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Novel ABCA1 peptide agonists with antidiabetic action

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Abstract

Previously, apoE-derived ABCA1 agonist peptides have been shown to possess anti-atherosclerotic and possibly antidiabetic properties. Here we assessed the in vitro and in vivo actions of a second generation of ABCA1 peptide agonists, CS6253 and T6991–2, on glucose homeostasis. The results show that these two peptides improve glucose tolerance in a prediabetic diet-induced obesity mouse model by enhancing insulin secretion. It was further demonstrated that T6991–2 also improved glucose tolerance in leptin-deficient (ob/ob) mice. CS6253 increased insulin secretion both under basal conditions and in response to high glucose stimulation in pancreatic INS-1 β -cells rendered leptin receptor deficient with specific siRNA. Additional in vitro cell studies suggest that the CS6253 agonist attenuates hepatic gluconeogenesis and glucose transport. It also potentiates insulin-stimulated glucose uptake and utilization. These observed anti-diabetic actions suggest additional benefits of the CS6253 and T6991–2 ABCA1 peptide agonists for cardiovascular disease beyond their direct anti-atherosclerosis properties previously described.

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Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflict-of-interest/financial disclosure statement

The Authors declare no conflict of interest except that Jan O. Johansson is a compensated employee of Artery Therapeutics Inc.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mce.2018.09.011>.

Keywords

HDL mimetic; Type 2 diabetes; Atherosclerosis; Insulin secretagogue; Glucose homeostasis; Diet induced diabetes

1. Introduction

The prevalence of diabetes, including type 2 diabetes (T2D) (which comprises about 87–91% of the total diabetes burden), has reached an epidemic level worldwide (World Health Organization (WHO), 2016; Zimmet et al., 2016). It is estimated that between 415 and 425 million people are living with diabetes in the world (International Diabetes Federation, 2017; Jaacks et al., 2016). It is predicted by 2040 there will be 642 million people with diabetes worldwide (World Health Organization (WHO), 2016). According to the estimates of the Centers of Disease Control and Prevention (CDC), there are 30.3 million people in the U.S. with diabetes (9.4% of the U.S. population), including 23.1 million who are diagnosed and 7.2 million people (23.8%) undiagnosed, with 90–95% of them having T2D (Centers for Disease Control and Prevention (CDC), 2017; Colhoun et al., 2004), a prevalence projected to increase by 21% by 2050 among the US population (Boyle et al., 2010). Diabetes is an independent risk factor for cardio-vascular disease (CVD), and CVD is a leading cause of morbidity and mortality in patients with T2D (American Diabetes Association, 2015; Circulation, 1999; Fox et al., 2004; Fox et al., 2007; Gaede et al., 2003; Go et al., 2013; Grundy et al., 1999). In fact, it is associated with 2–4 times higher risk of CVD and 3-fold increase in mortality rate (Circulation, 1999; Go et al., 2013; Grundy et al., 1999; Meisinger et al., 2010). Moreover, women with diabetes, regardless of their menopausal status, are 4–7 times more likely to develop cardiovascular disease compared with men who are only 2 to 3 times at risk of developing the disease (Ford et al., 2007; Fox et al., 2004).

Despite a marked decline in CVD related deaths over the past several decades, because of improvement in CVD risk factors such as lowering of smoking prevalence, and total cholesterol and blood pressure (Fox et al., 2007), a smaller reduction has occurred in patients with diabetes than patients without diabetes (Gaede et al., 2003; Go et al., 2013; Griffin et al., 2017). Furthermore, although there is a strong link between hyperglycemia and cardiovascular disease, intensive glycemic control in diabetes patients with currently available diabetes drugs in large clinical trials aimed at the prevention of CVD events have not yielded generally favorable results (ACCORD Study Group et al., 2011; Action to Control Cardiovascular Risk in Diabetes Study Group et al., 2008; ADVANCE Collaborative Group et al., 2008; Duckworth et al., 2009; Ismail-Beigi et al., 2010). These negative results raised the possibility that intensive glucose control alone is not sufficient to cause improvement in major CVD events in patients with diabetes. However, some recently developed antihyperglycemic agents have displayed some clinical benefit. Overall, based on the existing literature, there is an urgent need for the development of newer, highly efficacious and preferably dual action drugs without significant safety concerns to effectively treat both CVD and hyperglycemia in patients with diabetes.

Previously, an HDL mimetic peptide-ABCA1 transporter agonist peptide, ATI-5261 was designed and synthesized by our laboratories based on the amino acid sequence of the C-terminal domain of apolipoprotein E (apoE), and we reported its potent anti-atherosclerotic properties in two genetic mouse models of atherosclerosis (Bielicki et al., 2010). Subsequent characterization of this lead mimetic peptide, however, demonstrated that it possesses acute muscle toxicity at high doses and promotes hypertriglyceridemia at supra-therapeutic doses, which halted its further clinical development. The specific amino acid motifs of ATI-5261 responsible for its toxic effects were identified and substituted with other relevant amino acids using an iterative screening process. These screening efforts lead to the discovery of a second generation ABCA1 agonist family with identification of two lead peptides, CS6253 and T6991–2, both of which were found to be non-toxic and did not cause an increase in triglyceride levels (Bielicki et al., manuscript in preparation). It was further observed that CS6253 retained the full anti-atherosclerotic properties of the parent ATI-5261 mimetic peptide as demonstrated by the attenuation of atherosclerosis in high-fat/Western diet fed apoE null mice (manuscript in preparation). While carrying out these studies, we were surprised to observe that CS6253 also possessed anti-hyperglycemic properties. Given the known association between plasma triglycerides, insulin resistance and glucose homeostasis (Leon and Maddox, 2015), the absence of stimulation of triglyceride secretion in vivo by the CS6253 peptide compared to the legacy peptide ATI-5261 prompted us to investigate more thoroughly its anti-hyperglycemic properties, as well as those of the T6991–2 peptide. In this manuscript we summarize our novel findings.

Initially, we characterized the glucose lowering actions of CS6253 and T6991–2 in several mouse models of atherosclerosis (apoE KO mice), prediabetes/diabetes (Diet-Induced Obesity [DIO] mice) and obesity/diabetes (ob/ob mice). We provide evidence that improved insulin secretion was a major contributor to the anti-hyperglycemic actions of both peptides. Furthermore, treatment of DIO mice with the peptide improved glucose tolerance in a time dependent manner suggesting anti-diabetic actions in addition to the stimulation of insulin secretion. Additionally, in vitro studies demonstrated that CS6253 can function as an insulin secretagogue in an insulin secreting pancreatic β -cell line, potentiate insulin-stimulated glucose uptake in a designer skeletal muscle cell line, and inhibit gluconeogenesis in two model hepatic cell lines, further delineating the anti-diabetic properties observed in various relevant mouse models. Together, our results suggest that these two peptides have the potential for their clinical development as a new drug to simultaneously treat atherosclerosis and diabetes.

2. Materials and Methods

2.1. Materials

Both CS6253 and T6991–2 are 26 mers and were designed from the legacy peptide ATI-5261 (Bielicki et al., 2010) and synthesized by solid phase F-moc synthesis (Biosynthesis Inc., Lewisville, Texas). They were > 95% pure as judged by HPLC and LC-MS/MS analyses. Both peptides are alpha-helical type-A amphipathic peptides. Circular dichroism shows that both peptides in native state (without lipid) have > 60% alpha-helical structure. Peptides 2,4,5,6 and 9 are also 26 mers and were constructed with less

conservative amino acid substitutions, therefore resulting in different primary structure. All the peptides have high solubilities in PBS and are potent and selective ABCA1 agonists. All general chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Silence[®]Select siRNA 1 (s128016), siRNA 2 (s217853), and siRNA 3 (s217852) against rat leptin receptor (Lepr) (catalog #4390771) and Silence[®]Negative Control No. 1 siRNA (catalog #4390843) were purchased from ThermoFisher Scientific/Life Technologies Corporation, Grand Island, NY. Deoxy-D-glucose, 2-[1,2-³H], 30–60 Ci/mmol (1.11–2.22 TBq/mmol) was supplied by American Radiolabeled Chemicals, Inc., (St. Louis, MO). MADKMAG-71K multiplex assay kit was purchased from Millipore Sigma (Bedford, MA, USA). Stanbio Triglyceride LiquiColor[®] Test (Enzymatic) was purchased from Stanbio Laboratory Inc. (Boren, TX).

2.2. Animals and peptide treatment

All studies were performed at the VA Palo Alto Health Care System, Palo Alto, CA. All animal experiments were performed per the procedures approved by the VA Palo Alto Health Care System Institutional Animal Care and Use Committee.

Seven to eight-week-old apoE KO mice (on C57BL/6J background strain B6.129P2-Apoetm1Unc/J stock number 002052) were purchased from Jackson Laboratory, Bar Harbor, ME. After arrival, these mice were acclimated for one week on pelleted laboratory rodent chow (Harlan, 2018) and later switched to a high-fat/Western diet (TD.88137, Harlan Teklad, Indianapolis, IN) ad libitum with continuously available water and maintained on this diet for up to 24 weeks. Seven to eight weeks old C57BL/6J (Stock No: 000664 | Black 6) were also obtained from Jackson Laboratory and either used directly in experimentation or used in the DIO model. To produce DIO mice, WT mice were fed a high-fat diet (#TD 06414, Envigo RMS, Indianapolis, IN) starting at 7–8 weeks of age and maintained on this diet for up to 24–26 weeks of age. *ob/ob* (B6.Cg-*Lep^{ob}*/J; Stock No: 000632 | B6 ob) were supplied by Jackson Laboratory at 7–8 weeks of age and used starting at 10 weeks of age. All animals were treated with vehicle or specific peptides as specified under each figure.

2.3. Intraperitoneal glucose tolerance tests (ipGTT)

Prior to glucose challenge, the mice were fasted overnight (~14–16 h) by transferring them to clean cages with no food in hopper or bottom of the cage. All mice had free access to drinking water. A volume of 20% glucose (0.2g/ml) in Milli Q water was injected ip to achieve a glucose dose of 1 or 2 g/kg. Blood glucose levels were measured using a Bayer Contour Glucometer and test strips immediately prior to (0 min) and 15, 30, 60 and 120 min post glucose injection. The area of the blood glucose response profile curve corresponding to each animal was calculated by the trapezoid method (Andrikopoulos et al., 2008), using as reference each individual baseline blood glucose measurement. The sum of the trapezoidal areas between the 0, 15, 30, 60 and 120 min time points corresponding to each mouse were added to obtain area under the curve (AUC).

2.4. Intraperitoneal insulin tolerance test (ipITT)

For ipITT, animals were either non-fasted or fasted for 6 h. Insulin (Humulin[®]-R-100, Eli Lilly) was injected ip with a dose of 0.75 U/kg and blood glucose levels were measured

immediately prior to (0 min) and 15, 30, 45 and 60 min post insulin injection. The dependent changes in glucose levels for each mouse were used to calculate AUC for insulin, as described above under GTT.

2.5. Measurement of plasma triglyceride, cholesterol, glucose, free-fatty acids, adipokines and liver triglyceride content

Plasma glucose, triglyceride, total cholesterol levels were determined with commercial assay kits (Stanbio Laboratory, Boerne, TX). Plasma free cholesterol and free-fatty acids levels were determined with kits from Wako Diagnostic (Richmond, VA). Serum HbA1c was measured using a kit from Crystal Chem Inc. (Elk Grove Village, IL, USA). Serum obtained from mice was analyzed for adipokines using a multiplex mouse adipokines assay (MADKMAG-71K from Millipore, Bedford, MA, USA), and detected by Luminex xMAP (Luminex 200, Millipore, Bedford, MA, USA). Adipokines including IL-6, insulin, leptin, MCP-1, PAI-1, resistin, and TNF- α were measured in high fat diet fed apoE KO mice that were treated with CS6253 or T6991-2. For the measurement of liver triglyceride content, aliquots of liver homogenates were extracted with a mixture of chloroform and methanol and quantified for their triglyceride content using an enzymatic colorimetric assay kit supplied by Stanbio laboratory.

2.6. Cell lines and cell culture

Human Huh-7 hepatocarcinoma cells were kindly provided by Dr. Ting-Ting Huang, GRECC, VA Palo Alto Health Care System, Palo Alto, CA and rat H4IIE hepatoma cells (ATCC; H-4-II-E [ATCC[®] CRL-1548[™]]) were purchased from American Type Culture Collection (ATCC, Manassas, VA). INS-1-derived rat insulinoma cell subline 832/13 was generously provided by Dr. Chris Newgard, Duke University. Immortalized rat skeletal muscle cells (L6) overexpressing GLUT4 with a myc-epitope (L6-GLUT4myc Rat Myoblast Cell line) generated by Dr. Amira Klip's group (Kanai et al., 1993) was purchased from Kerablast, Inc., Boston, MA. Huh-7 or H4IIE cells were cultured in 24-well plates (0.5×10^6 cells per well) in 1.0 ml of Dulbecco's modified Eagle's low glucose medium supplemented with 5% fetal bovine serum (DMEM-FBS), glutamine, pyruvate, penicillin (100U/ml) and streptomycin (100 μ g/ml) (Invitrogen[™]/Life Technologies Corporation, Long Island, NY; catalog number: 10567-022). INS-1832/13 cells were cultured in 24-well plates (0.5×10^6 cells per well) in 1.0 ml of RPMI 1640 with 10% fetal bovine serum, 11.1 mM glucose as described by (Hohmeier et al., 2000). L6-GLUT4myc rat myoblast cells were cultured in 24-well plates (0.5×10^6 cells per well) in 1.0 ml of Eagle's Minimum Essential Medium (MEM) (Alpha Modification) with deoxyribonucleosides, ribonucleosides, L-glutamine, sodium bicarbonate, 10% fetal bovine serum and 5 μ g/ml blasticidin. For glucose transport assays, myoblasts were differentiated into myotubes by switching confluent myoblast monolayers to MEM- α + 2% fetal bovine serum for 4–5 days.

2.7. Lepr-siRNA and/or CS6253 peptide treatment of INS-1832/13 cells and insulin secretion

The cells plated at a density of 0.5×10^6 cells in 1.0 ml of RPMI 1640 with 10% fetal bovine serum, containing 11.1 mM glucose and dishes were cultured overnight. Next day, the medium was removed from the dishes and, subsequently, aliquots of 25 μ l DMEM

containing 50 nM of a combination of lepr-siRNA1, -siRNA2, and -siRNA3 or scrambled control siRNA were mixed with 25 μ l DMEM +2.5 μ l Polyjet transfection reagent and added to the respective dishes. Following 6h of incubation, the transfection medium was replaced with 1.0 ml of standard tissue culture medium and dishes incubated for an additional 48h. At the end of incubation, the standard tissue culture medium containing 11.1 mM glucose was switched to a fresh medium containing 5 mM glucose and dishes incubated for 18 h. Insulin secretion was assayed in Hanks Balanced Salt Solution (HBSS) modified with 10 mM HEPES and 0.2% fatty acid-free bovine serum albumin. Cells were washed with 1.0 ml of HBSS-3 mM glucose, followed by a 2 h pre-incubation in the same buffer. Insulin secretion was next measured by incubating cells in 0.8 ml of HBSS containing either 3 mM glucose or 15 mM glucose $\pm$ 1 mg/ml CS6253 peptide for 2 h. Insulin concentration in the medium was assayed by a sensitive Human Insulin ELISA kit with 100% cross reactivity to rat insulin (ALPCO).

2.8. Glucose production assay in rat and human hepatic cell lines

Huh-7 or H4IIE cells were cultured in 24-well plates (0.5×10^6 cells per well) in 0.5–1.0ml of DMEM-FBS to near confluence. The dishes were washed and then incubated with 1 ml of DMEM-FBS culture medium DMEM (pH 7.4) $\pm$ 500 nM dexamethasone and 0.1 mM Bt₂cAMP $\pm$ 1 mg/ml CS6253 peptide or $\pm$ 5 nM insulin for 5 h at 37 °C in the presence of 5% CO₂ and 95% air. Following 5 h incubation, the dishes were washed with 1 ml/well of warm (37 °C) PBS twice. PBS was replaced with 1 ml of glucose production buffer consisting of glucose-free DMEM (pH 7.4) without phenol red supplemented with 20 mM sodium lactate, 2 mM sodium pyruvate, 2 mM L-glutamine, 15 mM HEPES $\pm$ 500 nM dexamethasone and 0.1 mM Bt₂cAMP $\pm$ 1 mg/ml CS6253 peptide or $\pm$ 5 nM insulin and dishes incubated for 3h at 37 °C in the presence 5% CO₂ and 95% air. A 0.2 ml aliquot of medium from each well was collected and analyzed for glucose concentration with a colorimetric glucose assay kit (Millipore/Sigma) following manufacturer's instruction.

2.9. Glucose uptake assay in differentiated L6-GLUT4myc myotubes

L6-GLUT4myc rat myoblast cells were cultured in 24-well plates (0.5×10^6 cells per well) in 1.0 ml of MEM-alpha supplemented with deoxyribonucleosides, ribonucleosides, L-glutamine, sodium bicarbonate, 10% fetal bovine serum and 5 μ g/ml blasticidin, and myoblasts were differentiated into myotubes by incubating confluent myoblast monolayers in MEM-alpha + 2% fetal bovine serum medium for 5 days. Radiolabeled 2-deoxyglucose uptake was measured in glucose-free KRH buffer (120 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 1.3 mM CaCl₂, 1.3 mM KH₂PO₄ and 25 mM HEPES, 7.4). The dishes were washed in KRH buffer and pre-incubated in the 300 μ l of the same buffer $\pm$ 1 mg/ ml CS6253 peptide for 60min or $\pm$ 100 nM insulin for 15min. The glucose transport assay was initiated by addition of 6 μ l of [1,2-³H] deoxy-D-glucose stock solution (10 mM, 83 μ Ci/ml) and dishes were incubated for 5 min. Following incubation, the dishes were rapidly washed four times with 1 ml of ice-cold KRH buffer and, after final wash, the attached cells were dissolved in in 300 μ l of 0.05 N NaOH and counted in a liquid scintillation for the determination of ³H radioactivity.

2.10. Statistical analysis

Statistical analysis was performed using the Prism software (Prism 6.0, GraphPad, San Diego, CA). All of the data are expressed as the means $\pm$ SE. ANOVA analyses were performed with Bonferroni's multiple comparison test; a p value of < 0.05 was considered statistically significant. In some cases, data were analyzed by Student's t -test, and $p < 0.05$ was considered significant.

3. Results

3.1. CS6253 improves glucose tolerance in a mouse model of atherosclerosis

The effect of CS6253 on glucose tolerance was determined in apoE KO mice fed a high-fat diet/Western diet (atherosclerosis assessment mice model) for 14 weeks. Mice were then treated with vehicle or CS6253 (40 mg/kg ip alternate days) for 3 weeks, while continuing the diet. Mice were fasted overnight, administered CS6253 or vehicle and one hour later subjected to glucose tolerance test (GTT). As can be seen in Fig. 1, the fasting glucose levels were significantly lower in CS6253 vs. control treated animals after 3-weeks treatment ($p < 0.001$) as well as after 6-weeks treatment ($p < 0.05$). The blood glucose levels at 15, 30 and 60 min after glucose challenge were all significantly lower in the CS6253 vs. the control treatment group ($p < 0.0001$ for 15 and 30 min; $p < 0.05$ for 60 min). The cumulative responses to the glucose challenge, represented by area under the curve (AUC), for both the 3-weeks treated and 6-weeks treated animals were significantly lower in the active vs. controls (both with $p < 0.01$). Therefore, CS6253 greatly improved the GTT profiles after 3 and 6-weeks of treatment. To test for potential changes in cytokines, serum samples were assayed for the level of IL-6, insulin, leptin, MCP-1, PAI-1, resistin and TNF- α using the mouse adipokine magnetic panel from Millipore. As shown in Supplemental Figure 1, treatment of apoE KO mice with CS6253 and T6991-2 did not change the level of IL-6, leptin, MCP-1 and TNF- α . There seemed to be a trend of lower levels of serum insulin, PAI-1 total and resistin in the apoE KO mice treated with CS6253, however it did not reach statistical significance.

3.2. A single bolus dose of CS6253, T6991-2 vs. peptide #2, #4, #5, #6, or #9 in WT mice improves glucose tolerance

To assess the specificity of glucose lowering effect of CS6253, we compared the relative efficacies of CS6253 with that of six other structurally related peptides derived from the C-terminal domain of apoE, i.e., peptide T6991-2, peptides 2, 4, 5, 6, and 9. A single dose of 300 mg/kg of these various peptides was administered (ip) to overnight fasted WT mice and 1h or 3h later GTT measurements were carried out. AUC measurements indicated that CS6253 and T6991-2 peptides were most effective in preventing the increases in plasma glucose in response to intraperitoneal glucose challenge (Fig. 2). Peptide 2 also caused a slight but significant lowering of plasma glucose levels. In contrast, peptides 4, 5, 6 and 9 had no discernible effect on plasma glucose levels. Furthermore, although peptides CS6253 and T6991-2 substantially reduced the plasma glucose levels in response to glucose challenge, neither of the two peptides caused hypoglycemia.

3.3. CS6253 improves glucose tolerance and HbA1c, while promoting insulin secretion in a DIO model

To further examine the anti-hyperglycemic actions of CS6253, C57BL/6J (WT) mice fed a 60% high-fat diet (DIO model) were treated with the peptide (30 mg/kg BW, *sc*) on alternate days for two weeks, and then on the day of experimentation, the animals were treated with this peptide for 1 or 3 h followed by GTT. As shown in Fig. 3 A, glucose tolerance was significantly improved both after 1 and 3 h of peptide treatment. Glucose tolerance was improved significantly at 15 ($p < 0.0001$), 30 ($p < 0.0001$), 60 ($p < 0.0001$), and 120 ($p = 0.012$) minutes with 1 h treatment, and 30 ($p < 0.0001$) and 60 ($p < 0.0001$) minutes with 3h treatment of the peptide. The 1 h and 3h treatments were significantly different at 15 ($p = 0.0014$), 30 ($p = 0.0010$), and 60 ($p = 0.0037$) minutes. However, the improvement was much more evident at 1 h compared to 3 h following peptide administration showing its high efficacy in a short period (1 h) as shown in AUC in Fig. 3 B. To assess a more chronic effect on blood glucose levels, we assessed the effect on HbA1c levels in the same mice following 4 weeks of treatment with CS6253 (40 mg/kg ip alternate days). We observed significantly lower HbA1c levels ($p < 0.01$) in CS6253-treated group ($4.93 \pm 0.14\%$) as compared to vehicle treated mice ($5.47 \pm 0.12\%$).

To examine the potential effect of CS6253 peptide on insulin secretion in vivo, we performed GTT measurements in the same mice after in total 5-weeks of treatment. Blood samples were collected by retro-orbital bleeding and analyzed for plasma glucose, insulin, and C-peptide levels (a gauge of insulin production in vivo). As before, during GTT, there was a significant decrease in plasma glucose levels in CS6253 treated vs. vehicle treated control mice. Interestingly, plasma insulin levels along with C-peptide levels during GTT were significantly elevated, indicative of increased insulin secretion by the pancreas (Fig. 3 C–E). The increase of insulin and C-peptide by CS6253 was particularly pronounced during the early phase of the GTT. Chronic treatment of DIO mice with CS6253 (30 mg/kg, ip alternate day treatment for 13 weeks), however, was not associated with any significant change in body weight as compared to vehicle (control) treatment Fig. 3F. At termination, a 17.5% lower liver weight ($p = 0.01$) was observed in the CS6253 treated mice vs. vehicle (Fig. 3F). There was no difference in kidney weight, i.e. CS6253 ($0.85 \pm 0.11\%$ of BW) vs. vehicle ($0.87 \pm 0.10\%$ of BW). To evaluate the potential effects of CS6253 and T6991–2 treatment on lipid accumulation in the liver, liver TG levels were assayed using total hepatic lipid extracts. As shown in Table 1, compared to the PBS treated animals, treatment with CS6253 resulted in decreased liver TG content ($p < 0.05$). Animals treated with T6991–2 showed a trend towards decreasing liver TG content, but did not achieve statistical significance ($p = 0.054$).

3.4. Short-term and long-term treatment of DIO mice with CS6253, T6991–2 or Exendin-4 improves glucose tolerance

To better understand glycemic control of ABCA1 agonist peptides, we employed a short-term (2 weeks) and long-term (15 weeks) treatment strategy to examine and compare the glucose lowering actions of CS6253 and T6991–2 with that of incretin/glucagon-1 peptide 1 (GLP-1)/glucose-dependent insulinotropic polypeptide (GIP) mimetic, ex-endin-4 using its synthetic version, exenatide, in DIO mouse model. Synthetic exenatide is a 39-amino acid

with insulin secretagogue and anti-hyperglycemic activity and used clinically to improve glycemic control in patients with T2D (Levin et al., 2017). It also promotes weight loss and improves cardiovascular risk factors. In addition, ex-endin-4 has been shown to promote β -cell proliferation (Wang et al., 2015; Young et al., 1999; Li et al., 2004) and inhibits β -cell apoptosis. The GTT data presented in Fig. 4 indicate that treatment of high-fat diet fed mice with CS6253, structurally related T6991–2, or exendin-4 significantly decreased the blood glucose levels during GTT both at 2 and 15 weeks, although the effect was more robust after 15 weeks of treatment. Furthermore, a single dose (100 mg/kg BW) bolus injection of T6991–2 produced similar reductions in blood glucose levels during GTT. Finally, both peptides lowered blood glucose levels (AUC) to the same extent as that was elicited by exendin-4 (Fig. 4).

3.5. Effect of treatment of DIO mice with two different doses of T6991–2 on glucose and insulin tolerance

We next characterized the glucose lowering actions of T6991–2, a peptide structurally related to CS6253. Again, we used a DIO model, where mice were fed a high-fat diet for 6 weeks and then treated them with 10 mg/kg or 30 mg/kg of T6991–2 on alternate days for 5 weeks, while continuing the diet. Some animals in each group were fasted overnight and subjected to GTT. A week later the same group of animals was fasted for 6h and subsequently used in ITT, followed by ITT under fed conditions one week later. The GTT data presented in Fig. 5 A and B demonstrate that the two doses of T6991–2 were equally effective in attenuating the blood glucose response to glucose overload during GTT. Likewise, insulin administration (ITT) to T6991–2 treated mice led to clearance (reduction) of glucose at an accelerated rate (i.e., improvement in hyperglycemia) and this effect was seen at both 10 and 30 mg/kg dosage of T6991–2 (Fig. 5 C–F).

3.6. Studies with CS6253 and T6991–2 peptides involving ob/ob mice

We next examined the plasma glucose lowering actions of CS6253 and T6991–2 in a genetic mouse model of obesity, *ob/ob* mice. *Ob/ob* mice on a C56BL/6J background are homozygous for the obese spontaneous mutation (*Lep^{ob}*), lack functional leptin, exhibit obesity, diabetes-like syndrome of hyperglycemia, glucose intolerance and hyperinsulinemia. Results presented in Fig. 6 demonstrate that treatment of *ob/ob* mice with T6991–2 (10 mg/kg BW, sc, alternate days) for 6 weeks, partially prevented the increases in blood glucose levels during the GTT further confirming the anti-hyperglycemic effect of T6991–2.

3.7. CS6253 stimulates insulin secretion in INS-1 cells in a leptin dependent manner

Given that pancreatic β -cells express leptin receptor (OBR) and a majority of studies suggest that leptin reduces insulin secretion in isolated islets, β -cells and in vivo (Pallet et al., 1997; Poitout et al., 1998; Seufert et al., 1999a; Seufert et al., 1999b; Natalicchio et al., 2013), we examined the separate and combined actions of leptin receptor and peptide on insulin secretion in INS-1 pancreatic β -cells. For these studies, INS-1 cells were treated with specific siRNAs to down-regulate endogenous levels of leptin receptor. Results presented in Fig. 7 demonstrate that treatment of INS-1832/13 cells with three leptin receptor specific siRNAs (siRNA1, siRNA2 and siRNA3) significantly decreased the basal (3 mM glucose)

secretion rates of insulin. In contrast, co-treatment of cells with CS6253 peptide robustly increased insulin secretion both under basal condition and in response to treatment with a stimulatory dose of glucose (15 mM) (Fig. 7). These studies demonstrate that CS6253 peptide can restore the leptin receptor deficiency-mediated inhibition of insulin secretion in INS-1832/13 β -cells.

3.8. Effects of CS6253 peptide on basal and insulin-stimulated glucose transport in a genetic model cell line

To determine whether CS6253 peptide regulates cellular glucose transport, we exposed differentiated (myotubes) rat myoblast cells (L6) over-expressing GLUT4 with a myc-epitope (L6-GLUT4myc myoblast). The results presented in Fig. 8 demonstrate that CS6253 peptide potentiated the stimulatory action of insulin on [1,2-³H]deoxy-glucose transport in L6-GLUT4myc myotubes. However, it showed no significant effect on basal rates of glucose transport in these cells.

3.9. Effect of CS6253 peptide on in vitro gluconeogenesis in hepatic cell lines

We next assessed whether CS6253 was effective in inhibiting in vitro glucose production in human (Huh-7) and rat (H4IIE) hepatic cell lines. The results, see Fig. 9, show CS6253 significantly decreased both basal and dexamethasone + Bt₂cAMP stimulated and pyruvate and lactate supported glucose production in both cell lines. Furthermore, fold-inhibition of gluconeogenesis was greater in cells stimulated with dexamethasone + Bt₂cAMP as compared to cells incubated with basal medium alone. Finally, the extent of CS6253 inhibition of dexamethasone + Bt₂cAMP stimulated glucose production was comparable to that seen with insulin.

4. Discussion

This study reveals that ABCA1 peptide agonists, CS6253 and T6991-2, with potent anti-atherosclerotic properties, also improve glucose tolerance in DIO and *ob/ob* mouse models, and stimulate insulin secretion in a pancreatic β -cell line, INS-1, both under basal condition and in response to high-glucose concentration. In addition, we provide evidence that CS6253 attenuates gluconeogenesis in vitro in Huh-7 or H4IIE hepatic cell lines and insulin-stimulated glucose uptake and utilization in an immortalized rat skeletal muscle cells (L6) overexpressing GLUT4. These are the first apoE derived peptides to show such effects. There are only a few previous reports showing that recombinant HDL, apoA-I or apoA-I mimetic peptides synthesized from either D (D-4F) or L (L-4F) amino acids can attenuate insulin resistance in cell/tissue or animal models (Dalla-Riva et al., 2013; Domingo-Espin et al., 2016; Peterson et al., 2008; Peterson et al., 2009; Neugebauer et al., 2013). The actions of recombinant HDL, apoA-I, and apoA-I-derived peptides are mainly attributed to the ability of these agents to induce glucose-stimulated insulin secretion (GSIS) by the pancreatic β -cells, promote insulin-independent peripheral tissue glucose uptake and improve insulin signaling. For example, it was shown that treatment of L6 myo-tubes with recombinant nascent discoidal HDL, results in enhanced glucose uptake, an effect comparable to that seen with insulin (Dalla-Riva et al., 2013; Domingo-Espin et al., 2016). Likewise, ex vivo exposure of flexor digitorum brevis fibers from HA-GLUT4-GFP

transgenic mice is associated with increased cell surface localization of GLUT4 (Dalla-Riva et al., 2013; Domingo-Espin et al., 2016). It was further demonstrated that in vivo treatment of insulin-resistant, DIO mice with apoA-I, a predominant apolipoprotein constituent of HDL, leads to increased GSIS along with enhanced glucose uptake in peripheral tissues including heart and skeletal muscle. In addition, treatment of obese mice with apoA-I mimetic peptide synthesized from L (L-4F) amino acids has been shown to prevent insulin resistance via increased expression of heme oxygenase-1 (HO-1), and phosphorylated forms of AMP kinase (pAMPK) and Akt (pAkt) (Peterson et al., 2009). However, except for 4F apoA-I mimetic peptides (Peterson et al., 2008; Peterson et al., 2009), there are no reports showing that other recombinant HDL or apoA-I mimetic peptides also directly ameliorate atherosclerosis.

Given that our initial in vivo studies indicated that acute treatment of high-fat fed apoE null mice with CS6253 improved glucose tolerance, we initially carried out a series of kinetic GTT and ITT studies using acute or chronic treatment of control mice, DIO mice (Wang and Liao, 2012) or *ob/ob* mice with either CS6253 or T6991–2 peptide. Interestingly, treatment of control mice with a single pharmacological dose of CS6253 or T6991–2 peptide greatly impacted the GTT profiles. AUC calculations indicated that both of these peptides effectively prevented the increases in plasma glucose levels in response to intraperitoneal glucose challenge, but they did not cause hypoglycemia. Other CS6253 or T6991–2 related peptides derived from the C-terminal domain of the apoE such as peptide #2, #4, #5, #6 or #9 had either modest or no effect on glucose levels during GTT measurements. Like control mice, acute treatment of DIO mice for a short duration with CS6253 or T6991–2 peptide significantly improved glucose tolerance. Furthermore, we observed that CS6253 treatment of DIO mice significantly decreased HbA1c levels; the levels of HbA1c, also known as glycosylated or glycated hemoglobin, are reflective of blood glucose levels over several weeks and the extent of change in HbA1c levels are indicative of the effectiveness of anti-diabetic medications (MAYO CLINIC). GTT measurements indicated that CS6253 and T6991–2 peptides treatment not only improved plasma glucose levels but also significantly increased the circulating levels of insulin and C-peptide levels indicative of increased insulin secretion by the pancreatic β -cells. This seminal finding was also confirmed in an insulin secreting pancreatic β -cell line, INS-1832/13 cells. Treatment of this cell line with CS6253 enhanced insulin secretion both under basal condition and in response to high glucose stimulation.

To further examine the glycemic effects of CS6253 and T6991–2 peptides, we compared the efficacy of these two peptides with that of exendin-4 in their ability to improve glucose tolerance in DIO mice. Exendin-4 (exenatide) is a 39-amino acid peptide incretin mimetic that exhibits glucoregulatory activities like mammalian incretin hormone glucagon-like peptide 1 (GLP-1) (Cho et al., 2014; Levin et al., 2017; Htike et al., 2012; Nelson et al., 2004; Young et al., 1999; Stenkula et al., 2014). The exendin-4 actions include glucose dependent enhancement of insulin secretion, glucose-dependent suppression of inappropriately high glucagon secretion, slowing of gastric emptying and reduction of food intake (Levin et al., 2017; Nauck and Meier, 2005). Our studies demonstrated that both CS6253 and T6991–2 are equally effective, and their effects more or less were as effective as exendin-4 in improving glucose tolerance.

Our studies using ob/ob mice and leptin receptor (OBR) siRNA treated INS-1 pancreatic β -cells suggest that leptin mediates peptide-induced insulin secretion. However, our results are at variance with the majority of previously published studies with the exception of one report (Tanizawa et al., 1997) showing that leptin is a negative regulator of insulin secretion in isolated islets, β -cells, and in vivo (Kieffer et al., 1997; Kulkarni et al., 1997; Lee et al., 2011; Pallet et al., 1997; Poitout et al., 1998; Seufert et al., 1999a; Seufert et al., 1999b). At present, we don't know the exact reason as to why our findings are different from that of existing literature about the role of leptin in insulin secretion by the pancreatic β -cells. Since most of the previous studies were carried out using either isolated islets or pancreatic β -cells, it is possible that some of the systemic factors that may be required for the stimulatory action of leptin on GSIS may be lost during the isolation of pancreatic islets or β -cells. It is also possible that INS-1 pancreatic β -cell line which we employed have all the accessory factor(s) required for leptin dependent GSIS. Obviously, more experimental evidence is needed to sort out these possibilities. In addition, we made a novel observation that our two peptides inhibit hepatic glucose production using two liver cell lines. Increase in hepatic glucose production is the major source of fasting hyperglycemia in patients with T2D (Sharabi et al., 2015). Thus, CS6253 and T6991-2 peptides have the potential to improve T2D-induced hyperglycemia by inhibiting hepatic glucose production.

The results of our study have important clinical implications because a close link exists between T2D and CVD, which is the most prevalent cause of morbidity and mortality in diabetic patients (Circulation, 1999; Fox et al., 2004; Fox et al., 2007; Gaede et al., 2003; Lundberg et al., 1997). Namely, it underscores the importance of the availability of new, highly efficacious dual function drugs to simultaneously treat diabetes and CVD as hitherto no glucose lowering therapies have been effective in preventing CVD events in post-acute coronary syndrome patients. So far, no such desired drugs have been developed or marketed. At present, lipid lowering drugs and anti-diabetic drugs are commonly employed to treat both diabetes and associated cardiovascular complications. However, use of lipid lowering drugs such as statins and/or ezetimibe is often associated with intolerance and/or insufficient effectiveness in patients with T2D and high CVD risk (Gaede et al., 2003) leaving high residual risk. Intensive glycemic control in diabetes patients with currently available anti-diabetic drugs in large clinical trials aimed at the prevention of CVD events have not yielded generally favorable results (ACCORD Study Group et al., 2011; Action to Control Cardiovascular Risk in Diabetes Study Group et al., 2008; ADVANCE Collaborative Group et al., 2008; Duckworth et al., 2009; Kumar et al., 2016; Gu et al., 1999; Ismail-Beigi et al., 2010; Leon and Maddox, 2015). These negative results raised the possibility that intensive glucose control alone is not sufficient to cause improvement in major CVD events in patients with diabetes. Besides, some commonly used anti-diabetic drugs also increase cardiovascular risk (ACCORD Study Group et al., 2011; Ismail-Beigi et al., 2010; Kumar et al., 2016). Only some recently developed antihyperglycemic agents, however, have displayed some clinical benefit (Bethel et al., 2018; Htike et al., 2012; Motta et al., 2012; Zinman et al., 2015). Our two peptides, CS6253 and T6991-2 have the potential to be developed into highly effective dual action drugs to simultaneously treat diabetes and CVD. Our pre-clinical studies clearly indicate that these two peptides exhibit no toxicity,

ameliorate experimental atherosclerosis, and improve diabetes, by improving insulin resistance, insulin sensitivity, and glucose homeostasis.

5. Conclusions

In conclusion, our study shows that treatment of both metabolic DIO and genetic *ob/ob* mouse models of obesity, insulin resistance, hyperglycemia, diabetes and dyslipidemia with potent anti-atherosclerotic ABCA1 agonists peptides, CS6253 and T6991–2 result in improved glucose tolerance. Based on the experimental data obtained, it seems that an intact leptin receptor signaling system is required for maximal glycemic actions of these two and possibly other structurally related peptides as well. Furthermore, our in vitro cell culture studies provide evidence that these peptides, and particularly CS6253, possess insulin secretory properties, can potentiate insulin-stimulated glucose transport in insulin sensitive muscle cells and can inhibit glucose production in hepatic cell lines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. CS6253 improves glucose tolerance in a mouse model of atherosclerosis.

Groups of apoE KO mice were fed a high fat diet/western diet for 14 weeks and subsequently treated $\pm$ CS6253 peptide (40 mg/kg, sc, on alternate days) while continuing the diet for 6 weeks. GTT was performed at 3 (A), and 6 (B) weeks of CS6253 treatment after an over-night fast, with blood collected by glucometer at 0 (prior to), and 15, 30, 60, and 120 min after glucose injection (1 g/kg BW). (C) Cumulative response to glucose challenge was calculated as area under the curve (AUC). (D) Body weight of the animals. Results are Mean $\pm$ SE of 6–8 individual measurements. Data were analyzed with multiple t-tests, NS = not significant, * p < 0.05, *** p < 0.001, **** p < 0.0001.

Fig. 2. A single bolus dose of CS6253, T6991-2 vs. peptide #2, #4, #5, #6 or #9 peptide in WT mice improves glucose tolerance.

Groups of WT mice were treated with a bolus of CS6253, T6991-2, peptide #2, #4, #5, #6, #9 (300 mg/kg, ip) 2h-prior to initiation of (A) GTT performed as described in Fig. 1 using a 2 g/kg BW glucose injection. (B) AUC for glucose was calculated using the trapezoidal rule. Results are Mean ± SE of 4 individual measurements. Data were analyzed using two-way (A) and ordinary one-way (B) ANOVA, * $p < 0.05$, **** $p < 0.0001$.

Fig. 3. CS6253 improves glucose tolerance in a model of diet induced obesity.

Groups of WT mice were fed high-fat diet for six weeks and treated $\pm$ CS6253 (30 mg/kg, sc) for two weeks (A–B) or five weeks (C–E) while continuing the diet. (A) GTT with 1 g/kg glucose/BW was performed 1 or 3 h after dosing with peptide. (B) AUC for glucose was calculated using the trapezoidal rule. Results are Mean $\pm$ SE of 7–10 individual measurements. Data was analyzed using 2way (A) and ordinary one-way (B) ANOVA (F) Body and liver weight of the animals on the high-fat diet. For the group of animals after 5 weeks treatment, GTT was performed with CS6253 treatment 1 h after dosing. Blood was collected by retro-orbital bleeding. Glucose (C), insulin (D), and C-peptide (E) levels quantified at each time point. Results are Mean $\pm$ SE of 3–4 individual measurements. Data were analyzed using multiple t-tests, (A)* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ PBS1 h v CS6253 1h, †††† $p < 0.0001$ PBS 3 h v CS6253 3h, ^^^ $p < 0.01$, ^^^ $p < 0.001$ CS6253 1 h v CS6253 3h. (B–F) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Fig. 4. CS6253 and T6991-2 improves glucose tolerance comparably to exendin-4.

Using a DIO model, WT mice began treatment with $\pm$ CS6253 (30 mg/kg, on alternate days, sc), T6991-2 (30 mg/kg, on alternate days, sc) or exendin-4 (10 μ g/kg, every day, ip) for 2 weeks (A and B) or 15 weeks (C and D) after 6 weeks on high-fat diet. One group of mice was given a bolus injection of T6991-2 (100 mg/kg, sc) to vehicle treated mice prior to GTT utilizing 1 g/kg glucose/BW. The group labels for B and D are the same. Results are Mean $\pm$ SE of 5–6 individual measurements. Data were analyzed using two-way (A and C), and ordinary one-way (B and D) ANOVA, **** p < 0.0001.

Fig. 5. T6991-2 improves glucose tolerance in a DIO model.

Using a DIO model, WT mice were treated on alternate days for 5 weeks with low-(10 mg/kg, sc) or high-doses (30 mg/kg, sc) of T6991-2 after 6 weeks on high-fat diet. (A) GTT was performed utilizing 1 g/kg glucose/BW. (B) The high dose of T6991-2 improved the AUC. Results are Mean $\pm$ SE of 5 individual measurements. Data was analyzed using two-way (A), and ordinary one-way (B) ANOVA. For ITT measurements were performed on animals fasted for 4 h (C and D) or without fasting (E and F). Mice were injected with insulin (0.75 U/kg BW), blood sampled at 0 (prior to), and 15, 30, 45 and 60 min after insulin

injection, and were quantified for blood glucose using a glucometer. Results are Mean $\pm$ SE of 5 individual measurements. Data were analyzed using two-way (C and D), and ordinary one-way (E and F) ANOVA, * $p < 0.05$, ** $p < 0.01$ PBS v 10 mg/kg, ^ $p < 0.05$, ^^ $p < 0.01$, ^^^ $p < 0.001$, ^^^^ $p < 0.0001$ PBS v 30 mg/kg.

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Fig. 6. T6991-2 improves glucose tolerance in *ob/ob* mice.

Groups of *ob/ob* mice at 10 weeks of age were treated with vehicle or CS6253 peptide (10 mg/kg, sc, on alternate days) for 6 weeks prior to GTT. GTT measurements were carried out as described under Fig. 1 utilizing 1 g/kg glucose/BW. Results are Mean $\pm$ SE of 5–6 individual measurements. Data were analyzed using student t-tests, * $p < 0.05$, ** $p < 0.01$.

Fig. 7. CS6253 restores insulin secretion while leptin receptor is suppressed.

Cultured INS-1832/13 pancreatic β -cells were transfected with a mixture of lepr-siRNA1, -siRNA2, and -siRNA3 combined or scrambled control siRNA as described under Materials and Methods. Forty-eight hours following transfection, glucose-stimulated insulin secretion (GSIS) was stimulated by HBSS medium containing 3 mM glucose and 15 mM glucose. Data are indicated as mean $\pm$ SE from 3 individual experiments. Insulin concentration in the medium samples was assayed by a sensitive Human Insulin ELISA kit. Data were analyzed using ordinary one-way ANOVA, *** p < 0.001, **** p < 0.0001.

Fig. 8. CS6253 potentiates the insulin stimulated glucose transport in L6-GLUT4myc rat myoblast cells.

L6-GLUT4myc rat myoblast cells were cultured in 24-well plates (0.5×10^6 cells per well) and subsequently differentiated into myotubes by incubating confluent myoblast monolayers in MEM-alpha + 2% fetal bovine serum medium for 5 days. At the end of incubation groups of dishes were pre-incubated in the 300 μ l of the same buffer $\pm$ 1 mg/ml CS6253 peptide for 60 min or $\pm$ 100 nM insulin for 15 min. The glucose transport assay was initiated by addition of [1,2- 3 H]deoxy-D-glucose and following incubation for 5 for 5 min, the cells dissolved in 0.05 N NaOH and counted in a liquid scintillation for the determination of 3 H radioactivity. Data are indicated as mean $\pm$ SE from 4 to 6 individual measurements. Data were analyzed using ordinary one-way ANOVA, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

Fig. 9. CS6253 suppresses in vitro glucose production in human Huh-7 and rat H4IIE cells. Cultured Huh-7 or H4IIE cells were incubated with 1 ml of glucose production buffer consisting of glucose-free DME (pH 7.4) without phenol red supplemented with 20 mM sodium lactate, 2 mM sodium pyruvate, 2 mM L-glutamine, 15 mM HEPES ± 500 nM dexamethasone and 0.1 mM Bt₂cAMP ± 1 mg/ml CS6253 peptide or ± 5 nM insulin and dishes incubated for 3h at 37 °C in the presence 5% CO₂ and 95% air. A 0.2 ml aliquot of medium from each well was collected and analyzed for glucose concentration with a colorimetric glucose assay kit (MilliporeSigma) following manufacturer's instruction. Values are shown as mean ± SE of 6 individual measurements. Data were analyzed using ordinary one-way ANOVA, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

Table 1

Liver triglyceride content.

	TG mg/g liver
PBS	17.8 ± 4.8
CS6253	5.5 ± 0.9 *
T6991-2	6.2 ± 1.0

Seven to eight-week-old apoE KO mice were fed a high-fat/Western diet for six weeks before treatment with PBS, CS CS6253 or T6991-2 for additional six weeks. Livers were weighed and aliquots of liver were extracted with a mixture of chloroform and methanol and quantified for their triglyceride content. Results are Mean ± SE of 5–7 animals in each group and measurements were performed in quadruplicate. Data were analyzed with multiple t-tests,

*
 $p < 0.05$.