Increased Cytochrome P4502E1 Expression and Altered Hydroxyeicosatetraenoic Acid Formation Mediate Diabetic Vascular Dysfunction

Rescue by Guanylyl-Cyclase Activation

Andreas Schäfer, Paolo Galuppo, Daniela Fraccarollo, Christian Vogt, Julian D. Widder, Julia Pfraang, Piet Tas, Eduardo Barbosa-Sicard, Hartmut Ruetten, Georg Ertl, Ingrid Fleming, and Johann Bauersachs

OBJECTIVE—We investigated the mechanisms underlying vascular endothelial and contractile dysfunction in diabetes as well as the effect of HMR1766, a novel nitric oxide (NO)-independent activator of soluble guanylyl cyclase (sGC).

RESEARCH DESIGN AND METHODS—Two weeks after induction of diabetes by streptozotocin, Wistar rats received either placebo or HMR1766 (10 mg/kg twice daily) for another 2 weeks; thereafter, vascular function was assessed.

RESULTS—Endothelial function and contractile responses were significantly impaired, while vascular superoxide formation was increased in the aortae from diabetic versus healthy control rats. Using RNA microarrays, cytochrome P4502E1 (CYP2E1) was identified as the highest upregulated gene in diabetic aorta. CYP2E1 protein was significantly increased (16-fold) by diabetes, leading to a reduction in levels of the potent vasoconstrictor 20-hydroxy-eicosatetraenoic acid (20-HETE). Induction of CYP2E1 expression in healthy rats using isoniazide mimicked the diabetic noncontractile vascular response while preincubation of aortae from STZ-diabetic rats in vitro with 20-HETE rescued contractile function. Chronic treatment with the sGC activator HMR1766 improved NO sensitivity and endothelial function, reduced CYP2E1 expression and superoxide formation, enhanced 20-HETE levels, and reversed the contractile deficit observed in the diabetic rats that received placebo.

CONCLUSIONS—Upregulation of CYP2E1 is essentially involved in diabetic vascular dysfunction. Chronic treatment with the sGC activator HMR1766 reduced oxidative stress, decreased CYP2E1 levels, and normalized vasomotor function in diabetic rats. Diabetes 59:2001–2009, 2010
mitogenesis—an effect enhanced by inhibition of phosphodiesterase 5 (13,14). An NO-independent stimulator of sGC (15), 3-(5'-hydroxymethyl-3' furyl)-1-benzylindazole (YC-1), also exerts vascular protection through inhibition of smooth muscle cell proliferation (16). HMR1766 (at- aciguat), a novel antranilic acid derivative (17), belongs to a new structural class of sGC activators capable of activating the oxidized or heme-free forms of sGC independent from NO bioavailability (17). Thus, HMR1766 is active under conditions of oxidative stress (18), when other sGC stimulators or NO itself are ineffective because of sGC dysfunction (19,20).

The aim of the present study was to determine the mechanisms underlying vascular contractile dysfunction in streptozotocin (STZ)-induced, insulin-deficient type 1 diabetes. We hypothesized that improvement of cGMP signaling by chronic treatment with HMR1766 would beneficially affect vasomotor function in diabetes.

**RESEARCH DESIGN AND METHODS**

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication no. 85-23, revised 1996) and current guidelines at the University of Würzburg. Unless stated otherwise, all chemicals were obtained from Sigma (Deisenhofen, Germany) in the highest purity available.

Male Wistar rats (250–300 g obtained from Harlan-Winkelmann, Borchen, Germany) were housed in temperature-controlled cages (20–22°C) with a 12-h light-dark cycle and given free access to water and formulated diet.

**Induction of diabetes by STZ injection.** A single dose of STZ was used to induce pancreatic islet cell destruction and persistent type 1 diabetes–like hyperglycemia. STZ (10 mg/kg; Sigma, Deisenhofen, Germany) was freshly dissolved in sterile sodium citrate buffer (25 mmol/l; pH 4.5) and used within 10 min. Rats received a single 50 mg/kg intravenous injection of STZ or citrate buffer (control). Blood glucose was monitored using a one-touch blood glucose meter (Ascensia Elite; Bayer-Vital, Leverkusen, Germany). Hyperglycemia was defined as random blood glucose level ≥20 mmol/l at 2 and 4 weeks after injection. Rats were randomized to placebo or HMR1766 (15 mg/kg twice daily; sanofi-aventis, Frankfurt/Main, Germany) at day 14. Two weeks later, vasomotor function was assessed.

**Vascular reactivity studies.** The descending thoracic aorta was dissected following removal of the heart and cleaned of connective tissue. One section containing the entire aorta was assessed with BioAnalyzer 2100 (Agilent Technologies). RNA samples were converted to biotinylated cRNA and hybridized to GeneChip arrays (Rat Expression Array 230, version 2.0; Affymetrix, Santa Clara, CA) according to the manufacturer’s directions. Microarray data analysis was performed using R packages of Bioconductor (open-source software for Bioinformatics [http://www.bioconductor.org]).

**Immunoblot.** Rat aorta smooth muscle cells were cultured with 50 μmol/l methanol/water (1:1 vol/vol) and eicosanoids were determined with a Sciex API4000 mass spectrometer operating in the multiple reaction monitoring mode. Chromatographic separation was performed on a Gemini C18 column (150 × 2 mm inner diameter, 5-μm particle size; Phenomenex, Aschaffenburg, Germany).

**Microarray.** Total RNA from aorta tissues was extracted using a microRNA isolation kit (miR/Vana; Ambion) following the manufacturer’s instructions. RNA quality was assessed with BioAnalyzer 2100 (Agilent Technologies). RNA samples were converted to biotinylated cRNA and hybridized to GeneChip arrays (Rat Expression Array 230, version 2.0; Affymetrix, Santa Clara, CA) according to the manufacturer’s directions. Microarray data analysis was performed using R packages of Bioconductor (open-source software for Bioinformatics [http://www.bioconductor.org]).

**Immunohistochemistry.** For immunohistochemical analysis, frozen aortic 5-μm sections were stained using primary antibodies against smooth muscle actin (VPS281; Vector Laboratories, Burlingame, CA). Briefly, sections were fixed in cold acetone for 5 min followed by pretreatment with 0.3% hydrogen peroxide for 20 min to inhibit endogenous peroxidase activity. Subsequently, sections were blocked with 2% horse serum for 30 min and incubated with the primary antibody for 2 h at room temperature. After rinsing with PBS, the secondary sections were incubated for 30 min with a biotinylated secondary antibody. Staining was performed using a VECTASTAIN Elite ABC kit (PK-6145; Vector Laboratories) and 3,3’diaminobenzidine tetrahydrochloride (DAB) (SK4100; Vector Laboratories).
TABLE 1

|                    | Control placebo | STZ placebo | STZ HMR1766 |
|--------------------|-----------------|-------------|-------------|
| N                  | 35              | 35          | 35          |
| Blood glucose (mmol/l) | 141 ± 7         | 500 ± 10*   | 489 ± 13*   |
| Body weight (g)    | 354 ± 6         | 243 ± 4*    | 245 ± 5*    |
| Acetylcholine EC50 (nmol/l) | 20.6 ± 3.4     | 157.6 ± 44.1* | 17.3 ± 1.9† |
| Rmax (%)           | 95.7 ± 2.5      | 81.7 ± 2.8b | 99.5 ± 0.2† |
| DEA EC50 (nmol/l)  | 2.8 ± 0.4       | 20.6 ± 3.2* | 3.8 ± 0.7†  |
| Rmax (%)           | 100.0 ± 0.0     | 99.0 ± 0.7  | 100.0 ± 0.0 |

STZ rats were administered either placebo or HMR1766. *P < 0.01 vs. control. †P < 0.01 vs. STZ placebo.

Statistics. Data are means ± SEM. Relaxant responses are given as percentage relaxation relative to the preconstriction level. Statistical analysis was performed by repeated-measures ANOVA followed by Tukey-Kramer multiple comparison test. O2− formation was analyzed by ANOVA followed by a Tukey post hoc test where appropriate; P < 0.05 was considered statistically significant.

RESULTS

Vasomotor function: relaxant responses. In aortic rings from control animals, the cumulative administration of acetylcholine, which was used to elicit the Ca2+-dependent activation of eNOS, induced an endothelium-dependent vasorelaxation. This response was significantly impaired in vessels from diabetic animals but was preserved by treatment with HMR1766 (Table 1, where blood glucose levels and body weights are also shown, and Fig. 1A). Endothelium-independent vasorelaxation induced by 2-(N,N-diethylamino)-diazenolate-2-oxide was also diminished in diabetic rats but was normalized by HMR1766 treatment (Fig. 1B and Table 1). We further assessed the relaxant response to acetylcholine in HMR1766-treated STZ-diabetic rats in the presence of L-NNA and absence of diclofenac (supplemental Fig. 1A, available in an online appendix [http://diabetes.diabetesjournals.org/cgi/content/full/db09-1668/DC1]), which indicated that neither prostacyclin nor an endothelium-derived hyperpolarizing factor plays a substantial role in vasorelaxation in diabetic rat aorta during HMR1766 treatment. Furthermore, impaired smooth muscle cell sensitivity toward NO in STZ-induced diabetes was not modulated by the presence or absence of the endothelium (supplemental Fig. 1B).

We assessed the stretch-induced release of NO by adding L-NNA to slightly preconstricted aortic rings as previously described (21). This protocol elicits the generation of NO by a Ca2+-independent mechanism similar to that activated by shear stress in vivo and can be functionally detected as an NOS inhibitor–induced vasoconstriction. Whereas a normal response was observed in arteries from control animals, the response was significantly attenuated in animals with diabetes. Though we observed a slower onset of vasoconstriction to L-NNA in aortae from diabetic animals receiving HMR1766, the absolute maxi-
Vascular SGC expression and activity. The expression of sGC protein was not modified by diabetes or treatment with HMR1766. (Values normalized for GAPDH in arbitrary units were as follows: control 1.73 ± 0.13, STZ placebo 1.72 ± 0.10, and STZ HMR1766 1.63 ± 0.16; P > 0.05.) As expected, the in vitro sensitivity of the sGC to the NO donor SNP was markedly attenuated in diabetes—a phenomenon previously attributed to the oxidation of the sGC (4). However, HMR1766 enhanced aortic sGC activity in vessels from control as well as diabetic animals to approximately the same extent (Fig. 3A). Similarly, HMR1766 itself induced comparable relaxant responses in diabetic and nondiabetic aortae (Fig. 3B). Furthermore, the downstream signaling cascade for cGMP was not modified by the presence of diabetes or the treatment with HMR1766, as shown by incremental relaxations to 8-bromo-cGMP (Fig. 3C).

Vasomotor function: contractile responses. Next, vasoconstrictions in aortae from placebo and HMR1766-treated diabetic rats were systematically evaluated and compared with the contractile response in aortae from healthy, nondiabetic rats. The response to angiotensin II was significantly impaired in diabetes and improved by chronic treatment with HMR1766 (Fig. 4A). Phenylephrine-evoked vasoconstriction was highly significantly attenuated in diabetic rats and nearly normalized by chronic treatment with HMR1766 (Fig. 4B). Receptor-dependent as well as -independent vasoconstriction was also impaired in endothelium-denuded aortae from diabetic rats and similarly improved by HMR1766 (supplemental Fig. 1C and D). The fact that the response to phenylephrine was unchanged in the presence of l-NA or following endothelial denudation indicates that the impaired vasoconstriction observed cannot be attributed to an excessive production of NO in the diabetic animals (Fig. 4C).

Aortic CYP2E1 expression and HETE formation in diabetes. The marked differences in the contractile response and its modulation by chronic treatment with the sGC activator HMR1766 prompted the systemic profiling of gene expression in the aortae using microarrays (Fig. 5A). Several highly upregulated mRNAs detected in the aortae from diabetic rats were of particular interest. These included PCNA (mRNA upregulation by 3.2-fold vs. control), which is a marker for cells in the early G1 and S phase of the cell cycle. These values were reflected at the protein levels; PCNA protein levels were significantly increased in STZ rats and reduced by chronic sGC activation (Fig. 5B). Furthermore, histological assessment of aortae from the three different groups demonstrated aortic medial thickening accompanied by interrupted and deformed elastic fibers in aortae from diabetic animals,
which appeared less pronounced in HMR1766-treated animals (supplemental Fig. 2) (26).

The gene most affected by diabetes was, however, CYP2E1 (mRNA upregulation by 42-fold vs. control). This was also observed at the protein level, where CYP2E1 protein expression (Western blot) was significantly increased in the aortae from diabetic rats and significantly attenuated after HMR1766 treatment (Fig. 5C). To further elucidate whether CYP2E1 overexpression contributes to the impaired contractile response in the rat aorta, we induced CYP2E1 expression by repeated injection of isoniazide (27). This procedure resulted in significantly higher aortic CYP2E1 protein levels (Fig. 6A) and a rightward shift in the contractile response to phenylephrine (EC50: STZ-placebo 53.9 ± 6.1 nmol/l and STZ-isoniazide 112.6 ± 14.7 nmol/l; P < 0.01), which was similar to that recorded in diabetic animals (Fig. 6B).

CYP2E1 generates lipid mediators such as 18- and 19-HETE, which in turn inhibit CYP4A enzymes. The latter enzymes are of particular pathophysiological importance because the ω-hydroxylases are the source of 20-HETE, an important vasoconstrictor eicosanoid (as summarized in Fig. 7) (28,29). We therefore determined aortic 18-, 19-, and 20-HETE levels and found that 20-HETE was markedly reduced in the aortae from diabetic versus control animals, whereas treatment with HMR1766 significantly increased 20-HETE levels in diabetic aortae (Fig. 6C). In line with the hypothesis that CYP2E1 products contribute to lowered 20-HETE levels, 18- and 19-HETE levels were increased in aortae from diabetic rats and 19-HETE was substantially lowered by HMR1766 treatment (Fig. 6D and E). Preincubation of isolated aortic rings from control and diabetic animals with 1 μmol/l 20-HETE (30) augmented the contractile

FIG. 3. sGC activity in isolated aortic smooth muscle cells from control (*) and diabetic (STZ) (□) rats was determined under basal conditions and after in vitro stimulation with HMR1766 and/or the NO donor SNP (A). Vasorelaxation induced by HMR1766 was determined in aortic rings from control and diabetic rats (B). C: Concentration-response curves for sGC-independent cGMP-mediated vasorelaxation elicited by cumulative application of 8-bromo-cGMP in isolated aortic rings from control and diabetic rats (STZ) administered either placebo or HMR1766. Data are representative of n = 6 experiments. *P < 0.05 vs. control.

FIG. 4. Contractile vasomotor function was assessed by cumulative application of angiotensin II (A) and phenylephrine (B and C) in isolated aortic rings from control and diabetic rats administered either placebo or HMR1766. Concentration responses to phenylephrine were performed in the absence (B) and presence (C) of the NOS inhibitor L-NNA (100 μmol/l) for 45 min. Data are means ± SEM from 10–16 different animals. **P < 0.01 vs. control. ###P < 0.01 vs. STZ placebo.

diabetes.diabetesjournals.org DIABETES, VOL. 59, AUGUST 2010 2005
response to phenylephrine in diabetes to an extent observed in healthy control rats (Fig. 6F).

**DISCUSSION**

The results of the present investigation demonstrate that upregulation of CYP2E1 within the diabetic aorta results in deficient synthesis of the vasoconstrictor eicosanoid 20-HETE. Moreover, it seems that the decrease in 20-HETE production is a central mechanism underlying the impaired contractile function in diabetes. Chronic activation of sGC by HMR1766-enhanced NO/cGMP signaling preserved endothelial and contractile function in diabetes.

Endothelial dysfunction in diabetes is generally characterized by an imbalance between NO and ROS production and was documented in our study by impaired vasorelaxation in response to two distinct stimuli that activate eNOS by completely different intracellular mechanisms (21). The pronounced rightward shift of the concentration response curve to exogenous NO further indicates either or both of the following possibilities: that smooth muscle sensitivity to NO is reduced or that NO is scavenged by ROS before relaxing smooth muscle cells. Oxidative stress is the major cause of reduced NO bioavailability in diabetes (1). Indeed, we recorded significantly higher $O_2^-$ formation in aortic segments from diabetic rats compared with that in healthy controls. Chronic sGC activation enhanced smooth muscle cell sensitivity toward NO (31), improved overall vascular relaxation, and reduced vascular $O_2^-$ levels. While the assessment of $O_2^-$ production by lucigenin-enhanced chemiluminescence has been criticized, the measurement of 2-hydroxyethidium formation using high-performance liquid chromatography provides a sensitive and specific determination of superoxide anions (23). Similar to long-term enhancement of eNOS expression (32), chronic sGC activation with HMR1766 reduced $O_2^-$ formation and thereby improved NO bioactivity in the present study. Whereas enhancing NO formation or decreasing its degradation does not counteract impaired NO sensitivity on the sGC level, direct sGC activation with HMR1766 causally modifies oxidized sGC and thereby improves endothelial function.

A diminished contractile response has previously been observed in insulin-deficient models of diabetes (33,34), but the molecular mechanisms underlying this effect are currently obscure. To address this point, we used a microarray-screening approach to assess the major differences in aortic gene expression between diabetic and nondiabetic rats. The gene most influenced in the rat model of diabetes studied was **CYP2E1**. Given that **CYP2E1** is regulated by insulin, it seems plausible that during states of hyperinsulinemia (such as early type 2 diabetes), vascular hyperreactivity can be observed (35–37)—in contrast to the phenotype observed in insulin-deficient diabetes in the present study. A similar increase
in CYP2E1 gene expression and enzymatic activity has been described in peripheral blood mononuclear cells from diabetic patients (38) and in nonvascular tissues from STZ-diabetic rats, where it also increased mitochondrial oxidative stress (39). CYP2E1 is of potential interest in arteries because it can generate 19-HETE and 18-HETE and because, in spontaneously hypertensive rats, a decrease in 19-HETE and 18-HETE alleviates the intrinsic inhibition of a second class of CYP enzymes (CYP4A) that generates 20-HETE to either directly induce vasoconstriction or augment sensitivity to another vasoconstrictor such as phenylephrine (40). Mechanistically, 20-HETE modulates the vasoconstrictor effects of several mediators such as angiotensin II and phenylephrine (28,29) by affecting membrane potential (29) and α-kinase activity (30). In STZ-induced diabetes, the increase in CYP2E1 expression was paralleled by a decrease in 20-HETE levels and with a decreased sensitivity to phenylephrine and angiotensin II.

To demonstrate cause and effect between CYP2E1 expression and altered vasoreactivity, we chose to increase the expression of CYP2E1 in vivo by injecting rats with isoniazid (41). Indeed, 5 days’ treatment with isoniazid was sufficient to enhance CYP2E1 expression in the aorta and attenuate the vasoconstrictor effect of phenylephrine, thus mimicking the vascular consequences of diabetes. Moreover, the exogenous application of 20-HETE in vitro augmented the vasoconstriction to phenylephrine in aortae from diabetic rats, indicating that upregulation of CYP2E1 resulting in deficient synthesis of 20-HETE is a central mechanism underlying the impaired contractile function in diabetes.

Initially, NO was described as an inhibitor of CYP2E1 catalytic activity and ROS formation (42). In this study, chronic treatment with HMR1766 activating downstream NO signaling reduced vascular CYP2E1 expression, increased 20-HETE formation, and improved the contractile response to phenylephrine. The diabetes-induced increase in CYP2E1 expression has been attributed to the impaired insulin-mediated inhibition of CYP2E1 mRNA stabilization and elevated ketone bodies (43,44). While loss of insulin activity could explain the increased aortic expression of CYP2E1, leading to reduced levels of 20-HETE and impaired vasoconstriction in diabetes, the fact that chronic treatment with HMR1766 was able to reverse the noncontractile smooth muscle cell phenotype was initially surprising. However, cGMP regulates cell-cycle regulatory mechanisms in human smooth muscle cells and suppresses phenotype switching toward a noncontractile, synthetic phenotype (8). While the noncontractile smooth muscle cell phenotype is also characterized by a shift toward proliferation, chronic NOS inhibition leads to a prosynthetic shift in smooth muscle cell gene expression in healthy rats (11). High levels of cGMP also attenuate the
proliferative response of smooth muscle cells to many mitogens (12). Suggesting that impaired cGMP signaling contributes to proliferative changes in diabetes, in our study, the proliferation marker PCNA was significantly increased in aortic tissue from diabetic rats and suppressed by chronic sGC activation.

Impaired NO/cGMP signaling in diabetes induces endothelial dysfunction but also precipitates the switch of smooth muscle cells to a proliferative, noncontractile phenotype. Increased expression of vascular CYP2E1, a phenomenon also observed in other cells during insulin deficiency, reduces the availability of the potent co-vasoconstrictor 20-HETE. Our data provide evidence that improved NO/cGMP-mediated signaling using HMR1766 in diabetes results in the inhibition of vascular CYP2E1 expression. Thereby, formation of the potent and important co-vasoconstrictor 20-HETE is preserved in the vasculature of diabetic animals treated with HMR1766, preventing the shift toward a noncontractile smooth muscle cell phenotype. Furthermore, reduced CYP2E1 expression during HMR1766 treatment also attenuates CYP2E1-derived ROS formation, which contributes to enhanced NO bioavailability. Hence, stimulation of sGC in diabetes provides a useful therapeutic approach to improve vascular function, especially in diabetes.

ACKNOWLEDGMENTS

This study was partly supported by a research grant from sanofi-aventis, and H.R. is an employee of sanofi-aventis. No other potential conflicts of interest relevant to this article were reported.

A.S. researched data, contributed to discussion, and wrote the manuscript. P.G. researched data, contributed to discussion, and reviewed and edited the manuscript. D.F. researched data, contributed to discussion, and reviewed and edited the manuscript. C.V. researched data and contributed to discussion. J.D.W. contributed to discussion. J.P. researched data. P.T. researched data. E.B.-S. researched data and reviewed and edited the manuscript. H.R. contributed to discussion and reviewed and edited the manuscript. G.E. contributed to discussion and reviewed and edited the manuscript. I.F. contributed to discussion and reviewed and edited the manuscript. J.B. contributed to discussion and wrote manuscript.

The authors thank Ulrike Flierl and Melinda Hemberger for expert technical assistance.

REFERENCES

1. Schafer A, Bauersachs J. Endothelial dysfunction, impaired endogenous platelet inhibition and platelet activation in diabetes and atherosclerosis. Curr Vasc Pharmacol 2008;6:52–60
2. Fleming I, Busse R. NO: the primary EDRF. J Mol Cell Cardiol 1999;31:5–14
3. Guerci B, Bohme P, Kearney-Schwartz A, Zannad F, Drouin P. Endothelial dysfunction and type 2 diabetes. Part 2. Altered endothelial function and the effects of treatments in type 2 diabetes mellitus. Diabetes Metab 2001;27:436–447
4. Slach JP, Schmidt PM, Nedvetsky PI, Nedvetskaia TY, Arun Kumar HS, Meurer S, Deile M, Taye A, Knorr A, Lapp H, Muller H, Turgay Y, Rothkegel C, Tersteegen A, Kemper-Harper B, Muller-Esterl W, Schmidt HI. Targeting the heme-oxidized nitric oxide receptor for selective vasodilatation of diseased blood vessels. J Clin Invest 2006;116:2552–2561
5. Etienne P, Paré-HERBEUT E, Mani-Pouzet S, Gagnon J, Babin M, Lebel M, Fabien B, Monnier L. Phenotype modulation in primary cultures of aortic smooth muscle cells from streptozotocin-diabetic rats. Differentiation 1998;63:225–236
6. Pandolfi A, Grilli A, Cilli C, Patruno A, Giaccari A, Di Silvestre S, De Lutis MA, Pellegrini G, Capani F, Consoli A, Felaco M. Phenotype modulation in cultures of vascular smooth muscle cells from diabetic rats: association...
