

JPET # 211854

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

Kumiko Taguchi¹, Kimimasa Sakata¹, Wakana Ohashi, Takahiro Imaizumi, Joji Imura, and Yuichi Hattori

Department of Molecular and Medical Pharmacology (K.T., K.S., W.O., T.I., Y.H.) and Department of Diagnostic Pathology (J.I.), Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama 930-0194, Japan

JPET # 211854

A running title: GRK2 tonic inhibition of endothelial Akt/eNOS signaling

Corresponding author:

Yuichi Hattori, M.D., Ph.D.

Department of Molecular and Medical Pharmacology, Graduate School of Medicine and
Pharmaceutical Sciences, University of Toyama, Toyama 930-0194, Japan

Tel: 81-76-434-7260; Fax: 81-76-434-5021; E-mail: yhattori@med.u-toyama.ac.jp

The number of text pages: 31

The number of tables: 0

The number of figures: 8

The number of references: 38

The number of words in the Abstract: 250

The number of words in the Introduction: 565

The number of words in the Discussion: 1376

ABBREVIATIONS: GRK2, protein-coupled receptor kinase 2; GPCR, G protein-coupled receptor; ERK1/2, extracellular signal-regulated kinase 1/2; NO, nitric oxide; eNOS, endothelial NO synthase; HUVEC, human umbilical venous endothelial cell; HAEC, human aortic endothelial cell; HCAEC, human coronary artery endothelial cell; siRNA, small interfering RNA; PBS, phosphate buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAPI, 4', 6-diamidino-2-phenylindole dihydrochloride; MEK, mitogen-activated protein kinase kinase; PD98059, 2'-amino-3'-methoxyflavone; ROS, reactive oxygen species; CM-H₂DCFDA, 5-(and-6)-chloromethyl-2',7'-dichlorofluorescein diacetate, acetyl ester

SECTION: Cardiovascular Pharmacology

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) in order 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 and 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 HEK293T cells which overexpressed GRK2, Akt activity was unchanged, while 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 GPKs 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 provided 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, or 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, as those reported in a number of relevant cardiovascular, inflammatory, or 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 patients afflicted with different heart conditions (Harris et al., 2001; Reinkober et al., 2012). Moreover, it has been suggested that up-regulated 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 immunesystem 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 two- to fourfold 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 pathological 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 in order 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 μ g/ml hydrocortisone hemisuccinate, which were considered to have no affect on our outcome. Cells of passage 4-7 were used when 70-80% confluent. Then, cells were harvested and seeded into six-well plates. In order 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 to 72 h. 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 20-25 nucleotides scrambled sequence, 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 μ l of RIPA buffer (25 mM Tris-HCl, 150

mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, pH 7.4; Thermo, Rockford, IL) containing protease inhibitor cocktail (Nacalai, Kyoto, Japan) on ice. The lysates were centrifuged at $18,000 \times g$ for 10 min at 4°C and the resulting supernatants were collected. The proteins in the supernatant were measured using BCA Protein Assay Kit (Thermo). Where required, the membrane fractions were prepared; the supernatant was then spun at $100,000 \times g$ for 30 min at 4°C and the membrane pellet was resuspended in 50 μ l of lysis buffer and saved. The supernatants (~20 μ g 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 min 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 (PBS) 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 1:1,000~2,000 in Odyssey blocking buffer for 120 min at 38°C in dark. After being washed six times in PBS with 0.1% Tween 20, the blots were visualized using the Odyssey Infrared imaging system from LI-COR.

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 extracellular signal-regulated protein kinase 1/2 (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 min and incubated with anti-GRK2 rabbit polyclonal antibody (1:100; Bioss, Woburn, MA) for 2 h, followed by exposure to the anti-rabbit IgG conjugated to the high quality fluophore Alexa Fluor 488 for 1 h. The nucleus was counterstained with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI; Cell Signaling). Immunofluorescent images were observed using a fluorescence microscope (BZ-8100; Keyence, Osaka, Japan).

Measurement of NO_x Production.

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

Transient Transfection of GRK2 in HEK293T Cells.

HEK293T cells were maintained in 10% fetal bovine serum-supplemented Dulbecco's modified Eagle's medium. An expression plasmid encoding the human GRK2 (hGRK2), pCMV-SPORT6-hGRK2, was purchased from Dnaform (Yokohama, Japan). hGRK2 sequence is inserted into the NotI(774)/Sall(795) sites of pCMV-SPORT6 vector. Mock pCMV-SPORT6 vector was made by removing XhoI(755)/Sall(795) region including the hGRK2 sequence. Twenty-four h prior to transfection, 5×10^5 cells were seeded in a 6 well plate. Transfection of the cells was

performed using Lipofectamine2000 or LipofectamineLTX 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; final concentration 10 μ M) for 45 min in PBS at 37°C. At the end of an experiment, cells were rinsed with PBS, 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 \pm S.E. Data were analyzed by the use of Prism software (ver. 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. Fig. 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 h (Fig. 1B). Furthermore, treatment with insulin at a low (10 nM) or high concentration (100 nM) 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. The high glucose/high insulin-induced up-regulation of GRK2 expression was confirmed using confocal microscopy in HUVECs (Fig. 1C). The up-regulation of GRK2 expression after high glucose/high insulin was time-dependent (Fig. 1D). The up-regulation of GRK2 expression under high glucose/high insulin was also observed in HCAECs and HAECs (Fig. 1E). When HCAECs and HAECs responded to 72-h high glucose/high insulin exposure, the relative amounts of GRK2 protein were increased to $123 \pm 6\%$ ($n = 3$) and $134 \pm 14\%$ ($n = 4$) 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 Effect of GRK2 Inhibitor

High glucose/high insulin did not activate Akt and eNOS during 72 h after exposure, as indicated by no change in Ser-473 phosphorylation of Akt and Ser-1177 phosphorylation of eNOS (Fig. 3A 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 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, that provide a measure of its activation, were significantly increased after exposure to high glucose/high insulin (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 (MEK), 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 the previous report to note that Ser-670 represents a site of ERK1/2 phosphorylation (Pitcher et al., 1999).

GRK2 siRNA Behaved in the Same Way as 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. 4B 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 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). Interestingly, treatment with 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 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 immunoblot (Fig. 5B). Even when the β -arrestin 2 siRNA transfection experiments were performed at its 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). The decreasing effects of transfection of β -arrestin 2 siRNA were also observed on eNOS phosphorylation and NOx production (Fig. 6B and C). Furthermore, treatment with GRK2 inhibitor failed to increase Akt phosphorylation, eNOS phosphorylation, and NOx production when cells were transfected with β -arrestin 2 siRNA (Fig. 6A, B, and 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 pCMV-SPORT6-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 which overexpressed GRK2, Akt activity was unchanged, as revealed by no 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 h. However, high glucose/high insulin did not essentially modify the negative and positive effects of GRK2 overexpression on Akt and ERK1/2 phosphorylation levels, respectively, observed under normal conditions (Fig. 7D).

ROS Generation

JPET # 211854

When intracellular ROS were visualized using the fluorescence dye CM-H₂DCFDA, the exposure of HUVECs to high glucose for 72 h 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 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, but we suggest that such a GRK2 up-regulation, as those reported in several relevant cardiovascular, inflammatory, or 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 which overexpressed GRK2. Furthermore, the phosphorylated levels of eNOS, a target downstream of the Akt pathway, and eNOS-mediated NO_x 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 NO_x 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 which has been demonstrated in the previous report (Taguchi et al., 2011).

In endothelial cells exposed to high glucose/high insulin, 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 MEK 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 Ser-670 impairs the ability of GRK2 to phosphorylate their 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. In addition, 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 reports have demonstrated that vascular levels of ERK1/2 activation are elevated in type 2 diabetic mice (Tian et al., 2011; Choi et al., 2012), leading to vascular endothelial dysfunction. The results of those studies might be due to increased expression and activity of vascular GRK2 in the

disease state (Taguchi et al., 2011, 2012b).

The arrestin family consists of four members. Visual arrestins, arrestin 1 and 4, are exclusively expressed in the retina, β -arrestin 1 and β -arrestin 2 are ubiquitously expressed in most tissues (Sterne-Marr and Benovic, 1995). Although the two widely coexpressed isoforms of β -arrestins are highly similar in amino acid sequence, β -arrestin 2 appears to be much more potent than β -arrestin 1 in regulating receptor signaling and trafficking (Kohout et al., 2001). β -Arrestins, originally discovered for their role in terminating GPCR signaling by facilitating desensitization and internalization, are now appreciated for their additional functions as scaffold proteins to interact with several cytoplasmic proteins and link GPCRs to intracellular signaling pathways (Lefkowitz and Whalen, 2004; Lefkowitz and Shenoy, 2005). Moreover, 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. 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 and activity, both of which were 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 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 (Matsui-Hirai et al., 2011). We thus 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 which 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 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 pathological signal of vascular endothelial cells in insulin-resistant diabetes.

JPET # 211854

Acknowledgments

We thank Sayaka Aoyama and Toshio Fujimori for technical assistance.

Authorship Contributions

Participated in research design: Taguchi, Sakata, Ohashi, and Hattori.

Conducted experiments: Taguchi, Sakata, Ohashi, Imaizumi, and Imura.

Performed data analysis: Taguchi and Sakata.

Wrote the manuscript: Sakata and Hattori.

References

Aguirre V, Uchida T, Yenush L, Davis R, and White MF (2000) The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). *J Biol Chem* **275**: 9047-9054.

Bard-Chapeau EA, Hevener AL, Long S, Zhang EE, Olefsky JM, and Feng GS (2005) Deletion of Gab1 in the liver leads to enhanced glucose tolerance and improved hepatic insulin action. *Nat Med* **11**: 567-571.

Capellini VK, Celotto AC, Baldo CF, Olivon VC, Viaro F, Rodrigues AJ, and Evora PR (2010) Diabetes and vascular disease: basic concepts of nitric oxide physiology, endothelial dysfunction, oxidative stress and therapeutic possibilities. *Curr Vasc Pharmacol* **8**: 526-544.

Chen D, and Wang MW (2005) Development and application of rodent models for type 2 diabetes. *Diabetes Obes Metab* **7**: 307-317.

Choi SK, Galán M, Partyka M, Trebak M, Belmadani S, and Matrougui K (2012) Chronic inhibition of epidermal growth factor receptor tyrosine kinase and extracellular signal-regulated kinases 1 and 2 (ERK1/2) augments vascular response to limb ischemia in type 2 diabetic mice. *Am J Pathol* **180**: 410-418.

Chon HI, Xi Y, Pesant S, Harris DM, Hyslop T, Falkner B, and Eckhart AD (2009) G protein-coupled receptor kinase 2 expression and activity are associated with blood pressure in black Americans. *Hypertension* **54**: 71-76.

JPET # 211854

Cipolletta E, Campanile A, Santulli G, Sanzari E, Leosco D, Campiglia P, Trimarco B, and Iaccarino G (2009) The G protein coupled receptor kinase 2 plays an essential role in beta-adrenergic receptor-induced insulin resistance. *Cardiovasc Res* **84**: 407-415.

DeWire SM, Ahn S, Lefkowitz RJ, and Shenoy SK (2007) Beta-arrestins and cell signaling. *Annu Rev Physiol* **69**: 483-510.

Dresner A, Laurent D, Marcucci M, Griffin ME, Dufour S, Cline GW, Slezak LA, Andersen DK, Hundal RS, Rothman DL, Petersen KF, and Shulman GI (1999) Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. *J Clin Invest* **103**: 253-259.

Garcia-Guerra L, Nieto-Vazquez I, Vila-Bedmar R, Jurado-Pueyo M, Zalba G, Díez J, Murga C, Fernández-Veledo S, Mayor F Jr, and Lorenzo M (2010) G protein-coupled receptor kinase 2 plays a relevant role in insulin resistance and obesity. *Diabetes* **59**: 2407-2417.

Gros R, Chorazyczewski J, Meek MD, Benovic JL, Feurguson SS, and Feldman RD (2000) G-protein-coupled receptor kinase activity in hypertension: increased vascular and lymphocyte G-protein receptor kinase-2 protein expression. *Hypertension* **35**: 38-42.

Harris CA, Chuang TT, and Scorer CA (2001) Expression of GRK2 is increased in the left ventricles of cardiomyopathic hamsters. *Basic Res Cardiol* **96**: 364-368.

JPET # 211854

Inoguchi T, Li P, Umeda F, Yu HY, Kakimoto M, Imamura M, Aoki T, Etoh T, Hashimoto T, Naruse M, Sano H, Utsumi H, and Nawata H (2000) High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C-dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes* **49**: 1939-1945.

Jimenez-Sainz MC, Muga C, Kavelaars A, Jurado-Pueyo M, Krakstad BF, Heijnen CJ, Mayor F Jr, and Aragay AM (2006) G protein-coupled receptor kinase 2 negatively regulates chemokine signaling at a level downstream from G protein subunits. *Mol Biol Cell* **17**: 25-31.

Kim J, Ahn S, Guo R, and Daaka Y (2003) Regulation of epidermal growth factor receptor internalization by G protein-coupled receptors. *Biochemistry* **42**: 2887-2894.

Kirpichnikov D, and Sowers JR (2001) Diabetes mellitus and diabetes-associated vascular disease. *Trends Endocrinol Metab* **12**: 225-230.

Kohout TA, Lin FS, Perry SJ, Conner DA, and Lefkowitz RJ (2001) β -Arrestin 1 and 2 differentially regulate heptahelical receptor signaling and trafficking. *Proc Natl Acad Sci USA* **98**: 1601-1606.

Lefkowitz RJ, Shenoy SK (2005) Transduction of receptor signals by β -arrestins. *Science* **308**: 512-517.

JPET # 211854

Lefkowitz RJ, and Whalen EJ (2004) β -Arrestins: traffic cops of cell signaling. *Curr Opin Cell Biol* **16**: 162-168.

Liu S, Premont RT, Kontos CD, Zhu S, and Rockey DC (2005) A crucial role for GRK2 in regulation of endothelial cell nitric oxide synthase function in portal hypertension. *Nat Med* **11**: 952-958.

Luan B, Zhao J, Wu H, Duan B, Shu G, Wang X, Li D, Jia W, Kang J, and Pei G (2009) Deficiency of a b-arrestin-2 signal complex contributes to insulin resistance. *Nature* **457**: 1146-1150.

Matsui-Hirai H, Hayashi T, Yamamoto S, Ina K, Maeda M, Kotani H, Iguchi A, Ignarro LJ, and Hattori Y (2011) Dose-dependent modulatory effects of insulin on glucose-induced endothelial senescence in vitro and in vivo: A relationship between telomeres and nitric oxide. *J Pharmacol Exp Ther* **337**: 591-599.

Montagnani M, Golovchenko I, Kim I, Koh GY, Goalstone ML, Mundhekar AN, Jahansen M, Kucik DF, Quon MJ, and Draznin B (2002) Inhibition of phosphatidylinositol 3 kinase enhances mitogenic actions of insulin in endothelial cells. *J Biol Chem* **277**: 1794-1799.

Ozawa K, Whalen EJ, Nelson CD, Mu Y, Hess DT, Lefkowitz RJ, and Stamler JS (2008) S-nitrosylation of beta-arrestin regulates beta-adrenergic receptor trafficking. *Mol Cell* **31**: 395-405.

JPET # 211854

Penela P, Murga C, Ribas C, Lafarga V, and Mayor F Jr (2010) The complex G protein-coupled receptor kinase 2 (GRK2) interactome unveils new physiopathological targets. *Br J Pharmacol* **160**: 821-832.

Penn RB, Pronin AN, and Benovic JL (2000) Regulation of G protein-coupled receptor kinases. *Trends Cardiovasc Med* **10**: 81-89.

Pitcher JA, Tesmer JJG, Freeman JLR, Capel WD, Stone WC, and Lefkowitz RJ (1999) Feedback inhibition of G protein-coupled receptor kinase 2 (GRK2) activity by extracellular signal-regulated kinases. *J Biol Chem* **274**: 34531-34534.

Premont RT, and Gainetdinov RR (2007) Physiological roles of G protein-coupled receptor kinases and arrestins. *Proc Natl Acad Sci USA* **95**: 14082-14087.

Reinkober J, Tscheschner H, Plegler ST, Most P, Katus HA, Koch WJ, and Raake PW (2012) Targeting GRK2 by gene therapy for heart failure: benefits above β -blockade. *Gene Ther* **19**: 686-693.

Ren RB, Pronin AN, and Benovic JL (2000) Regulation of G protein-coupled receptor kinases. *Trends Cardiovasc Med* **10**: 81-89.

Shen N, Yu X, Pan F-Y, Gao X, Xue B, and Li C-J (2011) An early response transcription factor, Egr-1, enhances insulin resistance in type 2 diabetes with chronic hyperinsulinism. *J Biol Chem* **286**: 14508-14515.

JPET # 211854

Sterne-Marr R, and Benovic JL (1995) Regulation of G protein-coupled receptors by receptor kinases and arrestins. *Vitam Horm* 51: 193-234.

Taguchi K, Kobayashi T, Matsumoto T, and Kamata K (2011) 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.

Taguchi K, Matsumoto T, Kamata K, Kobayashi T (2012a) Inhibitor of G protein-coupled receptor kinase 2 normalizes vascular endothelial function in type 2 diabetic mice by improving β -arrestin 2 translocation and ameliorating Akt/eNOS signal dysfunction. *Endocrinology* **153**: 2985-2996.

Taguchi K, Matsumoto T, Kamata K, Kobayashi T (2012b) G protein-coupled receptor kinase 2, with β -arrestin 2, impairs insulin-induced Akt/endothelial nitric oxide synthase signaling in *ob/ob* mouse aorta. *Diabetes* **61**: 1978-1985.

Tian XY, Wong WT, Xu A, Chen ZY, Lu Y, Liu LM, Lee VW, Lau CW, Yao X, and Huang Y (2011) Rosuvastatin improves endothelial function in db/db mice: role of angiotensin II type 1 receptors and oxidative stress. *Br J Pharmacol* **164**: 598-606.

Vroon A, Heijnen CJ, and Kavelaars A (2006) GRKs and arrestins: regulators of migration and inflammation. *J Leukoc Biol* **80**: 1214-1221.

Zhang J, Ferguson SS, Barak LS, Aber MJ, Giros B, Lefkowitz RJ, and Caron MG (1997) Molecular mechanisms of G protein-coupled receptor signaling: role of G

JPET # 211854

protein-coupled receptor kinases and arrestins in receptor desensitization and resensitization. *Receptors Channels* **5**: 193-199.

JPET # 211854

FOOTNOTES

¹Drs. Taguchi and Sakata contributed equally to this work.

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

Figure Legends

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 h after exposure to normal (NG), high (HG), and extremely high (EHG) glucose conditions without or with low (LI) and high (HI) insulin levels ($n = 6$). *, $P < 0.05$ versus NG without insulin. NG, 5.5 mM; HG, 22 mM; EHG, 31 mM of glucose in the culture medium. LI, 10 nM; HI, 100 nM of applied insulin concentration. (C) Confocal images of HUVECs stained with anti-GRK2 antibody (green). Cells were exposed to NG or HG/HI for 72 h. Nuclei are counterstained in blue by DAPI. Original magnification: $\times 40$. (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.

Fig. 2. Membrane translocation of GRK2 in HUVECs. Increased GRK2 translocation to the plasma membrane under high glucose/high insulin. Cells were harvested at 72 h after 5.5 mM glucose or 22 mM glucose/100 nM insulin exposure, lysed, fractionated, and subjected to Western blotting with anti-GRK2 antibody as described in *Materials and Methods*. GRK2 inhibitor at a concentration of 1 μM was given simultaneously with exposure to normal glucose or high glucose/high insulin. The data are shown as ratio of GRK2 relative to GAPDH ($n = 6-8$). **, $P < 0.01$; ***, $P < 0.001$ versus the respective value obtained under normal glucose. ##, $P < 0.01$; ###, $P < 0.001$.

Fig. 3. Effects of GRK2 inhibitor treatment on Ser-473 phosphorylation of Akt (A),

Ser-1177 phosphorylation of eNOS (B), NO_x levels in the culture medium (C), and Thr-202/Tyr-204 phosphorylation of ERK1/2 (D) in HUVECs at 1-72 h after exposure to 22 mM glucose and 100 nM insulin. GRK2 inhibitor at a concentration of 1 μM was given simultaneously with exposure to high glucose/high insulin. *, *P*<0.05; **, *P*<0.01 versus the respective value at time 0 (*n* = 4-12). In each top trace of (A), (B), and (D), typical Western blots are shown. (E) Typical Western blots include the data showing the effects of PD98059 on phosphorylation levels of ERK1/2 at Thr-202/Tyr-204 and of GRK2 at Ser-670 in HUVECs exposed to high glucose/high insulin (HG + HI). These experiments were repeated twice.

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. Then, Ser-473 phosphorylation of Akt (B), Ser-1177 phosphorylation of eNOS (C), NO_x levels in the culture medium (D), and Thr-202/Thy-204 phosphorylation of ERK1/2 (E) were examined at 72 h 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.

Fig. 5. Interactions between GRK2 and β-arrestin 2 in HUVECs. (A) Effects of GRK2 inhibitor treatment on β-arrestin translocation to the plasma membrane. Cells were

harvested at 72 h after 5.5 mM glucose or 22 mM glucose/100 nM insulin exposure, lysed, fractionated, and subjected to Western blotting with anti- β -arrestin 2 antibody as described in *Materials and Methods*. GRK2 inhibitor at a concentration of 1 μ M was given simultaneously with exposure to normal glucose or high glucose/high insulin. The data are shown as ratio of β -arrestin 2 relative to GAPDH ($n = 6$). *, $P < 0.05$; ***, $P < 0.001$ versus the respective value obtained under normal glucose. #, $P < 0.05$; ##, $P < 0.01$. (B) Transfection of β -arrestin 2 siRNA decreased β -arrestin 2 protein expression in a concentration-dependent manner. (C) One day after β -arrestin 2 siRNA (100 nM) or control siRNA was transfected, cells were exposed to normal glucose or high glucose/high insulin for 72 h. Then, GRK2 expression was evaluated ($n = 6$). *, $P < 0.05$; **, $P < 0.01$ versus the respective value obtained under normal glucose without siRNAs. In the top trace, typical Western blots are shown. GAPDH served as loading control in Western blot analysis.

Fig. 6. Transfection of β -arrestin 2 siRNA in HUVECs. One day after β -arrestin 2 siRNA, cells were exposed to normal glucose or high glucose/high insulin for 72 h. Then, Ser-473 phosphorylation of Akt (A), Ser-1177 phosphorylation of eNOS (B), NOx levels in the culture medium (C), and Thr-202/Thy-204 phosphorylation of ERK1/2 (D) were evaluated. For comparison, the data obtained with any siRNA transfection protocol (NT) are set out. *, $P < 0.05$; **, $P < 0.01$ versus the respective white column ($n = 4$). In each top trace of (A), (B), and (D), typical Western blots are shown.

Fig. 7. Transfection of GRK2 into HEK293T cells. (A) Western blots probed by anti-GRK2 antibody showing ample GRK2 expression in HEK293T cells transfected

JPET # 211854

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 include the 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 h. 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 h ($n = 5$). ***, $P < 0.001$ 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.

Figure 1

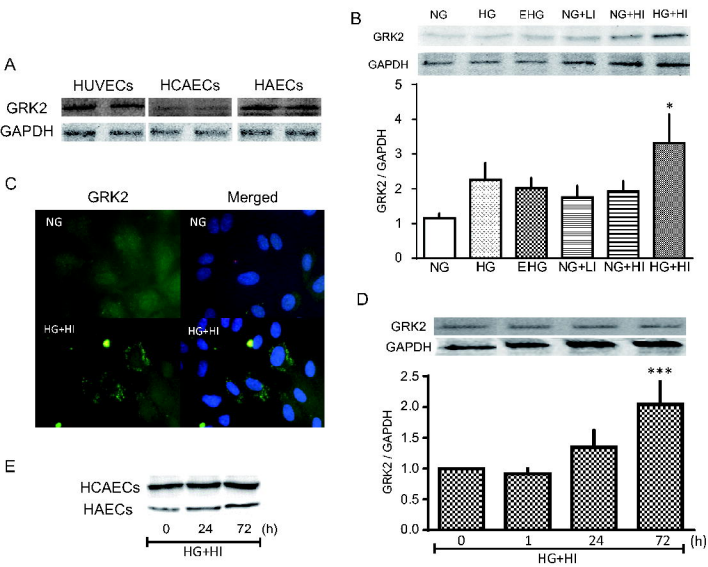


Figure 2

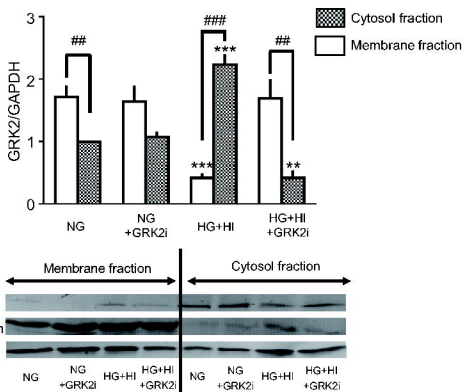


Figure 3

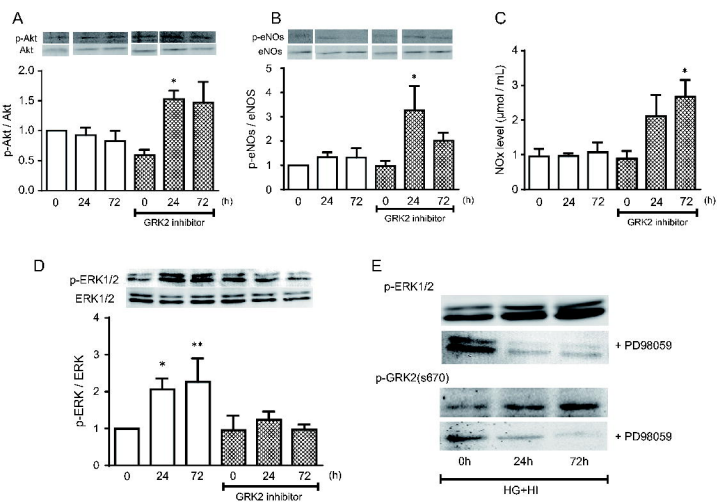


Figure 4

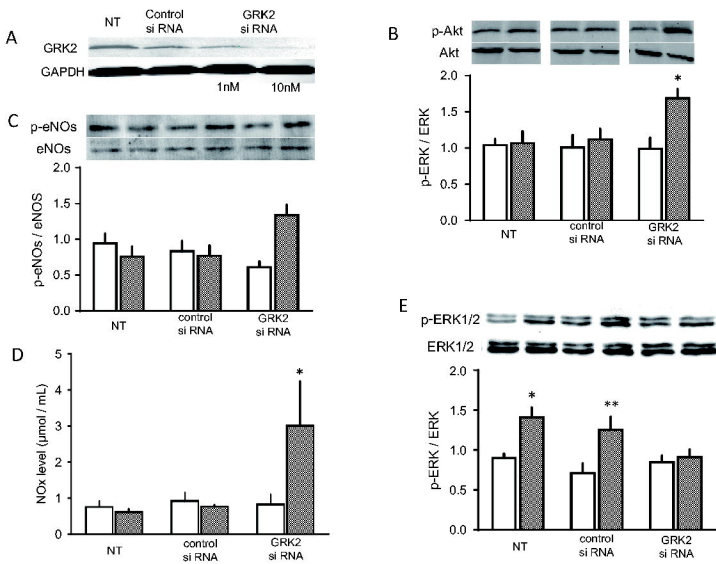


Figure 5

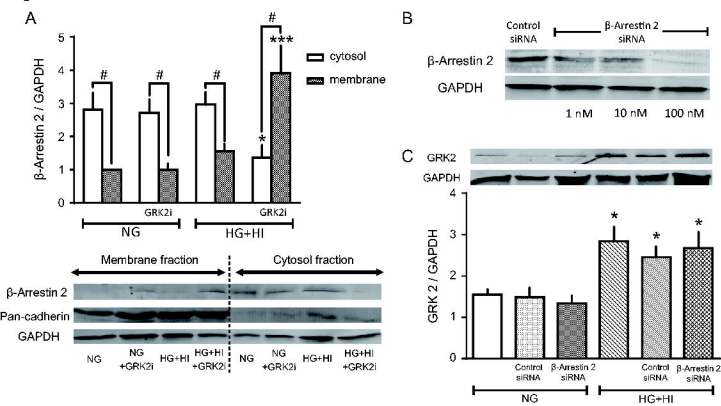


Figure 6

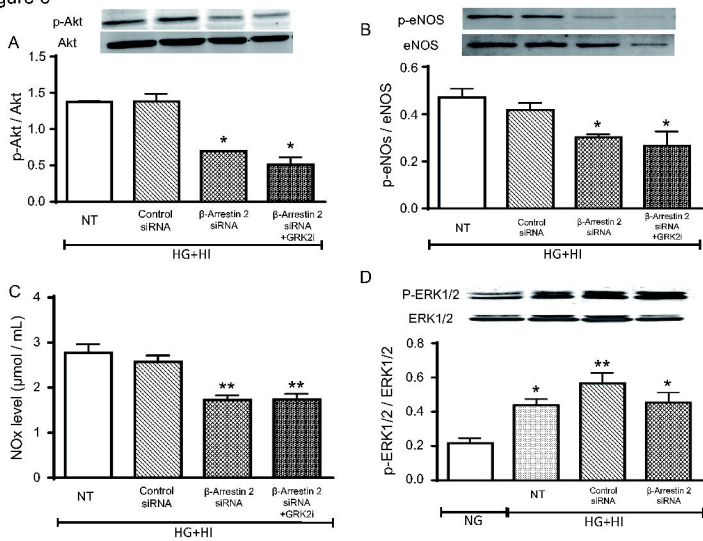


Figure 7

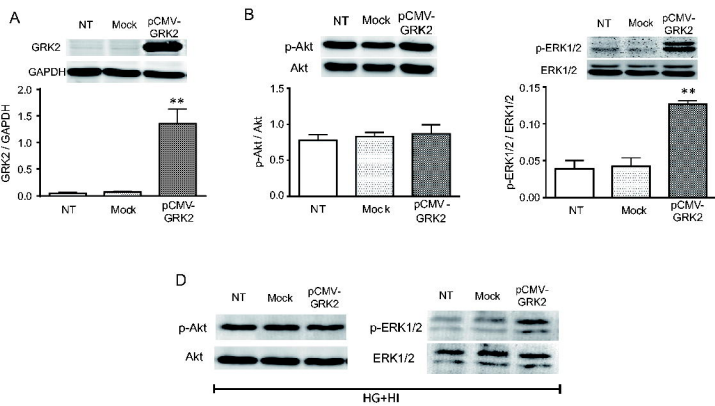
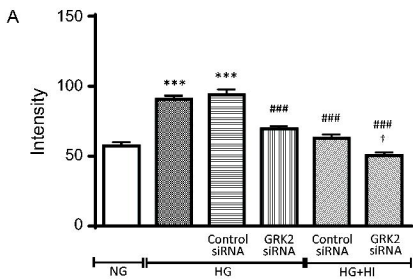


Figure 8



B

