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# Nitric oxide dynamics and endothelial dysfunction in type II model of genetic diabetes☆

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**Abstract**

Although diabetes is a major risk factor for vascular diseases, e.g., hypertension and atherosclerosis, mechanisms that underlie the "risky" aspects of diabetes remain obscure. The current study is intended to examine the notion that diabetic endothelial dysfunction stems from a heightened state of oxidative stress induced by an imbalance between vascular production and scavenging of reactive oxygen/nitrogen species. Goto-Kakizaki (GK) rats were used as a genetic animal model for non-obese type II diabetes. Nitric oxide (NO) bioavailability and $O_2^-$ generation in aortic tissues of GK rats were assessed using the Griess reaction and a lucigenin–chemiluminescence-based technique, respectively. Organ chamber-based isometric tension studies revealed that aortas from GK rats had impaired relaxation responses to acetylcholine whereas a rightward shift in the dose–response curve was noticed in the endothelium-independent vasorelaxation exerted by the NO donor sodium nitroprusside. An enhancement in superoxide ($O_2^-$) production and a diminuation in NO bioavailability were evident in aortic tissues of GK diabetic rats. Immunoblotting and high-performance liquid chromatography (HPLC)-based techniques revealed, respectively, that the above inverse relationship between $O_2^-$ and NO was associated with a marked increase in the protein expression of nitric oxide synthase (eNOS) and a decrease in the level of its cofactor tetrahydrobiopterin ($BH_4$) in diabetic aortas. Endothelial denudation by rubbing or the addition of pharmacological inhibitors of eNOS (e.g. $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME)), and NAD(P)H oxidase (e.g. diphenyleneiodonium, apocynin) strikingly reduced the diabetes-induced enhancement in vascular $O_2^-$ production. Aortic contents of key markers of oxidative stress (isoprostane F2α III, protein-bound carbonyls, nitrosylated protein) in connection with the protein expression of superoxide generating enzyme NAD(P)H oxidase (e.g. $p47^{phox}$, $pg91^{phox}$), a major source of reactive oxygen species in vascular tissue, were elevated as a function of diabetes. In contrast, the process involves in the vascular inactivation of reactive oxygen species exemplified by the activity of CuZnSOD was reduced in this diseased state. Our studies suggest that diabetes produces a cascade of events involving production of reactive oxygen species from the NADPH oxidase leading to oxidation of $BH_4$ and uncoupling of NOS. This promotes the oxidative inactivation of NO with subsequent formation of peroxynitrite. An alteration in the balance of these bioactive radicals in concert with a defect in the antioxidant defense counteracting mechanism may favor a heightened state of oxidative stress. This phenomenon could play a potentially important role in the pathogenesis of diabetic endothelial dysfunction.


*Keywords:* Superoxide anion; Nitrotyrosine; Superoxide dismutase; NADPH oxidase; Endothelial nitric oxide synthetase; Type II diabetes

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## 1. Introduction

Endothelial cells play a cardinal role in the control of vascular homeostasis through the release of a variety of vasoconstricting and vasodilating autocoids including thromboxane, prostacyclin, nitric oxide and an, as yet, elusive endothelium-derived hyperpolarizing factor (Feletou and Vanhoutte, 1999). An impairment in endothelium-dependent vasodilation, mediated largely by the loss of nitric oxide (NO) may represent an important feature of vascular disease, not only in subjects with established atherosclerosis but also in those with hyperlipoproteinemia, a positive family history of coronary artery disease and diabetes mellitus (De Vriese et al., 2000; Kojda and Harrison, 1999).

Diabetes constitutes one of the major independent cardiovascular risk factors, and patients with this disease suffer from premature cardiovascular morbidity and mortality (De Vriese et al., 2000; Kojda and Harrison, 1999; Gutterman, 2002). The role of endothelial dysfunction in the development of macro- and microvascular disease has been studied during diabetes. In this context, attenuated endothelium-dependent acetylcholine-induced relaxation was reported in different vascular beds of human and animal models of diabetes (De Vriese et al., 2000). Other authors observed a more pronounced deficit of the acetylcholine-induced relaxation in mesenteric arteries in the presence of inhibitors of nitric oxide synthetase and cyclo-oxygenase (Taylor et al., 1992) or a decrease in nitric oxide synthetase-resistant acetylcholine-induced relaxation in isolated renal arteries of diabetic rats (Taylor et al., 1992).

A number of cellular mechanisms have been suggested to account for impaired endothelium-dependent vasodilation including the production of cyclo-oxygenase constrictor substances, a deficit in substrate (L-arginine) or co-factor (biopterin) for nitric oxide synthetase, an increased production of advanced glycosylation end products, and an actual synthesis/release of hydroxyl radicals (De Vriese et al., 2000; Kojda and Harrison, 1999). While the above processes represent viable mediators to the development of vascular disturbances, a single unifying mechanism to account for endothelial dysfunction in diabetes has yet to emerge.

Insulin resistance and endothelial dysfunction appear to exist in a variety of metabolic and cardiovascular disorders, including atherosclerosis and type II diabetes (Pinkney et al., 1997). Drugs that enhance insulin sensitivity (e.g. troglitazone, vitamin C) lower blood pressure in both human and animal studies (Paolisso et al., 1994; Ogihara et al., 1995). Likewise, agents that lower peripheral vascular resistance in hypertensive subjects (e.g. angiotensin converting enzyme inhibitors) also improve insulin sensitivity (Torlone et al., 1991).

Because insulin sensitivity is susceptible to changes in whole body redox balance, oxidative stress may be involved in the development of insulin resistance. Indeed, insulin-resistant obese Zucker rats rapidly develop a type II diabetes-like state when exposed to a pro-oxidative insult (Laight et al., 1999). In view of the above information, a hypothesis was formulated stating that endothelial dysfunction and insulin resistance are associated with a heightened state of oxidative stress in diabetes mellitus. As an initial step towards testing this premise, we investigated endothelial function in isolated thoracic aortas obtained from the Goto-Kakizaki (GK) rat, a genetic animal model for non-obese type II diabetes, by assessing the production of both NO and $O_2^-$, in connection with oxidative stress markers (isoprostane, protein-bound carbonyls, protein nitration) and the protein expression of endothelial nitric oxide synthetase (eNOS) and NAD(P)H oxidase. In addition, vascular responses to endothelium-dependent and -independent vasodilators during diabetes were also considered.

## 2. Materials and methods

### 2.1. Animals

Animal studies were performed in accordance with the National Institute of Health Guidance for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, Revised 1996). Type II diabetic GK rats were produced by selective inbreeding of glucose-intolerant Wistar rats. All offsprings of GK animals are similarly affected by mild hyperglycemia within the first two weeks after birth. In 1996, we initiated a colony of GK rats at Kuwait University, from breeding stock kindly provided by Dr. Samy Abdel-Halim (Karolinska Institute, Sweden). Weight matched male Wistar rats serve as control (Kuwait University breeding colony).

### 2.2. Isometric tension studies

The thoracic aorta was excised from pentobarbital anesthetized rats (50 mg/kg, ip.), and immediately placed in ice-cold Krebs–Henseleit buffer (KHB, pH 7.4) of the following composition in mM; NaCl 120, KCl 5.6, $MgCl_2$ 1.2, $NaH_2PO_4$ 1.2, dextrose 11, $NaHCO_3$ 25, $CaCl_2$ 2.0. The aortic rings (4 mm) were then connected to isometric force transducer in a 10 ml organ chamber filled with KHB (37 °C and bubbled with 95% $O_2$:5% $CO_2$). Aortic rings from Wistar and GK rats were studied in parallel. They were set under a resting tension of 1 g and after equilibration for 60 min; all vessels were preconstricted with norepinephrine $10^{-7}$ M. Ligand-stimulated receptor-mediated NO bioavailability was assessed by a dose-dependent relaxation to (acetylcholine, $10^{-9}$ to $10^{-6}$ M), whereas (sodium nitroprusside, $10^{-9}$ to $10^{-6}$ M) was used as an endothelium-independent agonist. Relaxation responses to acetylcholine and sodium nitroprusside were expressed as percentage of relaxation from submaximal norepinephrine-induced constriction