Catestatin reduces hyperglycemia in insulin-resistant mice by redirecting glucose-6-phosphate from the gluconeogenic to the glycogenic pathway

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ABSTRACT

Objective: Defects in hepatic glycogen synthesis contribute to postprandial hyperglycemia in type 2 diabetic (T2D) patients. Chromogranin A (CgA) peptide Catestatin (CST: $hCgA_{352-372}$) inhibits dephosphorylation of glucose 6-phosphate (G6P) and improves glucose tolerance in insulin-resistant mice. Here, we seek to determine whether CST also reduces hyperglycemia by increasing hepatic glycogen synthesis.

Methods: We determined liver glycogen, G6P, and UDP glucose (UDPG); plasma insulin, glucagon, norepinephrine (NE), and epinephrine (EPI); glycogen synthase (GYS) and phosphoglucomutase (PGM) enzyme activities in fed and fasted liver of lean and obese wild-type and genetically obese CST knockout (KO) mice after treatments with saline, CST or insulin. We determined glycogen synthesis and glycogenolysis in cultured hepatocytes. We analyzed phosphorylation signals of GYS2 and GSK-3β by immunoblotting.

Results: CST stimulated glycogen accumulation in fed and fasted liver and in cultured hepatocytes. CST reduced plasma NE and EPI levels, suggesting that CST promotes glycogenesis by inhibiting catecholamine-induced glycogenolysis. CST also directly stimulated glycogenesis and inhibited NE and EPI-induced glycogenolysis in hepatocytes. CST elevated the levels of G6P and UDPG and increased GYS and PGM activities, redirecting G6P to the glycogenic pathway. CST-KO mice had decreased liver glycogen that was restored by treatment with CST, reinforcing the crucial role that CST plays in hepatic glycogenesis

Conclusions: We conclude that CST directly promotes the glycogenic pathway and reduces plasma glucose levels in insulin-resistant mice by (i) reducing glucose production from G6P, (ii) increasing glycogen synthesis from G6P via formation of UDPG, and (iii) reducing glycogenolysis.

1. INTRODUCTION

Obesity, a major risk factor for type 2 diabetes (T2D) [1], results in the development of insulin resistance and post-prandial hyperglycemia [2-4]. In the United States, >50% of the population have either diabetes or prediabetes [5]. Obesity-induced T2D is thus emerging as a global health problem threatening to reach pandemic levels by 2030 [6; 7]. Chronic hyperglycemia causes glucotoxicity [8; 9], which results in decreased secretion of insulin and increased insulin resistance [8; 10]. The liver plays a central role in maintenance of blood glucose homeostasis. In the postprandial state, the liver responds to an increase in portal vein blood glucose and insulin levels by absorbing glucose via glucose transporter 2 (GLUT2) and converting it to glycogen. The stimulation of net glycogen synthesis is a major, direct physiological function of postprandial insulin on hepatocytes [11]. In the postabsorptive state, the liver produces glucose by glycogenolysis and gluconeogenesis [12]. In the liver, glucose metabolism is determined largely by the glucose concentration in the portal vein (substrate supply) and is regulated by feed-forward activation mechanisms [13; 14]. Liver glycogen synthesis is impaired in T2D patients [14; 15]. Glycogen synthesis is also also impaired in other organs, as for instance the reduced glucose uptake in skeletal muscle in T2D subjects is partly accounted for by reduced glycogen synthesis [16]. Defects in the hepatic glycogen synthesis have been shown to contribute to postprandial hyperglycemia of patients (i) with poorly controlled insulin-dependent diabetes mellitus [15; 17; 18] and non-insulin-dependent diabetic subjects [19; 20], and glycogen storage disease [21; 22]. Therefore, targeting hepatic glucose storage or production is a potential therapeutic strategy for T2D.

Glucose homeostasis is controlled by several hormones, with insulin being the most important anabolic hormone and glucagon and catecholamines being the most catabolic hormones. Glucagon and catecholamines act by the following mechanisms: (i) inhibiting secretion of insulin via alpha adrenergic mechanism [23], (ii) by stimulating glucagon secretion [24], (iii) stimulating hepatic glucose production (HGP) via both beta- and alpha-adrenergic regulation of hepatic glycogenolysis and gluconeogenesis [25-28], (iv) by mobilizing gluconeogenic substrates such as lactate, alanine, and glycerol from extrasplanchnic tissues to the liver [29], and (v) by decreasing glucose clearance by direct inhibition of tissue glucose uptake [30; 31]. In addition, both glucagon and the catecholamines counteract insulin-induced hypoglycemia by stimulating glycogenolysis and gluconeogenesis and augmenting HGP [32; 33].

Chromogranin A (CgA), a ~49 kDa proprotein, gives rise to peptides with opposing regulatory effects [34-37]. While pancreastatin (PST: $hCgA_{250-301}$) is an anti-insulin and proinflammatory peptide [38; 39], catestatin (CST: $CgA_{352-372}$) is a proinsulin and anti-inflammatory peptide [40; 41]. The lack of all CgA peptides make the CgA knockout mice (CgA-KO) more sensitive to insulin despite being

obese [42]. In addition, opposite to wild-type (WT) mice, CgA-KO mice can maintain insulin sensitivity even after 4 months of high fat diet (HFD) [43]. In contrast, CST knockout (CST-KO) mice are obese and display insulin resistance and hyperinsulinemia even on normal chow diet (NCD) [41]. We have recently shown that supplementation of CST-KO mice with CST not only restored insulin sensitivity but also normalized plasma insulin levels in CST-KO mice [41]. In HFD-induced obese (DIO) mice, CST improved insulin sensitivity by attenuating inflammation, inhibiting infiltration of proinflammatory macrophages into the liver, and inhibiting gluconeogenesis [41]. We have also shown that CST improves insulin sensitivity in DIO mice by attenuating HFD-induced endoplasmic reticulum stress [44]. Another mechanism by which CST could potentially control hyperglycemia in insulin-resistant mice is by inducing synthesis of glycogen in the postabsorptive state.

Since CST inhibits both catecholamine secretion [40; 45-48] and gluconeogenesis (by inhibiting expression of glucose 6-phosphatase gene) [41], we reasoned that glucose-6-phosphate (G6P), the substrate for glucose 6-phosphatase, would accumulate after CST treatment, leading to stimulation of glycogenesis. In view of the above findings, we have measured plasma insulin, glucagon, NE and EPI and determined their effects on glycogen levels. This enabled us to determine whether the improved glucose tolerance that we reported in DIO mice by CST [41], could be due to CST's interaction with pancreatic and adrenomedullary hormones leading to mobilization of G6P from the gluconeogenic to the glycogenic pathway. Therefore, in the present communication, we have tested glycogenic effects of CST on genetically obese and insulin-resistant CST-KO mice as well as in insulin-resistant DIO mice and compared the effects of CST with insulin.

2. MATERIALS AND METHODS

2.1. Animals, diets and treatments

Diet-induced obese (DIO) mice were created by feeding male wild-type (WT) mice (C57BL/6), starting at 8 weeks of age, with HFD (Research diets D12492, 60% of calories from fat) for 16 weeks. Mice were kept in a 12:12 hour dark/light cycle; food and water were always available. CST knockout (CST-KO) mice with C57BL/6 background were also used. Both control WT mice as well as CST-KO mice were fed NCD (14% of calories from fat). Mice were injected intraperitoneally with CST (2 µg/g body weight for 14-15 days) after 11 weeks of HFD feeding when weight gains practically levelled off. A group of mice were fasted for 12 hours. Both fed and fasted mice were treated with saline or insulin (0.4 mU/g body weight) for 30 minutes before sacrificing for tissue collection. Tissues were rapidly frozen in liquid nitrogen and kept in -80°C freezer. Previously, we reported that CST treatment did not reduce body weight and did not change food intake in WT mice [41]. In accordance with NIH animal care guidelines, all procedures and animals were housed and handled with the approval of The Institutional Animal Care and Utilization Committee.

2.2. Hepatocyte isolation, culture and assay for glycogen synthesis.

Male mice (16-week-old) were fed NCD and used for perfusion of liver. Mice were perfused for 5 minutes with a calcium free buffer and followed by collagenase perfusion in a calcium containing buffer for another 5 minutes. Perfusion was carried out by inserting a catheter through inferior vena cava and passing buffer through a tube and allowing buffer to come out through portal vein which was cut for this purpose. The procedure was a modified version [41] of a published article [49]. Livers, after collagenase digestion, were excised out, hepatocytes were squeezed out in a petri dish inside a culture hood, filtered through 100-micron nylon filter, centrifuged at 50 x g for 5 minutes, and pellets were collected. The suspensions of cell pellets were then passed through 30% isotonic percoll by centrifuging at 100 x g for 10 minutes. Pellets were washed in buffer and suspended in culture medium (Williams E) containing glutamax, 10% FBS, 10 nM dexamethasone and antibiotics. Hepatocytes were seeded on collagen I coated plates. Mice were cultured in Williams E medium and then depleted of glycogen store by incubating in Hepes Krebs Ringer Bicarbonate buffer for 8 hours followed by switching to serum-free Williams E medium (25 mM glucose) with or without the presence of insulin (10 nM), CST (100 nM) or insulin+CST, incubated for 24 hours in presence of ^{14}C-glucose (10 µCi/ml, 5 mM). At the end of incubation, cultures were washed, and cells were dissolved in 30% KOH, boiled for 20 minutes, glycogen was precipitated by 60% ethanol (in presence of 5 µg glycogen as carrier), pellets washed with 70% ethanol, dried free of traces of ethanol, dissolved in water and counted.

2.3. Glycogen and enzyme analysis in liver

Liver (25-30 mg tissue) glycogen was extracted by boiling with 30% KOH solution as described previously [50]. Extracted glycogen was precipitated in cold by 66% ethanol and washed with 70% ethanol. After drying to remove traces of ethanol, the pellets were dissolved in water and then subjected to colorimetric determination of glycogen using Anthrone reagent (0.05% anthrone and 1% thiourea) in concentrated H_2SO_4.

A group of lean and obese mice were fasted for 12 hours and treated with saline or insulin for 15 minutes. Mice were sacrificed and livers were subjected to analysis of GYS activity and immunoblotting. GYS activity was measured by incorporation of UDP-^{14}C-glucose into glycogen in presence of 10 mM and 0.1 mM G6P, an activator, and ^{14}C-glycogen formed was spotted on GF/A filter paper, washed with 70% ethanol, dried and counted for radioactivity. Counts were subsequently normalized against protein. Results are presented as activation ratio (+G6P (10 mM)/-G6P (0.1 mM)). PGM assay was carried out using a commercial kit (Abcam, Cambridge, MA) which measures G6P concentration as the product. In this assay we measured intrinsic PGM activity and followed reaction 2 (Fig. 7D).

2.4. Catecholamine analysis

Blood was drawn from the heart under deep isoflurane-induced anesthesia and kept in potassium-EDTA tubes. Plasma catecholamines were measured by ACQUITY UPLC H-Class System fitted with Atlantis dC18 reversed-phase column (100A, 3 µm, 2.1 mm x 150 mm) and connected to an electrochemical detector (ECD model 2465) (Waters Corp, Milford, MA). The mobile phase (isocratic: 0.3 ml/min). consisted of phosphate-citrate buffer and acetonitrile at 95:5 (vol/vol). For determination of plasma catecholamines, DHBA (2 ng) was added to 150 µl plasma and adsorbed with ~15 mg of activated aluminum oxide for 10 min in a rotating shaker. After washing with 1 ml water adsorbed catecholamines were eluted with 100 µl of 0.1N HCl. 10 µl of the eluate was injected into UPLC where the ECD range was set at 500 pA. The data were analyzed using Empower software 3 (Waters Corp, Milford, MA). Catecholamine levels were normalized with the recovery of the internal standard. Plasma catecholamines are expressed as nM.

2.5. Analysis of glycogenolysis

Radiolabeled glycogen was synthesized in hepatocytes from ^{14}C-glucose (10 µCi/ml) as described above in presence of insulin only (no CST). Cultures were washed (Krebs-Ringer bicarbonate buffer) to remove external radioactivity and then exposed to buffer (basal), NE (10 µM), EPI (10 µM), insulin (100 nM) and CST (100 nM) alone and in various combinations and incubated for 20 minutes. Incubation was terminated by washing the cultures with cold buffer followed by cell lysis. An aliquot of each lysate was saved for protein assay and the rest was treated with 0.2 M barium hydroxide and

5% zinc sulphate (to precipitate protein) and centrifuged at 10,000 x g for 10 minutes. Supernatants were passed through 100 mg mixed bed ion-exchange resins (both cationic and anionic). Resins were washed twice with water and counted for bound radioactivity (glucose phosphates liberated from hydrolysis of radiolabeled glycogen will remain bound to the resins).

2.6. G6P and UDPG analysis

G6P levels were measured by a commercial kit (Sigma-Aldrich, St. Louis, MO) and UDPG levels were measured by an UPLC-UV detection method in the UC core facility.

2.7. Immunoblotting

Homogenization of livers were made in a lysis buffer supplemented with phosphatase and protease inhibitors. Homogenates were subjected to 10% SDS-PAGE and immunoblotted. The following primary antibodies were obtained from Cell Signaling Technology (Danvers, MA): GSK-3β (Catalog #9315) and pSer9-pGSK-3β (Catalog #5558), rabbit monoclonal GYS (Catalog #3886), and rabbit monoclonal, pSer641/640-GS (Catalog #3891). According to Cell Signaling Technology, this phospho-GYS antibody (Catalog #891) works to detect both muscle (pSer640) and liver (pSer641) signals.

2.8. Statistical analysis

Statistics were performed with PRISM 8 (version 8.4.3) software (San Diego, CA). Data were analyzed using either unpaired two-tailed Student's *t*-test for comparison of two groups or one-way or two-way analysis of variance (ANOVA) for comparison of more than two groups followed by Tukey's *post hoc* test if appropriate. All data are presented as mean ± SEM and significance were assumed when $p<0.05$.

3. RESULTS

3.1. CST induces hepatic glycogen synthesis in an insulin-independent pathway.

One of the major pathways by which liver removes glucose after a meal is through insulin-induced conversion of glucose into glycogen (glycogenesis) [10; 11]. This is reflected by the presence of >3.5-fold more glycogen in fed liver compared to fasted liver (Fig.1A&B). Since CST decreases hyperglycemia in DIO mice [41], we asked whether CST promotes glycogenesis in the postprandial state (with high insulin level) to reduce hyperglycemia. We found that chronic treatment of WT-NCD mice with CST resulted in a >2-fold increase in liver glycogen content in fed state (Fig. 1A). In the fasted state, insulin secretion is downregulated, leading to inhibition of glycogen synthesis and activation of glycogen breakdown (glycogenolysis). Since CST promoted glycogenesis in the fed state, we asked whether CST can maintain glycogen stores in the fasted state. Despite low plasma insulin in fasted state (Fig. 1D), chronic CST treatment of fasted WT-NCD mice caused a >5-fold increase in liver glycogen content (Fig. 1B). Administration of both CST and insulin resulted in an additive effect on hepatic glycogen levels (Fig. 1B), indicating that CST and insulin use divergent signaling pathways to induce hepatic glycogen synthesis.

We also measured the acute effect of insulin (30 min treatment) on liver glycogen content and plasma catecholamine levels in fed and fasted lean WT-NCD mice. In the fed state, exogenous administration of insulin caused a small (>1.13-fold) but significant increase in liver glycogen content without affecting the plasma catecholamines (Figs. 1C and 2A&B). In contrast in the fasted state, insulin not only increased liver glycogen content by >6-fold, but also increased plasma catecholamines (Figs. 1C and 2A&B) indicating that insulin inhibition of glycogenolysis dominates over catecholamine induction of glycogenolysis.

Since glycogenolysis, controlled by the counterregulatory hormones such as glucagon, NE and EPI [51], helps the liver to maintain blood glucose concentrations during the early stage of fasting, we measured plasma counterregulatory hormones and found decreased plasma NE and EPI levels upon CST treatment level in both fed and fasted state, but no changes in glucagon levels (Fig. 2C&D). These findings indicate that one of the mechanisms by which CST restores and increases hepatic glycogen content in the early stage of fasting is by inhibiting catecholamine-induced glycogenolysis. Interestingly, acute insulin treatment (for 30 minutes) raised plasma NE and EPI levels (Fig. 2A&B) only in the fasted state, not in the fed state. Exogenously administered insulin therefore seems to play a dual role in the fasting state to maintain glucose levels, stimulating gluconeogenesis by catecholamines but also promoting glycogenesis (Fig. 1C) at the same time.

Since the glycogenic function of liver is compromised in T2D [19; 20], we tested the effects of CST on the hepatic glycogen content in insulin-resistant DIO mice. Chronic treatment of DIO mice

with CST caused a >1.5-fold increase in liver glycogen content (Fig. 3A). Unlike in lean mice, CST caused a decrease in only NE levels with no changes in EPI and glucagon levels in fed DIO mice (Fig. 3B). In the fasted state, CST treatment increased liver glycogen content more than insulin in DIO mice: >3-fold increase by CST and >1.7-fold increase by insulin (Fig. 3C). Thus, CST was more effective in inducing glycogenesis than insulin in DIO mice. Like NCD mice, the effects of combined CST and insulin in hepatic glycogen content were additive, indicating that both promote glycogenesis via divergent and synergistic pathways (Fig. 3C). In fasted mice, the effects of CST on counterregulatory hormones were comparable between NCD and DIO mice (Fig. 2D&3D). The important point here is that insulin-induced glycogen synthesis is reduced in DIO mice, but CST is equally effective in lean or DIO mice (Figs. 1B&3C).

3.2. CST-KO mice display lower hepatic glycogen content compared to WT mice that is elevated by CST supplementation.

To gain a better understanding of the role CST in hepatic glucose homeostasis we examined CST-KO mice, which are genetically obese and insulin-resistant [41]. Hepatic glycogen content in fed CST-KO mice was lower than in WT mice (Fig. 4A&1A). Chronic treatment of CST-KO mice with CST resulted in ~3-fold increase in hepatic glycogen content (Fig. 4A). Plasma glucagon levels in CST-KO mice were higher than WT mice and were lowered after CST treatment (Figs. 2C&D and 5A&B) Despite the presence of higher plasma insulin levels in the fasted state (>2.4-fold higher than WT mice) (Fig. 4D&1D), fasted CST-KO mice had a lower hepatic glycogen content than fasted WT mice (Fig. 4B&1B). Chronic supplementation of CST-KO mice with CST resulted in >4.2-fold increase in hepatic glycogen content in fasted mice (Fig. 4B). These effects of CST correlated with decreased levels of plasma counterregulatory hormones (Fig. 5A&B).

We also tested the acute effects of insulin on hepatic glycogen content and plasma catecholamines in fed and fasted CST-KO mice to dissect the role of plasma catecholamines in CST-induced glucose homeostasis in the liver. In the fed state, insulin failed to increase the hepatic glycogen content (Fig. 4C), supporting the hepatic insulin resistance in CST-KO mice [41]. In the fasted state, insulin caused a ~1.4-fold increase in liver glycogen content in CST-KO mice (Fig. 4C) as compared to ~6.5-fold increase in WT-NCD mice (Fig. 1C). This reduced liver glycogen content in fasted CST-KO mice is possibly caused by the decreased insulin sensitivity despite a reduction of plasma NE levels (Fig. 5C).

Like in WT mice, CST treatment reduced plasma NE and EPI levels in both fed and fasting CST-KO mice (Fig. 5A&B). However, CST treatment of CST-KO mice also lowered glucagon levels (Fig. 5A&B), suggesting that CST might have aided glycogen accumulation by reducing glucagon-

induced glycogenolysis. Acute insulin treatment only reduced NE (in contrast to WT mice) in fasted CST-KO mice (Fig. 5C) but had no effects on NE or EPI in fed mice (Fig. 5C&D).

3.3. CST enhances glycogen synthesis and suppresses glycogenolysis

To confirm the *in vivo* studies and assess the direct effect of CST on glycogenesis and glycogenolysis on hepatocytes, we measured glycogen synthesis from radiolabeled glucose and glycogenolysis from prelabeled stored glycogen in primary hepatocytes cultured *ex vivo*. We found that CST and insulin alone caused a modest (>1.4-fold by CST; ~1.8-fold by insulin) increase in glucose incorporation into glycogen and in combination resulted in >2.6-fold increase (Fig. 6A), consistent with a previous report [12]. We also found that counterregulatory hormones NE + EPI caused a ~2.3-fold increase in glucose phosphate formation from prelabeled glycogen which was inhibited by both CST and insulin (Fig. 6B), indicating that both CST and insulin can induce glycogen synthesis and inhibit catecholamine-induced glycogenolysis.

3.4. CST increases G6P and UDPG levels

After entry of glucose into hepatocytes, glucose is immediately phosphorylated to G6P. G6P can then be converted to G1P by PGM, and then to UDPG by UDP-glucose pyrophosphorylase to provide the substrate for glycogen synthesis. Glycogen synthase (GYS) enzyme activities including GYS2 (liver GYS) catalyzes the transfer of glucosyl units from UDPG to glycogen by synthesis of α-1,4 bonds. A strong positive correlation exists between activation of GYS and intracellular G6P [52; 53]. Upon binding to GYS, G6P causes allosteric activation of the enzyme through a conformational rearrangement that simultaneously converts it into a better substrate for protein phosphatases [54-56]. Dephosphorylation results in activation of GYS.

CST increased G6P levels in both fed (>1.68-fold) and fasted (>1.88-fold) WT-NCD mice (Fig. 7A). In contrast, insulin had no effect on G6P levels. Unlike NCD mice, neither CST nor insulin caused a significant increase in G6P levels in both the fed and fasted state in DIO mice. The combined treatments of CST and insulin, however, resulted in a significant increase in G6P levels in both fed (>1.45-fold) and fasted (>1.28-fold) DIO mice (Fig. 7B). In DIO mice, the G6P levels in the fed state was 1.5-fold higher than the fasted levels (Fig. 7B). Interestingly, the fasted DIO mice showed decreased UDPG levels (by 27%) compared to fasted NCD mice (Fig. 7C) without lowering glycogen content (Fig. 1C&2C). Although UDPG levels were increased in fasted NCD-fed WT mice by both CST (>1.67-fold) and insulin (>1.71-fold), only CST increased UDPG levels in fasted DIO mice (Fig. 7C)

3.5. CST increases hepatic glycogen production by augmenting PGM activity

PGM is a key enzyme that determines the fate of G6P by directing it away from gluconeogenesis and glycolysis towards G1P and glyconeogenesis [57] (Fig. 7D). Hyperglycemia increases the flux through G6P, G1P and UDPG to glycogen (via GYS2 in liver) [58; 59]. Glycogen phosphorylase acts in the other direction during fasting [60]. PGM catalyzes a reversible reaction that is controlled by local G6P or G1P levels. We measured intrinsic PGM activity using a commercial kit that relies on the reverse reaction (G1P to G6P) in the presence of saturating concentration of G1P, and measures G6P (Fig. 7D). As the reaction is reversible, at equilibrium the forward reaction rate is the same as the reverse reaction. We found attenuated PGM activity in fasted WT-NCD mice in response to CST, compared to saline treatment (Fig. 7E). In contrast, CST did not modulate PGM activity in fed WT-NCD mice (Fig. 7E). Like fed WT-NCD mice, CST also diminished PGM activity compared to saline in fasted WT-DIO mice but did not modulate PGM activity in fed WT-DIO mice (Fig. 7F).

3.6. CST augments GYS activity by inactivating GSK-3β through phosphorylation.

GYS activity is controlled by covalent modification of the enzyme complex [61], allosteric activation by G6P [55; 62], and enzymatic translocation by insulin [63]. Therefore, we measured GYS activity by following incorporation of UDP-^{14}C-glucose into glycogen in presence and absence of saturating concentrations of G6P.

Basal activation of GYS in fed WT-NCD and WT-DIO mice was much higher than in fasted mice (Fig. 8A&B). CST did not modulate GS activity in fed WT-NCD or WT-DIO mice but did activate GYS activity in the fasted state (Fig. 8A&B).

In addition to the allosteric activation of GYS by G6P, GYS is regulated by multi-site phosphorylation which causes inactivation. GYS2 is phosphorylated on nine serine residues [64; 65], by kinases such as GS kinase (GSK)-3β, which, in turn is inactivated by phosphorylation at Ser9 site by PKA, AKT and p90RSK and activated by Tyr216 by autophosphorylation [66-69]. Phosphorylation of GYS2 results in its progressive inactivation and decreased sensitivity to allosteric activators [70]. GYS2 dephosphorylation is regulated by hormones like insulin and is mediated by GYS2 phosphatase like protein phosphatase 1 [55].

Immunoblotting revealed that in fasted WT-NCD or WT-DIO mice, CST and insulin alone increased GSK-3β Ser9 phosphorylation (Fig. 8C&D). The combination of CST and insulin was additive in WT-DIO mice but not in WT-NCD mice (Fig. 8C&D). Serine 641 is one of the GSK-3β target sites in GYS2 so we also measured Ser641 phosphorylation by immunoblotting. In fasted lean WT-NCD mice, CST and insulin alone caused dephosphorylation of Ser641 and in combination caused a small additive effect (Fig. 8C&E). However, in WT-DIO mice, neither CST nor insulin reduced Ser641 phosphorylation, but the combination did (Fig. 8C&E). In summary, CST increases glycogen

production in liver by increasing G6P levels, increasing phosphorylation of GSK-3β, and dephosphorylation of GYS2.

4. DISCUSSION

In the postprandial state, a major pathway that contributes to the removal of glucose from the portal vein by the liver is conversion of glucose into glycogen [71; 72] and defects in hepatic glycogen synthesis contribute to postprandial hyperglycemia in patients with poorly controlled insulin-dependent diabetes mellitus [15].

The present study shows that CST induced hepatic glycogenesis in both the fasted and fed state in both lean and DIO mice, but insulin was only able to stimulate glycogenesis in the fasted state. Insulin control of hepatic glycogenesis is mediated by induction of glucokinase, activation of GYS2 and suppression of glycogen phosphorylase. Insulin was not able to increase G6P levels in either the fed or fasted state, but CST caused an almost doubling of G6P levels. In the fed state, PGM and GYS activity are high, so the G6P can be efficiently converted to G1P and hence glycogen, and these enzymes are not stimulated by CST. However, likely the elevated G6P allosterically activates GYS to allow greater glycogen synthesis with CST in the fed state [52; 54; 55]. In the fasted state, basal PGM and GYS2 activities are lower, but PGM is further suppressed by CST while GYS2 activity is increased, thus CST spares glycogen stores in the fasted state and reduces HGP. In the early stage of fasting, CST or insulin alone induced >5-fold increase in hepatic glycogen content and in combination caused an additive effect, indicating that CST and insulin use divergent signaling pathways to induce hepatic glycogenesis in the postabsorptive state.

Part of the CST effect *in vivo* could be indirect through changes in catecholamines. Indeed, CST decreased the levels of plasma catecholamines in the fed state, while insulin had no effect. However, CST did not change plasma glucagon levels, indicating that CST restores and increases hepatic glycogen content in the early stage of fasting is by inhibiting catecholamine-induced glycogenolysis. In contrast, insulin-induced hepatic glycogenesis is associated with increased plasma catecholamines in the fasted mice. The contribution of catecholamine-indued glycogenolysis to glucose production is well known [25; 73]. Our results in hepatocytes showed that CST and insulin inhibits catecholamine-induced glycogenolysis. Although, under stressed condition (starvation), insulin elevates catecholamine levels, its inhibitory effects on glycogenolysis may still dominate allowing accumulation of glycogen. In hepatocytes, both glucose and insulin suppress glycogenolysis by inhibiting glycogen phosphorylase a, in a process possibly involving protein phosphatase 1 [74]. Protein phosphatase 1 inactivates glycogen phosphorylase via dephosphorylation of phosphorylase kinase (phosphorylated by PKA) as well as activates GYS2 by dephosphorylation [75]. The net result of this is increased glycogen accumulation. Suppression of catecholamine levels by CST can have

dual indirect impacts as it could reduce both the gluconeogenic as well as the glycogenolytic effects of catecholamines [73].

Studies in humans show a dichotomy of EPI and NE responses during the oral glucose tolerance test: decreased plasma EPI and increased plasma NE [76]. The authors did not find a correlation between changed NE and EPI with glucose tolerance in mice. CST deficiency in CST-KO mice creates glucose intolerance and insulin resistance even in NCD-fed CST-KO mice. As would be predicted, CST-KO mice stored less glycogen in liver than WT mice. In the absence of CST, elevated pancreatic glucagon and higher plasma NE may contribute to the glucose intolerance which could be corrected by insulin or CST.

Although the glycogenic function of liver has been shown to be compromised in T2D subjects [19; 20], CST increased hepatic glycogen content in fed DIO mice, and was more effective than insulin in inducing hepatic glycogenesis in fasted DIO mice. These findings indicate that increased hepatic glycogenesis in both fed and fasted DIO mice by CST may be due to the increased insulin sensitivity that we have reported earlier [41]. Besides indirect effects through catecholamines, CST seems to exert a direct effect on glycogenesis in hepatocytes. Both CST and insulin inhibit glycogenolysis in isolated hepatocytes but there is no additive effect suggesting a common signaling pathway. In contrast, CST and insulin have an additive effect on glycogen accumulation that may indicate a divergence of CST and insulin effects at the level of glycogen synthesis. The fact that CST alone stimulates glycogen synthesis in insulin-resistant DIO mice and in isolated hepatocytes, supports a direct, insulin-independent regulatory role of CST in glycogenesis. CST treatment increased cellular G6P levels which is consistent with our previous finding that CST suppresses of expression of the *G6pc* gene [41] thus reducing hydrolysis of G6P back to glucose. The present findings demonstrated that G6P was also directed by CST into the glycogenic pathway by stimulation of GYS and inhibition of glycogenolysis. We have previously shown that CST-KO mice are glucose intolerant [41], and we show here that the CST-KO mice have a reduced ability to convert glucose to glycogen owing to lack of CST.

We have shown here three ways CST causes glycogen accumulation. The first is to raise substrate levels (G6P and UDPG), one of which (G6P) is a known allosteric activator of GYS. The second is to enhance dephosphorylation of GYS by phosphorylating GSK-3β, and the third is to inhibit glycogenolysis. The insulin-independent stimulatory effect of CST in hepatocytes on glycogenesis but not glycogenolysis suggests that stimulation of glycogenesis by CST is the major pathway through which CST controls hepatic glucose production and improves glucose intolerance in obese mice. Given these characteristics, CST could be a new therapeutic peptide to treat both glucose intolerance and inflammation (in diabetes) as well as hypertension (due to its anti-adrenergic activity).

5. CONCLUSION

CST improves glucose tolerance in obese mice by three mechanisms: (i) reduced HGP from G6P, (ii) increased glycogen synthesis from G6P via formation of UDPG, and (iii) reduced glycogen breakdown (due to suppression of plasma catecholamine levels). UDPG formation was facilitated by increased GYS and PGM activities. The net result is reduced free available glucose. These conclusions were verified in CST-KO mice and in hepatocyte cultures.

AUTHOR CONTRIBUTIONS

GB researched the data, contributed to the design and discussion, and wrote the manuscript. KT generated the data, contributed to the design and discussion. NJW and GvdB edited the MS and provided intellectual inputs. SKM conceived the idea, generated glycogen data, supervised the studies, analyzed the data and made the graphics, wrote the MS, provided financial support. GB and SKM are the guarantor of this study.

DATA AVAILABILITY

The datasets generated during this study are available from the corresponding author on reasonable request.

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CONFLICT OF INTEREST

The authors declare no competing interests.

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Figure Legends

Figure 1. Effects of chronic CST and acute insulin treatments on hepatic glycogen contents in both fed and fasted lean WT mice. Mice were treated with CST (2 μg/g body weight/day for 15 days; intraperitoneal) and acutely (30 min) with saline or insulin (0.4 mU/g body weight; intraperitoneal) in fasted (12 hr) mice before harvesting tissues under deep anesthesia. (A) Glycogen content in fed WT-NCD mice (n=31-43); (B) in fasted WT-NCD mice (n=7-14); (C) Glycogen content in fed and fasted WT-NCD mice (n=27-47); (D) Plasma insulin levels in fed and fasted WT-NCD mice (n=7-12). ***p<0.001; ns, not significant.

Figure 2. Effects of chronic CST and acute insulin treatments on counterregulatory hormone levels in plasma in both fed and fasted lean WT mice. Mice were treated with CST (2 μg/g body weight/day for 15 days; intraperitoneal) and acutely (30 min) with saline or insulin (0.4 mU/g body weight; intraperitoneal) in fasted (12 hr) and fed mice before harvesting tissues under deep anesthesia. NE, EPI and glucagon levels in fed (A) and fasted (B) WT-NCD mice. NE (C) and EPI (D) levels in saline and insulin treated fed and fasted WT-NCD mice. **p<0.01; ***p<0.001; ns, not significant.

Figure 3. CST increases hepatic glycogen content in both fed and fasted obese WT mice coupled with decreased NE level in fed and NE and EPI in fasted mice. Mice were fed 60% high fat diet for 16 weeks. Diet-induced obese (DIO) mice were treated with CST (2 μg/g body weight/day for 15 days; intraperitoneal) and acutely (15 min) with saline or insulin (0.4 mU/g body weight; intraperitoneal) in fasted mice before harvesting tissues under deep anesthesia. (A) Glycogen in fed obese mice (n=16-28); (B) Counterregulatory hormones in fed WT-DIO mice (n=9-27); (C) Glycogen in fasted WT-DIO mice (n=16-28); (D) Counterregulatory hormones in fasted WT-DIO mice (n=9-27). ***p<0.001.

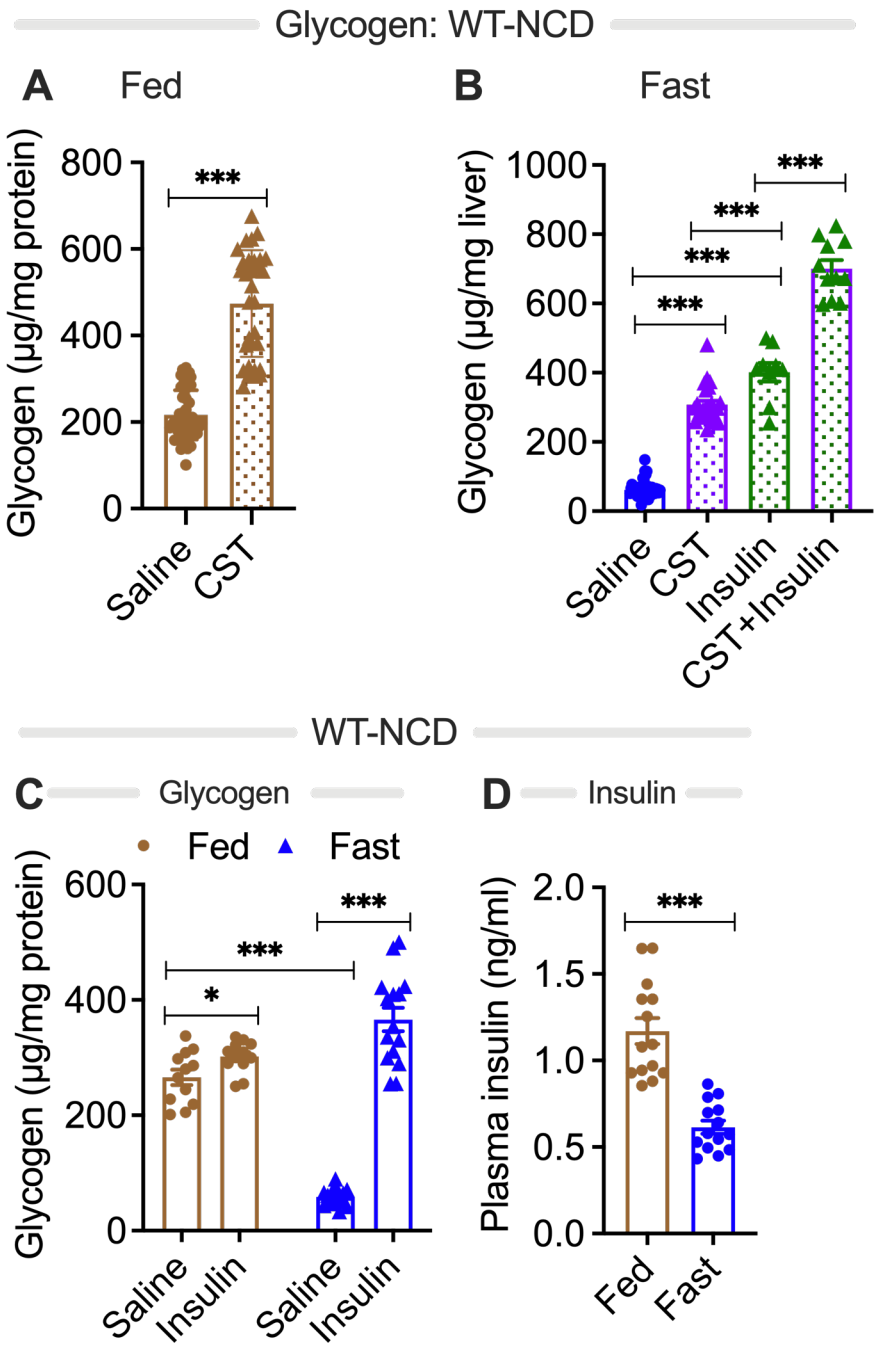
Figure 4. Supplementation of NCD-fed CST-KO mice with CST and insulin induces glycogen synthesis in both fed and fasted mice. WT-NCD mice were injected with CST (2 μg/g body weight/day for 15 days; intraperitoneal) and insulin (o.4 mU/g body weight) for 30 minutes. Tissues were harvested from fed or fasted (12 hr) mice under deep anesthesia. (A) Glycogen in fed CST-KO-NCD mice (n=14-16); and (B) fasted CST-KO-NCD mice (n=6-16) after CST treatment; (C) Glycogen in fasted and fed CST-KO-NCD mice (n=19-22); after insulin treatment (D) Plasma insulin levels in fasted and fed CST-KO-NCD mice. *p<0.05; ***p<0.001 ns, not significant.

Figure 5. Effects of chronic **CST and acute insulin treatments on counterregulatory hormone levels in plasma of both fed and fasted lean CST-KO mice.** CST-KO-NCD mice were treated with CST (2 μg/g body weight/day for 15 days; intraperitoneal) and acutely (15 min or 30 min) with saline or insulin (0.4 mU/g body weight; intraperitoneal) in fasted (12 hr) and fed mice before harvesting tissues under deep anesthesia. NE, EPI and glucagon levels in fed (A) and fasted (B) CST-KO-NCD mice. NE (C) and EPI (D) levels in saline and insulin treated fed and fasted CST-KO-NCD mice. *p<0.05; ***p<0.001, ns, not significant.

Figure 6. CST induces glycogenesis and inhibits catecholamine-induced glycogenolysis in lean hepatocytes. (A) Glycogenesis from 14C-glucose in cultured primary hepatocytes (n=6). (B) Glycogenolysis from preloaded radiolabeled glycogen (n=5). *p<0.05; ***p<0.001.

Figure 7. CST increases hepatic G6P and UDPG in fed and fasted lean WT mice as well as UDPG in fasted lean and obese WT mice. (A) G6P levels in liver of fed and fasted WT-NCD mice (n=6). (B) G6P levels in liver of fed and fasted WT-DIO mice (n=6). (C) UDPG in fed and fasted WT-NCD and WT-DIO mice (n=4). UDPG was measured by a UPLC method using UV detector at a UCSD core facility. (D) Schematic diagram showing determination of PGM activity by measuring G6P and G1P in reactions 1 & 2. (E) PGM activity in fed and fasted WT-NCD mice (n=6); (F) PGM activity in fed and fasted WT-DIO mice (n=6). Tissues were from the same experiments shown in Figs.1 & 2. *p<0.05; **p<0.01; ***p<0.001; ns, not significant.

Figure 8. CST augments hepatic GYS activity in fasted lean and obese WT mice coupled with decreased GSK-3β activity. (A) GYS activities in fed and fasted WT-NCD mice. For GS activity, activation ratio in presence or absence of G6P was measured (n=6); (B) GYS activities in fed and fasted WT-DIO mice (n=6); (C) Immunoblots showing total and phosphorylated signals for GSK-3β and GYS2 in WT-NCD and WT-DIO mice; (D) Densitometry values of GSK-3β shown in panel (C) (n=4); (E) Densitometry values of GYS2 shown in panel (C) (n=4). Tissues were from the same experiments shown in Figs.1 & 2. *p<0.05; ***p<0.001; ns, not significant.


Glycogen: WT-NCD
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Fed
Glycogen (µg/mg protein)
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Glycogen (µg/mg liver)
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Figure 1

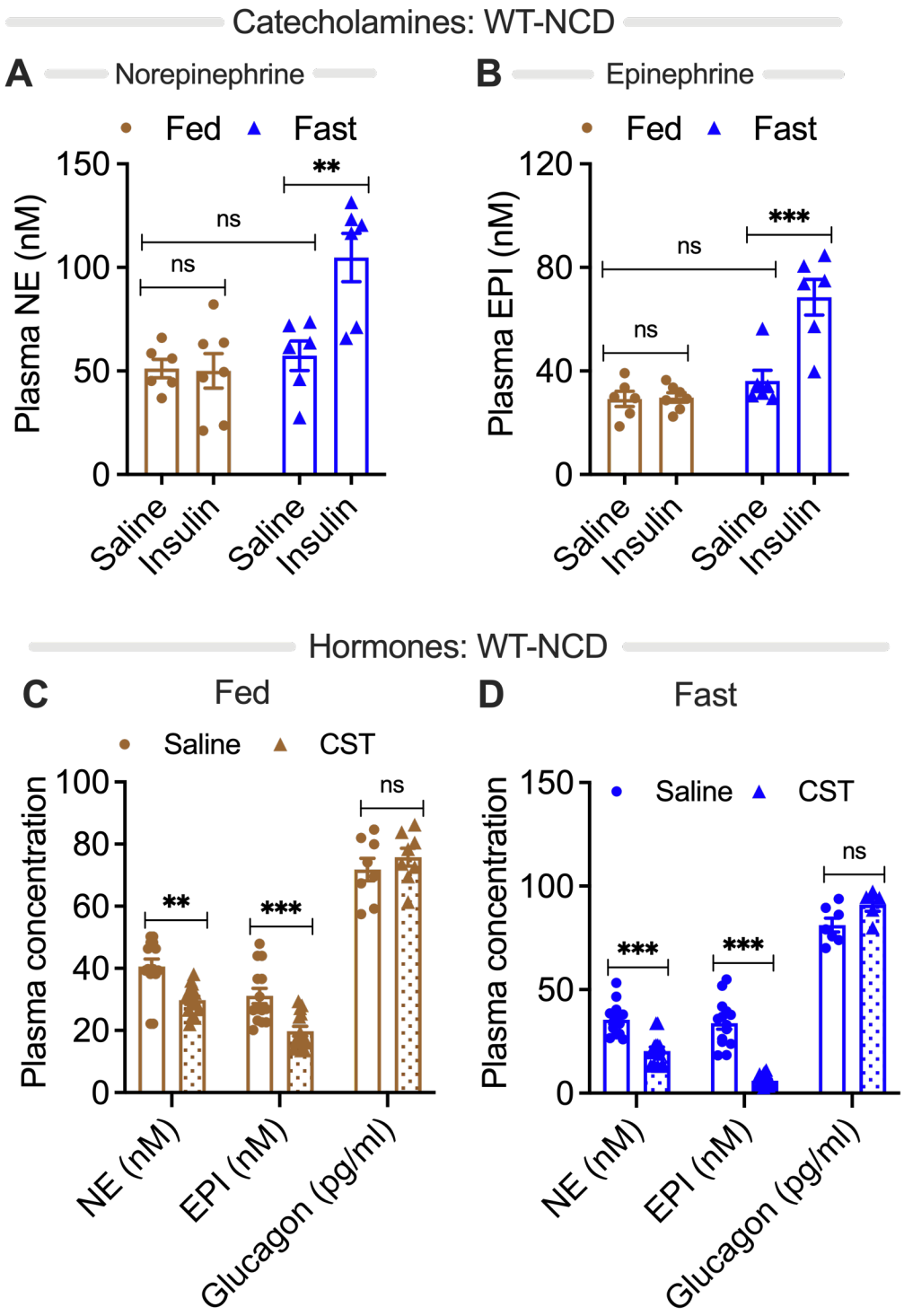
Catecholamines: WT-NCD
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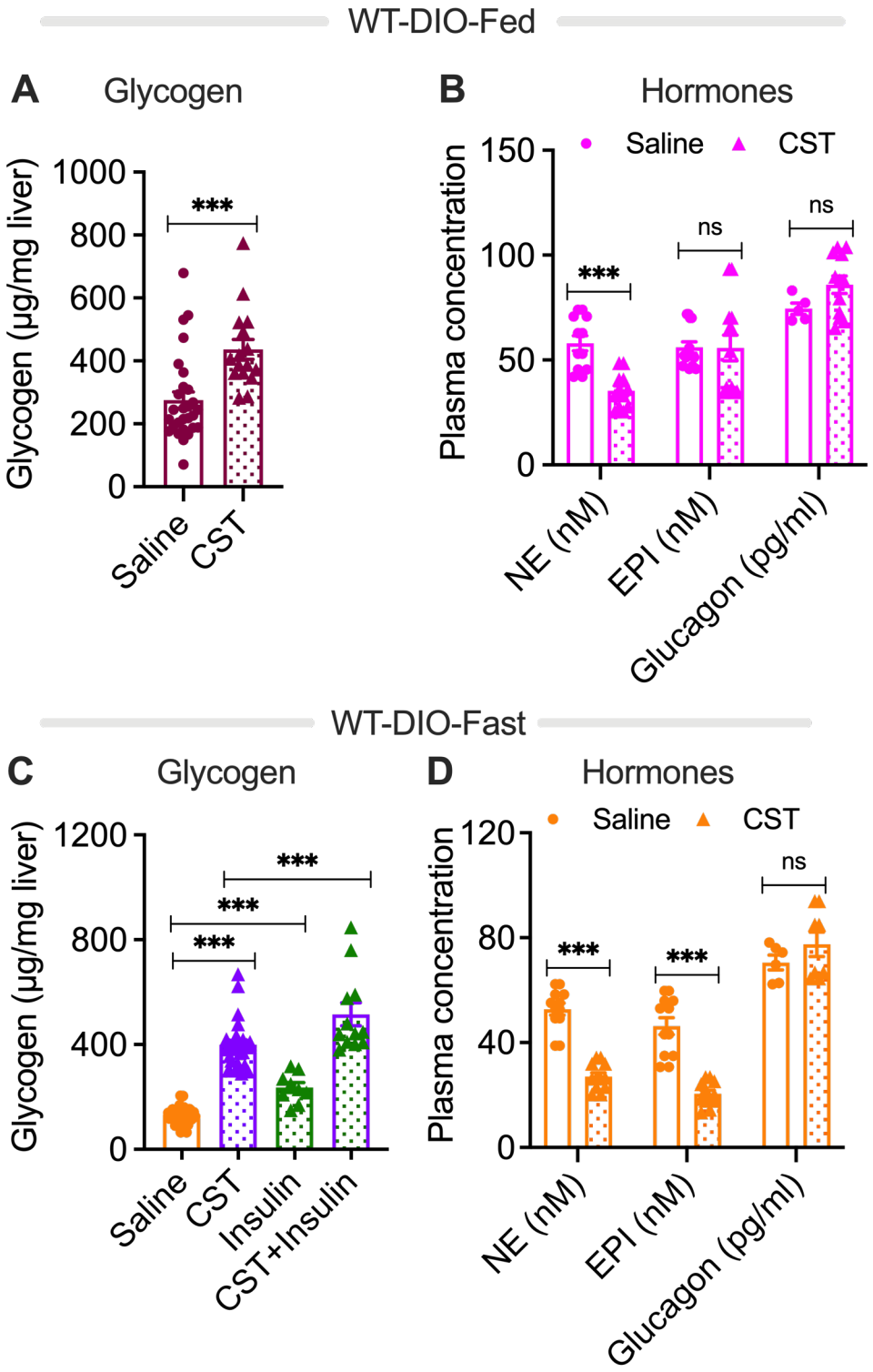
Figure 2


WT-DIO-Fed
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Glycogen
Glycogen (µg/mg liver)
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Hormones
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Plasma concentration
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NE (nM)
EPI (nM)
Glucagon (pg/ml)
WT-DIO-Fast
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Glycogen
Glycogen (µg/mg liver)
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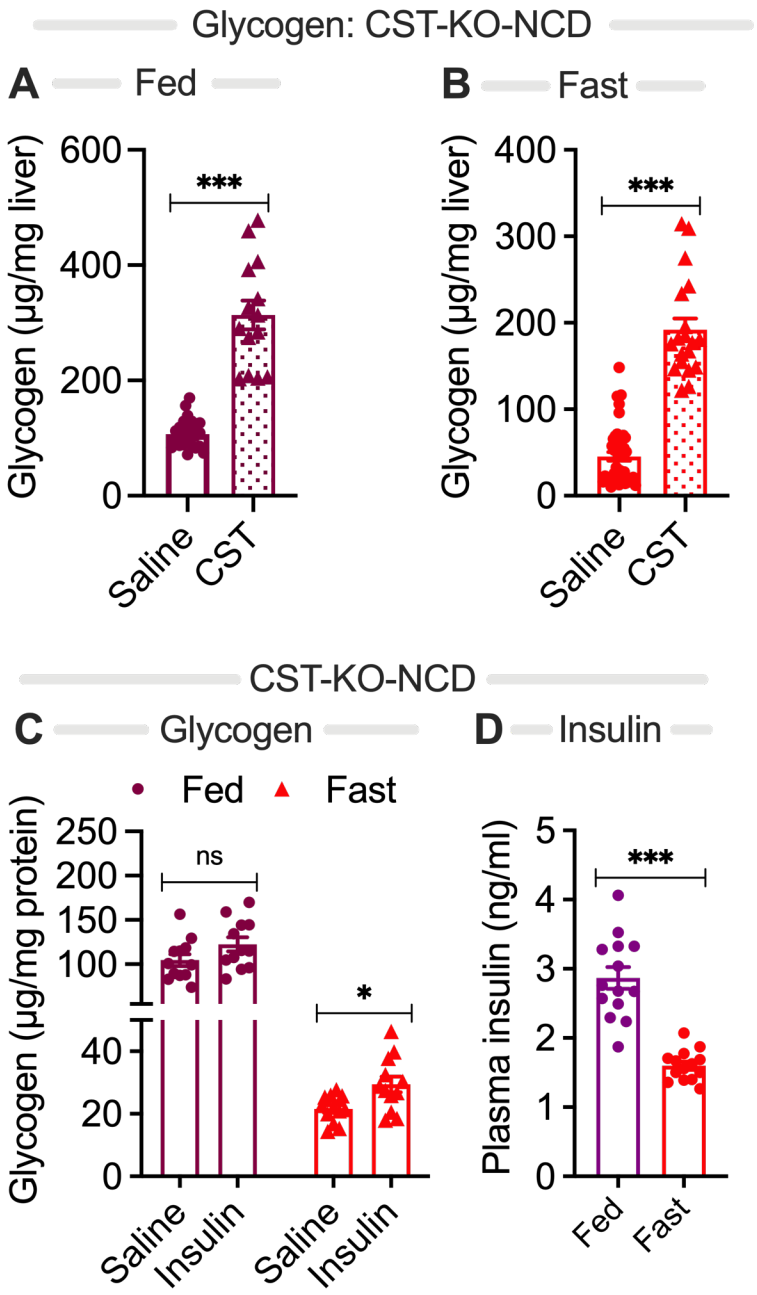
Figure 3


Glycogen: CST-KO-NCD
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Glycogen (µg/mg protein)
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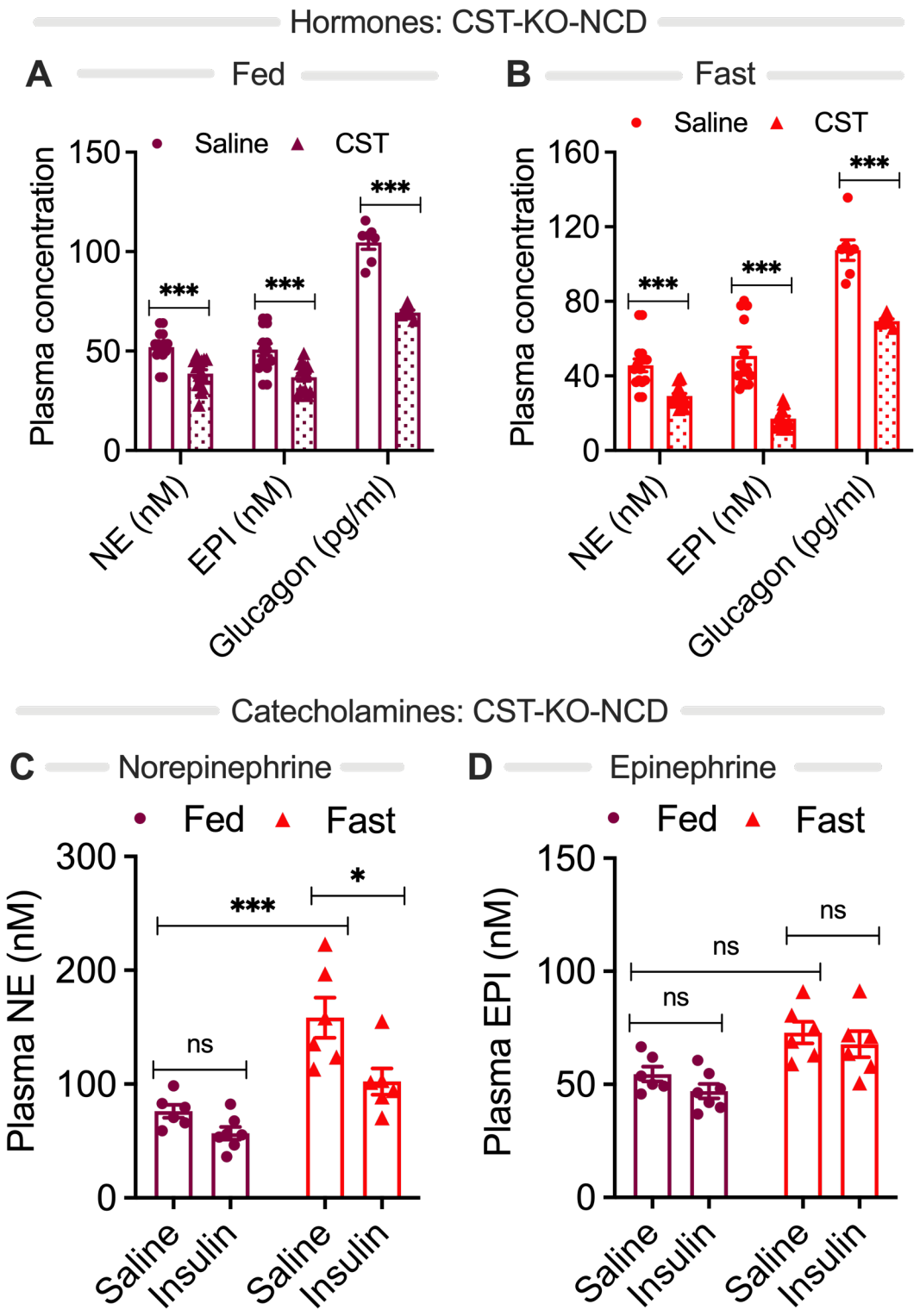
Figure 4


Hormones: CST-KO-NCD
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Plasma concentration
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NE (nM)
EPI (nM)
Glucagon (pg/ml)
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Saline
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Plasma concentration
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NE (nM)
EPI (nM)
Glucagon (pg/ml)
Catecholamines: CST-KO-NCD
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Norepinephrine
Fed
Fast
Plasma NE (nM)
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***
*
ns
Saline
Insulin
Saline
Insulin
D
Epinephrine
Fed
Fast
Plasma EPI (nM)
150
100
50
0
ns
ns
ns
Saline
Insulin
Saline
Insulin

Figure 5

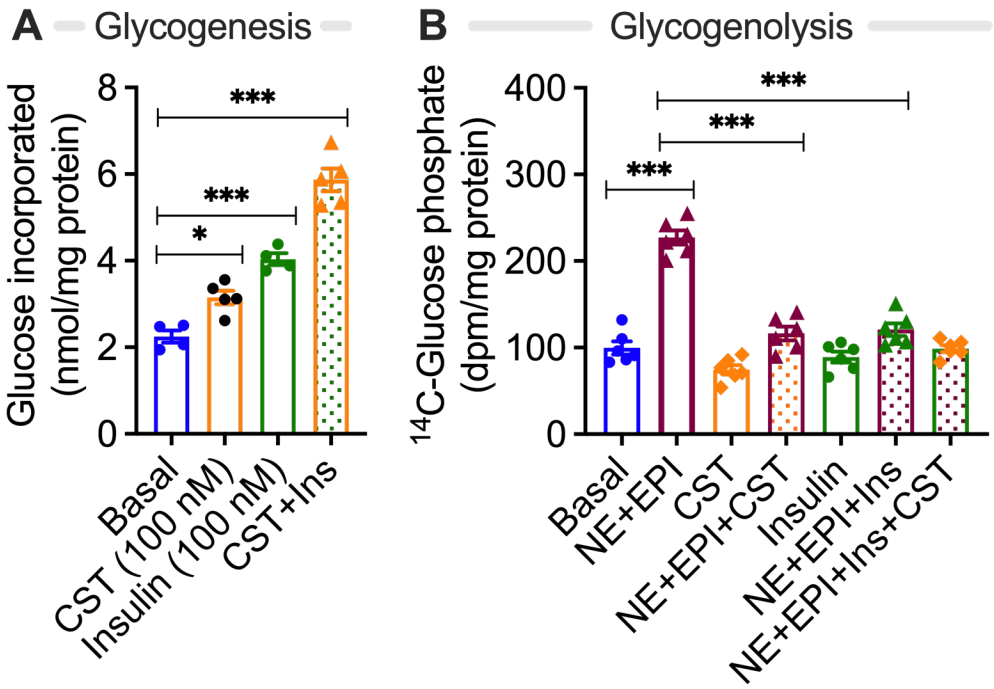
A
Glycogenesis
Glucose incorporated (nmol/mg protein)
8
6
4
2
0
***
***
*
Basal
CST (100 nM)
Insulin (100 nM)
CST+Ins
B
Glycogenolysis
14C-Glucose phosphate (dpm/mg protein)
400
300
200
100
0
***
***
***
Basal
NE+EPI
CST
NE+EPI+CST
Insulin
NE+EPI+Ins
NE+EPI+Ins+CST

Figure 6

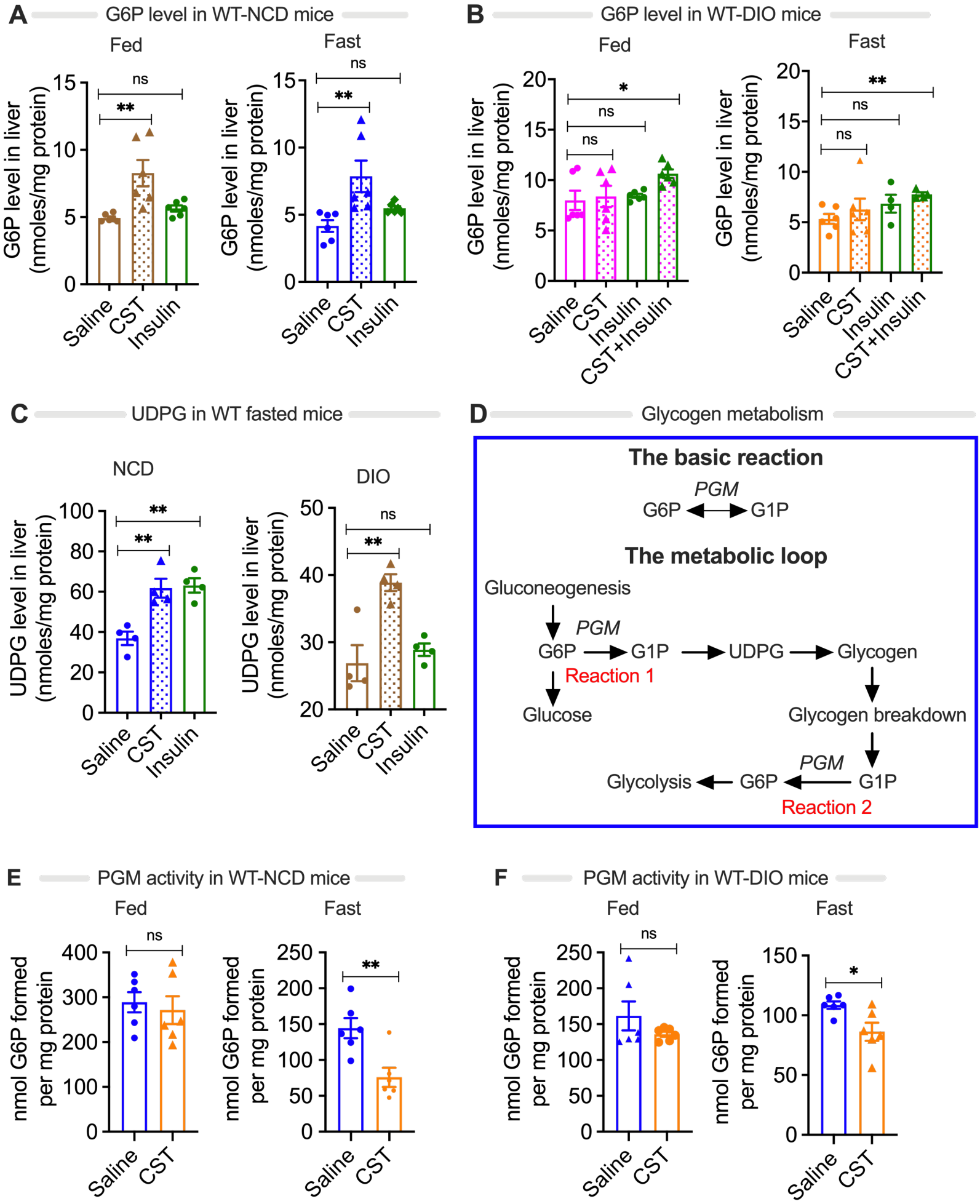
A
G6P level in WT-NCD mice
Fed
Fast
G6P level in liver (nmoles/mg protein)
ns
**
Saline
CST
Insulin
B
G6P level in WT-DIO mice
Fed
Fast
*
ns
**
CST+Insulin
C
UDPG in WT fasted mice
NCD
DIO
UDPG level in liver (nmoles/mg protein)
D
Glycogen metabolism
The basic reaction
PGM
G6P
G1P
The metabolic loop
Gluconeogenesis
Reaction 1
UDPG
Glycogen
Glucose
Glycogen breakdown
Glycolysis
Reaction 2
E
PGM activity in WT-NCD mice
nmol G6P formed per mg protein
F
PGM activity in WT-DIO mice

Figure 7

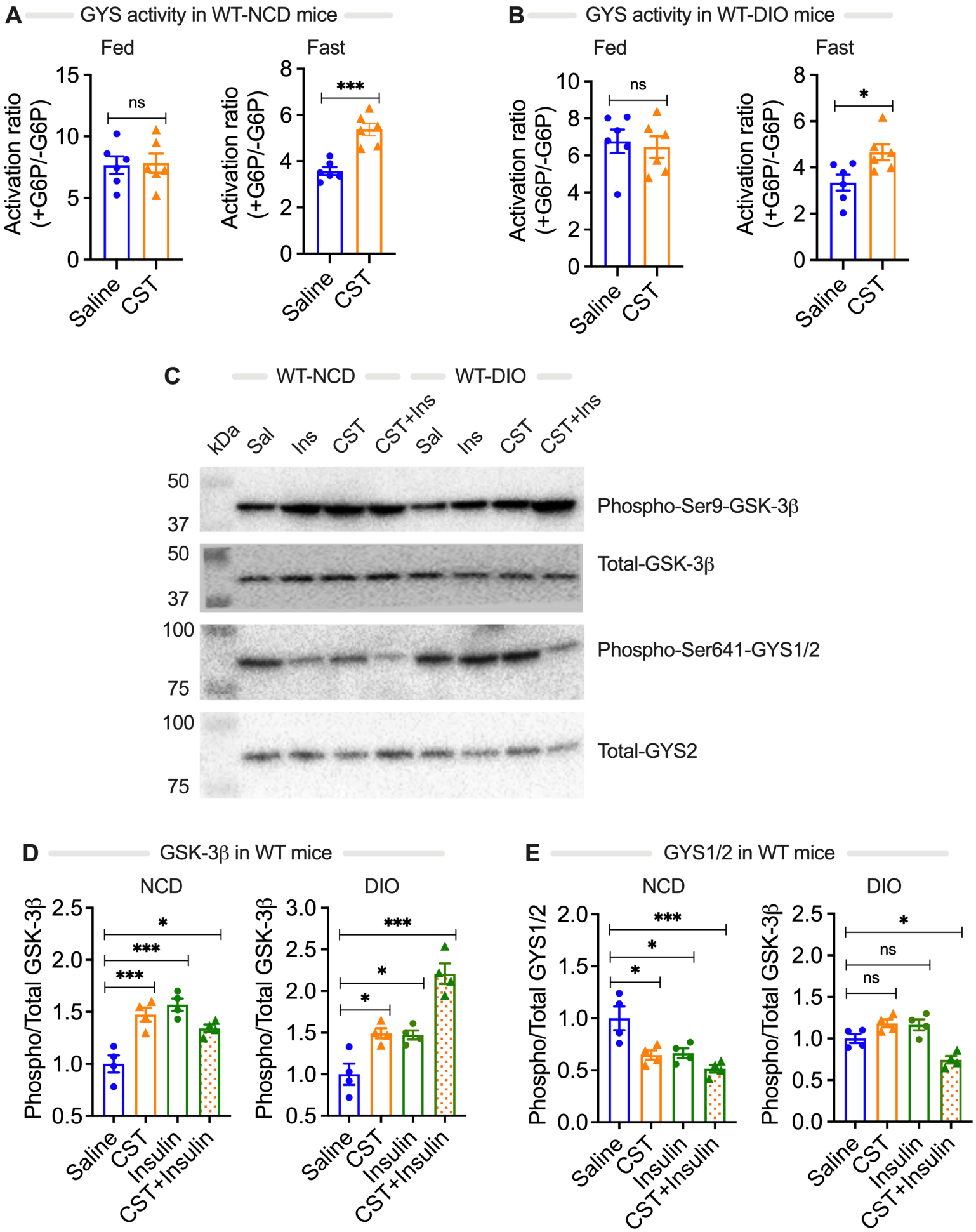
A
GYS activity in WT-NCD mice
Fed
Fast
Activation ratio (+G6P/-G6P)
ns
***
Saline
CST
B
GYS activity in WT-DIO mice
Fed
Fast
ns
*
C
WT-NCD
WT-DIO
kDa
Sal
Ins
CST
CST+Ins
Phospho-Ser9-GSK-3β
Total-GSK-3β
Phospho-Ser641-GYS1/2
Total-GYS2
D
GSK-3β in WT mice
NCD
DIO
Phospho/Total GSK-3β
Saline
CST
Insulin
CST+Insulin
E
GYS1/2 in WT mice
Phospho/Total GYS1/2

Figure 8