Cardiac-restricted Expression of the Carboxyl-terminal Fragment of GRK3 UnCOVERs Distinct Functions of GRK3 in Regulation of Cardiac Contractility and Growth

GRK3 CONTROLS CARDIAC α₁-ADRENERGIC RECEPTOR RESPONSIVENESS*

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G protein-coupled receptor kinase-2 and -3 (GRK2 and GRK3) in cardiac myocytes catalyze phosphorylation and desensitization of different G protein-coupled receptors through specificity controlled by their carboxyl-terminal pleckstrin homology domain. Although GRK2 has been extensively investigated, the function of cardiac GRK3 remains unknown. Thus, in this study cardiac function of GRK3 was investigated in transgenic (Tg) mice with cardiac-restricted expression of a competitive inhibitor of GRK3, i.e. the carboxyl-terminal plasma membrane targeting domain of GRK3 (GRK3ct). Cardiac myocytes from Tg-GRK3ct mice displayed significantly enhanced agonist-stimulated α1-adrenergic receptor-activated membrane-stimulated Ca2+ entry in vitro as compared to control hearts. Thus, inhibition of GRK3 apparently reduces tolerance to elevation of preload. In conclusion, inhibition of cardiac GRK3 causes hypertension because of hyperkinetic myocardium and increased cardiac output relying at least partially on cardiac myocyte α1-adrenergic receptor hyper-responsive. The reduced tolerance to elevation of preload may cause impaired ability to withstand pathophysiological mechanisms of heart failure.

G protein-coupled receptor kinases (GRK)3 are serine/threonine kinases that play crucial roles in the regulation of transmembrane signaling efficacy of G protein-coupled receptors (GPCR). The principal action of GRKs is as catalysts of phosphorylation of agonist-activated GPCRs leading to desensitization of receptor function. Seven G protein-coupled receptor kinase isoforms (GRK1–7), each encoded by a separate gene, have been cloned and characterized. Four of these have been reported to be expressed in myocardial tissue, i.e. GRK2 (βARK1), GRK3 (βARK2), GRK5, and GRK6. A report from our laboratory suggested functional diversity among the different GRKs in the heart based on the findings of distinct differences of distribution of the GRK isoforms among the cellular elements of the heart. Briefly, myocardial expression of GRK3 in several species (man, porcine, and rat) was found to be restricted to cardiac myocytes (CM). GRK2 and GRK5, on the other hand, were ubiquitously expressed among the cellular elements of myocardial tissue. Although GRK2 was predominantly expressed in microvascular endothelial cells, similar levels of GRK2 and GRK3 were found in fully differentiated rat CMs (3).

* This work was supported by grants from the Norwegian Research Council, the Norwegian Council on Cardiovascular Disease, and Center for Heart Failure Research, Faculty of Medicine, University of Oslo. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: GRK, G protein-coupled receptor kinase; AR, adrenergic receptor; BDM, 2,3-butanedione monoxime; CM, cardiac myocyte; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; LV, left ventricular; LVEDD, LV end-diastolic diameter; NLC, nontransgenic littermate control; PH, pleckstrin homology; Tg, transgenic; ERK1/2, extracellular signal-regulated kinase 1/2; ET-R, endothelin receptor; LVE5D, LV end-systolic diameter; LVEDP, LV end-diastolic pressure.
Function of Myocardial GRK3

Unique to GRK2 and GRK3, which constitute a separate sub-group within the GRK family, is a carboxyl-terminal pleckstrin homology domain (PH domain) that targets these kinases to the membrane and cytosolic surface of their cognate receptors (4, 5). These PH domains provide binding interfaces with liberated Gβγ subunits and phosphatidylinositol bisphosphates at the plasma membrane and may also directly interact with the receptor (6). Thus, the GPCR substrate specificities of GRK2 and GRK3 may be determined by binding interfaces with both Gβγ and receptor. Several studies have employed mini-gene-directed expression of a carboxyl-terminal polypeptide fragment of GRK2, i.e. GRK2ct (βARK1ct), which confers competitive inhibition of endogenous GRK2 (7, 8). Among these are studies that demonstrate that GRK2 controls β-adrenergic receptor (β-AR) signaling in CMs. Although GRK2-mediated regulation of β-ARs in CMs has been extensively studied, the substrate specificities of GRK2 and GRK3 have until recently been poorly understood. The diverging primary structures of the carboxyl-terminal regions of GRK2 and GRK3 recently led us to investigate potential differences between GRK2 and GRK3 with respect to substrate specificities at GPCRs in CMs. By means of adenovirus-mediated overexpression of GRK2 or GRK3, or their respective competitive inhibitors, i.e. GRK2ct or GRK3ct in CMs, the substrate specificities of GRK2 and GRK3 at GPCRs that have key roles in regulation of cardiac function were unraveled (3). An important observation in this study was that GRK2ct mirrored the specificity of GRK2 whereas GRK3ct mirrored the specificity of GRK3. Briefly, the novel findings uncovered in these studies were that GRK3 has substantially higher potency and efficacy than GRK2 at endogenous endothelin receptors (ET-R) and α1,-ARs on CMs. At cardiac β-AR, however, GRK3ct efficacy appeared much weaker than for the ET-R (3). Thus, GRK3 emerges as a primary regulator of ET-R and α1,-AR signaling, i.e. signaling pathways involved in regulation of cardiac contractility, growth, and survival signaling. Indeed, these findings are consistent with studies of hybrid transgenic mice in which cardiac-specific overexpression of GRK3, but not that of GRK2, was found to attenuate myocardial hypertrophy in mice with concomitant cardiac expression of a constitutively active mutant of α1B,-AR (9). However, myocardial overexpression of GRK3 did not invoke alterations of cardiac β-AR signaling either in vitro or in vivo (10). Although cardiac restricted overexpression of GRK3 has provided some novel insights into the function of GRK3, strategies aimed at inhibiting endogenous GRK3 are imperative. In the case of GRK2, competitive inhibition of endogenous activities by cardiac-restricted expression of the carboxyl-terminal region of the kinase (GRK2ct/βARK1ct) has been extensively investigated. These studies have revealed that GRK2 plays an essential role in the regulation of cardiac β-adrenergic receptor function. Nevertheless, many of the conclusions from these studies are limited by the fact that we do not know to what extent expression of the GRK2ct/βARK1ct peptide confers selective inhibition of endogenous GRK2. Yet employment of a similar principle for inhibition of endogenous GRK3, i.e. expression of the carboxyl-terminal region of GRK3, is an intriguing strategy in terms of unraveling selective functions of the carboxyl-terminal fragment of GRK2 and GRK3.

Furthermore, neither transgenic mice with cardiac-specific overexpression of GRK3 nor GRK3 knock-out mice were reported to display a distinct cardiac phenotype (10, 11). Thus, rigorous investigation of cardiac structure and function in mice with cardiac-restricted inhibition of GRK3 is clearly needed.

In this study we have generated transgenic mice with cardiac-specific expression of a presumptive competitive inhibitor of endogenous GRK3, i.e. GRK3ct, to investigate the physiological function of myocardial GRK3. Inhibition of endogenous GRK3 results in a surprising phenotype with elevated blood pressure because of hyperkinetic myocardium and thus uncovers novel important roles of GRK3 in the regulation of cardiac function and maintenance of cardiovascular function.

EXPERIMENTAL PROCEDURES

Generation of Transgenic GRK3ct Mice—A mini-gene construct (GRK3ct) encoding the carboxyl terminus of rat GRK3 (GenBankTM accession number M87855; amino acids 495–687) under control of the mouse α1,-myosin heavy chain promoter was constructed. The GRK3ct cDNA was preceded by the Kozak consensus sequence for initiation of transcription and flanked at the 3′-end by SV40 splice and poly(A) signals. This DNA construct was microinjected into fertilized oocytes from C56BL/6-CBA hybrid mice before implantation of the oocytes in pseudopregnant mice. Incorporation of transgene in the resultant offspring was confirmed by Southern blot analysis of genomic DNA using an SV40 cDNA probe (SV40 splice and poly(A)+ DNA; nucleotides 3792–4094 of pcDNA1 plasmid (InVitrogen)). Two transgenic founder lines, i.e. Tg-GRK3ct (1) and Tg-GK3ct (2) (demonstrating the highest myocardial expression of GRK3ct by Western blot analysis), were established and propagated. The founder mice (F0) were backcrossed with C56BL/6-CBA hybrid mice to generate the F1 generation. Further expansion was performed by mating transgenic siblings within the F1 generation. Nontransgenic littermate controls (NLC) were generated from noninjected C56BL/6-CBA mice (siblings of the mice employed for pronuclear injection) and bred similar to the transgenic lines, i.e. similar background as Tg-GRK3ct mice. Unless otherwise indicated, mice of the Tg-GRK3ct(1) line were employed in all experiments.

Histochemical Analysis of Myocardial Tissue and Immunohistochemical Analysis of Myocardial GRK2 and GRK3—Myocardial tissue sections (male, 9 months) were stained with hematoxylin/eosin or picrosirius red (van Gieson’s solution). Immunohistochemical analysis of myocardial distribution of endogenous GRK2 or GRK3 was performed using purified anti-GRK2- or anti-GRK3-specific IgG (Santa Cruz Biotechnology) as described previously (2).

Analysis of GRK3ct mRNA and Peptide Expression—To assess the specificity of α1,-myosin heavy chain promoter-driven expression of GRK3ct, several tissues from Tg-GRK3ct mice (male, 3 months) were sampled for isolation of total RNA and subsequent Northern blot analysis using the 32P-labeled SV40 cDNA probe described above.

Lysates of isolated CMs from Tg-GRK3ct and nontransgenic littermate control (NLC) mice (male, 3 months) were prepared and subjected to Western blot analysis as described previously using anti-GRK3-specific IgG (Santa Cruz Biotechnology) (2).
Echocardiographic Examination—Transthoracic echocardiography was performed using the Vivid 7 System (GE Vingmed Ultrasound, Horten, Norway) and a 13-MHz linear array transducer. Two-dimensional guided M-mode recordings of the left ventricle (LV) in the short axis view at the level of the papillary muscle were obtained, and interventricular septum and posterior wall thickness at end-diastole, respectively, were measured. Internal LV end-diastolic and end-systolic diameters (LVEDD and LVESD, respectively) were recorded as the largest antero-posterior diameter. Fractional shortening was determined as fractional shortening = (LVEDD − LVESD)/LVEDD. All echocardiographic recordings were performed under sedation with midazolam (6.25 mg/kg subcutaneously). All dimensions were analyzed off line by using the EchoPac software analysis program (GE Vingmed Ultrasound, Horten, Norway) by a trained specialist who had no knowledge of the study groups.

Tail-cuff Plethysmography—Tail-cuff plethysmographic recording of blood pressure was performed with a system for noninvasive digital recording of blood pressure in mice (Harvard Apparatus, Holliston, MA) (12). The mice (male, 3 and 9 months) were familiarized with the procedure by daily training sessions for 7 days before recording of blood pressure in order to reduce stress and the associated variability that may occur upon immobilization of mice in the restraining units required to perform tail-cuff plethysmography. The recorded blood pressure was average of seven successful readings from sessions on 3 consecutive days.

Telemetry—The components of the radiotelemetry system (Data Sciences International, St. Paul, MN) have been described previously (13). Miniature radiotelemetric pressure transducers (PhysioTel PA-C20) were implanted in the aortic arch via catheterization of the left carotid artery in male (3 months) Tg-GRK3ct mice (n = 7) and corresponding NLC mice (n = 6) as described previously (14). The mice were kept on a 12-h light/dark cycle and allowed to recover for 10 days before recording of blood pressure, and other variables were commenced. Data were subsequently analyzed by the analysis software module (Dataquest A.R.T. 2.3 Gold, Data Sciences International, St. Paul, MN).

Isolation of CMs and Maintenance of Primary Cultures—CMs were isolated from Tg-GRK3ct and NLC hearts (male, 3 months) by Ca\(^{2+}\)-free retrograde perfusion and enzymatic digestion as described previously (15). Isolated CMs were plated in wells pre-coated with mouse laminin (Invitrogen) and maintained in Joklik’s minimum essential medium supplemented with 25 mmol/liter HEPES, pH 7.0, 23.8 mmol/liter sodium bicarbonate, 0.6 mmol/liter MgSO\(_4\), 10 mmol/liter BDM, 1 mmol/liter dl-carnitine, 10 mmol/liter creatine, 20 mmol/liter taurine, 0.5 mmol/liter CaCl\(_2\), 0.1 mg/ml bovine serum albumin, 0.1 mmol/liter insulin, and 0.1 mmol/liter thyrroxin in humified atmosphere containing 5% CO\(_2\).

Assay of Receptor-generated Responses in Isolated CMs—Six hours before assay of receptor-generated responses, the cell culture medium was replaced with minimum essential medium containing the supplements indicated above except insulin and thyrroxin. The cells were subsequently stimulated with agonists (100 nmol/liter endothelin-1, 5 min; 100 nmol/liter angiotensin II, 5 min; 1 μmol/liter isoproterenol, 5 min; or 10 μmol/liter phenylephrine, 15 min). In the case of phenylephrine stimulation, minimum essential medium was also supplemented with 1 μmol/liter timolol. The assays were stopped at the indicated time points, and cells were harvested in sample buffer (50 mmol/liter Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, 1 mmol/liter sodium orthovanadate, 5 mmol/liter EDTA, and 1 mmol/liter phenylmethylsulfonyl fluoride). The cell lysates were separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes as described previously (2). The filter membranes were subjected to immunoblot analysis with anti-phospho-ERK1/2-specific IgG (anti-phosphothreonine 202/phophorytrosine 204 ERK1/2, Cell Signaling Technology, Inc.) or anti-phosphoserine 16 phospholamban IgG (Upstate Biotechnology, Inc.) according to the manufacturer’s instructions. To confirm similar levels of total ERK1/2 and total phospholamban, parallel filter membranes were subjected to immunoblot analysis with anti-ERK1/2 IgG (Upstate Biotechnology, Inc.) or anti-phospholamban IgG (Upstate Biotechnology, Inc.) according to the manufacturer’s instructions.

Cardiac Responses to Continuous Exposure to α₁-AR or β₁-AR Agonists—Tg-GRK3ct and NLC mice (male, 9 months) were randomized to continuous infusion of phenylephrine (Sigma; 170 μmol/kg \(^{-1}\)day\(^{-1}\) subcutaneously, i.e. 30 mgkg\(^{-1}\)day\(^{-1}\) subcutaneously), isoproterenol hydrochloride (120 μgkg\(^{-1}\)day\(^{-1}\) subcutaneously), or vehicle (saline) for 14 days (n = 10 mice in each treatment group for both Tg-GRK3ct and NLC mice) delivered by subcutaneously implanted micro-osmotic pumps (Alzet®). The dose of phenylephrine employed was based on previous reports and was verified in pilot experiments to be a subpressor dose (16). Blood pressure was monitored by tail-cuff plethysmography at day 0, 7, and 14, and echocardiography was performed at day 0 and 14 after implantation of the micro-osmotic pumps.

Analysis of Contractility of Isolated Papillary Muscles—Tg-GRK3ct and NLC mice (male, 3 months) were anesthetized with sodium pentobarbital (10 mg intraperitoneally) and euthanized by exsanguination of the heart. The aorta was cannulated, and the heart was subjected to retrograde perfusion with relaxing buffer (118.3 mmol/liter NaCl, 3.0 mmol/liter KCl, 0.5 mmol/liter CaCl\(_2\), 4.0 mmol/liter MgSO\(_4\), 2.4 mmol/liter KH\(_2\)PO\(_4\), 24.9 mmol/liter NaHCO\(_3\), 10.0 mmol/liter glucose, 2.2 mmol/liter mannitol) containing 20 mmol/liter BDM and equilibrated with 95% O\(_2\), 5% CO\(_2\) to pH 7.4 at 31 °C. The posterior LV papillary muscle was ligated at each end, carefully excised and mounted in organ bath, and allowed to adapt for 20 min before BDM was washed out. Ca\(^{2+}\) was gradually increased to 1.8 mmol/liter and Mg\(^{2+}\) lowered to 1.2 mmol/liter. The muscles were field-stimulated with alternating polarity at 1 Hz with impulses of 5-ms duration and current about 20% above individual threshold (10–15 mA, determined in each experiment). The isometrically contracting muscles were stretched to the maximum of their length-tension curve. The force was recorded and analyzed as described previously (17). After equilibration in the presence of prazosin (0.1 μmol/liter), isoproterenol was added directly to the organ baths at increasing concentrations until supramaximal concentration of agonist was obtained with respect to inotropic response. Signal averaged
Function of Myocardial GRK3

FIGURE 1. Distribution of GRK2 and GRK3 in mouse myocardial tissue. Representative photomicrographs of immunohistochemical staining of GRK2 and GRK3 in myocardial tissue sections (5 μm) of NLC mice. Left panel, myocardial sections stained with nonimmune rabbit IgG. Middle and right panel, myocardial section stained with rabbit anti-GRK2 IgG or anti-GRK3 IgG, respectively. GRK2 immunoreactivity was predominantly localized in microvascular endothelial cells (arrowheads), whereas GRK3 immunostaining was restricted to CMs (arrowheads). Sections were counterstained with hematoxylin. Magnification ×200.

contraction-relaxation cycles were calculated for the different experimental periods and used to determine the inotropic response expressed as increase in (dF/dt)max at increasing concentrations of isoproterenol.

Analysis of Cardiac Function in Vivo and ex Vivo—In vivo cardiac function was determined as described elsewhere (18) after trans-carotid catheterization of the LV of anesthetized Tg-GRK3ct and NLC (male, 3 months) mice using a Millar pressure-conductance catheter (SPR-853, Millar Instruments, Houston, TX). The mice were anesthetized with isoflurane (1% isoflurane) and pressure-volume parameters were recorded at heart rate stabilized above 450 beats/min. Analyses of cardiac function in ex vivo perfused (working mode) hearts from Tg-GRK3ct and NLC mice (male, 9 months) were obtained by inserting the catheter into the LV via the apex. All hearts were electrically paced (8 Hz) and pressure-volume parameters analyzed at different filling pressures (5–17.5 mm Hg) according to a previously described protocol with minor modifications (19).

Statistical Analysis—Echocardiographic alterations from starting point to end point in the phenylephrine infusion study were determined by two-tailed paired Student’s t test, followed by two-tailed nonpaired Student’s t test to assess intergroup differences. In the ex vivo perfused working heart experiments, dependent variables were assessed by two-way analysis of variance followed by two-tailed unpaired Student’s t test. Post hoc analysis with Bonferroni’s test was applied in the case of multiple comparisons. All other experiments were analyzed by two-tailed unpaired Student’s t test. All data are presented as mean ± S.E., p values <0.05 were considered statistically significant.

RESULTS

Distribution of GRK2 and GRK3 Immunoreactivities in Mouse Myocardial Tissue—As demonstrated in Fig. 1, GRK2 immunostaining was found predominantly in the endothelial lining of the microvasculature (middle panel), whereas GRK3 immunoreactivity appeared restricted to CMs (right panel). No immunostaining with nonimmune IgG could be detected (Fig. 1, left panel).

Cardiac-specific Expression of GRK3ct in Transgenic Mice—Expression of GRK3ct mRNA (Fig. 2A) could only be detected in myocardial tissue from transgenic mice. Similarly, Western blot analysis revealed expression of GRK3ct only in CMs of Tg-GRK3ct mice (Fig. 2B). The contents of GRK3ct in CMs from the two transgenic lines Tg-GRK3ct(1) and Tg-GRK3ct(2) were similar and estimated by quantitative Western blot analysis to be 1–2 pmol of GRK3ct/mg of CM protein (data not shown; for methodology, see Ref. 3).

Characterization of the Phenotype of GRK3ct Transgenic Mice—Tg-GRK3ct mice had normal appearance, and no differences in body weight of Tg-GRK3ct mice and corresponding NLC mice were detected at any age group. Furthermore, no differences in mortality of Tg-GRK3ct versus NLC mice were detected within the initial observation period of 12 months. No significant differences in heart weight to body weight ratios (mg/g) could be discerned between NLC and Tg-GRK3ct mice (3.54 ± 0.08 versus 3.64 ± 0.10 for NLC versus Tg-GRK3ct, respectively). The latter observation was corroborated by echocardiographic analysis demonstrating similar end-diastolic interventricular septum thickness in Tg-GRK3ct mice and NLC mice (Table 1). However, 3-month-old Tg-GRK3ct mice displayed increased fractional shortening as compared with age-matched NLC mice (Table 1). Histological analysis of myocardial tissue sections (hematoxylin/eosin staining) did not reveal overt differences of myocardial structure at any age group (Fig. 3, A–D). Furthermore, staining with picrosirius red (van Gieson’s solution) as shown in Fig. 3, E and F, did not disclose quantitative differences of extracellular matrix contents as determined by morphometric analysis.

Analysis of Blood Pressure and Heart Rate in Conscious Mice—Analysis of blood pressure in conscious mice by tail-cuff ple-
Tail-cuff plethysmography revealed significantly increased systolic blood pressure in Tg-GRK3ct mice as compared with NLC mice at both 3 and 9 months of age (Table 1). Similar elevations of systolic blood pressure were found in both GRK3ct transgenic lines. Blood pressure was elevated despite no alterations of heart rate in Tg-GRK3ct versus NLC mice. Radiotelemetric recording of blood pressure confirmed elevated blood pressure in Tg-GRK3ct compared with NLC mice, with normal diurnal variations in both groups (Fig. 4). Both diastolic and systolic blood pressures were significantly elevated in Tg-GRK3ct versus NLC mice despite no significant alterations of heart rate and activity (Table 2). Systolic and diastolic blood pressures were

### TABLE 1
Blood pressure, heart rate, and echocardiographic parameters of male age-matched Tg-GRK3ct and NLC mice (3 and 9 months of age)
Values are means ± S.E.; Tg-GRK3ct versus NLC mice. bpm indicates beats/min.

| Parameter                  | 3 months     | 9 months     |
|----------------------------|--------------|--------------|
|                           | NLC          | Tg-GRK3ct    | NLC          | Tg-GRK3ct    |
|                           | n = 12       | n = 11       | n = 40       | n = 39       |
| BP systolic, mm Hg         | 118.3 ± 4.7  | 136.8 ± 3.6* | 119.8 ± 3.1  | 130.8 ± 3.0* |
| BP mean, mm Hg             | 99.7 ± 4.1   | 110.5 ± 3.5* | 95.6 ± 3.3   | 104.9 ± 3.3* |
| Heart rate, bpm            | 574 ± 14     | 527 ± 16     | 523 ± 8      | 529 ± 11     |
| Echocardiography           | n = 26       | n = 32       | n = 19       | n = 20       |
| LVEDD, mm                  | 3.0 ± 0.1    | 3.1 ± 0.1    | 4.2 ± 0.1    | 4.4 ± 0.1    |
| LVESD, mm                  | 1.8 ± 0.1    | 1.7 ± 0.1    | 2.5 ± 0.1    | 2.6 ± 0.1    |
| IVST, mm                   | 0.82 ± 0.02  | 0.82 ± 0.02  | 1.04 ± 0.03  | 1.10 ± 0.02  |
| Fractional shortening, %   | 41.2 ± 1.1   | 46.2 ± 1.2*  | 40.1 ± 1.5   | 41.0 ± 1.6   |

* p < 0.05.

** FIGURE 3. Histochemical analysis of myocardial tissue of Tg-GRK3ct and NLC mice.** A–D, photomicrographs of hematoxylin/eosin-stained myocardial sections of male Tg-GRK3ct mice (A and C) and NLC mice (B and D) at 9 months of age. Panels demonstrate longitudinal (A and B) and transverse sections (C and D) at magnification of ×200 and ×400, respectively. E and F, photomicrographs of picrosirius red (van Gieson’s solution)-stained myocardial sections of Tg-GRK3ct (E) and NLC (F) mice. Magnification, ×200.

** FIGURE 4. Telemetric recording of systolic and diastolic blood pressures in Tg-GRK3ct and NLC mice.** Representative 24-h circadian rhythm of locomotor activity (top panel), heart rate (middle panel), and systolic and diastolic blood pressures (bottom panel) in Tg-GRK3ct (○) and corresponding NLC mice (●) recorded by radiotelemetry. Shaded area represents dark (night) period. The heart rate and blood pressure data are average of each 1-h period and are mean ± S.E. of n = 6 Tg-GRK3ct mice and n = 7 NLC mice.
**TABLE 2**

Radiotelemetric recording of systolic and diastolic blood pressures and heart rate of conscious age-matched male Tg-GRK3ct and NLC mice.

Values are mean ± S.E. of 1-h moving averages during a 12-h dark and light period; Tg-GRK3ct versus NLC mice. BP indicates blood pressure; bpm indicates beats/min; NS indicates nonsignificant.

|          | Tg-GRK3ct, n = 7 | NLC, n = 6 |
|----------|------------------|------------|
| **Dark** |                  |            |
| BP systolic, mm Hg | 131.2 ± 1.2 | 125.4 ± 0.7^a |
| BP diastolic, mm Hg | 101.2 ± 1.2 | 97.6 ± 0.7^a |
| Heart rate, bpm | 567 ± 13 | 560 ± 9 NS |
| **Light** |                  |            |
| BP systolic, mm Hg | 124.5 ± 1.4 | 118.1 ± 0.8^a |
| BP diastolic, mm Hg | 96.1 ± 1.1 | 90.8 ± 0.8^a |
| Heart rate, bpm | 528 ± 9 | 516 ± 7 NS |

^a p < 0.0001.

^p < 0.01.

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**FIGURE 5.** β-Adrenergic receptor-stimulated responses in isolated papillary muscles and CM of Tg-GRK3ct and NLC mice. A–C, concentration-effect curves of isoproterenol-stimulated contractility of isolated papillary muscles from Tg-GRK3ct and NLC control mice. A, semi-logarithmic plot of isoproterenol-stimulated maximal inotropic responses expressed as increase in \((dF/df)_{max}\) as percent above basal \((dF/df)_{max}\). The data are mean ± S.E. of independent observations from Tg-GRK3ct (C, n = 6) and NLC control mice (D, n = 8). C, representative scan of original calibrated force tracing. D and E, isoproterenol-stimulated phospho-Ser-16-phospholamban activities in isolated CMs from Tg-GRK3ct and NLC mice. D, photomicrograph of representative immunoblot of phospho-Ser-16-phospholamban immunoreactivities and total phospholamban immunoreactivities in CMs stimulated in the absence or presence of isoproterenol (1 μmol/liter) for 5 min. Immunoblot of total phospholamban levels in the same samples serves as control of equal loading of the lanes. E, histogram demonstrating densitometric analysis of phospho-phospholamban immunoreactivities. The values (optical density × mm²) are plotted as fold change relative to basal levels and represent mean ± S.E. of independent observation of n = 6 Tg-GRK3ct and n = 6 NLC mice. Cells from each heart were assayed in triplicate.

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Function of Myocardial GRK3

Contractile Responses to Isoproterenol—Contractile responsiveness to β-AR stimulation in isolated papillary muscles from Tg-GRK3ct and NLC mice was assessed by concentration-effect experiments of isoproterenol-stimulated increase in isometric contractility (Fig. 5, A–C). There were no statistically significant differences in basal contractile force between Tg-GRK3ct and NLC mice (mean ± S.E., 215.3 ± 3.3 mN/mm² versus 19.1 ± 2.2 mN/mm², respectively). Furthermore, no statistically significant differences in amplitude of isoproterenol-generated force (∆ maximal isoproterenol-stimulated force versus basal contractile force) in Tg-GRK3ct mice and NLC mice (mean ± S.E., 27.4 ± 5.9 mN/mm² and 24.8 ± 7.6 mN/mm², respectively) were observed. In addition, no statistical differences in maximal inotropic response \((dF/df)_{max}\) nor in sensitivity (EC₅₀) to isoproterenol were observed between the groups \((dF/df)_{max}\) 127.7 ± 10.1% versus 129.8 ± 16% above basal in Tg-GRK3ct versus NLC mice; EC₅₀, 73.8 ± 0.7 mmol/liter versus 74.6 ± 0.7 mmol/liter in Tg-GRK3ct versus NLC), indicating that inhibition of endogenous cardiac myocyte GRK3 does not affect cardiac β-AR responsiveness.

Cardiac Responsiveness to Continuous Infusion of α₁-Adrenergic or β-Adrenergic Receptor Agonists—Myocardial hypertrophy in response to continuous infusion of subpressor doses of the α₁-AR agonist phenylephrine or the β-AR agonist isoproterenol was assessed in Tg-GRK3ct versus NLC mice. Consecutive echocardiographic examinations of individual mice at day 0 and day 14 revealed that the hypertrophic responses to infusion of phenylephrine were enhanced in Tg-GRK3ct mice versus NLC mice, as evidenced both by determination of cardiac mass at day 14 and determination of percent increase of end-diastolic interventricular septum thickness from day 0 to day 14 (p < 0.05; Table 3). No significant changes of external LVEDD in either Tg-GRK3ct hearts or NLC hearts were found after phenylephrine infusion (Table 3). Continuous infusion of isoproterenol also elicited hypertrophic responses, as evidenced by increased cardiac mass and increased left ventricular wall thickness. As opposed to phenyleph-
Tg-GRK3ct mice, assays of signaling responses through several putative receptor substrates in isolated CMs from Tg-GRK3ct and NLC mice were performed.

As demonstrated in Fig. 5, D and E, stimulation of β-ARs with isoproterenol elicited robust phosphorylation of phospholamban (7.5-fold above basal activities). However, the response to isoproterenol was similar in CMs from Tg-GRK3ct mice and NLC mice. Similarly, ET-R- and AT₁ angiotensin receptor-mediated activation of ERK1/2 in CMs of Tg-GRK3ct versus NLC mice did not display significant differences (Fig. 6, C and D). On the other hand, α₁-AR-mediated activation of ERK1/2 as shown in Fig. 6, A and B, displayed robust responses that were significantly enhanced in CMs of Tg-GRK3ct versus NLC mice (fold stimulation 2.5 ± 0.26 versus 1.4 ± 0.17 in CMs from Tg-GRK3ct versus NLC, respectively; p < 0.05).

Myocardial Function of Tg-GRK3ct and NLC Mice as Assessed by in Vivo and ex Vivo Pressure-Volume Relationships—Hemodynamic parameters from simultaneous LV pressure-volume analysis in vivo of Tg-GRK3ct and NLC mice are provided in Table 4. As shown, Tg-GRK3ct hearts demonstrated significantly higher stroke work and cardiac output as compared with NLC mice, indicating increased systolic function. Variables of diastolic function were similar between Tg-GRK3ct and NLC mice. The above data were corroborated by LV pressure-volume analysis of ex vivo perfused, electrically paced (8 Hz) working Tg-GRK3ct and NLC hearts. At filling pressures in the physiological range (7.5 mm Hg; Table 5), Tg-GRK3ct hearts displayed significantly higher stroke work and stroke volume as compared with NLC hearts, corroborating the evidence from the integrated physiologic analysis in vivo of enhanced cardiac contractility and systolic function. However, at elevated supraphysiological filling pressures stroke work was decreased, the time constant of relaxation was prolonged, and LV end-diastolic pressure (LVEDP) was increased in

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Tg-GRK3ct hearts providing evidence of reduced diastolic tolerance (Fig. 7).

DISCUSSION

This study unravels novel cardiac functions of GRK3, a GRK isofrom that has been the subject of little investigation. As demonstrated, transgenic mice with CM-specific expression of the carboxyl-terminal domain of GRK3 displayed elevated blood pressure. The mechanism of the elevated blood pressure was a hyperkinetic LV that generated increased cardiac output and stroke work. This is in contrast to hypertension caused by elevated arterial resistance, which is associated with either maintained or reduced cardiac output.

Hyperkinetic heart, often associated with increased sympathetic nervous activity, is a recognized clinical cause of hypertrophy (20). Consonant with the mechanisms of GRK action, expression of GRK3ct would conceivably attenuate GRK3-mediated desensitization of cardiac α₁-ARs is a plausible mechanism of the hypercontractile phenotype of GRK3ct transgenic mice. Although cardiac hyper-responsiveness to α₁-AR agonist in transgenic GRK3ct mice is consistent with the observed selectivity of GRK3 for the α₁-AR, inhibition of GRK-mediated desensitization of other GPCRs on CMs may also conceivably contribute to the observed phenotype. However, altered responsiveness of other GPCRs known to play a role in the regulation of CM function, for example the AT₁ angiotensin receptor, ET-R, and the β-ARs, could not be discerned. Thus, only α₁-AR-mediated responses appeared to be enhanced by GRK3ct. In this respect, our findings are consistent with a previous report of hybrid transgenic mice in which cardiac-specific overexpression of GRK3 was found to inhibit the hypertrophic phenotype of CM-specific expression of constitutively active α₁B-AR (9). All three α₁-AR subtypes have been identified in mouse CMs, although the major subtypes appear to be the α₁A- and α₁B-AR subtypes. A study of transgenic mice with cardiac-specific overexpression of the α₁A-AR deserves particular notice (21), as these mice display hypercontractile hearts in the absence of myocardial hypertrophy, a finding similar to Tg-GRK3ct mice. Cardiac-specific overexpression of α₁B-AR, on the other hand, was found to induce left ventricular dysfunction and dilated cardiomyopathy in mice evident at 3 months of age, eventually progressing toward overt heart failure at 9 months (22). Interestingly, decreased tolerance to increased work load as reported in this study of Tg-GRK3ct mice was also a characteristic finding in young α₁B-AR-overexpressing mice before onset of dilated cardiomyopathy (23). Although the substrate specificities of GRK3 at α₁A-AR versus α₁B-AR are not known, the phenotypic findings of GRK3ct mice would be consonant with predominant enhancement of α₁A-AR-mediated responses.

One of the major phenotypic findings of this study was that Tg-GRK3ct mice had elevated blood pressure as compared with NLC mice. Increased blood pressure was consistently found in both transgenic lines established and at all age groups investigated. In addi-
tion, elevated blood pressure was also observed in unrestrained, freely moving mice after implantation of telemetric blood pressure transducers in the left carotid artery. Although increased blood pressure may result from either increased systemic peripheral resistance or increased cardiac output, evidence presented in this study strongly points to the latter as the unequivocal cause of elevated blood pressure. Demonstration of cardiac hypercontractility both in in vivo- and in ex vivo-perfused working hearts (the latter excluding potential confounding effects of alterations in peripheral vasculature function) clearly targets the mechanism of elevated blood pressure to the heart. GRK3ct-mediated inhibition of α₁-AR desensitization would elicit hyper-responsiveness to this positively inotropic signaling pathway. Furthermore, this signaling pathway is also documented to elicit hypertrophic responses in CMs, both in vitro and in vivo (24, 25). Thus, hyperkinetic myocardium is a plausible mechanism as far as the putative substrate specificity and mechanism of action of GRK3 are concerned. Transgenic GRK3ct mice did not display enhanced isoproterenol-stimulated phosphorylation of phospholamban nor increased isoproterenol-stimulated GPCR signalling. These arguments have also been maintained in the case of α₁b-ARs and ET-Rs, which all display varying degrees of cardiac dysfunction upon advancing age (25, 26).

In conclusion, transgenic mice with cardiac myocyte-restricted inhibition of endogenous GRK3 display cardiac phenotype with increased blood pressure because of hypercontractile myocardium relying, at least partially, on enhanced α₁-AR signaling. Although the pathophysiological significance of altered GRK3 activity in the human clinical setting is as yet unresolved, the position of the GRK3 gene in a locus on chromosome 22 associated with hypertension is intriguing (27). Studies investigating the role of GRK3 in pathophysiological models of cardiac disease are clearly needed.

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A limitation of this study is that the pleckstrin homology domain situated in the GRK3ct peptide may not only confer inhibition of endogenous GRK3 but also of other PH domain-containing proteins. Conceptually, GRK3ct may sequester significant quantities of Gi/βγ enough to affect Gi/βγ-mediated signalling. These arguments have also been maintained in the case of the GRK2ct (βARK1ct) transgenic mouse. However, the distinct properties of GRK3ct versus GRK2ct at different GPCR in isolated CMs, as well as the distinct phenotypes of GRK3ct transgenic mouse versus GRK2ct (βARK1ct) transgenic mice, do not favor nonspecific sequestration of Gi/βγ. Indeed, the specificities of GRK3ct versus GRK2ct at different GPCRs in CMs were mirrored by similar specificity of the corresponding kinases GRK3 versus GRK2 (3). The latter findings argue against nonspecific alterations of effector activities because of sequestration of Gi/βγ.

Analysis of electrically paced, ex vivo-perfused working hearts from Tg-GRK3ct versus NLC mice revealed subtle cues of LV diastolic dysfunction at increased filling pressures in Tg-GRK3ct hearts. The pathophysiologic significance of such ex vivo findings is uncertain and can only be solved by subjecting Tg-GRK3ct mice to pathophysiological settings with increased preload, for example by generating transgenic hybrids with transgenic models of heart failure. Yet the subtle findings of cardiac dysfunction of Tg-GRK3ct mice at elevated filling pressure would be consistent with other models of enhanced Gi signaling pathways, for example transgenic mice with cardiac-specific overexpression of the α₁b-adrenergic receptor or constitutively active Goq, which all display varying degrees of cardiac dysfunction upon advancing age (25, 26).
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