

Nitric Oxide–Sensitive Soluble Guanylyl Cyclase Activity Is Preserved in Internal Mammary Artery of Type 2 Diabetic Patients

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Vascular reactivity to nitric oxide (NO) is mediated by NO-sensitive soluble guanylyl cyclase (sGC). Since a diminished activity of vascular sGC has been reported in an animal model of type 2 diabetes, the sGC activity was assayed *in vitro* in internal mammary artery specimens obtained during bypass surgery from patients with and without type 2 diabetes. The sensitivity of sGC to NO, which is dependent on Fe²⁺-containing heme, was measured *in vitro* using stimulation with diethylamine NONOate (DEA/NO). In addition, the novel cyclic guanosine monophosphate-elevating compound HMR-1766 was used to test the stimulation of the oxidized heme-Fe³⁺-containing form of sGC. Basal activity of sGC and its sensitivity to stimulation by DEA/NO and HMR-1766 were not different between control and type 2 diabetic patients: maximum stimulation by DEA/NO amounted to 475 ± 67 and 418 ± 59 pmol · mg⁻¹ · min⁻¹ in control and type 2 diabetic patients, respectively. The maximum effects of HMR-1766 were 95 ± 18 (control subjects) and 83 ± 11 pmol · mg⁻¹ · min⁻¹ (type 2 diabetic patients). Hypertension, hyperlipidemia, drug treatment with statins, ACE inhibitors, or nitrates had no effect on sGC activity. In conclusion, the present findings do not support the hypothesis that desensitization of sGC contributes to the pathogenesis of diabetic vascular dysfunction in humans. *Diabetes* 53:2640–2644, 2004

Cardiovascular complications are the main cause of the reduced life expectancy of diabetic patients. Cardiovascular morbidity and mortality in diabetic subjects are due to a variety of mechanisms, including damage to the vascular endothelium by hyperglycemia. The endothelium-dependent vasodilation of forearm resistance vessels is blunted in patients with type 1 (1) and type 2 (2–4) diabetes. In addition, the vascular response to exogenous nitric oxide (NO) is also

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cGMP, cyclic guanosine monophosphate; DEA/NO, diethylamine NONOate; sGC, soluble guanylyl cyclase.

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reduced in type 2 diabetic subjects, suggesting a defective signaling pathway downstream of NO synthesis (2–4). In the genetically diabetic Goto-Kakizaki rat, the blunted vasorelaxation in response to exogenous NO is correlated with a decrease in the *in vitro* activity of vascular soluble guanylyl cyclase (sGC) (5). Since the expression of the guanylyl cyclase protein was unchanged, we concluded that the enzyme was functionally desensitized in the aortic tissue of the diabetic rats, e.g., by oxidation of its heme-bound Fe²⁺. Because the diabetes in Goto-Kakizaki rats shares many characteristics with human type 2 diabetes, this rat strain is a widely accepted animal model of human type 2 diabetes. Our *in vitro* findings in the vascular tissue of Goto-Kakizaki rats, and the fact that blunted vasorelaxation in response to exogenous NO donors was found in both diabetic patients and this animal model, led us to hypothesize that desensitization of vascular sGC might be a general finding in the vasculature of type 2 diabetic subjects. Desensitization of the enzyme could be the consequence of an increased formation of reactive oxygen species, which occurs in insulin-resistant states (6). Moreover, a recent study showed an upregulation of NADPH oxidase subunits and an increased formation of superoxide radicals in the internal mammary artery of type 2 diabetic patients (7), in agreement with previous findings in streptozotocin-induced diabetic rats (8). Thus, data in type 2 diabetic patients and in various animal models of the disease point to the possibility of a superoxide-mediated desensitization of sGC as a contributing factor to diabetic vascular dysfunction.

In the present study, we tested the hypothesis that the activity of NO-sensitive sGC is reduced in patients with type 2 diabetes. The activity of this enzyme was assayed *in vitro* in internal mammary artery specimens obtained during coronary artery bypass surgery from patients with and without type 2 diabetes. The sensitivity of sGC to NO, dependent on Fe²⁺-containing heme, was measured by *in vitro* stimulation with the NO donor diethylamine NONOate (DEA/NO). In addition, the stimulation of the oxidized heme-Fe³⁺-containing enzyme was assessed by stimulation with the novel cyclic guanosine monophosphate (cGMP)-elevating compound HMR-1766, which is known to predominantly activate the oxidized heme-Fe³⁺-containing form of sGC (9).

RESEARCH DESIGN AND METHODS

We included consecutive patients scheduled for elective coronary artery bypass surgery if they fulfilled the following inclusion criteria: age between 40 and 75 years, coronary artery disease without diabetes or coronary artery

TABLE 1
Characterization of the study population

	Nondiabetic patients	Diabetic patients
<i>n</i>	17*	18
Age (years)	64.0 ± 1.0	66.5 ± 1.4
Height (cm)	171.0 ± 2.5	171.3 ± 2.4
Weight (kg)	80.5 ± 3.4	83.7 ± 3.2
Sex (women)	6 (35)	3 (17)
Coronary artery disease severity		
One vessel	0 (0)	1 (6)
Two vessel	6 (35)	2 (11)
Three vessel	11 (65)	15 (83)
Impairment of left ventricular function		
No	11 (65)	9 (50)
Mild	1 (6)	2 (11)
Moderate	3 (18)	4 (22)
Severe	2 (12)	3 (17)
Concomitant diseases		
Hyperlipidemia	8 (47)	9 (50)
Hypertension	12 (71)	13 (72)
Peripheral artery disease	3 (18)	4 (22)
Chronic obstructive pulmonary disease/asthma	1 (6)	1 (6)
Renal failure	1 (6)	4 (22)
Medication		
Oral antidiabetic drugs	0 (0)	12 (67)†
Insulin	0 (0)	6 (33)†
ACE inhibitors	14 (82)	13 (72)
Nitrates	3 (18)	10 (56)†
Statins	14 (82)	10 (56)‡
β-Blockers	15 (88)	15 (83)
Aspirin	8 (47)	12 (67)

Data are means ± SE or *n* (%). *One patient of the control group (originally *n* = 18) was excluded because of low protein content of the tissue preparation. †*P* < 0.05, ‡*P* < 0.1 vs. control subjects (Fisher's exact test).

disease and type 2 diabetes of at least 2 years duration, and written informed consent. Patients were excluded if one of the following criteria was fulfilled: age <40 or >75 years, type 1 diabetes, or emergency surgery. The medical history of patients eligible for the study was recorded using a standardized questionnaire that contained the following items: type of coronary artery disease (one, two, or three vessels), left ventricular function, additional vascular diseases/events, standard laboratory parameters, and regular medication. If the internal mammary artery needed shortening for technical reasons (e.g., to avoid kinking), samples of the vessel were taken, immediately frozen in liquid nitrogen, and stored at -80°C until the assay of sGC was performed. The study was carried out in adherence to the Declaration of Helsinki and approved by the ethics committee of the University of Heidelberg (no. 128/2001).

Guanylyl cyclase assay. On the day of the biochemical experiments, vessel segments were thawed in cold (4°C) Ringer solution containing 1 mmol/l DL-dithiothreitol, freed from surrounding tissue, and homogenized in ice-cold assay buffer (50 mmol/l Tris, 5 mmol/l MgCl₂, 1 mmol/l DL-dithiothreitol, pH 7.4). After centrifugation at 25,000*g* and 4°C, the supernatant was separated and used in the guanylyl cyclase assay (5). The enzyme reaction was started by addition of the respective supernatant to prewarmed (37°C, pH 7.4) assay buffer containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (1 mmol/l), and a guanosine triphosphate-regenerating system (0.5 mmol/l guanosine triphosphate, 10 mmol/l phosphocreatine, 0.1 g/l creatine phosphokinase). Under these assay conditions, basal and stimulated cGMP formation was linear for up to 10 min (not shown). NO-mediated stimulation of guanylyl cyclase was studied by addition of DEA/NO (Calbiochem, Bad Soden, Germany) in a concentration range of 3 × 10⁻⁶ to 10⁻⁴ mol/l. For stimulation of the oxidized enzyme, HMR-1766 was added in concentrations of 10⁻⁷ up to 3 × 10⁻⁵ mol/l. Because HMR-1766 is poorly water soluble, the stock solution and all diluting steps of the compound were done in DMSO. The final DMSO concentration in the assay was 2% vol/vol. Comparison of basal cGMP

formation with and without DMSO 2% did not show any differences. After 6 min of incubation, tubes were heated at 120°C and centrifuged at 10,000*g*, and cGMP was measured in the supernatant by radioimmunoassay (TRK 500; Amersham Pharmacia Biotech, Freiburg, Germany). Protein content of the tissue preparation was determined using the Coomassie Plus assay (Pierce, Oud Beijerland, the Netherlands), with BSA as standard.

Data analysis and statistics. Concentration-response curves to DEA/NO and HMR-1766 were analyzed using the Pharmfit program (10), which calculates the maximum stimulation (E_{max}), the half-maximally active concentration (EC₅₀), and the corresponding pD₂ value (negative decadic logarithm of EC₅₀). Statistical comparison between groups was done by two-tailed Student's *t* test (sGC activity, calculated parameters from concentration-response curves) and Fisher's exact test (patient characteristics) at an error level of *P* < 0.05, and with *P* < 0.1 representing a statistical trend. Data are shown as mean ± SE.

RESULTS

The groups of nondiabetic and type 2 diabetic subjects included in the study were similar with regard to age, severity of coronary artery disease, left ventricular function, and the presence of concomitant diseases (Table 1). Control patients and those with type 2 diabetes were 64.0 ± 1 vs. 66.5 ± 1.4 years of age. Body weight was slightly but not significantly higher in diabetic patients (83.7 ± 3.2 kg) than in nondiabetic control subjects (80.5 ± 3.4 kg). Hyperlipidemia was present in 50% and hypertension in 70% of the patients, without differences between the groups. Regular medication showed the expected difference in the intake of antidiabetic drugs. None of the nondiabetic control group, but 16 of 18 type 2 diabetic patients, were regularly treated with antidiabetic drugs. Three of the diabetic patients received both oral antidiabetic drugs and subcutaneous insulin treatment. Prescription of nitrates was more frequent in diabetic patients (Fisher's exact test, *P* < 0.05), while statin therapy tended to be more frequent in nondiabetic control patients (Fisher's exact test, *P* < 0.1).

Basal and stimulated cGMP formation. Neither the basal rate of cGMP formation nor its stimulation by DEA/NO or HMR-1766 was different between nondiabetic and type 2 diabetic subjects (Table 2). In vitro stimulation

TABLE 2
Basal cGMP formation and stimulation by DEA/NO and HMR-1766

	Nondiabetic patients	Diabetic patients
DEA/NO		
Basal (pmol · mg ⁻¹ · min ⁻¹)	12.7 ± 1.9 (17)	13.2 ± 1.7 (18)
E _{max} (pmol · mg ⁻¹ · min ⁻¹)	475.0 ± 67.2 (16)	418.0 ± 58.5 (15)
E _{max} (×basal)	36.4 ± 2.4 (16)	35.9 ± 3.3 (15)
EC ₅₀ (μmol/l)	14.1 ± 3.0 (16)	8.9 ± 1.2 (15)
pD ₂ (-log mol/l)	5.0 ± 0.1 (16)	5.1 ± 0.1 (15)
HMR-1766		
Basal (pmol · mg ⁻¹ · min ⁻¹)*	14.4 ± 2.4 (17)	15.0 ± 2.1 (18)
E _{max} (pmol · mg ⁻¹ · min ⁻¹)	95.4 ± 18.3 (17)	83.0 ± 11.1 (17)
E _{max} (×basal)	6.4 ± 0.4 (17)	5.8 ± 0.5 (17)
EC ₅₀ (μmol/l)	1.4 ± 0.2 (17)	1.3 ± 0.3 (17)
pD ₂ (-log mol/l)	5.9 ± 0.1 (17)	6.0 ± 0.1 (17)

Data are means ± SE (*n*). *In the presence of 2% DMSO, *n* values for basal activity and parameters of stimulation are different because it was not possible to calculate a concentration-response curve in some preparations.

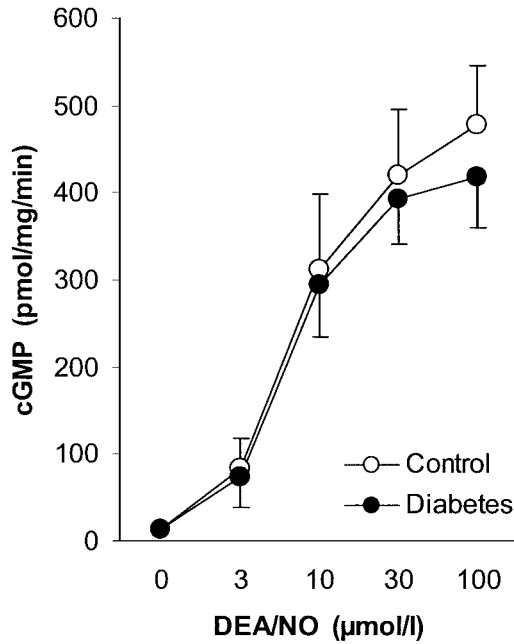


FIG. 1. Increase in cGMP formation by the NO donor, DEA/NO, in tissue preparations of the internal mammary artery from nondiabetic (○) and type 2 diabetic (●) patients with coronary artery disease. Potency and efficacy of DEA/NO were not different between the groups. Means ± SE, *n* = 17 (control subjects) and *n* = 18 (type 2 diabetic subjects)

by DEA/NO led to a concentration-dependent increase in cGMP formation (Fig. 1), with an EC₅₀ of 14.1 ± 3.0 and 8.9 ± 1.2 µmol/l in control and diabetic patients, respectively. The maximum stimulation (E_{max}) by DEA/NO amounted to 475.0 ± 67.2 and 418.0 ± 58.5 pmol · mg⁻¹ · min⁻¹ in control and diabetic tissues, representing a relative increase above basal enzyme activity by a factor of 36 in both groups (Table 2). The NO-independent sGC-activator HMR-1766 led to a sixfold increase in cGMP formation in both groups (Fig. 2), with EC₅₀ values of 1.4 ± 0.2 and 1.3 ± 0.3 µmol/l in arteries of control and diabetic patients.

Analysis of confounding factors. Since no differences in cGMP formation were found between control and type 2 diabetic patients, we evaluated the influence of potential confounding factors. Due to the limited sample size, it was not possible to do a multivariate factor analysis. Therefore, we grouped the patients according to other denominators, e.g., sex, hypertension, nitrate treatment, and we calculated the respective group means and 95% CIs. As shown in Figs. 3 and 4, only sex had an influence on vascular sGC activity, with women showing significantly (*P* < 0.05) less cGMP formation under basal conditions (Fig. 3) and a statistical trend (*P* < 0.1) for less effective stimulation by DEA/NO (Fig. 4) or HMR-1766 (not shown). Interestingly, the relative stimulation of sGC by the NO donor, DEA/NO, and the direct sGC activator, HMR-1766, was not different between female and male patients. The stimulation (fold increase over basal) by DEA/NO was 35.7 ± 2.3 in men and 37.4 ± 3.9 in women, and HMR-1766 increased cGMP formation by factors of 6.1 ± 0.4 and 6.0 ± 0.7, respectively.

DISCUSSION

The present study shows no difference in vascular sGC activity between type 2 diabetic patients and nondiabetic

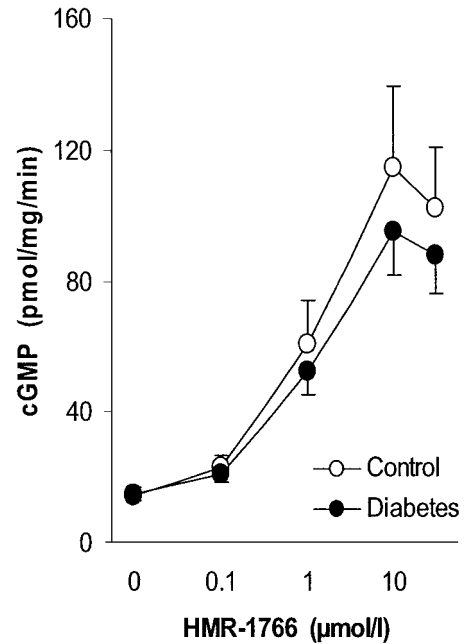


FIG. 2. Increase in cGMP formation by the direct sGC activator, HMR-1766, in tissue preparations of the internal mammary artery from nondiabetic (○) and type 2 diabetic (●) patients with coronary artery disease. Potency and efficacy of HMR-1766 were not different between the groups. Means ± SE, *n* = 17 (control subjects) and *n* = 18 (type 2 diabetic subjects)

control subjects in either the basal rate of cGMP formation or the efficacy of NO-dependent (DEA/NO) and -independent stimulation of the sGC (HMR-1766). This argues against a desensitization of this enzyme during increased

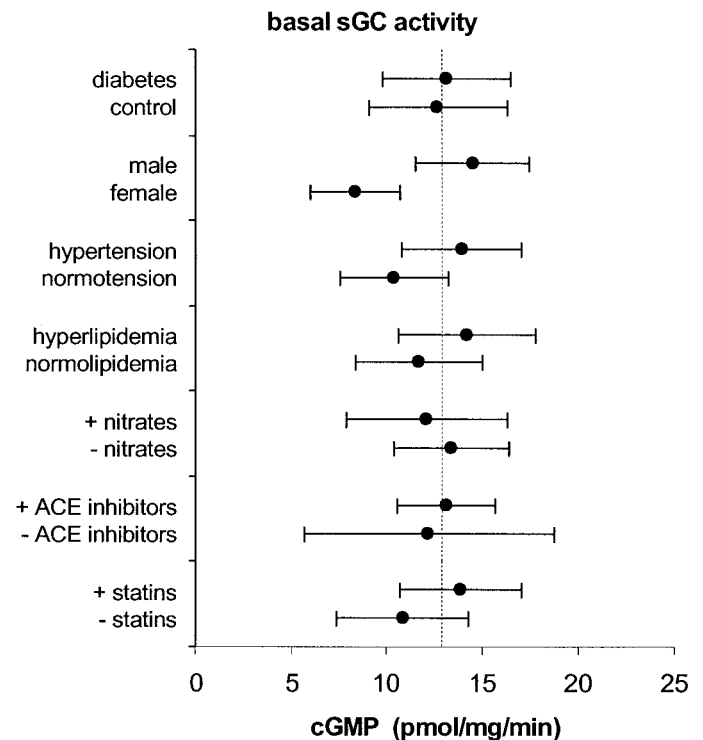


FIG. 3. Influence of possible confounding factors on the basal rate of cGMP formation in tissue preparations of the internal mammary artery. Basal sGC activity in the different subgroups is shown as means ± 95% CI. Sex (9 women and 26 men) was the only factor related to cGMP formation.

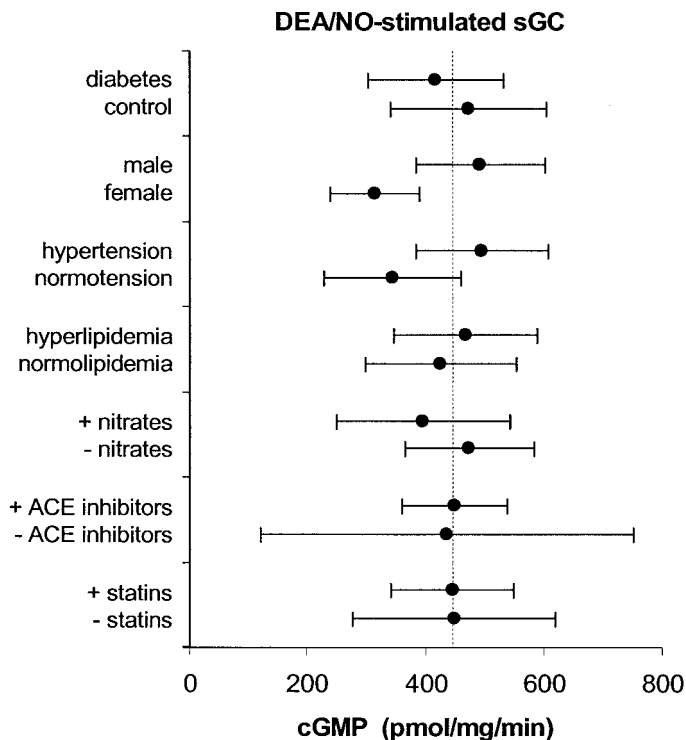


FIG. 4. Influence of possible confounding factors on DEA/NO-stimulated cGMP formation in tissue preparations of the internal mammary artery. Basal sGC activity in the different subgroups is shown as means \pm 95% CI. Sex (8 women and 23 men) was the only factor related to cGMP formation.

oxidative stress, as may occur in diabetes (6). Reactive oxygen species could induce the oxidation of the catalytic Fe^{2+} in the sGC molecule with consequent insensitivity toward NO. However, HMR-1766, a direct activator predominantly of oxidized sGC (9), was equally active in diabetic and control vessels in the present study. Thus, an increase in the oxidized form of sGC in diabetic vasculature is unlikely. The vasodilation in response to exogenous NO donors in type 2 diabetic subjects may be blunted by other mechanisms, e.g., downregulation of the cGMP-dependent protein kinase G (11) and/or increased activity of the cGMP-hydrolyzing phosphodiesterase PDE5 (12).

In our type 2 diabetic group, 16 of 18 diabetic patients were on treatment with oral antidiabetic drugs and/or insulin. Prolonged antidiabetic treatment can prevent vascular dysfunction in streptozotocin-induced diabetes in the rat (13). Therefore, vascular cGMP formation and its sensitivity to NO donors could be reduced in untreated and/or hyperglycemic type 2 diabetic patients.

Since, for obvious reasons, we could not study artery preparations from healthy subjects, potential changes of vascular sGC in type 2 diabetes could have been obscured by similar changes in our nondiabetic "control" group, suffering from coronary artery disease due to other risk factors. This hypothesis is supported by the unexpectedly high EC_{50} value of DEA/NO in both groups of patients ($\sim 10 \mu\text{mol/l}$), while the purified human sGC was reported to be more sensitive to DEA/NO, with an EC_{50} value $< 1 \mu\text{mol/l}$ (14).

Our in vitro study was carried out in segments of the internal mammary artery, which is less prone to atherosclerosis (15) and, by inference, may also be less sus-

ceptible to vascular dysfunction. However, the internal mammary artery is still a representative vessel, since its endothelium-dependent relaxation is blunted with high cholesterol concentrations (15) and in type 2 diabetes (16), when superoxide anion formation is also increased (7).

In our in vitro assay, basal and stimulated cGMP formation was measured in the presence of the antioxidant dithiothreitol, which in theory could have prevented the detection of differences in the oxidation status of the sGC's heme iron. However, in the same in vitro assay, the DEA/NO-stimulated sGC activity was blunted in aortic tissue from type 2 diabetic rats (5) and was restored after prolonged treatment with the radical scavenger tempol (17). These previous results demonstrate that the in vitro assay detects changes in the oxidant status of the heme iron.

One limitation of the present study could be that the sample size was too small to detect differences between control and type 2 diabetic patients. Based on an expected overall standard variation of 30% and a minimum relevant difference between control and type 2 diabetic patients of 30%, the number of patients needed was calculated as $n = 17$ per group. Because the interindividual variation in sGC activity was greater than expected, a 40% difference would have been needed to detect a statistical trend for a difference between groups. However, the present data show $< 15\%$ reduction in DEA/NO-stimulated sGC activity in vessels from type 2 diabetic patients, and the relative stimulation was not different. If sGC was desensitized by oxidation of the heme iron, as originally hypothesized, stimulation by HMR-1766 should have been more effective in the type 2 diabetic group. In fact, stimulation by HMR-1766 showed the same minor reduction in the type 2 diabetic group as DEA/NO. Therefore, the hypothesis of an oxidized vascular sGC in type 2 diabetes is rejected, even though a minor difference in vascular sGC activity between control and type 2 diabetic patients cannot be completely excluded.

None of the postulated confounding factors, i.e., hypertension, hyperlipidemia, previous treatment with nitrates, statins, and ACE inhibitors, had a significant effect on NO-sensitive sGC activity in vitro. Tissue preparations from women showed lower rates of cGMP formation than vessels from men, both under basal conditions and after stimulation by DEA/NO and HMR-1766. Vascular cGMP formation was blunted under all experimental conditions, i.e., in the absence and presence of both agonists, suggesting less enzyme protein in the tissue preparation of female than male patients. This could be due to downregulation of the enzyme in the vasculature of female patients or to a different proportion of smooth muscle cells in the female patient's vessel wall. However, the findings in female patients should be interpreted with caution because the sex-specific analysis was done post hoc and the sample size was small.

In conclusion, the present study shows that sGC activity and its sensitivity to NO-dependent and -independent stimulation is preserved in the internal mammary artery of type 2 diabetic patients. Therefore, it is unlikely that vascular dysfunction observed in diabetic patients is caused by desensitization of sGC in the vascular wall. Future studies are warranted to elucidate the targets downstream

of the sGC that impair vasoreactivity to NO in patients with type 2 diabetes in vivo.

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