Tonic Inhibition by G Protein–Coupled Receptor Kinase 2 of Akt/Endothelial Nitric-Oxide Synthase Signaling in Human Vascular Endothelial Cells under Conditions of Hyperglycemia with High Insulin Levels

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Received November 30, 2013; accepted February 20, 2014

ABSTRACT

G protein–coupled receptor kinase 2 (GRK2) participates together with β-arrestins in the regulation of G protein–coupled receptor signaling, but emerging evidence suggests that GRK2 can interact with a growing number of proteins involved in signaling mediated by other membrane receptor families under various pathologic conditions. We tested the hypothesis that GRK2 may be an important contributor to vascular endothelial dysfunction in diabetes. Human umbilical venous endothelial cells (HUVECs) were exposed to high glucose and high insulin (HG/Hi) to mimic insulin-resistant diabetic conditions. GRK2 expression and membrane translocation were up-regulated under HG/Hi conditions. HG/Hi did not modify activation of Akt or endothelial nitric-oxide synthase (eNOS), but GRK2 inhibitor or small interfering RNA (siRNA) resulted in an increase in Akt and eNOS activation in HUVECs exposed to HG/Hi. Extracellular signal-regulated kinase 1/2 (ERK1/2) activation was increased after exposure to HG/Hi, which was prevented by GRK2 inhibitor or siRNA. ERK1/2-mediated GRK2 phosphorylation at Ser-670 confirmed that ERK1/2 participated in a negative feedback regulatory loop. In human embryonic kidney 293T cells that overexpressed GRK2, Akt activity was unchanged, whereas ERK1/2 activity was raised. The effect of GRK inhibitor treatment on Akt/eNOS signaling was associated with membrane translocation of β-arrestin 2. The experiments with β-arrestin 2 siRNA showed that β-arrestin 2 may act as a positive modulator of Akt/eNOS signaling. Our studies reveal that GRK2, which is up-regulated by HG/Hi, leads to a tonic inhibition of the insulin Akt/eNOS pathway in endothelial cells. We provide new insights into the pathogenesis of diabetes–associated vascular endothelial dysfunction.

Introduction

G protein–coupled receptor kinase 2 (GRK2) is a member of the family of GRKs that are serine/threonine kinases and are widely distributed in different tissues (Penn et al., 2000). GRK2 was originally discovered to restrain cellular activation by phosphorylating specific agonist-occupied G protein–coupled receptors (GPCRs), leading to receptor desensitization and internalization together with β-arrestins (Zhang et al., 1997). However, recent evidence has shown that GRK2 can restrain signaling via direct interaction with downstream intracellular kinases, including Akt, extracellular signal-regulated kinase 1/2 (ERK1/2), phosphatidylinositol 3-kinase, and p38, leading to modulation of their activity (Liu et al., 2005; Jimenez-Sainz et al., 2006). Furthermore, GRK2 can also regulate signaling mediated by other membrane receptor families, such as tyrosine kinase receptors for insulin, insulin-like growth factor 1, platelet-derived growth factor, and epidermal growth factor (Kim et al., 2003; Cipolletta et al., 2009). Thus, it should be stressed that the cellular role of GRK2 is not limited to promoting β-arrestin binding to activated GPCRs. Alterations in GRK2 levels and/or activity, such as those reported in a number of relevant cardiovascular, inflammatory, and cancer pathologies, may play an important role in the development and/or progression of such human diseases (Penela et al., 2010). The relationship between up-regulated cardiac GRK2 levels and heart failure has been well established in animal models and in patients afflicted with different heart conditions (Harris et al., 2001; Reinkober et al., 2006). Furthermore, GRK2 can also regulate signaling mediated by other membrane receptor families, such as tyrosine kinase receptors for insulin, insulin-like growth factor 1, platelet-derived growth factor, and epidermal growth factor (Kim et al., 2003; Cipolletta et al., 2009). Thus, it should be stressed that the cellular role of GRK2 is not limited to promoting β-arrestin binding to activated GPCRs. Alterations in GRK2 levels and/or activity, such as those reported in a number of relevant cardiovascular, inflammatory, and cancer pathologies, may play an important role in the development and/or progression of such human diseases (Penela et al., 2010). The relationship between up-regulated cardiac GRK2 levels and heart failure has been well established in animal models and in patients afflicted with different heart conditions (Harris et al., 2001; Reinkober et al., 2006). Moreover, it has been suggested that up-regulated

This study was supported by a Grant-in-Aid for Young Scientists from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, the Astellas Foundation for Research on Metabolic Disorders, the Nishinomiya Basic Research Fund, and the Tamura Science Technology Foundation.

K.T. and K.S. contributed equally to this work.

dx.doi.org/10.1124/jpet.113.211854

ABBREVIATIONS: CM-H2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorofluorescein diacetate, acetyl ester; ERK1/2, extracellular signal-regulated kinase 1/2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPCR, G protein–coupled receptor; GRK2, G protein–coupled receptor kinase 2; HAEC, human aortic endothelial cell; HCAEC, human coronary artery endothelial cell; HEK, human embryonic kidney; HUVEC, human umbilical venous endothelial cell; NO, nitric oxide; eNOS, endothelial NO synthase; NOx, nitrite and nitrate; PD98059, 2′-amino-3′-methoxyflavone; ROS, reactive oxygen species; siRNA, small interfering RNA.
expression of GRK2 in the vasculature might be associated with the pathogenesis of human essential hypertension (Gros et al., 2000; Cohn et al., 2009). In the meantime, GRK2 is highly expressed in different cellular types of the immune system and emerges as an important regulator of cell responses in a variety of inflammatory disorders (Vroon et al., 2006).

Diabetes mellitus is marked by increased blood glucose levels and represents a major risk factor for cardiovascular morbidity and mortality. In particular, subjects with type 2 diabetes mellitus, which is characterized by insulin resistance, are at 2- to 4-fold increased risk of cardiovascular disease compared with those without diabetes (Kirpichnikov and Sowers, 2001). Vascular endothelial cells play a major role in maintaining cardiovascular homeostasis, and endothelial dysfunction is regarded as an important factor in the pathogenesis of diabetic vascular complications (Capellini et al., 2010). Insulin resistance is associated with endothelial dysfunction. Dysfunction of vascular endothelial cells in insulin-resistant states could be explained by alterations in insulin intracellular signaling that affect the production of nitric oxide (NO) (Dresner et al., 1999; Inoguchi et al., 2000; Montagnani et al., 2002).

GRK2 has been described to be involved in impaired endothelium-dependent relaxations in aorta of the ob/ob mouse (Taguchi et al., 2011, 2012a), a model of severe obesity, insulin resistance, and diabetes caused by leptin deficiency (Chen and Wang, 2005). Despite the degree of obesity and hyperinsulinemia, this type 2 diabetic model displays milder hyperglycemia. Given the negative regulation by vascular GRK2 of the insulin downstream effectors, Akt and endothelial NO synthase (eNOS), leading to the impairment of aortic endothelium-dependent relaxations in the ob/ob mouse (Taguchi et al., 2012b), a further exploration of the potential impact of GRK2 on endothelial cells under pathologic hyperglycemic/hyperinsulinemic conditions is important and necessary. In this study, we present evidence for a critical role of GRK2 in vascular endothelial pathology using human umbilical venous endothelial cells (HUVECs) exposed to high glucose and high insulin to mimic insulin-resistant diabetic conditions.

Materials and Methods

Cell Culture. HUVECs, human aortic endothelial cells (HAECs), and human coronary artery endothelial cells (HCAECs) were purchased from Cambrex Bioscience (Walkersville, MD) and cultured in endothelial cell growth media until the start of the experiment. The culture media contained 10 ng/ml human epidermal growth factor, 5 ng/ml human fibroblast growth factor B, and 1.34 μg/ml hydrocortisone hemisuccinate, which were considered to have no affect on our outcome. Cells of passages four through seven were used when 70% to 80% confluent. Then, cells were harvested and seeded into six-well plates. To mimic hyperglycemic or hyperglycemic/hyperinsulinemic conditions, confluent HUVECs were stimulated with 22 or 31 mM glucose in the presence or absence of insulin for 1–72 hours. Mannitol was used to rule out the effect of osmotic pressure. When cells were treated with GRK2 inhibitor methyl 5-[2-(5-nitro-2-furyl)vinyl]-2-furoate (Calbiochem, San Diego, CA), it was added simultaneously with exposure to the high glucose/high insulin medium.

Transfection of siRNAs. All small interfering RNAs (siRNAs) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The negative control (sc-37007) consists of a scrambled sequence of 20–25 nucleotides, which does not target any known mRNA. Introduction of siRNAs into cells was performed in siRNA transfection medium (Santa Cruz Biotechnology) according to the manufacturer's protocol.

Western Blot Analysis. Cells were harvested and lysed in 300 μl of radioimmunoprecipitation assay buffer (25 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate , pH 7.4) (Thermo Fisher Scientific, Rockford, IL) containing protease inhibitor cocktail (Nacalai, Kyoto, Japan) on ice. The lysates were centrifuged at 18,000 g for 10 minutes at 4°C, and the resulting supernatants were collected. The proteins in the supernatant were measured using the BCA Protein Assay Kit (Thermo Fisher Scientific). Where required, the membrane fractions were prepared; the supernatant was then spun at 100,000 g for 30 minutes at 4°C, and the membrane pellet was resuspended in 50 μl of lysis buffer and saved. The supernatants (~20 μg of protein) were run on 10% polyacrylamide gel and electrotransferred onto polyvinylidene fluoride filter membrane. To reduce nonspecific binding, the membrane was blocked for 90 minutes at room temperature in Odyssey blocking buffer, followed by overnight incubation with primary antibody at 4°C. The membrane was washed four times with phosphate-buffered saline with 0.1% Tween 20 and incubated with goat anti-rabbit IRDye 680, goat anti-mouse IRDye 800 CW, or goat anti-chicken IRDye 680 diluted in 1:1000–2000 in Odyssey blocking buffer for 120 minutes at 38°C in the dark. After being washed six times in phosphate-buffered saline with 0.1% Tween 20, the blots were visualized using the Odyssey Infrared Imaging System from LI-COR (Lincoln, NE).

The following antibodies, which are commercially available, were used: anti-human GRK2 mouse monoclonal antibody (Santa Cruz Biotechnology); anti-human phospho-GRK2 (Ser-670) rabbit polyclonal antibody (GeneTex, Irvine, CA); anti-human Akt rabbit polyclonal antibody (Cell Signaling, Danvers, MA); anti-human phospho-Akt (Ser-473) rabbit monoclonal antibody (Cell Signaling); anti-human eNOS mouse monoclonal antibody (BD Biosciences, San Jose, CA); anti-human phospho-eNOS (Ser-1177) mouse monoclonal antibody (BD Biosciences); anti-human ERK1/2 mouse monoclonal antibody (Cell Signaling); anti-human phospho-ERK1/2 (Thr-202/Tyr-204) rabbit monoclonal antibody (Cell Signaling); anti-human β-arrestin 2 mouse monoclonal antibody (Abcam, Cambridge, MA); and anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) chicken polyclonal antibody (Millipore, Billerica, MA).

Immunofluorescence. Cells were fixed using 95% ethanol for 30 minutes and incubated with anti-GRK2 rabbit polyclonal antibody (1:100; Bios, Woburn, MA) for 2 hours, followed by exposure to the anti-rabbit IgG conjugated to the high-quality fluorophore Alexa Fluor 488 for 1 hour. The nucleus was counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (Cell Signaling). Immunofluorescent images were observed using a fluorescence microscope (BZ-8100; Keyence, Osaka, Japan).

Measurement of NOx Production. To assess NOx (nitrite and nitrate) production by HUVECs, NOx content of the medium was measured with NO2/NO3 Assay Kit-C II (Colorimetric [Griess Reagent Kit]), (Dajindo, Kumamoto, Japan) according to the manufacturer's instructions.

Transient Transfection of GRK2 in Human Embryonic Kidney 293T Cells. Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. An expression plasmid encoding the human GRK2 (hGRK2), pCMV-SPORT6-hGRK2, was purchased from DNAform (Yokohama, Japan). The hGRK2 sequence was inserted into the NotI (774)/SalI (795) sites of pCMV-SPORT6 vector. Mock pCMV-SPORT6 vector was made by removing the XhoI (755)/SalI (795) region, including the hGRK2 sequence. Twenty-four hours prior to transfection, 5 × 104 cells were seeded in a six-well plate. Transfection of the cells was performed using Lipofectamine2000 or Lipofectamine LTX and Plus reagent (Invitrogen, Carlsbad, CA).

Imaging of Intracellular ROS by Fluorescence Microscopy. To detect intracellular reactive oxygen species (ROS), we loaded endothelial cells with the fluorescent probe 5-(and-6)-chloromethyl-
2′,7′-dichlorofluorescein diacetate, acetyl ester (CM-H2DCFDA; Invitrogen) to a final concentration of 10 μM for 45 minutes in phosphate-buffered saline at 37°C. At the end of an experiment, cells were rinsed with phosphate-buffered saline and then placed in culture medium. Imaging was conducted using a Cell Observer microscope with the AxoVision software (Carl Zeiss, Oberkochen, Germany). ImageJ was used for image analysis. H2O2 (50 mM) served as a positive control.

**Statistical Analysis.** Results are presented as the mean ± S.E. Data were analyzed by the use of Prism software (version 6; GraphPad Software, San Diego, CA). Statistical analysis was performed by one-way analysis of variance followed by Tukey’s multiple comparison test. A P value less than 0.05 was considered significant.

**Results**

**GRK2 Expression and Membrane Translocation.** We initially investigated whether GRK2 is constitutively expressed in three different types of human vascular endothelial cells. Figure 1A shows that HUVECs, HCAECs, and HAEcs all expressed GRK2 in a standard cell culture medium, although its expression levels by cell types were somewhat different. The expression level of GRK2 was not significantly changed when HUVECs were exposed to high glucose (22 or 31 mM) for 72 hours (Fig. 1B). Furthermore, treatment with insulin at a low (10 nM) or high (100 nM) concentration was without effect on GRK2 expression under normal glucose conditions. However, GRK2 expression was significantly up-regulated when HUVECs were treated with 100 nM insulin under 22 mM glucose. Confocal microscopy in HUVECs (Fig. 1C) confirmed that high glucose/high insulin conditions induced up-regulation of GRK2 expression. The up-regulation of GRK2 expression under high glucose/high insulin conditions was time-dependent (Fig. 1D). The up-regulation of GRK2 expression under high glucose/high insulin conditions was also observed in HCAECs and HAEcs (Fig. 1E). When HCAECs and HAEcs responded to 72-hour high glucose/high insulin exposure, the relative amounts of GRK2 protein were increased to 123 ± 6% (n = 3) and 134 ± 14% (n = 4) of control, respectively. In addition, treatment with high glucose/high insulin led to a significant induction of translocation of GRK2 to the plasma membrane in HUVECs (Fig. 2).

**Unmasking the Effect of GRK2 Inhibitor.** High glucose/high insulin conditions did not activate Akt or eNOS during the 72 hours after exposure, as indicated by the absence of change in Ser-473 phosphorylation of Akt or Ser-1177

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**Fig. 1.** Expression of GRK2 in human vascular endothelial cells. (A) Western blots showing that GRK2 was definitely expressed in HUVECs, HCAECs, and HAEcs, GAPDH served as loading control. (B) Changes in GRK2 expression in HUVECs at 72 hours after exposure to normal (NG), high (HG), and extremely high (EHG) glucose conditions with or without low (LI) or high (HI) insulin levels (n = 6). *P < 0.05 versus NG without insulin. NG, 5.5 mM glucose; HG, 22 mM glucose; EHG, 31 mM glucose in the culture medium. LI, 10 nM applied insulin concentration; HI, 100 nM applied insulin concentration. (C) Confocal images of HUVECs stained with anti-GRK2 antibody (green). Cells were exposed to NG or HG/HI for 72 hours. Nuclei are counterstained in blue by 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). Original magnification: ×40. (D) Time-dependent changes in GRK2 expression in HUVECs after exposure to HG/HI (n = 4). ***P < 0.001 versus the value at time 0. (E) Western blots depicting HG/HI-induced increases in GRK2 expression in HCAECs and HAEcs. In each top trace of (B) and (D), typical Western blots are shown.
phosphorylation of eNOS (Fig. 3, A and B). In the presence of GRK2 inhibitor, however, a significant activation of both Akt and eNOS occurred under high glucose/high insulin conditions. In line with the finding that inhibition of GRK2 was required for increased eNOS activation in a high glucose/high insulin environment, an increase in NOx in the culture medium after HUVECs were exposed to high glucose/high insulin conditions was detectable in the presence of GRK2 inhibitor (Fig. 3C). These data suggest that GRK2, which was up-regulated under high glucose/high insulin conditions, resulted in a tonic inhibition of the insulin downstream effectors, Akt and eNOS, in human vascular endothelial cells.

On the other hand, the phosphorylation levels of ERK1/2, which provide a measure of its activation, were significantly increased after high glucose/high insulin exposure (Fig. 3D). The increased ERK1/2 activation was abrogated by treatment with GRK2 inhibitor. These findings imply that the high glucose/high insulin–induced up-regulation of GRK2 mediated the increase in ERK1/2 activation in human vascular endothelial cells. The increase in ERK1/2 phosphorylation levels under high glucose/high insulin conditions was completely prevented by PD98059 (2′-amino-3′-methoxyflavone), an inhibitor of mitogen-activated protein kinase kinase, an ERK1/2 upstream activator (Fig. 3E). Moreover, PD98059 abrogated phosphorylation of GRK2 at Ser-670 when cells were exposed to high glucose/high insulin (Fig. 3E). This is in line with a previous assertion that Ser-670 represents a site of GRK2 inhibition of the insulin downstream effectors, Akt and eNOS, in human vascular endothelial cells.

**GRK2 siRNA Behaved as a GRK2 Inhibitor.** To further define the involvement of GRK2 in tonic inhibition of Akt/eNOS and activation of ERK1/2 in endothelial cells exposed to high glucose/high insulin, the knockdown of GRK2 gene expression was performed in HUVECs using siRNAs. Our transfection of GRK2 siRNA effectively silenced endothelial expression of GRK2 protein in a concentration-dependent manner (Fig. 4A). The ablation of GRK2 by siRNAs resulted in a significant increase in phosphorylated levels of Akt and eNOS in HUVECs exposed to high glucose/high insulin (Fig. 4, B and C). In addition, NOx production was significantly increased under high glucose/high insulin conditions when GRK2 siRNA was transfected (Fig. 4D). Meanwhile, the increase in ERK1/2 phosphorylation observed in a high glucose/high insulin environment was prevented by GRK2 siRNA transfection (Fig. 4E).

**Role of β-Arrestin 2 in GRK2-Mediated Endothelial Derangement.** It is well established that β-arrestins, whose recruitment is mediated by GRKs, can act as scaffold molecules that bring different signaling molecules into a receptor complex (Lefkowitz and Whalen, 2004; DeWire et al., 2007; Premont and Gainetdinov, 2007). Our Western blotting analysis showed that HUVECs constitutively expressed β-arrestin 2 (see Figs. 5B and 6). It is noteworthy that treatment with a GRK inhibitor induced translocation of β-arrestin 2 from the cytoplasm to the plasma membrane when cells were exposed to high glucose/high insulin but not to normal glucose (Fig. 5A), suggesting that the up-regulation of GRK2 under high glucose/high insulin conditions resulted in a tonic inhibition of membrane translocation of β-arrestin 2. However, the expression levels of β-arrestin 2 did not affect the high glucose/high insulin–induced GRK2 up-regulation. We introduced siRNAs targeting the β-arrestin 2 gene into HUVECs. β-Arrestin 2 protein expression was inhibited by β-arrestin 2 siRNA in a concentration-dependent manner and was strikingly repressed by 100 nM β-arrestin 2 siRNA, as assessed by immunoblotting (Fig. 5B). Even when the β-arrestin 2 siRNA transfection experiments were performed at the concentration of 100 nM, GRK2 expression, which was up-regulated under high glucose/high insulin conditions, remained unchanged regardless of whether β-arrestin 2 siRNA was present (Fig. 5C).

Transfection of β-arrestin 2 siRNA resulted in a significant decrease in Akt phosphorylation levels (Fig. 6A).
decreasing effects of transfection of β-arrestin 2 siRNA were also observed on eNOS phosphorylation and NOx production (Fig. 6, B and C). Furthermore, treatment with GRK2 inhibitor failed to increase Akt phosphorylation, eNOS phosphorylation, or NOx production when cells were transfected with β-arrestin 2 siRNA (Fig. 6, A–C). On the other hand, the increase in ERK1/2 phosphorylation observed in a high glucose/high insulin environment was unaffected by β-arrestin 2 siRNA transfection (Fig. 6D).

Overexpression of GRK2 in Transiently Transfected HEK293T Cells. Transfection of HEK293T cells with pCMVSPORT6-GRK2 led to successfully huge expression of GRK2 (Fig. 7A). Mock-transfected HEK293T cells showed the same level of GRK2 expression as native cells. In transiently transfected HEK293T cells that overexpressed GRK2, Akt activity was unchanged, as revealed by the absence of a significant difference in Akt phosphorylation levels from native and mock-transfected cells (Fig. 7B). In contrast, overexpression of GRK2 substantially heightened ERK1/2 activity in HEK293T cells (Fig. 7C). GRK2-overexpressed HEK293T cells were exposed to high glucose/high insulin for 72 hours. However, high glucose/high insulin conditions did not essentially modify the negative or positive effects of GRK2 overexpression on Akt and ERK1/2 phosphorylation levels, respectively, observed under normal conditions (Fig. 7D).

ROS Generation. When intracellular ROS were visualized using the fluorescence dye CM-H$_2$DCFDA, the exposure of HUVECs to high glucose for 72 hours resulted in a significant increase in intracellular fluorescence (Fig. 8). Transfection of GRK2 siRNA prevented this increase in ROS-induced intracellular fluorescence under high glucose conditions. However, a high glucose–induced increase in ROS generation was blunted when high insulin was present. In a high glucose/high insulin environment, ROS generation was further reduced by GRK2 siRNA transfection.

Discussion

GRK2 is widely distributed in mammalian tissues. We confirmed that GRK2 is ubiquitously expressed in all three types of human vascular endothelial cells examined here. GRK2 expression levels were increased when human vascular endothelial cells were cultured under conditions of hyperglycemia with high insulin levels. Furthermore, exposure of endothelial cells to high glucose/high insulin conditions resulted in a significant membrane translocation of GRK2.
Thus, our study shows that GRK2 is highly up-regulated in human vascular endothelial cells under pathologic high glucose/high insulin conditions. At the present time, we do not have a clear understanding of the mechanism(s) underlying the high glucose/high insulin–induced up-regulation of GRK2 in endothelial cells. However, we suggest that such a GRK2 up-regulation, as those reported in several relevant cardiovascular, inflammatory, and cancer pathologies (Penela et al., 2010), may have potentially deleterious effects in insulin-resistant diabetes.

Even though GRK2 was up-regulated in endothelial cells under high glucose/high insulin conditions, Akt phosphorylation levels were unchanged. In agreement with this result, no change in Akt phosphorylation was found in HEK293T cells that overexpressed GRK2. Furthermore, the phosphorylated levels of eNOS, a target downstream of the Akt pathway, and eNOS-mediated NOx production were scarcely changed in high glucose/high insulin–exposed endothelial cells. However, treatment with GRK2 inhibitor or transfection of GRK2 siRNA resulted in striking increases in Akt phosphorylation and eNOS phosphorylation, as well as NOx production. We interpret this finding to indicate that GRK2, which was up-regulated in a high glucose/high insulin environment, led to a tonic inhibition of the Akt/eNOS pathway in endothelial cells. This could fully explain the theoretical basis of the beneficial effect of GRK2 inhibitor on impaired endothelium-dependent relaxation to insulin in aorta of insulin-resistant diabetic ob/ob mouse, an effect that has been demonstrated in an earlier report (Taguchi et al., 2011).

In endothelial cells exposed to high glucose/high insulin conditions, increased phosphorylation of ERK1/2 was observed. The increased level of ERK1/2 phosphorylation was negated by treatment with GRK2 inhibitor or transfection of GRK2 siRNA, implying that the up-regulation of GRK2 is involved in the increase in ERK1/2 activation. This idea can be supported by the finding that HEK293T cells overexpressing GRK2 had substantially high levels of phosphorylated ERK1/2. On the other hand, the mitogen-activated protein kinase inhibitor PD98059 abrogated phosphorylation of GRK2 at Ser-670, which is considered to represent a site of ERK1/2 phosphorylation (Pitcher et al., 1999), when HUVECs were exposed to high glucose/high insulin. Phosphorylation at

![Fig. 4. Transfection of GRK2 siRNA in HUVECs. (A) Transfection of GRK2 siRNA, but not of the negative control siRNA, concentration-dependently decreased GRK2 protein expression. GAPDH served as loading control. One day after GRK2 siRNA (10 nM) or control siRNA was transfected, cells were exposed to 5.5 mM glucose or 22 mM glucose/100 nM insulin. Ser-473 phosphorylation of Akt (B), Ser-1177 phosphorylation of eNOS (C), NOx levels in the culture medium (D), and Thr-202/Thr-204 phosphorylation of ERK1/2 (E) then were examined at 72 hours after normal glucose (white columns) or high glucose/high insulin exposure (hatched columns). For comparison, the data obtained without any siRNA transfection protocol (NT) are presented on the left side of each panel. *P < 0.05; **P < 0.01 versus the respective value obtained under normal glucose without siRNAs (n = 4–12). In each top trace of (B), (C), and (E), typical Western blots are shown.](image-url)
Ser-670 impairs the ability of GRK2 to phosphorylate its substrates (Pitcher et al., 1999). Thus, ERK1/2-mediated GRK2 phosphorylation at Ser-670 confirmed that ERK1/2 participated in a negative feedback regulatory loop. By contrast, ERK1/2 has been implicated in the attenuation of the insulin signal. It has been reported that insulin-stimulated ERK1/2 is able to modulate the phosphorylation of insulin receptor substrate 1 (Aguirre et al., 2000). ERK1/2 has also been shown to attenuate insulin signaling via Ser-612 phosphorylation of insulin receptor substrate 1 (Bard-Chapeau et al., 2005). Thus, augmenting Akt signaling and blocking ERK1/2 signaling may prevent the development of insulin resistance and increase insulin sensitivity (Shen et al., 2011). Interestingly, recent evidence suggests that eNOS indirectly down-regulates GPCR function via interactions with β-arrestin 2 (Ozawa et al., 2008). In the present study, we demonstrated that human endothelial cells constitutively expressed β-arrestin 2. Expression and membrane translocation of β-arrestin 2 in endothelial cells were substantially unaffected when endothelial cells were exposed to high glucose/high insulin conditions. However, treatment with GRK inhibitor induced translocation of β-arrestin 2 from the cytoplasm to the plasma membrane in endothelial cells under high glucose/high insulin conditions, suggesting that the up-regulation of GRK2 under high glucose/high insulin led to a tonic inhibition of membrane translocation of β-arrestin 2. On the other hand, endothelial expression levels of β-arrestin 2 did not affect the high glucose/high insulin–induced GRK2 up-regulation. Thus, even when β-arrestin 2 siRNA was transfected into endothelial cells, GRK2 expression, which was up-regulated under high glucose/high insulin conditions, remained unchanged.

Transfection of β-arrestin 2 siRNA was found to down-regulate Akt/eNOS signaling in human endothelial cells.
under high glucose/high insulin conditions. We interpret these findings to suggest that β-arrestin 2 may act as a positive modulator of the Akt/eNOS signaling cascade. It has been documented that β-arrestin 2 plays a pivotal role in scaffolding the active insulin receptor/Akt/β-arrestin 2/Src signaling complex after insulin stimulation (Luan et al., 2009). The up-regulation of Akt/eNOS signaling observed in the presence of GRK2 inhibitor was completely abrogated when β-arrestin 2 siRNA was transfected. This suggests that the GRK2 inhibitor–induced up-regulation of Akt/eNOS signaling required β-arrestin 2. Alternatively, the tonic inhibitory effect of GRK2 on the Akt/eNOS signaling pathway may result from its prevention of β-arrestin 2 membrane translocation. In contrast, we showed that the increased activity of ERK1/2 in an high glucose/high insulin environment was unaffected by β-arrestin 2 transfection, implying that the mechanism(s) by which GRK2 can activate ERK1/2 would be independent of its interaction with β-arrestin 2.

eNOS-ROS interactions may be critical for eNOS signaling dysfunction under hyperglycemic/hyperinsulinemic conditions. We found that, despite a significant increase in intracellular ROS generation after exposure of HUVECs to high glucose, high glucose/high insulin conditions failed to lead to increased ROS generation. Our results are in agreement with the previous report showing that treatment with insulin reduced ROS generation in HUVECs under high glucose conditions (Matsui-Hirai et al., 2011). Thus, we suggest that ROS is not responsible for the altered Akt/eNOS pathway under high glucose/high insulin conditions. However, it is interesting that transfection of GRK2 siRNA prevented the increase in ROS generation under high glucose conditions. This finding suggests that GRK2 may play a regulatory role in high glucose–induced ROS generation in endothelial cells, although the exploration of its regulatory mechanism is beyond the scope of this study.

We acknowledge that there is a noteworthy limitation in the research design that allows criticism to be voiced. Most of the data in this study were obtained using HUVECs. Although HUVECs are widely used as a laboratory model system for the study of the function and pathology of endothelial cells, limitations remain in that they cannot fully represent the metabolic properties and the responses in the pathophysiology related to the different types of endothelial cells distributed in the entire organism. It would be preferable to focus on the data using endothelial cells from the arterial system, HCAECs and HAECs, in light of a specific pathologic entity of diabetes. However, we believe the present results, using HUVECs, can guide future studies directed to understanding the role of GRK2 in vascular pathology in insulin-resistant diabetes.

In conclusion, the present study identifies GRK2 as an important negative regulator of insulin signaling in human vascular endothelial cells. This view is in good agreement with the recent report obtained in adipocytes and skeletal
myocytes (Garcia-Guerra et al., 2010). GRK2 may be key to contributing to a vicious cycle for the development of insulin-resistant conditions. Our studies could uncover this molecule as a potential target in the prevention and treatment of vascular disorders in insulin-resistant individuals. Although several questions remain concerning the mechanism for the

Fig. 7. Transfection of GRK2 into HEK293T cells. (A) Western blots probed by anti-GRK2 antibody showing ample GRK2 expression in HEK293T cells transfected with pCMV-SPORT5-GRK2 but not with mock plasmid (n = 3). (B) Western blots of phospho-Akt at Ser-473 and of total Akt (n = 3). (C) Western blots of phospho-ERK1/2 and of total ERK1/2 (n = 3). For comparison, the data obtained without any plasmid transfection protocol (NT) are presented on the left side of each panel. **P < 0.01 versus NT. In each top trace, typical immunoblots are depicted. (D) Typical Western blots including data showing total and phosphorylation levels of Akt and ERK1/2 in GRK2-overexpressed HEK293T cells exposed to 22 mM glucose/100 nM insulin for 72 hours. This experiment was repeated twice.

Fig. 8. ROS generation in HUVECs. (A) Effects of GRK2 siRNA (10 nM) or control siRNA transfection on ROS generation when cells were exposed to normal glucose (NG) (5.5 mM), high glucose (HG) (22 mM), or high glucose/high insulin (HG/HI) for 72 hours (n = 5). ***P < 0.001 versus the value under NG. ###P < 0.001 versus the value under HG. †P < 0.05 versus the value under HG/HI. (B) Images of intracellular ROS visualization using CM-H2DCFDA.
hyperglycemic/hyperinsulinemic-dependent up-regulation of GRK2, the identification of the role of GRK2 in the Akt/eNOS pathway will provide new insight into the understanding of the pathologic signal of vascular endothelial cells in insulin-resistant diabetes.

Acknowledgments

The authors thank Sayaka Aoyama and Toshio Fujimori for technical assistance.

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