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Circulating cathepsin S improves glycaemic control in mice

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Abstract

Cathepsin S (CTSS) is a cysteine protease that regulates many physiological processes and is increased in obesity and type 2 diabetes. While previous studies show that deletion of CTSS improves glycaemic control through suppression of hepatic glucose output, little is known about the role of circulating CTSS in regulating glucose and energy metabolism. We assessed the effects of recombinant CTSS on metabolism in cultured hepatocytes, myotubes and adipocytes, and in mice following acute CTSS administration. CTSS improved glucose tolerance in lean mice and this coincided with increased plasma insulin. CTSS reduced *G6pc* and *Pck1* mRNA expression and glucose output from hepatocytes but did not affect glucose metabolism in myotubes or adipocytes. CTSS did not affect insulin secretion from pancreatic β -cells, rather CTSS stimulated glucagon-like peptide (GLP)-1 secretion from intestinal mucosal tissues. CTSS retained its positive effects on glycaemic control in mice injected with the GLP1 receptor antagonist Exendin (9–39) amide. The effects of CTSS on glycaemic control were not retained in high-fat-fed mice or db/db mice, despite the preservation of CTSS' inhibitory actions on hepatic glucose output in isolated primary hepatocytes. In conclusion, we unveil a role for CTSS in the regulation of glycaemic control via direct effects on hepatocytes, and that these effects on glycaemic control are abrogated in insulin resistant states.

Key Words

- ▶ CTSS
- ▶ glucose metabolism
- ▶ liver
- ▶ insulin sensitivity
- ▶ lipid metabolism

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Introduction

Hepatokines are liver-secreted proteins that can signal to, and induce, metabolic effects in the liver and other peripheral tissues including adipose tissue, skeletal muscle and pancreas (Meex *et al.* 2015). Altered hepatokine secretion has been implicated in the development of the metabolic syndrome, non-alcoholic fatty liver disease (NAFLD), type 2 diabetes and cardiovascular disease by

regulating a multitude of signalling cascades and inducing transcriptional changes in target tissues (Pal *et al.* 2012, Meex *et al.* 2015, Jung *et al.* 2016, Oh *et al.* 2016). Changes in hepatokine secretion in NAFLD can influence glycaemic control, insulin action, lipid metabolism and inflammation (Meex & Watt 2017), and in this respect, several hepatokines including fetuin-A, fetuin-B, adropin,

selenoprotein P and fibroblast growth factor 21 (FGF21) have been shown to directly impact these metabolic processes and contribute to associated pathologies (Meex & Watt 2017, Watt *et al.* 2019).

Cathepsin family proteins are lysosomal peptidases categorised to the cysteine, serine and aspartic protease classes that have been shown to be secreted by some tissues, including the liver (Kuester *et al.* 2008). Within this family, cathepsin S (CTSS) is a potent cysteine protease that has been associated with several pathological states including arthritis, cancer, and cardiovascular disease (Wilkinson *et al.* 2015). A pathological feature of these disease states is increased CTSS secretion and enhanced enzymatic activity towards extracellular substrates (Wilkinson *et al.* 2015). In contrast to the other cathepsins, CTSS remains active and stable at neutral pH, allowing for its activity to be conserved when secreted and thereby suggesting potential endocrine functions (Jordans *et al.* 2009). Circulating CTSS levels have been shown to be increased in individuals with type 2 diabetes (T2D), obesity and dyslipidemia (Liu *et al.* 2006, Taleb *et al.* 2006, Verrijn Stuart *et al.* 2012, Chen *et al.* 2013), and are reduced following weight loss (Taleb *et al.* 2006). In this respect, increased circulating CTSS levels may contribute to defects in glycaemic control, as deletion of *Ctss* or administration of orally active small-molecule CTSS inhibitors in mice improved glucose tolerance by reducing hepatic glucose production (HGP) (Lafarge *et al.* 2014) and attenuated the onset of type 1 diabetes (Hsing *et al.* 2010). While these studies are informative, the direct impact of circulating CTSS on glycaemic control and systemic metabolism is currently unknown. Accordingly, this study focused on understanding the endocrine role of CTSS and examined the effects of recombinant (circulating) CTSS in regulating glucose metabolism in cultured cells, tissues and mice.

Materials and methods

Recombinant CTSS protein production and validation

Murine CTSS recombinant protein was generated at Monash University Protein Production Unit (Melbourne, Australia) using a mammalian expression system (Expi293 cells) and purified under native conditions in 100 mM NaPO₄ and 150 mM NaCl. Recombinant protein was freeze dried, resuspended at 0.5 mg/mL, and stored at –80°C, avoiding repeated freeze-thawing. The presence of glycosylation was tested by immunoblotting of recombinant protein following PNGase F treatment (P7367, Sigma). Circular dichroism measurements

were performed on an Applied Photophysics Chirscan spectrometer (Surrey, UK), using a 0.1 cm pathlength quartz cuvette. Measurements were performed at 20°C, and samples were scanned from 190 nm to 260 nm in 1 nm step intervals and with 2 s averaging time. The data was analysed using DICHROWEB (Whitmore & Wallace 2004), using the K2D algorithm (Andrade *et al.* 1993). CTSS protein activity was assessed using a fluorescence-based assay with the synthetic CTSS substrate VVR-AFC (Abcam, ab65307).

Animal studies

C57BL/6 male mice were housed at 22°C on a 12 h light:12 h darkness cycle and were fed either a rodent chow (5% energy from fat) or a high-fat diet (HFD; 43% energy from fat) (High Fat Rodent Diet SF04-001; Specialty Feeds) starting at 8–9 weeks of age for a total of 8 weeks. Diabetic db/db mice (Jackson Laboratory strain: BKS.Cg-Dock7m *+/+* Lepr^{db}/J) were used at 8 weeks of age. Mice were fasted from 07:00 to 11:00 h before all experiments. Experimental procedures were approved by the University of Melbourne Anatomy & Neuroscience, Pathology, Pharmacology, and Physiology Animal Ethics Committee (ethics no. 1814403) and conformed to the National Health and Medical Research Council of Australia guidelines regarding the care and use of experimental animals.

Energy expenditure

Whole-body energy expenditure, the respiratory exchange ratio, food intake and physical activity were assessed using a 16-chamber metabolic system (Promethion Metabolic System, Sable, Nevada, USA). Studies were commenced after 8 h of acclimation to the metabolic chamber. Expired gases were assessed at 30 min intervals for 48 h. Mice received CTSS (1 mg/kg body weight) or saline injection i.p. 24 h following acclimatisation and were monitored for 24 h thereafter.

Glucose and insulin tolerance tests

Mice were fasted for 4 h prior to experiments. Recombinant CTSS (or saline control) was injected i.p. at 1.0 mg/kg body weight after 3 h fasting (i.e. 60 min before the oral glucose or i.p. insulin challenge). Mice were either gavaged orally with glucose (2 g/kg body weight) or injected i.p. with insulin (0.75 U/kg body weight, Actrapid). During the oral glucose tolerance test (OGTT), blood glucose levels

were assessed at 0, 15, 30, 45, 60 and 90 min (Accu-Chek II glucometer; Roche Diagnostics), and additional blood was collected at 0, 15 and 30 min for later assessment of serum insulin and C-peptide levels (Ultra-Sensitive Mouse Insulin ELISA/Mouse C-peptide ELISA Kit; Crystal Chem, Elk Grove Village, IL, USA). During the insulin tolerance test (ITT), blood glucose levels were assessed at -10, 0, 10, 20, 30 and 45 min after insulin injection. In separate experiments, the GLP1 receptor antagonist Exendin (9–39) amide (Abcam) was administered to mice (i.p. 4 µg/g body weight) in the presence or absence of CTSS (i.p. 1.0 mg/kg body weight), and a GTT was performed as described previously.

Assessment of CTSS and Cystatin C by ELISA

CTSS secretion from murine liver slices, plasma CTSS and Cystatin C were assessed by ELISA (CTSS – Aviva System Biology, San Diego, USA; Cystatin C – Crystal Chem, Elk Grove Village, IL, USA). Plasma was obtained from mice following a 4 h fast. For secretion experiments, 300 µm thick precision-cut liver slices were obtained from 4 h fasted db+ and db/db mice using a Krumdieck tissue slicer (TSE Systems GmbH, Germany) and secreted proteins were collected after 16 h incubation in EX-CELL® 325 PF CHO Serum-Free Medium (Sigma Aldrich).

Plasma CTSS levels in mice

Mice were fasted for 4 h, recombinant CTSS was injected (i.p.) at 1 mg/kg body weight, and blood samples were taken from a tail vein at 0, 15, 30, 45, 120, 240 and 480 min. Plasma CTSS was assessed by immunoblotting (see below).

Assessment of GLP1 in human intestinal samples

Ex vivo secretion assay procedures

Surgical specimen collection Procedures were approved by the Southern Adelaide Clinical Human Research Ethics Committee. Tissue from the terminal ileum was collected from consented patients undergoing right hemicolectomy for colon cancer at Flinders Medical Centre and Flinders Private Hospital. Specimens from patients that were indicated for any form of inflammatory bowel disease were not collected for this study. Characteristics of the patients (4 men and 4 women) are: age 68 ± 3.6 years, BMI 29 ± 2.1 kg/m². Three patients had type 2 diabetes (one was diet-controlled, two were metformin-treated). The specimens were immediately placed in oxygenated iced-cold Krebs buffer (in mM, NaCl 138, KCl 4.5, CaCl₂ 2.6, NaHCO₃

4.2, MgCl₂ 1.2, NaH₂PO₄ 1.2, HEPES 10, Glucose 5) and transported to the laboratory for dissection within 15 min with continuous carbogen bubbling. The specimens were rinsed with iced-cold Krebs buffer to remove any luminal content and dissected clear of adipose, muscular and connective tissue. The mucosae were gently separated from the submucosa using spring scissors, cut into pieces and weighed individually.

***Ex vivo* secretion assay** Mucosal tissues were incubated with 100 µL of assay buffer (modified Krebs buffer as above, supplemented with 1 µM sitagliptin and 0.1% BSA) or assay buffer containing different stimulants in a 96-well plate for 15 min at 37°C in 95% O₂/5% CO₂. As such, secretion upon CTSS exposure was compared to that under control conditions (assay buffer only) in tissue from the same donor. Supernatants were collected and stored in aliquots at -20°C until analysis. Active GLP1 levels were quantitated using a commercially available ELISA (EZGLPHS-35K, Merck Millipore, USA). 300 mM glucose was used as a positive control as we previously showed high extracellular glucose reliably stimulates GLP1 release from human small intestine (Kady *et al.* 2018).

Cell culture

C2C12 myoblasts were grown in DMEM (Gibco), containing 10% foetal bovine serum (Thermo Fisher Scientific) and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin), as previously described (Montgomery *et al.* 2013). Differentiation was induced by serum starvation with 2% horse serum for 5–7 days.

3T3-L1 adipocytes were cultured in DMEM supplemented with 10% foetal bovine serum, 4 mM L-glutamine and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin), and once fully confluent, differentiation was induced with DMEM containing 10% FBS, 0.4 µM insulin, 0.25 µM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX). After 3 days, the medium was replaced with DMEM containing 10% FBS and 0.4 µM insulin for another 3 days, followed by overnight incubation in DMEM containing 0.5% FBS. Primary murine hepatocytes were isolated from 8-week-old chow mice and 12-week-old db/db mice by collagenase digestion, as previously described (Montgomery *et al.* 2015).

Assessment of substrate metabolism *in vitro*

C2C12 myotubes, 3T3-L1 adipocytes cells and primary murine hepatocytes were incubated with CTSS (100 pg/mL,

10 ng/mL and 1 µg/mL) for 2 h prior to the assessment of glucose metabolism and glucose uptake using radio-labelled substrates.

Glucose oxidation and glycogen synthesis

Cells were incubated in high-glucose DMEM containing 0.2% BSA and 1 µCi/mL U-¹⁴C-glucose (Perkin Elmer) for 2 h. Thereafter, the media was acidified in 0.5 mL of 1 M perchloric acid and the escaped CO₂ captured in 200 µL 1 M NaOH. The radioactivity within the NaOH fraction (representing the rate of glucose oxidation) was counted on a scintillation counter (TRI-CARB 4910TR 110 V Liquid Scintillation Counter, Perkin Elmer). The tissue homogenate was incubated in 100 µL 2 M KOH at 70°C for 20 min. Following digestion, 75 µL of saturated Na₂SO₄ and 1.3 mL of 98% ethanol was added on ice in order to precipitate glycogen. Samples were centrifuged at 16,000 *g* for 10 min at 4°C to pellet glycogen. The pellet was dissolved in 200 µL ddH₂O, glycogen precipitated again through the addition of 98% ethanol and centrifugation. The glycogen pellet was air-dried and resuspended in 200 µL of a solution containing 0.3 mg/mL amylo-glucosidase in 0.25 M acetate buffer, pH 4.75, and incubated overnight at 37°C to ensure full digestion of glycogen into glucosyl units. This digest was used for assessment of glycogen synthesis (i.e. presence of radioactivity) and for assessment of total glycogen (i.e. presence of glucosyl units) using a glucose oxidase kit (Infinity™ Glucose (Ox), Fisher Scientific, USA).

Glucose output

Primary murine hepatocytes were incubated without or with CTSS (10 ng/mL) in glucose-free DMEM, supplemented with 10 mM pyruvate for 24 h, followed by the assessment of glucose in the media using a glucose oxidase assay kit (Infinity™ Glucose (Ox), Fisher Scientific, USA). In separate experiments, hepatocytes were incubated for 8 h under the same conditions for the determination of *G6pc* and *Pck1* expression by qRT-PCR.

Glucose-stimulated insulin secretion (GSIS)

MIN6 pancreatic β-cells were cultured in DMEM containing 10% FBS and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin). Prior to GSIS assessment, the cells were starved in Krebs Ringer Buffer (KRB) containing 0.25% BSA and 2.8 mM glucose for 30 min, followed by incubation in KRB with 2.8 mM (low) or 16.8 mM (high) glucose for 1 h. Insulin secreted into the media was measured by ELISA (Ultra-Sensitive Mouse

Insulin ELISA Crystal Chem, Elk Grove Village, IL, USA) and corrected for cell protein content.

Immunoblot analysis

Cell lysates and plasma were prepared in RIPA buffer, proteins were resolved by SDS-PAGE electrophoresis and conventional immunoblotting procedures were conducted as described previously (Mohktar *et al.* 2016). Proteins were visualised using Image Lab software version 6.0.1 (Bio-Rad Laboratories), and density was quantified by ImageJ software (version 1.52r). Antibodies used were Akt (Cell Signalling, 9272S), Akt Ser 473 (Cell Signalling, 4058S) and CTSS (Abcam, Ab232740). All antibodies were used as 1/1,000 dilution (primary antibodies) or 1/10,000 dilution (secondary antibodies).

Gene expression analysis

Total RNA was extracted from murine primary hepatocytes using TRI reagent (Sigma), treated with DNase (Ambion DNA free kit, Thermo Fisher) and reverse transcribed with iScript Reverse Transcriptase (Invitrogen). Gene expression was determined by CFX Connect™ Real-Time PCR Detection System (Bio-rad) using SYBR Green PCR Master Mix (Quantinova® SYBR Green PCR kit, Qiagen). Samples were normalised using *Hprt* as a housekeeping gene. Primer sequences were: *Pck1* forward: 5'-AATATGACAACTGTTTGCTG reverse: 5'-AATGCTTTCTCAAAGTCCTC; *G6p* forward: 5'-ACTGTGCATACATGTTTCATC reverse: 5'-TGAATGTTT TGACCTAGTGC; *Hprt* forward: 5'-AGGGATTTGAA TCACGTTTG reverse: 5'-TTTACTGGCAACATCAAGAG.

Statistical analysis

Data are expressed as mean ± S.E.M. Statistical analysis was performed by two-tailed unpaired t-test, one-way or two-way ANOVA followed by Bonferroni *post hoc* analysis (GraphPad Prism Version 7.04). The analysis used for specific experiments is outlined in the figure legends. Statistical significance was set *a priori* at *P* < 0.05.

Results

Liver CTSS secretion and plasma CTSS levels in mice

We first confirmed that CTSS is a *bone fide* hepatokine. CTSS was secreted from precision-cut liver slices of lean db/+ mice and CTSS secretion was significantly increased in liver slices from db/db mice with type 2

diabetes (Fig. 1A). CTSS was readily detected in the plasma of mice using a commercial ELISA. Consistent with previous studies reporting increased CTSS in obesity and type 2 diabetes (Taleb *et al.* 2006, Chen *et al.* 2013), plasma CTSS tended to be increased in obese high-fat-fed compared with chow-fed C57BL/6J mice (34%, $P=0.06$; Fig. 1B), and in db/db mice compared with lean normoglycaemic db/+ littermates (25%, $P=0.06$; Fig. 1C). We also assessed plasma levels of cystatin C, which is the most abundant and potent CTSS inhibitor (Wilkinson *et al.* 2015). Plasma cystatin C was unaltered in obese high-fat-fed mice compared with chow-fed C57BL/6J mice (Fig. 1D) and tended to be decreased in db/db mice compared with db/+ littermates (19%, $P=0.06$; Fig. 1E).

CTSS improves glucose tolerance in lean mice

To assess the effects of circulating CTSS on systemic metabolism, we generated recombinant murine CTSS protein. Protein structure assessment using circular dichroism spectrometry estimated CTSS to be 9% α -helix, 43% β -sheet and 48% random coil, which is close to the predicted secondary structure elements and indicative of correct protein folding (Fig. 2A). In addition, we confirmed that recombinant CTSS was glycosylated (Fig. 2B) and, most importantly, enzymatically active (Fig. 2C). CTSS injected into lean C57BL/6 mice at 1 mg/kg body weight led to a two-fold increase in circulating CTSS 45 min after ip administration, which remained elevated for the 8 h duration of the experiment (Fig. 2D and E).

To assess the metabolic impact of circulating CTSS, we injected lean C57BL/6 mice with recombinant CTSS 1 h prior to metabolic assessments. Administration of CTSS did not affect fasting blood glucose levels but significantly

improved glucose tolerance when compared to control mice (Fig. 3A). This improvement was accompanied by a transient increase in glucose-stimulated insulin secretion (Fig. 3B). CTSS administration did not affect insulin tolerance in lean mice (Fig. 3C).

To assess the effects of circulating CTSS on whole-body energy metabolism, mice were housed in Promethion metabolic cages for 48 h. Whole-body oxygen consumption was increased 20 min after CTSS administration (Fig. 3D) and this increase was maintained throughout the experiment (Fig. 3E). The respiratory exchange ratio (RER) was not affected by CTSS administration (Fig. 3F and G), indicating no change in the proportion of carbohydrate and fatty acids being oxidised. Food intake (Fig. 3H) and physical activity (Fig. 3I) were not affected by CTSS.

CTSS does not affect glucose metabolism in myotubes or adipocytes

We utilised cell models to determine how CTSS improves glucose metabolism. CTSS did not affect glucose oxidation (Fig. 4A) or glycogen synthesis under basal conditions or following insulin stimulation (Fig. 4B) in C2C12 myotubes. CTSS increased Akt Ser473 phosphorylation in the basal state, however, CTSS did not affect Akt Ser473 following insulin stimulation (Fig. 4C and D). Consistent with a lack of effect of CTSS on glucose metabolism in skeletal myotubes, CTSS did not affect basal or insulin-stimulated glucose uptake in 3T3-L1 adipocytes (Fig. 4E).

CTSS reduces hepatic glucose production

Studies in primary murine hepatocytes showed that CTSS significantly reduced hepatic glucose output when compared to control under basal conditions. There were

Figure 1

Liver CTSS secretion and plasma CTSS and Cystatin C levels in mice. (A) CTSS secretion from liver slices collected from lean db/+ ($n = 5$) and db/db mice with T2D ($n = 5$). (B) Plasma CTSS in 4 h fasted chow-fed ($n = 15$) and HFD-fed mice ($n = 8$). (C) Plasma CTSS in 4 h fasted lean db/+ mice ($n = 8$) and db/db mice with T2D ($n = 8$). (D) Plasma Cystatin C in 4 h fasted chow-fed ($n = 7$) and HFD-fed mice ($n = 10$). (E) Plasma Cystatin C in 4 h fasted lean db/+ mice ($n = 8$) and db/db mice with T2D ($n = 10$). Statistical analysis was performed by two-tailed unpaired Student's t test. Values are means $\pm$ S.E.M. * $P < 0.05$.

Figure 4

CTSS does not affect glucose metabolism in myotubes or adipocytes. CTSS was applied to C2C12 myotubes and 3T3-L1 adipocytes at 10 ng/mL, unless otherwise stated, for 2 h prior to metabolic assessment. (A) Glucose oxidation ($n = 8$), (B) basal and insulin-stimulated glycogen synthesis ($n = 3$), and (C and D) Akt (Ser 473) signalling ($n = 6$) in C2C12 myotubes. (E) Basal and insulin-stimulated glucose uptake in 3T3-L1 adipocytes ($n = 9$). Statistical analysis was performed by one-way (A) and two-way ANOVA (B, C, D and E) with Bonferroni *post hoc* tests. Values are means $\pm$ s.e.m. * $P < 0.05$.

no significant differences between CTSS and control with insulin stimulation (Fig. 5A). Consistent with these findings, the expression of the gluconeogenic gene *G6pc* (Fig. 5B) was significantly reduced by 50% with CTSS compared with control under basal conditions. *Pck1* tended to be reduced by CTSS under basal conditions ($P = 0.052$) (Fig. 5C). Insulin reduced *G6pc* and *Pck1* expression to similar levels in control and CTSS treated hepatocytes (Fig. 5B and C). CTSS did not affect the rate of glycogen synthesis under basal or insulin-stimulated conditions (Fig. 5D) or glucose oxidation (Fig. 5E). Together, these data indicate that CTSS suppresses glucose

output by reducing hepatic gluconeogenesis under non-insulin stimulated conditions. Consistent with this finding CTSS significantly increased Akt S473 phosphorylation in primary murine hepatocytes under basal conditions (i.e. not insulin stimulated) (Fig. 5F and G).

CTSS does not affect insulin secretion in murine pancreatic β -cells

Acute CTSS administration increased circulating insulin levels in lean mice (Fig. 3B). To determine whether CTSS directly affects pancreatic insulin secretion, we administered

Figure 5

CTSS reduces hepatic glucose production in primary murine hepatocytes. Basal and insulin-stimulated (A) glucose output, mRNA expression of (B) *G6pc* and (C) *Pck1* ($n = 9$); (D) glycogen synthesis ($n = 9$) and (E) glucose oxidation ($n = 9$). (F and G) Akt (Ser 473) phosphorylation/total Akt protein content ($n = 3$). Statistical analysis was performed by two-way ANOVA (A, B, C and D) or unpaired two-tailed t-test (E and F). Values are mean $\pm$ s.e.m. * $P < 0.05$.

CTSS to murine MIN6 pancreatic β -cells and assessed basal and glucose-stimulated insulin secretion. While glucose significantly increased insulin secretion, CTSS did not have any effect on basal or glucose-stimulated insulin secretion, at any dose examined (Fig. 6A).

CTSS increases GLP1 secretion, but this effect is dispensable for glycaemic control

Based on the apparent contrasting results in cultured β -cells and mice, we next asked whether CTSS could be driving insulin secretion by regulating incretin hormone secretion, which enhances insulin secretion following carbohydrate ingestion (Nauck & Meier 2018). To test this, we incubated human intestinal samples with CTSS or glucose. GLP1 was reliably detected in all secretion supernatants and 300 mM glucose significantly increased GLP1 secretion (Fig. 6B). CTSS administration increased GLP1 secretion to a greater extent than glucose, with effects seen at 10 ng/mL CTSS (Fig. 6B), a concentration that approximates plasma values in mice (Fig. 1B and C). Of note, we were not able to detect gastric inhibitory polypeptide (GIP) in the human

intestine secretion supernatants, which was expected as human tissues were obtained from the terminal ileum where enteroendocrine GIP-releasing K-cells have low abundance (Latorre *et al.* 2016).

CTSS improves oral glucose tolerance concomitant with increased insulin secretion, and the *ex vivo* experiments in intestinal samples indicate that this may be mediated through CTSS-regulation of GLP1. To test this possibility *in vivo*, we injected lean mice with the GLP1 receptor antagonist Exendin (9–39) amide (Schirra *et al.* 1998), in the absence or presence of CTSS prior to an oral glucose challenge. Consistent with previous studies (Chambers *et al.* 2017), Exendin (9–39) caused marked glucose intolerance in mice whereas co-administration of CTSS significantly improved glucose tolerance in Exendin (9–39) treated mice (Fig. 6C and D). These results suggest that, while CTSS has the capacity to induce GLP1 secretion in an *ex vivo* system, the CTSS-GLP1 axis does not appear to be the primary mechanism mediating CTSS' positive effects on glycaemic control in mice.

CTSS does not improve glycaemic control in mice with T2D

Based on the finding that CTSS improved glucose tolerance in lean mice, we next assessed the effects of acute CTSS administration on glucose metabolism and glycaemic control in obese db/db mice with T2D. CTSS regulated glucose metabolism in cultured primary hepatocytes isolated from db/db mice. Specifically, CTSS reduced basal HGP (Fig. 7A), increased glucose oxidation (Fig. 7B), and increased glycogen synthesis and glycogen content when compared to untreated hepatocytes (Fig. 7C and D). This demonstrates that CTSS effects on glucose metabolism are maintained in hepatocytes obtained from mice with type 2 diabetes. Insulin did not impact any of these measures which is indicative of the pronounced insulin resistance in db/db hepatocytes (Fig. 7A, B, C and D). In contrast to these pronounced effects *in vitro*, acute CTSS administration did not affect glucose tolerance or plasma insulin levels in db/db mice (Fig. 7E and F). This response was conserved in a second model of insulin resistance, where CTSS administration had no effect on glycaemic control in high-fat-fed mice that were obese and insulin resistant (Fig. 7G and H).

Figure 6

CTSS has GLP1- dependent and -independent effects on glycaemic control. (A) Insulin secretion in murine MIN6 pancreatic beta cells with low (2.8 mM) and high-glucose (16.8 mM) ($n = 9$). (B) Basal and glucose-stimulated (300 mM glucose) GLP1 secretion in human intestinal explants ($n = 8$). (C and D) Exendin (9-39) and/or CTSS were injected 60 min before an oral glucose tolerance test in lean chow-fed C57BL/6 mice ($n = 10$ per group). Statistical analysis was performed by two-way ANOVA (A and C), a paired one-way ANOVA with Fishers Least Significant Difference *post hoc* test (B), or an unpaired two-tailed t-test (D). Values are mean $\pm$ S.E.M. * $P < 0.05$.

Discussion

Cathepsin S plays an important role in the degradation of damaged proteins within the endo-lysosomal pathway.

Figure 7

CTSS does not improve glycaemic control in mice with type 2 diabetes. (A, B, C and D) Primary hepatocytes were isolated from db/db mice, followed by assessment of CTSS effects on (A) glucose output ($n = 6$), (B) glucose oxidation ($n = 9$), (C) glycogen synthesis ($n = 9$) and (D) glycogen content ($n = 6$). (E and F) db/db mice were injected with CTSS (1 mg/kg body weight, i.p.) or saline control 60 min before (E) an oral glucose tolerance test (OGTT) ($n = 14$ per group). (F) Plasma insulin during OGTT ($n = 14$ per group). (G and H) C57BL/6 mice were fed a high-fat diet for 8 weeks followed by acute CTSS injection (1 mg/kg body weight, i.p.) 60 min before (G) OGTT ($n = 8$ per group). (H) Plasma insulin during OGTT ($n = 8$ per group). Statistical analysis was performed by two-way ANOVA and Bonferroni *post hoc* analysis. Values are mean $\pm$ s.e.m. * $P < 0.05$ vs control.

Extending beyond this critical function in protein turnover, recent evidence suggests that CTSS contributes to a wide range of physiological processes (Wilkinson *et al.* 2015). Here, we show that CTSS is a *bona fide* liver-secreted protein that improves glycaemic control by reducing hepatocyte gluconeogenesis and glucose output. CTSS increased GLP1 secretion but this effect appears to be dispensable for CTSS effects on glycaemic control.

While CTSS is primarily localised to the lysosome, several lines of evidence support its role as an extracellular protein including the presence of a N-terminal signal peptide in its protein sequence, evidence that it is secreted from cells (Vidak *et al.* 2019), and stability at physiological pH, which distinguishes CTSS from other cathepsins (Wilkinson *et al.* 2015). Consistent with this notion, we report secretion from murine liver, although our studies cannot distinguish between secretion from hepatocytes and non-parenchymal cells. With previous studies demonstrating no secretion of CTSS from isolated hepatocytes and limited expression in antigen-presenting cells (Rawlings & Salvesen 2012), it is likely that CTSS is secreted from liver resident sinusoidal endothelial cells, stellate cells, various myeloid and lymphoid cell population, cholangiocytes, mast cells or neutrophils (Mehrfeld *et al.* 2018). We also show that CTSS secretion is increased by 2.5-fold from livers of db/db mice and that circulating CTSS levels are increased in obese insulin

resistant db/db and high-fat-fed mice, which agrees with previous reports in mice (Xu *et al.* 2013) and humans (Kuester *et al.* 2008, Hsing *et al.* 2010, Rogers *et al.* 2014, Maimaitiyiming *et al.* 2015). Together, this suggests that the liver may be a significant contributor to systemic CTSS levels, particularly in states of metabolic disease.

The magnitude of crosstalk between liver cells and other tissues in regulating hepatic and systemic metabolism is becoming better understood (Watt *et al.* 2019, Xiong *et al.* 2019). A notable finding from the present study is that CTSS improves glycaemic control in lean mice, which is associated with suppression of hepatic glucose output. The suppression of glucose output in isolated hepatocytes coincided with reduced expression of *G6pc* and *Pck1*, key regulators of gluconeogenesis, with no evidence that CTSS regulates other processes of hepatic or skeletal muscle carbohydrate metabolism including glycogen synthesis or glucose oxidation. It is presently unclear how CTSS suppresses hepatocyte glucose output, or whether CTSS suppresses hepatic glucose output *in vivo*, but this may be related to the known role of CTSS in proteolytic cleavage of various extracellular and cell surface proteins including CD47, E-cadherin, protease-activated receptor 2 (PAR-2), collagen, elastin or integrins (Wilkinson *et al.* 2015). PAR2 is abundantly expressed in hepatocytes and stellate cells of the liver (Shearer *et al.* 2016), and its deletion in mice leads to an increase in glycogen synthesis, a decrease in

glycolysis and gluconeogenesis, and overall reduction in blood glucose levels (Wang *et al.* 2015, Rana *et al.* 2019). Similarly, CD47 knockout mice show improved glucose tolerance (Maimaitiyiming *et al.* 2015) and CD47 signalling was shown to inhibit VEGFR-mediated Akt phosphorylation (Rogers *et al.* 2014). While we did not assess CTSS effects on hepatic PAR2 or CD47 signalling, it is unlikely that CTSS improves glucose tolerance through these receptors as previous studies showed that CTSS activates PAR2 (Chung *et al.* 2019) and CD47-mediated (Lee *et al.* 2014) signalling pathways, with activation of such pathways being associated with impairments in glycaemic control.

A previous study showed that germline whole-body deletion of *Ctss* or chronic pharmacological inhibition of CTSS reduced fasting blood glucose and improved glucose tolerance in mice, which was associated with reduced expression of gluconeogenic genes and reduced hepatic glucose output, but no change in insulin sensitivity (Lafarge *et al.* 2014). Chronic *Ctss* deletion and pharmacological inhibition also increased insulin secretion, with the authors concluding that CTSS promotes 'pro-diabetic' alterations in the liver. It is perplexing that we have described a strikingly similar phenotype despite inducing the opposite effect; that is, acute upregulation of CTSS compared with long-term deletion/inhibition. The discrepancies between these data sets are difficult to reconcile but may relate to transcriptome/proteome adaptations with longer compared with acute interventions, as well as alterations induced by intracellular *Ctss* deletion compared with extracellular effects of the circulating protein. These data sets could therefore potentially distinguish the extracellular metabolic actions of CTSS and CTSS enzymatic activity within the lysosomal compartment.

GLP1 is produced and secreted by intestinal enteroendocrine L-cells and exerts powerful effects on insulin secretion, inhibits glucagon secretion at glucose concentrations above fasting levels and decelerates gastric emptying; factors that contribute to the blood glucose-lowering effects of GLP1 (Drucker 2018). Our unexpected finding that GLP1 secretion was induced by CTSS is, to the best of our knowledge, the first evidence that a cathepsin family protein can regulate incretin hormone secretion. Indeed, our functional studies in human intestinal explants showed that CTSS potently induced GLP1 secretion to a similar magnitude as that induced by glucose administered at concentrations usually seen by enteroendocrine L-cells after a meal (i.e. 50–500 mM)

(Ferraris *et al.* 1990). While we speculate that CTSS most likely acts on enteroendocrine L-cells through cleavage and subsequent activation of CTSS substrates, such as PAR2, which is present in the intestinal epithelium and is highly expressed in the gastrointestinal tract (Sébert *et al.* 2019), the exact mode of action requires further, detailed mechanistic studies. Furthermore, while CTSS increased GLP1 secretion *ex vivo*, this mechanism appears to be dispensable for CTSS effects on glycaemic control in mice, as mice administered Exendin (9–39), a specific and competitive antagonist of GLP1, retained CTSS-mediated improvements during an oral glucose tolerance test. The increase in GLP1 secretion with CTSS *ex vivo* was associated with CTSS-induced hyperinsulinemia during an oral glucose challenge in mice, supporting the notion that CTSS likely increases GLP1 secretion *in vivo*. However, as murine GLP1 is rapidly degraded following intestinal release as a result of endoproteolytic cleavage, with a peak at 1 min (Windeløv *et al.* 2017), we were not able to reliably assess GLP1 secretion in mice *in vivo* despite using two commercial ELISAs. The importance of GLP1 for glycaemic control is more broadly highlighted by our studies demonstrating marked impairments during an oral glucose tolerance test with administration of Exendin (9–39).

While this work identifies CTSS as a circulating protein that improves glycaemic control in lean mice, an unexpected finding was the complete ablation of these effects in obese, insulin resistant mice and db/db mice with type 2 diabetes. This observation was intriguing in light of the established association between circulating CTSS levels and metabolic disease (Taleb *et al.* 2005, 2006, Liu *et al.* 2006, Naour *et al.* 2010), the reduction in CTSS following bariatric surgery and weight loss (Naour *et al.* 2010), and the improvement in glycaemic control in insulin resistant, high-fat-fed mice with *Ctss* deletion (Lafarge *et al.* 2014). It is unlikely that obese mice were refractory to CTSS effects due to 'CTSS resistance' in peripheral tissues, akin to insulin or leptin resistance in peripheral tissues (Steinberg & Dyck 2000), because CTSS reduced glucose output and increased glucose oxidation and glycogen storage in hepatocytes isolated from db/db mice. Interestingly, CTSS increased glycogen synthesis in hepatocytes isolated from db/db mice but not lean mice. The lack of CTSS effects in hepatocytes from lean mice might be due to relatively high glycogen synthesis rates that are 10–15 times higher than hepatocytes from insulin-resistant db/db mice. In this respect, previous studies have similarly reported reduced hepatic glycogen

content in db/db mice (Gao *et al.* 2010, Yang *et al.* 2019). Finally, repeated administration of CTSS may be required to improve glycaemic control in diabetes and will be the focus of future investigation.

CTSS activity in the circulation is regulated by its inhibitor cystatin C (Wilkinson *et al.* 2015), but it is unlikely that increased CTSS degradation explains our *in vivo* observations, as plasma cystatin C was either unchanged or reduced in obese, insulin-resistant mice and db/db mice, respectively. These findings align with previous studies suggesting either unchanged (Nørgaard *et al.* 2019) or decreased (Takiyama *et al.* 2018) plasma cystatin C levels in db/db mice. It is possible that higher levels of circulating CTSS might be required to induce positive effects on glycaemic control in obese, insulin-resistant mice or mice with T2D. However, it should be noted that the same relative amount of CTSS was injected into all mice (1 mg/kg mass), suggesting that circulating levels were likely to be higher in the heavier, obese mice whose excess weight is mostly adipose tissue.

In conclusion, we report an unexpected role for CTSS in the regulation of glycaemic control in mice, which appears to be mediated by direct suppression of glucose output from hepatocytes. We further show that CTSS induces GLP1 secretion, but this effect is dispensable for the beneficial effects of CTSS on glycaemic control. Future studies are warranted to assess CTSS substrates and mode of action within the intestine and liver.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

H K and M K M designed research, performed research, analysed data and wrote the paper. S N K, E W S, D A W and D K performed research and analysed data. M J W designed research, analysed data and wrote the paper. M M and M W are co-last authors. All authors edited the manuscript and agreed to the final version of the manuscript.

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