

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

Rescue by Guanylyl-Cyclase Activation

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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

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Diabetes is associated with accelerated development of cardiovascular disease, which is the primary cause of morbidity and mortality among patients, accounting for more than 80% of deaths. Subjects with diabetes develop abnormal endothelial function, platelet hyperreactivity, aggressive atherosclerosis, and adverse arterial remodelling relatively early on (1).

The endothelium plays a crucial role in the control of vascular homeostasis by releasing a spectrum of endothelium-derived autocooids, the most important of which is nitric oxide (NO) generated by the endothelial NO synthase (eNOS) (2). The generation of reactive oxygen species (ROS) within the vascular wall scavenges NO, thereby decreasing its bioavailability for intracellular receptors (e.g., the soluble guanylyl cyclase [sGC]). Increased radical production has been proposed as an important contributor to impaired endothelial function in hyperglycemia and diabetes (3). Enhanced oxidative stress decreases the expression and impairs NO-induced activation of heme-containing sGC, making vasodilator therapy with NO donors less effective. Under conditions of enhanced oxidative stress in vivo in several disease states including diabetes, sGC is indistinguishable from the in vitro oxidized/heme-free enzyme. The oxidized/heme-free sGC variant is unresponsive to NO and prone to degradation (4). In addition to its effects on vascular tone, the healthy endothelium plays a pivotal role in preserving the normal contractile or differentiated smooth muscle cell phenotype. In diabetes, the smooth muscle cell phenotype changes to a noncontractile or synthetic phenotype, resulting in a loss of contractility and enhanced proliferation (5–7). Indeed, cyclic guanosine monophosphate (cGMP) generated by sGC regulates cell cycle molecules in smooth muscle cells and suppresses proliferation and migration, thus preventing the switch toward a noncontractile, synthetic phenotype (8). NO inhibits the angiotensin II-induced migration of smooth muscle cell (9) and gene transfer of cGMP-dependent protein kinase sensitized smooth muscle cells for the antiproliferative effects of NO/cGMP (10). Chronic inhibition of NOS in vivo alters smooth muscle cell gene expression in favor of cell proliferation (11).

Decreased levels of cGMP appear to be a prerequisite for smooth muscle cell proliferation after vessel damage (12). In fact, organic nitrates suppress proliferation and

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-acigat), a novel anthranilic 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, Borcheln, 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 diets.

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/ml; 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 was used for measurement of O_2^- production, and the other was cut into 3-mm rings, which were mounted in an organ bath (Föhr Medical Instruments, Seeheim, Germany) for isometric force measurements. The rings were equilibrated for 30 min under a resting tension of 2 g in oxygenated (95% O_2 ; 5% CO_2) Krebs-Henseleit solution (in millimoles per liter: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.6, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 12; pH 7.4, 37°C) containing diclofenac (1 μ mol/l). Rings were repeatedly contracted by KCl (with a maximum of 100 mmol/l) until reproducible responses were obtained. A contractile response curve using cumulative doses of phenylephrine was performed in the absence and presence of the NOS inhibitor N^G -nitro-L-arginine (L-NNA) (100 μ mol/l). In separate experiments, contractile responses to angiotensin II were assessed.

The relaxant response to cumulative doses of acetylcholine was assessed after precontraction with phenylephrine to comparable levels. Afterward, aortic rings were slightly precontracted to ~20% of the maximal constriction with low, incremental doses of phenylephrine, and the additional contraction to L-NNA was measured as a marker of physiological stretch-induced, calcium-independent NO formation (21). Furthermore, relaxant responses to the endothelium-independent vasodilator 2-(N,N-diethylamino)-diazolol-2-oxide (DEA-NONOate; Alexis Biochemicals, San Diego, CA) were determined after precontraction with phenylephrine in the presence of L-NNA.

sGC activity assay. Aliquots of thoroughly rinsed aortae from control or STZ-injected rats were added to four parts of TED-buffer (50 mmol/l TrisHCl, 1 mmol/l EDTA, and 1 mmol/l dithiothreitol and protease-inhibitor mix [Complete mini; Roche, Mannheim, Germany]). The aorta was macerated and homogenized with an Ultraturax; all steps were performed on ice. The homogenate was centrifuged at 100,000g for 60 min at 4°C. The supernatant containing soluble sGC was hence used in the sGC activity assay. Protein concentration of the rat aorta supernatant was determined with the Lowry method.

A 10- μ l aliquot of rat aortic sGC was added to the reaction mixture containing 50 mmol/l tetraethylammonium/HCl (pH 7.5), 3 mmol/l $MgCl_2$, 3

mmol/l glutathione, 0.1 mmol/l GTP, and 1 mmol/l isobutylmethylxanthine to a final volume of 100 μ l. The reaction mixture was incubated for 60 min at 25°C in the presence of either solvent, 10 μ mol/l HMR1766 (dissolved in dimethyl sulfoxide/water), 100 μ mol/l sodium nitroprusside (SNP), or HMR1766 plus SNP. The reaction was stopped by adding 100 μ l stop reagent (50 mmol/l EDTA, pH 8). These reaction mixtures (50 μ l) were used to determine the cGMP content according to the nonacetylation protocol (EIA kit; Amersham, Munich, Germany). Absorbance at 450 nm was read in a microtiter plate reader with the reference wavelength set at 620 nm. Specific activity of sGC is expressed in picomoles of cGMP formed per milligram protein per minute.

Measurement of superoxide anion (O_2^-) formation. Vascular O_2^- formation was measured using lucigenin-enhanced chemiluminescence (22). The light reaction between O_2^- and 5 μ mol/l lucigenin was detected in a luminometer (Wallac, Freiburg, Germany) during incubation of rings in a HEPES-modified Krebs buffer (pH 7.40). The specific chemiluminescence signal was expressed as counts per minute per milligram dry weight of tissue.

The oxidative fluorescent dye hydroethidine was used to evaluate in situ production of superoxide as previously described (22). Unfixed frozen ring segments were cut into 10- μ m-thick sections and placed on a glass slide. Hydroethidine (2 μ mol/l) was topically applied to each tissue section and coverslipped. Slides were incubated in a light-protected humidified chamber at 37°C for 30 min. Images were obtained with a Bio-Rad MRC-1024 laser scanning confocal microscope equipped with a krypton/argon laser. Aortic rings from STZ animals and control tissues were processed and imaged in parallel. Laser settings were identical for acquisition of images from STZ and control specimens. Fluorescence was detected with a 585-nm long-pass filter.

To achieve more specific determination of superoxide formation, aortic segments were incubated with dihydroethidium and 2-hydroxyethidium formation was measured using high-performance liquid chromatography as recently described (23,24).

Liquid chromatography-mass spectrometry/mass spectrometry measurements. The aortae were homogenized and resuspended in 100 μ l of 0.1 mol/l potassium phosphate buffer (pH 7.2), hydrolyzed for 15 min at 37°C using NaOH (10 N), and neutralized with glacial acetic acid, and deuterated internal standard for 20-HETE-d6 was added. A liquid-liquid extraction was performed twice using 0.5 ml ethyl acetate. After evaporation of the solvent in a vacuum block under a gentle stream of nitrogen, samples were reconstituted with 50 μ 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 \times 2 mm inner diameter, 5- μ m 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]).

Immunoblot. Rat aorta samples with endothelium were homogenized in ice-cold Tris-buffer containing 30 mmol/l Tris-HCl, pH 7.6; 5 mmol/l $MgCl_2$; 2 mmol/l EDTA; 1 mmol/l dithiothreitol; 1% sodium cholate; 1% Triton-X-100; 0.025% sodium dodecyl sulfate; and 250 mmol/l sucrose). The homogenates were centrifuged at 8,000g for 10 min at 4°C. The resulting supernatants were mixed with sample blue loading buffer (category no. 7722; Cell Signaling Technology, Denver, CO) and separated on 15% sodium dodecyl sulfate-polyacrylamide gels under reducing conditions. Proteins were electrotransferred onto polyvinylidene difluoride membrane (0.2 μ m Immun-Blot; Bio-Rad, Munich, Germany). The bands were detected using chemiluminescence assay (ECL; Amersham). The primary antibodies used recognize cytochrome P4502E1 (ab28146; Abcam, Cambridge, U.K.), proliferating cell nuclear antigen (PCNA) (ab29; Abcam), gp91phox (611414; BD Bioscience, Heidelberg, Germany), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab8245; Abcam).

Immunohistochemistry. For immunohistochemical analysis, frozen aortic 5- μ m 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 sections were incubated for 30 min with a biotinylated secondary antibody. Staining was performed using a VECTASTAIN Elite ABC kit (PK-6100; Vector Laboratories) and 3,3'-diaminobenzidine tetrahydrochloride (DAB) (SK4100; Vector Laboratories).

TABLE 1

Blood glucose levels, body weight, acetylcholine- and 2-(N,N-diethylamino)-diazonolate-2-oxide (DEA)-induced relaxations in phenylephrine-precontracted aortic rings from diabetic STZ rats compared with nondiabetic controls

	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			
EC ₅₀ (nmol/l)	20.6 ± 3.4	157.6 ± 44.1*	17.3 ± 1.9†
R _{max} (%)	95.7 ± 2.5	81.7 ± 2.8*	99.5 ± 0.2†
DEA			
EC ₅₀ (nmol/l)	2.8 ± 0.4	20.6 ± 3.2*	3.8 ± 0.7†
R _{max} (%)	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 precontraction level. Statistical analysis was performed by repeated-measures ANOVA followed by Tukey-Kramer multiple comparisons 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)-diazonolate-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 precontracted 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-

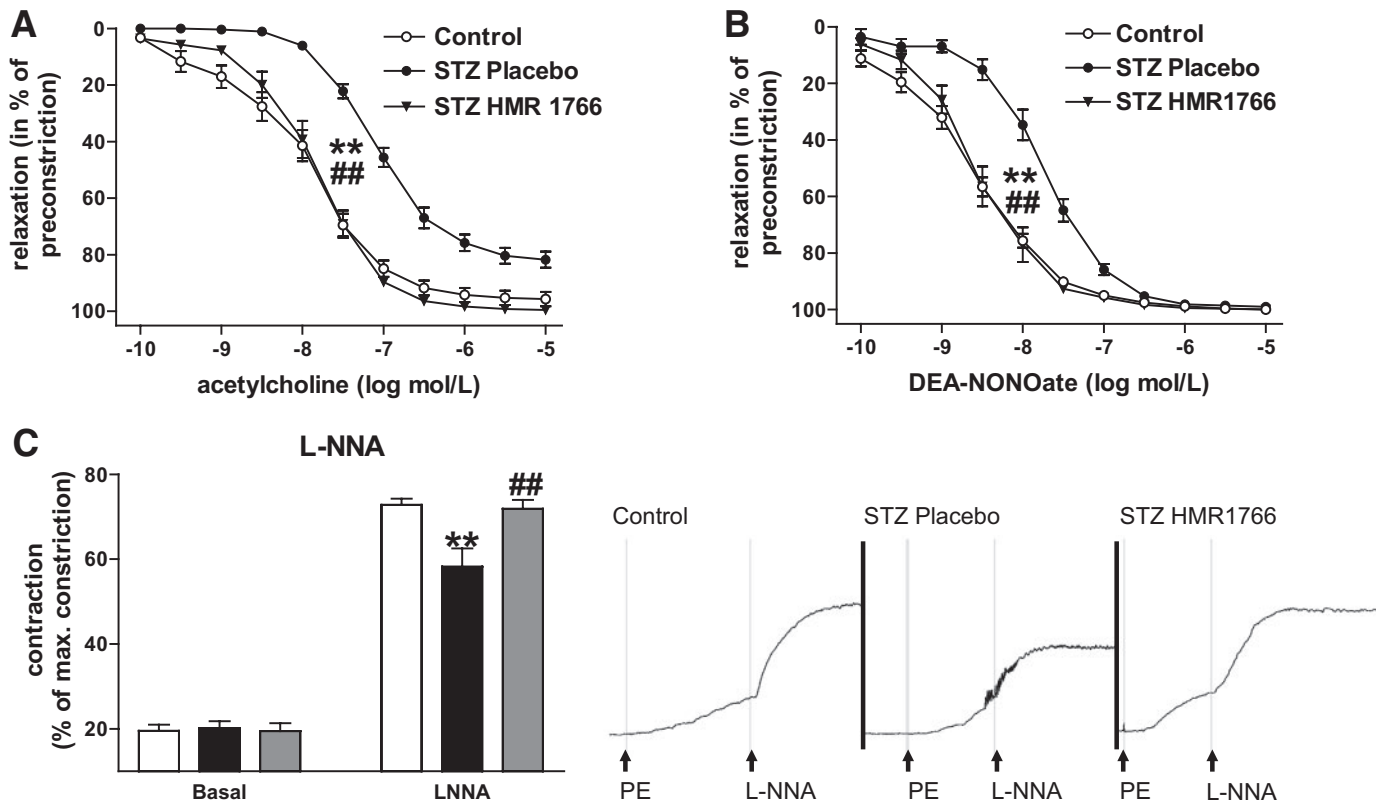


FIG. 1. Concentration-response curves for endothelium-dependent, NO-mediated vasorelaxation elicited by cumulative application of acetylcholine (A) and endothelium-independent relaxation by incremental concentrations of 2-(N,N-diethylamino)-diazonolate-2-oxide (DEA-NONOate) (B) in isolated aortic rings from control and diabetic rats (STZ) treated either with placebo or HMR1766. C: Additional increments in vasomotor tone in slightly precontracted aortic rings (~20% of maximal constriction) following NO synthase inhibition with N^G-nitro-L-arginine were used as an index of calcium-independent NO formation induced by vascular contraction. White bars, control; black bars, STZ placebo; grey bars, STZ HMR1766. Data are means ± SEM from 10–16 different animals. ***P* < 0.01 vs. control. ##*P* < 0.01 vs. STZ placebo.

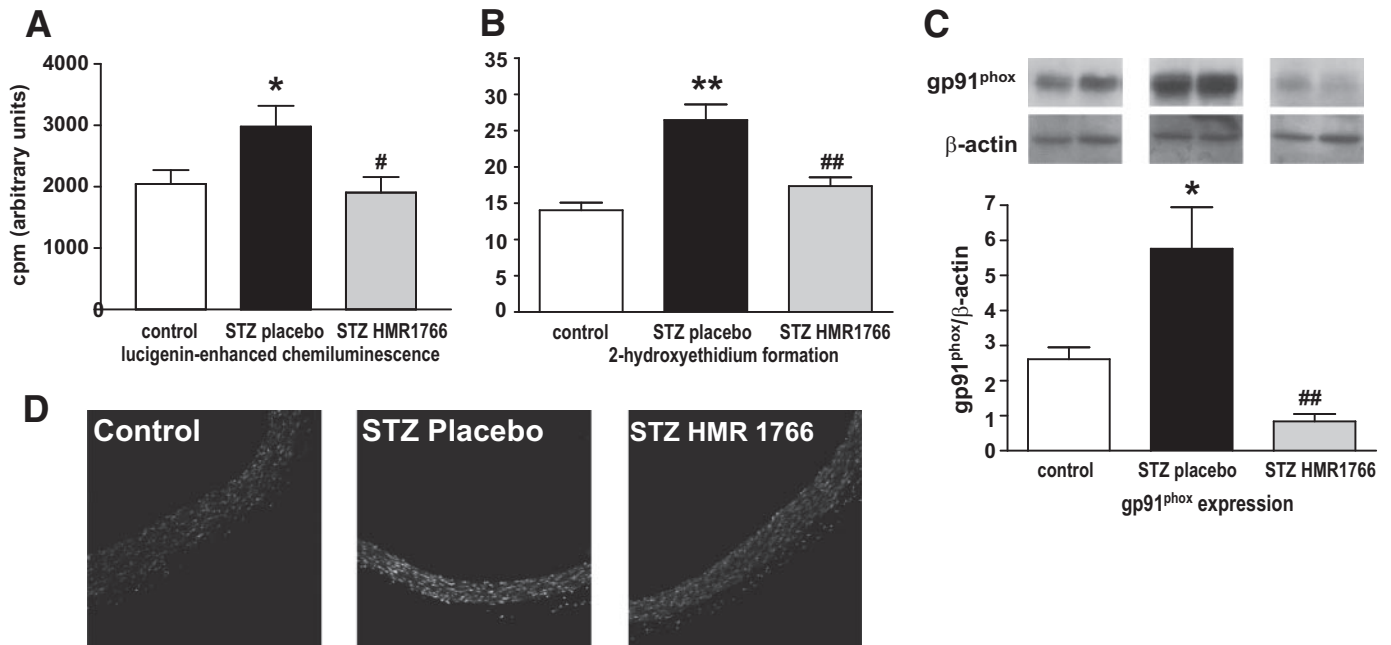


FIG. 2. Superoxide production in aortic rings from control and diabetic rats (STZ) treated either with placebo or HMR1766 was detected and quantified by lucigenin-enhanced chemiluminescence (A) and 2-hydroxyethidium formation (B). Expression of the NADPH oxidase subunit gp91^{phox} (C) was assessed by Western blot. Data are means \pm SEM from 6–10 separate experiments. D: Representative images of vessels labeled with the O₂⁻-sensitive fluorescent dye hydroxyethidium, which produces fluorescence when oxidized to hydroxyethidium by O₂⁻, are shown. **P* < 0.05, ***P* < 0.01 vs. control; #*P* < 0.05, ##*P* < 0.01 vs. STZ placebo. cpm, counts per minute.

mal contractile response achieved was similar to the one in healthy control animals (Fig. 1C). These findings indicate that the novel sGC activator is able to prevent the diabetes-associated decrease in NO release in response to receptor-dependent and mechanical stimuli.

Vascular ROS. Because the excessive formation of ROS significantly contributes to reduced NO sensitivity in diabetes, aortic O₂⁻ production was assessed by several techniques. Lucigenin-enhanced chemiluminescence (Fig. 2A) and high-performance liquid chromatography (Fig. 2B) demonstrated significantly increased superoxide formation in rats with diabetes, which was reduced by chronic treatment with HMR1766. One major source of O₂⁻ in diabetes is the NADPH oxidase (25), and protein expression of the gp91^{phox} (Nox2) subunit was significantly increased in aortae from diabetic rats and reduced by chronic treatment with HMR1766 (Fig. 2C). Representative microtopographic images of O₂⁻ formation demonstrated increased signal intensity throughout the vessel wall in diabetic versus control animals and was markedly reduced in rats treated with HMR1766 (Fig. 2D).

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-NNA 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 systematic 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 media thickening accompanied by interrupted and deformed elastic fibers in aortae from diabetic animals,

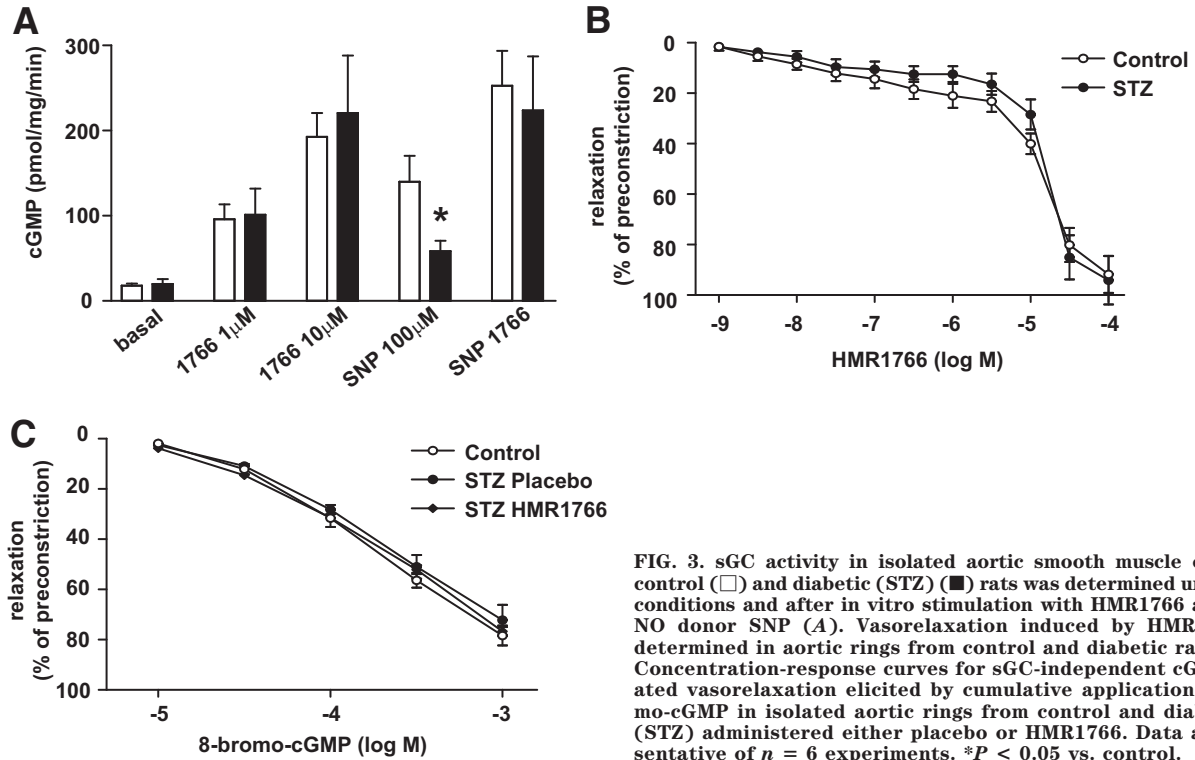


FIG. 3. sGC activity in isolated aortic smooth muscle cells from control (□) and diabetic (STZ) (■) rats was determined under basal conditions and after in vitro stimulation with HMR1766 and/or the NO donor SNP (A). Vasorelaxation induced by HMR1766 was determined in aortic rings from control and diabetic 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.

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 (EC_{50} : 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 μ mol/l 20-HETE (30) augmented the contractile

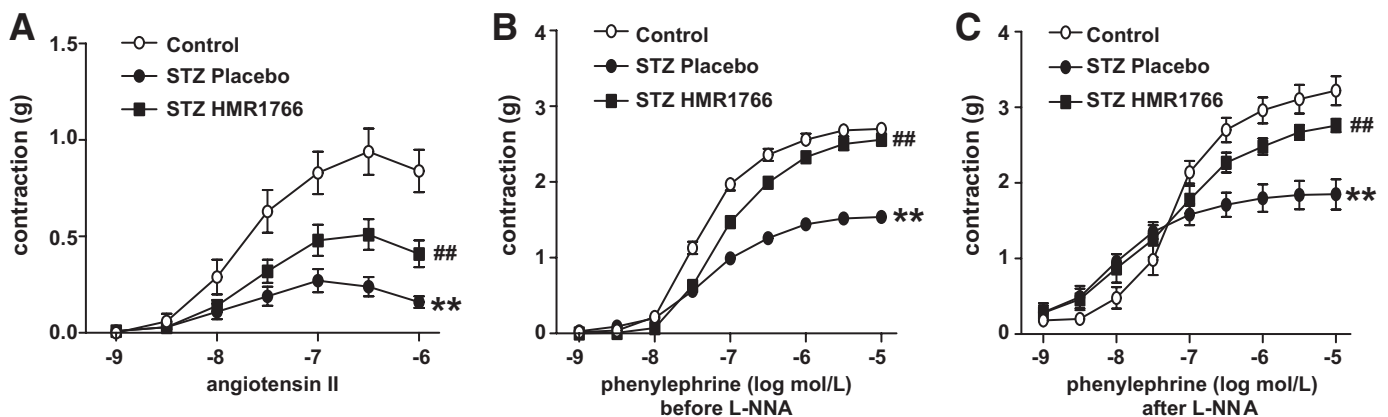


FIG. 4. Contractile vasomotor function was assessed by cumulative application of angiotensin II (A) and phenylephrine (B and C) in isolated aortic rings from control and diabetic rats administered either placebo or HMR1766. Concentration responses to phenylephrine were performed in the absence (B) and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

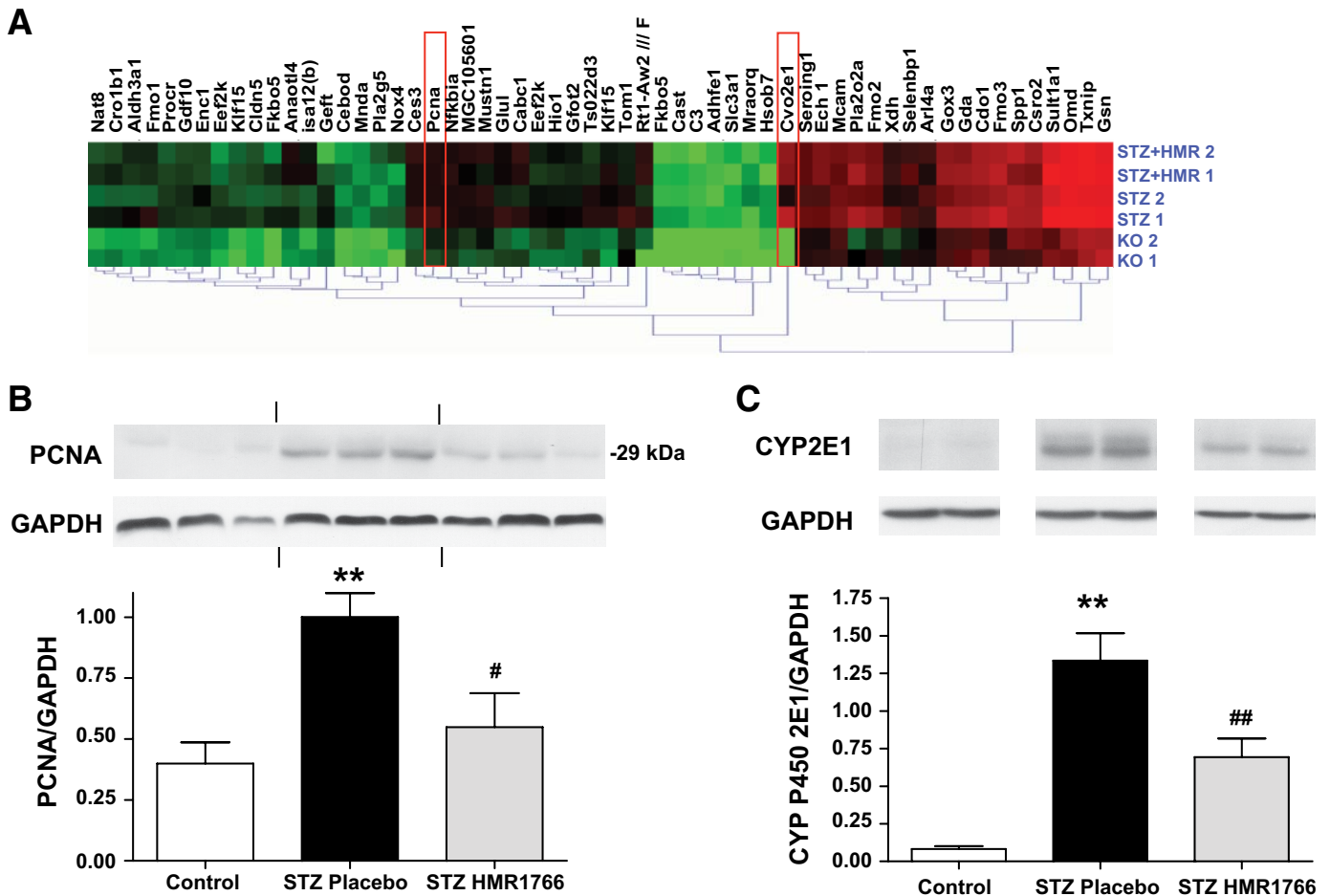


FIG. 5. Heat map of strongly regulated mRNAs in diabetic vs. nondiabetic aortae (A). Aortic protein expression (Western blot) of PCNA (B) and CYP2E1 (C), the highest upregulated mRNA in diabetic aortae, in control and diabetic rats (STZ) administered either placebo or HMR1766. Data are means \pm SEM from 6–10 separate experiments. ** $P < 0.01$ vs. control. # $P < 0.05$, ## $P < 0.01$ vs. STZ placebo. (A high-quality digital representation of this figure is available in the online issue.)

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

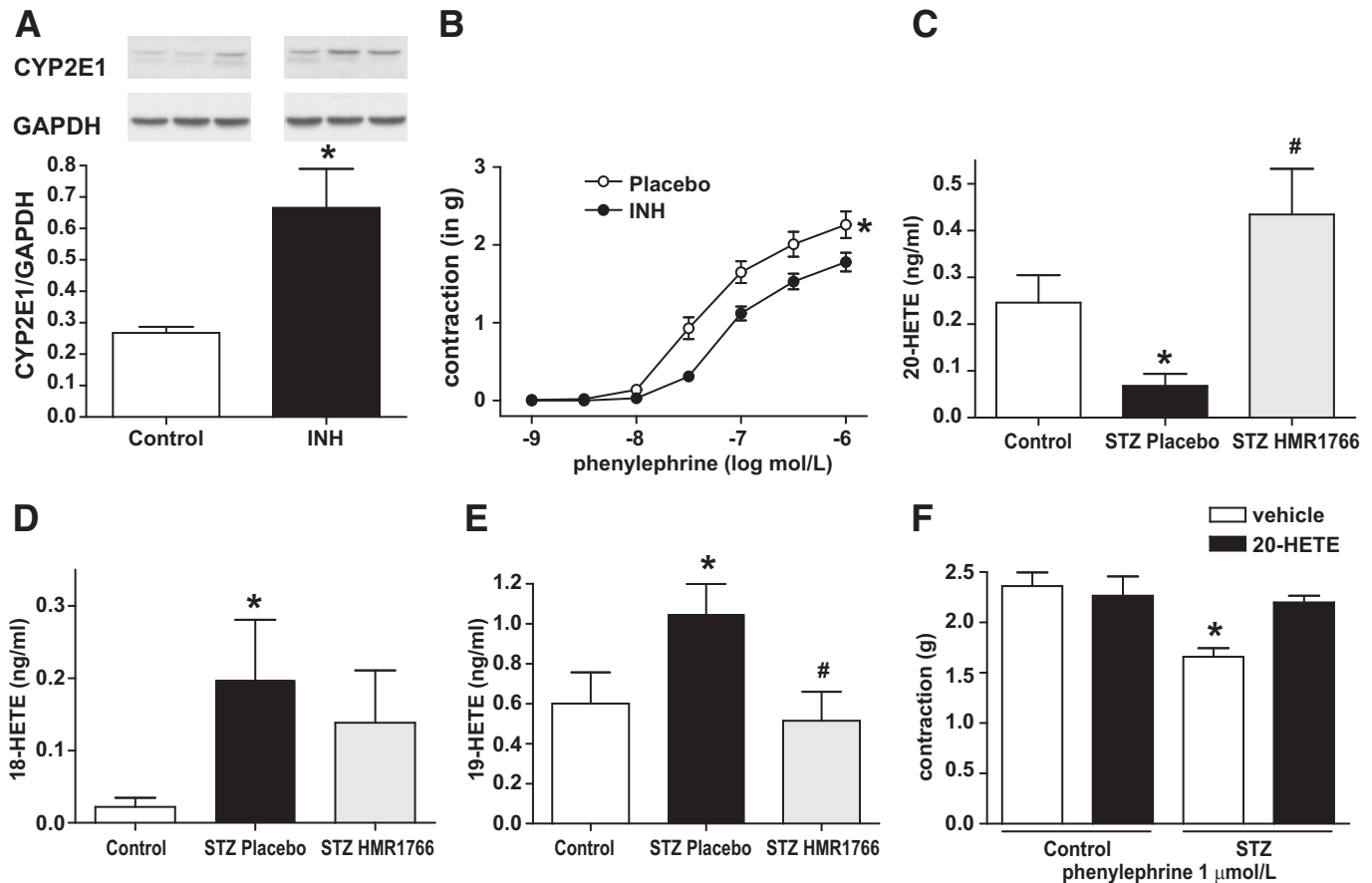


FIG. 6. Protein expression of CYP2E1 (A) and contractile vasomotor function following cumulative application of phenylephrine (B) in aortae from rats administered either placebo or 200 mg/kg i.p. isoniazide (INH) for 5 days. C–E: aortic levels of the potent vasoconstrictor 20-HETE (C), which is decreased following CYP2E1-dependent release of 18-HETE (D) and 19-HETE (E) in aortae from control and diabetic rats (STZ) administered either placebo or HMR1766. F: contraction in response to 1 μmol/l phenylephrine in isolated aortic rings from control and diabetic rats following preincubation with 1 μmol/l 20-HETE for 30 min. Data are means ± SEM from 6–10 separate experiments. * $P < 0.05$ vs. control/vehicle. # $P < 0.05$ vs. STZ placebo.

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 (Fig. 7) (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 ρ -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

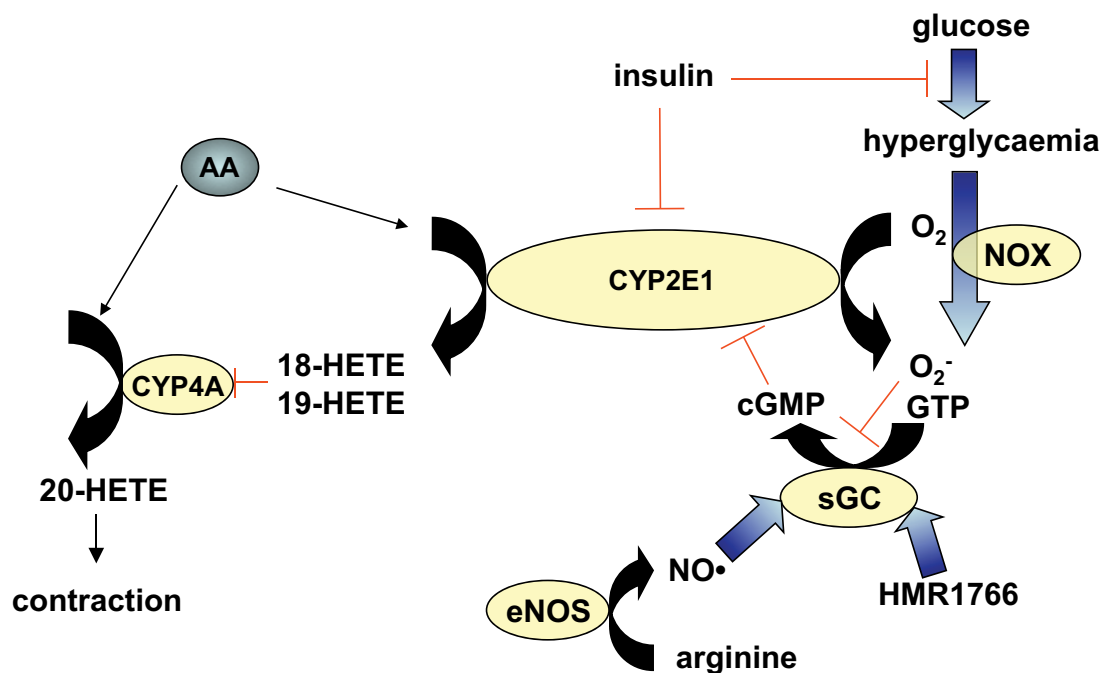


FIG. 7. Arachidonic acids (AAs), which are increased in diabetes, are substrates for cytochrome CYP2E1 and are transformed to HETEs, e.g., 18-HETE and 19-HETE. Those inhibit CYP4A enzymes, which are the source of 20-HETE, a potent and essential vasoconstrictor. Insulin inhibits CYP2E1 expression. During insulin deficiency in diabetes, 18- and 19-HETE formation is increased, leading to suppression of CYP4A activity and 20-HETE production. During hyperglycemia, oxidative stress is increased, driven by activation of CYP2E1 and NADPH oxidases (NOXs). Superoxide (O_2^-) reduces NO-mediated relaxation by scavenging the product of eNOS and inhibits the NO target enzyme sGC. The novel anthranilic acid derivative HMR1766 activates heme-oxidized sGC and thereby compensates for the reduced stimulation of sGC by NO in diabetes and increases the levels of the sGC product cGMP, which in turn inhibits CYP2E1. (A high-quality digital representation of this figure is available in the online issue.)

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 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.

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