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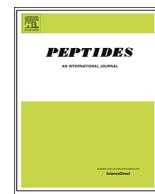
Efficacy of lanthionine-stabilized angiotensin-(1-7) in type I and type II diabetes mouse models

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ABSTRACT

Native angiotensin-(1–7) exerts many therapeutic effects. However, it is rapidly degraded by ACE and other peptidases. This drawback is largely eliminated for lanthionine-stabilized angiotensin-(1–7), termed cAng-(1–7), which is fully resistant to ACE and has strongly increased resistance to other peptidases. Goal of the present study was to test whether cAng-(1–7) has therapeutic activity in diabetes mouse models: in a multiple low dose streptozotocin-induced model of type I diabetes and / or in a *db/db* model of type II diabetes. In the type I diabetes model cAng-(1–7) caused in an increase in the insulin level of 133% in week 4 ($p < 0.001$) compared to vehicle, and in the type II diabetes model an increase of 55% of the insulin level in week 8 ($p < 0.05$) compared to vehicle. cAng-(1–7) reduced blood glucose levels in the type I model by 37% at day 22 ($p < 0.001$) and in the type II diabetes model by 17% at day 63 of treatment ($p < 0.001$) and in an oral glucose tolerance test in a type II diabetes model, by 17% at week 4 ($p < 0.01$). cAng-(1–7) also caused a reduction of glycated hemoglobin levels in the type II diabetes model of 21% in week 6 ($p < 0,001$). These data are consistent with therapeutic potential of cAng-(1–7) in type I and II diabetes.

1. Introduction

The heptapeptide hormone angiotensin-(1–7) plays an important role in the Renin Angiotensin System [15,28]. Ang-(1–7) is mainly formed by the C-terminal cleavage of AngII by the angiotensin converting enzyme ACE2 [13,50,53]. Ang-(1–7) stimulates the Mas receptor [46]. Ang-(1–7) actions counterbalance AngII-mediated stimulation of the AT_1 receptor [16,44]. Mas receptor deficiency resulted amongst others in cardiac dysfunction [45], increased blood pressure, endothelial dysfunction [58] and thrombogenesis [17]. The ACE2/Ang-(1–7)/MasR axis has a protective role in diabetes mellitus [1,12,41,42]. Therefore, MasR stimulation might be an important strategy in the therapy of diabetes mellitus.

The *in vivo* half-life of natural Ang-(1–7) is as short as seconds due to fast degradation in plasma and tissue, predominantly by ACE [7,59]. In contrast, 4,7 D,L lanthionine-stabilized Ang-(1–7), also termed cAng-(1–7) (Fig. 1), was fully resistant against ACE and had 34-fold enhanced survival in rats. cAng-(1–7) also showed a higher resistance to breakdown by peptidases than natural Ang-(1–7) in pig plasma [27], homogenates of pig organs, pancreas, liver [54] and kidney [27]. This resistance is due to two aspects: 1) the thioether bridge and 2) the D-

configuration at position 4 of the D,L lanthionine. The introduction of a lanthionine (dAla-S-Ala) is a non-bulky modification and the thioether bridge is much more stable than a disulfide bridge. cAng-(1–7) stimulates the MasR as its activity was abolished / reduced by the MasR antagonists D-Pro7 / D-Ala7, respectively. Extensive dose-response studies demonstrated that cAng-(1–7) had a significantly greater capacity than native Ang-(1–7) to induce relaxation of precontracted rat aorta rings [27]. Moreover, cAng-(1–7) offered perspectives for oral and pulmonary delivery [54]. cAng-(1–7) demonstrated therapeutic efficacy in non-diabetic animal models of myocardial infarct [14], lung fibrosis [57], neonatal lung injury [55] and pulmonary arterial hypertension [4]. The goal of this study was to evaluate the therapeutic potential of cAng-(1–7) in the MLD-STZ-induced mouse model of type I diabetes, and in the *db/db* mouse model of type II diabetes.

2. Materials and methods

2.1. Peptide

4,7 D,L lanthionine-stabilized Angiotensin-(1–7), also termed cAng-(1–7), was chemically synthesized from disulfide-bridged DRVdCIHC
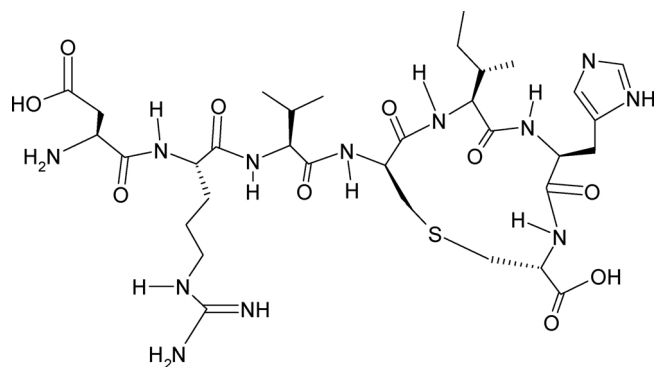
Fig. 1. 4,7 D,L lanthionine-angiotensin-(1–7).

via base-assisted sulphur extrusion [20]. PK data on this peptide have been previously obtained in rat [54]. In preliminary experiments daily injection with 5 mg/kg, 50 mg/kg and 300 mg/kg of cAng-(1–7), the dose of 50 mg/kg, used in this study, gave the best effect. At high or frequent dosing some extent of desensitization, common to GPCRs, might occur [26].

2.2. Animals

The effect of cAng-(1–7) was studied in C57Bl/6 female mice, which received multiple low doses of streptozotocin to which this strain is adequately sensitive allowing for the disease model to develop appropriately. Upon arrival, mice were 6 weeks of age. In a separate study *db/db* (BKS.Cg-m +/+ *Lepr^db*) male mice were used. Upon arrival, mice were 5 weeks of age. Both mice strains were obtained from Jackson Laboratories (Bar Harbor, ME, USA). The mice were housed in a room with a photo cycle of 12 h of light and 12 h of dark and an ambient temperature of 70–72 °F. Mice were fed on regular diet and water *ad libitum* for seven days. Studies were performed at INVITEK Inc., according to INVITEK's Standard Operating Procedures.

2.2.1. Multiple low dose STZ mice

After acclimatization, 7–8 weeks old mice were dosed intraperitoneally with 50 mg/kg STZ solubilized in 0.05 M sodium citrate at a volume of 100 μl per mouse for five consecutive days. Blood glucose levels were monitored beginning day 4 post STZ by monitoring with the Accu check glucose meter (Roche, CA). On day six, after five days of STZ injection, mice were grouped into three groups of 10 mice each having average blood glucose levels of 200 mg/dL. During 28 days, mice were daily injected with a subcutaneous dose volume per mouse of 100 μl PBS containing 50 μg/kg or 500 μg/kg cAng-(1–7).

2.2.2. C57Bl/6 db/db (BKS.Cg-m +/+ Lepr^db) mice

Following acclimatization, mice were grouped in three groups of eight mice each according to their blood glucose levels of 220–270 mg/dL. The groups of vehicle control and 50 μg/kg cAng-(1–7) were injected subcutaneously with a dosing volume of 150 μL. cAng-(1–7) was prepared daily in saline and administrated daily for 84 days. On day 0, week 4, 8, and 12 from overnight fasted *db/db* mice before the oral glucose tolerance tests (OGTT) approximately 50 μL of blood samples were collected through tail nip in a tube containing lithium heparin as anticoagulant and processed for plasma. On week 12, twenty-four hours post OGTT mice were fasted again and some blood samples were drawn. Plasma samples of *db/db* mice were used for the analysis of insulin as described above and for determining the levels of triglyceride, cholesterol and FFA by using the triglycerides kit, the cholesterol kit and the NEFA C kit purchased all from Wako Chemicals USA, Inc. Richmond, VA.

2.3. Body weight

Body weights of *db/db* and MLD-STZ-induced mice were measured two and three times a week, respectively, using a laboratory balance.

2.4. Blood glucose

In MLD-STZ induced mice, blood glucose levels were monitored three times a week between 10:00 a.m. to 10:30 a.m. using the Accu check glucose meter by placing a drop of blood directly on to the meter strip, and measuring according to manufacturer's instructions. In *db/db* mice, blood glucose levels were measured twice a week between 9:30 a.m. and 10:00 a.m. using the Accu check glucose meter before administration of test compounds.

2.5. HbA1c measurement

HbA1c measurement is primarily used for diagnosis of type II diabetes. In this study glycated hemoglobin A1c of the *db/db* mice was measured on weeks 6 and 12 using Bayer A1c Now (Bayer Healthcare, LLC., Sunnyvale, CA, USA) as per manufacturer's instructions.

2.6. Oral glucose tolerance

Oral Glucose Tolerance Tests (OGTT) performed with the *db/db* mice on day 0, week 4, 8, and 12. Mice were fasted overnight and blood glucose levels were measured at time zero. Subsequently each mouse received by oral gavage a single dose of 250 μl glucose solution consisting of 2 g/kg body weight of D-(+)-Glucose (Sigma) solubilized in deionized water. Thereafter decreasing blood glucose levels were measured with the Accu check glucose meter at 30, 60, 90, and 120 min. The test compounds were administrated after the completion of oral glucose tolerance test.

2.7. Insulin measurement

Blood samples of MLD-STZ induced mice were collected on days 14 and 28 and processed for plasma by centrifugation. Plasma insulin levels were measured using mouse ultrasensitive insulin ELISA kit (ALPCO Diagnostic, USA). Blood samples from *db/db* mice were collected at day 0, week 4, 8 and 12 and insulin was measured using the ultrasensitive insulin mouse ELISA kit (Crystal Chem Inc. Downers Grove, IL).

2.8. Data analysis and statistics

Data are presented as the mean ± standard error (S.E.M.). For measurements at different time points p values were calculated with Tukey multiple comparisons testing following two-way ANOVA when three groups were involved or with Sidak multiple comparisons testing when two groups were involved. For statistic calculations with the AUC, the triglyceride, the cholesterol and the FFA data the unpaired *t*-test was used. Calculations were performed using GraphPad Prism 7 software (Graph Pad Software, San Diego California USA). Differences between groups were considered significant at $p < 0.05$.

3. Results

3.1. cAng-(1–7) does not significantly reduce body weight

Neither in experiments involving MLD-STZ mice nor in experiments with *db/db* mice were any significant differences in body weight between the cAng-(1–7) and vehicle-treated groups observed (Fig. 2A,B).
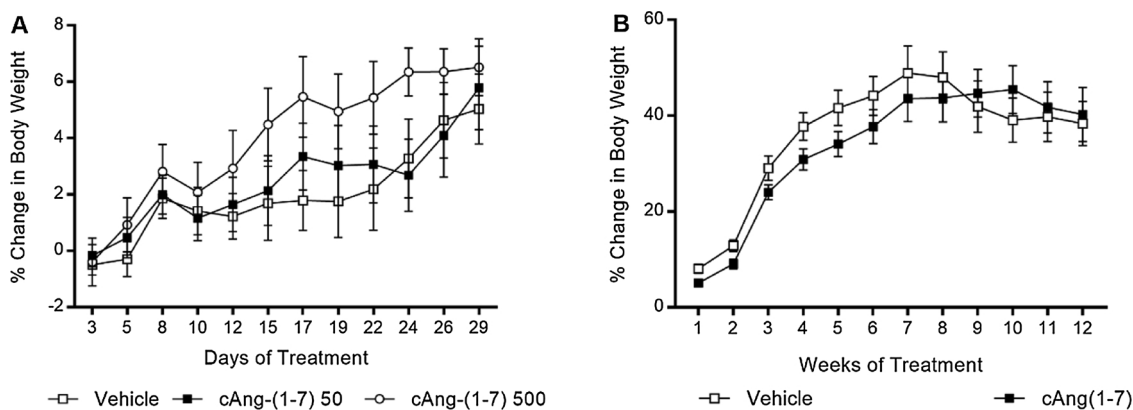
Fig. 2. AB. cAng-(1–7) does not significantly reduce body weight. (A): Body weight of STZ-mice, treated with vehicle, 50 μg/kg cAng-(1–7), or 500 μg/kg cAng-(1–7). (B) Body weight of *db/db* mice treated with vehicle or 50 μg/kg cAng-(1–7). No significant change in body weight compared to vehicle was measured (neither in A nor in B).

3.2. cAng-(1–7) lowers blood glucose

Blood glucose levels were measured in MLD-STZ mice (Fig. 3A) and in *db/db* mice (Fig. 3B). Fig. 3A very clearly demonstrates highly significant reductions of glucose levels upon treatment with either 50 μg/kg or 500 μg/kg of cAng-(1–7) in MLD-STZ mice as compared to the vehicle treated group. cAng-(1–7) also significantly lowered blood glucose levels in *db/db* mice compared to vehicle (Fig. 3B). As the statistical significance of the effect of cAng-(1–7) in the *db/db* mice slightly varied in time (Fig. 3B), HbA1c was measured in the *db/db* mice.

3.3. cAng-(1–7) improves HbA1c levels

When glucose levels are elevated, relatively more haemoglobin within the red blood cells is glycated, an irreversible non-enzymatic coupling. HbA1c is thereby an indicator of plasma glucose concentration over prolonged periods of time. The percentage glycated hemoglobin in *db/db* mice was measured at the end of week 6 and 12 (Fig. 4). In both cases, cAng-(1–7) caused significant reduction in HbA1c levels as compared to vehicle.

3.4. cAng-(1–7) improves oral glucose tolerance

To further evaluate the effect of cAng-(1–7) in *db/db* mice, the capacity of cAng-(1–7) to improve oral glucose tolerance in *db/db* mice was investigated (Fig. 5ABCD). The cAng-(1–7) treated group revealed
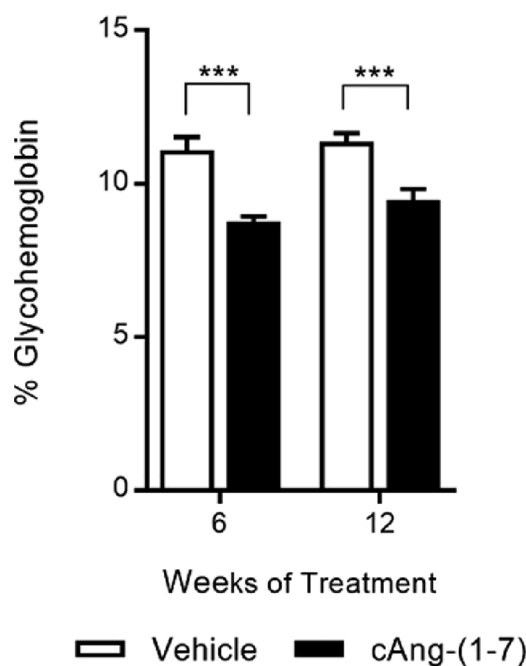
Fig. 4. cAng-(1–7) reduces glycated hemoglobin levels in db/db mice. Percentages HbA1c in blood samples from *db/db* mice treated with either vehicle or 50 μg/kg cAng-(1–7) were determined (*** $p < 0.001$).
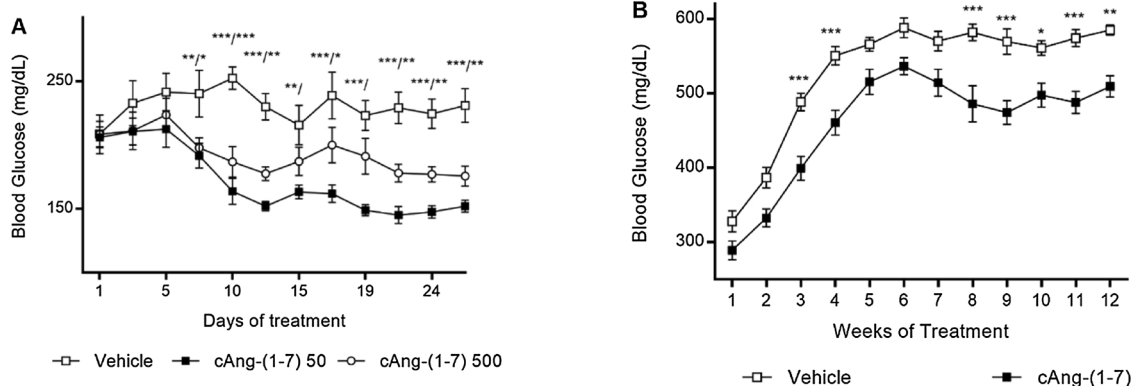
Fig. 3. AB. cAng-(1–7) reduces blood glucose levels. (A) Blood glucose level in MLD-STZ mice treated with either vehicle (□), 50 μg/kg (■), or 500 μg/kg cAng-(1–7) (○). Significant differences, compared to vehicle, for 50 μg/kg cAng-(1–7) left of the slash and for 500 μg/kg after the slash, are indicated (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). (B) Blood glucose level of *db/db* mice treated with either vehicle (□) or with 50 μg/kg cAng-(1–7) (■). Significant differences are indicated (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).
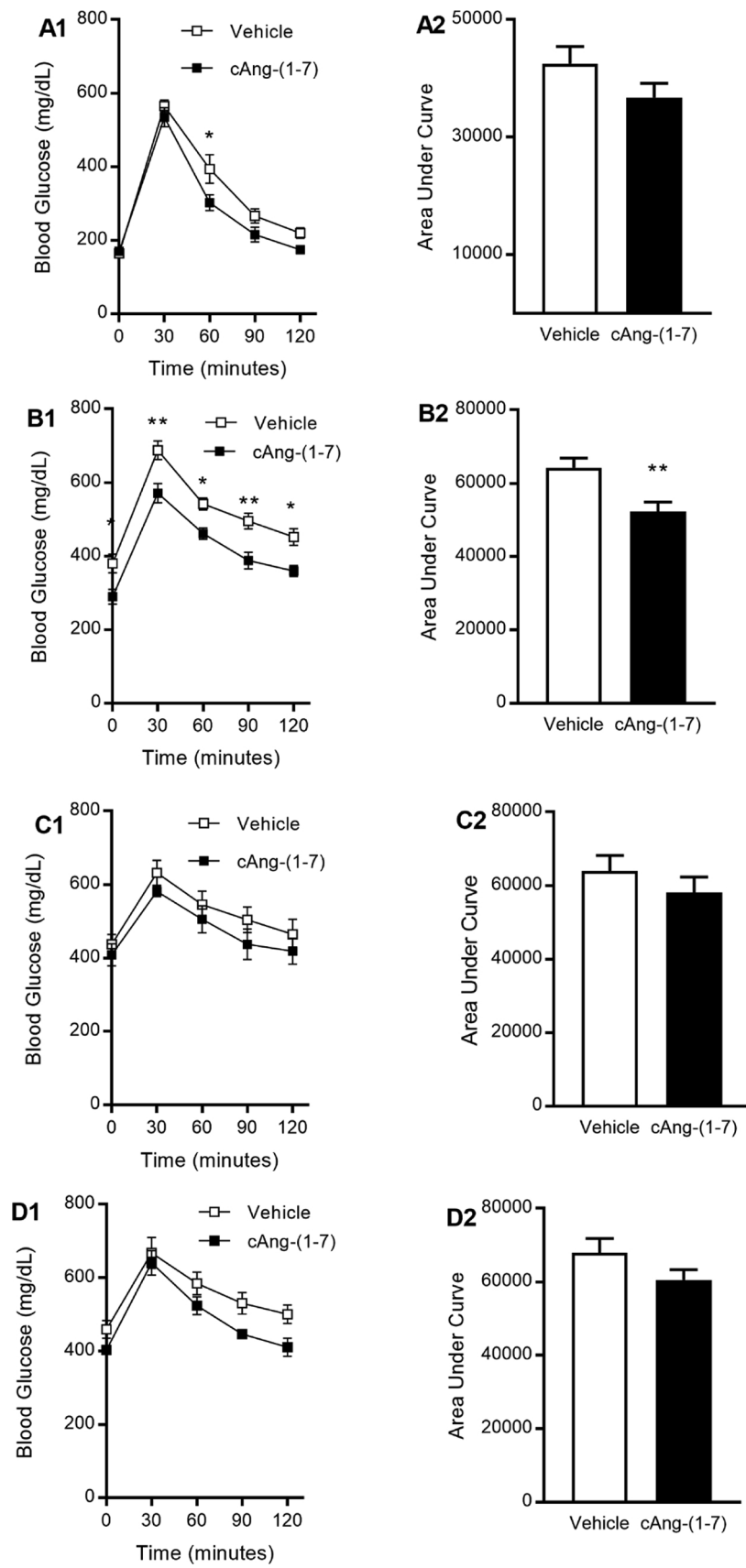
Fig. 5. ABCD. cAng-(1–7) lowers blood glucose in Oral Glucose Tolerance Test. Db/*db* mice were treated with either vehicle or 50 μg/kg of cAng-(1–7). The oral glucose tolerance test was performed on day 0 (A1, A2), at week 4 (B1, B2), at week 8 (C1, C2) and at week 12 (D1, D2). Figures A2, B2, C2 and D2 represent areas under the curve (AUC) of Figures A1, B1, C1 and D1, respectively. Significant differences are indicated (* p < 0.05; ** p < 0.01; *** p < 0.001).

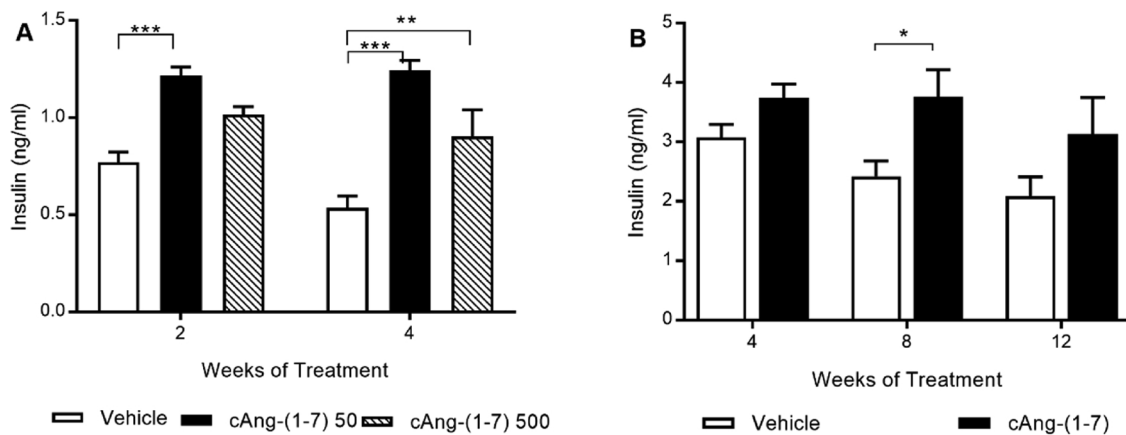**Fig. 6.** AB. cAng-(1–7) raises insulin levels. A: MLD-STZ mice were treated with either vehicle (open bar), or 50 µg/kg cAng-(1–7) (A: black bar) or 500 µg/kg cAng-(1–7) (A: \\\ bar). B: *db/db* mice were treated with either vehicle (open bar) or 50 µg/kg cAng-(1–7) (B: black bar). The insulin concentration in plasma samples was measured (* p < 0.05; ** p < 0.01; *** p < 0.001).

significant improvement, as compared to vehicle, at t = 60 min (A1), on week 4 at each time point (B1) and as area under the curve (B2), and on week 12 at time t = 90 min (D1).

3.5. cAng-(1–7) causes an increase in insulin

Plasma insulin levels in STZ-treated mice (Fig. 6A) were lower than in *db/db* mice (Fig. 6B). In STZ-treated mice (Fig. 6A), cAng-(1–7) at 50 µg/kg in week 2 and 4, and at 500 µg/kg in week 4 significantly elevated insulin levels as compared to vehicle. In *db/db* mice (Fig. 6B), cAng-(1–7) at 50 µg/kg caused a significant increase in insulin concentration on week 8 as compared to vehicle.

3.6. cAng-(1–7) did not alter lipid levels in db/db mice

As the *db/db* mouse model displays high plasma triglyceride and cholesterol levels [35] it was investigated in this model whether cAng-(1–7) might affect the levels of these lipids. Levels of triglyceride (Fig. 7A), total cholesterol (Fig. 7B) and non-esterified fatty acids (NEFA) (Fig. 7C) were measured at week 12 in *db/db* mice treated with cAng-(1–7). Neither triglycerides nor cholesterol nor non-esterified fatty acids were significantly affected by cAng-(1–7) as compared to vehicle.

4. Discussion

The two major components of the renin angiotensin system,

angiotensinII and the mostly protective Ang-(1–7) are involved in the pathogenesis of diabetes mellitus and its consequences such as nephropathy, retinopathy and cardiomyopathy [41,42].

Ang-(1–7) clearly has beneficial effects on glucose metabolism [23,43,49]. Ang-(1–7) increased expression of glucose transporters [5] and increased glucose uptake into tissues [5,41,48]. Overexpression of ACE2 enhanced glucose tolerance in db/db mice when tested at day 7 post ACE2 injection [6]. Ang-(1–7) protected against high glucose-induced injuries in cardiomyocytes by suppressing overexpression levels of leptin and p-p38 MAPK/p-extracellular signal-regulated protein kinase 1/2 (ERK1/2) [30]. In a rat model of type II diabetes oral Ang-(1–7) inhibited the development of hyperglycemia [47].

Ang-(1–7) also clearly has protective effects with respect to the function of insulin and the function of pancreatic β cells. Ang-(1–7) protected against STZ-induced diabetes by reducing insulin resistance [46], enhancing insulin secretion, and improving pancreatic β cell survival [23]. Ang-(1–7) reduced hepatic insulin resistance by stimulating via the Akt/PI3K/IRS-1/JNK insulin signaling pathway [5]. Ang-(1–7) protects the function of pancreatic β cells by improving the function of islet microvascular endothelial cells [18,33].

It would be tempting to speculate that Ang-(1–7) might lower glucagon levels. Ablation of the MasR caused an increase of glucagon-producing α-cells and glucagon in mice [3]. Therefore it would be of interest to study whether MasR stimulation would cause lowering of glucagon levels further contributing to glucose resistance.

In addition to its regulation of glucose homeostasis Ang-(1–7) exerts other therapeutic effects in diabetes. In man, Ang-(1–7) is an important
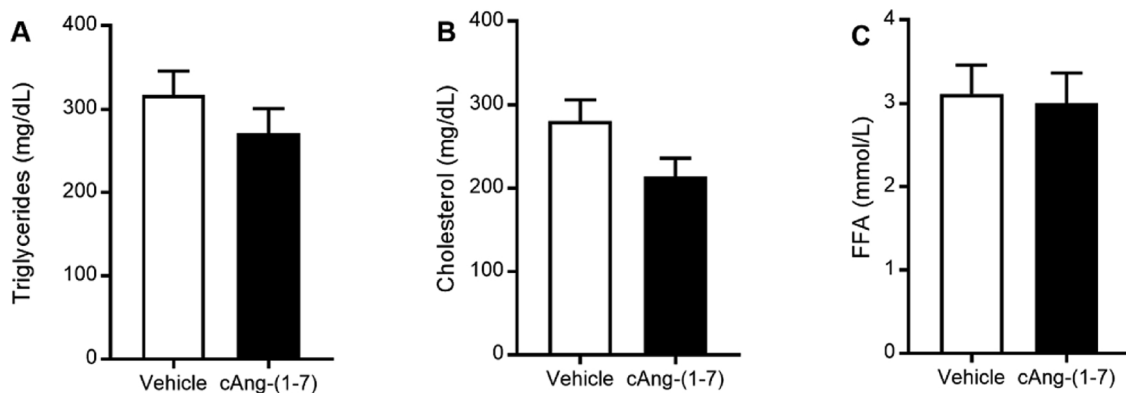

Fig. 7. ABC. cAng-(1–7) does not significantly reduce levels of triglyceride, cholesterol or free fatty acid. *Db/db* mice were treated with either vehicle (open bar) or 50 µg/kg cAng-(1–7) (black bar). Concentrations triglyceride (A), cholesterol (B) and free fatty acids (C) were measured in week 12. No significant differences were measured.

regulator of epicardial adipose tissue inflammation and cardiac dysfunction in obesity [39]. Ang-(1–7) enhanced vascular repair [51], exerted anti-inflammatory effects in the STZ-treated rat model indicating potential for treating atherosclerosis in diabetes [2] and reduced right ventricular fibrosis and dysfunction [21]. In the *db/db* model, Ang-(1–7) even completely rescued the diastolic dysfunction [34]. STZ treatment of SHR rats decreased Ang-(1–7) levels in cerebral cortex and hippocampus [31]. Intraventricularly administered Ang-(1–7) attenuated cognitive impairment in STZ-treated rats [9]. Ang-(1–7) may via several mechanisms reduce hypertension and nephropathy in diabetes [36]. By reducing oxidative stress and macrophage infiltration Ang-(1–7) prevented lung dysfunction in *db/db* mice [38]. Treatment of *db/db* mice with Ang-(1–7) caused an increase in bone marrow and circulating endothelial progenitors, as well as bone marrow mesenchymal stem cells, were increased in mice treated with Ang-(1–7) [37]. Ang-(1–7) reverses bone marrow mobilopathy in diabetic mice [51]. Ang-(1–7) shows beneficial effects in attenuation of diabetes-induced phosphodiesterase activity [11]. Ang-(1–7) confers protection against the development of diabetic retinopathy [52].

In view of the above data and the very high susceptibility of Ang-(1–7) to breakdown by ACE, research on *in vivo* efficacy of a stable angiotensin-(1–7) analog in diabetes models is relevant. In this study, diabetic mice were treated with the fully ACE-resistant, lanthionine-stabilized cAng-(1–7) to evaluate its therapeutic potential. Two different animal models were used. A model for type I diabetes involved C57Bl/6 mice which are diabetic due to selective destruction of pancreatic islet β-cells by low dosing with streptozotocin (STZ) [24,25,40]. The resultant pathology of this animal model resembles human type I diabetes mellitus with chronic pancreatic islet inflammation, insulitis and insulin deficiency, causing hypoinsulinemia and hyperglycemia [19]. Type I diabetes is associated with lack of insulin rather than with insulin resistance. The STZ-induced diabetic mice treated with cAng-(1–7) showed a significant raise in insulin level when compared with the vehicle-treated group.

As a model for type II diabetes *db/db* (BKS.Cg-m +/+ *Lepr^{db}*) diabetic mice were used. These mice are leptin-receptor-deficient by a single autosomal recessive mutation in the leptin receptor gene which results in reduced insulin receptor sensitivity [8,29]. In a C57BLKS/J genetic background, *db/db* mice express morbid obesity, chronic hyperglycemia, and pancreatic beta cell atrophy and become hypo-insulinemic. This strain is used to test glucose lowering agents, insulin sensitizers, insulin secretagogues, and anti-obesity agents [56]. cAng-(1–7) caused also in this model an increase in insulin level, although less significantly than in the STZ model.

Glucose and glycated hemoglobin levels are both important in diabetic research aiming at glycemic control. In the present study, both type I and type II diabetic mice models showed elevated blood glucose levels consistent with the diabetic pathology. cAng-(1–7) strongly lowered glucose levels in the STZ-treated mice. In *db/db* mice, the observation of cAng-(1–7)-induced reduction of glucose levels was confirmed by measuring HbA1c levels, mostly used for diagnosis of type II diabetes, and the oral glucose tolerance test. In future experiments it might be of interest to measure insulin levels during the oral glucose tolerance test for cAng-(1–7)-treated *db/db* mice [22].

Clinical and basic studies have demonstrated that AT$_1$R blockers, downregulating the ACE / AngII / AT$_1$R receptor arm of the RAS, improve β-cell function, glucose tolerance and delay the onset of type II diabetes [10,32]. Consistently the stimulation of the protective arm of the RAS, the ACE2 / Ang-(1–7) / MasR arm, in this study initiated by the administration of cAng-(1–7), would be predicted to protect in the case of type II diabetes [18,33], as indeed observed in this study.

5. Conclusion

In conclusion, cAng-(1–7) induced recovery of insulin levels in type I and type II diabetes mouse models. cAng-(1–7) has a significantly positive impact on the glycemic control. Taken together, cAng-(1–7) may have a beneficial role in treatment of diabetic diseases, provided its effect when combined with standard of care, such as AT$_1$R blockers and ACE inhibitors, shows sufficient additivity/synergy for clinical significance.

Conflict of interest statement

Author contributions

AK wrote the manuscript and performed statistical analyses, GM contributed to writing the manuscript, LW and RF designed the experiments. All authors have approved the final article.

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