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Angiotensin II causes endothelial dysfunction via the GRK2/Akt/eNOS pathway in aortas from a murine type 2 diabetic model

Kumiko Taguchi, Tsuneo Kobayashi, Yasuhiro Takenouchi, Takayuki Matsumoto, Katsuo Kamata*

Department of Physiology and Morphology, Institute of Medicinal Chemistry, Hoshi University, Shinagawa-ku, Tokyo 142-8501, Japan

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ABSTRACT

Nitric oxide (NO) production and endothelial function are mediated via the Akt/eNOS pathway. We investigated the reductions of these mechanism(s) in type 2 diabetes. Diabetic model (nicotinamide + streptozotocin-induced) mice were fed for 4 weeks on a normal diet either containing or not containing losartan, an AT_1 R antagonist. Relaxations and NO productions were measured in isolated aortas. G-protein coupled receptor kinase 2 (GRK2) protein levels and activities in the Akt/eNOS signaling-pathway were mainly assayed by Western blotting. Clonidine- and insulin-induced relaxations and NO productions, all of which were significantly decreased in aortas isolated from the diabetics, were normalized by 4 weeks' losartan administration. Plasma angiotensin II (Ang II) and GRK2 protein levels were increased in diabetes, and each was normalized by 4 week's losartan administration. Additionally, there was a direct correlation between the plasma Ang II and aortic GRK2 protein levels. In the diabetics, the clonidine-induced responses (but not the insulin-induced ones) were enhanced by GRK2-inhibitor. Akt phosphorylation was markedly below control in the clonidine-stimulated diabetes. The phosphorylation of Akt at Thr^{308} was significantly normalized and the phosphorylation of eNOS at Ser^{1177} tended to be increased by GRK2-inhibitor in the clonidine-stimulated diabetics. Our data suggest that (a) the Akt/eNOS pathway is downstream of GRK2, and that GRK2 inhibits Akt/eNOS activities, and (b) this pathway underlies the impaired NO production seen in type 2 diabetes, in which there are defective phosphorylations of Akt and eNOS that may be caused by an upregulation of GRK2 secondary to a high plasma Ang II level. Inhibitors of GRK2 warrant further investigation as potential new therapeutic agents in diabetes.

1. Introduction

Diabetes mellitus is an important risk factor for hypertension and other cardiovascular diseases, and impaired vasodilation has been described in diabetic humans and in animal models of this disease [1–5]. Recent investigations suggest that in diabetes, the potent vasodilator NO is underproduced by endothelial cells and that the impaired NO production results, in part, from endothelial dysfunction [6]. Recent reports have suggested that for efficient NO production in response to a variety of stimuli, eNOS phosphorylation via Akt is required [7–9]. By phosphorylating eNOS, Akt enhances NO production. We and others have found that activation of the Akt/eNOS pathway by clonidine or insulin is impaired in the aorta in diabetes [10–12], resulting in endothelial dysfunction.

The experimental model employed here (mice given STZ and partially protected with a suitable dose of nicotinamide) was devised a few years ago (10). In this model, the diabetic syndrome shares a number of features with human type 2 diabetes: viz. it is characterized by stable moderate hyperglycemia, glucose intolerance, and hypertension. The presence of such features in this model has yet to be explained.

GRK2, the best-characterized GRK, is ubiquitously expressed, and can phosphorylate many different GPCRs [13,14]. This enzyme plays a particularly vital role in the heart, where it regulates the force and rate of muscle contraction by phosphorylating β-adrenergic receptors (β-AR) [15,16]. However, biochemical and transgenic studies have strongly implicated GRK2 in the progression of cardiovascular disease [15], and GRK2 has been found to be elevated in cardiac myocytes after congestive heart failure [17–19]. One example of its possible involvement in cardiovascular disease is as follows. It has been proposed that impaired vascular β-adrenergic responsiveness may play an important role in the development or maintenance, or both, of essential hypertension, and that underlying this defect may be enhanced GRK2 expression,

Abbreviations: α-AR, α-adrenergi receptor; ACh, acetylcholine; AngII, angiotensin II; DM, diabetic mice; eNOS, endothelial oxide synthase; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; IGTT, intravenous glucose tolerance test; IRS-1, insulin receptor substrate-1; KHS, Krebs–Henseleit solution; NO, nitric oxide; PH, pleckstrin homology; $PGF_{2\alpha}$, prostaglandin; $F_{2\alpha}$RGS, regulators of G-protein signaling; SNP, sodium nitroprusside; STZ, streptozotocin; vWF, von Willebrand factor.

* Corresponding author. Tel.: +81 3 5498 5856; fax: +81 3 5498 5856.
E-mail address: kamata@hoshi.ac.jp (K. Kamata).

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leading both to reduced receptor responsiveness and to impaired coupling of the receptor and G-protein [17]. However, there have as yet been no reports linking GRK2 to endothelial dysfunction in diabetes.

The α_2-AR is a known GRK2 substrate *in vitro* [19,20]. We hypothesized (a) that there is a link between α_2-AR-stimulated GPCR signaling (specifically G$\beta\gamma$) and Akt/eNOS activity/NO production, and (b) that a defect in the upstream kinase, GRK2, is associated with impaired eNOS activity in diabetes.

Angiotensin II (Ang II) alters vascular tone by inducing vascular smooth muscle cell contraction [21,22]. Two known GPCRs, designated AT_1 R and AT_2 R, mediate the physiological effects of Ang II, and there is evidence that Ang II-receptor signaling may be negatively regulated by GRK2 [23]. Vascular GRK2 is increased in human hypertension and in animal models of the disease (leading to reduced GPCR signaling in hypertension) [24,25] and administration of an Ang II-receptor blocker results in a normalization of upregulated cardiac GRK2 in a rabbit model of myocardial infarction-induced heart failure [26].

Akt is known to be activated by factors in the cell other than α_2-AR, including insulin, ET-1, and VEGF [7,9], and it has been demonstrated that GRK2 mitigates the effects of multiple Akt activators [27]. Interestingly, the Akt/eNOS pathway is impaired in the endothelial cells of several diabetic models [10–12]. Against that background, the aim of the present study was to test the following hypotheses: (1) Ang II causes endothelial dysfunction in diabetes via the Akt/eNOS pathway, and an increase in plasma Ang II increases the vascular GRK2 level; (2) GRK2 interacts negatively with Akt, and the Akt/eNOS pathway is downstream of GRK2 and (3) in aortas from diabetic mice, an increase in GRK2 leads to an impairment of the Akt/eNOS pathway.

2. Materials and methods

2.1. Chemicals and drugs

Clonidine, insulin, N^G-nitro-L-arginine (L-NNA), monoclonal β-actin antibody, anti-von Willebrand factor (vWF), and anti-mouse IgG CY3 conjugate antibody were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Losartan (Nulotan) was from Banyu Co. Ltd. (Tsukuba, Japan). Akt inhibitor (=1L-6-hydroxymethyl-chiro-inositol 2-[(R)-2-O-methyl-3-O-octadecylcarbonate]), GRK2-inhibitor (=methyl[(5-nitro-2-furyl)vinyl]-2-furoate), and Gallein (=4,5-dihydroxyfluorescein dihydrate,2-(3,4,5-trihydroxy-6-oxoxanthen-9-yl)-benzoic acid dehydrate) were from CALBIOCHEM (La Jolla, CA, USA). All drugs were dissolved in saline, except. GRK2-inhibitor and Gallein, which were dissolved in DMSO. All concentrations are expressed as the final molar concentration of the base in the organ bath. HRP-linked secondary anti-mouse or anti-rabbit antibody was purchased from Promega (Madison, WI, USA), while antibody against GRK2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Akt, phosphorylated Akt (pS473/pT308), and phosphorylated eNOS (pS1177) were obtained from Cell Signaling Technology (Danvers, MA, USA), while antibody against eNOS was from BD Bioscience (San Jose, CA, USA). Anti-rabbit IgGDyLight™ 488 was obtained from KPL (Gaithersburg, MD, USA).

2.2. Animals and experimental design

To induce diabetes, Institute of Cancer Research (ICR; Tokyo Animal Laboratories, Tokyo) mice (5-week-old males) received an intraperitoneal injection of 1.5 g/kg of nicotinamide dissolved in saline 15 min before an injection via the tail vein of streptozotocin (STZ; 200 mg/kg) dissolved in a citrate buffer [10]. Age-matched control mice received saline, followed by the citrate buffer (via the routes mentioned above). At 12 weeks after these administrations, the mice were assigned to various experiments. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology, Japan). Starting 8 weeks after these administrations, diabetic model mice were fed for 4 weeks on a normal diet either containing or not containing losartan (Nulotan; 50 mg/kg/day). These mice are referred to as the DM and losartan-treated DM groups, respectively.

2.3. Chronic GRK2 inhibitor administration

For continuous inhibition of GRK2 recruitment to membranes, some DM or age-matched control mice (17 weeks old, $n = 4$) were treated with either high- or low-dose GRK2-inhibitor (high-dose, 56.4 ng/kg/day; low-dose, 5.64 ng/kg/day). This treatment was given by way of an osmotic mini-pump (2ML1; Alzet, Palo Alto, CA, USA) for 2 weeks.

2.4. Intravenous glucose tolerance test (IGTT)

Glucose (2.0 g/kg) was given as a 30% solution by intraperitoneal injection in conscious fasting animals, as previously described [10]. Blood samples were collected sequentially before and at 15, 30, 60, and 120 min after the injection.

2.5. Assessment of blood parameters and blood pressure

Plasma glucose, insulin, and Ang II levels, and systolic blood pressure were measured as described previously [5,22,28]. Briefly, plasma glucose was determined by the use of a commercially available enzyme kit (Wako Chemical, Osaka, Japan). Plasma insulin was measured by enzyme immunoassay (Shibayagi, Shibukawa, Japan). Plasma Ang II was eluted with methanol using C-18 columns (Cayman Chemical, MI, USA), then measured using a commercially available Ang II enzyme immunoassay (SPI-BIO, Massy Cedex, France) according to the manufacturer's instructions. 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) at least 5 min after the mouse had been put in a restrainer for the purpose of measuring.

2.6. 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 ring connected to a tissue holder, and the other to a force–displacement transducer, as previously described [10]. For the relaxation studies, rings were precontracted with an equieffective concentration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-6} M). When the $PGF_{2\alpha}$-induced contraction had reached a plateau level, ACh (10^{-9} to 10^{-5} M), SNP (10^{-10} to 10^{-5} M), clonidine (10^{-9} to 10^{-6} M), or insulin (10^{-8} to 3×10^{-6} M) was added in a cumulative manner. To examine the effects of Akt inhibitor (7×10^{-7} M), L-NNA (NOS inhibitor; 10^{-4} M), Gallein (βγ subunit inhibitor; 10^{-7} M), GRK2-inhibitor (10^{-6} M), or Ang II (10^{-6} M) on the responses to these relaxant agents, one of these inhibitors was added to the bath 30 min before the application of $PGF_{2\alpha}$.