

# Intracardiac Injection of AdGRK5-NT Reduces Left Ventricular Hypertrophy by Inhibiting NF- $\kappa$ B-Dependent Hypertrophic Gene Expression

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**Abstract**—Several studies underline the role of the transcription factor NF- $\kappa$ B in the development of left cardiac hypertrophy (LVH). We have demonstrated recently that the RGS homology domain within the amino terminus of GRK5 (GRK5-NT) is able to inhibit NF- $\kappa$ B transcription activity and its associated phenotypes. The aim of this study was to evaluate the ability of GRK5-NT to regulate LVH through the inhibition of NF- $\kappa$ B both in vitro and in vivo. In cardiomyoblasts, GRK5-NT inhibits phenylephrine-induced transcription of both NF- $\kappa$ B and atrial natriuretic factor promoters, assessed by luciferase assay, thus confirming a role for this protein in the regulation of cardiomyocyte hypertrophy. In vivo, we explored 2 rat models of LVH, the spontaneously hypertensive rat and the normotensive Wistar Kyoto rat exposed to chronic administration of phenylephrine. Intracardiac injection of an adenovirus encoding for GRK5-NT reduces cardiac mass in spontaneously hypertensive rats and prevents the development of phenylephrine-induced LVH in Wistar Kyoto rats. This associates with inhibition of NF- $\kappa$ B signaling (assessed by NF- $\kappa$ B levels), transcriptional activity and phenotypes (fibrosis and apoptosis). Such phenomenon is independent from hemodynamic changes, because adenovirus encoding for GRK5-NT did not reduce blood pressure levels in spontaneously hypertensive rats or in Wistar Kyoto rats. In conclusion, our study supports the regulation of LVH based on the GRK5-NT inhibition of the NF- $\kappa$ B transduction signaling. (*Hypertension*. 2010;56:696-704.)

**Key Words:** cardiac hypertrophy ■ intracardiac injection ■ spontaneously hypertensive rats ■ NF- $\kappa$ B ■ transcription factors

NF- $\kappa$ B is an ubiquitously expressed transcription factor that modulates the expression of genes involved in the regulation of cell functions, such as survival, apoptosis, growth, division, innate immunity, differentiation, and cellular responses to stress, hypoxia, and ischemia.<sup>1-4</sup> The classic cellular model in which this factor is studied is the immune system for its central role in cytokine production.<sup>4,5</sup> It has been reported recently that NF- $\kappa$ B is relevant in the development of left ventricular hypertrophy (LVH) and remodeling through mechanisms independent from inflammation. NF- $\kappa$ B mediates hypertrophic growth of cardiomyocytes in response to G protein-coupled receptor agonists, including norepinephrine, endothelin 1, and angiotensin II.<sup>6,7</sup> Also, NF- $\kappa$ B inhibition attenuates LVH in different animal models of disease.<sup>8,9</sup> This evidence suggests that NF- $\kappa$ B blockade may be an effective strategy to inhibit LVH and remodeling. The family of G protein-coupled receptor kinases (GRKs) and, in particular GRK2 and GRK5, possesses the ability to bind both NF- $\kappa$ B and its inhibitor, I $\kappa$ B $\alpha$ .<sup>10,11</sup> In particular,

GRK5, by means of its RGS homology (RH) domain within the amino terminus, interacts with I $\kappa$ B $\alpha$  leading to the stabilization and accumulation of the I $\kappa$ B $\alpha$ /NF- $\kappa$ B complex in the nucleus and, consequently, to the inhibition of NF- $\kappa$ B transcriptional activity.<sup>11</sup> This feature of the RH domain of GRK5 leads to the hypothesis that the use of peptides that are designed and engineered on this sequence of the kinase may induce NF- $\kappa$ B inhibition. When applied to cardiac myocytes, this strategy could efficiently reduce hypertrophic responses. To test this hypothesis, we evaluated whether an adenovirus that encodes for the RH domain within the amino terminal of GRK5 (AdGRK5-NT) is able to regulate hypertrophic responses of the cardiac myocyte, both in vitro and in vivo, by means of its ability to inhibit NF- $\kappa$ B transcription activity.

## Methods

### Cell Culture

A cell line of cardiac myoblasts (H9C2) was maintained in culture in DMEM supplemented with 10% FBS at 37°C in 95% air-5% CO<sub>2</sub>.

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## Luciferase Assay

Cells were transfected with plasmid expression vectors containing the luciferase reporter gene linked to 5 repeats of an NF- $\kappa$ B binding site ( $\kappa$ B-Luc) or atrial natriuretic factor (ANF) promoter and infected with  $10^{10}$  pfu/mL of an adenovirus encoding the amino-terminal region of GRK5 (AdGRK5-NT) that comprises the RH domain. AdGRK5-NT was a kind gift of Prof Walter J. Koch (Thomas Jefferson University).<sup>12</sup> Transient transfection was performed using the Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Cells were stimulated with the alpha 1 adrenergic receptor agonist phenylephrine (PE;  $10^{-7}$  M) for 24 hours. Lysates were analyzed using the luciferase assay system with reporter lysis buffer from Promega and measured by liquid scintillation. Luciferase activity was normalized against the coexpressed  $\beta$ -galactosidase activity to overcome variations in transfection efficiency between samples.

## In Vivo Study

Experiments were carried out in accordance with the Federico II University Ethical committee on 12-week-old normotensive Wistar Kyoto (WKY;  $n=13$ , subdivided as follows: untreated  $n=4$ , PE  $n=3$ , PE+AdLac-Z  $n=3$ , and PE+AdGRK5-NT  $n=3$ ) and spontaneously hypertensive (SHR;  $n=12$ , subdivided as follows: untreated  $n=3$ , AdLac-Z  $n=3$ , and AdGRK5-NT  $n=6$ ) male rats (Charles River, Calco, LC, Italy), which had access to water and food ad libitum. The animals were anesthetized by vaporized isoflurane (4%). After the induction of anesthesia, rats were orotracheally intubated, the inhaled concentration of isoflurane was reduced to 1.8%, and lungs were mechanically ventilated (New England Medical Instruments Scientific, Inc) as described previously.<sup>13</sup> The chest was opened under sterile conditions through a right parasternal minithoracotomy to expose the heart. Then, we performed 4 injections (50  $\mu$ L each) of AdGRK5-NT ( $10^{10}$  pfu/mL) or AdLac-Z ( $10^{10}$  pfu/mL), as control, into the cardiac wall (anterior, lateral, posterior, and apical). Finally, the chest wall was quickly closed in layers using 3-0 silk suture, and animals were observed and monitored until recovery. In the WKY group, we implanted under the skin a miniosmotic pump (ALZET 2004) releasing PE (100 mg/kg).<sup>14</sup>

## Echocardiography

Transthoracic echocardiography was performed at days 0, 7, 14, and 28 after surgery using a dedicated small-animal high-resolution imaging system (VeVo 770, Visualsonics, Inc) equipped with a 17.5-MHz transducer (RMV-716). The rats were anesthetized by isoflurane (4%) inhalation and maintained by mask ventilation (isoflurane 2%). The chest was shaved with a depilatory cream (Veet, Reckitt-Benckiser).<sup>15</sup> Left ventricular (LV) end-diastolic and LV end-systolic diameters (LVEDD and LVESD, respectively) were measured at the level of the papillary muscles from the parasternal short-axis view as recommended.<sup>16</sup> Intraventricular septal (IVS) and LV posterior wall thickness (PW) were measured at end diastole. LV fractional shortening (LVFS) was calculated as follows:  $LVFS = (LVEDD - LVESD) / LVEDD \times 100$ . LV ejection fraction (LVEF) was calculated using a built-in software for the VeVo770.<sup>13</sup> LV mass (LVM) was calculated according to the following formula, representing the M-mode cubic method:  $LVM = 1.05 \times [(IVS + LVEDD + LVPW)^3 - (LVEDD)^3]$ ; LVM was corrected by body weight. All of the measurements were averaged on 5 consecutive cardiac cycles and analyzed by 2 experienced investigators blinded to treatment (G.S. and A.A.).

## Blood Pressure Measurement and Evaluation of Cardiac Performance

Blood pressure (BP) was measured as described previously,<sup>17,18</sup> and the record of both systolic (SBP) and diastolic (DBP) BPs was taken using a pressure transducer catheter (Mikro-Tip, Millar Instruments, Inc). The catheters already placed in the ascending aorta were then advanced further into LV to record the maximal and minimal first derivatives of pressure over time (dP/dt maximum and dP/dt minimum), as indices of global cardiac contractility and relaxation.<sup>19</sup> To include the effects of differences of SBP on cardiac contractility, we

also corrected dP/dt maximum by peak systolic pressure (dP/dt maximum/SBP), as described previously.<sup>20</sup> These maneuvers were performed at week 4 after surgery.

## Histology

Four weeks after AdGRK5-NT or AdLac-Z intracardiac injection, the hearts were immersion fixed in 10% buffered paraformaldehyde. The tissues were embedded in paraffin, cut at 5  $\mu$ m, and processed. For Masson's trichrome staining of collagen fibers, slides were stained with Weigert hematoxylin (Sigma-Aldrich) for 10 minutes, rinsed in PBS (Invitrogen), and then stained with Biebrich scarlet-acid fuchsin (Sigma-Aldrich) for 5 minutes. Slides were rinsed in PBS and stained with phosphomolybdic/phosphotungstic acid solution (Sigma-Aldrich) for 5 minutes, then stained with light green (Sigma-Aldrich) for 5 minutes. Slides were rinsed in distilled water, dehydrated with 95% and absolute alcohol, and a coverslip was placed. To evaluate adenovirus expression, paraffined sections were analyzed directly at fluorescent microscopy or the green fluorescent protein (GFP) tag was evaluated by immunohistochemistry, as previously described.<sup>12</sup> For the analysis of cardiomyocytes size, Masson's trichrome staining sections were used.<sup>21</sup> The areas (squared micrometers) of 100 cardiac myocytes per heart were measured with the public domain Java image processing program ImageJ.<sup>22</sup>

## Western Blot

The experiments were performed as described previously.<sup>11</sup> The antibodies anti-I $\kappa$ B $\alpha$ , actin, and histone 3 were from Santa Cruz Biotechnology, Inc; anticaspase 3, NF- $\kappa$ B, p-NF $\kappa$ B (p65), and phospho-cAMP responsive element binding (p-CREB) antibodies were from Cell Signaling Technology. Densitometric analysis was performed using Image Quant software (Molecular Dynamics, Inc). Blots from 3 independent experiments were quantified and corrected for appropriate loading control. Densitometric analysis was adapted to fit a graph scale between 0 and 10 000 arbitrary densitometric units (ADUs). Results are reported as mean  $\pm$  SEM.

## Real-Time PCR

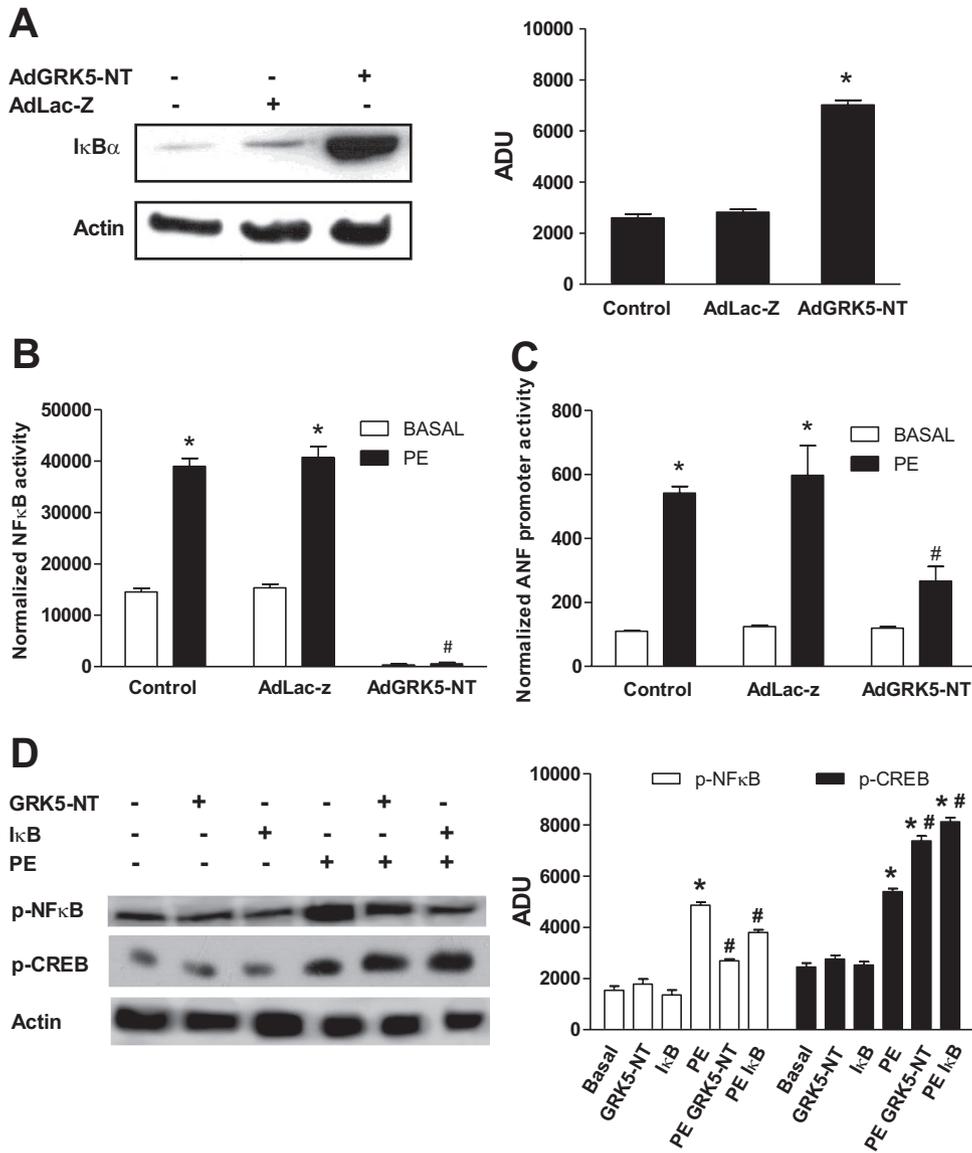
Total RNA was isolated using TRIzol reagent (Invitrogen) and cDNA was synthesized by means of a Thermo-Script RT-PCR System (Invitrogen), following the manufacturer's instruction. After reverse transcription, real-time quantitative PCR was performed with the SYBR Green real-time PCR master mix kit (Applied Biosystems) and quantified by built-in SYBR Green Analysis (Applied Biosystem) on a StepOne instrument (Applied Biosystem). Primers for gene analysis were as follows: ANF: forward 5'cgtgccccgacccacgccagcatggctcc3', reverse 5'ggctccgaggccagcagcagagcctca3'; tumor necrosis factor alpha (TNF- $\alpha$ ): forward 5'cag-gagaaagtcagcctct3', reverse 5'cgataaagggtcagagtaat3'; and 18S: forward 5'gtaacccttgtaaccatt3', reverse 5'catccaatcgtagtagc3'.

## Electrophoretic Mobility-Shift Assay

Nuclear proteins were isolated from heart samples, and NF- $\kappa$ B binding activity was examined by electrophoretic mobility-shift assay, as described previously.<sup>11</sup> For the competition, assay nuclear extracts were incubated with a 50-fold excess of unlabeled oligos for 20 minutes before adding the labeled oligo. Electrophoretic mobility-shift assay for organic cation transporter 1 (OCT-1) binding was performed as a loading control (5'tgtcaatgcaaacctctctct3').

## Statistical Analysis

For each parameter we compared the values observed in the AdGRK5-NT-treated SHRs ( $n=6$ ) with a control SHR group, which was composed of untreated ( $n=3$ ) and AdLacZ-treated ( $n=3$ ) SHRs. This was possible because within these groups there was no statistical difference. For the same reason, in WKY rats we compared the effect of PE between AdGRK5-NT-treated rats ( $n=3$ ) with a control WKY PE group, composed of PE-treated ( $n=3$ ) and PE/AdLac-Z-treated ( $n=3$ ) rats. Both SHRs and PE-treated WKY rats were compared with untreated WKY rats ( $n=4$ ). All values are



**Figure 1.** The effect of AdGRK5-NT on hypertrophy in vitro. A, In H9C2 cardiomyoblasts, GRK5-NT overexpression was induced by adenovirus-mediated gene transfer (AdGRK5-NT), and total IκBα levels were analyzed by Western blot. GRK5-NT causes an increase of IκBα levels. AdLac-Z and noninfected cells were used as controls. The graph shows densitometric analysis after normalization according to Methods from 3 independent experiments expressed in ADUs; \* $P < 0.05$  vs control. B, H9C2s were infected with AdGRK5-NT and stimulated with PE. NF-κB activity was evaluated by luciferase assay and normalized according to the Methods section. PE increases NF-κB activity, and AdGRK5-NT inhibits this activity both in basal condition and after PE stimulation (\* $P < 0.05$  vs basal, # $P < 0.05$  vs control). Results are expressed as mean  $\pm$  SEM from 5 independent experiments. C, H9C2s were infected with AdGRK5-NT and stimulated with PE. ANF promoter activity was evaluated by luciferase assay and normalized as indicated in the Methods section. PE induces ANF promoter activity, and AdGRK5-NT inhibits such response (\* $P < 0.05$  vs Basal, # $P < 0.05$  vs Control). Results are expressed as mean  $\pm$  SEM from 5 independent experiments. D, H9C2s were transfected with either GRK5-NT or IκB and stimulated with PE. Control cells were transfected with an empty plasmid. p65 and CREB phosphorylations were evaluated by Western blot. Overexpression of either GRK5-NT or IκB inhibits NF-κB but not CREB activation. Actin was used as loading control. The graph shows the densitometric analysis after normalization according to methods of 3 independent experiments (\* $P < 0.05$  vs basal, # $P < 0.05$  vs PE).

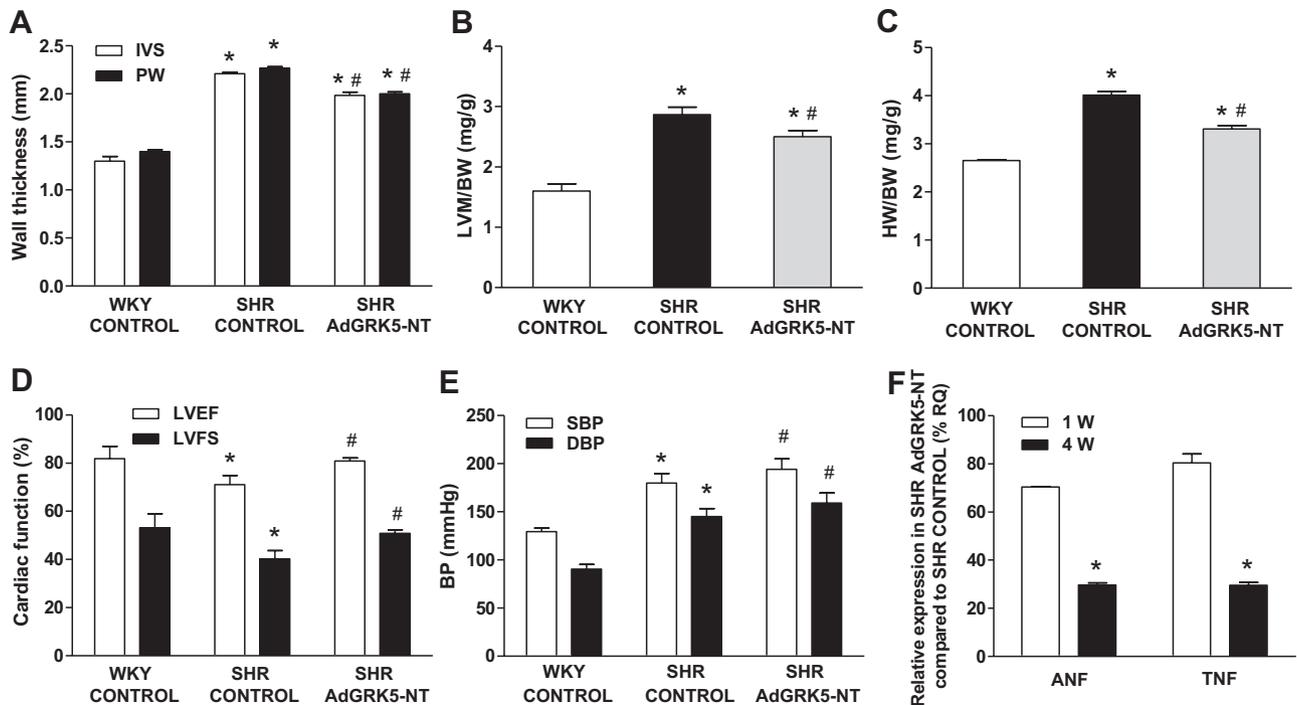
presented as mean  $\pm$  SEM. Two-way ANOVA was performed to compare the different parameters between the different groups. A  $P$  value  $< 0.05$  was considered to be significant. Statistics were computed with GraphPad Prism version 5.01 (GraphPad Software).

## Results

### In Vitro Study

We evaluated in vitro the effect of GRK5-NT, comprising the RH domain, on the regulation of NF-κB signaling and transcriptional activity. In H9C2 cardiomyoblasts, AdGRK5-NT

caused an increase of total IκBα levels (Figure 1A), analyzed by Western blot, as described previously in other cellular types.<sup>11,12</sup> The effect of GRK5-NT on NF-κB activity was evaluated by luciferase assay. PE increased NF-κB transcription activity, and the overexpression of GRK5-NT reduced such effect both in basal conditions and after PE stimulation (Figure 1B), suggesting that NF-κB is induced by hypertrophic stimuli in cardiac cells and that GRK5-NT is able to inhibit such phenomenon. To assess the ability of GRK5-NT



**Figure 2.** The effect of intracardiac injection of AdGRK5-NT in vivo in SHR. A through C, Cardiac hypertrophy was evaluated by echocardiography in 4 untreated WKY rats, 6 AdGRK5-NT SHR, and 6 control SHR, as indicated in the Methods section. Four weeks after injection, a reduction of IVS and PW (A), LVM/BW ratio (B), and HW/BW ratio (C) was found in AdGRK5-NT versus SHR control hearts ( $*P < 0.05$  vs WKY;  $\#P < 0.05$  vs SHR control). D, Interestingly, LVFS and LVEF were also ameliorated in SHR by AdGRK5-NT as compared with SHR control ( $*P < 0.05$  vs WKY;  $\#P < 0.05$  vs SHR control). E, To exclude the involvement of BP values in GRK5-NT-dependent regulation of cardiac hypertrophy, we assessed in treated rats SBP and DBP values. AdGRK5-NT-treated rats had higher SBP and DBP than SHR controls ( $*P < 0.05$  vs WKY;  $\#P < 0.05$  vs SHR control). F, The ability of AdGRK5-NT to reduce LVH was further confirmed on myocardial samples by a reduction of ANF and TNF- $\alpha$  gene expression evaluated by real-time PCR. The values found in AdGRK5-NT-treated LVs were corrected by those found in the SHR control and expressed as the percentage of SHR control at 1 week and 4 weeks from starting treatment ( $*P < 0.05$  vs 1 week). Results are expressed as mean  $\pm$  SEM from 3 to 5 independent experiments.

to modulate hypertrophy in vitro, we performed an ANF promoter-driven luciferase assay. Figure 1C shows that GRK5-NT overexpression inhibits PE-induced ANF promoter activity. These in vitro data suggest that GRK5-NT can regulate the expression of hypertrophic genes by modulating NF- $\kappa$ B activity. To evaluate whether GRK5-NT effect is selective for NF- $\kappa$ B signaling, we assessed its ability to regulate an NF- $\kappa$ B-independent transcription factor, CREB, by Western blot. We overexpressed in cardiomyoblasts GRK5-NT or the main inhibitor of NF- $\kappa$ B, I $\kappa$ B, by transient transfection. Control cells were transfected with an empty plasmid. The overexpression of either GRK5-NT or I $\kappa$ B inhibited NF- $\kappa$ B phosphorylation but did not affect CREB activation both in basal condition and after stimulation with PE (Figure 1D). These results indicate that GRK5-NT selectively inhibits NF- $\kappa$ B signaling and does not affect the signaling of other transcription factors.

## In Vivo Study in SHR

### Echocardiographic Parameters

To confirm the role of GRK5-NT in the regulation of LVH, we analyzed in vivo the SHR, an animal model of hypertension-induced LVH. AdGRK5-NT or AdLac-Z was injected in the cardiac wall of the SHR. LVH was evaluated weekly for 4 weeks by echocardiography. AdGRK5-NT treatment efficiently reduced IVS and PW thickness (Figure

2A), LVM/body weight (BW; Figure 2B) and the heart weight (HW)/BW ratio (Figure 2C). Cardiac function assessed by ultrasound changed accordingly. Indeed, LV ejection fraction and LVFS, which are depressed in the SHR control group with respect to the WKY rat, returned to the levels of normotensive rats in the AdGRK5-NT-treated SHR (Figure 2D). To rule out changes in hemodynamics as a possible mechanism of regulation of LVH, we measured BP in rats at 4 weeks. BP was slightly but significantly increased in AdGRK5-NT-treated rats with respect to controls (Figure 2E). This phenomenon might be attributed to an amelioration of LV dysfunction observed by cardiac ultrasounds and also

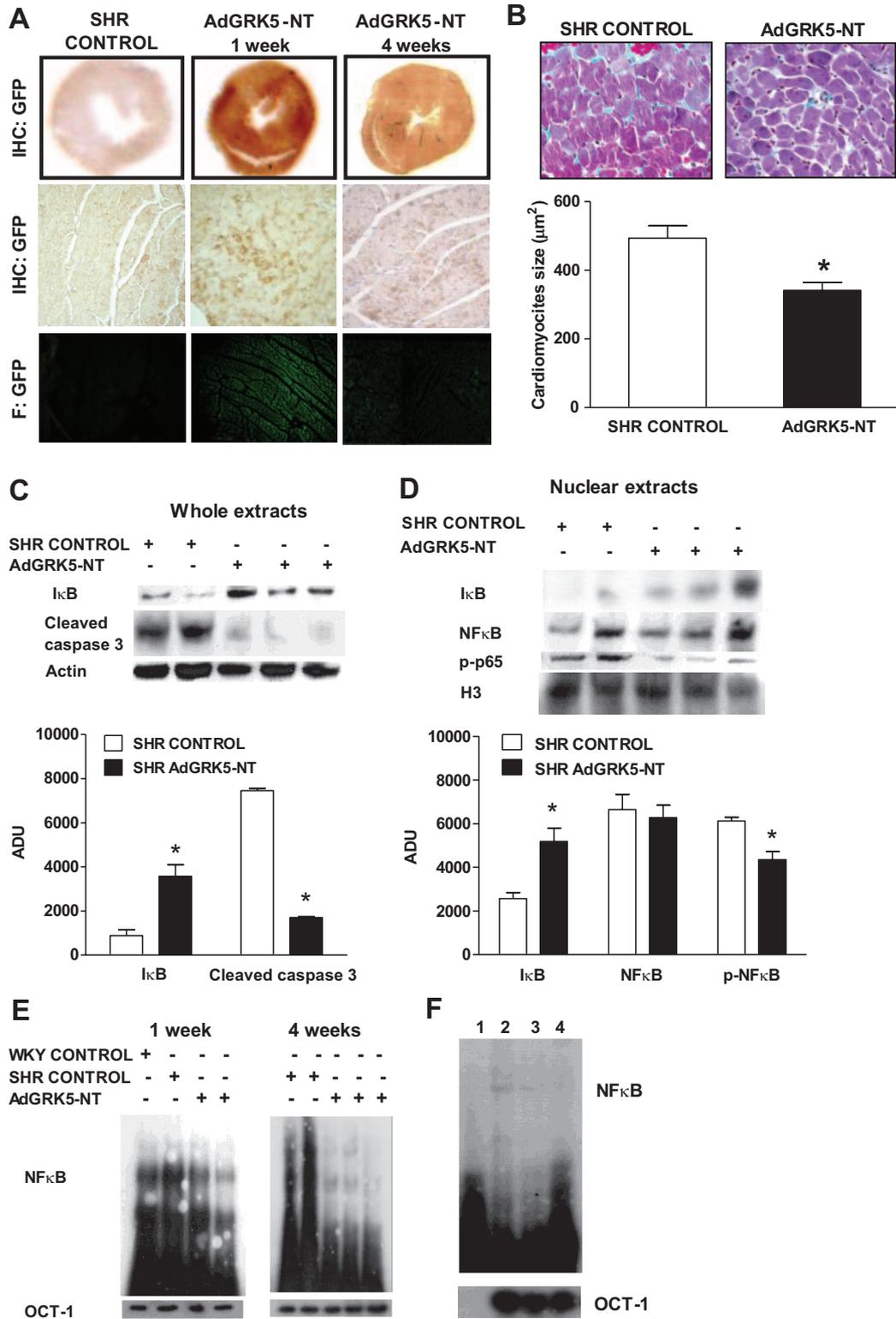
**Table. Cardiac Contractility Assessed In Vivo**

Group	dP/dt Maximum	dP/dt Minimum	dP/dt Maximum/SBP
WKY control (n=4)	+7989 $\pm$ 486	-6728 $\pm$ 452	59.35 $\pm$ 9.84
SHR control (n=6)	+4187 $\pm$ 296*	-4761 $\pm$ 398*	24.02 $\pm$ 5.08*
SHR AdGRK5-NT (n=6)	+7543 $\pm$ 683†	-6754 $\pm$ 554†	41.87 $\pm$ 8.13†

Cardiac contractility was assessed by intra-LV positioning through the carotid artery of a pressure transducer catheter. WKY control indicates untreated WKY rats; SHR control, SHR control group; SHR AdGRK5-NT, SHR hearts infected with the adenovirus encoding for the amino-terminus region of GRK5. Data are mean  $\pm$  SEM.

\* $P < 0.05$  vs WKY.

† $P < 0.05$  vs SHR control.



**Figure 3.** Evaluation of AdGRK5-NT treatment on NF-κB signaling. A, AdGRK5-NT expression in treated hearts was evaluated by GFP expression by both immunohistochemistry (IHC) and fluorescence (F) 1 week and 4 weeks from injection. In the top lane, IHC shows that GFP is homogeneously diffused in the whole heart at 1 week, and its expression is decreased but still present at 4 weeks from intracardiac injection. In the middle lane, panels show an upper magnification of IHC. In the bottom lane, panels show that also by fluorescence the distribution of the GFP localizes to the cardiac myocytes after 1 week and 4 weeks. Images are representative of 3 independent experiments. B, Paraffin-embedded sections of control and treated hearts were stained by trichrome staining to evaluate collagen fibers. AdGRK5-NT treatment reduces fibrosis compared with SHR control. Cardiomyocytes size was measured by means of ImageJ software, and means of areas are showed in the histogram (\* $P < 0.05$  vs SHR control). Images are representative of 3 independent experiments. C, In LV whole extracts, IκBα and cleaved caspase 3 levels were evaluated by Western blot. Four weeks after injection, AdGRK5-NT treatment increases the levels of IκBα and reduces apoptosis with respect to controls (\* $P < 0.05$  vs SHR control).

confirmed by invasive hemodynamics, which showed the hastening of both systolic and diastolic performance: indeed, both positive and negative  $dP/dt$  were ameliorated by AdGRK5-NT (Table). Biochemical analysis confirmed the reduction of LVH. We evaluated the expression of the hypertrophy marker gene ANF and the inflammation marker gene TNF- $\alpha$  by means of real-time PCR in rat hearts. After 1 week, ANF expression was significantly reduced in AdGRK5-NT-treated hearts, and after 4 weeks a further reduction was observed (Figure 2F). A similar pattern is shown for the TNF- $\alpha$  gene expression (Figure 2F).

### Histological Analysis

At 1 or 4 weeks from injection, hearts were harvested, and AdGRK5-NT expression was assessed by histological analysis. GRK5-NT was homogeneously expressed in the heart at 1 week and, although decreased, was still expressed at 4 weeks from injection as assessed by both immunohistochemistry and fluorescence analysis (Figure 3A). LVH caused by chronic hypertension is accompanied by 2 key pathological processes, myocyte hypertrophy, because of increased cell size, and fibrosis. Thus, to confirm the inhibitory effect of GRK5-NT on LVH, we analyzed cardiomyocyte size and fibrosis by immunohistochemistry. Both myocyte size and collagen fiber staining were reduced in AdGRK5-NT versus SHR control hearts (Figure 3B).

### NF- $\kappa$ B Signaling

We have shown previously that GRK5-NT causes cellular and nuclear accumulation of the negative regulator of NF- $\kappa$ B signaling, I $\kappa$ B.<sup>11</sup> To verify the ability of AdGRK5-NT to induce the same phenomenon also in the hypertrophic SHR heart, we analyzed I $\kappa$ B $\alpha$  levels both in whole and nuclear extracts by Western blot. AdGRK5-NT increased cellular (Figure 3C) and nuclear (Figure 3D) I $\kappa$ B $\alpha$  levels with respect to the SHR control group. The regulation of apoptosis is an event under the control of NF- $\kappa$ B activity. Although NF- $\kappa$ B is commonly found to be cytoprotective, there are a number of instances, depending on stimuli and cell context, where it is proapoptotic. LVH is such an example.<sup>23</sup> Thus, we evaluated apoptosis in hypertrophic hearts by analyzing cleaved caspase 3 levels. In agreement with the inhibition of NF- $\kappa$ B, we found that AdGRK5-NT reduced apoptosis in hypertrophic SHR hearts (Figure 3C). To demonstrate that I $\kappa$ B accumulation results in the inhibition of NF- $\kappa$ B, we assessed NF- $\kappa$ B nuclear localization by Western blot. NF- $\kappa$ B levels in the nucleus were increased both in SHR control and AdGRK5-NT hearts, but the activated form of NF- $\kappa$ B (phosphorylated p65) was reduced only in the latter, suggesting that NF- $\kappa$ B accumulates in the nucleus with I $\kappa$ B $\alpha$  but cannot activate gene transcription. To confirm such a result, we

evaluated NF- $\kappa$ B transcriptional activity by electrophoretic mobility-shift assay. NF- $\kappa$ B activity was enhanced in hypertrophic SHR hearts with respect to nonhypertrophic WKY rats. AdGRK5-NT, on the contrary, reduces NF- $\kappa$ B activity at 1 week from starting treatment, and prolonged treatments further decreased such activity (Figure 3E). To assess the specificity of NF- $\kappa$ B binding, a competition assay was performed. The DNA binding of NF- $\kappa$ B was reduced by 10-fold excess of unlabeled oligos, and it was completely inhibited by 20-fold excess (Figure 3F).

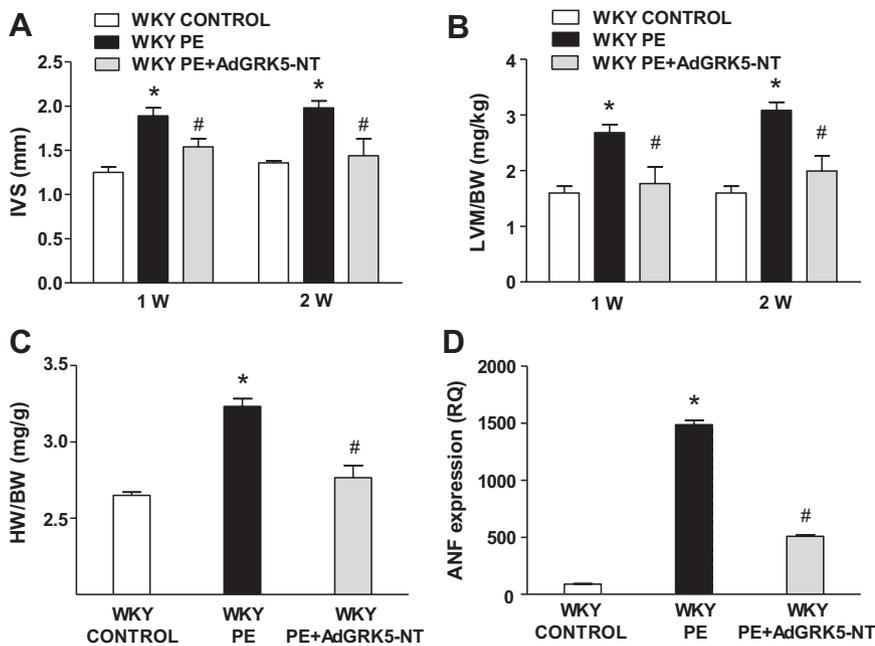
### In Vivo Study in WKY Rats

We then verified whether AdGRK5-NT was able to prevent the development of LVH. To this aim, we chronically (14 days) injected PE in WKY rats infected previously with AdGRK5-NT (n=3) or AdLac-Z (n=3) in the left ventricle. As a control we used untreated WKY rats (n=4). Cardiac remodeling was evaluated by echocardiographic analysis once a week for 2 weeks. AdGRK5-NT significantly prevented the development of PE-induced LVH, because we found a smaller increase of IVS (Figure 4A), LVM/BW (Figure 4B), and HW/BW (Figure 4C). This finding is associated with a reduction of ANF gene expression, evaluated by real-time PCR (Figure 4D).

### Discussion

In the present study we offer the compelling evidence that the RH domain within GRK5-NT inhibits LVH in 2 animal models of the disease. This effect is attributed to the sterical inhibition of NF- $\kappa$ B activity in the cardiac myocyte through means of the stabilization of I $\kappa$ B $\alpha$ . Indeed, GRK5-NT contains the kinase RH domain, which is sufficient for GRK5-NT effects<sup>12</sup> but lacks both the catalytic domain and the nuclear localization sequence of GRK5. Thus, this mutant cannot participate in the regulation of hypertrophy that depends on GRK5-mediated phosphorylative events.<sup>24</sup> Rather, we show NF- $\kappa$ B inhibition induced by I $\kappa$ B $\alpha$  accumulation in cardiac myocytes, a mechanism demonstrated previously in other cell types.<sup>11,12</sup> In the in vivo study, we used a genetic model of LVH, the SHR, in which LVH is sustained by high BP levels. It has been described that the inhibition of NF- $\kappa$ B reduces LVH in SHRs in a BP-independent manner.<sup>25</sup> Indeed, the antihypertensive drug hydralazine has no effect on cardiac mass or NF- $\kappa$ B activity in hypertrophic SHRs.<sup>25</sup> On the contrary, the pharmacological inhibition of NF- $\kappa$ B with pyrrolidine dithiocarbamate or the treatment with an angiotensin-converting enzyme inhibitor (captopril) significantly reduces heart size and inhibits NF- $\kappa$ B activity.<sup>25</sup> Our present data are, therefore, in agreement with the literature, because we found that GRK5-NT is able to inhibit NF- $\kappa$ B activity

**Figure 3 (Continued).** Graph summarizes normalized ADUs from 3 independent experiments. D, In nuclear extracts, 4 weeks after injection AdGRK5-NT causes accumulation of I $\kappa$ B $\alpha$ . NF- $\kappa$ B levels in the nucleus are increased both in controls and treated hearts, but the phosphorylated, active form of p65 is reduced in GRK5-NT-treated hearts (\* $P$ <0.05 vs SHR control). Histone 3 (H3) was used as loading control. Graph summarizes normalized ADUs from 3 independent experiments. E, NF- $\kappa$ B activity was evaluated by electrophoretic mobility-shift assay in control and treated hearts. NF- $\kappa$ B activity is enhanced in hypertrophic SHR hearts with respect to nonhypertrophic WKY rats; GRK5-NT treatment reduces such activity in a time-dependent manner. Organic cation transporter 1 was used as the loading control. Images are representative of 3 independent experiments. F, A competition assay was performed adding unlabeled oligos. A 10-fold excess of unlabeled oligos reduced and a 20-fold excess completely inhibited binding of NF- $\kappa$ B. Lane (Ln) 1, empty; Ln 2, no unlabeled oligos; Ln 3, 10-fold excess of unlabeled oligos; Ln 4, 20-fold excess of unlabeled oligo. Images are representative of 3 independent experiments.



**Figure 4.** The effect of AdGRK5-NT treatment on PE-induced cardiac hypertrophy was evaluated in WKY rats. We studied a group of WKY PE+AdGRK5-NT-treated rats ( $n=3$ ) and a group of WKY PE-treated rats ( $n=6$ ), as indicated in the Methods section. As control we used untreated WKY ( $n=4$ ). After 2 weeks, AdGRK5-NT treatment causes a reduction of IVS (A), LVM/BW ratio (B), and HW/BW ratio (C). Accordingly, we found a reduction of ANF gene expression evaluated by real-time PCR (D) (\* $P<0.05$  vs WKY control; # $P<0.05$  vs WKY PE).

and, consequently, to reduce LVH without reducing BP levels. Indeed, GRK5-NT effect on LVH in SHR does not associate with a reduction but rather causes a slight increase of BP levels. Although we do not have a direct proof of it, we believe that the increased BP is the result of the ameliorated cardiac contractility, as shown by dP/dt maximum and LV ejection fraction, which occurs without modification of total peripheral resistance.<sup>26–28</sup> Total peripheral resistances are increased by the hypertensive status and are a hallmark of SHRs, and our treatment, being localized in the heart, cannot affect them. Therefore, increased systolic function in the presence of unchanged total peripheral resistances, results in increased systolic BP. In turn, the ameliorated cardiac contractility can be the result of reduced cardiac fibrosis and hastened diastolic dysfunction typical of the SHR.<sup>19,28</sup> As a consequence, GRK5-NT-induced inhibition of LVH does not depend on systemic hemodynamics but rather is an effect of myocyte biology and intracellular signal transduction. We also performed a confirmatory *in vivo* study in a different animal model, the WKY rats, in which LVH was induced pharmacologically by means of chronic PE administration. This model differs from the SHR because it is not genetically determined, and the phenotype is acutely induced by treatment. Also in this model, AdGRK5-NT is able to reduce the development of LVH by inhibiting NF- $\kappa$ B activity. Thus, our data demonstrate that GRK5-NT exerts its effect on cardiac LVH independently from the animal model and that NF- $\kappa$ B activation is an intrinsic determinant of the cardiac hypertrophic response. We also showed that GRK5-NT inhibition of LVH is attributed to the inhibition of apoptosis and fibrosis that characterize the hypertrophic phenotype. This could appear in contrast with our previous data, when we demonstrated that GRK5-NT-dependent inhibition of NF $\kappa$ B causes an increase of apoptosis both in endothelial and tumor cells.<sup>11,12</sup> In fact, NF- $\kappa$ B has contradictory effects on apoptosis and cell survival that largely depend on cell type.<sup>29</sup> In

particular, NF- $\kappa$ B is antiapoptotic in tumor cells<sup>30</sup> and proapoptotic in cardiac cells.<sup>23</sup> Therefore, the different reported effects of GRK5-NT on apoptosis can be ascribed to differences in cell types.<sup>29</sup>

LVH is a complex event that depends on the activation of different signaling pathways.<sup>31</sup> Several mechanisms are involved in this response, including cardiomyocyte stretching, as well as circulating neurohormones, such as catecholamines, endothelin 1, angiotensin II, insulin and growth factors, and cytokines released locally by the myocardial cells.<sup>32–34</sup> These, in turn, activate second messengers that regulate nuclear transcription factors activity (NF- $\kappa$ B, CREB, NFAT, and GATA-4) modifying the expression of hypertrophic genes.<sup>35,36</sup> Our data support the relevance of NF- $\kappa$ B to the hypertrophic phenotype. This further underlines the general application of GRK5-NT treatment versus a transduction signaling that is involved not only in LVH but also in other physiological and pathological conditions in different tissues. From our data, we cannot exclude the possibility that GRK5-NT could have multiple cellular targets and that its effect on LVH could also involve other signaling pathways.<sup>37</sup> We, therefore, tested the effects of GRK5-NT on CREB, a transcription factor activated by cAMP signaling that is involved in the development of LVH by enhancing the expression of pivotal genes for efficient oxidative capacity and resistance to apoptosis.<sup>38</sup> Because AdGRK5-NT does not change on CREB activation, we can rule out that the effect on NF- $\kappa$ B is attributable to a nonspecific inhibition of nuclear transcriptional activity. Rather, AdGRK5-NT is selective in its inhibition of NF- $\kappa$ B.

For the *in vivo* study, we used the intracardiac injection of the adenovirus AdGRK5-NT. We opted for adenovirus-mediated gene delivery to obtain GRK5-NT expression in the heart, because the adenovirus homogeneously diffuses throughout the treated tissue, also far from directly injected sites,<sup>39</sup> and is maintained for as long as 30 days.<sup>40</sup> One way

of directly obtaining cardiac delivery is the intracoronary way,<sup>13</sup> but it causes high mortality and is not easily achievable in small animals, such as the rat. Otherwise, the systemic administration through the intravenous route does not guarantee a good expression of a protein in the heart, because, in large part, the adenovirus targets other organs and tissues. Moreover, systemic treatment could be toxic for animals. The advantage of the intracardiac injection directly into the wall is that this maneuver is less toxic for the whole body and leads to a selective localization of the adenovirus throughout the heart to obtain a specific overexpression of the encoded protein in the cardiac tissue.

In conclusion, our study demonstrates the ability of GRK5-NT to reduce myocardial NF- $\kappa$ B activity and LVH. Moreover, our data suggest the efficiency of such a mechanism of regulation of LVH based on the inhibition of a specific transduction signaling that is independent from BP overload, the main stimulus for LVH.

### Perspectives

Our data suggest NF- $\kappa$ B as a target for LVH. This inhibition could be used in combination with BP-lowering strategies to better achieve the target of organ protection in hypertensive states. Furthermore, it is possible that, in other conditions with reduced cardiac performance, such as heart failure, it may be useful to increase cardiac contractility and reduce adverse remodeling. Further studies need to confirm this hypothesis.

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### Disclosures

None.

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**Intracardiac Injection of AdGRK5-NT Reduces Left Ventricular Hypertrophy by Inhibiting NF- $\kappa$ B-Dependent Hypertrophic Gene Expression**  
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