Pancreastatin-dependent inflammatory signaling mediates obesity-induced insulin resistance

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ABSTRACT

Chromogranin A knockout (Chga-KO) mice exhibit enhanced insulin sensitivity despite obesity. Here we probed the role of the Chromogranin A-derived peptide pancreastatin (PST: CHGA_{273-301}), by investigating the effect of diet-induced obesity (DIO) on insulin sensitivity of these mice. We found that on a high fat diet (HFD), Chga-KO mice (KO-DIO) remain more insulin sensitive than wild-type DIO (WT-DIO) mice. Concomitant with this phenotype is enhanced Akt and AMPK signaling in muscle and white adipose tissue (WAT) as well as increased FoxO1 phosphorylation and expression of mature Srebp-1c in liver and downregulation of the hepatic gluconeogenic genes, PePck and G6pase. KO-DIO mice also exhibited downregulation of cytokines and pro-inflammatory genes and upregulation of anti-inflammatory genes in WAT, and peritoneal macrophages from KO mice displayed similarly reduced pro-inflammatory gene expression. The insulin-sensitive, anti-inflammatory phenotype of KO-DIO mice is masked by supplementing PST. Conversely, a PST variant peptide PSTv1 (PST-NΔ3: CHGA_{276-301}), lacking PST activity, simulated the KO phenotype by sensitizing WT-DIO mice to insulin. In summary, the reduced inflammation due to PST deficiency prevented the development of insulin resistance in KO-DIO mice. Thus, obesity manifests insulin resistance only in the presence of PST, and in its absence obesity is dissociated from insulin resistance.
INTRODUCTION

The Chromogranin A (human CHGA/mouse Chga) proprotein (1-4) undergoes proteolysis and gives rise to bioactive peptides including the antihypertensive catestatin (CHGA352-372) (5-8) and the diabetogenic pancreastatin (PST: CHGA250-301) (9-12). We have shown that Chga deficient mice (Chga-KO) are obese, hyperadrenergic and hypertensive. They display elevated levels of circulating leptin and catecholamines but lower levels of IL-6 and Mcp-1 (11; 13-16). Despite these abnormalities, Chga-KO mice exhibit enhanced insulin sensitivity (11), a phenotype masked by supplementing PST. PST regulates hepatic insulin signaling through cPKC and Srebp-1c (11). Increased plasma PST levels in diabetic populations correlate with insulin resistance (10). Similarly, increased circulating levels of PST in diet-induced obese (DIO) and diabetic db/db mice are associated with insulin resistance. Despite high levels of plasma leptin and catecholamines, Chga-KO mice are obese owing to peripheral leptin and catecholamine resistance (17).

Since normal chow diet (NCD)-fed Chga-KO mice displayed increased insulin sensitivity (11), we hypothesized that Chga-KO mice may be able to maintain insulin sensitivity when exposed to the dysglycemic stress of a high fat diet (HFD). The hallmarks of insulin resistance in DIO mice are obesity, hyperinsulinemia and increased inflammation (18-22). Suppression of inflammation in DIO mice can improve insulin sensitivity (23-25). For example, rosiglitazone can improve inflammation and insulin sensitivity in DIO mice without reducing obesity significantly (23-25). Chga-KO mice are obese and presumably would become more obese after HFD feeding. Here, we address the following questions: (i) does HFD suppress the insulin-sensitive phenotypes of Chga-KO mice? (ii) does PST-deficiency in Chga-KO mice confer protection against HFD-induced insulin resistance? (iii) is endogenous PST a pro-inflammatory
factor that is responsible for or contributes to the development of insulin resistance after HFD feeding?
RESEARCH DESIGN AND METHODS

Animals. Male WT (control) and Chga-KO mice on a stable mixed genetic background (50% 129svJ; 50% C57BL/6J), for >50 generations were used. Only in experiments shown in Fig. 1F and 8H, mice were on a C57BL/6J background. Animals were kept in a 12 hours dark/light cycle. The Institutional Animal Care and Utilization Committee approved all the procedures.

Diets. Mice were fed ad libitum for 12-16 weeks with a high fat diet (HFD: 60% of calories from fat; Research Diets, Inc. D12492). Some mice were also fed a normal chow diet (NCD: 14% calorie from fat; LabDiet 5P00).

Synthetic peptides. Wild-type human PST (CHGA_{273–301}: PEGKGEQEHSQQKEEEEEMAVVPQGLFRG-amide) and PST variant PSTv1 (also known as PSTN\Delta3; CHGA_{276–301}: KGEQEHSQQKEEEEEMAVVPQGLFRG-amide) were synthesized by the solid phase method and purified by reverse phase HPLC to at least 95% homogeneity (GenScript Corporation, NJ).

GTT, ITT and Clamp studies. For glucose tolerance tests (GTT), glucose (1 mg/g) was injected intraperitoneally (IP-GTT) (at time zero) or gavaged orally (O-GTT) after a 12-hours fast. Tail-vein glucose levels were measured at -30, 0, 15, 30, 60, 90, and 120 minutes. For insulin tolerance tests (ITT), insulin (0.4 mU/g) was injected intraperitoneally and blood glucose levels were measured at the indicated time points. Chronic PST (5 \mu g/g/day) and PSTv1 (10 \mu g/day) treatments were carried out for 15 days. For acute treatment, either peptides or saline were injected 30 minutes before glucose or insulin injection. Synergy Software Kaleidagraph Version 4.0 calculated the area under the curve (AUC) for each line curve.
The hyperinsulinemic-euglycemic clamp protocol was adapted from the description by Kim (26) and described previously by us (11). We calculated insulin-stimulated glucose disposal rate (IS-GDR) as: IS-GDR = glucose disposal rate (GDR; during the clamp) – basal GDR. This value represents a measurement of the increase in GDR from the basal value due to insulin infusion. Since basal hepatic glucose production [HGP] = basal GDR, we calculated the total GDR during the clamp as glucose infusion rate (GIR) + HGP (during the clamp). During the clamps, insulin was infused at a constant rate of 12 mU/kg/min. The suppression of HGP by insulin was calculated as described by Kim (26) from these data.

**Immunoblotting.** Tissues were homogenized in a buffer containing phosphatase and protease inhibitors as described (11; 17). Homogenates were subjected to SDS-PAGE and immunoblotted. Primary antibodies for phosphorylated and total Akt, AMPK, FoxO1, and JNK were from Cell Signaling Technology (Beverly, MA). Actin and Srebp-1 antibodies were from Santa Cruz Biotechnology (CA).

**Preparation and culture of intraperitoneal macrophages.** Peritoneal macrophages were isolated after thioglycollate (3% solution in water) stimulation as described in detail by Zhang et al (27). Isolated macrophages were cultured in DMEM with 10% FBS for 48 hours with daily medium changes. Cells were then serum-starved overnight in DMEM with low FBS (0.5%) and then exposed to saline, PST (100 nM), or LPS (100 ng/ml) for 4 hours. At the end of incubation, the cultures were subjected to RNA extraction.

**In vitro Chemotaxis Assay.** In vitro chemotaxis assays were performed as previously described (28). Briefly, thioglycollate-activated peritoneal macrophages were isolated from 8 week-old male C57BL/6J mice and pretreated with the indicated concentrations of PST for 2 hours at 37°C
in serum-free RPMI. For migration analysis, 200,000 peritoneal macrophages were placed in each of the upper chamber of a 4-µm polycarbonate filter (24-transwell format; Corning, Tewksbury, MA), whereas compounds resuspended in serum-free RPMI at the indicated concentrations were placed in the lower chamber. After 3 hours of migration at 37°C, cells were fixed in 4% paraformaldehyde, stained with DAPI and counted under the microscope.

**Real Time PCR.** RNA from tissues was extracted using a kit (RNeasy Plus, Qiagen, Valencia, CA). After DNase digestion, 200 ng of RNA was transcribed into cDNA in a 20-µl reaction using a High Capacity cDNA Archive kit and this cDNA was amplified. PCR (25-µl reactions) contained 5 µl of cDNA, 2× SYBR Green PCR Master Mix, and 400 nM of each primer. Differences in the threshold cycle number (ΔCt) between the target gene, the housekeeping gene *Gapdh* and the ribosomal protein gene *36B4* were used to calculate differences in expression. Primers sequences are provided as a supplement.

**Determination of plasma cytokines.** Plasma cytokine concentrations were determined by a multiplex system from Quansys Biosciences (Logan, UT).

**Statistics.** Data are expressed as mean ± SEM. Statistical analyses were performed using Student’s *t* tests, as well as one- and two-way ANOVA followed by Dunnett’s *post hoc* test when appropriate. Statistical significance was concluded at *p*<0.05. Statistics were computed with the program InStat (GraphPad Software, Inc. San Diego, CA).
RESULTS

Effects of PST deficiency and supplementation on obesity.

The deficiency of Chga proprotein causes obesity in Chga-KO mice. At 7 weeks of age on normal chow diet (NCD), Chga-KO mice were heavier than WT mice (31.28g vs. 23.08g). From week 7 to 22, there was an additional increase in body weight gain compared to WT mice (from 23.08g to 32.53g for WT mice versus 31.28g to 45.96g for Chga-KO mice) during the same period of growth (Fig. 1A-B). A similar trend holds true for mice on a high fat diet (HFD). After 15 weeks of HFD feeding, Chga-KO-DIO (KO-DIO) mice gained more weight than WT-DIO mice (from 24.4g to 44.84g for WT-DIO versus from 30.72g to 58.59g for Chga-KO mice) (Fig. 1C-D). PST deficient KO-DIO mice also ate more on a HFD (2.94 vs. 2.11gm/day, p<0.0001) than WT-DIO mice. PST administration to Chga-KO mice on NCD for 2 weeks did not change body weight (Fig. 1E). With the increase in obesity, plasma levels of PST were elevated in WT-DIO mice, similar to the levels seen in obese and diabetic db/db mice (Fig. 1F). Since obesity in Chga-KO mice occurred in the absence of PST expression and PST supplementation did not change body weight, we concluded that PST is not involved in the development of obesity.

Plasma leptin level increased in both WT-DIO and KO-DIO mice compared to NCD-fed mice (Fig. 1G) but the level in KO-DIO was the highest due to the (i) increase in adipose mass (compared to WT-DIO mice) and (ii) absence of PST-mediated negative regulation of leptin production (29).

Obese Chga-KO mice display improved glucose tolerance and insulin sensitivity, dependent upon the absence of PST.

Despite increased obesity, KO-DIO mice maintained insulin sensitivity as shown by GTT and ITT (Fig. 2A-F). Both IP-GTT (Fig. 2A&B) and O-GTT (Fig. 2C&D) demonstrated
improved glucose tolerance, whereas IP-ITT (Fig. 2E & F) showed increased insulin sensitivity in KO-DIO mice. Decreased AUC (area under the curve) for corresponding GTT (Fig. 2B & D) and ITT (Fig. 2F) established that KO-DIO mice were more glucose tolerant and insulin sensitive than WT-DIO mice. Of note, fasting glucose levels (at time zero) in KO-DIO mice were consistently lower than WT-DIO mice (Fig. 2A, C, E and Fig. 3A & C). It appears that the lack of PST in KO-DIO mice helps to maintain their insulin sensitivity on HFD, as this phenotype was abolished upon chronic supplementation of PST (Fig. 2E & F). Similarly, when PST was supplemented to WT or Chga-KO mice on a NCD, PST caused insulin resistance (Fig. 3E & F). These data emphasize the point that PST treatment causes insulin resistance without causing obesity, and that obesity occurring in the absence of PST does not induce insulin resistance. These results in mice with a mixed genetic background were also corroborated in mice with a C57/BL6J background (see supplement Fig. s1).

The GTT and ITT findings of insulin sensitivity in KO-DIO mice were reinforced by hyperinsulinemic-euglycemic clamp studies, where KO-DIO mice displayed an increased GIR and suppressed HGP by insulin. The basal GDR was similar between WT-DIO and KO-DIO mice but IS-GDR was higher in KO-DIO compared to WT-DIO mice (Fig. 2G-J). Fasting plasma insulin concentrations in KO-DIO mice were lower than that of WT-DIO mice (Fig. 2K). Since supplementation of PST raised fasting glucose (Fig. 2E & F) and insulin levels in KO-DIO mice over WT-DIO levels (Fig. 2L), our results support the notion that the deficiency of PST in Chga-KO mice prevented the development of hyperglycemia and hyperinsulinemia in KO-DIO mice.

The effects of PST, much like anti-insulin, were reversed by a PST-variant peptide.
To combat anti-insulin like effects of PST, we created a variant, PSTv1, by deleting three N-terminal residues of native PST. PSTv1 treatment of WT-DIO mice (10 µg/g/day) for 15 days lowered fasting plasma glucose levels (Fig. 3A&C) as well as improved glucose tolerance (Fig. 3A&B) and insulin sensitivity (Fig. 3C&D) possibly by competing against endogenous PST. While chronic PST supplementation to both WT and Chga-KO mice on a NCD induced insulin resistance (Fig. 3E&F and Fig. 4A-D), chronic PSTv1 improved insulin sensitivity (Fig. 3G&H). In NCD-fed Chga-KO mice (KO-NCD), the hyperglycemic effect of PST was attenuated by concomitant injection of PSTv1 (Fig. 4A&B). Moreover, chronic supplementation of PSTv1 reversed the effects of chronic PST treatment (Fig. 4C&D). Of note, PSTv1 alone had no effect on Chga-KO mice, presumably because of the lack of endogenous PST with which PSTv1 could compete (Fig. 4A-D). These results demonstrate PST deficiency as the primary reason for insulin sensitivity and protection against diet-induced insulin resistance in Chga-KO mice.

Effects of PST deficiency and HFD on metabolic regulation and insulin signaling.

Consistent with our metabolic characterization described thus far, we found that KO-DIO mice displayed increased hepatic glycogen storage and reduced gluconeogenic gene expression. Under the hyperinsulinemic condition during clamps, KO-DIO mice assimilated more glycogen in the liver than WT-DIO mice (Fig. 4E), which helped remove circulating glucose. The mRNA levels of gluconeogenic genes (Pepck and G6pase) in the liver were significantly lower in KO-DIO mice (Fig. 4F), and were reversed by treatment with PST (Fig. 4F). In addition, analysis of lipid metabolism gene expression revealed no difference in the mRNA levels of genes for lipogenesis (Acc and Srebp-1) and lipid oxidation (Ppara, Cpt-1 and Acox) between WT-DIO and KO-DIO mice (Fig. 4G), whereas PST supplementation increased the expression of Acox-1 and Cpt-1.
Among the metabolic signals that were significantly improved in *Chga*-KO mice were Akt and Srebp1c, which are likely to be involved in producing the insulin sensitive phenotype of these animals. We analyzed phosphorylation and activation of Akt, an important component of the insulin signaling pathway, by Western blot. Akt phosphorylation in muscle (Fig. 5A) and white adipose tissue (WAT; Fig. 5B) was not significantly increased by insulin in WT-DIO mice (due to insulin resistance), but was robustly stimulated by insulin in KO-DIO mice (Fig. 5A&B). In the liver, insulin significantly enhanced Akt phosphorylation in KO-DIO mice when compared with the saline-treated controls (Fig. 5C). The fold response to insulin was reduced in WT-DIO livers (because of insulin resistance) but was higher in KO-DIO livers compared to WT-DIO livers (Fig. 5C). In contrast, PST supplementation to KO-DIO mice completely inhibited stimulation of phosphorylation by insulin (Fig. 5A-C).

In addition to Akt, we analyzed the activity of other signaling pathway components, including AMPK, FoxO1, and Jnk. AMPK phosphorylation was improved in the muscle and WAT of KO-DIO mice (but not in the liver) (Fig. 5D-F), which might have contributed to an increase in glucose disposal and decrease in inflammation in KO-DIO mice. However, PST supplementation to KO-DIO mice did not change the overall pattern of AMPK signaling. As expected from increased Akt activation, the insulin-stimulated phosphorylation of FoxO1, downstream of Akt (82 kDa pFoxO1 and 95 kDa pFoxO1), in KO-DIO mice was higher in WAT and the liver than corresponding insulin-treated WT-DIO tissues (Fig. 6A&B). However, PST supplementation modulated FoxO1 signaling in WAT but not in the liver (Fig. 6A&B). In addition, the basal phosphorylation of p46 and p54 Jnk was lower in KO-DIO WAT compared to WT-DIO, suggesting persistent activation of inflammatory signals in WT-DIO mice (Fig. 6C&D). Interestingly, insulin seems to stimulate these negative Jnk signals in WAT for self-
regulation. However, PST treatment did not alter hepatic AMPK, FoxO1 or Jnk signaling, suggesting additional PST-independent regulatory mechanisms.

Consistent with reduced gluconeogenesis, the expression level of mature Srebp-1c p68 protein in the liver of insulin-treated KO-DIO mice was higher than the WT-DIO controls (Fig. 6E), suggesting that the processing of the precursor p125 to mature p68 was facilitated by insulin in KO-DIO livers. Compared to WT-DIO mice, basal levels of p125 and p68 were also elevated in the liver (Fig. 6E) of KO-DIO mice. PST treatment reversed the expression pattern of p68 in KO-DIO livers by raising basal levels and decreasing insulin sensitivity (Fig. 6E). As a result, PST treatment decreased the p68 to p125 ratio compared to untreated KO-DIO livers. In other words, PST treatment reduced insulin-stimulated processing of the Srebp-1c precursor p125 to mature p68 in KO-DIO livers.

**PST Modulates Adipose Tissue Inflammation**

The increased activation of Akt and AMPK in PST-deficient KO-DIO mice led us to wonder whether these mice may display reduced inflammation, which is regulated by these factors (30-40). Indeed, mRNA levels of the pro-inflammatory cytokines *IL-1β*, *Tnfa*, *IL-6*, and *Mcp-1* were reduced in adipose tissue of KO-DIO mice compared to WT-DIO mice (Fig. 7A). Consistent with this trend, the expression of the pro-inflammatory genes *Cd11c* and *IL-12p40* (Fig. 7B) and *iNos* (Fig. 7C) were also lower in KO-DIO adipose tissue than WT-DIO mice. Accordingly, PST treatment caused increased expression of the pro-inflammatory genes *IL-1β*, *Tnfa*, *IL-6*, *Mcp-1* and *iNos* (Fig. 7A-C). Conversely, expression of anti-inflammatory genes such as *Arg-1*, *IL-10*, *Mgl-1*, *Mgl-2*, and *Ym1* in adipose tissue was higher in KO-DIO mice than WT control animals (Fig. 7D&E). PST treatment significantly reduced the expression of *Arg-1* and *IL-10*, and displayed a trend toward downregulating *Ym-1*, but had no effect on *Mgl-1* or *Mgl-2*.
The adipose tissue expression of \( Tnfa, \ IL-6 \) and \( Mcp-1 \) genes was reduced in KO mice in either NCD or DIO conditions compared to WT, although DIO increased levels in both backgrounds (Fig. 7F). We speculate that the subdued state of inflammation found in KO mice contributes to their improved insulin sensitivity.

Corresponding to reduced inflammatory gene expression, several cytokines were detected at lower levels in the plasma of KO-DIO compared to WT-DIO mice. Levels of IL-12p70, Ifn\( \gamma \), Mip-1, IL-6 and Kc showed significant decreases, whereas Mcp-1 levels showed a trend to be lower, and Tnf\( \alpha \) levels were unchanged (Fig. 7G&H). PST treatment of KO-DIO mice raised plasma levels of IL-12p70 and Mcp-1 but had no effect on Ifn\( \gamma \), IL-6, Mip-1\( \alpha \), Kc and Tnf\( \alpha \) (Fig. 7G&H).

Overall, expression of most pro-inflammatory markers were reduced whereas anti-inflammatory markers were elevated in KO-DIO mice compared to WT-DIO mice, likely due to deficiency of PST and other \( Chga \)-derived peptides.

**PST Promotes Macrophage Inflammation and Chemotaxis**

As PST influenced inflammatory gene expression in adipose tissue, we investigated its role in macrophages. Elevated numbers of adipose tissue macrophages are associated with obesity, increased inflammation and reduced insulin sensitivity (41-44). \( Chga \) mRNA expression was identified in peritoneal macrophages of WT mice (Fig. 8A). LPS (lipopolysaccharide) caused robust expression of the pro-inflammatory gene \( iNos \) (~100-fold) in both WT and \( Chga \)-KO macrophages (Fig. 8B). This was our reference point for comparing PST effects on expression of other inflammatory markers. The basal expression of \( iNos, Tnfa, Mcp-1, IL-6, \) and \( IL-12p40 \) and \( iNos \) was lower in \( Chga \)-KO macrophages, and PST treatment caused a smaller increase in expression than in WT macrophages (Fig. 8B-F). Specifically, PST increased the
expression of \textit{Tnfa} and \textit{Mcp-1}, but had no effect on the expression of \textit{IL-6}, \textit{IL-12p40} and \textit{iNos} genes in \textit{Chga-KO} macrophages (Fig. 8C-G). Developmental adjustments in \textit{Chga-KO} mice may have lowered the potential for induction of these genes by PST. It is also possible that supplementation of other \textit{Chga}-derived peptides may be necessary to produce the full complement of expression in \textit{Chga-KO} mice.

Consistent with induction of inflammatory gene expression, PST induced chemotaxis of macrophages \textit{in vitro}. Using peritoneal macrophage cultures, we compared the chemotactic ability of the chemokine \textit{Mcp-1} versus PST. PST treatment led to significantly higher levels of chemotaxis compared to saline-treated controls, although with lower potency than \textit{Mcp-1} (chemotaxis induced by 10 nM PST was 44\% that of 10 nM \textit{Mcp-1}) (Fig. 8H).

In summary, these results reinforce the notion that (i) PST alone negatively regulates insulin sensitivity in several tissues, and as a result, PST supplementation can produce insulin resistance, and (ii) along with another \textit{Chga}-produced peptide, PST upregulates proinflammatory signals that are lacking in \textit{Chga-KO} mice.


**DISCUSSION**

PST deficiency renders NCD-fed *Chga-KO* mice more insulin sensitive than WT mice (11). A GTP-binding protein-coupled-receptor mediated signaling pathway leading to activation of conventional DAG/Ca\(^{2+}\)-dependent PKC and downregulation of mature Srebp-1c (p68), is thought to play a role in the anti-insulin effects of PST (11). Recently, we observed that PST could also modulate ER-stress by interacting with BiP/GRP78 (45). Feeding a HFD creates obesity, leading to hyperinsulinemia and inflammation (18-22). PST-deficient KO-DIO mice are more obese than WT-DIO mice but remain more insulin sensitive as assessed by GTT, ITT and clamp studies (Fig. 2). Insulin sensitivity was compromised by PST replacement, suggesting that the absence of PST in *Chga-KO* mice not only enhanced insulin action but also prevented further damage by dietary fat.

The most important aspect of this study is that obesity can be dissociated from insulin resistance as long as inflammation is suppressed. The presence of supraphysiological levels of PST can reconnect obesity with insulin resistance by inducing inflammation. In the absence of PST, animals could be insulin sensitive despite obesity. This is reminiscent of rosiglitazone-treated WT-DIO mice, which are insulin sensitive but obese (23-25).

**PST and insulin resistance.** We can now provide answers to the questions we raised in the introduction; (i) HFD feeding does not suppress the insulin-sensitive phenotype of *Chga-KO* mice, (ii) PST-deficiency in *Chga-KO* mice provides protection against HFD-induced insulin resistance, and (iii) PST is an endogenous pro-inflammatory factor that affects insulin action independent of obesity. Obesity caused by dietary fat cannot induce inflammation and insulin resistance in the absence of PST and another Chga-produced peptide.
It appears that PST deficiency provides benefits to obese mice by (i) enhancing hepatic glycogen storage and decreasing glucose production through stimulation of Akt signaling and suppression of gluconeogenic genes via increased expression of Srebp-1c proteins (46; 47), and (ii) increasing glucose disposal by muscle via increased AMPK and insulin-stimulated Akt signaling (Fig. 2&5), and by (iii) suppressing macrophage-mediated inflammation in adipose tissue (Fig. 7&8). Moreover, PST deficiency may decrease ER stress (31).

PST deficiency also increased insulin-stimulated pFoxO1-p95 signals in WAT of KOT-DIO mice, which was reversed after PST supplementation (Fig. 6A). Since increased phosphorylation of FoxO1 (inactive form) in WAT favors adipogenesis through PPARγ (48; 49), our results are consistent with the greater adiposity of Chga-KO mice.

**PST and Inflammation.** The increased Akt and AMPK activities in WAT in KO-DIO mice (Fig. 5) could potentially provide the benefit of dampening the inflammation induced by a HFD. This is because both Akt and AMPK can suppress the inflammatory responses of resident macrophages in adipose tissue (30-35; 40) and modulate cytokine production by adipocytes, macrophages and neutrophils (32; 36-39). Our results corroborate this notion and suggest that supraphysiological levels of PST can impose an inflammatory burden and thereby disrupt glucose homeostasis. Our profiling of pro- and anti-inflammatory markers and cytokines suggests that the adipose tissue of KO-DIO mice was less inflamed than WT-DIO mice. Since PST treatment of peritoneal macrophage cultures (obtained from WT and Chga-KO mice on NCD) increased the expression of *Tnfa, Mcp-1, IL-6, IL-12p40* and *iNos* (Fig. 8C-G), this suggests a direct effect of PST on the behavior of peritoneal macrophages. As expected, the expression of these genes was much lower in Chga-KO macrophages. Treatment of Chga-KO macrophages with PST elevated their expression but not to the extent of PST-treated WT
macrophages (Fig. 8C-G). Moreover, PST provoked macrophage chemotaxis in vitro (Fig. 8H). It is possible that other Chga-derived peptides also contributed to the regulation of inflammatory gene expression, leading to a partial PST-stimulated response in Chga-KO macrophages compared to WT macrophages. However, in terms of insulin resistance, PST alone can fully account for the insulin resistance conferred to KO-DIO mice. It is possible that the diabetogenic effects of a supraphysiological level of PST may result from the combined stimulation of two different pathways; inflammation and ER stress as we have shown that PST reduces the adaptive unfolded protein response through binding to the ER chaperone, GRP78 (31).

Insulin sensitization by antagonism of PST action. Finally, to implicate a direct in vivo role of PST in the regulation of insulin sensitivity, we injected WT-DIO mice with the PST variant, PSTv1, to block the effect of native PST (which is upregulated in DIO mice). PSTv1 lacks the first three N-terminal residues of native PST and was designed to block PST-mediated inhibition of glucose uptake and leptin secretion in 3T3-L1 adipocytes (manuscript in preparation). Chronic PSTv1 treatment lowered fasting plasma glucose levels in WT-DIO mice and improved glucose tolerance (Fig. 3A&B) and insulin sensitivity (Fig. 3C&D). These results suggested that in WT-DIO mice where the level of PST is high, PSTv1 administration could compete with the native PST and thereby phenocopy Chga-KO mice. From this perspective, PSTv1 could serve as an anti-diabetic agent.

Conclusion. The deficiency of PST in Chga-KO mice provides an anti-inflammatory environment leading to prevention of HFD-induced insulin resistance. As a result, insulin sensitivity is maintained even in obese KO-DIO mice. Thus, obesity manifests insulin resistance only in the presence of PST, and in its absence obesity is dissociated from insulin resistance. We visualize the chain of events following suppression of PST action that contributes to improved
glucose homeostasis as (i) promotion of insulin signaling and suppression of inflammatory cytokine production through increased PI-3-K/Akt/Foxo-1 signaling and (ii) suppression of hepatic gluconeogenesis by increased expression of mature Srebp-1c. Future studies will be directed to determine whether administration of PSTv1 to diabetic animals will improve insulin sensitivity by reducing PST-induced inflammation and ER stress.

**AUTHOR CONTRIBUTIONS.** G.K.B. researched data and wrote the manuscript. M.L., E.A., J.M.S., J.R.G., J.W. and C.U.V. researched data. N. -W.C. participated in discussion and edited the manuscript. D.T.O.C. participated in discussion and reviewed/edited the manuscript. S.K.M. conceived, researched and analyzed the data, made the graphics and reviewed/edited the manuscript.

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FIGURE LEGENDS

**Fig. 1: Chga-KO mice display elevated body weight gain on NCD and HFD.**

(A) Body weights of WT and Chga-KO mice (with mixed genetic background) on NCD were taken every week starting from week 7 till week 22 (2-way ANOVA: Strain, p<0.0001; Age, p<0.0001; Interaction, p<0.0004; n=9), and (B) the initial weight at week 7 and final weight at week 22 were compared. (C) Similarly, body weights of WT and Chga-KO mice on HFD were taken every week starting from week 7 till week 22 (2-way ANOVA: Strain, p<0.0001; Age, p<0.0001; Interaction, p=0.16; n=13), and (D) the initial weight at week 7 and final weight at week 22 were compared. (E) Effects of PST administration to WT and Chga-KO mice on NCD for 2 weeks (from week 19 to week 21) on body weight gain were shown. (F) Plasma PST levels were measured in 6 month old WT-NCD mice, or WT-DIO or in obese-diabetic db/db mice (all mice with C57/BL6 background) (n=6). (G) Four month old WT and Chga-KO mice with mixed genetic background were fed NCD or HFD for 12 weeks. Plasma leptin levels were measured by ELISA (n=7). “*” Indicates comparison between the indicated groups. “*”: P<0.05, “**”: p<0.01, “***”: p<0.001.

**Fig. 2: Chga-KO mice on HFD display improved glucose tolerance and insulin sensitivity, dependent upon the absence of PST.** (A-F) WT-DIO and KO-DIO male mice with mixed genetic background were fasted for 12 hrs and subjected to (A) IP-GTT (2-way ANOVA: Strain, p<0.0001; Time, p<0.0001; Interaction, p<0.045; n=8), (C) oral GTT (2-way ANOVA: Strain, p<0.0001; Time, p<0.0001; Interaction, p=0.09; n=8), or (E) IP-ITT (2-way ANOVA: Treatment (WT), p<0.0001; Time, p<0.0001; Interaction, p=0.56; Treatment (KO), p<0.0001; Time, p<0.0001; Interaction, 0.43; n=9), and AUC for glucose excursions were determined (B, D&F). (E) WT-DIO and KO-DIO mice were treated with intraperitoneal PST (5µg/g BW) for 15 days, fasted for 12 hrs after the last day of injection and subjected to GTT. GTT and the corresponding AUC were shown in (E) and (F), respectively. Body weight-matched WT-DIO and KO-DIO mice were fasted for 12 hrs and subjected to clamp studies to determine (G) glucose infusion rate (GIR), (H) glucose disposal rate (GDR), (I) insulin-stimulated GDR (IS-GDR) and (J) % suppression of hepatic glucose production (HGP) (n=8-9). Fasting (12 hrs) basal levels of plasma insulin before (K) and after (L) chronic PST treatment (5 µg/g BW/day, IP for 15 days) (n=6) were shown. “&” Indicates comparison between WT-DIO+Sal and KO-DIO+Sal, “$” indicates comparison between WT-DIO+Sal and WT-DIO+PST, “#” indicates comparison between KO-DIO+Sal and KO-DIO+PST, and “$$” indicates comparison between the indicated groups. “*, $ or #”: P<0.05, “**, &&, ## or $$”: p< 0.01, “***, ### or &&&”: p<0.001.

**Fig. 3: Treatment with the PST variant PSTv1 improves, whereas PST worsens, glucose tolerance and insulin sensitivity in WT-DIO and WT-NCD mice.** WT-DIO mice were treated with saline or PSTv1 (10 µg/g BW/day) for 15 days. Weight-matched DIO mice were fasted for 12 hrs and subjected to (A) IP-GTT (2-way ANOVA: Treatment, p<0.0001; Time, p<0.0001;
Interaction, p=0.17, n=6) and (C) IP-ITT (2-way ANOVA: Treatment, p<0.0001; Time, p<0.0001; Interaction, p=0.62 n=9) and AUCs for glucose excursions were determined (B&D). NCD fed WT mice (WT-NCD) were treated with saline or PST (5 µg/g BW) for 15 days, fasted for 12 hrs and subjected to (E) IP-ITT (2-way ANOVA: Treatment, p<0.0001; Time, p<0.0001; Interaction, p=0.62 n=8). The corresponding AUC was shown in (F). WT-NCD mice were treated with saline or PSTv1 (10 µg/g BW) for 15 days, fasted for 12 hrs and subjected to (G) IP-ITT (2-way ANOVA: Treatment, p<0.0001; Time, p<0.0001; Interaction, p<0.02 n=8). The corresponding AUC was shown in (H). “*” Indicates comparison between the indicated groups. “*”: P<0.05, “**”: p< 0.01, “***”: p<0.001.

Fig. 4: The PST variant PSTv1 blocks the ability of PST to suppress the glucose tolerance of KO-NCD mice, and PST treatment reversed reduced gluconeogenic and lipid metabolic gene expression in KO-DIO mice. Acute effects: NCD fed Chga-KO mice (KO-NCD) were fasted for 12 hrs, treated with saline, PST (5 µg/g BW), PSTv1 (10 µg/g BW) or PST+PSTv1 for 30 min (-30 min) before injecting glucose (0 min) for (A) IP-GTT (2-way ANOVA: Treatment (Sal vs PST), p<0.0001, Time, p<0.0001; Interaction, p<0.002; Treatment (PST vs PSTv1), p<0.0001; Time, p<0.0001; Interaction, p<0.005; n=7). The corresponding AUC is shown in (B). Chronic effects: KO-NCD mice were treated with saline, PST (5 µg/g BW), PSTv1 (10 µg/g BW) or PST+PSTv1 for 15 days, fasted for 12 hrs after the last of injection and subjected to (C) GTT (2-way ANOVA: Treatment (Sal vs PST), Time, p<0.0001; Interaction, p<0.05; Treatment (PST vs PSTv1), p<0.0001; Time, p<0.0001; Interaction, p<0.01; n=7). The corresponding AUC is shown in (D). (E) Liver glycogen at basal and during clamp in DIO mice (n=6). (F) Chronic effects of PST (5 µg/g BW for 15 days) on expression of hepatic gluconeogenic genes (Pepck and G6pase) in DIO mice (n=6). (G) Chronic effects of PST (5 µg/g BW for 15 days) on expression of hepatic lipid metabolic (Acc, Ppara, Cpt-1, Acox-1 and Srebp-1) genes in DIO mice (n=6). “$” Denotes comparison between saline and PST, “#” denotes comparison between PSTv1 and PST+PSTv1, and “*” denotes comparison between the indicated groups. “*, $ or #”: P<0.05, “**, $$ or ## ”: p< 0.01, “*** or $$$”: p<0.001.

Fig. 5: KO-DIO mice display improved Insulin-induced Akt and AMPK phosphorylation, dependent upon the absence of PST. Weight-matched saline-treated WT-DIO or KO-DIO and PST-treated KO-DIO mice were fasted for 12 hrs, injected with insulin (0.4mU/g BW, IP) for 10 min and sacrificed for tissue collection. Tissues were homogenized, lysates were subjected to SDS-PAGE and immunoblotted for p-Akt in muscle (A, n=4), WAT (B, n=4) and liver (C, n=4) and for p-AMPK in muscle (D), WAT (E) and liver (F). “*” Denotes comparison between the indicated groups. “*: P<0.05, “*”: p<0.01, “***”: p<0.001.

Fig. 6: FoxO1, Srebp-1 and JNK signaling is altered in KO-DIO mice. Weight-matched saline-treated WT-DIO or KO-DIO and PST-treated KO-DIO mice were fasted for 12 hrs, injected with insulin (0.4mU/g BW, IP) for 10 min and sacrificed for tissue collection. Tissues
were homogenized, lysates were subjected to SDS-PAGE and subsequently immunoblotted to
detect phospho-p95-FoxO1 and phospho-p82-FoxO1 signals in WAT (A, n=4) and phospho-
p82-FoxO1 signals in liver (B, n=4), phospho-p54-Jnk and phospho-p46-Jnk signals in WAT (C,
n=4) and liver (D, n=4), and p125-Srebp-1 and p-68-Srebp-1 (mature) signals in liver (E,
n=4). “*” Denotes comparison between the indicated groups. “*”: P<0.05, “**”: p< 0.01, “***”: p<0.001.

Fig. 7: PST Modulates Adipose Tissue Inflammation. A group of KO-DIO mice were injected
with saline or PST (5 µg/g BW) for 15 days. Weight-matched saline-treated WT-DIO, KO-DIO
and PST-treated KO-DIO mice were fasted for 12 hr and sacrificed. Blood was collected to
measure plasma cytokine levels. Tissues were subjected to RNA extraction, cDNA preparation
and RT-qPCR analysis for cytokines (A, n=6), pro-inflammatory genes (B: Cd11c & IL-12p40,
n=6; C: iNos, n=6), and anti-inflammatory genes (D: Arg-1 & IL-10, n=6; E: Mgl-1, Mgl-2 and
Ym1, n=6). Plasma cytokine levels were shown in G & H (n=5). “*” Denotes comparison
between the indicated groups. “*”: P<0.05, “**”: p< 0.01, “***”: p<0.001.

Fig. 8: PST Promotes Macrophage Inflammation and Chemotaxis. Peritoneal macrophages
were isolated from WT-NCD and KO-NCD after thioglycollate injection. After 4-hr exposure to
saline, LPS (100 ng/ml) or PST (100 nM), RNAs were extracted and cDNAs were prepared for
for qPCR analyses of Chga (A, n=8), iNos (B, n=8), Tnfa (C, n=8), Mcp-1 (D, n=8), IL-6 (E,
n=8), IL-12p40 (F, n=8) and iNos genes (G, n=8). Effects of LPS stimulation on expression of
iNos are shown in (B) and the effects of PST are shown in C-G. (H) Effects of PST on
chemotaxis of activated macrophages from NCD-fed C57BL/6J mice (n=5). “*” Denotes
comparison between the indicated groups. “*”: P<0.05, “**”: p< 0.01, “***”: p<0.001.
Fig. 1
Fig. 2

203x266mm (288 x 288 DPI)
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 7
Figure 8

203x258mm (288 x 288 DPI)
Quantitative PCR Primer sequences:

Acc
F: TAA TGG GCT GCT TCT GTG ACT C
R: CTC AAT ATC GCC ATC AGT CTT G

Acox
F: GTC GAC CTT GTT CGC CA
R: GGT TCC TCA GCA CGG CTT

Arg-1
F: ATG GAA GAG ACC TTC AGC TAC
R: GCT GTC TTC CCA AGA GTT GGG

36b4
F: AGA TGC AGC AGA TCC GCA T
R: GTT CTT GCC CAT CAG CAC C

Cd11c
F: ACA CAG TGT GCT CCA GTA TGA
R: GCC CAG GGA TAT GTT CAC AGC

Chga
F: AGG AGC GTC TGT CCA GAG AA
R: CAG GCT CTC TAG CTC CTG GT

Cpt1
F: CAG GAT TTT GCT GTC AAC CTC
R: GAG CAT CTC CAT GGC GTA G

Gapdh
F: TAT GTC GTGAG TCT ACT GGT GT
R: GTC ATC ATA CTT GGC AGG TTT CT

G6Pase
F: GTT GAA CCA GTC TCC GAC CA
R: CGA CTC GCT ATC TCC AAG TGA

IL-1b
F: AAA TAC CTG TGG CCT TGG GC
R: CTT GGG ATC CAC ACT CTC CAG

IL-6
F: CCA GAG ATA CAA AGA AAT GAT GG
R: ACT CCA GAA GAC CAG AGG AAA T

IL-10
F: TGA ATT CCC TGG GTG AGA AG
R: TCA CTC TTC ACC TGC TCC ACT

IL-12p40
F: CCA GAG ACA TGG AGT CAT AG
R: AGA TGT GAG TGG CTC AGA GT

iNos
F: GAG GCC CAG GAG GAG AGA GAT CCG
R: TCC ATG CAG ACA ACC TTG GTG TTG

Mcp-1
F: AGG TCC CTG TCA TGC TTC TG
R: GCT GCT GGT GAT CCT CTT GT
Mg1–1       F: ATG ATG TCT GCC AGA GAA CC
           R: ATC ACA GAT TTC AGC AAC CTT A

Mg1–2       F: CAG AAC TTG GAG CGG GAA GAG
           R: TTC TTG TCA CCA TTT CTC ATC TCC T

Pepck       F: CTG CAT AAC GGT CTG GAC TTC
           R: CAG CAA CTG CCC GTA CTC C

Srebp–1c     F: GGA GCC ATG GAT TGC ACA TT
           R: CTT CCA GAG AGG AGG CCA G

Tnfa        F: CCA GAC CCT CAC ACT CAG ATC
           R: CAC TTG GTG GTT TGC TAC GAC

Yml         F: GGG CAT ACC TTT ATC CTG AG
           R: CCA CTG AAG TCA TCC ATG TC
SUPPLEMENTAL FIGURE LEGEND

Fig. s1: GTT and ITT in mice with C57BL/6 genetic background. Three month old WT and Chga-KO mice were fed HFD for 12 weeks. Body weight-matched mice were fasted for 12 hrs and subjected to IP-GTT (A) and ITT (C) and the corresponding AUCs for glucose excursions were determined (B&D). (E) Four month old Chga-KO mice were fed HFD for 12 months, fasted for 12 hrs, injected with PST (5 µg/g BW, IP) at -30 min and subjected to GTT after injecting glucose at 0 min. *: P<0.05, **: p< 0.01, ***: p<0.001.