Endothelial G Protein–Coupled Receptor Kinase 2 Regulates Vascular Homeostasis Through the Control of Free Radical Oxygen Species

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Objective—The role of endothelial G protein–coupled receptor kinase 2 (GRK2) was investigated in mice with selective deletion of the kinase in the endothelium (Tie2-CRE/GRK2$^{fl/fl}$).

Approach and Results—Aortas from Tie2-CRE/GRK2$^{fl/fl}$ presented functional and structural alterations as compared with control GRK2$^{fl/fl}$ mice. In particular, vasoconstriction was blunted to different agonists, and collagen and elastic rearrangement and macrophage infiltration were observed. In primary cultured endothelial cells deficient for GRK2, mitochondrial reactive oxygen species was increased, leading to expression of cytokines. Chronic treatment with a reactive oxygen species scavenger in mice corrected the vascular phenotype by recovering vasoconstriction, structural abnormalities, and reducing macrophage infiltration.

Conclusions—These results demonstrate that GRK2 removal compromises vascular phenotype and integrity by increasing endothelial reactive oxygen species production. **(*Arterioscler Thromb Vasc Biol*. 2013;33:2415-2424.)**

Key Words: endothelial cells ■ G protein-coupled receptor kinases ■ mitochondria ■ vascular medicine

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Endothelium is a cellular monolayer separating blood from parenchymal tissue. Despite this anatomic simplicity, its physiological complexity allows the control of several vascular functions, including permeability, tone, and angiogenesis, by integrating the multiple stimuli from the bloodstream and elaborating a response through release of specific factors. Adrenergic mechanisms controlling endothelial function have been studied during these years, culminating in the observation that endothelium produces catecholamines[1] through which to regulate specific functions, such as vascular tone and angiogenesis,[2] endothelial nitric oxide synthase (eNOS) activity,[3] and intracellular specific pathways by activation of β adrenergic receptor (βAR) signaling.[4,5] βAR signaling is in turn heavily regulated, as verified in other tissues, and, in particular, βARs undergo G protein-coupled receptor kinase (GRK)–mediated desensitization.[6–8] Interestingly, GRK2 has also been demonstrated to regulate eNOS phosphorylation in AKT-dependent manner.[9] The role of GRK2 in physiopathology has been mainly studied at cardiac level, where both biochemical and transgenic studies have demonstrated that during congestive heart failure[10–12] its increased levels contribute to the progression of the disease. Recent studies have also shown that increased GRK2 levels contribute to the endothelial dysfunction and defective vasorelaxation observed in transgenic model of type II diabetes mellitus.[13] Incoming evidence, however, indicates that GRK2 is able to regulate different molecules and cellular functions, such as inflammation, cellular proliferation, glucose uptake, and metabolism, by kinase-dependent and -independent mechanisms,[14–16] raising questions about the multiple roles played in the cell. In particular, a recent observation shows that GRK2 localizes at mitochondria level, where it plays a protective role during hypoxic/ischemic conditions by promoting mitochondria biogenesis and increasing ATP production.[17] Given the importance of mitochondria increased reactive oxygen species (ROS) and ATP production in endothelial dysfunction,[18,19] the mitochondrial effects of GRK2 for endothelium appear to be particularly relevant. Hereby, to ascertain the function of endothelial GRK2, we used transgenic mice with selective deletion of GRK2 gene in the endothelium. Our results propose a protective role of GRK2 in the endothelium with effects on inflammation and macrophages and lipids infiltration in the vascular wall.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

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Results

GRK2 Endothelial Removal Affects Aorta's Receptor-Dependent and -Independent Vasoconstriction

First of all, we confirmed by reverse transcriptase polymerase chain reaction that Tie2-CRE/GRK2$^{fl/fl}$ aortas present selective endothelial GRK2 removal. As shown in Figure IA in the online-only Data Supplement, GRK2 expression is reduced in Tie2-CRE/GRK2$^{fl/fl}$ aorta compared with GRK2$^{fl/fl}$, which is indicative of GRK2 deletion in the endothelium and presence in the other layers (media and adventitia). This result was confirmed by the finding of no difference in GRK2 expression, after removing endothelium from both GRK2$^{fl/fl}$ and Tie2-CRE/GRK2$^{fl/fl}$.

In our previous studies, to evaluate vasorelaxation to the different agonists, aortas were preconstricted with increasing concentration of the α1 adrenergic agonist phenylephrine. However, this was the case only for GRK2$^{fl/fl}$, whereas Tie2-CRE/GRK2$^{fl/fl}$ showed an impaired response to this drug (Figure 1A). To further evaluate this result, we used other different compounds known to increase vascular tone such as Ser, Oxy, and KCl; this last one allows the evaluation of receptor-independent vasoconstriction. As shown in Figure 1B to 1D, all these agonists present a defective vasoconstriction as compared with GRK2$^{fl/fl}$ mice. To find an explanation for these results, in isolated GRK2$^{fl/fl}$ endothelial cells (ECs), we evaluated the possibility that GRK2 may coimmunoprecipitate with Akt or eNOS, as suggested by previous studies[9,13] that indicate the ability of GRK2 to affect the Akt/eNOS axle. Therefore, GRK2 removal may potentially enhance vasodilation through increased eNOS activation and inhibit vasoconstriction. As shown in Figure IB in the online-only Data Supplement, none between Akt and eNOS appeared to directly or indirectly interact with GRK2. Moreover, GRK2 removal by infection adenovirus encoding for CRE recombinase (Figure IC in the online-only Data Supplement) does not modify levels of eNOS phosphorylation (Figure ID in the online-only Data Supplement). Finally, we also evaluated the effects of endothelial removal on vasoconstriction. Effective endothelial removal was confirmed by observing paradox vasoconstriction to acetylcholine (data not shown). With this maneuver, aortas displayed a partial recovery of vasoconstriction, which, however, was not completely restored as compared with GRK2$^{fl/fl}$.

All together, these observations indicate that the defective vasoconstriction observed in Tie2-CRE/GRK2$^{fl/fl}$ mice is not related to a functional alterations involving eNOS activation but rather to structural abnormalities, leading to the need of histological studies on aorta sections.

Increased Vascular Inflammation and Tissue Degeneration in Tie2-CRE/GRK2$^{fl/fl}$ Mice

The tunica media of the aorta consists of regular concentric elastic lamellae, between which are smooth muscle cells, collagen, and elastic fibers. Histological studies by Masson trichrome showed that aorta from GRK2$^{fl/fl}$ mice, used as

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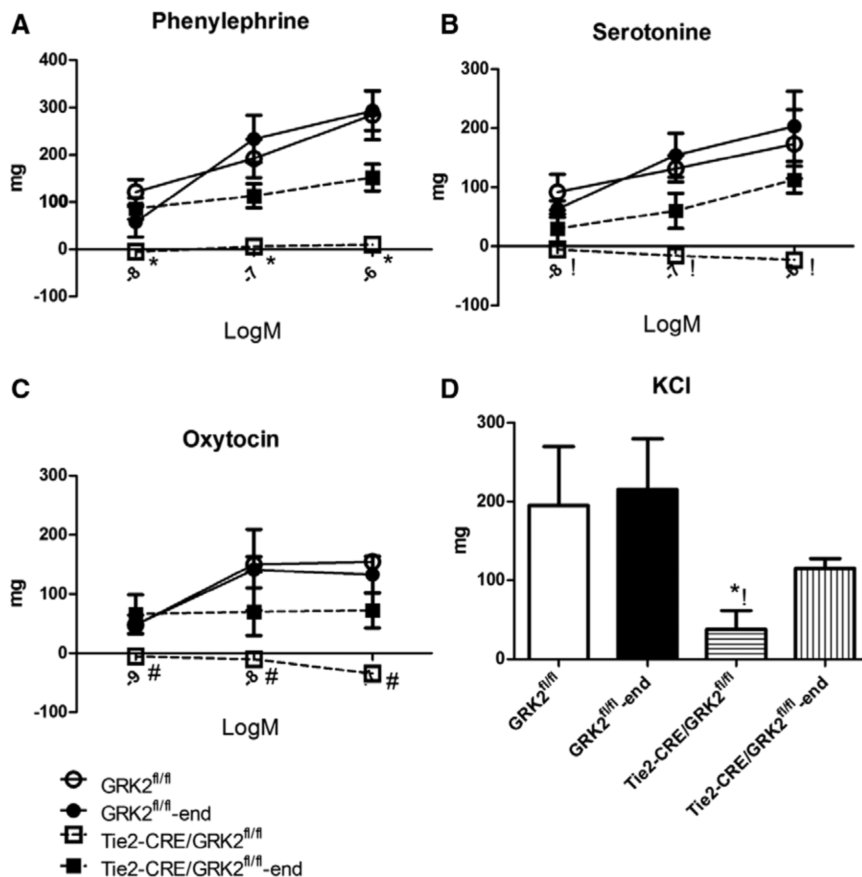

Figure 1. A–D, Effect of selective endothelial G protein–coupled receptor kinase 2 (GRK2) deletion on aortic vasoconstriction. Vasocontriction was tested in response to α_1 adrenergic agonist phenylepherine (10^{-8} to 10^{-6} mol/L), 5-hydroxytryptamine receptor 1 agonist serotonine (10^{-8} to 10^{-6} mol/L), oxytocin (10^{-7} to 10^{-5} mol/L), and to potassium chloride (KCl; 12.5 mmol/L). Defective vasoconstriction can be observed for all the above agonists in Tie2-CRE/GRK2$^{fl/fl}$ (*, !, #, *! vs GRK2$^{fl/fl}$; $P<0.05$; 2-way ANOVA). Endothelium removal from Tie2-CRE/GRK2$^{fl/fl}$ aortas (Tie2-CRE/GRK2$^{fl/fl}$-end) produces partial and not significant recover after endothelium removal of vasoconstriction to the above drugs (vs Tie2-CRE/GRK2$^{fl/fl}$; ns).

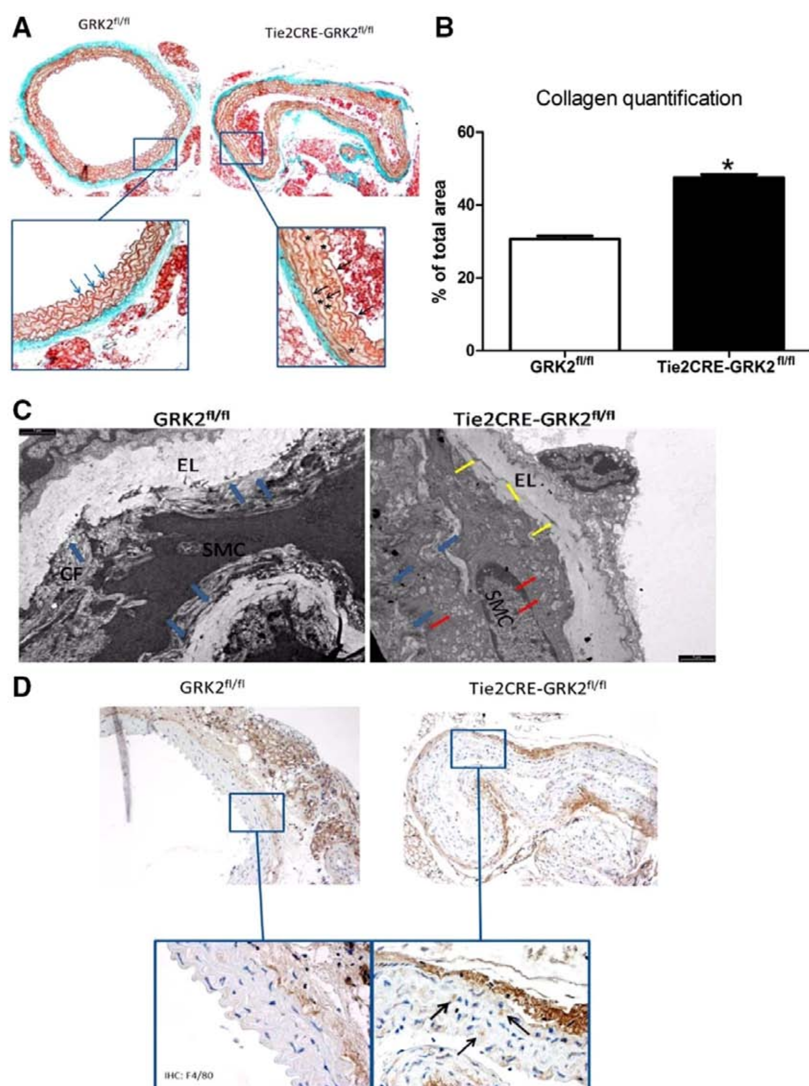

Figure 2. G protein–coupled receptor kinase 2 (GRK2) endothelial deletion induces an inflammatory phenotype in the aorta of transgenic mice. **A**, Masson trichrome staining of thoracic aorta from GRK2$^{fl/fl}$ and Tie2-CRE/GRK2$^{fl/fl}$. GRK2$^{fl/fl}$ aorta shows conserved morphology with wavy elastic lamina and lamellae (blue arrows). This latter appears collapsed with straight elastic lamina and lamellae (black arrows) and increased interlaminae matrix deposition (black star), which is quantified in **B** as a percentage of total aortic wall (*vs GRK2$^{fl/fl}$; *P*<0.05). **C**, Transmission electron microscopy on GRK2$^{fl/fl}$ and Tie2-CRE/GRK2$^{fl/fl}$ aortas. In GRK2$^{fl/fl}$ aorta, elastic lamina (EL) is thick and compact with tightly adhering bundles of collagen fibers (CF). Matrix between laminae is mostly occupied by smooth muscle cells (SMCs). Tie2-CRE/GRK2$^{fl/fl}$ has a thinner EL (yellow arrows) with detached CF that appears mostly fragmented (blue arrows). Pycnotic SMC nucleus and presence of matrix vesicles can be also noted. **D**, Immunohistochemistry analysis performed on thoracic aorta. Detection of the F4/80 macrophage fragment reveals an extensive macrophage infiltration in Tie2-CRE/GRK2$^{fl/fl}$ (black arrows) aorta as compared with GRK2$^{fl/fl}$ aorta.

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control, has a normal tunica media with intact elastic lamellae characterized by their peculiar wavy look (Figure 2A, blue arrows). Instead, Tie2-CRE/GRK2$^{fl/fl}$ aorta is collapsed and the internal elastic lamina and the elastic lamellae within the tunica media are mostly stretched (Figure 2A, black arrows). Also, matrix between elastic lamellae is increased (black stars), indicating an altered deposition of collagen (Figure 2B). To deeply investigate the modifications in Tie2-CRE/GRK2$^{fl/fl}$ aorta, transmission electron microscopy analysis was performed. Control GRK2$^{fl/fl}$ aorta has a preserved media structure, which is composed of smooth muscle cells with normal appearance and parallel orientation with respect to the thick and compact elastic lamina and lamellae, which confers the ability to equally transmit contractile force to the entire vessel. Moreover, complex bundles of collagen fibers (CF) tightly adhere to the lamellae (Figure 2C, blue arrows). In Tie2-CRE/GRK2$^{fl/fl}$ aortas, instead, we observed presence of matrix vesicles and degeneration and altered deposition of CFs (Figure 2C, blue arrows), which appear fragmented and mostly detached from the elastic lamellae. These latter also are thinner and less compact than in controls (Figure 2C, yellow arrows). Noteworthy, smooth muscle cells display a pyknotic nucleus with marked chromatin condensation and peripheral margination, indicating presence of an apoptotic process. This morphology prompted us to evaluate presence of inflammation and, in particular, macrophage infiltration into the vessels. Immunohistochemistry revealed an extensive infiltration of macrophages in Tie2-CRE/GRK2$^{fl/fl}$ aortas with respect to controls (Figure 2D, black arrows), which may be responsible for the observed alterations of the media. Indeed, we found increased mRNA and protein expression of metalloproteinase 2 and 9 (MMP2-9), which are produced by macrophages and known to be involved in collagen matrix degradation (Figure 3A and 3B). Furthermore, the macrophage chemoattractant protein-1 resulted to be increased in Tie2-CRE/GRK2$^{fl/fl}$ with respect to control (Figure 3A and 3B). Because the alterations observed in vascular endothelium of the Tie2-CRE/GRK2$^{fl/fl}$ may be extended to other organ and tissues, we performed histological analysis by Masson and immunohistochemistry on lungs from Tie2-CRE/GRK2$^{fl/fl}$ and control mice. We found collagen degradation (Figure IIIA in the online-only Data Supplement, black arrows) and macrophage infiltration (Figure IIIB in the online-only Data Supplement, blue arrows) in Tie2-CRE/GRK2$^{fl/fl}$ mice but not in GRK2$^{fl/fl}$.

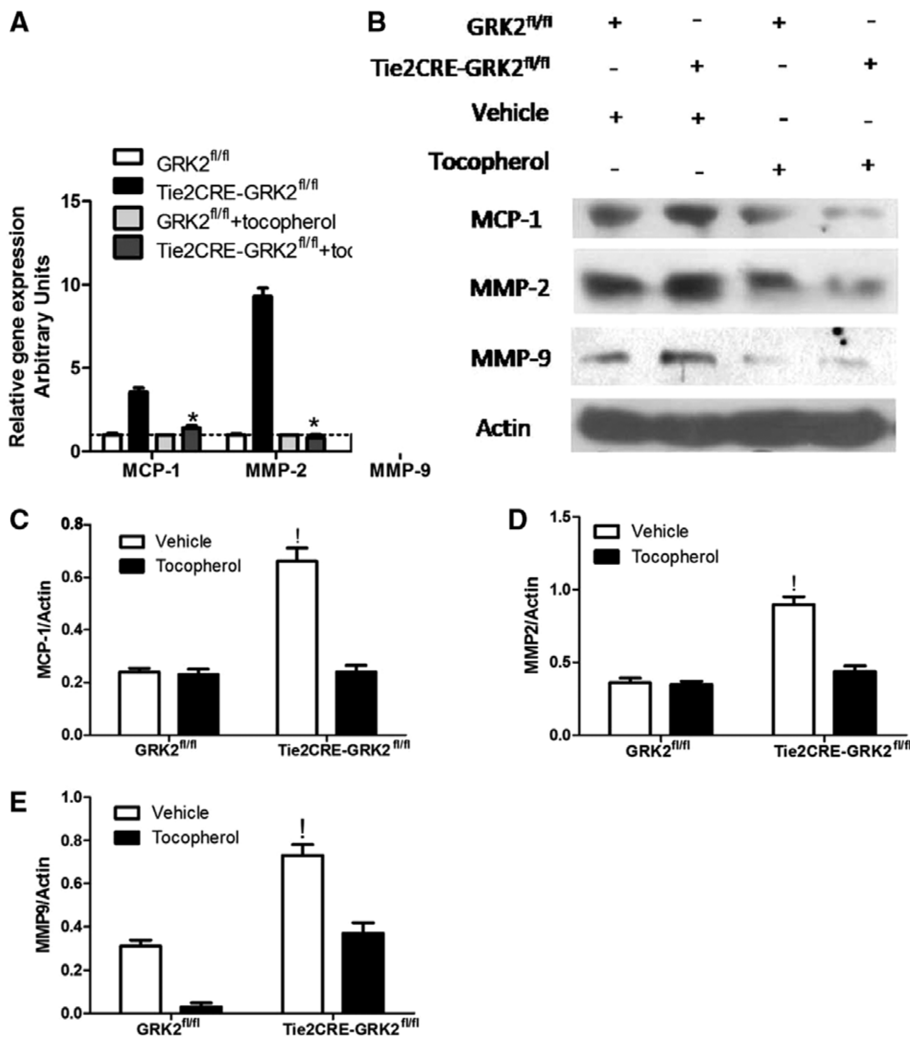

Figure 3. G protein–coupled receptor kinase 2 (GRK2) endothelial deletion increases mRNA expression and level of inflammatory proteins. **A**, mRNA levels of the cytokine macrophage chemoattractant protein-1 (MCP-1) and metalloproteinase-2 and -9 evaluated by reverse transcriptase polymerase chain reaction on thoracic aorta samples. Increased expression of MCP-1, metalloproteinase (MMP)-2, and MMP-9 can be observed in Tie2-CRE/GRK2$^{fl/fl}$ as compared with GRK2$^{fl/fl}$ samples (*P<0.01 vs GRK2$^{fl/fl}$), which are significantly reduced by tocopherol chronic treatment. Data are expressed as relative gene expression. **B**–**E**, Protein levels of MCP-1, MMP-2, and MMP-9 were evaluated by Western blot on total aortas lysates. Increased protein levels of MCP-1, MMP-2, and MMP-9 are observed in Tie2-CRE/GRK2$^{fl/fl}$ as compared with GRK2$^{fl/fl}$ samples (!P<0.01 vs GRK2$^{fl/fl}$), which are significantly reduced by tocopherol as shown by representative images in **B** and densitometry in **C**–**E**.

Endothelial cell and macrophages have the same myeloid origin, which means that during embryogenesis the Tie2 promoter could be activated in both cell lines. To ascertain that *grk2* gene is not removed also in leukocytes, we isolated macrophages of Tie2-CRE/GRK2$^{fl/fl}$ and GRK2$^{fl/fl}$ mice from peritoneum and performed Western blot analysis to measure GRK2 protein expression. We did not observe any significant difference in GRK2 expression in macrophages from the 2 groups of animals (data not shown).

GRK2 Gene Deletion From Endothelial Cells Induces Increased ROS Production

The above data indicate that selective knockout of GRK2 in the endothelium produces a dramatic modification of the phenotype of these transgenic mice, characterized by vascular inflammation and defective vascular reactivity. To identify the specific alteration of the endothelial cells, we performed in vitro studies using intact mitochondria isolated from GRK2$^{fl/fl}$ EC and bovine aortic endothelial cells (BAEC), respectively treated with adenovirus encoding for CRE recombinase and small interfering RNA for bovine GRK2, to induce GRK2 knockdown (Figure IC and IE in the online-only Data Supplement). In particular, we evaluated mitochondrial ROS production basing on 2 evidences: the recent discovery of GRK2 localization in mitochondria, where it exerts a positive effect on ATP production and biogenesis,[17] and the known role of endothelial ROS in physiopathology of vascular diseases.[20] GRK2 removal from EC by adenovirus encoding for CRE recombinase infection produces a typical senescent morphology of the cell (Figure IIC in the online-only Data Supplement), which may be related to an altered ROS production. Indeed, we observed in both GRK2$^{fl/fl}$ EC and BAEC that GRK2 reduction by adenovirus encoding for CRE recombinase and small interfering RNA significantly increases ROS production (Figure 4A and 4B). Increased ROS production was also observed in the aorta from Tie2-CRE/GRK2$^{fl/fl}$ mice (Figure IID in the online-only Data Supplement) stained with 2',7'-dichlorfluorescein-diacetate. To pin down the role of ROS in the above described modifications, we treated endothelial GRK2$^{fl/fl}$ cells and BAEC with the antioxidant α-tocopherol (vitamin E), which significantly attenuated ROS production when GRK2 gene was deleted (Figure 4A and 4B).

Moreover, GRK2 knockout in GRK2$^{fl/fl}$ cells increased the expression of interleukin 1, interleukin 10, and macrophage chemoattractant protein-1, as evaluated by reverse transcriptase polymerase chain reaction and ELISA assay, indicating that endothelial cells are directly involved in leukocyte

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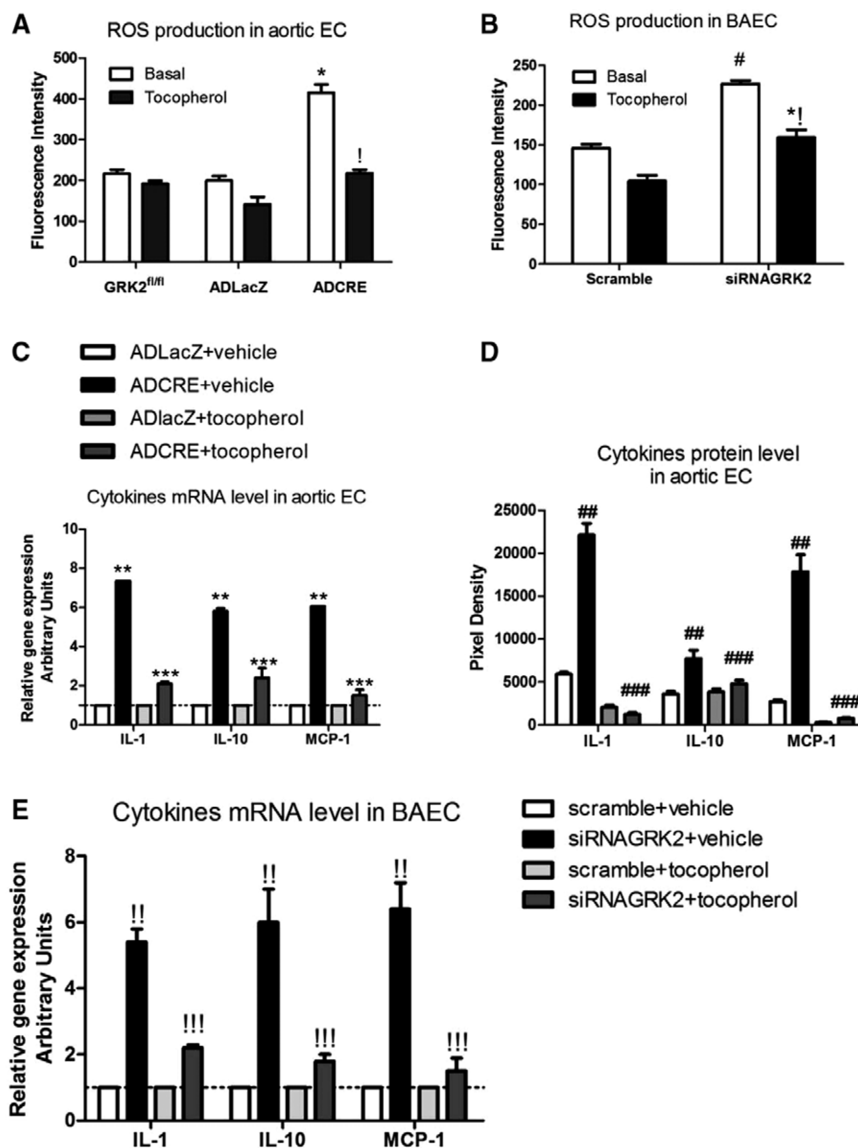

Figure 4. Endothelial G protein–coupled receptor kinase 2 (GRK2) reduced level increases reactive oxygen species (ROS) production and cytokine expression. **A**, ROS production was evaluated in isolated mitochondria from GRK2$^{fl/fl}$ endothelial cells (ECs) infected with adenovirus encoding for CRE recombinase (ADCRE) and ADLacZ, with or without tocopherol treatment. ROS production has been evaluated by incubation of mitochondria with DCFH as described in the Materials and Methods in the online-only Data Supplement. ADCRE increases ROS production as compared with ADLacZ (*vs ADLacZ; $P<0.05$) but is significantly attenuated by antioxidant treatment (!vs ADCRE+vehicle; $P<0.05$). Data are expressed as total fluorescence intensity. **B**, Similarly to **A**, ROS production was evaluated in bovine aortic endothelial cell (BAEC) transfected with small interfering RNA (siRNA) for GRK2 and scramble, in presence or not of tocopherol treatment. GRK2 knockdown enhances mitochondrial ROS production (#vs scramble; $P<0.05$) but is reduced by tocopherol treatment (*!vs siRNA-GRK2+vehicle; $P<0.05$). **C**, mRNA levels of macrophage chemoattractant protein-1 (MCP-1), interleukin-1 (IL-1), and IL-10 were evaluated on GRK2$^{fl/fl}$ endothelial cells, with or without tocopherol treatment. GRK2$^{fl/fl}$ ECs were infected with ADCRE, to induce GRK2 gene deletion, and ADLacZ used as control. ADCRE+vehicle increases MCP-1, IL-1, and IL-10 gene expression as compared with ADLacZ (**vs ADLacZ+vehicle; $P<0.05$). Tocopherol treatment significantly reduces cytokine expression (***vs ADCRE+vehicle; $P<0.05$). **D**, Cytokine production was evaluated in culture medium from GRK2$^{fl/fl}$ EC infected with ADLacZ or ADCRE. ADCRE infection significantly increases MCP-1, IL-1, and IL-10 protein levels (##vs ADLacZ+vehicle) but they are decreased by tocopherol treatment (###vs ADCRE+vehicle). Data are expressed as pixel density. **E**, As above, mRNA levels of MCP-1, IL-1, and IL-10 were evaluated on BAEC transfected with bovine siRNA for GRK2 or scramble, in presence of tocopherol or vehicle treatment. siRNA-GRK2 produces increased cytokine gene expression (!!vs scramble+vehicle; $P<0.05$), which is then significantly reduced by tocopherol treatment (!!!vs siRNA-GRK2+vehicle; $P<0.05$).

migration (Figure 4C and 4D), and the same result was obtained in BAEC treated with GRK2 small interfering RNA (Figure 4E). However, α-tocopherol treatment significantly reduced cytokine expression in both cell lines with GRK2 removal (Figure 4C–4E), indicating potential therapeutic effects of α-tocopherol on vascular inflammation.

Chronic Treatment With α-Tocopherol Ameliorates Vascular Inflammation and Restores Aortic Vasoconstriction

Because the antioxidant α-tocopherol has shown the ability to cut down mitochondrial ROS production in vitro, we tested whether the same treatment could ameliorate the inflammation

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affecting aorta and lung of Tie2-CRE/GRK2$^{fl/fl}$ mice. First of all, we evaluated the effects of α-tocopherol chronic treatment on in vivo inflammation by measuring nitrate production, which is expression of increased inducible nitric oxidase synthase and leukocytes activity, in blood and urine samples from GRK2$^{fl/fl}$ and Tie2-CRE/GRK2$^{fl/fl}$ mice. As expected, Tie2-CRE/GRK2$^{fl/fl}$ mice showed increased nitrate production in blood and urine samples, which were reduced by α-tocopherol treatment (Figure IIA and IIB in the online-only Data Supplement). Thus, we evaluated the possible ability of the antioxidant to attenuate or recover vascular damage induced by increased endothelial ROS production. α-Tocopherol treatment significantly blunted the increased expression of macrophage chemoattractant protein-1 and MMP-2–9 in the aorta of Tie2-CRE/GRK2$^{fl/fl}$ mice, which was no longer different as compared with that of GRK2$^{fl/fl}$ aorta (Figure 3A–3E).

Immunohistochemistry on aortas revealed that the robust macrophage infiltration in Tie2-CRE/GRK2$^{fl/fl}$ (Figure 5A and 5B, black arrows) was reduced by α-tocopherol treatment, and morphological analysis with Masson showed that elastic lamellae were newly folded, recovering the typical wavy look (Figure 5C, black arrows), whereas matrix between lamaelle was reduced (Figure 5C, black star), indicating regression of collagen deposition, as also quantified in Figure 5D. Similarly, in lungs we observed absence of macrophage infiltration after α-tocopherol treatment (Figure IIIC and IIID in the online-only Data Supplement). Inflammatory modifications observed in the aortas of Tie2-CRE/GRK2$^{fl/fl}$ are typical of several vascular diseases, including atherosclerosis. Because vascular inflammation and atherosclerosis are both related to the presence of endothelial dysfunction and loss of vascular integrity, we evaluated lipid deposition in thoracic aorta wall by Oil Red O staining. Tie2-CRE/GRK2$^{fl/fl}$ shows a significant increase in lipid deposition with respect to GRK2$^{fl/fl}$ aorta (Figure 5E). Also, aorta cross-sections (Figure IIE in the online-only Data Supplement) show that lipid accumulation is not organized in an atherosclerotic plaque that indicates presence of an early atherosclerotic lesion. Indeed, proteoglycan accumulation, which is a typical alteration of advanced stage of atherosclerosis, was not observed in both aorta

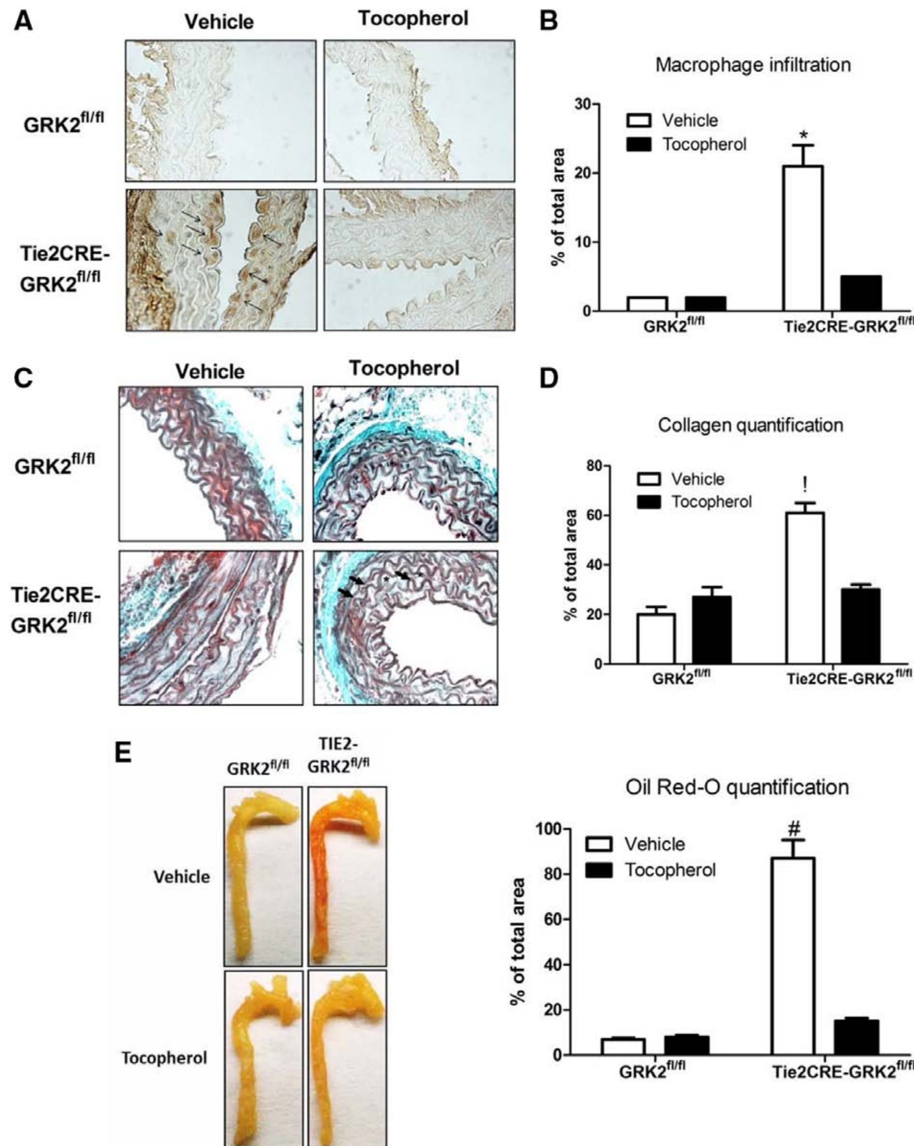

Figure 5. Effect of α-tocopherol chronic treatment on vascular inflammation. **A** and **B**, Immunohistochemistry analysis performed on thoracic aorta. Detection of the F4/80 macrophage fragment reveals an extensive macrophage infiltration in Tie2-CRE/GRK2$^{fl/fl}$ (black arrows) aorta as compared with GRK2$^{fl/fl}$ aorta, which is reduced by tocopherol chronic treatment as shown in the representative images in **A** and in **B** by quantitative analysis with ImageJ. Data are expressed as percentages of brown spots with respect to total aortic area. **C** and **D**, Masson trichrome staining of thoracic aorta from GRK2$^{fl/fl}$ and Tie2-CRE/GRK2$^{fl/fl}$. GRK2$^{fl/fl}$ has a preserved morphology, which is modified in particular at tunica media level as described above. Here, chronic tocopherol treatment mostly recovers typical aortic appearance, with folded elastic lamina and lamellae (black arrows) and reduced matrix deposition (black stars), as also quantified in **D** (*vs Tie2-CRE/GRK2$^{fl/fl}$+vehicle; $P<0.05$). **E**, Oil Red O staining of thoracic aortas. Tie2-CRE/GRK2$^{fl/fl}$ shows increased lipid deposition with respect to GRK2$^{fl/fl}$ as quantified in the bargraph as a percentage of total aortic area (#vs GRK2$^{fl/fl}$; $P<0.05$). However, treatment with α-tocopherol dramatically reduced lipid deposition in the aorta (*!vs Tie2-CRE/GRK2$^{fl/fl}$+vehicle; $P<0.01$).

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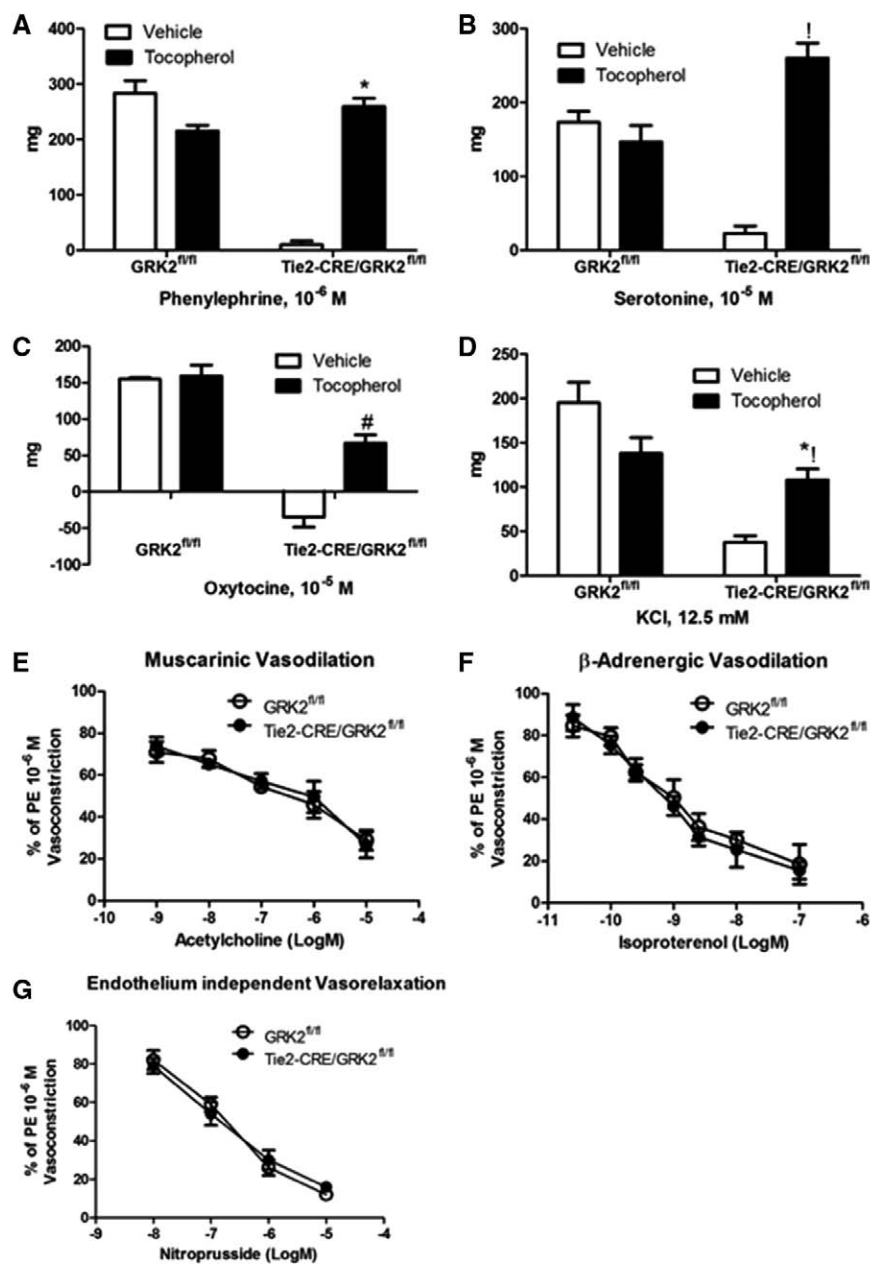

Figure 6. Effect of α-tocopherol treatment on vasomotor responses. **A**–**D**, Vasocontriction was evaluated as above to the maximal concentrations tested: phenylepherine (Phe; 10^{-6} mol/L), serotonine (10^{-6} mol/L), oxytocin (10^{-5} mol/L), and to potassium chloride (KCl; 12.5 mmol/L). Significant recovery of vasoconstriction can be observed in Tie2-CRE/GRK2$^{fl/fl}$ treated with the antioxidant with respect to vehicle (*, !, #, *!vs Tie2-CRE/GRK2$^{fl/fl}$+vehicle; P<0.05; 2-way ANOVA). **E**–**G**, Vasodilation was evaluated in GRK2$^{fl/fl}$ and Tie2-CRE/GRK2$^{fl/fl}$ mice treated with α-tocopherol in response to acetylcholine (10^{-9} to 10^{-5} mol/L) and isoproterenol (3×10^{-10} to 10^{-7} mol/L) for endothelial-dependent vasorelaxation, and sodium nitroprusside (10^{-9} to 10^{-5} mol/L) for endothelial-independent vasorelaxation, after maximal vasoconstriction induced by Phe 10^{-6} mol/L. No significant differences were found between the 2 groups (2-way ANOVA).

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samples from GRK2$^{fl/fl}$ and Tie2-CRE/GRK2$^{fl/fl}$ mice (data not shown). Of note, aorta Oil Red O staining showed a significant reduction in lipid deposition after tocopherol versus not treated animal samples as evaluated in both gross morphology (Figure 5E) and cross-sections of the aorta (Figure IIE in the online-only Data Supplement). GRK2$^{fl/fl}$ and Tie2-CRE/GRK2$^{fl/fl}$ do not show significant difference in serum total cholesterol level (Figure IIF in the online-only Data Supplement), indicating that the above observations are not attributable to differences in lipid profile between the 2 groups of animals.

Finally, we evaluated whether the beneficial effects brought by α-tocopherol at structural and biochemical levels can be also extended to the vasomotor responses. As evidenced in Figure 6, α-tocopherol administration significantly restored receptor-dependent and -independent vasoconstriction to the different compounds used in this study (Figure 6A–6D) and, notably, at same levels of GRK2$^{fl/fl}$ mice. Nonetheless, restored vasoconstriction is also accompanied by receptor-dependent (acetylcholine and isoproterenol) and -independent (sodium nitroprusside) vasodilation as shown in Figure 6E–6G. All together, these indicate that inflammatory modifications induced by GRK2 endothelial removal can be significantly attenuated by a ROS scavanger.

Discussion

Vascular integrity and function are preserved and regulated by endothelium. This accepted knowledge underlies complex molecular mechanisms, which allow the endothelium to regulate functions such as cellular migrations and metabolite passages. Our data for the first time show that the presence of GRK2 in the endothelial cell is necessary for the proper functioning of the endothelium. GRK2 removal from the

endothelium associates to an inflammatory state of the vessel wall, as evidenced by elastic and CF degeneration, defective vasoconstriction, and increased lipid deposition into the aortic wall of Tie2-CRE/GRK2$^{fl/fl}$ mice. This feature is generalized because inflammation can be found also in other tissues rich in endothelium, such as lung. The underlying mechanism appears to be the increased endothelial ROS production because chronic treatment of Tie2-CRE/GRK2$^{fl/fl}$ with tocopherol, a ROS scavenger, reduces aorta and lung inflammation and restores aortic receptor-dependent and -independent vasoconstriction. These observations unveil the crucial role played by GRK2 in the vascular homeostasis, indicating that its role is fundamental not only for the embryonic development as previously described[21] but also for the adult life. Our previous report has described the ability of GRK2 to localize at the mitochondria level, in particular, when the cells are exposed to hypoxia. This phenomenon has favorable effect on cell metabolism, by preserving ATP production and mitochondrial biogenesis. In pathological models such as hyperglycemia or hypertension, mitochondrial ROS generation is increased, and targeting ROS by antioxidants such as α-tocopherol[20] improves endothelium function[22] and reduces expression of adhesion molecules, such as vascular cell adhesion protein 1, on endothelial surface.[22,23] In this study, we have observed that GRK2 removal from cell increases mitochondrial ROS production, which can be cut down using a lipophilic antioxidant, such as vitamin E. This mechanism appears quite specific for the transgenic model used in this study because we did not observe influence of GRK2 removal on eNOS activity, which is a known regulator of vascular responses. Indeed, previous studies have identified GRK2 as an important regulator of eNOS phosphorylation and activity.[9] Specifically, GRK2 appears to be able to bind and sequester Akt, rendering it unavailable for eNOS activation. Then, lacking of GRK2 would remove a potential brake on Akt activity and leading to eNOS overactivation and vasodilation, which may explain the defective vasoconstriction in Tie2-GRK2$^{fl/fl}$ aortas. However, this was not the case in our system, where GRK2 does not show ability to interact either with Akt or eNOS, and also GRK2 removal from EC does not produce any significant modification in eNOS phosphorylation and activation.

Our data add new knowledge along the way to explore GRK2 role in mitochondria, concluding that it participates in the mitochondrial redox reactions and contributes to the physiological functioning of the cell. Even if this finding is not supported by a complete explanation of the mechanisms through which GRK2 interferes with the mitochondrial functions, however, basing on our previous finding that GRK2 localizes in mitochondria and promotes mitogenesis, we can hypothesize that GRK2 deals with mitochondrial integrity and regeneration. In particular, GRK2 may favor mitochondrial localization of important proteins belonging to the mitochondrial complexes, and its absence will then reduce ATP production, as previous demonstrated, and increase ROS production, as evidenced here. Nonetheless, we here demonstrate that GRK2 is not only fundamental for cardiovascular development during embryogenesis but is also important for cellular integrity and function during adult life by regulating a mitochondrial function such as ROS production, which in turn is important for development of several vascular diseases.

Indeed, GRK2 has been associated with cardiovascular diseases such as congestive heart failure and hypertension,[10,11] but through the years this kinase has been related also to other complex diseases such as type II diabetes mellitus, Alzheimer, rheumatoid arthritis, and atherosclerosis.[24–26] Of note, these conditions share the common feature of being initiated and perpetrated by an inflammatory process, which, as known, involves release of cytokines and migration of leukocytes and lymphocytes from the bloodstream to the parenchymal tissue. Here we observe a similar phenotype, because removing GRK2 from endothelium induces cytokine production and macrophage migration into the vascular media, where they release metalloproteinases, such as MMP-2 and MMP-9, corrupting the elastic fibers and CFs of the vessel. Moreover, the physical interlinkages between components of the tunica media, made by CFs that extend through fenestration of the parallel elastic lamellae, are important in maintaining structural integrity and physicomechanical properties of the aortic wall,[27] and their disruptions, such as those observed in Tie2-CRE/GRK2$^{fl/fl}$ mice, are implicated in disease processes, such as atherosclerosis,[28,29] aneurysm formation,[30] and in ageing.[31] In our study, we were able to rescue these vascular alterations and recover defective vasoconstriction in Tie2-CRE/GRK2$^{fl/fl}$ by chronic administration of vitamin E, which reduces endothelial ROS production from mitochondria. Besides the specific cellular lineage used in this study, this and our previous studies raise the importance for GRK2 in mitochondrial regulation, indicating that the optimal therapeutic strategy for treatment of chronic inflammatory diseases would be to restore and increase GRK2 localization at mitochondria level, to attenuate the increased ROS production observed during chronic inflammation.

Apparently, our study contradicts the knowledge that accumulating GRK2 is deleterious for the cell and it may appear provocative as it shows that GRK2 is protective in the vasculature. On the contrary, our data are in agreement with previous observations that GRK2 gene deletion is detrimental for embryonic cardiac development,[21] and in adult life, cardiac-selective GRK2 removal alters the cardiac hypertrophic response to chronic βAR stimulation, leading to an eccentric dilatation of the heart similar to that observed in intermediate–advanced phases of heart failure.[32] To reconcile these apparently contradictive evidence, we need to take in account 2 findings: first, ours is a gene deletion model, which cannot be considered the reciprocal of GRK2 accumulation observed in pathological conditions; second, GRK2 is a complex molecule, which was demonstrated to interact and regulate several substrates through its kinase activity, regulator of G-protein signaling and pleckstrin homology domains, giving it the ability to have multiple regulatory roles into the cells.[33,34] Moreover, the only effective therapeutic strategy aiming to counteract GRK2-mediated receptor desensitization at plasma membrane level, for example, on beta adrenergic and insulin signaling, has used the carboxyl terminal portion of GRK2 (βARKct), whose mechanisms in animal models of cardiovascular diseases are not completely unveiled. This molecule represents the carboxyl terminal portion of GRK2, which retains the ability to localize at plasma membrane–binding Gβγ subunit through the PH domain, but lacks the kinase activity.[35,36]

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βARKct displaces from plasma membrane but does not inhibit GRK2 activity, which is then theoretically free to move into other compartments like mitochondria where it can accomplish its protective role.

Therefore, an integrative and fascinating hypothesis would provide that GRK2 plays different roles into the cell according to its subcellular localization or compartmentalization, furnishing also a complete explanation for the positive effects of βARKct for cell functioning during disease.

Of course, this hypothesis needs to be confirmed with further studies by evaluating the roles of GRK2 in different cellular compartments, but it is already becoming evident that we need to move in a different way to target GRK2 in a therapeutic strategy. Indeed, if increased levels of GRK2 are negative for cell function, on the other side a complete reduction or inhibition of the kinase level or activity is dangerous as well. Thus, a new perspective therapy includes a tight modulation of GRK2 activity and level into the cell, taking also into account the specific role played by GRK2 in the different cellular compartments.

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Disclosures

None.

References

1. Sorriento D, Santulli G, Del Giudice C, Anastasio A, Trimarco B, Iaccarino G. Endothelial cells are able to synthesize and release catecholamines both in vitro and in vivo. *Hypertension*. 2012;60:129–136.
2. Iaccarino G, Ciccarelli M, Sorriento D, et al. Ischemic neoangiogenesis enhanced by beta2-adrenergic receptor overexpression: a novel role for the endothelial adrenergic system. *Circ Res*. 2005;97:1182–1189.
3. Iaccarino G, Cipolletta E, Fiorillo A, Annecchiarico M, Ciccarelli M, Cimini V, Koch WJ, Trimarco B. Beta(2)-adrenergic receptor gene delivery to the endothelium corrects impaired adrenergic vasorelaxation in hypertension. *Circulation*. 2002;106:349–355.
4. Ciccarelli M, Cipolletta E, Santulli G, Campanile A, Pumiglia K, Cervero P, Pastore L, Astone D, Trimarco B, Iaccarino G. Endothelial beta2 adrenergic signaling to AKT: role of Gi and SRC. *Cell Signal*. 2007;19:1949–1955.
5. Ciccarelli M, Santulli G, Campanile A, Galasso G, Cervèro P, Altobelli GG, Cimini V, Pastore L, Piscione F, Trimarco B, Iaccarino G. Endothelial alpha1-adrenoceptors regulate neo-angiogenesis. *Br J Pharmacol*. 2008;153:936–946.
6. Bristow MR, Ginsburg R, Minobe W, Cubicciotti RS, Sageman WS, Lurie K, Billingham ME, Harrison DC, Stinson EB. Decreased catecholamine sensitivity and beta-adrenergic-receptor density in failing human hearts. *N Engl J Med*. 1982;307:205–211.
7. Hausdorff WP, Hnatowich M, O'Dowd BF, Caron MG, Lefkowitz RJ. A mutation of the beta 2-adrenergic receptor impairs agonist activation of adenylyl cyclase without affecting high affinity agonist binding. Distinct molecular determinants of the receptor are involved in physical coupling to and functional activation of Gs. *J Biol Chem*. 1990;265:1388–1393.
8. Ungerer M, Böhm M, Elce JS, Erdmann E, Lohse MJ. Altered expression of beta-adrenergic receptor kinase and beta 1-adrenergic receptors in the failing human heart. *Circulation*. 1993;87:454–463.
9. Liu S, Premont RT, Kontos CD, Zhu S, Rockey DC. A crucial role for GRK2 in regulation of endothelial cell nitric oxide synthase function in portal hypertension. *Nat Med*. 2005;11:952–958.
10. Izzo R, Cipolletta E, Ciccarelli M, Campanile A, Santulli G, Palumbo G, Vasta A, Formisano S, Trimarco B, Iaccarino G. Enhanced GRK2 expression and desensitization of betaAR vasodilatation in hypertensive patients. *Clin Transl Sci*. 2008;1:215–220.
11. Iaccarino G, Barbato E, Cipolletta E, De Amicis V, Margulies KB, Leosco D, Trimarco B, Koch WJ. Elevated myocardial and lymphocyte GRK2 expression and activity in human heart failure. *Eur Heart J*. 2005;26:1752–1758.
12. Napolitano R, Campanile A, Sarno L, Anastasio A, Maruotti GM, Morlando M, Trimarco B, Martinelli P, Iaccarino G. GRK2 levels in umbilical arteries of pregnancies complicated by gestational hypertension and preeclampsia. *Am J Hypertens*. 2012;25:366–371.
13. Taguchi K, Kobayashi T, Matsumoto T, Kamata K. Dysfunction of endothelium-dependent relaxation to insulin via PKC-mediated GRK2/Akt activation in aortas of ob/ob mice. *Am J Physiol Heart Circ Physiol*. 2011;301:H571–H583.
14. Cipolletta E, Campanile A, Santulli G, Sanzari E, Leosco D, Campiglia P, Trimarco B, Iaccarino G. The G protein coupled receptor kinase 2 plays an essential role in beta-adrenergic receptor-induced insulin resistance. *Cardiovasc Res*. 2009;84:407–415.
15. Ciccarelli M, Chuprun JK, Rengo G, Gao E, Wei Z, Peroutka RJ, Gold JI, Gumpert A, Chen M, Otis NJ, Dorn GW 2nd, Trimarco B, Iaccarino G, Koch WJ. G protein-coupled receptor kinase 2 activity impairs cardiac glucose uptake and promotes insulin resistance after myocardial ischemia. *Circulation*. 2011;123:1953–1962.
16. Usui I, Imamura T, Babendure JL, Satoh H, Lu JC, Hupfeld CJ, Olefsky JM. G protein-coupled receptor kinase 2 mediates endothelin-1-induced insulin resistance via the inhibition of both Galphaq/11 and insulin receptor substrate-1 pathways in 3T3-L1 adipocytes. *Mol Endocrinol*. 2005;19:2760–2768.
17. Fusco A, Santulli G, Sorriento D, Cipolletta E, Garbi C, Dorn GW 2nd, Trimarco B, Feliciello A, Iaccarino G. Mitochondrial localization unveils a novel role for GRK2 in organelle biogenesis. *Cell Signal*. 2012;24:468–475.
18. Dikalova AE, Bikineyeva AT, Budzyn K, Nazarewicz RR, McCann L, Lewis W, Harrison DG, Dikalov SI. Therapeutic targeting of mitochondrial superoxide in hypertension. *Circ Res*. 2010;107:106–116.
19. Wang D, Luo Z, Wang X, Jose PA, Falck JR, Welch WJ, Aslam S, Teerlink T, Wilcox CS. Impaired endothelial function and microvascular asymmetrical dimethylarginine in angiotensin II-infused rats: effects of tempol. *Hypertension*. 2010;56:950–955.
20. Lass A, Sohal RS. Effect of coenzyme Q(10) and alpha-tocopherol content of mitochondria on the production of superoxide anion radicals. *FASEB J*. 2000;14:87–94.
21. Jaber M, Koch WJ, Rockman H, Smith B, Bond RA, Sulik KK, Ross J Jr, Lefkowitz RJ, Caron MG, Giros B. Essential role of beta-adrenergic receptor kinase 1 in cardiac development and function. *Proc Natl Acad Sci U S A*. 1996;93:12974–12979.
22. Abdala-Valencia H, Berdnikovs S, Cook-Mills JM. Vitamin E isoforms differentially regulate intercellular adhesion molecule-1 activation of PKCα in human microvascular endothelial cells. *PLoS One*. 2012;7:e41054.
23. Catalán U, Fernández-Castillejo S, Pons L, Heras M, Aragonés G, Anglès N, Morelló JR, Solà R. Alpha-tocopherol and BAY 11-7082 reduce vascular cell adhesion molecule in human aortic endothelial cells. *J Vasc Res*. 2012;49:319–328.
24. Vroon A, Kavelaars A, Limmroth V, Lombardi MS, Goebel MU, Van Dam AM, Caron MG, Schedlowski M, Heijnen CJ. G protein-coupled receptor kinase 2 in multiple sclerosis and experimental autoimmune encephalomyelitis. *J Immunol*. 2005;174:4400–4406.
25. Lombardi MS, Kavelaars A, Schedlowski M, Bijlsma JW, Okihara KL, Van de Pol M, Ochsmann S, Pawlak C, Schmidt RE, Heijnen CJ. Decreased expression and activity of G-protein-coupled receptor kinases in peripheral blood mononuclear cells of patients with rheumatoid arthritis. *FASEB J*. 1999;13:715–725.
26. Otten JJ, de Jager SC, Kavelaars A, Seijkens T, Bot I, Wijnands E, Beckers L, Westra MM, Bot M, Busch M, Bermudez B, van Berkel TJ, Heijnen CJ, Biessen EA. Hematopoietic G-protein-coupled receptor kinase 2 deficiency decreases atherosclerotic lesion formation in LDL receptor-knockout mice. *FASEB J*. 2013;27:265–276.
27. Ogeng'o JA, Malek AA, Kiama SG. Structural organisation of tunica intima in the aorta of the goat. *Folia Morphol (Warsz)*. 2010;69:164–169.
28. Cimmino G, Ragni M, Cirillo P, Petrillo G, Loffredo F, Chiariello M, Gresele P, Falcinelli E, Golino P. C-reactive protein induces expression of matrix metalloproteinase-9: a possible link between inflammation and plaque rupture. *Int J Cardiol*. 2012;12:415–420.

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29. Lavezzi AM, Ottaviani G, Matturri L. Biology of the smooth muscle cells in human atherosclerosis. *APMIS*. 2005;113:112–121.
30. Hayashi T, Morishita E, Ohtake H, Oda Y, Asakura H, Nakao S. Expression of annexin II in experimental abdominal aortic aneurysms. *Int J Hematol*. 2009;90:336–342.
31. Greenwald SE. Ageing of the conduit arteries. *J Pathol*. 2007;211:157–172.
32. Matkovich SJ, Diwan A, Klanke JL, Hammer DJ, Marreez Y, Odley AM, Brunskill EW, Koch WJ, Schwartz RJ, Dorn GW 2nd. Cardiac-specific ablation of G-protein receptor kinase 2 redefines its roles in heart development and beta-adrenergic signaling. *Circ Res*. 2006;99:996–1003.
33. Ribas C, Penela P, Murga C, Salcedo A, García-Hoz C, Jurado-Pueyo M, Aymerich I, Mayor F Jr. The G protein-coupled receptor kinase (GRK) interactome: role of GRKs in GPCR regulation and signaling. *Biochim Biophys Acta*. 2007;1768:913–922.
34. Ciccarelli M, Cipolletta E, Iaccarino G. GRK2 at the control shaft of cellular metabolism. *Curr Pharm Des*. 2012;18:121–127.
35. Koch WJ, Rockman HA, Samama P, Hamilton RA, Bond RA, Milano CA, Lefkowitz RJ. Cardiac function in mice overexpressing the beta-adrenergic receptor kinase or a beta ARK inhibitor. *Science*. 1995;268:1350–1353.
36. Rengo G, Lymperopoulos A, Zincarelli C, Donniacuo M, Soltys S, Rabinowitz JE, Koch WJ. Myocardial adeno-associated virus serotype 6-betaARKct gene therapy improves cardiac function and normalizes the neurohormonal axis in chronic heart failure. *Circulation*. 2009;119:89–98.

Significance

Maintenance of endothelium integrity is fundamental in preventing development of vascular diseases, such as atherosclerosis. Increased reactive oxygen species is often associated with the loss of endothelial homeostasis; however, mechanisms that lead to the alteration of the cellular redox reactions are poorly understood. Here we demonstrated that G protein–coupled receptor kinase 2 is important for the physiological functioning of the cell, because its deletion induces increased mitochondrial reactive oxygen species and a dramatic alteration of the vascular phenotype and function. Here we add new knowledge on the complex role played by G protein–coupled receptor kinase 2 into the cell, which can be different, detrimental, or protective, according to its subcellular localization.

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