

G Protein–Coupled Receptor Kinase 2, With β -Arrestin 2, Impairs Insulin-Induced Akt/Endothelial Nitric Oxide Synthase Signaling in *ob/ob* Mouse Aorta

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In type 2 diabetes, impaired insulin-induced Akt/endothelial nitric oxide synthase (eNOS) signaling may decrease the vascular relaxation response. Previously, we reported that this response was negatively regulated by G protein–coupled receptor kinase 2 (GRK2). In this study, we investigated whether/how in aortas from *ob/ob* mice (a model of type 2 diabetes) GRK2 and β -arrestin 2 might regulate insulin-induced signaling. Endothelium-dependent relaxation was measured in aortic strips. GRK2, β -arrestin 2, and Akt/eNOS signaling pathway proteins and activities were mainly assayed by Western blotting. In *ob/ob* (vs. control [Lean]) aortas: 1) insulin-induced relaxation was reduced, and this deficit was prevented by GRK2 inhibitor, anti-GRK2 antibody, and an siRNA specifically targeting GRK2. The Lean aorta relaxation response was reduced to the *ob/ob* level by pretreatment with an siRNA targeting β -arrestin 2. 2) Insulin-stimulated Akt and eNOS phosphorylations were decreased. 3) GRK2 expression in membranes was elevated, and, upon insulin stimulation, this expression was further increased, but β -arrestin 2 was decreased. In *ob/ob* aortic membranes under insulin stimulation, the phosphorylations of Akt and eNOS were augmented by GRK2 inhibitor. In mouse aorta, GRK2 may be, upon translocation, a key negative regulator of insulin responsiveness and an important regulator of the β -arrestin 2/Akt/eNOS signaling, which is implicated in diabetic endothelial dysfunction. *Diabetes* 61:1978–1985, 2012

D iabetes mellitus is an important risk factor for hypertension and other cardiovascular diseases, and impaired endothelial function has been described in diabetic humans and animal models of this disease (1,2). One of the most important functions of the endothelium is the production of nitric oxide (NO), and impaired NO production can result from endothelial dysfunction (3).

Endothelium is an insulin target tissue: in endothelial cells, insulin activates a signaling pathway involving insulin receptor (IR) and Akt, and this leads to endothelial NO synthase (eNOS) activation, NO synthesis, and vasodilation (4,5). We and others (6,7) have supported such a role for the Akt/eNOS pathway in the endothelium on the grounds that inhibition of agonist-induced activations of the Akt/eNOS pathway leads to impaired NO availability. Recently, Kubota et al. (8) reported that insulin signaling in endothelial cells plays a pivotal role in the regulation of

glucose uptake by skeletal muscle, that the Akt/eNOS pathway might be particularly susceptible to the adverse effects of conditions such as obesity and insulin resistance and that insulin-stimulated Akt activated eNOS to a degree that was proportional to the amount of eNOS protein available. Molecular defects in this upstream pathway are therefore likely to affect not only insulin-stimulated glucose uptake in typical target tissues, but also insulin-stimulated eNOS, and such defects may thereby contribute to both altered glucose homeostasis and endothelial dysfunction (9).

G protein–coupled receptor kinases (GRKs) were initially identified as serine/threonine kinases that participate, together with β -arrestins, in the regulation of multiple G protein–coupled receptors (GPCRs). The GRKs constitute a group of protein kinases that specifically recognize and phosphorylate agonist-activated GPCRs (10,11). Among the GRKs, GRK2 has attracted interest as a ubiquitous GRK family member that appears to play a central, integrative role in signal-transduction pathways known to modulate intracellular effectors involved in cardiac and endothelial function (10,11). GRK2-mediated phosphorylated GPCR promotes the binding of β -arrestin 2, which is reportedly ubiquitously expressed, and then mediates various signal-transduction pathways such as Akt (12).

Recently, Luan et al. (13) reported that insulin stimulates the formation of a new β -arrestin 2 signal complex in which β -arrestin 2 acts as a scaffold for translocation of Akt to IR, even though IR is not a GPCR. We previously reported that an upregulation of GRK2 and a decrease in β -arrestin 2 inhibit insulin-induced stimulation of Akt/eNOS signaling and that GRK2 overactivation may result from an increase in PKC activity in aortas from diabetic mice with hyperinsulinemia (14). Alongside the above negative regulatory role of GRK2/ β -arrestin 2, emerging evidence indicates that GRK2 and β -arrestin 2 are each able to interact with Akt. Against the above background, we investigated whether/how in aortas from *ob/ob* mice (a model of type 2 diabetes with hyperinsulinemia): 1) GRK2 and β -arrestin 2 might regulate insulin-induced Akt/eNOS signaling, and 2) GRK2 upregulation might hinder the establishment of a normal relationship between β -arrestin 2 and Akt under insulin stimulation.

RESEARCH DESIGN AND METHODS

Male *ob/ob* (diabetic) and age-matched Lean (control) C57BL/6J mice (27–32 weeks old) were obtained at the age of 5 weeks. This study was carried out in accordance with the guide issued by the Hoshi University Animal Care and Use Committee, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology. In *ob/ob* mice (vs. Lean mice): 1) body weight, plasma insulin, and plasma parameters relating to lipid metabolism and systolic blood pressure were all significantly elevated, 2) plasma leptin

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was significantly lower, and 3) plasma glucose and triglyceride were not different (Supplementary Table 1).

Studies using small interfering RNA and Chariot-mediated antibody delivery in organ culture of aorta. A small interfering RNA (siRNA) against GRK2 and one against β -arrestin 2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). We used the appropriate siRNA. For specific silencing of GRK2 or β -arrestin 2 expression, we used a well-established method for the organ culture (15,16). After transfection with a given siRNA, aortas were incubated for 48 h, as described by Chen et al. (17). A macromolecular protein delivery system, Chariot, was used as described by Morris et al. (18).

Measurement of isometric force and nitrite plus nitrate. The aorta (cut into rings) was placed in a bath containing modified Krebs-Henseleit solution, with one end of each strip connected to a tissue holder and the other to a force-displacement transducer, then subjected to measurement of the agonist-induced relaxation response, as previously described (7,14). The concentration of nitrite plus nitrate (NOx) in the effluent from each tissue was sampled and assayed by the method described previously (ENO20; Eicom, Kyoto, Japan) (7).

Measurement of protein expressions (GRK2, Akt, and eNOS and their phosphorylated forms). Aortic membranes were prepared as described by Marwaha et al. (19). Aortas were homogenized in lysis buffer, and protein levels and activities were assayed by Western blotting, as previously described (14,20).

Statistical analysis. Each relaxation response is expressed as a percentage of the contraction induced by prostaglandin F_{2 α} . Values are means \pm SE. When appropriate, statistical differences were assessed by Dunnett test for multiple comparisons after a one- or two-way ANOVA, with $P < 0.05$ being regarded as significant. Statistical comparisons between concentration-response curves were made using a one-way ANOVA, with post hoc correction for multiple comparisons by Bonferroni's test, with $P < 0.05$ again being considered significant.

RESULTS

GRK2 and the endothelial relaxation and NO production induced by insulin in *ob/ob* mice. To evaluate endothelial function, the vasorelaxation responses to insulin and acetylcholine were examined in aortas from *ob/ob* and Lean mice (Fig. 1A and Supplementary Figs. 1 and 2). Next, we examined insulin-induced relaxation and/or Western blots in the absence and presence of endothelium or in the absence and presence of N^G-nitro-L-arginine, a NO synthase inhibitor, in aortic rings from *ob/ob* and Lean mice to determine whether the changes in Akt/eNOS are occurring specifically in the endothelium (Supplementary Fig. 1). As revealed previously (7,14), the above results suggest that the insulin-induced relaxation, but not the acetylcholine-induced one, is mediated through the endothelium-dependent Akt/eNOS pathway and that in aortas from *ob/ob* mice, this pathway is impaired (Supplementary Figs. 1 and 2).

To examine the involvement of GRK2 in the insulin-induced relaxation response, we used GRK2 inhibitor, anti-GRK2 antibody, and GRK2 siRNA. In the presence of GRK2 inhibitor, anti-GRK2 antibody, or GRK2 siRNA, the insulin-induced relaxation was enhanced in *ob/ob*, but not in Lean aortas (Fig. 1B–F). The efficiencies of the delivery condition, siRNA transfection, and anti-GRK2 antibody were assessed by measuring GRK2 expression by Western blotting (Fig. 1G and H). As shown in Fig. 2B and C, in such antibody-treated *ob/ob* and GRK2 siRNA-transfected *ob/ob* aortas, NOx production under insulin stimulation was significantly greater than that seen in aortas treated with GRK2 antibody alone, or with Chariot alone, or with *ob/ob* control siRNA. In contrast, no such effects were seen in Lean aortas (Fig. 1C and E). No effects of GRK2 siRNA were observed on the basal expression levels of total Akt or eNOS protein (Fig. 1G). Two GRKs, GRK2 and GRK5, have been specifically investigated in studies of hypertension (21). So, we explored by Western blotting whether GRK2 inhibitor

affects GRK5. In our experiment, GRK5 expression was not affected by GRK2 inhibitor treatment (data not shown).

We examined whether insulin-induced NO release might be regulated by GRK2 (Fig. 2A). The insulin-stimulated release of NOx from *ob/ob* aortas was significantly below that from Lean ones. Following preincubation with Akt inhibitor or both Akt inhibitor and GRK2 inhibitor, insulin-stimulated NOx release did not differ between the groups due to the response in the Lean group being much reduced. Interestingly, following preincubation with GRK2 inhibitor, insulin-stimulated NOx production was significantly increased only in *ob/ob* aortas.

β -Arrestin 2 and insulin-induced endothelial function in *ob/ob* mice. β -Arrestin 2 siRNA appeared to be highly selective for the target (Fig. 3G). To determine the effects of β -arrestin 2 depletion on insulin signaling, the insulin-induced relaxation response and NO production were examined in aortas from *ob/ob* and Lean mice following β -arrestin 2 siRNA transfection. We observed that both responses were significantly reduced in Lean aortas with β -arrestin 2 siRNA transfection (Fig. 3A, B, and E). We next used Chariot for selective overexpression of β -arrestin 2 (Fig. 3H), but such overexpression did not alter insulin-induced relaxation or NO production (Fig. 3C, D, and F).

Effects of reduced GRK2 expression on insulin signaling via Akt/eNOS. Neither eNOS nor Akt expression differed between *ob/ob* and Lean aortas (Fig. 4B).

We investigated whether insulin would induce GRK2-linked eNOS phosphorylation in the aortas transfected with GRK2 siRNA (Supplementary Fig. 3). Stimulation with insulin led to a significant increase in eNOS phosphorylation at Ser¹¹⁷⁷ in Lean aortas transfected with GRK2 siRNA, but not in *ob/ob* ones. Interestingly, after transfection with GRK2 siRNA, insulin-stimulated eNOS phosphorylation at Thr⁴⁹⁵ was increased only in the *ob/ob* aorta. Next, we examined whether insulin-induced Akt phosphorylation might be controlled by GRK2 (Supplementary Fig. 3). Upon insulin stimulation, Akt phosphorylation was significantly lower in *ob/ob* aortas transfected with GRK2 siRNA than in their Lean counterparts. Importantly, the insulin-stimulated Akt phosphorylation at Thr³⁰⁸ was increased in *ob/ob* aortas transfected with GRK2 siRNA. The above effects of GRK2 siRNA were similar to those of the chemical blocker of GRK2 (Supplementary Fig. 4).

GRK2 expression and activity. We studied aortic GRK2 expression, and we confirmed a significantly higher expression level in the *ob/ob* group than in the Lean one (Fig. 4A).

There was a greater expression of GRK2 in membranes than in the cytosol in the *ob/ob*, but not in the Lean aorta (Fig. 4C). We confirmed that expression of caveolin, which is known to exist only on the inner surface of the plasma membrane, was detected only in the membrane fraction in *ob/ob* and Lean mice (Supplementary Fig. 6).

In the basal condition, GRK2 activity was significantly, about threefold, higher in *ob/ob* aortas than in Lean ones (Fig. 4E).

Insulin-stimulated translocation of GRK2. We studied GRK2 expression under insulin-stimulation in aortic membranes (Fig. 4D). In aortic membranes, the GRK2 expression was significantly increased by insulin stimulation in the *ob/ob* group, and this increase was completely prevented by GRK2 inhibitor pretreatment. Interestingly, in Lean aortas, there were no such significant differences.

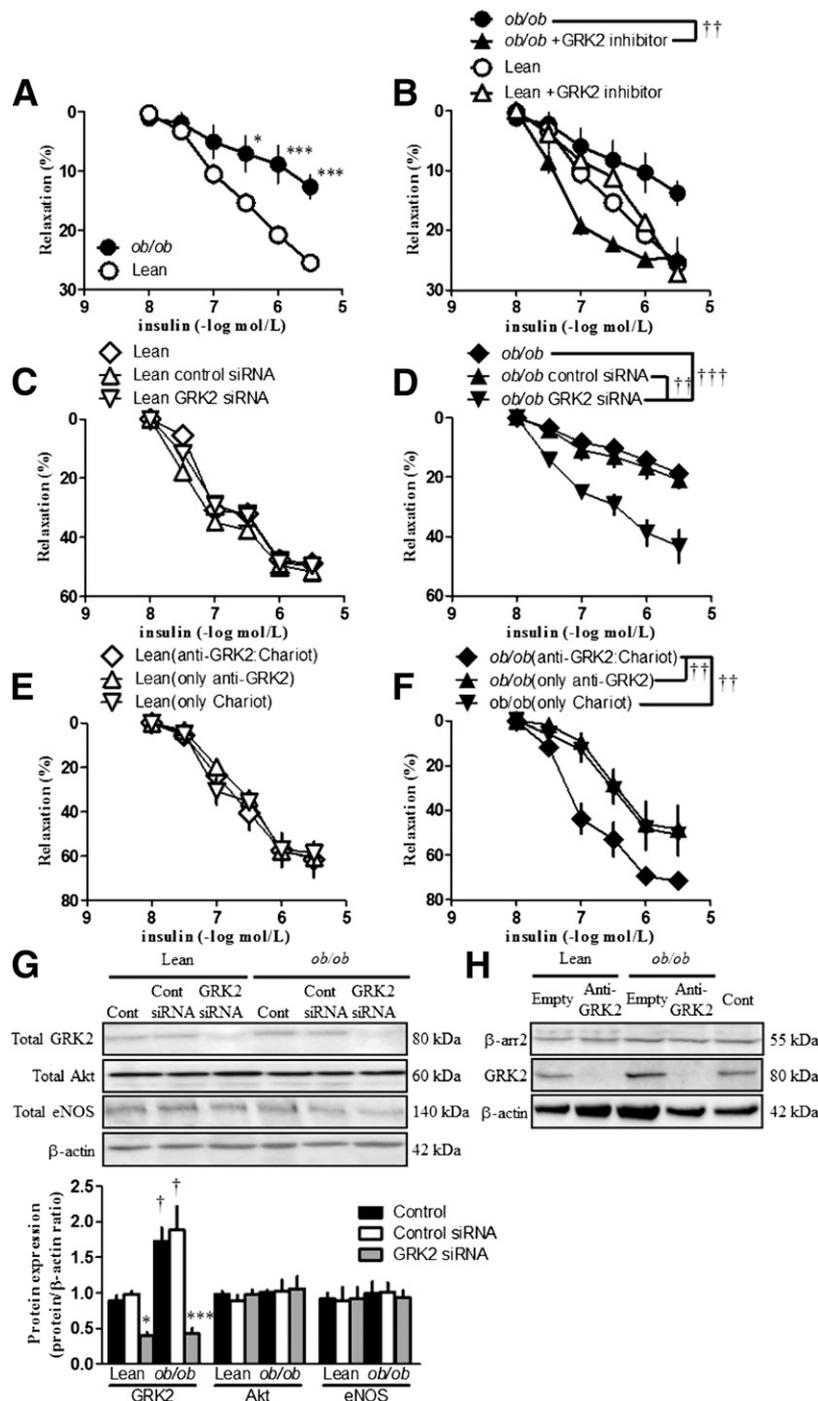


FIG. 1. Concentration-response curves for insulin-induced relaxations of aortic rings from *ob/ob* and Lean mice. **A:** Insulin-induced relaxation. **B:** Effect of GRK2 inhibitor (10⁻⁶ mol/L). **C** and **D:** Effects of siRNA against GRK2 (300 nmol/L). **E** and **F:** Effects of Chariot-mediated delivery of GRK2 antibody (4 μg), together with its controls (GRK2 antibody alone or Chariot alone). Western blots showing efficiencies of siRNA transfection (**G:** GRK2 siRNA) and Chariot protein delivery (**H:** anti-GRK2 antibody) in aortas. No effects were seen on either Akt or eNOS expression. Lanes were run on different gels, but under the same conditions. Values are mean ± SE; *n* = 4–6. **P* < 0.05 vs. Lean; ****P* < 0.001 vs. Lean; ††*P* < 0.01 vs. *ob/ob*, *ob/ob* GRK2 siRNA, or *ob/ob* (anti-GRK2:Chariot); †††*P* < 0.001 vs. *ob/ob* GRK2 siRNA. In **G:** **P* < 0.05 vs. control siRNA; ****P* < 0.001 vs. control siRNA; †*P* < 0.05 vs. Lean control.

We next examined the β-arrestin 2 expression in aortic membranes (Fig. 4*F*). The β-arrestin 2 in the membrane fraction decreased significantly in *ob/ob* aortas upon insulin stimulation.

Finally, we focused on insulin-induced Akt and eNOS phosphorylation levels in the membrane fraction of aortas after Akt inhibitor or GRK2 inhibitor treatment (Fig. 5).

The significant changes in phosphorylated Akt and phosphorylated eNOS levels induced by insulin in the membrane and cytosol fractions were similar to those induced in the whole aorta (Supplementary Figs. 4 and 5). Akt inhibitor pretreatment prevented insulin-induced Akt and eNOS phosphorylations in the membranes and cytosol. Notably, GRK2 inhibitor pretreatment significantly increased

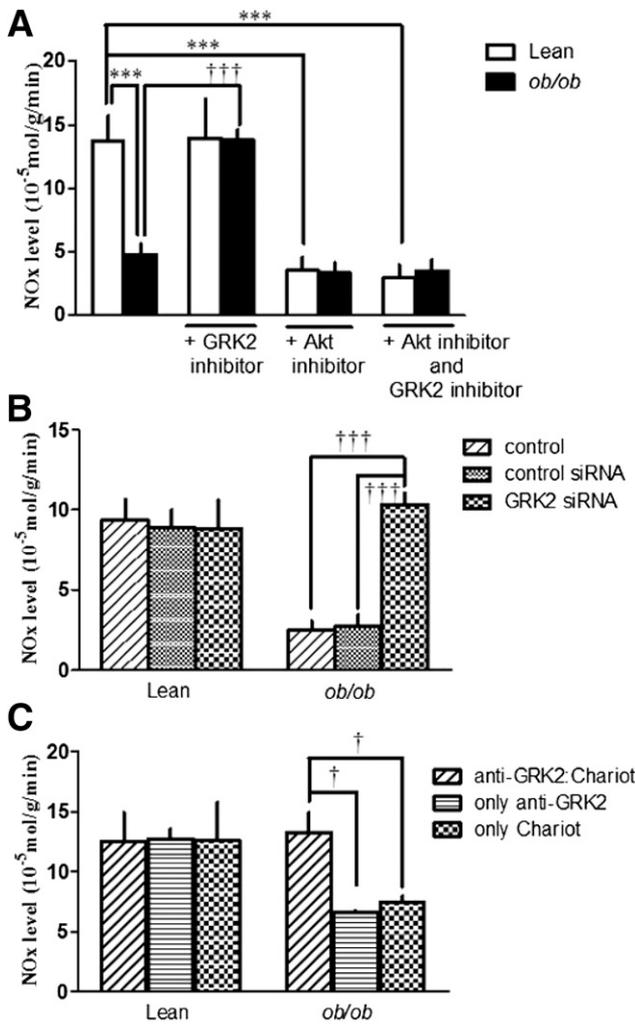


FIG. 2. Analysis of NO_x production under insulin stimulation. **A:** Aortic strips from *ob/ob* and Lean mice were treated with insulin (10^{-6} mol/L for 20 min), insulin + GRK2 inhibitor (10^{-6} mol/L), insulin + Akt inhibitor (7×10^{-7} mol/L), or insulin + both GRK2 inhibitor and Akt inhibitor. **B:** Effects of siRNA against GRK2. **C:** Effects of Chariot-mediated delivery of GRK2 antibody. Values are mean \pm SE; $n = 6-8$. *** $P < 0.001$ vs. Lean; † $P < 0.05$ vs. *ob/ob* (anti-GRK2:Chariot); ††† $P < 0.001$ vs. *ob/ob* or *ob/ob* GRK2 siRNA.

the effects of insulin on the following, but only in *ob/ob* aortas: *Thr*³⁰⁸-phosphorylated Akt (whole aortas and membranes), *Ser*¹¹⁷-phosphorylated eNOS (membranes), and *Thr*⁴⁹⁵-phosphorylated eNOS (whole aortas and membranes). Moreover, in the *ob/ob* group, GRK2 inhibitor pretreatment significantly reduced the eNOS membrane-to-cytosol ratio under insulin stimulation (Supplementary Fig. 5F).

DISCUSSION

In the current study, the most important question was whether GRK2 negatively controlled the insulin-induced Akt/eNOS pathway in aortas from diabetic mice with hyperinsulinemia. An important vascular action of insulin is its vasodilator effect, which is associated with increased NO production by endothelial cells (4,5,22,23). The results shown in the present Fig. 1A and B are consistent with our previous report (14). In that previous report, we discussed why GRK2 was increased in the diabetic aorta and how

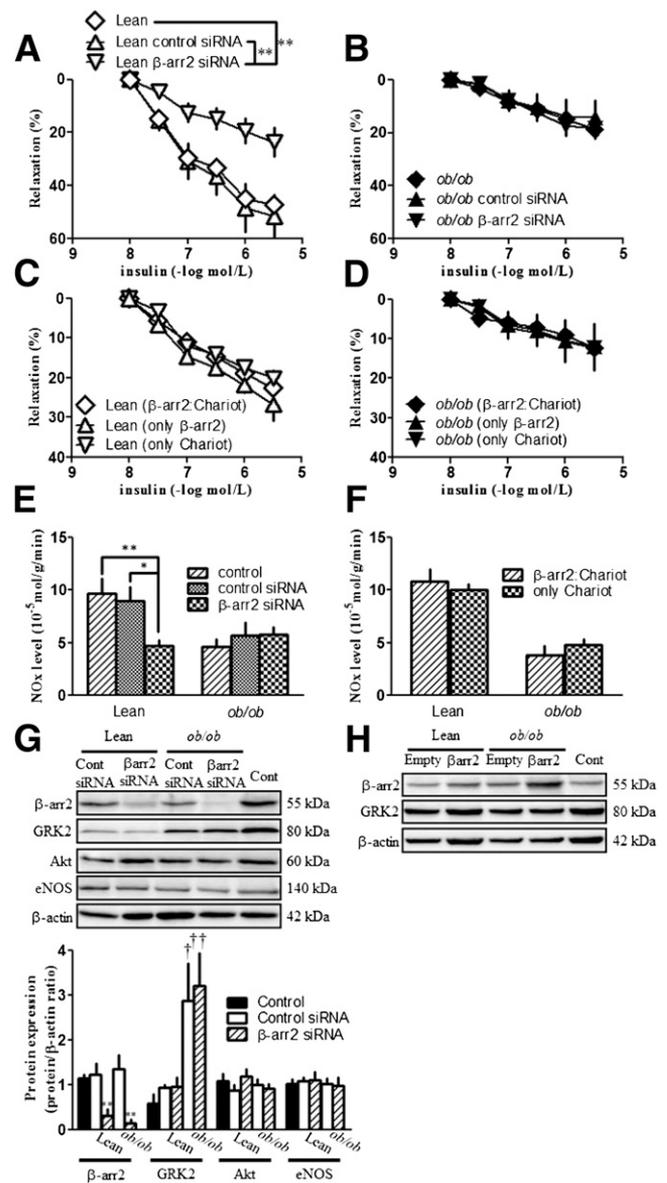


FIG. 3. Effects of Chariot-mediated delivery of β -arrestin 2 antibody (6 μ g), together with its controls (β -arrestin 2 antibody alone or Chariot alone), and siRNA against β -arrestin 2 (300 nmol/L) on insulin-induced relaxation and insulin-stimulated NO production. Insulin-induced relaxations of aortic rings from *ob/ob* (B and D) and Lean mice (A and C). E and F: NO_x production under insulin stimulation. Aortic strips were treated with insulin (10^{-6} mol/L for 20 min). Western blots showing efficiencies of siRNA transfection (G: β -arrestin 2 siRNA) and Chariot protein delivery (H: β -arrestin 2) in aortas. No effects were seen on either Akt or eNOS expression. Lanes were run on different gels, but under the same conditions. Values are mean \pm SE; $n = 5$. * $P < 0.05$ vs. Lean; ** $P < 0.01$ vs. Lean β -arrestin 2 siRNA. In G, ** $P < 0.01$ vs. control siRNA; † $P < 0.05$; †† $P < 0.01$ vs. Lean control.

it affected the dysfunction of the endothelium-dependent relaxation to insulin that is mediated via the Akt pathway. We drew the conclusion that PKC activation mediated GRK2 overactivation and that the upregulation of GRK2 led to inhibition of the insulin-induced stimulation of the Akt/eNOS pathway (14). In the current study, we were interested in the pathway downstream of GRK2. From the results, we can propose that GRK2 acts by competing for β -arrestin 2 upon insulin-induced Akt/eNOS activation in the control or diabetic aorta.

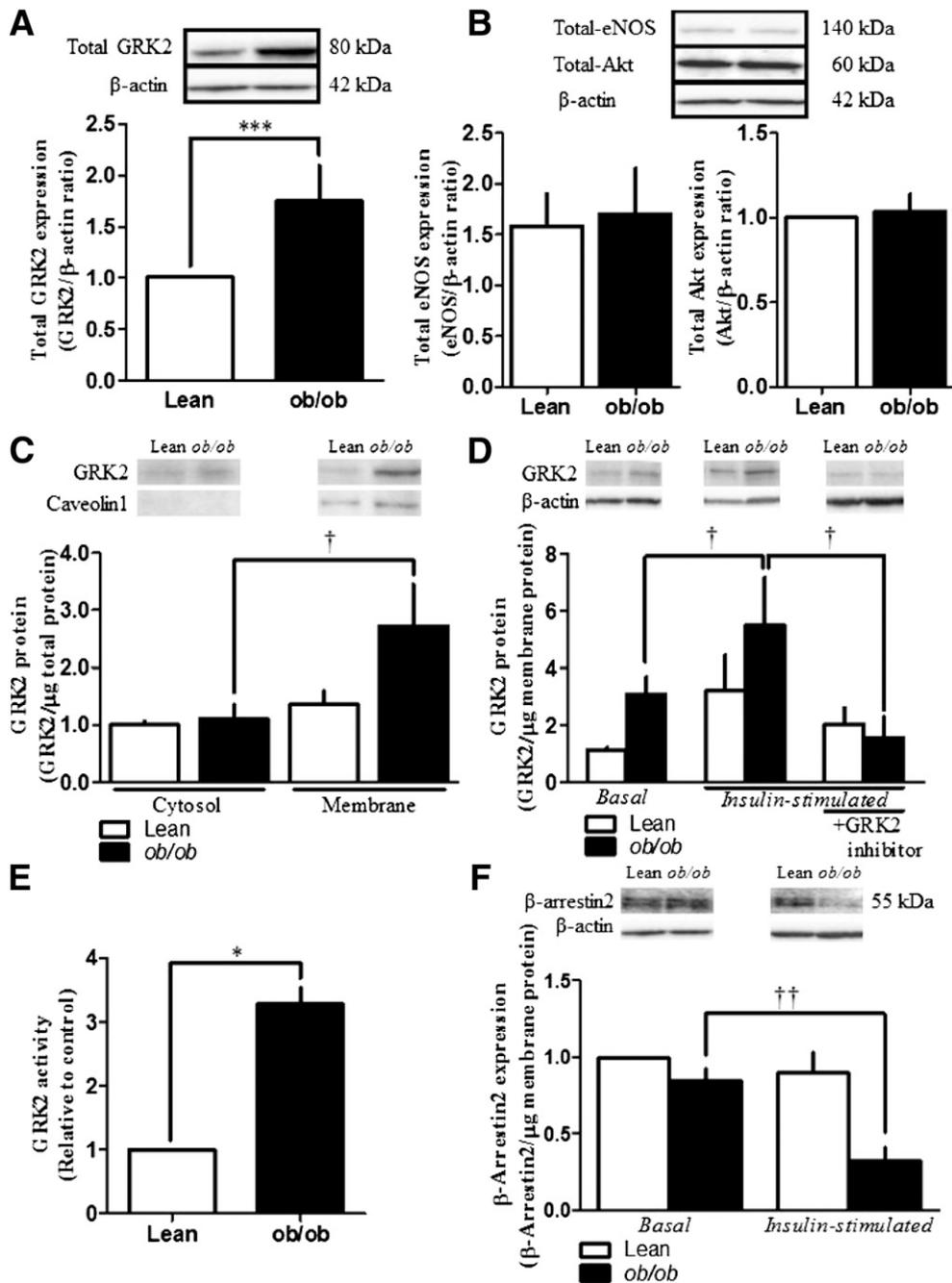


FIG. 4. Characterization of GRK2 expression in aortic strips from Lean and *ob/ob* mice. **A:** Total GRK2 expression. **B:** Total eNOS and Akt expressions. Several bands were quantified by scanning densitometry. Ratios were calculated for the optical density of GRK2 over that of β -actin. **C:** GRK2 subcellular localization. **E:** GRK2 activity. GRK2 (**D**) and β -arrestin 2 (**F**) expressions in membrane fraction. Aortic strips were treated for 24 h with vehicle (basal), insulin (10^{-6} mol/L), or insulin + GRK2 inhibitor (10^{-6} mol/L). All lysates (20 μ g protein) were prepared from aortas and used in Western blotting for GRK2 or β -arrestin 2. Lanes were run on the same gel, but were noncontiguous (except, in **E**, the GRK2 inhibitor/insulin-stimulated lane was run on a different gel). Values are means \pm SE; $n = 4$. * $P < 0.05$ vs. Lean; *** $P < 0.001$ vs. Lean; † $P < 0.05$ vs. insulin-stimulated or -nonstimulated *ob/ob*; †† $P < 0.01$ vs. *ob/ob*.

In this study, we used a GRK2 inhibitor. The efficacy and specificity of this inhibitor are unknown. However, we decided to use the inhibitor in the current study because Iino et al. (24) reported that it is selective. Indeed, in our experiment, the GRK2 inhibitor at a concentration of 10^{-6} mol/L had no effect on the insulin-induced relaxation seen in the Lean group (Fig. 1B), and the results of the experiments we performed using GRK2 siRNA and Chariot-mediated anti-GRK2 antibody were similar to the results obtained in the experiments in which we used the GRK2

inhibitor. In addition, we have already used the same GRK2 inhibitor in a study in which we showed that in *ob/ob* mice, the level of GRK2 activity was significantly reduced by pretreatment with the GRK2 inhibitor (14). Nevertheless, we look forward to the development of an inhibitor with known high specificity. In the current study, to facilitate the intracellular inhibition of GRK2, we used a newly developed carrier, Chariot (18). This noncovalent reagent allows the delivery of proteins, including antibodies, to the inside of the cell compartment. The Chariot

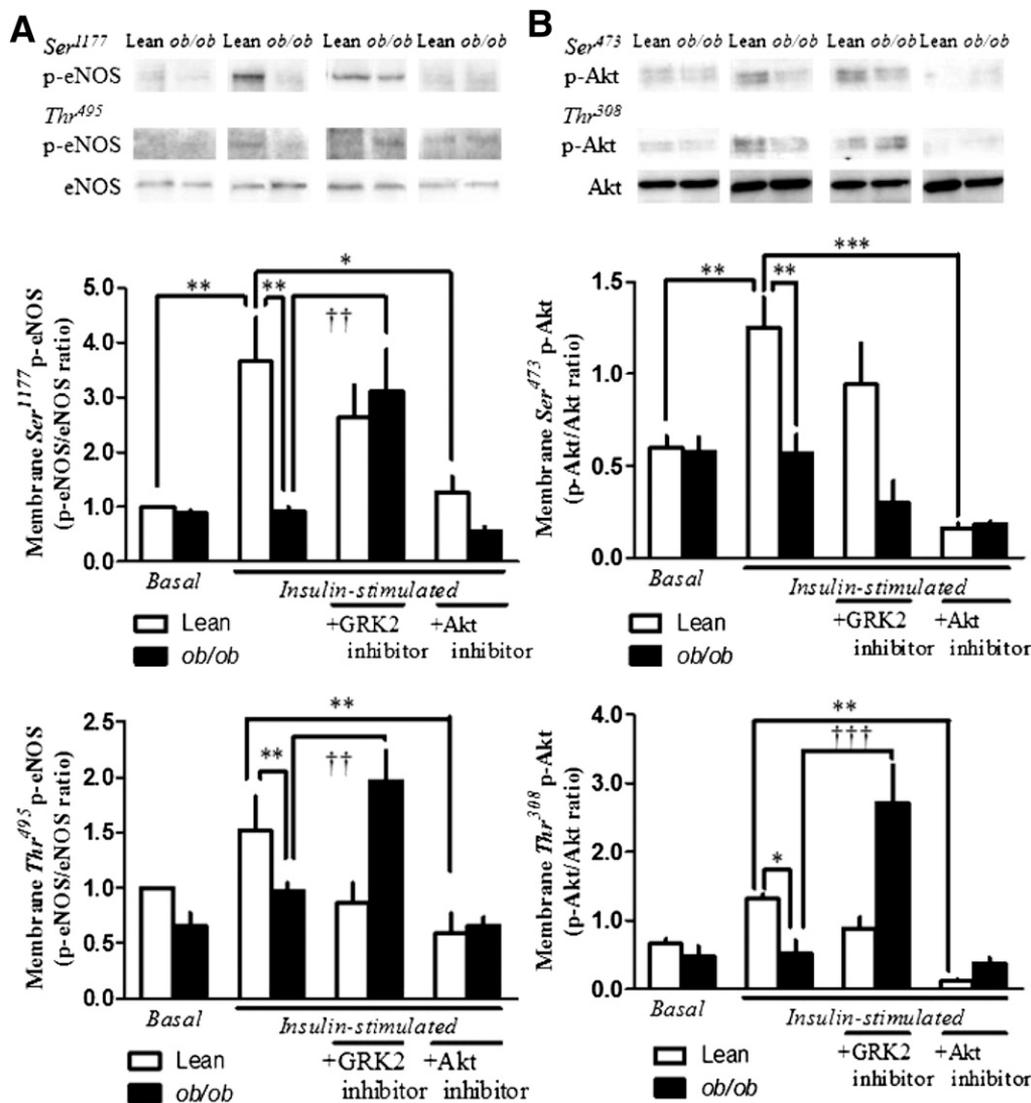


FIG. 5. Akt/eNOS pathway activity in the aortic membrane fraction. Aortic strips were treated for 24 h with vehicle (basal), insulin (10^{-6} mol/L), insulin + GRK2 inhibitor (10^{-6} mol/L), or insulin + Akt inhibitor (7×10^{-7} mol/L). All lysates (20 μ g protein) were prepared from aortas and used in Western blotting for eNOS phosphorylation at Ser¹¹⁷⁷ or Thr⁴⁹⁵ (A) or Akt phosphorylation at Ser⁴⁷³ or Thr³⁰⁸ (B). Lanes were run on the same gel but were noncontiguous. Values are means \pm SE; $n = 4$. * $P < 0.05$ vs. insulin-stimulated Lean; ** $P < 0.01$ vs. insulin-stimulated Lean; *** $P < 0.001$ vs. insulin-stimulated Lean; †† $P < 0.01$ vs. *ob/ob*; ††† $P < 0.001$ vs. *ob/ob*.

peptide then localizes to the cell nucleus, where it is degraded, leaving the macromolecule free to proceed to its target organelle. By directly delivering protein, peptide, or antibody, Chariot completely bypasses the transcription-translocation process associated with gene expression. Following its penetration through the cell membrane, the delivered Chariot-antibody complex dissociates and so liberates the antibody inside the cell. The liberated, functional antibody then diffuses throughout the cell and interacts with the target protein and would thus, for example, allow identification and/or inhibition of GRK2. The findings made by several independent groups provided a solid rationale for the use of Chariot in our experiments (25,26). Furthermore, we obtained confirmatory results by the use of a well-established method, organ culture of aortas transfected with an siRNA against GRK2. Because inhibition of GRK2 via Chariot-mediated antibody delivery and transfection with GRK2 siRNA significantly enhanced insulin-induced relaxation and NO production in the diabetic

aorta, we inferred that blocking GRK2 by these maneuvers mimics the effects of the GRK2 inhibitor. This therefore supports the specificity of the GRK2 inhibitor.

Probably the most important basic findings made in the current study were that GRK2 protein was increased in the membrane fraction of the *ob/ob* aorta and that in the *ob/ob* aorta, GRK2 activity was greatly enhanced. That aortic GRK2 levels were increased in our *ob/ob* mice is consistent with studies on other pathologic states; for instance, GRK2 is reportedly increased in hypertension and failing human hearts (27,28). GRK2 is a cytosolic protein kinase that is translocated to the membrane upon activation. Our findings may indicate a possible mechanism present in established diabetes: namely, persistent GRK2 upregulation in the aortic membrane fraction. Importantly, increased GRK2 activity is generally indicative of an inhibition of agonist-bound GPCR activity. Furthermore, we detected a shift of GRK2 from cytosol to membrane under insulin stimulation in *ob/ob* aortas, most likely indicating translocation to Akt

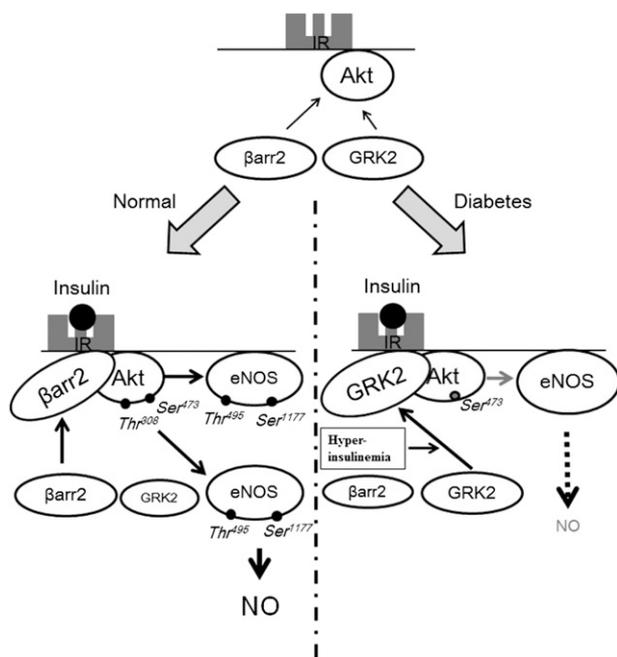


FIG. 6. Proposed model of GRK2-mediated inhibition of Akt/eNOS pathway in diabetes. In normal aortas, binding of insulin to IR activates Akt/eNOS pathway via β -arrestin 2 (β arr2) activation, and GRK2 is not translocated. In diabetes, however, GRK2, which is present in increased quantities, binds to Akt (and β arr2 is not activated). This leads to reductions of Akt/eNOS signaling and NO production in the membrane.

and eNOS. An association of GRK2 with Akt appears to participate in the control of GRK2 activity and in determining the complex subcellular distribution of the kinases (10). Our study is the first to indicate eNOS localization under insulin stimulation in the aorta, although the significance of our finding for GPCR signaling and vascular physiology remains to be established. The present data suggest that insulin regulates GRK2 in the membrane fraction in aortas from *ob/ob* mice and that such inhibition in the membrane leads to inhibition of eNOS phosphorylation via Akt phosphorylation at Thr^{308} and thereby to impairments of eNOS activation and NO production. However, further research is needed for a fuller understanding of why GRK2 activity is not normalized in established diabetic states.

Finally, we addressed the association among GRK2, β -arrestin 2, and insulin signaling. In this study, β -arrestin 2 siRNA significantly reduced insulin-induced relaxation and NO production, and no effects of excess β -arrestin 2 were observed on these responses, suggesting that insulin signaling is mediated through β -arrestin 2 and that GRK2 may stimulate formational changes in β -arrestin 2, Akt, and IR. Moreover, in the *ob/ob*, but not in Lean aortas, β -arrestin 2 was severely downregulated under insulin stimulation.

Recent data highlight a potential mechanism by which GRK2 might couple with other proteins, in particular with Akt (10). That study found that GRK2 binds directly to Akt and inhibits Akt signaling (10). There are many reports of involvements of GRK2 in multiple interactions with nonreceptor proteins such as Akt (10,29). In particular, Jiang et al. (29), using a two-hybrid system, reported interaction between GPCR-mediated signaling (for example, GRK2) and the Akt signaling pathway. Moreover, Liu et al. (10), using the immunoprecipitation method, found that

GRK2 binds directly to Akt and inhibits its activation. In the current study, we found that in *ob/ob* mice, insulin caused translocation of GRK2 to the membrane and inhibited Akt phosphorylation at Thr^{308} and that upon insulin stimulation, cytosolic β -arrestin 2 is not translocated to the membrane in *ob/ob* mice. We think that taken together, the above reports and our present results strongly suggest direct interaction between GRK2 and Akt in the *ob/ob* aorta.

From the above, our data are consistent with the following scenarios (Fig. 6). In the normal aorta, β -arrestin 2 binds to Akt under insulin stimulation. In diabetes, in contrast, insulin causes translocation of GRK2 to the membrane, where it binds to Akt, preventing β -arrestin 2 binding to Akt because GRK2 remains bound. GRK2 has been identified as serine/threonine kinases that participate, together with β -arrestin 2, in the regulation of multiple GPCRs. In contrast, insulin receptor is one of the tyrosine kinase type, not a GPCR, and insulin activates a signaling pathway involving insulin receptor phosphatidylinositol 3-kinase and Akt, and this leads to eNOS activation. So, we think that GRK2 does not act directly on insulin receptor, and others have reported that GRK2 does not affect insulin receptor phosphorylation (30). However, Luan et al. (13) reported that insulin stimulated the formation of a new β -arrestin 2 signal complex, in which β -arrestin 2 scaffolds Akt to insulin receptor. Further, GPCR phosphorylation by GRK2 induces β -arrestin 2, and recently, we and others (12,14,19) have reported that GRK2 is induced by insulin stimulation. So, we speculate that GRK2 and β -arrestin 2 compete for the insulin receptor upon insulin stimulation. However, whether the induction by insulin of Akt/eNOS activation is related to this agonist's potential cleaving effect on GRK2 is an issue that remains to be investigated. We can say that in our study, the insulin-stimulated Akt and eNOS phosphorylations were reduced in the membrane fraction from the aortas of *ob/ob* mice and improved by GRK2 inhibitor (Fig. 5). In this context, further research is needed for a better understanding of how GRK2 and β -arrestin 2 activities might be orchestrated and their dependence on insulin receptor. It is our aim to examine this in future papers (involving phosphorylation of insulin receptor after insulin stimulation under treatment with GRK2 inhibitor). In our scheme (Fig. 6), the interaction between GRK2 and Akt directly inhibits Akt phosphorylation at Thr^{308} , resulting in decreased eNOS phosphorylation and a consequent reduction in NO production. Although we do not yet know precisely how GRK2 binds to Akt or how β -arrestin 2 forms a complex with GRK2 and Akt, it seems likely that these interactions can take place in the absence of a larger binding complex, as already proposed (10,13). In summary, we have provided important new evidence highlighting the possible role played by GRK2 in negatively regulating insulin/ β -arrestin 2/Akt/eNOS-mediated NO production in type 2 diabetes. Our results suggest that a maneuver that inhibits GRK2 might counteract the endothelial dysfunction that occurs via the Akt/eNOS pathway in *ob/ob* mice. If this is true in human type 2 diabetes, GRK2 inhibition may find a place in advanced vascular therapeutic strategies.

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No potential conflicts of interest relevant to this article were reported.

K.T. developed the project, researched data, and wrote the manuscript. T.M. and T.K. helped develop the project, performed some experiments, and wrote the manuscript. K.K. helped plan the project. T.K. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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