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Dysfunction of endothelium-dependent relaxation to insulin via PKC-mediated GRK2/Akt activation in aortas of *ob/ob* mice

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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* 301: H571–H583, 2011. First published May 13, 2011; doi:10.1152/ajpheart.01189.2010.—In diabetic states, hyperinsulinemia may negatively regulate Akt/endothelial nitric oxide synthase (eNOS) activation. Our main aim was to investigate whether and how insulin might negatively regulate Akt/eNOS activities via G protein-coupled receptor kinase 2 (GRK2) in aortas from *ob/ob* mice. Endothelium-dependent relaxation was measured in aortic rings from *ob/ob* mice (a type 2 diabetes model). GRK2, β-arrestin2, and Akt/eNOS signaling-pathway protein levels and activities were mainly assayed by Western blotting. Plasma insulin was significantly elevated in *ob/ob* mice. Insulin-induced relaxation was significantly decreased in the *ob/ob* aortas [vs. age-matched control (lean) ones]. The response in *ob/ob* aortas was enhanced by PKC inhibitor or GRK2 inhibitor. Akt (at Thr^{308}) phosphorylation and eNOS (at Ser^{1177}) phosphorylation, and also the β-arrestin2 protein level, were markedly decreased in the membrane fraction of insulin-stimulated *ob/ob* aortas (vs. insulin-stimulated lean ones). These membrane-fraction expressions were enhanced by GRK2 inhibitor and by PKC inhibitor in the *ob/ob* group but not in the lean group. PKC activity was much greater in *ob/ob* than in lean aortas. GRK2 protein and activity levels were increased in *ob/ob* and were greatly reduced by GRK2 inhibitor or PKC inhibitor pretreatment. These results suggest that in the aorta in diabetic mice with hyperinsulinemia an upregulation of GRK2 and a decrease in β-arrestin2 inhibit insulin-induced stimulation of the Akt/eNOS pathway and that GRK2 overactivation may result from an increase in PKC activity.

hyperinsulinemia; insulin signaling; Akt/endothelial nitric oxide synthase pathway; membrane fraction; G protein-coupled receptor kinase 2; protein kinase C

INSULIN RESISTANCE, A HALLMARK of type 2 diabetes, involves a relative inability of insulin to stimulate insulin-receptor signaling. This leads to impaired endothelial function, which has become a serious public-health threat because of its causal involvement in hypertension and other cardiovascular diseases (8, 10, 35). One of the most important functions of the endothelium is the production of nitric oxide (NO) in response to a variety of hormonal, mechanical, and chemical stimuli. A previous investigation (44) suggested that impaired NO production can result from endothelial dysfunction. Several reports (11, 13) have suggested that for efficient NO production in response to a variety of stimuli, phosphorylation of endothelial nitric oxide synthase (eNOS) via Akt is required, and we and others (21, 38) have supported such a role for the Akt/eNOS pathway in the endothelium on the grounds that inhibition of agonist-induced activation of the Akt/eNOS pathway leads to impaired NO availability.

Insulin has multiple physiological effects on vascular tissues, including vasodilation (2, 12, 40, 52), which may be endothelial cell dependent and which can be attenuated by inhibitors of Akt and NOS (2, 40, 48, 50). We and others (21, 53) have suggested that insulin can increase the production of NO by activating the Akt/eNOS pathway via insulin receptors.

Many factors present in insulin-resistant states may cause endothelial dysfunction (37), among them postprandial hyperglycemia, elevated plasma concentrations of free fatty acids, activation of the renin-angiotensin system, oxidative stress, and proinflammatory cytokines. Naruse et al. (32) suggested that activation of protein kinase C (PKC) may play an important role in the vascular complications of diabetes. The PKC superfamily consists of conventional, novel, and atypical isoforms of serine/threonine protein kinases. The lipid-regulated subgroup of novel PKCs has been broadly implicated in the development of insulin resistance (42), and functional inhibition of PKC improves glucose homeostasis in rodent models of type 2 diabetes (6, 43). Diabetes or high glucose concentrations will activate PKC in certain vascular cells, most likely via a de novo synthesis of diacylglycerol from glucose or free fatty acids (37). A selective PKC inhibitor has been shown to improve the decreased retinal blood flow seen in diabetic rats (17), the decreased endothelium-dependent vasodilation observed in humans during hyperglycemia (4), and the decreased vascular NO production seen in Zucker fatty rats (5). Moreover, *1*) PKC activators inhibit the insulin-induced activation of eNOS, *2*) overexpression of PKC within endothelial cells inhibits the insulin-induced stimulation of eNOS expression, and *3*) PKC activation selectively inhibits activation of phosphatidylinositol 3 (PI3)-kinase/Akt in the vasculature (14, 22). However, to date there has been no systematic demonstration indicating how PKC might be responsible for decreasing PI3-kinase/Akt-dependent eNOS activation in vascular cells.

G protein-coupled receptor (GPCR) kinases (GRKs) were initially identified as serine/threonine kinases that participate, together with β-arrestins, in the regulation of multiple GPCRs. The GRKs constitute a group of protein kinases that specifically recognize and phosphorylate agonist-activated GPCRs (24, 27, 36, 47). 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 (24, 27, 33). β-Arrestin2 binds to the phosphorylated receptor, leading to uncoupling from G proteins and consequent receptor desensitization. As a result of β-arrestin2 binding, phosphorylated receptors also become targets for endocytosis, a process that classically serves to

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resensitize receptors and recycle them back to the plasma membrane (39).

The GRK2/β-arrestin2 system plays a pivotal role in the desensitization of GPCR function. Alongside the negative inhibitory role of GRK2/β-arrestin2, emerging evidence indicates that GRK2 and β-arrestin2 are each able to interact with a variety of cellular proteins involved in signal transduction, thus contributing to signal propagation at defined cellular locations. In the case of β-arrestin2, several laboratories (36, 39) have shown that these proteins can act as scaffold molecules that bring different signaling molecules such as Akt, c-Src, JNK-3, and ERK1/2 into the receptor complex. Therefore, GRK2-mediated β-arrestin2 recruitment is critical for triggering the modulation of important intracellular signaling pathways, and it thereby contributes to the overall cellular response to the presence of an agonist. Recently, it was reported that insulin stimulates the formation of a new β-arrestin2 signal complex in which β-arrestin2 acts as a scaffold for the translocation of Akt and Src to the insulin receptor, even though the insulin receptor is not a GPCR (25). Moreover, the existence of G protein-independent, GRK/β-arrestin-mediated signal transduction has been established for several non-GPCRs (9, 23).

Against the above background, we designed the present study to test the following three hypotheses; that is, that in diabetes: *1*) GRK2 negatively regulates the Akt/eNOS pathway under insulin stimulation; *2*) hyperinsulinemia increases PKC activity, leading to increased GRK2 activity and translocation to membranes; and *3*) an overactivation of GRK2 can hinder the establishment of a normal relationship between β-arrestin2 and Akt under insulin stimulation.

MATERIALS AND METHODS

Animals and experimental design. Male *ob/ob* C57BL/6J mice and age-matched lean C57BL/6J mice were obtained at the age of 5 wk (Oriental Yeast Industry, Tokyo, Japan). All animals were allowed a standard laboratory diet and water ad libitum. This study was approved by the Hoshi University Animal Care and Use Committee (which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology, Japan). Mice were studied at 27–32 wk of age.

Measurement of blood parameters and blood pressure. Plasma parameters and systolic blood pressure were measured as described previously (29). Briefly, plasma glucose, cholesterol, HDL, nonesterified fatty acids, and triglyceride were determined by the use of a commercially available enzyme kit (Wako Chemical, Osaka, Japan). Plasma insulin was measured by enzyme immunoassay (Shibayagi, Shibukawa, Japan). The LDL level was derived from the above data using the Friedewald formula: LDL cholesterol = total cholesterol − HDL − (1/5) triglyceride. Plasma leptin was determined by ELISA (Morinaga Institute of Biological Science, Yokohama, Japan). For blood pressure measurements, a given mouse was kept in a constant-temperature hot-box at 37°C for 15 min. Then, its blood pressure was measured by the tail-cuff method using a blood pressure analyzer (BP-98A; Softron, Tokyo, Japan) ≥5 min after the mouse had been put in a restrainer for the purpose of measuring.

Intraperitoneal glucose tolerance test. Glucose (2.0 g/kg) was given as a 30% solution by intraperitoneal injection in conscious, fasting animals, as previously described (21). Blood samples were collected sequentially just before and at 15, 30, 60, and 120 min after the injection.

Measurement of isometric force. Mice were anesthetized with diethyl ether and killed by decapitation. A section of the thoracic aorta from the region between the aortic arch and the diaphragm was removed and placed in oxygenated, modified Krebs-Henseleit solution (KHS). The aorta (cut into rings) was placed in a bath containing 10 ml of KHS, with one end of each strip connected to a tissue holder and the other to a force-displacement transducer, as previously described (21). For the relaxation studies, rings were precontracted with an equieffective contraction of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-6}-3 × 10^{-6} M). The presence of stable and reproducible contractions in each aortic preparation were confirmed by comparison with the first contraction induced using 80 mM K^+, the latter being taken as 100%. When the $PGF_{2\alpha}$-induced contraction had reached a plateau level, insulin (10^{-8}-3 × 10^{-6} M) was added in a cumulative manner. When the effects of Akt inhibitor (10^{-6} M), GRK2 inhibitor {methyl[(5-nitro-2-furyl)vinyl]−2-furoate; 10^{-6} M}, N^G-nitro-L-arginine (L-NNA; 10^{-4} M), or PKC inhibitor (chelerythrine chloride; 10^{-6} M) on the responses to these relaxant agents were to examined, one of these inhibitors was added to the bath 30 min before the application of $PGF_{2\alpha}$. In some strips, the endothelium was removed by infusing a 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate solution (0.1%, for 60 s), successful removal being functionally confirmed by the absence of relaxation to 10^{-5} M acetylcholine.

Measurement of PKC activity. PKC activity was determined by means of a commercially available PKC assay kit (Assay Designs, Ann Arbor, MI).

Preparation of aortic membranes. Murine aortic membranes were prepared as described by Marwaha and Lokhandwala (28). Briefly, aortas were homogenized in a buffer (10 mM Tris·HCl, 250 mM sucrose, 2 mM PMSF, and protease inhibitor cocktail, pH 7.4) and then centrifuged at 20,000 *g* for 25 min at 4°C. The upper fluffy layer of the pellet was resuspended in the homogenization buffer. This layer was considered to represent the membrane fraction, and this was confirmed by Western blotting with an against caveolin-1, which exists only on the inner surface of the membrane.

Measurement of the protein expressions of GRK2, β-arrestin2, Akt, and eNOS (by Western blotting). Aortas were homogenized in ice-cold lysis buffer containing 50 mM Tris·HCl buffer (pH 7.5), 150 mM NaCl, 1% Triton-X, and protease inhibitor cocktail, as previously described (20). Homogenates were centrifuged at 13,000 *g* for 5 min. The supernatant was sonicated at 4°C, and the proteins were solubilized in Laemmli's buffer containing mercaptoethanol. The protein concentration was determined by means of a bicinchoninic acid protein assay regent kit (Pierce). These samples or the membrane fractions of samples (25 μg/lane) were resolved by electrophoresis on 10% SDS-PAGE gels and then transferred onto PVDF membranes. Briefly, after the residual protein sites on the membrane were blocked, the membrane was incubated with anti-GRK2 antibody (1:500), anti-β-arrestin2 antibody (1:100), anti-Akt antibody (1:1,000), anti-eNOS antibody (1:1,000), or β-actin antibody (1:5,000) in blocking solution. To normalize the data, we used β-actin as a housekeeping protein.

Measurements of Thr^{308} Akt, Ser^{670} GRK2, and Ser^{1177} eNOS phosphorylations. Phosphorylation of Akt at Thr^{308}, GRK2 at Ser^{670}, or eNOS at Ser^{1177} is coincident with Akt, GRK2, or eNOS activation, respectively, in vivo. Therefore, in vitro, such phosphorylations have been used as markers for Akt, GRK2, and/or eNOS activity (with the aid of phospho-specifc antibodies). Here, each phosphorylation was examined using Western blotting. Each aorta was set up under tension in an organ bath under the same conditions as those used for the measurement of isometric force, the bath containing 0.5 ml KHS at 37°C. Samples of effluent were collected on two occasions as follows: insulin-stimulated samples, for a 20-min period after application of 10^{-6} M insulin (with or without GRK2 inhibitor or PKC inhibitor); and basal samples, for a 20-min period without insulin stimulation. The samples were partitioned into the membrane fractions, and Western blotting was performed. The membrane was incubated with one of the following antibodies: anti-phospho Akt Thr^{308} (1:500), anti-phospho GRK2 Ser^{670} (1: 1,000), or anti-phospho eNOS Ser^{1177} (1:1,000) in blocking solution. The optical densities of the bands on

Table 1. *Values of various parameters in ob/ob and lean mice*

	Lean (n = 10)	*ob/ob* (n = 10)
Body weight, g	35.3 ± 0.6	71.4 ± 1.1†
SBP, mmHg	104.1 ± 2.4	122.6 ± 2.6†
Glucose, mg/dl	183.0 ± 10.8	185.4 ± 16.7
Insulin, ng/ml	1.9 ± 0.3	80.4 ± 13.7†
Total cholesterol, mg/dl	89.8 ± 6.0	211.9 ± 9.4†
HDL, mg/dl	50.5 ± 4.3	100.0 ± 5.0†
LDL, mg/dl	20.5 ± 5.5	78.8 ± 12.8*
NEFA, mEq/l	1.6 ± 0.1	1.8 ± 0.1
Triglyceride, mg/dl	143.2 ± 23.2	165.3 ± 12.4
Leptin, ng/ml	8.0 ± 1.6	0.5 ± 0.3†

Values are means ± SE; n = number of determinations. SBP, systolic blood pressure; NEFA, nonesterified fatty acids. *$P < 0.01$, †$P < 0.001$ vs. lean mice.

the film were quantified using densitometry, with correction for the optical density of the corresponding total protein band.

Statistical analysis. Each relaxation response is expressed as a percentage of the contraction induced by $PGF_{2\alpha}$. Values are means ± SE. When appropriate, statistical differences were assessed by Dunnett's 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. GRK2 activity was taken to be the reciprocal of the phospho-GRK2 expression.

Materials. Insulin from porcine pancreas, L-NNA, and monoclonal β-actin antibody were all purchased from Sigma Chemical (St. Louis, MO). Akt inhibitor {1L-6-hydroxymethyl-chiro-inositol 2-[(R)−2-*O*-methyl-3-*O*-octadecyl-*sn*-glycerocarbonate]}, GRK2 inhibitor {methyl[(5-nitro-2-furyl)vinyl]-2-furoate}, and PKC inhibitor (chelerythrine chloride) were manufactured by Calbiochem (La Jolla, CA). All drugs were dissolved in saline, unless otherwise noted. GRK2 inhibitor was dissolved in DMSO. All concentrations are expressed as the final molar concentration of the base in the organ bath. Horseradish-peroxidase-linked secondary anti-mouse or anti-rabbit antibody was purchased from Promega (Madison, WI), while antibodies against GRK2 and β-arrestin2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Akt, phosphorylated Akt at Thr^{308}, and phosphorylated eNOS at Ser^{1177} were obtained from Cell Signaling Technology (Danvers, MA), while the antibody against eNOS was from BD Bioscience (San Jose, CA). The antibody against phosphorylated GRK2 at Ser^{670} was obtained from Millipore (Temecula, CA).

RESULTS

General parameters. As shown in Table 1, at the time of the experiment (when the mice were 27- to 32-wk-old), the body weight of the *ob/ob* mice was higher than that of the lean mice (the age-matched nondiabetic control). Plasma insulin and plasma parameters relating to lipid metabolism (total cholesterol, HDL, and LDL) were each significantly elevated in the *ob/ob* mice. Plasma glucose, triglyceride, and nonesterified fatty acids were not different between the *ob/ob* mice and the lean mice. The plasma leptin level was significantly lower in the *ob/ob* mice than in the lean mice. We investigated glucose tolerance test. In the result, after an intraperitoneal glucose injection (2.0 g/kg) in lean mice, postload plasma glucose and insulin levels returned to basal values at ~120 min (Fig. 1). In *ob/ob* mice, the postload glucose disappearance was slower than in lean mice; plasma glucose reached 566 ± 45 mg/dl at 30 min after the glucose injection, and then slowly decreased but had not returned to the based level at 120 min. Glucose administration in *ob/ob* mice did not increase the circulating insulin level, which was still raised (vs. lean) at 120 min (Fig. 1), suggesting that *ob/ob* mice exhibited insulin resistance. Systolic blood pressure was significantly higher in the *ob/ob* mice than in the lean mice.

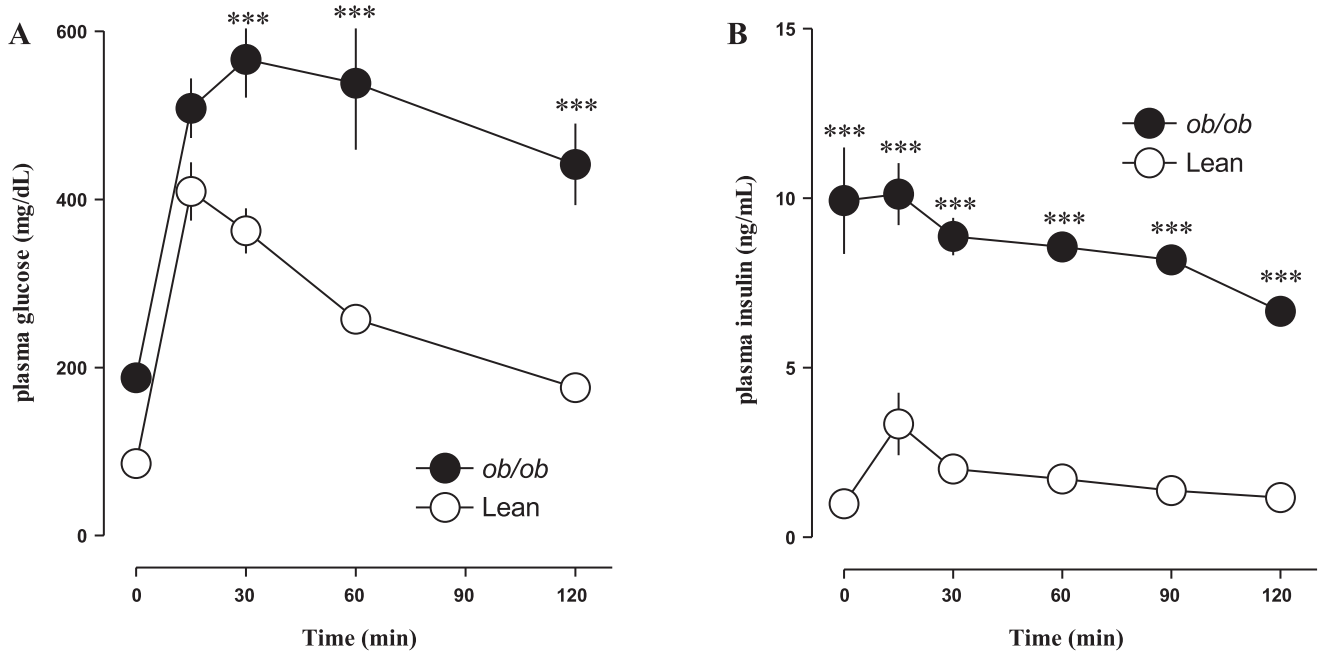


Fig. 1. Plasma glucose (*A*) and insulin (*B*) levels during glucose tolerance test in *ob/ob* and lean mice. Glucose was administered at 2.0 g/kg. Values are means ± SE; n = 6. ***$P < 0.001$ vs. lean.

Effects of PKC, Akt, eNOS, and GRK2 inhibitors on insulin-induced relaxation in ob/ob aorta. Endothelial dysfunction is an initial marker of several physiological conditions, such as altered anticoagulant and anti-inflammatory properties of the endothelium, impaired modulation of vascular growth, and dysregulation of vascular remodeling. The term endothelial dysfunction has been used to refer to an impairment of endothelium-dependent relaxation caused by a loss of nitric oxide (NO) bioactivity within the vessel wall. The bioactivity of NO depends, in part, on its interaction with Akt/eNOS pathway signaling. We (21) previously demonstrated that in aortas isolated from another type 2 diabetic model, nicotinamide + streptozotocin (STZ)-induced diabetic mice, insulin-induced relaxation is greatly attenuated and that this attenuation results from an impairment of the PI3-K/Akt signaling pathway (a major cause of endothelial dysfunction). Here, to evaluate endothelial function, the vasorelaxation responses to insulin and ACh were examined in *ob/ob* and lean aortas. The ACh-induced endothelium-dependent relaxation, a classical endothelium-dependent relaxation, was not different between *ob/ob* and lean mice (data not shown). However, previously we (21) reported that the new type 2 diabetic model mentioned above (nicotinamide + STZ-induced diabetic model) did not exhibit impairments of either ACh-induced relaxation or NO production (which are not mediated via the PI3-K/Akt signal pathway). Insulin-induced concentration-dependent relaxations of aortic rings were obtained from the *ob/ob* mice and lean mice, but the relaxation was significantly weaker in the *ob/ob* group (Fig. 2*A*), suggesting a reduction in Akt/eNOS-pathway activation. Indeed, as revealed previously, the responses to insulin in the lean group were significantly inhibited after either inhibition of Akt (using Akt inhibitor; 10^{-6} M) or inhibition of NOS (using L-NNA; 10^{-4} M; Fig. 2, *B* and *C*; Ref. 21). *Ob/ob* mice are similar to the new type 2 diabetic model mentioned

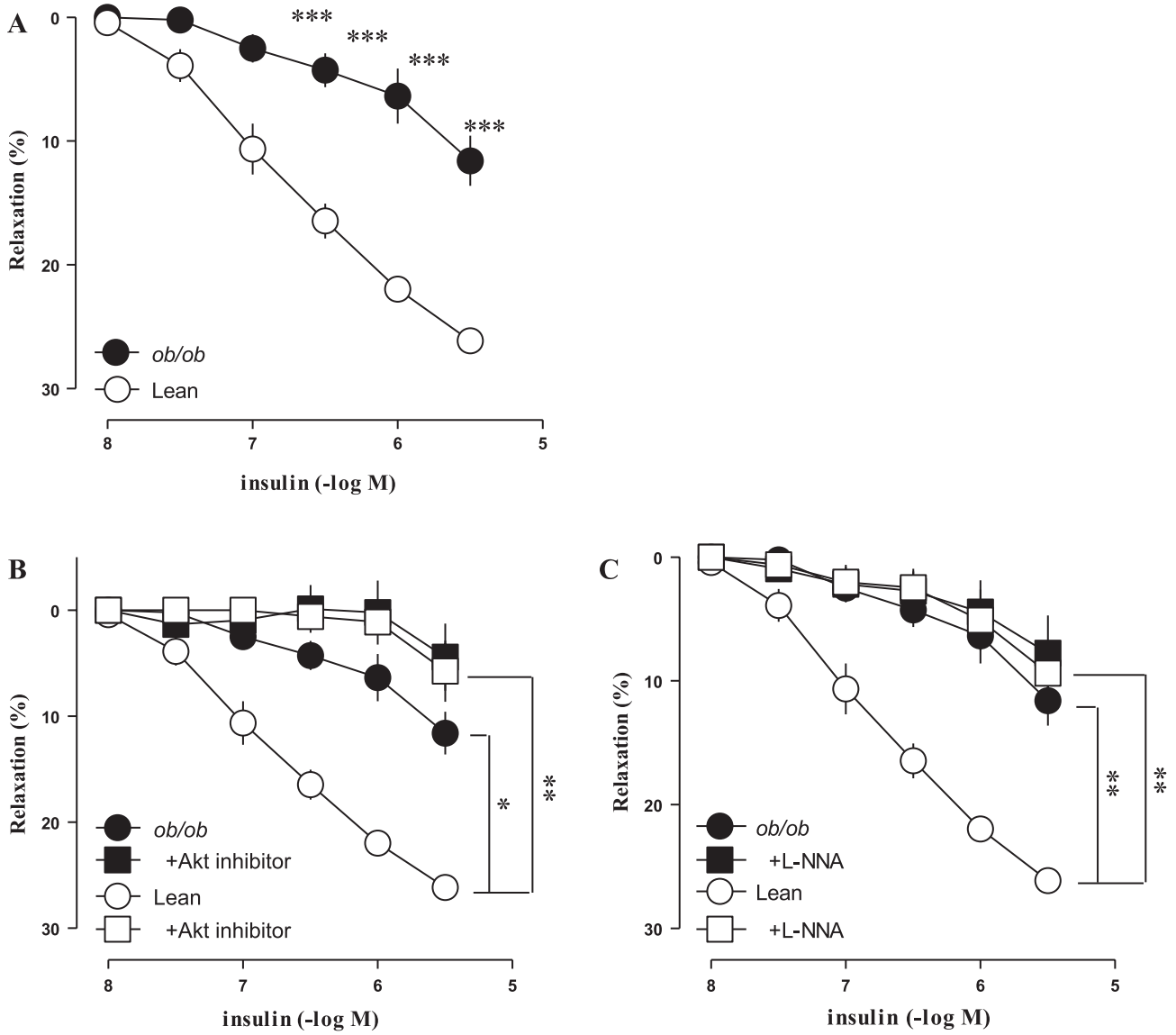


Fig. 2. Concentration-response curves for insulin-induced relaxations. *A*. insulin-induced relaxation of aortic rings from *ob/ob* and lean mice. *B* and *C*: effects of Akt inhibitor (*C*; 10^{-6} M) and N^G-nitro-L-arginine (L-NNA; *D*; 10^{-4} M) on insulin-induced relaxations of aortic rings from *ob/ob* and lean mice. Values are mean ± SE; $n = 4$. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. lean mice.

above. Insulin-induced relaxation was mostly blocked by endothelial denudation of aortic rings from both *ob/ob* and lean mice (data not shown), suggesting that the insulin-induced relaxation was an endothelium-dependent relaxation, and when taken together with Fig. 2*A*, that endothelial function was impaired in aortas from *ob/ob* mice.

Published evidence (22) suggests that PKC activation inhibits the activation of eNOS by insulin. Therefore, we examined the effects of the broad-spectrum PKC inhibitor chelerythrine chloride (10^{-6} M) on insulin-induced aortic relaxations (Fig. 3, *A* and *B*). In the lean group, the relaxation was significantly weaker after preincubation with the PKC inhibitor. By contrast, in the *ob/ob* group it was significantly enhanced. These results suggest that PKC may be involved in the regulation of the insulin-induced relaxation, with the PKC pathway exerting a negative regulatory influence in the *ob/ob* aorta.

Since GRK2 regulates the Akt/eNOS pathway (24), we examined the effect of GRK2 on the insulin-induced relaxation. This relaxation was not significantly altered by GRK2 inhibitor (10^{-6} M) in the lean aortas (Fig. 3*C*), but in the *ob/ob* aortas it was significantly increased (Fig. 3*D*). These results suggest that in the *ob/ob* aorta, GRK2 might downregulate the insulin-induced activation of the Akt/eNOS pathway.

Measurement of PKC activity in ob/ob mice. Published evidence suggests that PKC activation is consistently present in obese and insulin-resistant subjects (15). Therefore, we examined PKC activity. As illustrated in Fig. 4, the basal PKC activity was significantly higher (~3.5-fold) in the *ob/ob* aorta than in the lean aorta.

Characterization of GRK2 in ob/ob mice. From the above results, we hypothesized that GRK2 might downregulate Akt and eNOS activations in diabetes. In aortas from the present

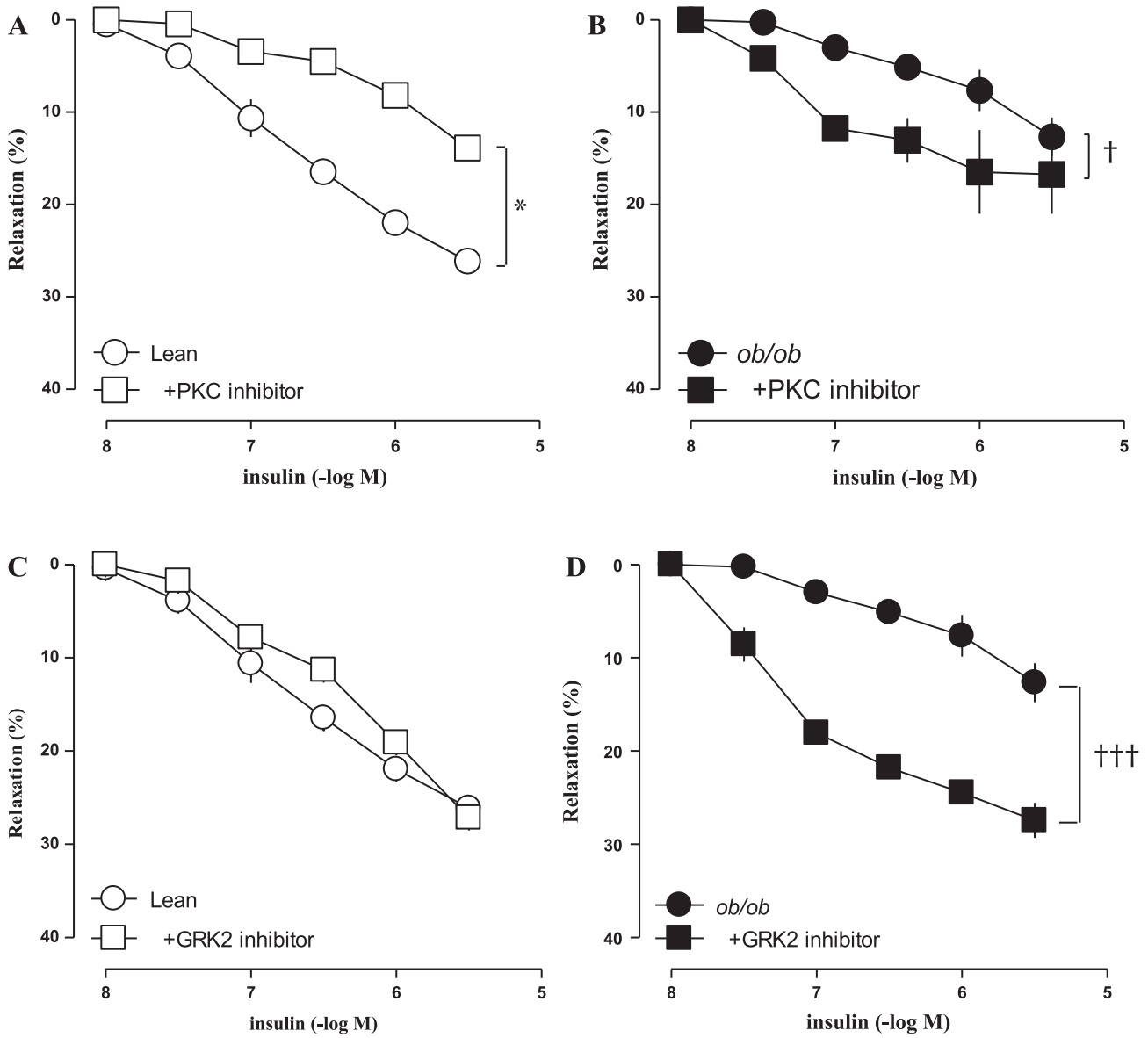


Fig. 3. Effects of PKC inhibitor (*A* and *B*) or G protein-coupled receptor kinase 2 (GRK2) inhibitor (*C* and *D*) on insulin-induced relaxations of aortic rings from *ob/ob* and lean mice. Aortic rings were preincubated with PKC inhibitor (10^{-6} M) or GRK2 inhibitor (10^{-6} M) for 30 min. Values are mean ± SE; $n = 4$. *$P < 0.05$ vs. lean mice. †$P < 0.05$ vs. *ob/ob* mice. †††$P < 0.001$ vs. *ob/ob* mice.

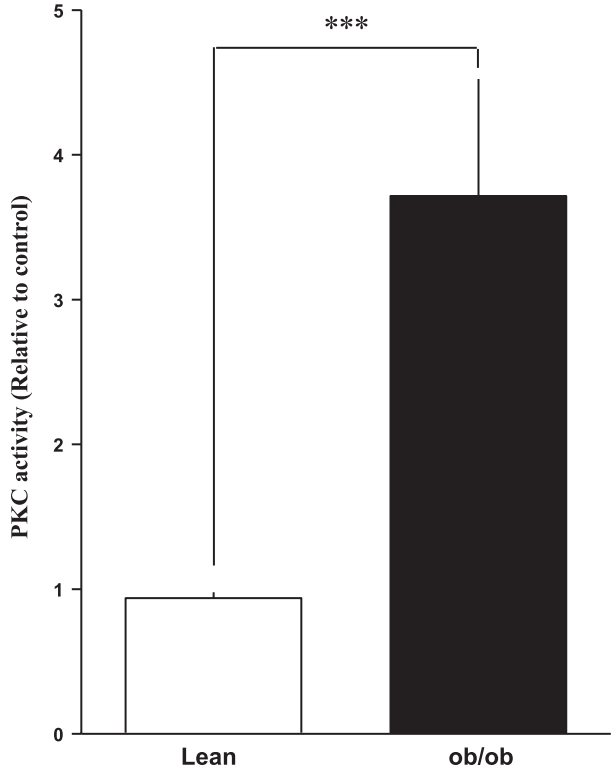


Fig. 4. PKC activity in the aorta. Basal PKC activity was determined in aortic strips from *ob/ob* and lean mice. Values are means ± SE; $n = 6$. ***$P < 0.001$ vs. lean mice.

ob/ob mice, total GRK2 expression was significantly increased (vs. lean aortas) (Fig. 5*A*).

Since GRK2 is a cytosolic protein kinase that is translocated to the membrane upon activation, we examined GRK2 activity and the subcellular distribution of GRK2 protein. Caveolin protein expression was detected only in the membrane fraction in *ob/ob* and lean mice (data not shown). Phosphorylation of GRK2 induces a decrease in the activity of this protein. Therefore, we assessed GRK2 activity by measuring its phosphorylation at Ser^{670} (51). In the basal condition, GRK2 activity was significantly higher (~3-fold) in the *ob/ob* aorta than in the lean aorta (Fig. 5*B*). There was a greater GRK2 expression in membranes isolated from *ob/ob* aortas than in those from lean ones (Fig. 5*C*). Long-term insulin treatment, leading to hyperinsulinemia, can enhance the sensitivity of blood vessels to vasoconstrictors and cause hypertension and arteriosclerosis (19). Therefore, we hypothesized that in diabetes insulin might cause GRK2 activation and also its translocation to the membrane. To test this idea, we studied GRK2 expression under insulin stimulation in aortic membranes (Fig. 5, *D* and *E*). Incubating aortas from lean mice with insulin increased GRK2 expression in the membranes. In the basal condition, membranes from the *ob/ob* aortas already exhibited increased GRK2 expression, and it was not increased further by insulin stimulation. Insulin did not increase translocation of GRK2 to the membrane in the presence of either the GRK2 inhibitor or PKC inhibitor in the lean group or in the *ob/ob* group.

Relationship between GRK2 activity and β-arrestin2 under insulin stimulation. In the lean aorta, GRK2 activity was significantly higher under insulin treatment than in the basal condition (Fig. 6*A*). As in the case of GRK2 expression in the membranes, in the *ob/ob* aorta GRK2 activity was already high in the basal condition, and it was not increased further by insulin stimulation (Fig. 6*B*). The level of GRK2 activity seen under insulin stimulation was significantly reduced by pretreatment with either GRK2 inhibitor or PKC inhibitor in the *ob/ob* group (Fig. 6*B*).

Since the GRK2-arrestin system plays a pivotal role in the signal-transduction cascade, we measured the β-arrestin2 expression in aortic membranes (Fig. 6*C*). β-Arrestin2 protein expression in the membrane fraction under insulin stimulation was lower in the *ob/ob* group than in the lean group. Insulin-induced β-arrestin2 expression was increased by pretreatment with GRK2 inhibitor or PKC inhibitor in *ob/ob* aortas (Fig. 6*C*).

Insulin leads to akt and eNOS phosphorylations via GRK2 in ob/ob aorta. Use of anti-eNOS antibody or anti-Akt antibody allowed detection of an immunoreactive protein with a molecular mass of 140 or 60 kDa, respectively. In the basal (unstimulated) condition, neither the eNOS protein nor Akt protein expression level differed between the *ob/ob* and lean groups (Fig. 7, *A–C*).

We next investigated whether insulin stimulation led to eNOS phosphorylation via changes in GRK2 and PKC in the aorta (Fig. 7, *D* and *E*). Insulin stimulation led to a significant increase in eNOS phosphorylation at Ser^{1177} in lean aortas but not in *ob/ob* aortas. Interestingly, pretreatment with GRK2 inhibitor or PKC inhibitor increased eNOS phosphorylation under insulin stimulation only in the *ob/ob* aortas.

Since eNOS is activated by phosphorylated Akt, we examined whether insulin-induced Akt phosphorylation might be controlled by GRK2 (Fig. 7, *D* and *F*). We confirmed that insulin treatment led to Akt phosphorylation at Thr^{308} in the lean group. In aortas stimulated with insulin, the level of Akt phosphorylation was significantly lower in the *ob/ob* aorta than in the lean aorta. Importantly, pretreatment with either GRK2 inhibitor or PKC inhibitor increased the insulin-stimulated level of Akt phosphorylation at Thr^{308} in the *ob/ob* group but not in the lean group.

DISCUSSION

We made several new findings in this study, and these lead us to propose the following. First, GRK2 negatively regulates insulin-induced vascular relaxation via the Akt/eNOS pathway in type 2 diabetic obese *ob/ob* mice. Second, hyperinsulinemia increases PKC activity and causes GRK2 activation and translocation to membranes. Third, the diabetes-associated reduction in the vascular response to insulin is attributable to GRK2 activation and translocation to membranes mediated via increased PKC activity. Fourth, the expression of β-arrestin2 in aortic membranes under insulin stimulation is lower in *ob/ob* mice than in lean mice, suggesting that an overactivation of GRK2 may impede the development of a normal relationship between β-arrestin2 and Akt under insulin stimulation. These results strongly suggest that changes in the PKC/GRK2 /β-arrestin2 pathway underlie the impairment of the insulin-induced relaxation response seen in aortas from type 2 diabetic *ob/ob* mice.

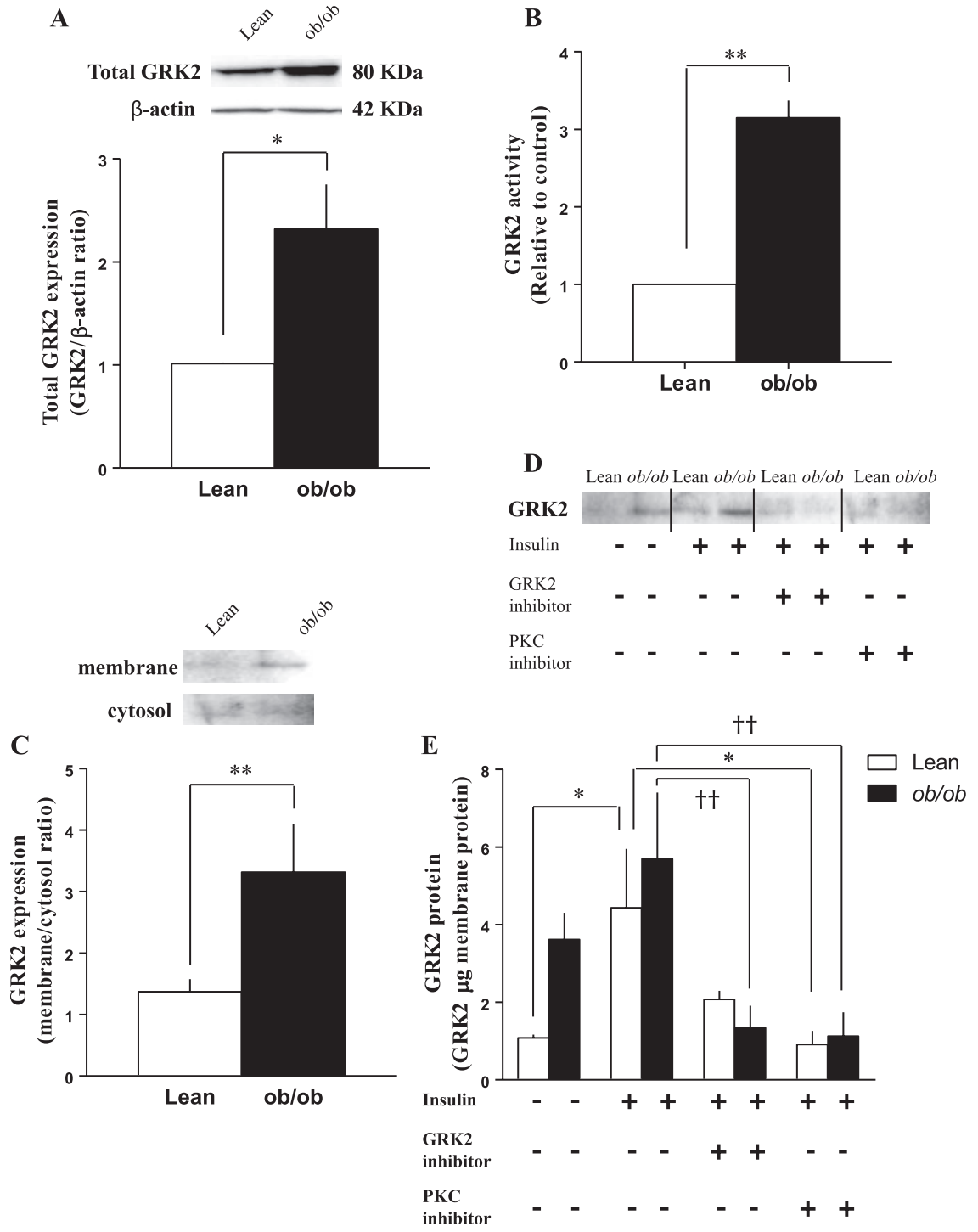
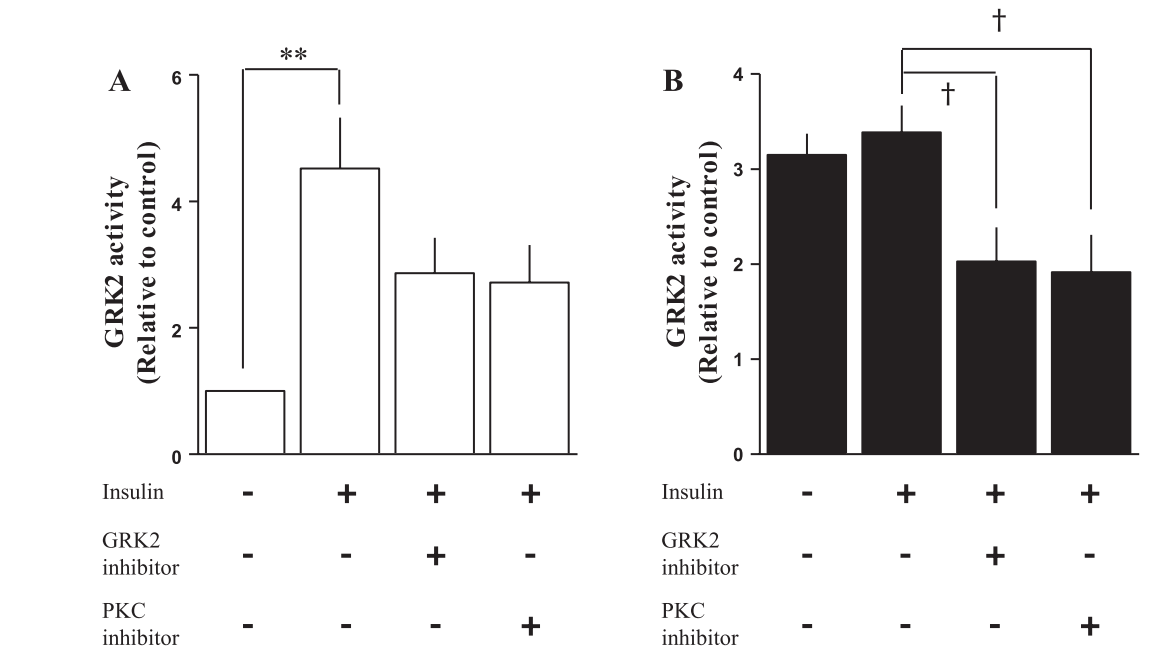


Fig. 5. GRK2 expression and activity in aortic strips from *ob/ob* and lean mice. *A*, *top*: representative Western blots for GRK2 protein. *Bottom:* total GRK2 expression (bands quantified by scanning densitometry). Ratios were calculated for the optical density of GRK2 over that of β-actin. *B*: basal GRK2 activity in aortic strips from *ob/ob* and lean mice. *C*: subcellular distribution of GRK2. *Top:* representative Western blots. *Bottom:* membrane/cytosol fraction ratios (bands quantified by scanning densitometry). Ratios were calculated for the optical density of GRK2 in the membrane fraction over that of GRK2 in the cytosol fraction. *D*: representative Western blots for GRK2 expression in the membrane fraction. Vehicle and insulin-stimulated lanes were run on the same gel but were noncontiguous, and GRK2 inhibitor/insulin-stimulated and PKC inhibitor/insulin-stimulated were run on a separated gel but under the same conditions. *E*: GRK2 expression in the membrane fraction. Aortic strips were treated for 24 h with vehicle (basal), insulin (10^{-7} M), insulin + GRK2 inhibitor (10^{-6} M), or insulin + PKC inhibitor (10^{-6} M). Values are means ± SE; $n = 5$. *$P < 0.05$ vs. lean or insulin-stimulated lean mice. **$P < 0.01$ vs. lean mice. $^{\dagger\dagger}P < 0.01$ vs. insulin-stimulated *ob/ob* mice.



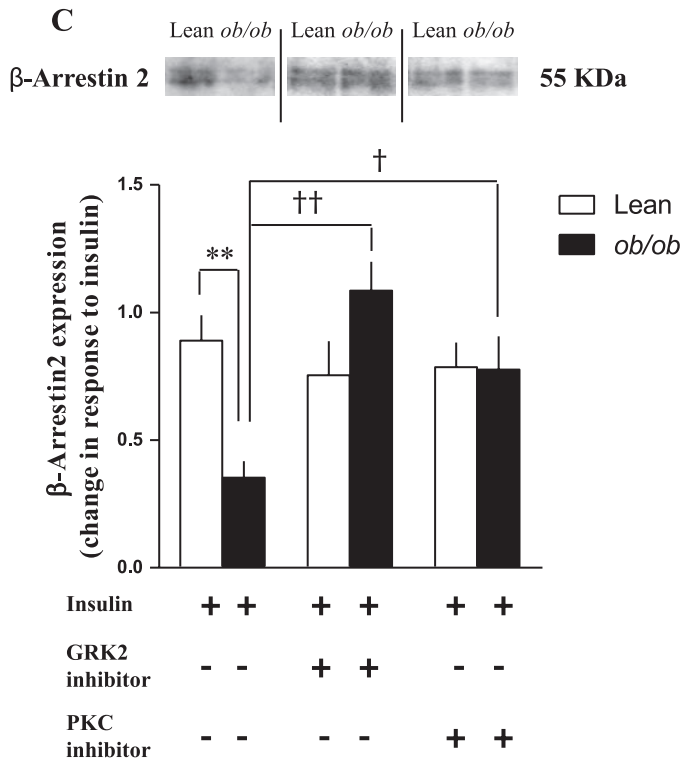


Fig. 6. Membrane-specific activation of GRK2 in *ob/ob* mice. Aortic strips were treated for 24 h with vehicle (basal), insulin (10^{-7} M), insulin + GRK2 inhibitor (10^{-6} M), or insulin + PKC inhibitor (10^{-6} M), then subjected to fractionation (to obtain the membrane fraction) by ultra-centrifugation. GRK2 activities in the membrane fraction were analyzed for lean mice (*A*) and *ob/ob* mice (*B*). *C*: insulin-stimulated β-arrestin2 expression in the membrane fraction (essential details as above). Lanes were run on the same gel but were noncontiguous. Values are means ± SE; $n = 4$. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. insulin-stimulated *ob/ob* mice. $^{**}P < 0.01$ vs. insulin-stimulated lean mice.

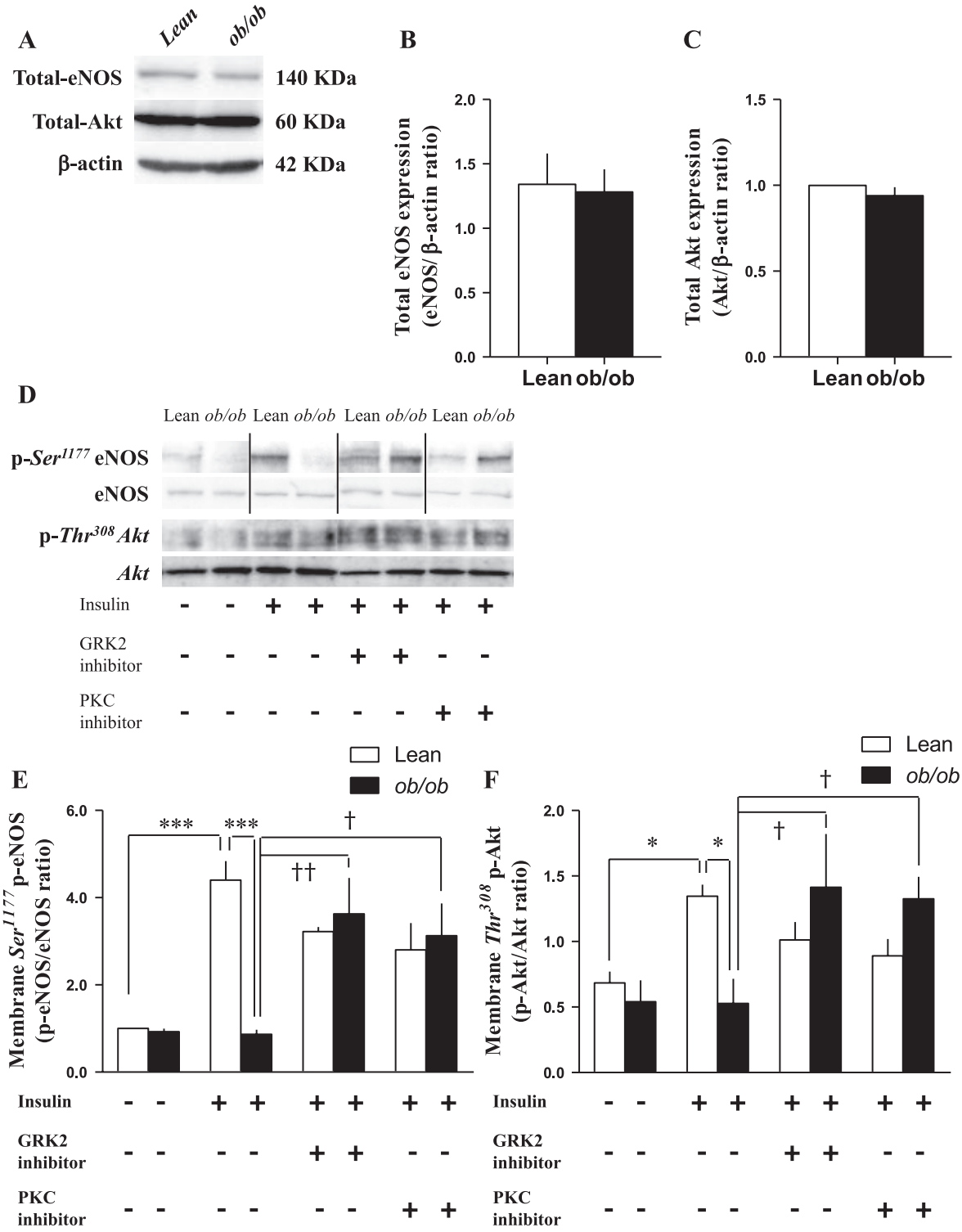


Fig. 7. Influence of GRK2 over Akt/endothelial nitric oxide synthase (eNOS) pathway. *A*. representative Western blots for total eNOS and total Akt expression. *B* and *C*: total protein expressions (bands quantified by scanning densitometry). Ratios were calculated for the optical density of eNOS or Akt over that of β-actin. *D*: representative Western blots for eNOS phosphorylation at Ser^{1177} and Akt phosphorylation at Thr^{308} in insulin-stimulated membrane fractions of aortas. *Top bands*: phosphorylation proteins. *Bottom bands*: total proteins. Lanes were run on a different gel. In eNOS phosphorylation at Ser^{1177}, vehicle, insulin-stimulated, and GRK2 inhibitor/insulin-stimulated lanes were run on the same gel but were noncontiguous; PKC inhibitor/insulin-stimulated lane was run on a separate gel but under the same conditions. In total eNOS, vehicle, insulin-stimulated, and PKC inhibitor/insulin-stimulated lanes were run on the same gel but were noncontiguous; GRK2 inhibitor/insulin-stimulated were run on a separate gel but under the same conditions. *E* and *F*: expressions of eNOS phosphorylation at Ser^{1177} and Akt phosphorylation at Thr^{308}. Aortic strips were treated for 24 h with vehicle (basal), insulin (10^{-7} M), insulin + GRK2 inhibitor (10^{-6} M), or insulin + PKC inhibitor (10^{-6} M), then subjected to fractionation (to obtain the membrane fraction) by ultra-centrifugation. Values are means ± SE; $n = 5$. *$P < 0.05$ vs. insulin-stimulated lean mice. †$P < 0.05$ vs. *ob/ob* mice. ††$P < 0.01$ vs. insulin-stimulated *ob/ob* mice. ***$P < 0.001$ vs. insulin-stimulated lean mice.

An important vascular action of insulin is vascular relaxation, which is associated with increased NO production by endothelial cells (12, 22, 40, 48, 50, 52). We and others (8, 10, 20, 21, 35) have suggested that diabetes and insulin resistance involve impaired endothelial function, a causal factor in hypertension and other cardiovascular diseases. Therefore, we first investigated insulin-induced responses in the *ob/ob* mouse model of type 2 diabetes. The amounts of insulin given by cumulative administration were very high compared with the normal circulating levels (see Table 1). However, excessive circulating insulin appears to be a contributor to insulin resistance via downregulation of insulin receptor and/or signaling. This downregulation occurs due to prolonged and repeated elevations of circulating insulin (41). In the *ob/ob* aorta, the insulin-induced relaxation response was reduced vs. that in the lean mice. In the lean group, the relaxation response to insulin was almost abolished by pretreatment with Akt inhibitor or NOS inhibitor, suggesting that it is mediated via activation of a signaling pathway involving Akt/eNOS. The presence of such a reduced response in diabetes is consistent with other data linking Akt to the eNOS pathway (21). Special attention should be paid to the following two results: *1*) the insulin-induced relaxation response was enhanced by pretreatment with GRK2 inhibitor in the *ob/ob* group; and *2*) in the *ob/ob* aorta, insulin-induced Akt/eNOS signaling was evidently enhanced by pretreatment with GRK2 inhibitor or PKC inhibitor. In the case of these two inhibitors, the attenuation of the insulin-induced relaxation seen in the *ob/ob* group (vs. the lean group) was specifically enhanced by the optimal concentration of a given inhibitor (i.e., the concentration of GRK2 inhibitor we employed had no effect on the insulin-induced relaxation seen in the lean group, and we employed the concentration of PKC inhibitor that is generally used; Ref. 30). We undertook the present study primarily to identify the underlying mechanism by which the relaxation response to insulin via the Akt/eNOS signaling pathway is impaired in the *ob/ob* aorta.

Many stimuli (including insulin, VEGF, α_2-agonists, endothelin-1, and shear-stress signals) regulate NO production by activating eNOS via Ser^{1177} phosphorylation through the Akt pathway (11, 13, 21, 31). The major inference we draw from the present data is that the mechanisms underlying endothelial dysfunction in the aorta of the *ob/ob* mouse may include an impairment of the GRK2/β-arrestin2/Akt (Thr^{308})/eNOS (Ser^{1177}) pathway. In the *ob/ob* aorta, the insulin-induced phosphorylations of Akt at Thr^{308} and eNOS at Ser^{1177} were decreased (vs. those in the insulin-stimulated lean aorta). Interestingly, in the *ob/ob* aortas treated with either GRK2 inhibitor or PKC inhibitor there was a marked increase in Akt phosphorylation at Thr^{308} and in eNOS phosphorylation at Ser^{1177} (Fig. 7, *D-F*). These results strongly suggest *1*) that the impairment of the insulin-induced relaxation response observed in the *ob/ob* aorta was due to decreases in Akt and NOS activities, and *2*) that the decreases in Akt/eNOS activities were due to increases in the activities of PKC and/or GRK2 in the *ob/ob* aorta.

There have been numerous studies on PKC that have suggested a role for oxidative stress (1, 6, 14, 17, 22, 28, 42, 43). Among these is a report (28) that in the proximal tubules of STZ-induced diabetic rats increases in PKC expression and activity lead to translocation of GRK2 and also a report (1) that insulin stimulation causes GRK2-mediated receptor phosphorylation via a mechanism(s) involving the PI3-kinase/PKC pathway. PKC activation appears to modulate the effect of insulin on eNOS expression since rapid PKC activation causes inhibitions of insulin-stimulated PI3-kinase activity and eNOS expression (22). Here, we found that in the lean aorta the insulin-induced relaxation response was inhibited by pretreatment with PKC inhibitor. This result supports the above previous report. Recently, it was reported that insulin stimulation causes GRK2-mediated receptor phosphorylation via a mechanism(s) involving PI3-kinase and PKC (1). In the present study, the plasma insulin was markedly elevated in the *ob/ob* mice. We therefore hypothesized that in type 2 diabetes insulin stimulation might increase PKC activity and cause GRK2 activation and translocation to membranes and that GRK2 might negatively regulate the Akt/eNOS signaling pathway. Interestingly, in the insulin-treated *ob/ob* aorta, pretreatment with either GRK2 inhibitor or PKC inhibitor increased both Akt phosphorylation at Thr^{308} and eNOS phosphorylation at Ser^{1177} to around the levels seen under insulin stimulation in the lean aorta (in which these inhibitors did not alter the insulin-induced effects on those phosphorylations). Furthermore, in insulin-treated *ob/ob* aortas, pretreatment with either GRK2 inhibitor or PKC inhibitor decreased GRK2 expression in the membranes, suggesting that the insulin-stimulated PKC activation caused GRK2 translocation to membranes in the *ob/ob* aorta. Actually, the basal GRK2 level was greater in the *ob/ob* aorta than in the lean aorta. Additionally, in the *ob/ob* aorta the basal GRK2 activity and GRK2 expression in the membrane fraction were enhanced (vs. the lean aorta), suggesting that in the basal condition GRK2 has undergone greater translocation to the membranes in the *ob/ob* aorta. Notably, the basal PKC activity was greater in the *ob/ob* aorta than in lean, and the insulin-induced GRK2 activation and translocation were PKC dependent (since PKC inhibitor blocked the insulin-induced GRK2 upregulation). Furthermore, these results suggest that GRK2 is activated downstream of PKC. In support of our data, there are reports (1, 18) that insulin can activate PKC and that PKC-induced phosphorylation of GRK2 causes its activation and translocation to membranes. These results there-

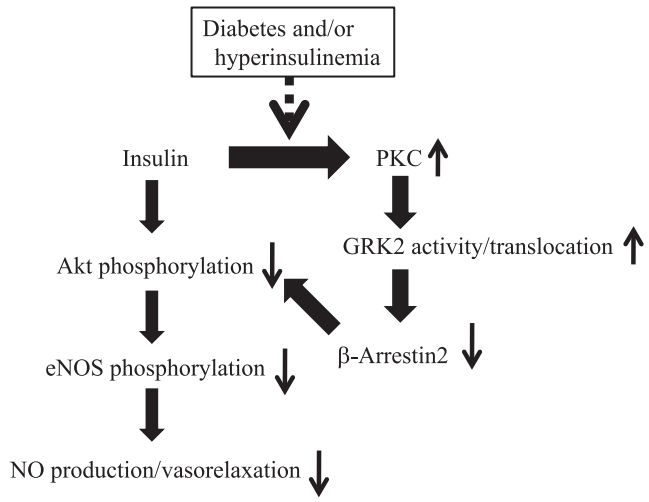


Fig. 8. Schema showing the proposed events underlying insulin-induced aortic relaxation in nondiabetic and diabetic mice. Thin arrows show changes due to diabetes and/or hyperinsulinemia.

fore indicate a signaling cascade for the insulin-induced phosphorylations of Akt and eNOS involving PKC/GRK2. Incidentally, we postulate that the hyperinsulinemia present in the *ob/ob* mouse may increase GRK2 signaling. The PKC inhibitor reduced insulin-stimulated GRK2 expression in membranes in *ob/ob* aortas, while the GRK2 inhibitor was ineffective in lean aortas (Fig. 5*E*). This idea is in agreement with other reports (1, 49) showing that in obese Zucker rats endothelial dysfunction coexists with hyperinsulinemia.

In this study, we mainly used insulin, PKC inhibitor, and GRK2 inhibitor. In the case of insulin, we investigated the concentration-dependent effects and confirmed the efficacy and specificity by making comparisons with our previous our reports (20, 21). In addition, we used the PKC inhibitor in the concentration previously reported (30). Probably, the most important issue in this context is the efficacy and specificity of the GRK2 inhibitor. The efficacy and specificity of the GRK2 inhibitor we used are unknown, but this is the only inhibitor yet available. We therefore await the development of an inhibitor with known high efficacy and specificity. In this study, we based our experimentation on the report of Iino et al. (16) that the inhibitor was selective. Indeed, in our experiment the GRK2 inhibitor at a concentration of 10^{-6} M had no effect on the insulin-induced relaxation seen in the lean group (Fig. 3*C*).

Finally, we addressed the association between GRK2 and β-arrestin2. GRK2 has been identified as serine/threonine kinase that participates, together with β-arrestin2, in the regulation of multiple GPCRs. A classic mechanism for desensitization is the binding of β-arrestin to GRK-phosphorylated receptors; this uncouples the receptor from its G proteins and prevents further signal transduction (34, 47). Moreover, β-arrestins mediate the biological functions of GPCR by linking activated receptors with distinct sets of accessory and effector proteins, thereby determining the specificity, efficiency, and capacity of signals (3, 26). Recently, the existence of G protein-independent, GRK/β-arrestin-mediated signal transduction has been established for several receptors (9, 23, 46). This represents a potentially important mechanism by which these receptors might modulate physiologic processes. Insulin receptors are of the tyrosine kinase-type, not GPCRs, and insulin activates a signaling pathway involving insulin receptor, insulin receptor substrate-1 (IRS-1), PI3-K, and Akt, and this leads to eNOS activation. In fact, we think that GRK2 does not act directly on insulin receptor, and we know of no reports suggesting that it does. Very recently, Luan et al. (25) revealed that insulin stimulated the formation of a new β-arrestin2 signal complex in which β-arrestin2 acts as a scaffold molecule for the translocation of Akt and Src to the insulin receptor, so that loss or dysfunction of β-arrestin2 in diabetes results in a deficiency of this signal complex and a consequent disturbance of insulin signaling and thereby to insulin resistance. Some studies (7, 45) have suggested that GRK2 binds IRS-1. Therefore, 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. However, in the present *ob/ob* mice insulin-stimulated Akt and eNOS phosphorylations were reduced, but they were improved by GRK2 inhibitor (Fig. 7). In this context, further research is needed for a better understanding of how GRK2 and β-arrestin2 activities might be orchestrated, and their dependence on insulin receptor and/or IRS-1. Here, we have shown that in the membranes of the *ob/ob* aorta, β-arrestin2 is severely downregulated under insulin stimulation. Furthermore, β-arrestin2 was increased to the level seen in the lean group by treatment of the *ob/ob* aortas with GRK2 inhibitor or PKC inhibitor, suggesting that in diabetes the PKC/GRK2 pathway negatively regulates β-arrestin2.

The most intriguing evidence to emerge from this study is that in diabetes with hyperinsulinemia and insulin resistance, the insulin-mediated endothelial dysfunction involved a novel signal transduction cross-talk (Fig. 8). In diabetes, insulin evidently increases PKC activity, leading to GRK2 translocation to membranes. It is possible that this in turn may inhibit β-arrestin2 binding to Akt, thereby decreasing Akt signal transduction. We postulate that the interaction between GRK2 and Akt directly inhibits Akt phosphorylation at Thr^{308}, resulting in decreased eNOS phosphorylation at Ser^{1177}. Although we do not yet know precisely how GRK2 binds to Akt or how β-arrestin2 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 proposed by Liu et al. (24) and Luan et al. (25).

In summary, our findings provide an insight into the part in insulin-induced endothelial dysfunction played by PKC/GRK2 activation (viz. as a negative regulator of β-arrestin2/Akt/eNOS-mediated endothelial function). Future investigations of the function and precise mechanism of this novel signal pathway should increase our understanding of hyperinsulinemia and type 2 diabetes and potentially uncover new molecular targets for the treatment of metabolic diseases.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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