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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^{ω}-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

(10^{-7} M). Regression analysis using three data points along the linear section of the concentration–response curve was applied to generate an equation from which the ED_{50} values were determined.

2.3. Assessment of key markers of oxidative stress and nitrative stress

2.3.1. Measurement of superoxide anion O_2^-

O_2^- concentration in aortic tissue was determined using a lucigenin enhanced chemiluminescence method (Li Y Zhu et al., 1998) and the resulting data were further confirmed by a cytochrome *c*-based technique (Liochev and Fridovich, 1997; Kuthan and Ulrich, 1982). Segments of the thoracic aorta were placed into 2 ml modified KHB solution, pH 7.4, and prewarmed to 37 °C for 1 h. Immediately before measurement, rings were transferred to scintillation vials containing KHB with 5 μmol/l lucigenin and the O_2^--generated chemiluminescence was recorded for 5 min with a scintillation counter. The amount of O_2^- produced was quantified using a standard curve of O_2^- generation by xanthine/xanthine oxidase and the data are expressed as nmol per min per mg of wet weight. In some experiments vessels were denuded of endothelium by gentle rubbing of the luminal surface, whereas in others, N^{ω}-nitro-L-arginine methyl ester (L-NAME) 0.1 mM, diphenylene iodonium 0.1 mM, or apocynin 3 mM were added 60 min before determining O_2^- generation.

Superoxide production was also determined using the superoxide dismutase (SOD)-inhibitable cytochrome *c* assay. Briefly, three to four aortic ring segments (2 mm) were placed in a buffer containing (in mM) NaCl 145, KCl 4.86, Na_2HPO_4 5.7, $CaCl_2$ 0.54, $MgSO_4$ 1.22, glucose 5.5, deferoxamine mesylate 0.1, and 1 U/ml catalase. Cytochrome *c* (50 μM) was added and the reaction mixture was incubated at 37 °C for 60 min with or without SOD (200 U/ml). Cytochrome c reduction was measured by reading absorbance at 550 nm. O_2^- formation in nmol/mg protein was calculated from the difference between absorbance with or without SOD, and the extinction coefficient for change of ferricytochrome *c* to ferrocytochrome *c*, i.e., 21 mM/cm^{-1}.

2.3.2. Isoprostane (iPF2a-III)

Aortic tissue level of isoprostane was measured using an enzyme immunoassay kit (Cayman Chemical Co. Ann Arber, MI, USA). Briefly, aortic samples were powdered under nitrogen and isoprostane was extracted overnight using 2:1 (v/v) chloroform–methanol with 0.0005% butylated hydroxytoluene as a chain-breaking antioxidant. Samples were filtered through glass wool and the organic phase was evaporated at 40 °C under nitrogen. The dried products was resuspended in methanol, saponified using 15% KOH followed by a 60-min incubation at 40 °C. Samples were applied to reverse-phase C-18 columns and eluted with 1:1 (v/v) ethyl acetate–heptane. The eluents were further purified on silica columns and eluted with 1:1 (v/v) ethylacetate–methanol. Samples were evaporated and the pellets were resuspended in an assay buffer and analyzed according to the manufacturer's instruction.

2.3.3. Protein oxidation

Protein-bound carbonyl content, as an index of protein oxidation, was measured according to previously published procedure with slight modification (Kolbeck et al., 1997; Levine et al., 1990). Briefly, sample tissues were homogenized in 50 mM phosphate buffer pH 7.4 containing protease inhibitors, leupeptin (1 μg/ml), pepstatin (1.4 μg/ml), and aprotinin (1.0 μg/ml). The homogenates were centrifuged at $500\times g$ for 5 min, and the supernatant (900 ml) was transferred to a microcentrifuged tube containing 100 μl of 10% streptomycin sulfate (in 50 mM HEPES) to remove nucleic acid, incubated for 15 min at room temperature and then centrifuged at $12{,}000\times g$ for 15 min at 4 °C. The resulting supernatants containing 2–5 mg of soluble proteins were used for reaction with 2, 4-dinitrophenylhydrazine. For each sample, the supernatants were divided into two equal volumes. Four volumes of 10 mM dinitrophenylhydrazine (DNPH) in 2 M HCl were added to one of the sample pair, and four volumes of 2 M HCl alone were added to the other one (for reagent blank assay). Samples were then incubated for 1 h at room temperature in the dark with continuous stirring and were precipitated with an equal volume of 20% trichloroacetic acid. After 15-min incubation on ice, samples were centrifuged at $5000\times g$ for 10 min and the supernatants were discarded. Protein pellets were washed in 10% trichloroacetic acid once and in ethanol/ethyl acetate (1:1) three times to remove free dinitrophenylhydrazine and additional lipid contaminants. Final protein precipitates were dissolved in 6 M guanidine hydrochloride solution. The difference in absorbance between the dinitrophenylhydrazine-treated and the HCl-treated samples were determined spectrophotometrically at 375 nm. Carbonyl content was calculated by using a molar absorption coefficient of 22,000 M^{-1} cm^{-1}.

2.3.4. Nitrotyrosine

Nitrotyrosine, a biomarker of peroxynitrite formation, was determined in the aorta by estimating the levels of protein nitration using immunochemical methods. Existing antibodies were removed from aortic homogenates by incubating first with protein A/G agarose, followed by an overnight incubation with a rabbit anti-nitrotyrosine antibody (Upstate Biotechnology, Lake placid, NY, USA). Protein A/G agarose was added to precipitate the nitrotyrosine complexed antibody. Proteins were separated on 8% denaturing polyacrylamide gels, and the resulting blots were incubated with mouse anti-nitrotyrosine antibody (Upstate Biotechnology, Lake placid, NY, USA). Detection of nitrotyrosine was achieved using peroxidase-conjugated secondary antibody.

2.4. Nitrite detection

Nitrite levels were determined as an index of NO generation in aortic homogenates by the Griess reaction after conversion of nitrate to nitrite by nitrate dehydrogenase (Green et al., 1982) An aliquot of the supernatant was mixed with an equal volume of Griess reagent (Sulfanilamide 1% w/v; naphtylethylenediamine dihydrochloride, 0.1% w/v; and orthophosphoric acid, 25% v/v) and incubated at room temperature for 10 min. The absorbance of the samples at 540 nm was determined and compared with those of known concentrations of sodium nitrite. The amount of nitrite formed was normalized to the protein content of the respective aorta.

2.5. Vascular content of biopterin

Aortic tissues derived from Wistar and GK rats were homogenized in 25 mM Tris–HCl buffer (pH 7.4) and measurement of biopterin content in aortic extract was achieved using high-performance liquid chromatography-based analysis and a differential oxidation method as described previously (Fukushima and Nixon, 1980). The amount of BH_4 was determined from the difference between total (BH_4 plus BH_2 plus biopterin) and the alkaline-stable oxidized biopterin (BH_2 plus biopterin). A C-18 column (5×250 mm, 5 μM) was used with 5% methanol/95% water as a solvent at a flow rate of 1.0 ml per min. The fluorescent detector was set at 350 nm for excitation and 450 nm for emission.

2.6. Vascular SOD activity

Aortas were homogenized in 0.05 M Tris buffer (pH 7.4) containing 0.25 M sucrose and a cocktail of protease inhibitors. After centrifugation (5000×*g* for 10 min at 4 °C), the supernatant was used for SOD activity testing. The SOD-dependent inhibition of cytochrome *c* reduction catalyzed by xanthine oxidase was assessed spectrophotometrically by monitoring the absorbance at 550 nm for 2 min at 37 °C (Okado-Matsumoto and Fridovich, 2001). Briefly, the reaction mixture contained 0.1 mM EDTA, 0.09 mM xanthine, 0.018 mM cytochrome *c*, 50 mM potassium buffer pH 7.8, and the cellular extract supernatant. The assay was calibrated by adding xanthine oxidase at a concentration that increased the absorbance of the mixture by 0.025 per min, as a result of cytochrome *c* reduction. One SOD unit is arbitrary defined as the amount of enzyme required to inhibit the reduction rate of cytochrome *c* by 50%. Enzymatic activity (in arbitrary units per milligram of total proteins) was determined by calculating from the slope of the absorbance curves, the percentage inhibition of cytochrome *c* reduction in the test samples relative to the controls. The CuZnSOD was differentiated from MnSOD by addition of 3 mM NaCN to eliminate the activity of CuZnSOD from total SOD activity.

2.7. Western blot analysis for eNOS and protein subunits of NADPH oxidase

Segments of endothelium-intact thoracic aortas were washed with cold PBS and chilled in buffer containing in mM: (Tris–HCl 50, NaCl 150, disodium EDTA 1, EGTA 0.1, as well as NP-40, 0.1%, SDS 0.1% and deoxycholate 0.5%). Phenylmethylsulfonyl fluoride (1 mmol/l), aprotonin (10 μg/ml), leupeptin (10 μg/ml), and pepstatin (10 μg/ml) all from Sigma Chemicals (St. Louis, MO, USA) were added as the protease inhibitors. Tissues were homogenized in a standard fashion, followed by centrifugation at 14,000×*g* for 20 min at 4 °C. The supernatants were collected and total protein concentration was determined. Samples containing 50 μg of protein were loaded on to a 7.5% (eNOS) and 12.5% ($p47^{phox}$, $pg91^{phox}$) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, run and electroblotted onto nitrocellulose membrane. Prestained molecular weight marker proteins were used as standards for the SDS-PAGE. A ponceau staining was performed to verify the quality of the transfer and to ensure equal protein loading. Blots were blocked in 5% skimmed nonfat milk in PBS for 1 h, treated overnight with antibodies to eNOS (Transduction Laboratory, USA) and NADPH Oxidase ($p47^{phox}$, $pg91^{phox}$, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and then incubated with peroxidase-conjugated secondary antibodies for 1 h. Immunoblots were developed with an ECL Western blotting detection system.

2.8. Assessment of in vivo insulin action

Insulin sensitivity was determined by the steady-state plasma glucose (SSPG) method with the use of somatostatin (Harano et al., 1981). An infusate containing somatostatin (120 μg/kg/h), glucose (1.0 g/kg/h), and insulin (2.0 U/kg/h, Human Actrapid, Novo Nordisk) was administered intravenously to control and GK rats at a flow rate of 2.8 ml/h for 120 min. The SSPG levels and the steady-state plasma insulin (SSPI) contents were determined at 120 min following the start of the infusion. Plasma glucose and free fatty acid concentrations were measured according to the standard enzymatic assays, while insulin level was determined using a radioimmunoassay with anti-rat insulin antibody.

2.9. Protein

Tissue protein content was determined as described previously (Lowry et al., 1951) using bovine serum albumin as a standard.

2.10. Calculations and statistical analysis

When applicable (comparison between two values) statistical analysis was done by Student's *t*-test. For multiple

comparisons, results were analyzed by analysis of variants (ANOVA) followed by a two tailed *t*-test. Dose response curves were fitted by nonlinear regression with simplex algorithm. Relaxation responses were given as the percentage of norepinephrine-preconstriction. Comparisons of dose–response curves were evaluated by 2-way ANOVA for repeated measures. Data are presented as means±S.E.M. Statistical significance was assumed at $P<0.05$.

3. Results

3.1. Animal characteristics

Wistar and GK rats exhibited similar gains in body weight over the indicated study period (e.g. 1 month, Table 1). Fasting and postprandial glucose levels in GK rats were elevated when compared to the corresponding Wistar controls values (Table 1). Similarly, plasma concentrations of insulin and free fatty acids were also increased in these animals. Insulin sensitivity as indicated by the SSPG level was diminished as a function of diabetes (Table 1). These data are consistent with the concept that this genetic animal model of type II diabetes exhibits alterations in lipid and carbohydrate profiles, with an evidence of insulin resistance.

3.2. Diabetes-induced impairment of NO-dependent vascular relaxation

NO-mediated endothelium-dependent relaxation to various concentrations of acetylcholine in GK rats was markedly reduced in terms of maximum response and ED_{50} compared with Wistar control values (Fig. 1A; Table 2). The concentration–response curve of the endothelium-independent vasodilator, sodium nitroprusside in aorta from GK rats was significantly shifted to the right, but no difference in the maximum relaxation response was observed between aortic rings from GK and Wistar rats (Fig. 1B; Table 2). The degree of aortic contraction in response to 0.1 μM of norepinephrine appears not to be affected by the diabetic state.

Table 1
Global physiological parameters of Wistar and GK rats

Parameter	Wistar	GK
Weight (g)	316±15	322±14
FBG (mg/100 ml)	78±10	138±12[a]
Non-FBG (mg/100 ml)	98±9	157±15[a]
Plasma insulin (ng/ml)	0.42±0.04	0.75±0.08[a]
Plasma free fatty acid (mmol/l)	0.78±0.05	1.30±0.09[a]
Insulin sensitivity test (n=4/group)		
SSPG (mg/100 ml)	125±8	189±12[a]
SSPI (ng/ml)	16±2.34	19±2.63

Values are expressed as means±S.E.M of at least 8 animals with the exception of SSPG/SSPI where n=4 for each group.
[a] Indicates significantly different from corresponding control values at $P<0.05$.

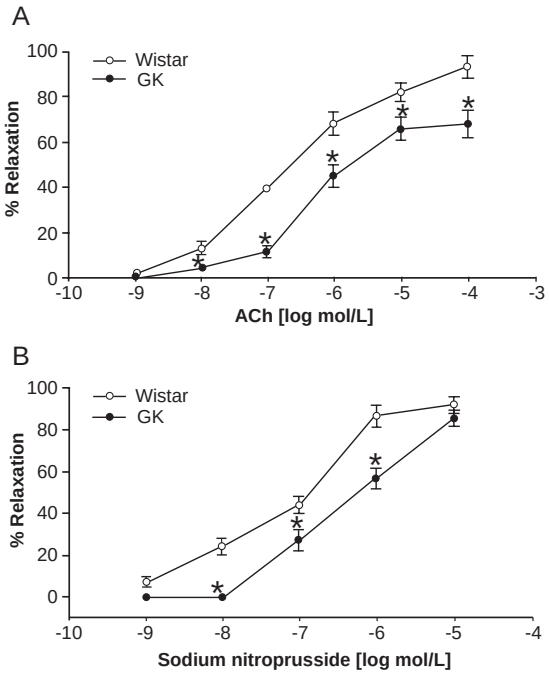

Fig. 1. Aortic vasorelaxation during diabetes. Aortic segments of Wistar (○) and GK (●) rats were isolated and their functional performance was assessed in an organ chamber. Drugs were added in increasing concentrations. Endothelial cell-dependent vasorelaxation was tested with acetylcholine (A), whereas endothelial cell-independent relaxation was investigated with nitroprusside (B). Graphs show force of contraction, expressed as percentage of maximum norepinephrine-induced vasoconstriction. Data are shown as means±S.E.M of at least seven animals/group. *Significantly different from corresponding control values at $P<0.05$.

3.3. Diabetes-associated alterations in the eNOS enzyme system

NO bioavailability in aortic tissue of GK rats were reduced when compared with corresponding Wistar control values (Fig. 2A). As NO in vascular endothelial cells is synthesized primarily by eNOS, we examined the possibility that a diabetes-related decrease in NO bioavailability might be due to a change in the rate of expression of the NO-producing enzyme. Unexpectedly, our data revealed that the expression of eNOS was elevated as a function of diabetes (Fig. 2B and C). Thus, there appears to be a sharp dissociation between eNOS and its product NO in the vascular tissue of GK rats. No attempt was made in the present study to confirm that the overexpression of eNOS was associated with a similar increase in its activity in the aortic tissue of diabetic rats.

3.4. Diabetes-dependent elevation in vascular superoxide formation

Superoxide anion constitutes the main oxidant of NO (Gryglewski et al., 1986). Accordingly, the premise that a diabetes-related decrease in NO bioavailability stems from an increase in O_2^- levels was examined using a lucigenin-

Table 2
Aortic vasorelaxation in Wistar and GK rats

Kinetic parameters	Wistar	GK
Acetylcholine		
$ED_{50(-Log\ M)}$	6.52±0.2	6.09±0.1[a]
Maximal relaxation (%)	93±7	68.5±6[a]
Sodium nitroprusside		
$ED_{50(-Log\ M)}$	−6.82±0.2	−6.22±0.3[a]
Maximal relaxation (%)	92±4	86±4

Values are expressed as means±S.E.M of at least 8 animals.
[a] Indicates significantly different from corresponding control values at $P<0.05$.

enhanced chemiluminescence-based technique. Basal O_2^- release from intact vessel rings was higher in GK rats compared with Wistar counterpart (Table 3, $p<0.05$). To confirm the validity of the lucigenin technique for measurement of O_2^- in our animal mode of diabetes, we also

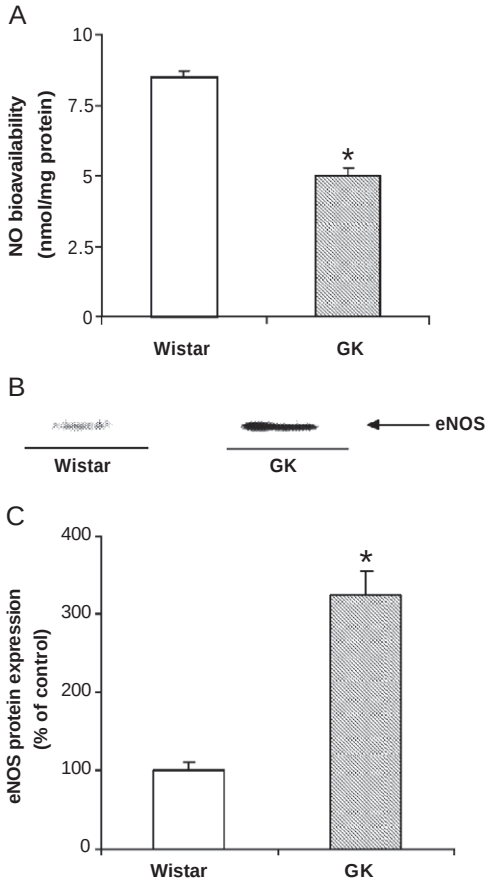

Fig. 2. Aortic NO bioavailability and eNOS expression during diabetes. (A) NO bioavailability was assessed in aortic homogenates using the Griess reaction. (B) Representative Western blot analysis of eNOS protein expression in aortas of Wistar and GK rats; 50 μg of protein from aortic lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-eNOS antibody as described in Materials and methods. (C) Averaged densitometry data for diabetic group expressed as a percentage of elevation over the control value established as 100%. Data are presented as means±S.E.M of at least 5 animals/group. *Significantly different from corresponding control values at $P<0.05$.

Table 3
Aortic superoxide production during diabetes

Treatment	Superoxide production (*counts/mg protein/min*)	
	Wistar	GK
Endothelium intact	178±21	316±27[a]
Endothelium denudation	145±17[b]	182±24[b]
L-NAME (100 μmol/l)	169±19	238±33[b]
Diphenyleneiodonium (100 μmol/l)	124±12[b]	173±28[b]
Apocynin (3 mmol/l)	127±15[b]	166±31[b]

Values are expressed as means±S.E.M of at least six animals/group.
[a] Indicates significantly different from corresponding Wistar control values at $P<0.05$.
[b] Indicates significant difference between basal (intact endothelium) and treatment groups within each strain at $P<0.05$.

assessed vascular basal O_2^- formation by the cytochrome *c* method. O_2^- production by aortic rings from the diabetic insulin-resistant animals (2.87±0.18 nmol/min/mg tissue) was significantly higher than that of corresponding control values 1.61±0.23). Accordingly, the findings harmonize with the notion that both of the above techniques appear to compare favorably with each other.

Identification of the source of O_2^- dictated the deendothelialization of the aortic segments or treating them with L-NAME, an inhibitor of NOS activity. The results revealed that denuding aortic endothelium in GK rats significantly reduced O_2^- generation (Table 3). This maneuver produced a much smaller effect on O_2^- production in Wistar control rats (Table 3). Because NO synthetase is abundant in the endothelium, we determined if this enzyme contributed to the formation of O_2^- during diabetes. The data revealed that the NO synthetase inhibitor L-NAME markedly reduced the rate of O_2^- formation in diabetic but not in control vessels (Table 3). Collectively, the above results suggest that vascular endothelium represents a pivotal source of O_2^- production during diabetes.

NADPH oxidase constitutes a major source of reactive oxygen species production in both the intact vessels and cultured vascular smooth muscle cells (Patrono and Fitzgerald, 1997; Pagano et al., 1995). We explored the degree of involvement of this enzyme system in aortic generation of O_2^- during diabetes. The resulting data showed that both diphenyleneiodonium and apocynin, inhibitors of NADPH oxidase, significantly reduced O_2^- formation in GK diabetic rats (Table 3).

3.5. Oxidation of tetrahydrobiopterin in aortas

L-NAME ability to reduce the rate of O_2^- generation in aortic tissue of GK rats together with the overexpression of eNOS raised a possibility that the NOS system is dysregulated during diabetes. Tetrahydrobiopterin plays a crucial role as a cofactor for all NO synthetases (Jones et al., 1996; Stuechr, 1997). In its absence, purified eNOS produces reactive oxygen species rather than NO (Vas-

quez-vivar et al., 1998; Wever et al., 1997), a phenomenon which is known as eNOS uncoupling. In view of these findings, we tested the preposition that diabetes-induced eNOS uncoupling may stem from an abnormality in vascular tissue metabolism of biopterin. The data revealed that the aortic content of tetrahydrobiopterin in GK rats was reduced by about 50% when compared with Wistar counterpart (Fig. 3). In contrast, a corresponding increase in the level of oxidized forms of tetrahydrobiopterin (7,8-dihydrobiopterin) was evident in these animals (Fig. 3). It is worthy to note that the total tissue content of biopterin was not altered as a function of diabetes (Fig. 3).

3.6. Protein subunits of NAD(P)H oxidase in aortas

Diphenyleneiodonium inhibits several reactive oxygen species-generating flavin-containing enzymes besides NADPH oxidase. Likewise, apocynin may also have other actions. Accordingly, we conducted an additional experiment involving the immunoblotting of measurement of the levels of expression of the membrane-bound ($pg91^{phox}$) and the cytosolic ($p47^{phox}$) subunits of the NADPH oxidase system. The obtained data showed that the protein expression of $pg91^{phox}$ and $p47^{phox}$ in aortic tissues of GK rats was elevated over their corresponding control values by 48% and 55%, respectively (Fig. 4). Together, the immunoblotting data in connection with the inhibitor studies (e.g. diphenyleneiodonium, apocynin) of the NADPH oxidase support the involvement of this enzyme system in the overproduction of vascular O_2^- during diabetes.

3.7. SOD activity

Local vascular levels of O_2^- reflect both the rate of O_2^- formation and the rate of removal by endogenous antioxidants (primarily SODs). Within blood vessels, the predominant isoform of SOD (when expressed as percent of total SOD activity) is CuZnSOD (Fukai et al., 1998; Stralin et al., 1995). Aortic activity of CuZnSOD in GK rats (5.23±0.4 U/mg protein, n=7) was reduced by about 33% (p<0.05) when compared to corresponding control values (7.85±0.8 U/mg protein, n=7). The above data are consistent with the proposition that heightened state of oxidative stress in vascular tissue of diabetics is a manifestation of not only an enhancement in the amount of O_2^- generation but also a diminuation in the rate of removal of this free radical by an SOD-based mechanism. The activity of total SOD was also reduced in GK diabetic rats (GK, 6.97±0.6 vs. control, 10.14±0.14).

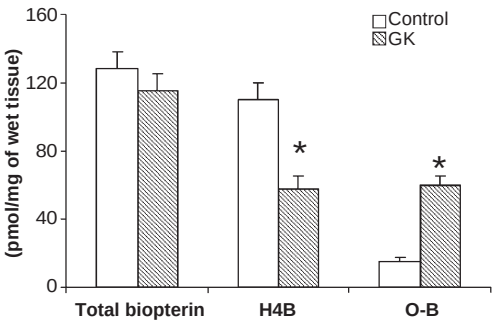

Fig. 3. Tetrahydrobiopterin and oxidized biopterin contents in aortas from Wistar and GK diabetic rats. Tetrahydrobiopterin (BH_4) and oxidized biopterin (O-B) were determined using HPLC-based technique after differential oxidation. Aortas from four to six animals were pooled for each measurement. Each data points represent the means±S.E.M of 3 measurements. *Significantly different from corresponding control values at P<0.05.

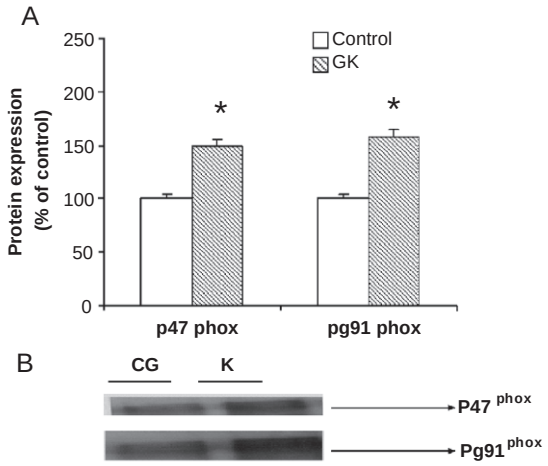

Fig. 4. Expression of protein subunits of NADPH oxidase in aortic tissue of diabetics. (A) Representative Western blot analysis of $p47^{phox}$, $pg91^{phox}$ protein expression in aortas of Wistar and GK rats; 50 μg of protein from aortic lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with antibody against $p47^{phox}$, $pg91^{phox}$ as described in Materials and methods. (B) Averaged densitometry data for diabetic group expressed as a percentage of elevation over the control value established as 100%. Data are presented as means±S.E.M of at least 5 animals/group. *Significantly different from corresponding control values at P<0.05.

3.8. Isoprostane and protein-bound carbonyl in aortic vessels

Further experimentation was conducted to support the premise that the state of oxidative stress is heightened in diabetic aortic vessels. In this vein, aortic contents of isoprostane and protein-bound carbonyls in GK rats were elevated, respectively, by 36% and 41% when compared to corresponding control values (Fig. 5). Isoprostane and protein-bound carbonyl levels are sensitive indicators for lipid peroxidation and protein oxidation, respectively (Levine et al., 1990; Patrono and Fitzgerald, 1997).

3.9. Deposition of 3-nitrotyrosinated proteins in Wistar and GK aortic segment

An elevation of O_2^- level is the prerequisite condition for increasing peroxynitrite formation (Gryglewski et al., 1986).

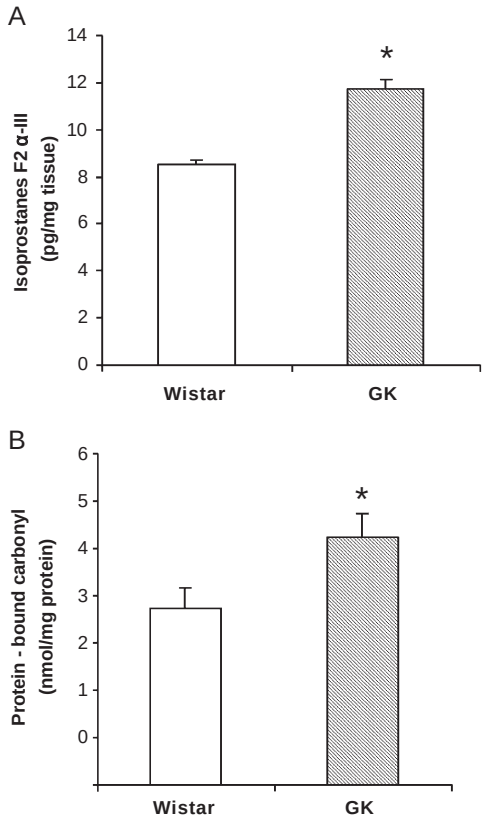

Fig. 5. Aortic contents of isoprostanes (A) and protein-bound carbonyls (B) during diabetes. Markers of the oxidative stress including isoprostanes F2α-III and protein-bound carbonyls were measured in aortic homogenates according to the procedures described in Materials and methods. Values are means±S.E.M of at least 7 animals/group. *Significantly different from corresponding control values at $P<0.05$.

Accordingly, we sought to investigate whether the increase in eNOS expression, together with enhanced O_2^- production in aortic tissue of GK rats, was associated with peroxynitrite formation and the nitration of tyrosine residues. A Western blot analysis of aortic proteins revealed that the immunoreactive nitrotyrosine levels, an index of peroxynitrite formation in vivo were elevated in GK rats compared with the Wistar counterpart (Fig. 6A and B). The data are consistent with the concept that the overproduction of superoxide during diabetes results in the formation of the peroxynitrite at the expense of NO. The latter oxidant is merely responsible for the nitration of aortic proteins.

4. Discussion

The central hypothesis of our research is consistent with the concept that a heightened state of oxidative stress is involved in the development of insulin resistance and this phenomenon constitutes an integral part of several cardiovascular risk factors including essential hypertension, dyslipidemia, obesity, glucose intolerance, and diabetes mellitus (Reaven, 1988). In this connection, a number of insulin resistance states appear to be associated with insulin's impaired ability to increase blood flow together with vasospastic angina and obstructive coronary artery disease (Shinozaki et al., 1995; Shinozaki et al., 1996). The current data revealed that the in vivo insulin action assessed by the steady-state plasma glucose method was reduced in GK rats when compared to corresponding Wistar control values. Similarly, skeletal muscle activities of two key enzymes, e.g. phosphatidylinositol (PI) 3 kinase and protein kinase B(Akt), in the insulin signaling pathway were also reduced in this genetic animal model of non-obese type II diabetes (Bitar et al., 2004). This insulin resistance state was associated with an impaired arterial response to acetylcholine and sodium nitroprusside indicating that GK rats exhibit a dysfunctional endothelium and decreased smooth muscle sensitivity to NO. Our findings are in accordance with previously reported results demonstrating diminution in acetylcholine-based vascular relaxation in human and animal model of diabetes (De Vriese et al., 2000; Watts et al., 1996; Heitzer et al., 2000).

The underlying cellular and molecular mechanisms associated with diabetes-related endothelial dysfunction were explored in the context of a number of possibilities including changes in the NO bioavailability or the expression of eNOS; increased breakdown of NO due to augmented production of O_2^- and an imbalance in the rate of reactive oxygen/nitrogen species production and disposal within the microenvironment of the vessels. In this connection, we have demonstrated a marked reduction in aortic NO bioavailability during diabetes. This phenomenon appears not to be due to a defect in NO-producing enzymes since the level of eNOS was upregulated in GK vascular tissues, compared with their corresponding Wistar control

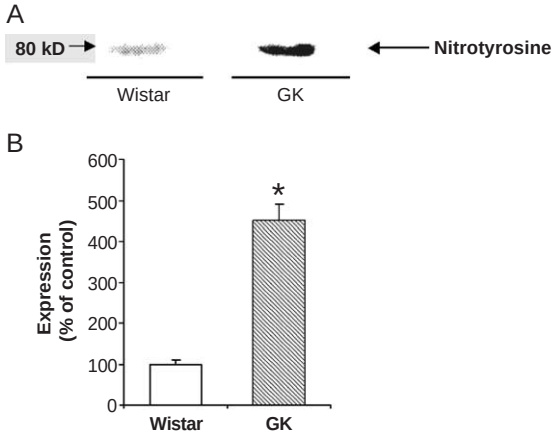

Fig. 6. Aortic contents of immunoreactive 3-nitrotyrosine during diabetes. (A) Representative western blot analyses of 3-nitrotyrosine protein expression in aortas of Wistar and GK rats; 50 μg of protein from aortic lysates was resolved by SDS-PAGE, transferred to nitrocellulose and probed with 3-nitrotyrosine antibody as described in Materials and methods. (B) Averaged densitometric data for diabetic group expressed as a percentage of elevation over the control value established as 100%. Data are presented as means±S.E.M; of at least five animals/group. *Significantly different from corresponding control values at $P<0.05$.

values. A similar finding of increased eNOS expression has recently been reported in streptozotocin diabetic rats (Hink et al., 2002). It is worthy of note that Sprague–Dawley rats rendered insulin resistant by a high fructose diet displayed a decrease in aortic activity of eNOS (Shinozaki et al., 2000). Understanding this apparent paradox of diabetes-induced super induction of eNOS concomitantly with a decrease in vascular NO bioavailability dictated the measurement of O_2^- anion, a well-known factor in the degradation of NO and its conversion to peroxynitrite (Gryglewski et al., 1986). Interestingly, we found that the level of this free radical was elevated in the aortic segment of the GK rats.

In the light of these data it is reasonable to speculate that the eNOS enzyme system is overactivated in diabetic vessels as a compensatory mechanism to counter balance endothelial dysfunction induced by diabetes-dependent oxidative stress. However, the excess O_2^- generation in diabetic aorta more than balances the increase in NO production leading to a net decrease in NO bioavailability. Alternatively or in a similar view, an upregulation of eNOS can also occur in response to a number of physiological stimuli including shear stress (Ranjan et al., 1995), cytokines, and growth-promoting polypeptides (Inoue et al., 1995; Kostyk et al., 1995) as in the case of transforming growth factor and basic fibroblast growth factor. Moreover, the GK rats used in our study exhibited a marked elevation in mean arterial blood pressure (unpublished observation).

NADH/NAD(P)H oxidase, xanthine oxidase, a dysfunctional NO synthetase, or mitochondrial flavoproteins represent an important source for reactive oxygen species generation within vascular endothelial and smooth muscle cells (Wever et al., 1998). These reactive oxygen species-based enzymatic sources are subject to alterations by a variety of physiological and pathophysiological states including hypercholesterolemia, hypertension, heart failure, aging, and diabetes (Cai and Harrison, 2000). Moreover mitochondrial flavoprotein-mediated increases in O_2^- generation have also been observed in bovine aortic endothelial cells cultured under hyperglycemic conditions (Nishikawa et al., 2000). The current study partially identified the enzymatic sources related to overproduction of superoxide anion during diabetes. In the GK but not the Wistar rats, both the removal of endothelium by rubbing and the L-NAME treatment caused a significant reduction in superoxide anion levels, thus substantiating our conclusion of the involvement of NOS in the overproduction of O_2^- during diabetes. This phenomenon of the so-called NOS uncoupling occurring in the GK rats may reflect a deficiency in arginine and/or tetrahydrobiopterin-dependent pathways. Indeed an increase in tetrahydrobiopterin oxidation shown in the aortic tissues of our animal model of diabetes may favor NAD(P)H oxidase activity of NOS with subsequent formation of reactive oxygen species (O_2 and H_2O_2) in lieu of NO (Vasquez-vivar et al., 1998; Wever et al., 1997). The exact mechanism whereby NOS generates O_2^- is uncertain. However molecular cloning of NO synthetase revealed close amino acid sequence homology between NO synthetase and cytochrome *P*450 reductase, a known cellular source of superoxide anion (Bredt et al., 1991).

The NAD(P)H oxidase system constitutes a pivotal signaling element in the genesis of endothelial dysfunction (Zalba et al., 2001; Brandes, 2003) and is widely accepted to account for the majority of superoxide generation in the vascular endothelial and smooth muscle cells (Patrono and Fitzgerald, 1997; Pagano et al., 1995; Jones et al., 1996). Accordingly, we examined the hypothesis that stimulation of NADH/ NAD(P)H oxidase contributes to a diabetes-related increase in vascular O_2^- production. This proposition has enjoyed support from the current findings, which demonstrate that the increased lucigenin chemiluminescence of diabetic vessels could be substantially inhibited by diphenyleneiodonium and apocyanin. Diphenyleneiodonium is frequently used as an inhibitor of NAD(P)H pathways, although it has other actions including inhibition of NOS (Wang et al., 1993). The vascular NAD(P)H oxidase consists of at least 3–5 subunits, with those they make-up the membrane-bound cytochrome *b*558, $P22^{phox}$, and $gp91^{phox}$ being important for electron transport or the reduction of molecular oxygen to O_2^-. Apocynin acts by interfering with NAD(P)H subunit assembly in the membrane and is therefore a more specific inhibitor than diphenyleneiodonium (Meyer and Schmitt, 2000).

Further experimentation using a Western blotting-based technique revealed that the protein abundance of $pg91^{phox}$ and $p47^{phox}$ subunits of NAD(P)H oxidase were elevated in aortic tissue of GK diabetic rats. A similar data were reported in the saphenous veins and internal mammary arteries of human type II diabetes (Guzik et al., 2002). Taken together, the inhibition of O_2^- production by diphenyleneiodonium and apocynin in connection with the overexpression of $gp91^{phox}$ and $p47^{phox}$ in aortic tissue of GK rats harmonize with the notion that the NAD(P)H oxidase in the diabetic state is hyperactive and this enzyme system like NOS may contribute, at least in part, for the overproduction of O_2^- in diabetic vessels. The reasons for the diabetes-induced activation of NAD(P)H oxidase were not identified in the current study. However, previous reports have shown that in both endothelial and vascular smooth muscle cells, mechanical stretch activates the NAD(P)H oxidase (Howard et al., 1997; Hishikawa et al., 1997) and it is possible that the stretch of vascular cells caused by hypertension may lead to increased O_2^- generation by this enzyme complex. Similarly, angiotensin II is also a potent stimulus of the NAD(P)H oxidase (Griendling et al., 1994). The GK rats are hypertensive and the vascular expression of angiotensin II receptor is upregulated as a function of diabetes (Candido et al., 2004). Accordingly, the possibility that the aforementioned parameters are involved in the diabetes-related activation of NAD(P)H oxidase deserves to be explored.

Cogent evidence indicates that when cellular levels of O_2^- or NO are increased, the potent oxidant, peroxynitrite is

quickly formed (Gryglewski et al., 1986; Rubanyi and Vanhoutte, 1986; Harrison, 1997). Indeed, the reaction rate for the formation of peroxynitrite is approximately six times faster than the scavenging of O_2^- by SOD, implying that peroxynitrite formation can occur in vivo (Beckman and Koppenol, 1996). Peroxynitrite has been described as a "cloaked oxidant" and repeated administration appears to induce vascular dysfunction and attenuates the endothelium-dependent dilation by a number of vasodilatory compounds. The present study revealed that aortic segments derived from GK rats exhibited an enhancement in their ability to generate O_2^- rather than diminished NO production. This aberration in aortic diabetic tissue appears to be associated with a marked elevation in protein 3-nitrotyrosine content, a biomarker of in vivo production of peroxynitrite (Reiter et al., 2000; Zou et al., 1999). Collectively these data may forge a link between peroxynitrite formed by NO-superoxide reaction and endothelial dysfunction in diabetes mellitus. How peroxynitrite is able to alter endothelial function is unclear, but it is possible that nitration of tyrosine residues plays a central role in this process. Peroxynitrite-mediated tyrosine nitration of specific proteins attenuates their tyrosine phosphorylation (Gow et al., 1996) and thus is able to inactivate proteins whose activity depends on phosphorylation of tyrosine residues. For example, agonist-induced calcium signaling in endothelial cells is regulated by tyrosine phosphorylation (Fleming et al., 1995) and peroxynitrite is able to inhibit this response (Elliott, 1996). Similarly, nitration of prostacyclin synthetase and Mn-superoxide dismutase induced by overproduction of peroxynitrite have been shown to contribute to the development of endothelial dysfunction in a number of disease states including atherosclerosis and aging (Beckman et al., 1994; Loo et al., 2000). To this end, in-depth studies are necessary for investigating the pathophysiological involvement of peroxynitrite and subsequent tyrosine nitration in the development of endothelial dysfunction during diabetes.

Vascular cells are endowed with antioxidant defense mechanism to buffer O_2^- and other reactive species. In addition to chemical antioxidants, the defense include SODs, which are present in mitochondria such as manganese SOD (MnSOD), cytosol such as CuZnSOD and plasma membrane, and extracellular spaces such as extracellular SOD, catalase, glutathione peroxidase, and thioredoxin (Sawyer et al., 2002; Sorescu and Griendling, 2002). The balance of reactive oxygen species production and disposal determines the level of oxidative stress within the vascular tissues. Our current data indicate that cytosolic CuZnSOD activity (predominant isoform of SOD in vessels) was reduced whereas isoprostane and protein-bound carbonyl levels were elevated in aortic tissues of GK diabetic rats. In view of these findings it is reasonable to suggest that diabetes-induced decrease in CuZnSOD activity contribute to the heightened state of oxidative stress within the microenvironment of the vessels. A credence for this proposition is provided by previous published reports showing that CuZnSOD-deficient mice exhibits a marked increase in reactive oxygen species production in vascular tissue (Didion et al., 2001). A similar finding was observed in animals receiving pharmacological inhibitors of CuZn-SOD (Didion et al., 2001; Wambi-Kiesse and Katusie, 1999). In contrast, a gene therapy with CuZnSOD or administration of conjugated form of this enzyme have been shown to improve NO-dependent arterial dilation via reducing reactive oxygen species bioavailability (Kinouchi et al., 1991; Mugge et al., 1991).

Overall, the development of endothelial dysfunction in aortic tissue of diabetic rats is likely to be linked to an exaggerated production of O_2^-. This enhancement in the production of O_2^- may result in inactivation of NO and generation of peroxynitrite as reflected by increased aortic content of 3-nitrotyrosine. The resulting decrease in NO availability might be involved in the impairment of NO-dependent relaxation. Accordingly, oxidative degradation of NO caused by increased O_2^- secondary to overactivity of NADH/NAD(P)H oxidase and the uncoupling of eNOS would provide a reasonable explanation for the diminished response to acetylcholine in the aorta of GK rats. Of course, our results do not exclude a role for other potential sources of O_2^- (e.g. xanthine oxidase, mitochondrial flavoproteins) within diabetic vascular cells. Furthermore, the observation that responses to sodium nitroprusside are altered in aortic tissue of GK rats suggests that other molecular mechanisms (e.g. diminished expression and activity of vascular smooth muscle cell guanylate cyclase) may also contribute to impaired vasodilatory responsiveness during diabetes.

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Further reading

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