL). 15 \(\mu\)g of total protein were electrophoresed per well on a 10% SDS-polyacrylamide gel and transferred onto Immobilon-FL PVDF membranes (Millipore, Billerica, MA). Immunoblotting was performed with the following primary antibodies: monoclonal anti-actin and smooth muscle \(\alpha\)-actin antibody, p44/p42 (ERK1/2) rabbit polyclonal antibody, phospho-p44/p42 (ERK1/2) rabbit polyclonal antibody, phospho-Akt (Ser-473) rabbit monoclonal antibody, Akt (pan) mouse monoclonal antibody (Cell Signaling Technology) and then probed with a mixture of IRDye polyclonal secondary antibodies (LI-COR Biosciences, Lincoln, NE). Images were read with an Odyssey infrared imaging system and the average density of each band was quantified using the Image Studio software (LI-COR Biosciences) (28).

Boyden Chamber Assay Analysis—VSMCs migration was assessed with Boyden chambers using Transwell\textsuperscript{®} inserts (Corning Life Sciences, Oneonta, NY). A 200-\(\mu\)l suspension of serum-starved cells at a concentration of 2 \(\times\) 10\(^5\)/ml was added in the upper chamber while various concentrations of agonists were added to serum-free medium in the lower chamber. The assembled chamber inserts were then incubated at 37 °C, 5% CO\(_2\) for 24 h. Direct microscopic counting at 40\(^\times\) magnification (Leica DFC 295, Leica Microsystems) of cells that have migrated to the lower side of the membrane was performed and a mean value for each sample was calculated.

Trypan Blue Exclusion Assay—VSMCs at 2 \(\times\) 10\(^5\)/ml were seeded into T25 flasks for 48 and 96 h and then harvested with trypsin-EDTA combined with medium, spun down, and resuspended with Trypan Blue (1:1). To generate a growth curve, an automated cell counter (Invitrogen) was used to calculate total cell number, live cells, dead cells, and viability.

Cell Proliferation ELISA, BrdU Assay Analysis—Serum-starved VSMCs cells to a final concentration of 2 \(\times\) 10\(^5\) cells/ml were seeded onto 96-well tissue-culture microplates, treated with different type of agonists for 24 h, and a cell proliferation
ELISA BrdU (colorimetric) assay (Roche Applied Science) was performed. For signaling studies, serum starved VSMCs were pretreated with various concentrations of PI3 kinase inhibitor (LY294002) (Cell Signaling Technology), Akt inhibitor (Akti-1/2) (Abcam, Cambridge, MA), and MEK 1/2 inhibitor (U0126) (Cell Signaling). After incubation, the reaction product was measured with a plate reader, Infinite M200 (TECAN, Morrisville, NC) at wavelength of 370 nm.

Statistical Analysis—Statistical calculations were performed using GraphPad Prism software version 5 (GraphPad Software, La Jolla, CA). All parametric data were analyzed with one-way Analysis of Variance followed by Tukey-Kramer or Dunnett post-hoc comparisons. When two data sets were compared, a Student’s t test was used. Statistically relevant differences between mean values were determined based on a p \leq 0.05 criterion.

RESULTS

shGRK2 Mice Are Smaller and Have Reduced Body Weight—We employed shRNA, using a U6 mouse polymerase III promoter as a transgene, to universally knockdown, but not knock-out, GRK2 protein expression in transgenic mice. The GRK2 shRNA transgene incorporated into the Y chromosome resulting in only viable transgenic male mice. Thus, age-matched C57BL/6 male mice were used as controls. The resulting shGRK2 transgenic mouse had reduced GRK2 protein expression in all tissues tested including: the aorta, heart, lungs, liver, kidneys, and brain (Fig. 1A). Male shGRK2 transgene positive pups at E17.5 were significantly smaller in size and had lower body weight as compared with female in utero wild-type littermates (Fig. 1, B and C). Despite the smaller body weight at E17.5 for shGRK2 positive male pups, the percentage of male versus female pups at this stage was equal (Fig. 1D). At the time of weaning (4 weeks of age), we observed that the percentage of male versus female was significantly lower suggesting a significant post-natal loss of shGRK2 mice (Fig. 1D). The lower body weight of shGRK2 male mice was still apparent at both 12 and 24 weeks of age (Fig. 1E). Similarly, assessment of body length (nose to tip of tail) revealed that shGRK2 mice are significantly smaller in body size at 12 weeks of age (shGRK2: 16.5 ± 0.1 cm, n = 6 versus C57Bl/6: 18.1 ± 0.1 cm, n = 5, p < 0.05).

Metabolic Parameters, Activity, and Inactivity Are Altered in shGRK2 Mice—We assessed indirect calorimetry, activity and inactivity measurements in both 12-week old shGRK2 and wild-type mice using metabolic cages. No significant differ-

\[\text{ELISA BrdU (colorimetric) assay (Roche Applied Science) was performed.}\]

\[\text{For signaling studies, serum starved VSMCs were pretreated with various concentrations of PI3 kinase inhibitor (LY294002) (Cell Signaling Technology), Akt inhibitor (Akti-1/2) (Abcam, Cambridge, MA), and MEK 1/2 inhibitor (U0126) (Cell Signaling). After incubation, the reaction product was measured with a plate reader, Infinite M200 (TECAN, Morrisville, NC) at wavelength of 370 nm.}\]

\[\text{Statistical Analysis—Statistical calculations were performed using GraphPad Prism software version 5 (GraphPad Software, La Jolla, CA). All parametric data were analyzed with one-way Analysis of Variance followed by Tukey-Kramer or Dunnett post-hoc comparisons. When two data sets were compared, a Student’s t test was used. Statistically relevant differences between mean values were determined based on a p ≤ 0.05 criterion.}\]

\[\text{RESULTS}\]

\[\text{shGRK2 Mice Are Smaller and Have Reduced Body Weight—We employed shRNA, using a U6 mouse polymerase III promoter as a transgene, to universally knockdown, but not knock-out, GRK2 protein expression in transgenic mice. The GRK2 shRNA transgene incorporated into the Y chromosome result-}\]

\[\text{ing in only viable transgenic male mice. Thus, age-matched C57BL/6 male mice were used as controls. The resulting shGRK2 transgenic mouse had reduced GRK2 protein expression in all tissues tested including: the aorta, heart, lungs, liver, kidneys, and brain (Fig. 1A). Male shGRK2 transgene positive pups at E17.5 were significantly smaller in size and had lower body weight as compared with female in utero wild-type littermates (Fig. 1, B and C). Despite the smaller body weight at E17.5 for shGRK2 positive male pups, the percentage of male versus female pups at this stage was equal (Fig. 1D). At the time of weaning (4 weeks of age), we observed that the percentage of male versus female was significantly lower suggesting a significant post-natal loss of shGRK2 mice (Fig. 1D). The lower body weight of shGRK2 male mice was still apparent at both 12 and 24 weeks of age (Fig. 1E). Similarly, assessment of body length (nose to tip of tail) revealed that shGRK2 mice are significantly smaller in body size at 12 weeks of age (shGRK2: 16.5 ± 0.1 cm, n = 6 versus C57Bl/6: 18.1 ± 0.1 cm, n = 5, p < 0.05).}\]

\[\text{Metabolic Parameters, Activity, and Inactivity Are Altered in shGRK2 Mice—We assessed indirect calorimetry, activity and inactivity measurements in both 12-week old shGRK2 and wild-type mice using metabolic cages. No significant differ-}\]
ences in O_2 consumption, CO_2 production, respiratory exchange ratio, energy expenditure, and food or water consumption between shGRK2 and wild-type mice were observed during the light cycle (Fig. 2, A–F). However, both O_2 consumption and CO_2 production were significantly increased in shGRK2 mice during the dark/active cycle (Fig. 2, A and B). Interestingly, assessment of inactivity (sleep) and activity revealed that shGRK2 mice had a significant reduction in their sleep time during the dark/active cycle, as well as a significant increase in both total and ambulatory activity measurements (Fig. 2, G–I).

*shGRK2 Mice Develop Hypertension with Age*—No significant differences in systolic and diastolic blood pressure or heart rate were observed between shGRK2 and wild-type mice at 4–5 weeks of age using the non-invasive tail-cuff method (Fig. 3A). Interestingly, systolic blood pressure was significantly higher in 8-week-old shGRK2 mice as compared with age-matched wild-type mice (Fig. 3B). Hypertension was established in 12–14-week (3 month)-old shGRK2 mice as compared with wild-type controls with significant increases in both systolic and diastolic blood pressures (Fig. 3C). The elevated blood pressures were still evident in shGRK2 mice at 6 months of age (Fig. 3D). To

**FIGURE 3.** Assessment of blood pressures and heart rates in wild-type and shGRK2 transgenic. Blood pressures and heart rates of C57Bl/6 and shGRK2 mice, as measured by non-invasive tail cuff CODA method at (A) 4–5 weeks, (B) 8 weeks, (C) 3 months and (D) 6 months of age. E, blood pressures and heart rates in lightly anesthetized 3 month old mice using the tail-cuff method. F, blood pressures and heart rates measured via invasive Millar catheterization at 3 months of age. G, blood pressure responsiveness in Millar cannulated 3-month-old mice after phenylephrine administration. Data are represented as the mean ± S.D. of five independent experiments. * indicates statistically significant differences versus C57Bl/6 mice (p < 0.05).
eliminate the possibility of a stress/anxiety-induced elevation in blood pressure, we obtained blood pressures and heart rates in lightly anesthetized mice using the tail-cuff methods and both systolic and diastolic blood pressures were significantly elevated in shGRK2 mice (Fig. 3E). We also assessed hemodynamic parameters via invasive Millar catheterization and found that both systolic and diastolic blood pressures were significantly higher in shGRK2 mice as compared with wild-type controls (Fig. 3F). In Millar cannulated mice, phenylephrine (PE, 1 mg/kg) injection significantly elevated systolic blood pressure in both wild-type and shGRK2, but the change in systolic blood pressure was significantly higher in shGRK2 mice as compared with wild-type controls (Fig. 3G).

**GRK2 Deficiency Results in Altered Vascular Reactivity**—Mesenteric arteries from shGRK2 mice demonstrated enhanced myogenic tone development with a significant increase in the extent of myogenic constriction (Fig. 4A). Additionally, the set-point of the myogenic response was significantly reduced in mesenteric arteries from shGRK2 mice when compared with wild-type controls (80 vs 100 mm Hg, p < 0.05) (Fig. 4A). Similarly, we observed a significant increase in the contractile responses to phenylephrine in arteries obtained from shGRK2 mice (EC$_{50}$: 85.2 ± 12.8 nM, n = 5) when compared with wild-type controls (EC$_{50}$: 247 ± 25 nM, n = 5) (Fig. 4B). To determine if the increase in receptor-mediated vaso-motor response was generalized to all constrictor agents, we examined the response to increasing doses of potassium chloride (KCl). No significant differences in KCl-mediated constriction were observed between shGRK2 and wild-type mouse arteries (Fig. 4C). To determine whether the enhanced vasomotor responses observed in arteries from shGRK2 mice was attributable to heightened Ca$^{2+}$ sensitivity of the contractile apparatus, we assessed KCl-mediated constriction in the presence of increasing concentrations of extracellular calcium. No
significant differences in Ca\(^{2+}\) sensitivity were observed in mesenteric arteries obtained from shGRK2 and wild-type mice (Fig. 4D). To determine if GPCR-stimulated vasodilator pathways were altered in shGRK2 mice, we examined receptor-mediated vasodilation in response to isoproterenol in phenylephrine pre-constricted arteries. Isoproterenol-mediated vasodilation was significantly enhanced at 100 nM and greater doses (Fig. 4E, wild-type control EC\(_{50}\): 107 ± 13 nM, \(n = 5\) versus shGRK2 EC\(_{50}\): 70 ± 7 nM, \(n = 5\), \(p < 0.05\)). However, the non-receptor mediated vasodilation in response to sodium nitroprusside was not significantly different between shGRK2 and wild-type mouse arteries (Fig. 4F).

**Heterotrimeric G Protein-mediated Signaling Is Altered in shGRK2 VSMCs**—Smooth muscle \(\alpha\)-actin was expressed at relative constant levels over six different passages (Fig. 5, A and B). To assess the effectiveness of shGRK2 knockdown with increasing passage in vitro, we examined GRK2 protein expression in passaged VSMCs and found that GRK2 knockdown was maintained in cultured shGRK2 VSMCs when compared with wild type VSMCs (Fig. 5, C and D).
To determine whether alteration in GRK2 expression could exert an effect in G\(_\alpha\)/H9251-mediated signaling, cyclic adenosine monophosphate (cAMP) accumulation in response to the \(\beta\)AR agonist isoproterenol (Iso) was assessed. The EC\(_{50}\) for Iso-stimulated cAMP accumulation in VSMCs prepared from 3-month-old mice was significantly reduced in shGRK2 VSMCs when compared with wild-type VSMCs (Fig. 5E). In contrast, forskolin-stimulated cAMP formation was unchanged in shGRK2 versus control VSMCs (Fig. 5F). Thus, a loss of GRK2 expression appeared to lead to decreased desensitization of \(\beta\)adrenergic receptor signaling. The release of Ca\(^{2+}\) from intracellular stores in response to treatment with 100 nM Ang II was assessed in VSMCs prepared from 3-month and 6-month shGRK2 and wild-type mice. We found that Ang II treatment elicited a significantly greater Ca\(^{2+}\) release from VSMCs cultured from 3 and 6-month-old shGRK2 mice, when compared with wild-type VSMCs (Fig. 5G and H). These results indicated that GRK2 expression not only played a significant role in regulating GPCR desensitization in VSMCs, receptor responsiveness was significantly altered with age.

**Effect of GRK2 Knockdown on ERK1/2 Phosphorylation**—We compared Iso-stimulated ERK1/2 phosphorylation with ERK1/2 phosphorylation responses elicited with the biased agonist carvedilol in VSMCs derived from 3-month-old shGRK2 and wild-type mice. Isoproterenol treatment (10 \(\mu\)M) of both wild-type and shGRK2 VSMCs elicited a rapid increase in ERK1/2 phosphorylation at 5 and 15 min of agonist treatment, but ERK1/2 phosphorylation responses were significantly greater in VSMCs derived from shGRK2 mice (Fig. 6A). Carvedilol treatment (10 \(\mu\)M) also resulted in an increase in ERK1/2 phosphorylation that was enhanced and prolonged in VSMCs derived from shGRK2 mice when compared with wild-type mice (Fig. 6B). In contrast, for VSMCs derived from 6 month old mice, the pattern of Iso-stimulated ERK1/2 phosphorylation was significantly altered, with increases in ERK1/2 phosphorylation observed at later time points of agonist treatment (Fig. 6C). Iso-stimulated ERK1/2 phosphorylation at later time points in VSMCs derived...
from 6-month-old wild-type mice was significantly elevated when compared with Iso-stimulated shGRK2 VSMC cultures (Fig. 6C). However, carvedilol did not elicit increased ERK1/2 phosphorylation in VSMCs derived from either 6-month-old shGRK2 or wild-type mice (Fig. 6D).

ERK1/2 phosphorylation in response to 100 nM Ang II treatment was also significantly increased and prolonged in shGRK2 versus wild-type VSMCs derived from 3-month-old mice (Fig. 7A). A similar response profile was observed in response to the treatment of 3-month-old shGRK2 and wild-type cultures with the biased angiotensin receptor agonist [Sar\(^1\),Ile\(^4\),Ile\(^8\)] Ang II (SII), except that a second later phase of ERK1/2 phosphorylation was observed in wild-type VSMCs that was not observed in response to Ang II treatment (Fig. 7B). Similar to what was observed for Iso treatment of VSMCs derived from 6-month-old shGRK2 mice, Ang II-stimulated ERK1/2 phosphorylation was delayed (Fig. 7C). However, unlike what was observed for carvedilol, SII treatment mediated a modest biphasic increase in ERK1/2 phosphorylation in shGRK2 VSMCs that was not observed in wild-type VSMCs (Fig. 7D). Taken together, these data indicated that changes in GRK2 expression modulate ERK1/2 activity in response to both full and biased agonists and that these responses were altered with age.

Effect of GRK2 Knockdown on Akt Phosphorylation—β-Arrestins also couple GPCRs to the activation of Akt (29). Therefore, because GRK2 knockdown altered ERK1/2 phosphorylation responses following either βAR or ATR activation, we assessed Akt signaling in VSMCs derived from 6-month-old wild-type and shGRK2 mice. We find that Iso treatment of wild-type VSMCs resulted in a modest increase in Akt phosphorylation following 15 and 30 min agonist stimulation, whereas Iso treatment of shGRK2 VSMCs resulted in a more robust, sustained and biphasic activation of Akt phosphorylation (Fig. 8A). In contrast, carvedilol did not induce Akt phosphorylation in wild-type VSMCs and provoked only a modest increase in Akt phosphorylation in shGRK2 VSMCs following 15, 30, and 180 min agonist treatment (Fig. 8B). Ang II and SII treatment of wild-type VSMCs did not significantly promote Akt phosphorylation (Fig. 8, C and D). The stimulation of

**FIGURE 7.** Assessment of Go\(_{q}\)-mediated ERK1/2 signaling in primary VSMCs. Representative immunoblots and densitometric analysis showing time course for ERK1/2 phosphorylation with corresponding total ERK1/2 immunoblots from VSMCs derived from 3-month-old wild-type (C57Bl/6) and shGRK2 mice in response to either (A) 100 nM Ang II or (B) 100 μM SII. Data are represented as the mean ± S.D. of four independent experiments expressed as percentage of basal ERK1/2 phosphorylation. Representative immunoblots and densitometric analysis showing time course for ERK1/2 phosphorylation with corresponding total ERK1/2 immunoblots from VSMCs derived from 6 month old wild-type (C57Bl/6) and shGRK2 mice in response to either (C) 100 nM Ang II or (D) 100 μM SII. Total ERK1/2 was used as internal control. Data are represented as the mean ± S.D. of four independent experiments expressed as percentage of basal ERK1/2 phosphorylation. † indicates statistically significant differences compared with unstimulated C57Bl/6 control (p < 0.05). * indicates statistically significant differences compared with time matched C57Bl/6 ERK1/2 (p < 0.05).
shGRK2 VSMCs with Ang II elicited the same robust, sustained and biphasic activation of Akt phosphorylation observed following Iso treatment, and SII treatment induced a modest, but significant, increase in Akt phosphorylation (Fig. 8D). Taken together, these observations indicated that a loss of GRK2 expression favored increased Akt phosphorylation selectively in response to full receptor agonists.

**Effect of GRK2 Knockdown on VSMC Proliferation**—We found that wild-type and shGRK2 VSMCs derived from 3-month-old mice exhibited a limited proliferation rate (Fig. 9A). In contrast, VSMCs derived from 6-month-old wild-type and shGRK2 mice exhibited a more robust proliferative phenotype than VSMCs derived from 3-month-old mice, with shGRK2 VSMCs exhibiting a significantly greater proliferation rate that wild-type VSMCs (Fig. 9A). Agonist treatment with either 10 μM Iso, 10 μM carvedilol, 100 nM Ang II, or 100 μM SII elicited a significant increase in 5-bromo-2-deoxyuridine (BrdU) incorporation in VSMCs derived from 3-month-old wild-type and shGRK2 mice when compared with serum-free medium (Fig. 9B). For cells derived from 6-month-old mice, neither Iso, carvedilol, Ang II, nor SII treatment increased BrdU incorporation into wild-type VSMCs, whereas only Ang II and SII treatment increased BrdU incorporation into shGRK2 VSMCs (Fig. 7C). Treatment of shGRK2 VSMCs with either an Akt inhibitor (Akti-1/2), phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002), or ERK1/2 inhibitor (U0126) inhibited Ang II-stimulated BrdU incorporation (Fig. 9D). These data suggested that the activation of ATRs in shGRK2 mice selectively activates cell proliferation of older VSMCs in a PI3K/Akt/ERK-dependent manner.

**Effect of GRK2 Knockdown on VSMC Migration**—Migration assays for wild-type and shGRK2 VSMCs derived from 3-month-old mice revealed that neither 100 nM Ang II or 100 μM SII stimulated cellular migration of VSMCs derived from 3-month-old wild-type and shGRK2 mice when compared with serum-free medium (Fig. 10, A and B). However, the treatment of VSMCs derived from 6-month-old wild-type and shGRK2 mice with either Ang II or SII significantly increased shGRK2, but not wild-type, VSMCs migration as compared with serum-free medium (Fig. 10, C and D). The treatment of shGRK2 VSMCs with either Akt or ERK1/2 inhibitors prevented Ang II-induced shGRK2 VSMCs migration (Fig. 10E). Thus, GRK2 knockdown increased VSMCs migration in response to Goq/11-coupled GPCR activation.
Because we observed alterations in ERK1/2 and Akt signaling that were associated with cellular age that may be correlated with alterations in VSMC activity associated with the development of hypertension, we examined whether ERK1/2 and Akt signaling in response to Ang II and SII were altered in VSMCs that were derived from mice that were pre-hypertensive. We found that Ang II treatment did not result in increased ERK1/2 phosphorylation in wild-type VSMCs, but stimulated a statistically significant increase in ERK1/2 phosphorylation in VSMCs derived from shGRK2 mice (Fig. 11A). However, SII treatment did not result in an increase in ERK1/2 phosphorylation in VSMCs derived from either wild-type or shGRK2 mice (Fig. 11B). Neither Ang II nor SII altered Akt signaling in either wild-type or shGRK2 mice (Fig. 11 C and D).

**DISCUSSION**

GRK2 contributes to the desensitization of GPCR signaling and generalized increases in GRK2 expression are linked to the development of essential hypertension in both humans and experimental animal models (7–17). To further understand the role of the balance between GRK2 expression and vascular reactivity, we developed a shGRK2 transgenic mouse. The shGRK2 transgenic mice are viable, show indications of intrauterine growth retardation and become spontaneously hypertensive at 8–12 weeks following birth. GRK2 knockdown both increases vasodilator and vasoconstrictor responses to GPCR activation in intact tissue and VSMC cultures, but increase in vasoconstrictor mechanisms appear to dominate the physiological phenotype. VSMCs isolated from older hypertensive mice also show alterations in ERK1/2 and Akt signaling, which are linked to altered cellular proliferation and migration responses to $G_{q/11}$-coupled GPCR signaling.

GRK2/3 are unique with respect to other GRK family members in that they mediate the GRK2 RGS homology domain-dependent phosphorylation-independent desensitization of only $G_{q/11}$-coupled GPCRs (22). Consequently, significant reductions in GRK2 protein expression may preferentially result in enhanced signaling via $G_{q/11}$-coupled GPCRs leading to increased vasoconstrictor activity and hypertension. Thus, the current results are not incongruent with previous studies linking reduced GRK2 expression to hypertension, as reduced GRK2 expression, given the importance of GRK2-phosphorylation-independent desensitization in the regulation of $G_{q/11}$-coupled GPCR signaling.

GRK2 expression is associated with both enhanced $\beta$AR desensitization in lymphocytes and impaired vascular reactivity in hypertensive humans and increased GRK2 expression in umbilical arteries is correlated with gestational hypertension.
VSMC-specific overexpression of GRK2 enhances βAR desensitization, attenuates βAR-mediated vasodilation, increases medial VSM thickness and results in cardiac hypertrophy (9). The reduction of GRK2 expression in hemizygous GRK2 knock-out mice also partially protects against Ang II-mediated hypertension and vascular remodeling via a mechanism involving the preservation of nitric oxide availability, but does not affect resting blood pressure (11). We observe an age-dependent onset of hypertension in shGRK2 knockdown mice with a full onset of hypertension observed at 12 weeks of age, which is maintained throughout the lifespan of the animals. The increases in blood pressure are not confounded by stress and/or anxiety as elevated blood pressure is also observed in lightly anesthetized mice. We also find that vasodilatation in response to βAR stimulation is enhanced in the shGRK2 mice, but consistent with the hypertensive phenotype vasoconstriction in response to PE is also increased and may predominate physiologically. In addition, myogenic response was increased in shGRK2 mouse mesenteric arteries, and this has been linked to Goq_{i/11}-mediated pathways. These observations suggest that extensive loss of GRK2 expression favors an increase in vasoconstriction associated with increases in peripheral resistance likely due to reduced Goq_{i/11} coupled receptor desensitization. This is consistent with the observation that inhibition of VSMC-GRK2 by either the overexpression of the GRK2-ct or VSMC-specific ablation of GRK2 protein expression increases α_{i/11}AR-stimulated vasoconstriction (14). Consequently, it appears that either increased or decreased GRK2 expression can lead to a dysregulation in the balance between vasoconstrictor- and vasodilator-regulated vessel tone. Goq_{i/11}-coupled GPCRs that mediate vasodilatation are regulated solely by GRK2-mediated phosphorylation, whereas Goq_{i/11}-coupled receptors are regulated by both GRK2 phosphorylation-dependent and -independent mechanisms (22). Therefore, Goq_{i/11}-coupled GPCRs may be more sensitive to small increases in GRK2 expression due to the loss of phosphorylation-dependent desensitization, whereas a loss of GRK2 expression increases both Goq_{i/11} and Goq_{i/11}-GPCR signaling. Thus, the shGRK2 hypertensive phenotype may be the consequence of extent of GRK2 suppression achieved in this model, due to the reduction of the Goq_{i/11}-selective uncoupling of Goq_{i/11}-coupled GPCRs by the, GRK2 RGS homology domain, which is not observed for Goq_{i/11}-coupled GPCRs (22). Consequently, therapeutic strategies that target GRK2 activity, as opposed inhibiting recep-

---

**FIGURE 10.** Assessment of migration responses for primary VSMCs. **A**, representative images and (**B**) quantification of the migration of VSMCs derived from wild-type (C57Bl/6) and 3-month-old shGRK2 mice treated for 24 h SFM, 100 nM Ang II, or 100 μM [Sar1,Ile4,Ile8] Ang II (SII). Bar graph data represent the mean ± S.D. of four independent experiments. **C**, representative images and (**D**) quantification of the migration of VSMCs derived from wild-type (C57Bl/6) and 6-month-old shGRK2 mice treated for 24 h with SFM, 100 nM Ang II, or 100 μM [Sar1,Ile4,Ile8] Ang II (SII). Bar graph data represent the mean ± S.D. of four independent experiments. **E**, quantification of cell migration responses of VSMCs cultured from 6 month old shGRK2 treated with SFM or 100 nM Ang II for 24 h in the presence or absence of either 1 μM Akti-1/2, or 1 μM U0126 inhibitors. Data represent the mean ± S.D. of four independent experiments. In **A**, the microphotographs were taken at 40× magnification and scale bar = 50 μm. * indicates statistically significant differences compared with unstimulated control (p < 0.05).
tor interactions, may be more effective for the treatment of hypertension.

Consistent with the observation that vasodilatation in response to βAR stimulation is increased in the shGRK2 mice, Iso-stimulated cAMP formation is enhanced shGRK2 VSMCs when compared with wild-type mice indicating that βAR desensitization is reduced. We also observe that Ang II-stimulated Ca²⁺ release is increased in VSMCs cultured from shGRK2 mice suggesting that there is reduced GRK2-mediated desensitization of Gαq/11 signaling. This is similar to what was previously observed for endothelin receptor signaling in VSMCs transfected with a GRK2-K220R/D110A mutant that does not mediated receptor desensitization (13). Thus, in general, VSMCs responses recapitulate responses observed in intact tissue.

The ERK1/2 pathway is coupled to enhanced contractile responses and increased VSMC proliferation and the inhibition of ERK1/2 activity significantly reduces smooth muscle cell growth (30–33). We also observe alterations in ERK1/2 and Akt signaling. Specifically, we find that reduced GRK2 expression results in a significant increase in the extent and duration of both Iso- and Ang II-stimulated ERK1/2 phosphorylation in VSMC cultures derived from 6-month-old wild-type (C57Bl/6) and shGRK2 mice. This is in alignment with previous observations that GRK2 expression exerts a negative effect on β-arrestin signaling by competing with GRK5 and GRK6 for receptor binding (6). The time course for ERK1/2 phosphorylation in response to Iso and Ang II shifts with age with more effective activation of ERK1/2 phosphorylation at early time points in 3 month cultures and a more prolonged and delayed activation in 6 month cultures. This supports the concept that β-arrestin-mediates the prolonged activation of ERK1/2 (34). Carvedilol and SII have been shown to activate ERK1/2 phosphorylation solely via the β-arrestin-dependent mechanism (35, 36). We find that both carvedilol and SII treatment results in increased ERK1/2 phosphorylation in VSMCs derived from 3- month-old wild-type and shGRK2 mice, but ERK1/2 phosphorylation is selectively increased in shGRK2 VSMCs. However, the treatment of
VSMCs derived from 6-month-old wild-type mice with either carvedilol or SII does not result in either ERK1/2 or Akt phosphorylation, whereas in shGRK2 cultures SII, but not carvedilol stimulates ERK1/2 phosphorylation. In contrast, both carvedilol and SII stimulation elicit Akt phosphorylation in VSMCS derived from 6-month-old shGRK2 mice. We observe increases ERK1/2, but not Akt signaling in response to Ang II in VSMCs derived from 6-week-old shGRK2 mice prior to the development of hypertension. Thus, it appears that alterations in both ERK1/2 and Akt are age-dependent and progress with the generation of the hypertensive phenotype, as pre-hypertensive mice do not exhibit a generalized alteration in both ERK1/2 and Akt activation in response to both Ang II and SII. Taken together, these observations indicate that GRK2 expression antagonizes ERK1/2 and Akt phosphorylation in VSMCs and the pattern of responsiveness to drug treatment changes with the development of hypertension. This suggests that the responsiveness to biased agonists may be reduced with age and might potentially limit their window of therapeutic effectiveness.

GRK2 plays an inhibitory role in cell cycle progression and GRK2 overexpression reduces smooth muscle, thyroid cancer, and hepato-carcinoma cell growth and that GRK2 and β-arrestin2 regulate cell migration (37–42). The proliferation and migration of VSMCs derived from older shGRK2 mice is significantly increased in response to Ang II stimulation via an ERK1/2- and Akt-dependent mechanism. β-Arrestin2 deletion reduces both VSMCs proliferation and migration and blocking GRK2 activity increases neutrophil migration (40, 42). Thus, increased β-arrestin signaling in the absence of GRK2 expression promotes VSMCs proliferation and migration, which may contribute to the age-dependent onset of hypertension in shGRK2 mice. These increased proliferative and migratory responses are only observed following ATR activation of VSMCs derived from shGRK2 mice. Thus, the context of the activation of the ERK/Akt pathway is likely also important in that Gaq/11, but not Gao, receptor activation leads to proliferation and may require a concomitant increase in Ca2+ release from intracellular stores.

In summary, global knockdown of GRK2 expression results in a mouse that spontaneously develops hypertension, due to alterations in the balance between mechanisms regulating vasodilatation and vasoconstriction. The loss of GRK2 expression not only results in increases in GPCR responsiveness to both vasodilators and constrictors, associated with reduced receptor desensitization, VSMC proliferation and migration are also increased as the consequence of what appears to be β-arrestin-mediated signaling. This highlights the complexity of the balance between G protein-dependent and -independent signaling pathways engaged by GPCRs. These observations indicate that, while partial inhibition of GRK2 may be of therapeutic benefit, total inhibition of GRK2 expression may lead to alterations in GPCR signaling that will lead to the development of hypertension or other pathological conditions.

Acknowledgments—We thank Cheryl Vander Tuin and Dr. Christie Godin for technical assistance.

REFERENCES

1. Feldman, R. D., and Gros, R. (1998) Impaired vasodilator function in hypertension: the role of alterations in receptor-G protein coupling. *Trends Cardiovasc. Med.* 8, 297–305

2. Ferguson, S. S. G., and Feldman, R. D. (2014) beta-Adrenoceptors as molecular targets in the treatment of hypertension. *Can. J. Cardiol.* 30, S3–S8

3. Lefkowitz, R. J. (2013) Arrestins come of age: a personal historical perspective. *Prog. Mol. Biol. Trans. Sci.* 118, 3–18

4. Brinks, H. L., and Eckhart, A. D. (2010) Regulation of GPCR signaling in hypertension. *Biochem. Biophys. Acta* 1802, 1268–1275

5. Luttrell, L. M., Ferguson, S. S. G., Daaka, Y., Miller, W. E., Maudsley, S., Delia Rocca, G. J., Lin, F., Kawakatsu, H., Owada, K., Luttrell, D. K., Caron, M. G., and Lefkowitz, R. J. (1999) Beta-arrestin-dependent formation of β2 adrenergic receptor-Src protein kinase complexes. *Science* 283, 655–661

6. Zidar, D. A., Violin, J. D., Whalen, E. J., and Lefkowitz, R. J. (2009) Selective engagement of G protein coupled receptor kinases (GRKs) encodes distinct functions of biased ligands. *Proc. Natl. Acad. Sci. U.S.A.* 106, 9649–9654

7. Gros, R., Benovic, J. L., Tan, C. M., and Feldman, R. D. (1997) G-protein coupled receptor kinase activity is increased in hypertension. *J. Clin. Invest.* 99, 2087–2093

8. Gros, R., Chorazyczewski, J., Meek, M. D., Benovic, J. L., Ferguson, S. S. G., and Feldman, R. D. (2000) G-Protein-coupled receptor kinase activity in hypertension: increased vascular and lymphocyte G-protein receptor kinase-2 protein expression. *Hypertension* 35, 38–42

9. Eckhart, A. D., Ozaki, T., Tevaaeiri, H., Rockman, H. A., and Koch, W. J. (2002) Vascular-targeted overexpression of G protein-coupled receptor kinase-2 in transgenic mice attenuates β-adrenergic receptor signaling and increases resting blood pressure. *Mol. Pharmacol.* 61, 749–758

10. Harris, D. M., Cohn, H. I., Pesant, S., Zhou, R. H., and Eckhart, A. D. (2007) Vascular smooth muscle G(α) signaling is involved in high blood pressure in both induced renal and genetic vascular smooth muscle-derived models of hypertension. *Am. J. Physiol. Heart Circ. Physiol.* 293, H3072–H3079

11. Avendaño, M. S., Lucas, E., Jurado-Pueyo, M., Martínez-Revelles, S., Vila-Bedmar, R., Mayor, F., Jr, Salaices, M., Briones, A. M., and Muruga, C. (2014) Increased nitric oxide bioavailability in adult GRK2 hemizygous mice protects against angiotension II-induced hypertension. *Hypertension* 63, 369–375

12. Napolitano, R., Campanile, A., Sarno, L., Anastasio, A., Maruotti, G. M., Morlando, M., Trimarco, B., Martinelli, P., and Iaccarino, G. (2012) GRK2 levels in umbilical arteries of pregnancies complicated by gestational hypertension and preeclampsia. *Am. J. Hypertension* 25, 366–371

13. Morris, G. E., Nelson, C. P., Standen, N. B., Challiss, R. A., and Willets, J. M. (2010) Endothelin signalling in arterial smooth muscle is tightly regulated by G protein-coupled receptor kinase 2. *Cardiovasc. Res.* 85, 424–433

14. Cohn, H. I., Harris, D. M., Pesant, S., Pfeiffer, M., Zhou, R. H., Koch, W. J., Dorn, G. W., and Eckhart, A. D. (2008) Inhibition of vascular smooth muscle G protein-coupled receptor kinase 2 enhances alpha1D-adrenergic receptor constrictor activity. *Am. J. Physiol. Heart Circ. Physiol.* 295, H1695–H1704

15. Cohn, H. I., Xi, Y., Pesant, S., Harris, D. M., Hyslop, T., Falkner, B., and Eckhart, A. D. (2009) G protein-coupled receptor kinase 2 expression and activity are associated with blood pressure in black Americans. *Hypertension* 54, 71–76

16. Gros, R., Tan, C. M., Chorazyczewski, J., Kelvin, D. I., Benovic, J. L., and Feldman, R. D. (1999) G-protein-coupled receptor kinase expression in hypertension. *Clin. Pharmacol. Therapeut.* 65, 545–551

17. Izzo, R., Cipolletta, E., Ciccarelli, M., Campanile, A., Santulli, G., Palumbo, G., Vasta, A., Formisano, S., Trimarco, B., and Iaccarino, G. (2008) Enhanced GRK2 expression and desensitization of βAR vasodilatation in hypertensive patients. *Clin. Trans. Sci.* 1, 215–220

18. Carotenuto, A., Cipolletta, E., Gomez-Monterrey, I., Sala, M., Vernieri, E., Limatola, A., Bertamino, A., Musella, S., Sorrentino, D., Grieco, P., Trimarco, B., Novellino, E., Iaccarino, G., and Campiglia, P. (2013) Design, synthesis and efficacy of novel G protein-coupled receptor kinase 2 inhibitors. *Eur. J. Med. Chem.* 69, 384–392
Altered Vascular Activity in GRK2 Knockdown Mice

19. Jaber, M., Koch, W. J., Rockman, H., Smith, B., Bond, R. A., Sulik, K. K., Ross, J. J., Lefkowitz, R. J., Caron, M. G., and Giros, B. (1996) Essential role of beta-adrenergic receptor kinase 1 in cardiac development and function. Proc. Natl. Acad. Sci. U.S.A. 93, 12974–12979

20. Zhang, J., Ferguson, S. S., Barak, L. S., Aber, M. J., Giros, B., Lefkowitz, R. J., and Caron, M. G. (1997) Molecular mechanisms of G protein-coupled receptor signaling: role of G protein-coupled receptor kinases and arrestins in receptor desensitization and resensitization. Recept. Chem. 5, 193–199

21. Luttrell, L. M., and Kenakin, T. P. (2011) Refining efficacy: alosterism and bias in G protein-coupled receptor signaling. Meth. Mol. Biol. 756, 3–35

22. Ferguson, S. S. G. (2007) Phosphorylation-independent attenuation of GPCR signalling. Trends Pharmacol. Sci. 28, 173–179

23. Roy, A., Fields, W. C., Rocha-Resende, C., Resende, R. R., Guatimosim, S., Gros, R., Ding, Q., Chorazyczewski, J., Pickering, J. G., Limbird, L. E., and Van Wert, R., You, X., Thorin, E., and Husain, M. (2002) Effects of age, gender, and blood pressure on myogenic responses of mesenteric arteries from C57BL/6 mice. Am. J. Physiol. Heart Circ. Physiol. 282, H380–H388

24. Gros, R., You, X., Baggio, L. L., Kabir, M. G., Sadi, A. M., Mungrue, I. N., Parker, T. G., Huang, Q., Drucker, D. J., and Husain, M. (2003) Cardiomyocyte-secreted acetylcholine is required for maintenance of homeostasis in the heart. FASEB J. 27, 5072–5082

25. Gros, R., Van Wert, R., You, X., Thorin, E., and Husain, M. (2002) Effects of age, gender, and blood pressure on myogenic responses of mesenteric arteries from C57BL/6 mice. Am. J. Physiol. Heart Circ. Physiol. 282, H380–H388

26. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) A new generation of indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450

27. Ribeiro, F. M., Paquet, M., Ferreira, L. T., Cregan, T., Swan, P., Cregan, Beaulieu, J. M., Sotnikova, T. D., Marion, S., Lefkowitz, R. J., Gainetdinov, R. R., and Caron, M. G. (2005) An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. Cell 122, 261–273

28. Metallic, A., Fields, W. C., Rocha-Resende, C., Resende, R. R., Guatimosim, S., Gros, R., Ding, Q., Chorazyczewski, J., Pickering, J. G., Limbird, L. E., and Van Wert, R., You, X., Thorin, E., and Husain, M. (2002) Effects of age, gender, and blood pressure on myogenic responses of mesenteric arteries from C57BL/6 mice. Am. J. Physiol. Heart Circ. Physiol. 282, H380–H388

29. Kim, J., Lee, Y. R., Lee, C. H., Choi, W. H., Lee, C. K., Bae, Y. M., Cho, S., and Kim, B. (2005) Mitogen-activated protein kinase contributes to elevated basal tone in aortic smooth muscle from hypertensive rats. Eur. J. Pharmacol. 514, 209–215

30. Mii, S., Khalil, R. A., Morgan, K. G., Ware, J. A., and Kent, K. C. (1996) Mitogen-activated protein kinase and proliferation of human vascular smooth muscle cells. Am. J. Physiol. 270, H142–H150

31. Touyz, R. M., Deschepper, C., Park, J. B., He, G., Chen, X., Neves, M. F., Virdis, A., and Schiffrin, E. L. (2002) Inhibition of mitogen-activated protein/extracellular signal-regulated kinase improves endothelial function and attenuates Ang II-induced contractility of mesenteric resistance arteries from spontaneously hypertensive rats. J. Hypertension 20, 1127–1134

32. Ahn, S., Shenoy, S. K., Wei, H., and Lefkowitz, R. J. (2004) Differential kinetic and spatial patterns of beta-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. J. Biol. Chem. 279, 35518–35525

33. Wei, H., Ahn, S., Shenoy, S. K., Karnik, S. S., Hunyady, L., Luttrell, L. M., and Lefkowitz, R. J. (2003) Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. Proc. Natl. Acad. Sci. U.S.A. 100, 10782–10787

34. Wisler, J. W., DeWire, S. M., Whalen, E. J., Violin, J. D., Drake, M. T., Ahn, S., Shenoy, S. K., and Lefkowitz, R. J. (2007) A unique mechanism of beta-blocker action: carvedilol stimulates beta-arrestin signaling. Proc. Natl. Acad. Sci. U.S.A. 104, 16657–16662

35. Métayé, T., Levillain, P., Krakim, M. J., and Perdrisot, R. (2008) Immunohistochemical detection, regulation and antiproliferative function of G-protein-coupled receptor kinase 2 in thyroid carcinomas. J. Endocrinol. 198, 101–110

36. Peppel, K., Jacobson, A., Huang, X., Murray, J. P., Oppermann, M., and Freedman, N. J. (2000) Overexpression of G protein-coupled receptor kinase-2 in smooth muscle cells attenuates mitogenic signaling via G protein-coupled and platelet-derived growth factor receptors. Circulation 102, 793–799

37. Grynkiewicz, G., Poonie, M., and Tsien, R. Y. (1985) A new generation of Ca2+ indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450

38. Ribeiro, F. M., Paquet, M., Ferreira, L. T., Cregan, T., Swan, P., Cregan, S. P., and Ferguson, S. S. G. (2010) Metabotropic glutamate receptor-mediated cell signaling pathways are altered in a mouse model of Huntington’s disease. J. Neurosci. 30, 316–324

39. Beaulieu, J. M., Sotnikova, T. D., Marion, S., Lefkowitz, R. J., Gainetdinov, R. R., and Caron, M. G. (2005) An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. Cell 122, 261–273

40. Kim, J., Zhang, L., Peppel, K., Wu, J. H., Zidar, D. A., Brian, L., DeWire, S. M., Exum, S. T., Lefkowitz, R. J., and Freedman, N. J. (2008) Beta-arrestins regulate atherosclerosis and neointimal hyperplasia by controlling smooth muscle cell proliferation and migration. Circ. Res. 103, 70–79

41. Lafarga, V., Mayor, F., Jr., and Penela, P. (2012) The interplay between G protein-coupled receptor kinase 2 (GRK2) and histone deacetylase 6 (HDAC6) at the crossroads of epithelial cell motility. Cell Adh. Migr. 6, 495–501

42. Liu, X., Ma, B., Malik, A. B., Tang, H., Yang, T., Sun, B., Wang, G., Minshall, R. D., Li, Y., Zhao, Y., Ye, R. D., and Xu, J. (2012) Bidirectional regulation of neutrophil migration by mitogen-activated protein kinases. Nat. Immunol. 13, 457–464