The Hemodynamic and Metabolic Profiles of Zucker Diabetic Fatty Rats Treated with a Single Molecule Triple Vasopeptidase Inhibitor, CGS 35601

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CGS 35601 is a triple vasopeptidase inhibitor (VPI) of angiotensin converting enzyme (ACE), neutral endopeptidase (NEP), and endothelin (ET) converting enzyme-1 (ECE-1), with respective IC_{50} values of 22, 2, and 55 nM. The aim of the present study was to establish the hemodynamic profile of Zucker diabetic fatty (Zdf)-Fatty rats, a high-fat diet gene-prone model developing spontaneous Type 2 diabetes (T2D) and the effects of CGS 35601. Male Zdf-Fatty (14 weeks, *n* = 17–23), Zdf-Lean (14 weeks, *n* = 8–10), and Wistar (14 weeks, *n* = 9–10) rats on distinct diets were implanted with a catheter in the left carotid and placed individually in a metabolic cage for 30 days. The hemodynamic profile and some metabolic biomarkers were assessed daily. After a 7-day stabilization period, the Zdf-Fatty rats were divided into two groups: Group 1, controls (*n* = 7–10) receiving vehicle-saline (250 μl/hr) and Group 2, (*n* = 10–13) receiving increasing doses of CGS 35601 (0.1, 1, and 5 mg/kg/day × 6 days each, intra-arterially) followed by a 5-day washout period. Mean arterial blood pressure (MABP) of young Zdf-Fatty rats was compared with age-matched Zdf-Lean and Wistar rats, which were found similar. MABP decreased by 5.9% (from baseline at 102 ± 5 to 96 ± 4 mmHg), 12.7% (to 89 ± 6 mmHg) and 21.6% (to 80 ± 4 mmHg), at 0.1, 1, and 5 mg/kg/day, respectively, in CGS 35601-treated Zdf-Fatty rats. Systolic and diastolic blood pressures were similarly reduced. The heart rate was not affected. Hyperglycemic status and insulin-resistance were not modulated by short-term treatment. CGS 35601 presented an excellent short-term safety profile. This novel molecule and class of VPI may be of interest for lowering vascular tone. Further long-term studies, once cardiovascular and renal complications have developed in this T2D rat model are warranted to define the efficacy of this class of VPI. Exp Biol Med 231:824–829, 2006

Key words: angiotensin-converting enzyme; endothelin converting enzyme; neutral endopeptidase 24.11; vasopeptidase inhibitor; CGS 35601

Introduction

Occurrence of non–insulin-dependent diabetes mellitus (NIDDM), or Type 2 insulin-resistant diabetes (T2D), is associated with the development of hypertension. Such complication requires effective antihypertensive therapy to prevent or delay further complications that increase overall morbidity and mortality. Thus far, blockade of the renin-angiotensin system (RAS), through angiotensin converting enzyme (ACE) inhibitors or angiotensin II (AII) type 1 receptor (AT1-R) antagonism, has proven partly effective in clinical settings against diabetes-related hypertension and other complications such as diabetic nephropathy. Although AII is implicated in diabetic nephropathy, RAS blockade does not prevent the progression of renal disease (1). Other vasoactive mediators may be implicated in the worsening of diabetes-related complications. Endothelins (ETs as ET-1) are potent vasoconstrictors, and promitogenic peptides may be among them. It has been demonstrated that circulating plasma concentrations of ET-1 were chronically elevated in patients with both T1D (2) and T2D (3), and that tissue and plasma concentrations of ET-1 were increased in spontaneously, gene-prone, T2D Goto-Kakizaki rats (4). Furthermore, ET-converting enzyme-1c (ECE-1c) isoform

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expression and/or activity is overexpressed or up-regulated in endothelial cells in response to high glucose levels (5) and in clinical diabetes (6). The fact that Bosentan, a dual ET receptor antagonist (ERA) at both ET receptor subtypes, completely prevented the development of hypertension and renal vasoconstriction in uninephrectomized streptozotocin (STZ)-induced diabetic rats supports a pathophysiological role of the ET system in renal disease (7). A dual neutral endopeptidase (NEP)/ECE inhibitor, CGS 26303, was also able to reduce glomerulonephritis in puromycin aminonucleotide-induced glomerular lesions in rats (8, 9). Moreover, blockade with a so-called selective ET_A antagonist (Darusentan) lowered blood pressure in spontaneously, gene-prone, T2D Goto-Kakizaki rats (10). The combination of a so-called selective ET_A antagonist (Darusentan) combined with an ACEi (Trandolapril) was better at lowering the systolic blood pressure (SBP) in Cohen-Rosenthal diabetic hypertensive rats than either drug alone (11), suggesting that both the RAS and ET pathways were responsible for elevated blood pressure in this preclinical model of T2D.

Recently, new strategies have emerged aimed at simultaneously inhibiting two major enzymatic systems involved in blood pressure homeostasis such as dual ACE/NEP inhibitors. Dual vasopeptidase inhibitors (VPIs) such as AVE7688 (12) and MDL-100,240 (13) are still under development, although Omapatrilat (OMP), a distinct molecule of this class of drugs, has not fulfilled clinical expectations. Yet, VPI remain very effective antihypertensive drugs (14), and dual ACE/NEP inhibitors were shown to have renoprotective effects (15, 16), even though they did not take into consideration the different roles played by ET-1 in both hypertension and its complications.

The purpose of the present study was to assess the efficacy of CGS 35601, a triple VPI (17), capable of simultaneously inhibiting ACE, NEP, and ECE-1, with respective IC_{50} values of 22, 2, and 55 n*M*, in a gene-prone experimental rat model of nonobese T2D (Zdf-Fatty) using chronically instrumented, conscious and unrestrained rats. Here, we demonstrated that CGS 35601 reduces blood pressure in young diabetic, hyperinsulinemic, but not yet hypertensive, Zdf-Fatty rats.

Materials and Methods

All procedures were previously approved by the local Ethics Animal Care Committee, and the guidelines on animal welfare established by the Canadian Council on Animal Care were followed.

Presurgical Setup, Anesthesia, Surgical Procedures, and Postoperative Care. Adult male Zdf-Fatty (ZDF/Crl-Lepr fa), Zdf-Lean (ZDF/Crl-Lepr fa/+), and Wistar rats (14 weeks; Charles River, Saint-Constant, Canada) were housed individually in modified metabolic cages (Nalgene, Rochester, NY), under a 12-hr cycle of day/night, with free access to food and drinking water. Wistar and Zdf-Lean rats were fed a normal rat chow (Charles River #5075, Charles River). Zdf-Fatty rats were on a high-fat diet since their weaning (FormulaLab #5008, Ren's, Oakville, Canada).

As described before (18), surgical procedures under inhaled anesthesia (2% isoflurane inhalation, Baxter Corp., Toronto, Canada) were performed in a strictly aseptic environment with sterilized materials. A urethane-coated antithrombogenic vascular catheter (PhysioCath, Data Sciences International, St. Paul, MN) was inserted into the left carotid and tunneled under the skin to the dorsal site at the neck. It was then connected to a low-flow peristaltic pump (Instech-Solomon, Plymouth Meeting, PA) through a stainless-steel spring stock protector and swivel via an adjustable counterbalance lever arm (Instech-Solomon). A constant infusion of sterile heparinized (4 U/ml) saline (250 µl/hr) prevented the formation of blood clots within the catheter.

Hemodynamic Profile Determination. The hemodynamic profile was assessed each day at 1300 hrs, on calm, resting, unrestrained and conscious rats, via direct measurement through the implanted vascular catheter using a blood pressure transducer (Harvard Apparatus, Montreal, Canada) connected to a precalibrated computerized system (PowerLab, ADInstrument, Colorado Springs, CO). MABP and heart rate (HR) were simultaneously derived from systolic and diastolic blood pressure (SBP; DBP) data and recorded.

Drug and Administration. CGS 35601 was kindly provided by Novartis Institutes for BioMedical Research (East Hanover, NJ). CGS 35601, a potent triple ACE, NEP, and ECE-1 inhibitor (L-tryptophan, *N*-[[1-[[(2*S*)-2-mercapto-4-methyl-1-oxopentyl] amino] cyclopentyl] carbonyl]) was dissolved at 10 mg/ml in sterile saline:NaOH (0.1 *N*) before its final dilution at 1 mg/ml in saline (Fig. 1a). The drug solution was then sterilized through a 0.22-µm filter and kept frozen until use. Seven days after surgery, the rats were administered intra-arterially (i.a.) through the vascular catheter, with CGS 35601 at 0.1, 1, 5, mg/kg/day for 6 days at each dose followed by a 5-day washout period (Fig. 1b).

Oral Glucose Tolerance Test (OGTT). Following an overnight fast (2000 to 0800 hrs), an OGTT was performed in untreated and CGS-treated conscious Zdf-Fatty rats. In brief, the OGTT consisted of a dextrose solution administered by oral gavage at a dose of 1.5 g of carbohydrate (CHO)/kg body wt. The initial dextrose solution was diluted to approximately 0.45 g/ml to administer no more than 1 to 1.5 ml to each rat. Blood samples were obtained via the i.a. implanted catheter at 0, 15, 30, 60, 120, and 180 minutes (200 µl each time) following the challenge, for determination of the circulating concentrations of blood glucose and plasma insulin.

Biochemical Assays. *Glucose.* Circulating plasma concentrations of glucose were measured via a colorimetric assay using a Vitros 950 from Beckman Coulter Inc. (Hialeah, FL), whereas blood glucose for the OGTT was measured via an automatic analyzer (Glucom-

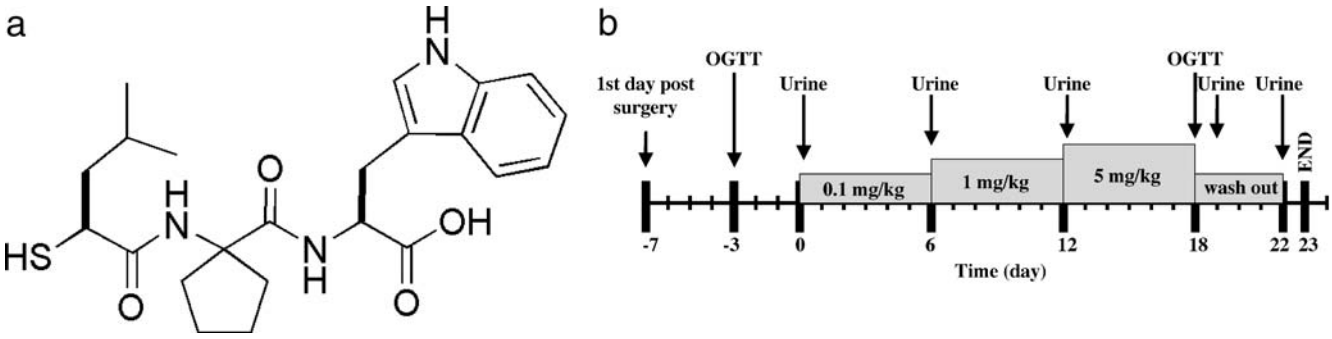

Figure 1. (a) Chemical structure of the single molecule triple ACE/NEP/ECE vasopeptidase inhibitor, CGS 35601. (b) Experimental timeframe and treatment protocol in chronically instrumented, unrestrained and conscious rats of various phenotypes.

eter Elite XL, Bayer Inc., Toronto, Canada) using glucose oxidase/potassium ferricyanide reagents strips.

Insulin. Circulating plasma concentrations of insulin were assessed using a rat/mouse EIA kit from Linco (cat# EZRMI-13K; St. Charles, MO) with a detection limit of 0.2 ng/ml, 100% cross-reactivity for rat insulin and intra- and inter-assay of $<3.5\%$ and $<9.5\%$, respectively.

Statistical Analyses. Results are expressed as mean $\pm$ SEM. Because of the unavailability of some samples/data at a given time point, a repeated measurement analysis of variance (ANOVA) was inapplicable to compare values obtained at different time period. Thus, for comparisons within groups, a randomized block design was applied using two factors defined for the analysis: the subject effect and the time period effect.

Comparison between groups was performed by a three-way ANOVA with a blocking factor representing subjects. Interaction between the time period factor and one used to compare groups was added to the model. When interaction was significant for parameters, comparisons at different time period were analyzed using Student's paired *t* test. The normality and variance assumptions were met for almost all data. All analyses were conducted using the statistical package SAS (SAS Institute Inc., Cary, NC).

Results

Age-matched, conscious unrestrained Wistar rats and Zdf-Lean rats, fed a normal diet, were used as normotensive controls with an average MABP of 108 $\pm$ 9 and 104 $\pm$ 5 mm Hg, respectively (Fig. 2c). It is noteworthy that young Zdf-Fatty rats used in this study, that were fed a high-caloric diet from weaning, did not develop high blood pressure (107 $\pm$ 7 mm Hg) at 14 weeks of age, even though they were hyperglycemic (glycemia at 23.0 $\pm$ 2.2 m*M*; Fig. 3a) and hyperinsulinemic (insulin at 8 $\pm$ 1 ng/ml) since the age of 10 weeks. Both Wistar and Zdf-Lean rats remained normoglycemic (glycemia at 7.4 $\pm$ 0.1 and 7.4 $\pm$ 0.2 m*M*, respectively).

The lowest dose of CGS 35601 (0.1 mg/kg/day i.a.) decreased the MABP in Zdf-Fatty rats by an average of 5.9% after 6 consecutive days of treatment (a 6-day average of 102 $\pm$ 5 to 96 $\pm$ 4 mm Hg; $P < 0.05$; Fig. 2c). Higher doses at 1 and 5 mg/kg/day further reduced MABP by an average of 12.7% (down to a 6-day average of 89 $\pm$ 6 mm Hg; $P < 0.01$) and 21.6% (down to a 6-day average of 80 $\pm$ 4 mm Hg; $P < 0.001$), respectively. The same dose-profile (0.1, 1, 5 mg/kg/day) attenuated SBP and DBP of Zdf-Fatty rats by an average of 7.7%, 14.6%, and 22.5%, and 6.9%, 12.7%, and 21.9%, respectively (Fig. 2a and b). Therefore, the overall efficacy for each dose over 6 days was similar in both cases to those observed for MABP. CGS 35601 did not affect the heart rate of Zdf-Fatty rats (Fig. 2d). Blood pressures gradually rose back to baseline following cessation of treatment (Fig. 2a–c).

Hyperglycemia measured in Zdf-Fatty rats was not affected by treatment with CGS 35601 (Fig. 3a). OGTTs conducted before (at 15 weeks of age) and after 18 days of dose-escalating treatment (at about 18 weeks of age) with CGS 35601 were similar, and the degree of insulin-resistance was not significantly modified (Fig. 3b and c).

Other parameters of renal and hepatic function were not affected (data not shown).

Discussion

Hypertension is the main risk factor for the progression of kidney damage in diabetes. Therefore, a vigorous control of blood pressure is necessary to avoid the worsening of diabetes-related complications such as diabetic nephropathy. ACE inhibition is currently the established treatment of choice in diabetic nephropathy. However, it was demonstrated that the ETS significantly contributes to the elevation in blood pressure (10) and has been implicated in kidney disease and end-stage renal failure (7). Therefore, the simultaneous blockade of both the RAS, through ACEi and/or AT1-R antagonist, and the ETS, through mixed $ET_{A/B}$ or ET_A or ECE inhibitor, would unravel a significant degree of additive or even synergistic effects that would contribute to a better management of hypertension and its complications.

The first attempt in inhibiting two major pathways or axes that modulate vascular tone and contribute to the regulation of blood pressure was initiated with dual ACE/NEP inhibitors, defining the concept of vasopeptidase

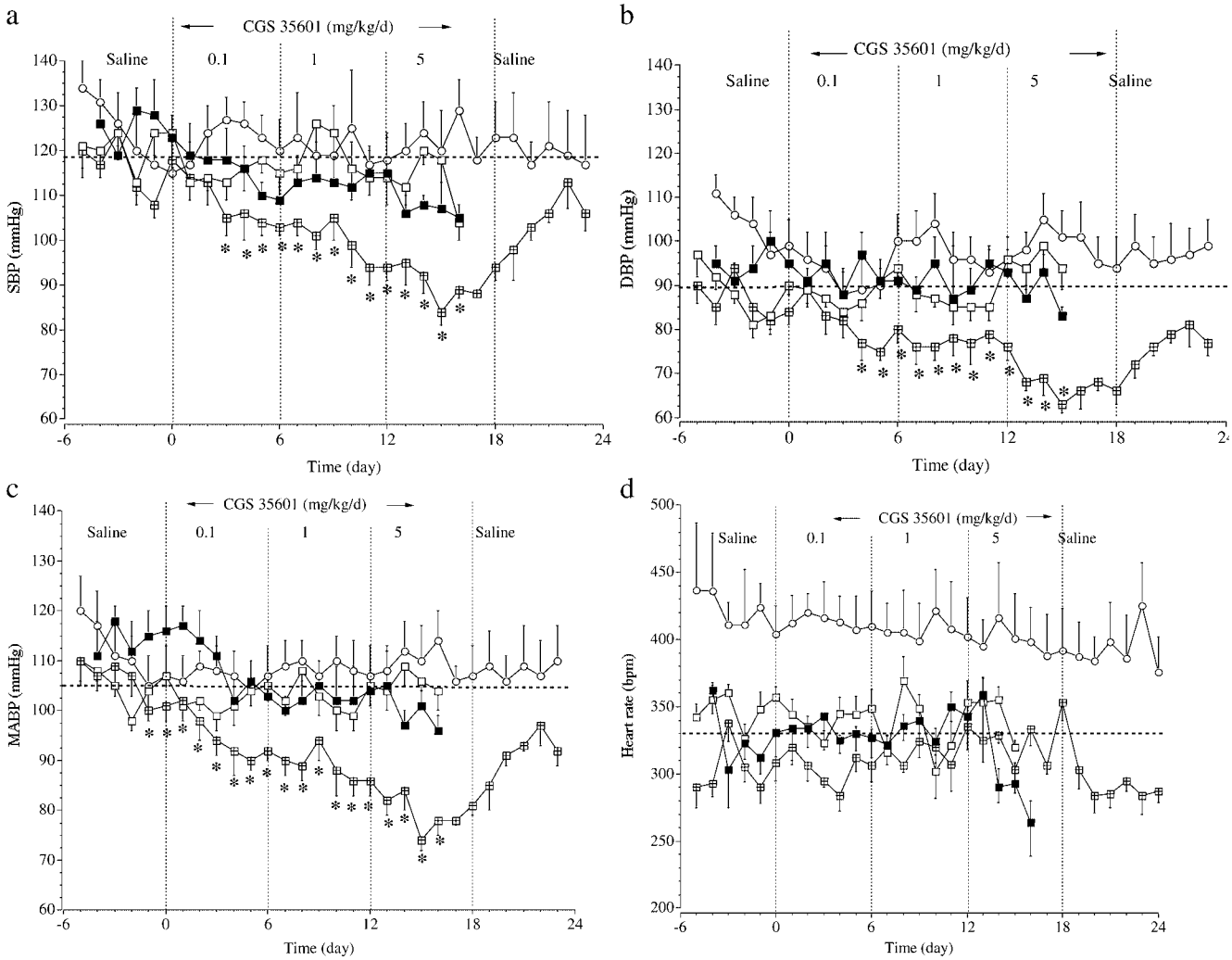

Figure 2. (a) Systolic blood pressure (SBP; mmHg); (b) Diastolic blood pressure (DBP; mm Hg); (c) Mean arterial blood pressure (MABP; mmHg), and (d) Heart rate (HR; bpm) in chronically instrumented, unrestrained and conscious Zdf-Fatty (Group 1, crossed square; $n = 10–13$) treated with increasing doses of the vasopeptidase inhibitor CGS 35601, (starting 7 days after surgery, renamed time 0 at 0.1, 1, and 5 mg/kg/day, continuous i.a. infusion, 6 days/dose) over 18 days followed by a 5-day washout period; untreated control Zdf-Fatty (Group 2, saline, ■, $n = 7–10$); Zdf-Lean (Group 3, saline, □, $n = 8–10$); Wistar (Group 4, saline, ○, $n = 9–10$) rats. *$P < 0.05–0.01$, significantly different between CGS 35601-treated Group 1 and untreated Group 2 over time.

inhibition. These VPIs are very effective antihypertensive, antifibrotic, and anti-inflammatory compounds offering nephroprotection and presented, at some point, a great therapeutic potential. However, safety concerns emerged with OMP with the incidence of angioedema. One can hypothesize that the limitation reported with OMP lies in the underestimation of the roles played by the ETs in modulating physiopathological responses. As a consequence, a new class of triple VPI that concomitantly inhibits ACE, NEP, and ECE-1 was developed (17, 19). It was demonstrated that the chronic infusion in spontaneous hypertensive rats (SHRs) of CGS 35601 at 5 mg/kg/day, was able to reduce MABP by 40% (from 156 ± 4 to 94 ± 5 mm Hg) (20). In instrumented Dahl salt-sensitive rats, a low renin model of hypertension, a 5 mg/kg/day dosage of CGS 35601 reduces MABP by 14%, down to 127 mm Hg, i.e., below the MABP in untreated, vehicle-administered, Dahl salt-sensitive rats (21).

As hypertension is generally present in patients with T2D, it was particularly interesting to evaluate the potential of CGS 35601 at lowering blood pressure in such a population. It is known that hyperglycemia increases circulating plasma concentrations of ET-1 in diabetic patients (3), which may contribute to the elevation in blood pressure (10) and the worsening of diabetic nephropathy (7). Our present data demonstrate that CGS 35601 is capable of reducing blood pressures in Zdf-Fatty rats in a dose-dependent manner without affecting heart rate. The highest dose of CGS 35601 reduces MABP by 22% in young 14-week-old normotensive Zdf-Fatty rats fed a high-caloric diet

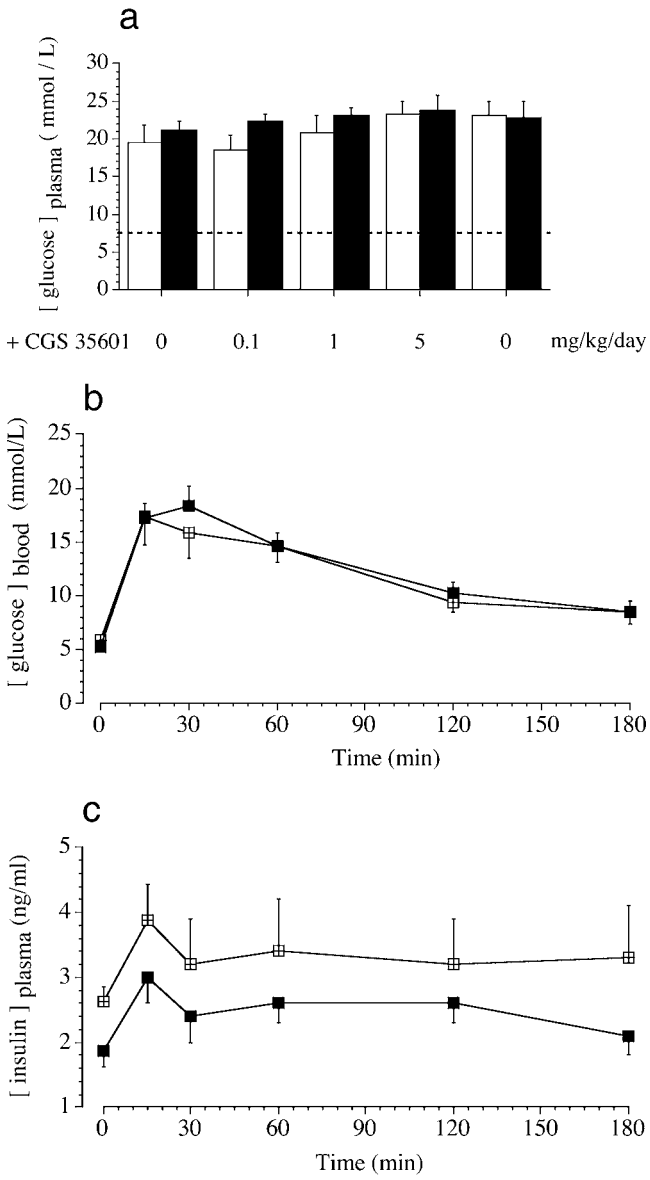

Figure 3. Circulating plasma concentrations of (a) glucose (m*M*) and profiles of blood glucose (b) and insulin (c) after an oral glucose tolerance test (OGTT) in untreated (open column or ■; $n = 7–10$) versus CGS 35601-treated (closed column or crossed square; $n = 10–13$) chronically instrumented, unrestrained and conscious T2D Zdf-Fatty rats. Treatment was initiated 7 days after surgery (renamed time 0) at 0.1, 1, and 5 mg/kg/day, continuous i.a. infusion, 6 days/dose) over 18 days followed by a 5-day washout period. OGTTs were conducted on Days −3 and +18 (Fig. 1b).

producing metabolic dysfunction, thus below baselines measured in Zdf-Lean and Wistar rats. This result suggests great efficacy upon that primary end point. But short-term treatment did not affect hyperglycemia or insulin-resistance. It remains to be determined if the efficacy over the present end point can be as potently sustained with selective or dual VPI, and if cardiorenal protection can be observed in aged T2D rat models that developed overt dysfunctions and complications. Complete profiles of pro-inflammatory and vasoactive mediators defining the mechanisms of action of such class of molecules will also be studied (have been studied in SHR).

Triple VPIs may constitute a promising new class of molecules whose benefits go beyond lowering elevated blood pressure. Their ability to interfere with multiple pathways or axes should subsequently prevent various diabetic complications and end-stage organ failures.

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