UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Mechanism of the Development of Hepatic Insulin Sensitivity in Chromogranin A Null Mice

A thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

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The Thesis of Sonia K Chokshi is approved, and it is acceptable in acceptable in quality and form for the publication on microfilm and electronically.

Co-Chair

Chair

University of California, San Diego

2010

DEDICATION

To my mother, father, and sister, for driving me to succeed in whatever I do, and always believing and supporting me. Your love and support has made be the person I am today and allowed me to achieve great things. Also to all those who have supported me this past year. I wouldn't have been able to do it without each and every one of you. I love you all!

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LIST OF ABBREVIATIONS

Acox - Peroxisomal acyl-coenzyme A oxidase

ANOVA - analysis of variance

ATGL - Adipose triglyceride lipase

CHGA/Chga - human/mouse chromogranin A

CHGA/*Chga* - human/mouse chromogranin A gene

Cpt-1- Carnitine palmitoyl transferase

CST - catestatin

Gpat - glycerol-3-phosphate acyltransferase gene

G6Pase - glucose-6-phosphatase

G6pase - glucose-6-phosphatase gene

HSL – Hormone-sensitive lipase

KO - knock-out

Pepck1 - phosphoenolpyruvate carboxykinase-1 gene

*Ppar_*, peroxisome proliferator-activated receptor _gene

PST - pancreastatin

SREBP1c - sterol-regulatory element-binding protein 1c

Srebp1c - sterolregulatory element-binding protein 1c gene

WT - wild type

ACKNOWLEGMENTS

Foremost, I would like to express my deep and sincere gratitude to my advisor Dr. Sushil Mahata for providing me with the opportunity to complete my Masters thesis in his lab; everybody part of Dr. Daniel T. O'Connor and Dr. Sushil Mahata lab at Stein and Skaggs for their continuous patience and guidance throughout this project.

I would like to thank Dr. Nigel Crawford and Dr. Laurie Smith for their generous support and being part of my committee.

ABSTRACT OF THE THESIS

The Mechanism of the Development of Hepatic Insulin Sensitivity in Chromogranin A Null Mice

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Sonia K Chokshi

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Chromogranin A (CHGA/*Chga*), a proprotein distributed in endocrine and neuroendorcrine tissues. Pancreastatin (PST), CHGA derivative, has been reported to

interfere with insulin action. *Chga* knock-out (KO) mouse was generated by targeted deletion of *Chga* gene in neuroendocrine tissues. KO mice displayed hypertension, decreased insulin level despite being euglycemic indicating increase insulin sensitivity, and inhibition of gluconeogenesis which was reversed after PST supplementation. Hepatic and plasma lipid concentrations in KO mice were lower than wild type mice. KO mice had increased adipose tissue mass suggesting hepatic and plasma lipids were sequestered and stored in adipose tissue, and PST deficiency influenced both glucose and lipid metabolism. To better understand the effect of PST on lipid metabolism, mRNA expression levels of lipogenic, oxidative and lipolytic genes were measured in WT, KO and KO plus PST (40µg/g bw ip for 7 days)-treated mice. Expression of gluconeogenic genes, phosphoenolpyruvate carboxykinase (*Pepck)* and glucose6-phosphatase (*G6pase),* were studied. The varying effect of catestatin and PST were compared to determine which active peptide of CHGA affects lipid metabolism and insulin sensitivity. Our results demonstrated that lipogenesis and fatty acid oxidation in adipose tissue of KO mice were lower than liver. PST reversed inhibition of expression of *Pepck* and *G6Pase* in KO mice. In conclusion, PST deficiency in KO mice enhanced hepatic insulin sensitivity towards glucose production and lipid disposal by suppressing *Pepck* and *G6Pase* expression and stimulating fatty acid oxidation. Decreased hepatic and plasma lipid concentrations in KO mice helped maintain higher insulin sensitivity in KO mice.

INTRODUCTION

Chromogranin A (CHGA=human/*Chga*=mouse), is a 48-kDa acidic protein genetically distinct member of the granin family of proteins which is present in secretory granules of chromaffin cells and post-ganglionic sympathetic neurons, where it is co-stored and co-released by exocytosis along with catecholamines (1-4). CHGA is required for formation of catecholamine secretory vesicles in chromaffin cells and its expression may be sufficient to induce a regulated secretory system even in non-secretory cells (5-8). CHGA is widely distributed in neuroendocrine and endocrine tissues (not expressed in muscle, liver and adipose tissues) (1-4), the processing of CHGA yields biologically active peptides such as pancreastatin (PST; human $CHGA_{250-301}$) (9) a dysglycemic hormone, catestatin (CST; human $CHGA_{352-372}$) (10-13) an inhibitory catecholamine, and vasostatin (human $CHGA_{1-76}$) (14) a vascular smooth muscle vasodilator with diverse and complex actions. However, the level of processing is generally low and varies with different tissues, and much of CHGA remains intact upon release.

There is a strong link between hypertension, insulin resistance (type 2 diabetes), and the metabolic syndrome in humans, where expression of CHGA is heritable and varies (2, 15). One of the prominent biologically derivatives of CHGA is catestatin. Inefficient processing of catestatin (CST) fragment leads to hypertension (15, 16). As a nicotinic cholinergic antagonist (10-13), CST inhibits secretion from dense core granule, therefore diminishing circulating levels of catecholamines. A central role of CST deficiency in hypertensive knockout mice, is supported by the 'rescue' by exogenous CST (17).

Pancreastatin (PST), a biologically active derivative of CHGA, was isolated from porcine pancreas (9, 18). Proteolysis of *Chga* may occur both inside and outside the cell to yield PST. Regulation of exocrine and endocrine of several glands is one of the biologically functions of PST (19). Thus, PST inhibits insulin secretion stimulated by physiologic activators such as glucose, arginine and glucagon (18). An endocrine pancreatic peptide, PST, modifies the insulin/glucagon ratio to produce a catabolic and mobilizing glucose effect (18). Furthermore, PST has been found to regulate glucose, lipid, and protein metabolism in the liver and adipose tissue providing fuel metabolites and promoting energy expenditure to the system (20, 21). The systemic null *Chga* (knockout, KO) mice display phenotypes of (i) higher systolic (SBP) and diastolic (DBP) blood pressure (BP), (ii) increase levels of plasma catecholamine, and (iii) decreased insulin level despite being euglycemic indicating insulin sensitivity, inhibition of gluconeogenesis and reversal of inhibition of gluconeogenesis after supplementation with PST (17, 22-25). Moreover the PST level is elevated in patients with Type 2 diabetes mellitus (26).

To further understand the role of PST involvement in lipid metabolism, we tested whether the removal of PST, a negative regulator of insulin action, affects lipid metabolism, gluconeogenesis in knock-out (KO) mice and protects against metabolic disorders. Thus, we characterized more extensively the phenotype of the global *Chga* KO mouse (20), which is found to be hypertensive and hyperadrenergic.

In conclusion, we report that by comparison with wild-type (WT), KO mice are insulin sensitive and remain euglycemic despite increased lipid storage in adipose tissue, resulting in increase in body weight (23). In comparison to WT mice,

significantly more hepatic lipids are disposed in KO mice and deposited in adipose tissue. It is possible that reduced fat accumulation in liver helped maintain higher insulin sensitivity in KO mice.

METHODS AND MATERIAL

Animals

Mice used in this study had mixed genetic background (129svJ X C57BL/6). Both Wild type (WT) and Chga-knockout (KO) mice were generated from the original founder carrying mixed genotype (50% 129svJ and 50% C57BL/6) and were maintained by brother sister mating. Six-month old mice were used for experiments. Mice were kept in a 12-hour dark/light cycle and fed a standard chow diet *ad libitum*. Mice weighing 30-35gm (6-8 per group) were used for each experiment. The IACUC approved all procedures.

Treatment of mice and incorporation of 2-14C-acetate and 1-14C-palmitate into lipids:

Mice (wild type and Chga-knockout) were treated with saline or PST or CST (5 microgram/gm body weight) by IP injected daily for 7 days. On the 8^{th} day, mice were injected IP with 100 microliter 10 microCurie 2-^{14}C-acetate (1mM) or 2 microCurie 1-^{14}C-palmitate (0.2 mM as BSA complex). After 2 hours of injection of radioactivity, mice were sacrificed and tissues were collected for lipid extraction and analysis.

Another group of mice, treated similarly with PST and CST but no radioactivity, were used to extract RNA from liver and adipose tissues for RT-QPCR.

Real-time-quantitative PCR:

RNA was extracted using an RNA purification kit (RNeasy Plus, QIAGEN,

Valencia, CA), according to the manufacturer's specifications. After DNAse digestion, 100 ng RNA was transcribed into cDNA in a 20-µl reaction using a High Capacity cDNA Archive kit, analyzed and amplified. PCR was performed in a 25-µl reaction, containing 5-µl cDNA (one-fifth diluted), 2 x SYBR Green PCR Master Mix and 400 nM of each primer. Cycle threshold (Ct) values were used to calculate the amount of amplified PCR product relative to GAPDH and 18S ribosomal RNA. The relative amount of mRNA was calculated as $2^{-\Delta Ct}$.

Lipid extraction:

50-100 mg tissue was subjected to lipid extraction by organic solvents following a procedure described by Bligh and Dyer (27) and Folch J. et al. (28). 50 mg tissue was homogenized by Polytron (3x 10sec) in a mixture of 0.8 ml ice-cold TBS, 2 ml methanol and I ml chloroform and then centrifuged at 10,000 xg for 10 minutes. The pellet is saved to extract protein later. The supernatant was collected and mixed with 1 ml 1M NaCl and 1ml Chloroform. The final mixture was vortexed and centrifuged at 10,000 x g for 10 min to get separation of solvent layers. The lower chloroform layer containing lipids was collected. The chloroform extraction of lipids from the upper aqueous layer was repeated one more time by adding 1 ml chloroform followed by vortexing and centrifugation. The combined chloroform phase was evaporated by passing air (inside a biohazard hood) and the lipids were dispersed in 1ml TBS containing 0.1% NP-40 by sonication. An aliquot was counted for radioactivity.

Fatty Acid Oxidation:

50-100 mg liver tissue was ground in liquid nitrogen and transferred to 1.0 ml Buffer 1 (Buffer 1: 100 mM KCl, 50 mM Tris-HCl, 1 mM EDTA, 5 mM MgCl2, 1mM ATP, pH. 7.4) and homogenized. Aliquots of homogenate equivalent to 100 microgram protein were acidified with100 microliter of 70% perchloric acid. The acidified reaction mixture from each incubation vial was transferred to a microfuge tube, neutralized with 100 microliter 3 N KOH and centrifuged at 10,000 x g for 10 min. The supernatants were transferred to scintillation vials for counting radioactivity. This represents Acid Soluble Metabolites (ASM) of oxidized palmitate.

Statistics:

Data are presented as the mean + one S.E. Multiple comparisons were made using either one-way or two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. Statistical significance was concluded at P less than 0.05.

RESULTS

Lipid Uptake and Storage in Liver and Adipose Tissue

1. De Novo Lipogenesis from 2C precursor

The amount ^{14}C-acetate into lipids through *de novo* lipogenesis was higher in liver and lower in adipose tissue of KO mice compared to WT tissues (~65%), where lipogenesis was similar in both tissues (Fig. 1A). In KO mice, *de novo* lipogenesis from precursor is higher in liver than adipose tissue, yet KO liver did not accumulate fat as demonstrated in previous work (23).

2. Lipogenesis from Fatty Acids

In contrast, lipogenesis from preformed fatty acids, measured by incorporation of preformed ^{14}C-palmitate fatty acid into liver and adipose tissue, showed an increase in both KO liver (~89%, $p<0.01$; WT vs. KO) and adipose tissue (55%, $p<0.001$ WT vs. KO) compared to WT mice (Fig. 1B). Comparison between KO liver and adipose tissue (Fig. 1B) showed an increase of ~45% in adipose tissue ($p<0.003$; liver vs. adipose). Overall, KO adipose tissue incorporates more preformed fatty acids compared to WT and KO liver. This supports results from previous studies (23) of increased adipose tissue mass in KO compared to WT mice due to increase in lipid uptake and storage (21).

3. Fatty Acid oxidation to ASM

Oxidation of fatty acids produces CO2 and short chain acid-soluble fatty acids and keto acids. Measurement of these acid soluble metabolites (ASM) gives a measure

of fatty acid oxidation. Therefore, breakdown of fatty acids were measured by radioactive acid soluble metabolites (ASM). Fatty acid oxidation is higher (7080 *versus* 4184 cpm/gm tissue in WT; $p<0.02$ WT vs. KO) in KO liver compared to WT mice (Fig. 1C). However, oxidation in KO adipose tissue was reduced by ~54% in comparison to WT adipose tissue (Fig 1C; $p<0.001$ WT vs. KO). Overall, both WT and KO adipose tissue had lower amount of ASM compared to liver. WT adipose tissue and KO liver is oxidizing more fatty acids than KO adipose tissue indicating storage of lipids in adipose tissue while fatty acid disposal is significantly diminished by 82% in KO adipose compared to KO liver.

Expression of Genes Involved in ***Lipid Metabolism in KO Mice***

1. Lipogenesis and lipolysis

Srebp is a transcription factor which regulates expression of number of lipogenic genes. The mRNA expression level of lipogenic genes, *Srebp and Ppar-g* was increased by a factor of 3.65 ($p<0.001$ vs. WT) and 1.8 ($p<0.05$ vs.WT) repectively in KO mice liver. The other lipogenic gene measured, *Gpat* showed no significant change in KO liver. However, in adipose tissue the treatment of PST showed an increase in both lipogenic genes, *Srebp* by ~50% ($p<0.05$; KO+PST vs. KO and WT) and *Gpat* by ~42% ($p<0.001$; KO+PST vs. KO and WT) compared to KO mice (Fig. 2B). Presence of PST increases lipogenesis in adipose tissue in KO compared to WT suggesting that PST exerts greater effects in the absence of precursor CHGA protein or other CHGA-derived peptides in KO mice. On the other hand, KO mice showed no significant change in the mRNA expression level of *HSL and ATGL*

genes in adipose tissue (Fig. 2C), which are involved in the hydrolysis of triglycerides and plasma lipids into fatty acids which are then processed through β-oxidation. Treatment with PST also did not cause a significant change in the expression levels of these genes, which are involved in lipolysis compare to KO mice (Fig. 2C). Together with the results from Figure 1, it further suggests that (i) increased uptake of fatty acids, and (ii) reduced fatty acid oxidation in KO adipose tissue facilitate redistribution and storage of lipids from liver to adipose tissue in KO mice.

2. Fatty Acid Oxidation

The mRNA level of lipogenic genes such as *Cpt-1,* and *Ppar-α* were lower in KO mice liver and were conversely increased by a factor of ~11.5 ($p<0.001$; KO+PST vs. WT and KO) and ~1.6 ($p<0.001$; KO+PST vs. WT and KO) respectively, with the treatment if PST compared to KO mice. The *Acox* mRNA levels are increased in KO mice and further increased by ~80% with the administration of PST from KO liver ($p<0.001$; KO+PST vs. WT and KO) (Fig. 3). Peroxisomal oxidation gene, *Acox* ,and mitochondrial oxidation gene, *Cpt-1*, increased a significant amount compared to transcription factor *Ppar-α* (only 56% increase) in KO mice with PST. Again, PST seems to be a more effective stimulator of gene expression (compared to WT) in absence precursor CHGA protein or other CHGA-derived peptides. Interestingly, contrasting expression pattern between *Cpt-1* and *Acox* in KO mice suggests that peroxisomal oxidation of fatty acids may play greater role in lipid disposal by liver in KO mice. Increase catabolism of lipids in the presence of PST in liver compared to

adipose tissue further supports that increase in adipose mass size in KO mice is due to gain in lipid storage in PST deficiency.

Hepatic Insulin Sensitivity in KO Mice

1. Effects of Insulin on hepatocytes

The presence of insulin in WT mice results in a decrease in mRNA levels of *Pepck* and *G6pase*, gluconeogenic genes in comparison to liver of WT mice. (Fig. 4). The inhibitory effect of insulin on these genes establishes a negative relationship between insulin and gluconeogenesis and glycogenolysis. KO mice mRNA expression levels were reduced by 45% and 76%, *Pepck* ($p<0.05$; KO vs. WT) and *G6pase* ($p<0.001$; KO vs. WT), respectively. Presence of insulin in KO mice further reduced *Pepck* by 65% ($p<0.001$; KO+INS vs. KO) and *G6pase* by 41% ($p<0.001$; KO+INS vs. KO) compared to KO mice. Addition of insulin causes KO mice hepatocytes to be more insulin sensitive, indicating *Chga* protein or *Chga*- derived peptides play a role in antagonizing insulin activity, and *Chga*-deficiency contributed to enhanced insulin sensitivity in KO mice.

2. Effects of PST on hepatocytes

KO compared to WT mice, KO mRNA levels of gluconeogenic genes, such as *Pepck* and *G6pase,* was 45% ($p<0.01$; KO+PST vs. KO) and 76% ($p<0.001$; KO+PST vs. KO) lower respectively. Conversely pretreatment of KO mice with PST increased *Pepck* and *G6pase* mRNA expression levels in comparison to KO mice (Fig. 5). Previous studies have shown KO mice have reduced hepatic gluconeogenesis marked

by decrease in glucose production in response to pyruvate, with PST restoring glucose response to pyruvate (23). Together with the reduction of gluconeogenic genes in KO mice with previous findings, emphasizes higher insulin sensitivity in KO mice as well as PST, the *Chga*-derived peptide, as the regulator of Pepck and G6pase expressions.

Comparison of PST and CST

Chga has many biologically active derivates, therefore the affects of PST and CST on mRNA expression level of hepatocytes were compared to get a better understanding which peptide specifically is causing change in expression levels of various genes. As noted before, the presence of PST increases the mRNA expression of gluconeogenic genes (Fig. 5), *Pepck* was raised above WT and *G6pase* levels were increase but not beyond WT expression levels. Despite the increase in *Pepck* levels with treatment of PST and CST beyond WT expression levels, *G6pase* expression levels do not respond the same way. Thus, indicating the KO phenotype seen is also regulated by other *Chga* peptides.

Since lipid metabolism is also effected in *Chga* KO mice liver (Fig. 1A, B, and C), we further tested how mRNA expression levels are affected by the two *Chga* derivatives, PST and CST. As shown before, *Srebp* expression was elevated in KO mice, compared to WT (Fig. 2A). PST supplementation to KO mice caused suppression of Srebp expression (Fig. 6A). In contrast, CST supplementation to KO mice did not influence *Srebp* expression (Fig. 6B). *Ppar-γ* ($p<0.05$; KO vs.WT), was elevated in liver, as seen before, in KO mice compared WT mice (Fig. 2A). But, in contrast *to Srebp, Ppar-g* expression level was not modulated by PST (Fig. 6A). The

addition of CST had no significant effect on *Srebp* and *Ppar-γ* in comparison to KO mice (Fig. 6B). Both PST and CST supplementation enhanced *Ppar-a* and *Cpt-1* (genes involved in fatty acid oxidation) expressions (Fig.6 A and B). Once again suggesting other *Chga* peptides are involved in the regulation of these genes. β-oxidation genes, *Acox, Cpt-1,* and *Ppar-α,* in liver mRNA expression increased in the presence of PST and CST above both WT and KO mice (Fig. 2A & 6) suggesting that in presence of Chga-precursor protein or is cleaved peptides, as in WT mice, PST and CST actions are lightly controlled whereas, in their absence, as in KO mice, these two peptides act as stimulator of gene expression except for *Srebp* and *Ppar-g* genes. *Srebp* expression is uniquely regulated by PST (not CST) whereas, *Ppar-g* expression is regulated by neither of them.

DISCUSSION

Both acetate and palmitate incorporation are increased (Fig. 1A and B) in KO liver and adipose tissue compared to WT. Stimulation of lipid uptake in the liver can be attributed to the increase in lipogenic genes in the liver (Fig. 2A), The expression of *Srebp* and *Ppar-g* have increased significantly in KO mice liver. The increase in lipogenic genes indicates that *Chga* plays an important role in lipogenic metabolism. Adipose tissue in KO mice have a decrease in acetate incorporation but an increase in palmitate uptake (Fig. 1A and B). The expression of lipogenic genes, *Srebp* and *Ppar-g*, and lipolytic genes (HSL and ATGL) are only slightly changed in adipose tissue (Fig 2 B and C). KO adipose tissue incorporates a significant amount more palmitate compared to liver (Fig 1B), however liver oxidizes a larger amount than adipose tissue (Fig. 1C). Genes involved in β-oxidation are increased in KO liver (Fig. 3), whereas genes for lipolysis in adipose tissue show no significant change (Fig. 2C). Combination of increased palmitate in adipose tissue with lower β-oxidation activity causes an overall accumulation of lipids in adipose tissue. Even though KO liver incorporates more acetate than adipose tissue, there is a decrease in plasma triglycerides in KO mice compared to WT (23). Therefore, despite the increase in lipid uptake in liver compared to adipose tissue, liver mass is not increasing nor is there an increase in plasma triglycerides (data presented in 23). Lipogenic genes activity are not increase in KO adipose tissue as they are in liver, which means the increase of lipids in adipose tissue is due to incorporation of pre-forms lipids rather than synthesis of lipids (Fig. 1B and 2B). Overall, adipose tissue in KO mice has an increase in lipid content (Fig. 1B), no significant change in lipogenic or lipolytic genes (Fig. 2 B & C)

and decrease in β-oxidation (Fig. 1C) so lipids produced in the liver are being redistributed to adipose tissue preventing a rise in plasma or liver lipid content. As a result there was an increase in adipose tissue mass in KO mice compared to WT (data shown in 23) because adipose tissue is accumulating more lipids and disposal of lipids is not greater. The increase in adipose tissue could be due to individual cells are increasing lipogenic activity causing increase in cells size leading to increase in adipose tissue mass or precursor adipocytes are recruited therefore increasing the number of cells performing lipogenesis and storage causing adipose tissue to increase in mass size. Another possibility of increase lipid storage in adipose tissue may be due to the fact that KO mice are more insulin sensitive as previous studies have shown, causing a reduction in the need of lipid breakdown for energy purposes. Further studies to explore whether increase in adipose mass size is due to individual adipocytes increasing in size or recruitment of new adipocytes, and energy usage would allow better understanding of *Chga* involved in lipid metabolism.

As stated *Chga* has many peptide biologically active derivates, as PST is one of them. The presences of PST on gene expression were quantified to better understand the role of PST in lipid metabolism. Increase in lipogenic liver genes (Fig. 2A) in KO mice were decreased with PST treatment (Fig. 6A). Insulin is known to have a positive effect on fatty acid synthesis, therefore the decrease in lipogenic genes are consistent with the antagonistic effect of PST on insulin activity (29, 30). Once again, adipose tissue responded to PST in opposite manner than liver. KO mice treated with PST caused a decrease in *Srebp* expression but no change in adipose tissue (Fig. 2B). The opposing responses of liver and adipose tissue in the presence of PST pattern

were also seen in lipid catabolism. Presence of PST suppressed *Srebp* (Fig. 6A) but increased the gene expression of *Cpt-1, Acox* and *Ppar-α* (Fig. 3) while having no signification effect on adipose genes, *HSL* and *ATGL* (Fig. 2C). CST on the other hand did not influence expression of lipogenic genes but stimulated expression genes for fatty acid oxidation (Fig. 6B). Overall, it appears that *Chga*-deficiency in KO mice caused increased lipogenesis and oxidation of lipids in liver (20) compared to adipose tissue (29-33). Decreased lipolytic activity and fatty acid oxidation led to accumulation of fat in adipose tissue in KO mice.

Gluconeogenic genes, *Pepck* and *G6pase* in WT mice with insulin treatment are reduced compare to WT (Fig 4). Reduction of *Pepck* and *G6pase* in the presence of insulin illustrates the inhibitory role insulin has on these gluconeogenic genes. As known from before glucose metabolism in KO mice is suppressed. KO mice have a lower amount of *Pepck* and *G6pase* expression indication of insulin sensitivity in the absence of *Chga*. Addition of insulin in KO mice, further increased their insulin sensitivity (Fig 4.) which means that *Chga* does have an effect on insulin metabolism.

Previous studies confirm increased insulin sensitivity in KO mice through GTT, ITT, clamp studies, and pyruvate tolerance test (23). Consistent with the antagonistic effect of the *Chga* peptide PST on insulin the current study demonstrated that *Chga* KO mice display *increased* insulin sensitivity as confirmed by decrease in *Pepck* and *G6pase*. Studies further suggest that the main antagonistic effect of PST on insulin action is to reduce the suppressive effect of insulin on hepatic gluconeogenic genes (Fig. 5). Administration of PST raised the mRNA levels of *Pepck* and *G6pase*

gluconeogenic genes. These findings identify a novel role for PST in the regulation of gluconeogenic gene transcription by insulin.

Chga is a proprotein with many active derivatives which can play a role on insulin sensitivity in KO mice. For better understanding of the roles of various peptides on insulin sensitivity, KO mice were also treated with CST. Contrast of PST and CST effects on mRNA levels of lipogenic and oxidative genes were also studied. The presence of PST did not increase *Srebp* levels equivalent to WT levels and treatment of CST did not have a significant effect. Expression levels of Ppar-γ were not significantly affected with the administration of PST or CST compared to KO mice (Fig. 6A and B). This suggests that other *Chga*-derived peptides also play an important role in regulation of lipogenic genes. Hepatic genes involved in oxidation, *Cpt-1, and Ppar-α*, mRNA levels were raised beyond WT levels with PST and CST treatment (Fig. 6A and B). This means the decrease in hepatic oxidation seen in KO mice is due to *Chga* deficiency and absence of peptide derivatives PST and CST are mainly responsible for the phenotype seen.

Chga deficiency increased lipid disposal and storage in adipose tissue and possible redistribution from liver since blood lipid parameters were decreased in KO mice (23) perhaps to increase insulin sensitivity. The elevated hepatic oxidation (Fig. 1C) in KO mice and decrease in gluconeogenic genes may lead to metabolic adaptation to fatty acid rather than glucose oxidation for fuel to prevent muscle protein breakdown. Although catecholamines stimulate transient gluconeogenesis and glycogenolysis (34, 35) glucose production is low and hepatic glycogen content is high in KO as compared with WT mice (23). This could be due to the absence of PST-

induced glycogenolysis (Fig. 5) in KO mice. Thus, glucose homeostasis in PST-deficient hypertensive *Chga* KO mice is maintained by (i) increase suppression of hepatic gluconeogenic genes, (ii) increase in β-oxidation in hepatocytes and (iii) decreased lipid concentrations in plasma and liver due to sequestration of lipids into adipose tissue.

In summary, lack of PST increases insulin sensitivity and helps maintain euglycemia in KO mice by increased sequestration of circulating and hepatic lipids into adipose tissue and increased suppression of hepatic gluconeogenesis. Despite the low level of insulin (only 20% of WT) and the development of hypertension, due to the loss of catestatin, KO mice remained euglycemic (23). PST is overexpressed by ~3.7 fold in patients with Type 2 diabetes mellitus (21, 26), our results may have implication for the treatment of diabetes. Our data suggests that disorders in lipid and glucose metabolism may improved by suppressing PST action.

APPENDIX

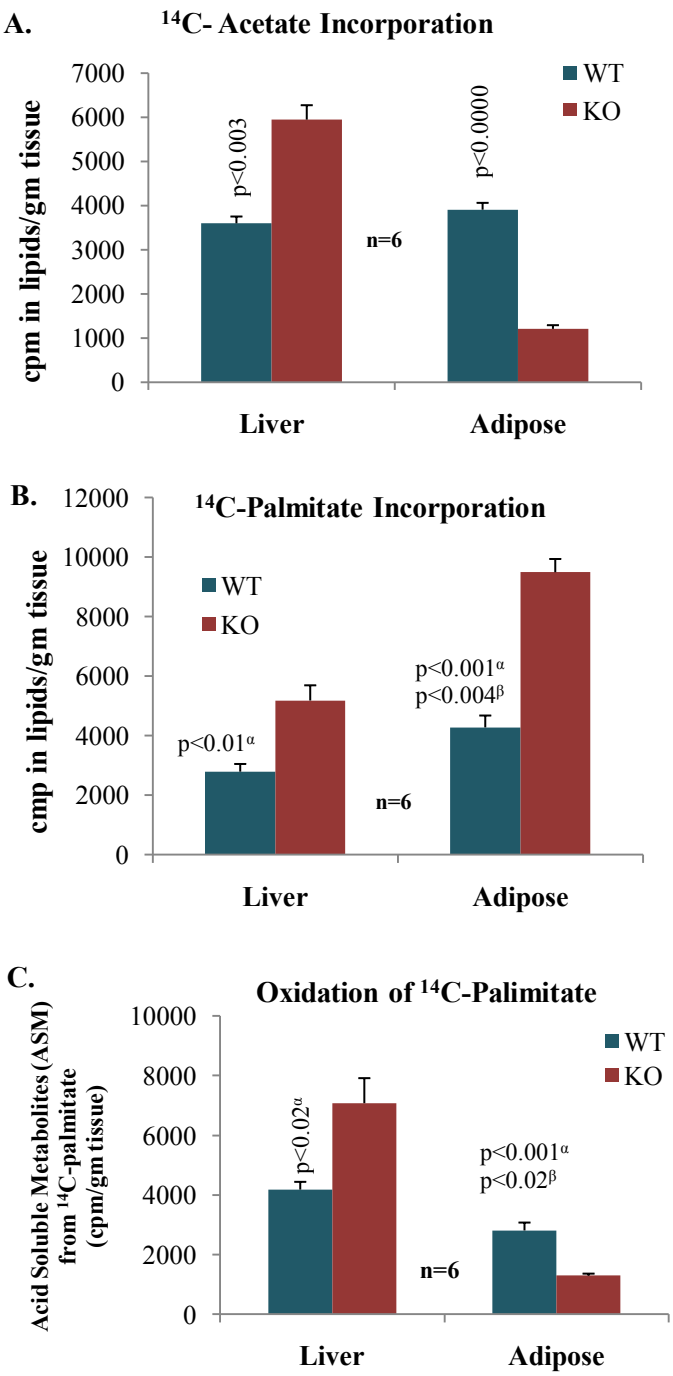

FIGURE 1. **Fatty acid synthesis and uptake in WT and KO mice.** WT and KO mice are fasted for 12 hr and subjected to radioactive *A,* de novo fatty acid synthesis. *B*, deposition of pre-formed fatty acids. *C,* fatty acid β-oxidation.

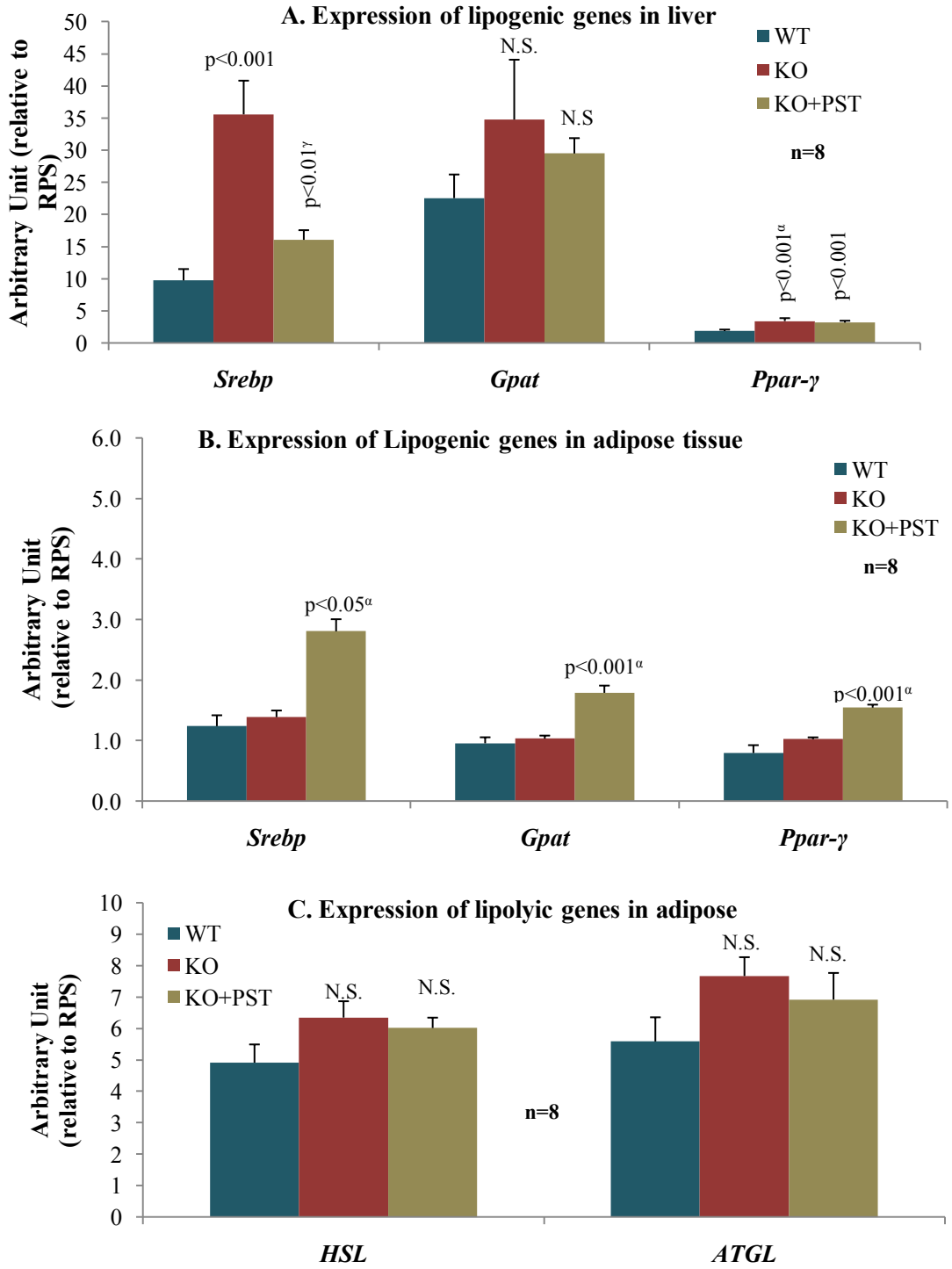

FIGURE 2. **Expression of lipogenic genes (A and B) and lipolytic genes (C).** mRNA expression levels of lipogenic genes (*Srebp, Gpat,* and *Ppar-γ)* before and after PST treatment in liver tissue (A) and adipose tissue (B). mRNA expression levels of lipolytic (*HSL* and *ATGL*) genes in adipose tissue (C). Real time PCR analysis used to measure gene expression levels. *α*, KO *versus* WT; *β*, KO+PST *versus* KO and WT; *γ,* KO+PST *versus* KO. Values are expressed as mean + S.E. *N.S.*, not significant.

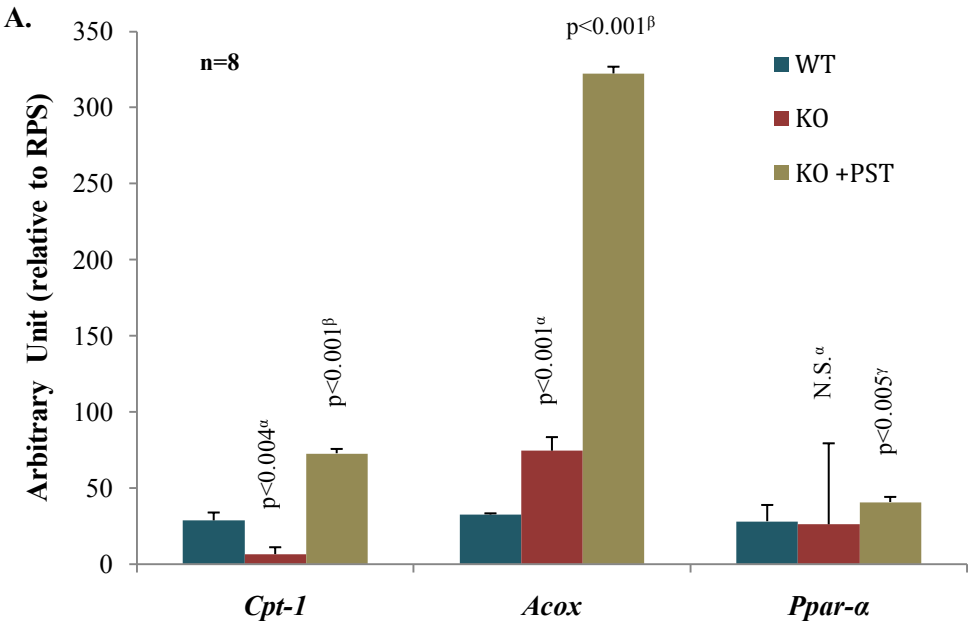

FIGURE 3. **Effects of PST on mRNA expression levels of genes involved in fatty acid oxidation in liver.** mRNA expression levels of β-oxidation genes (*Cpt-1,Acox,* and *Ppar-α)* before and after PST treatment in liver tissue. Real time PCR analysis used to measure gene expression levels. Values are expressed as mean + S.E. *N.S.*, not significant.

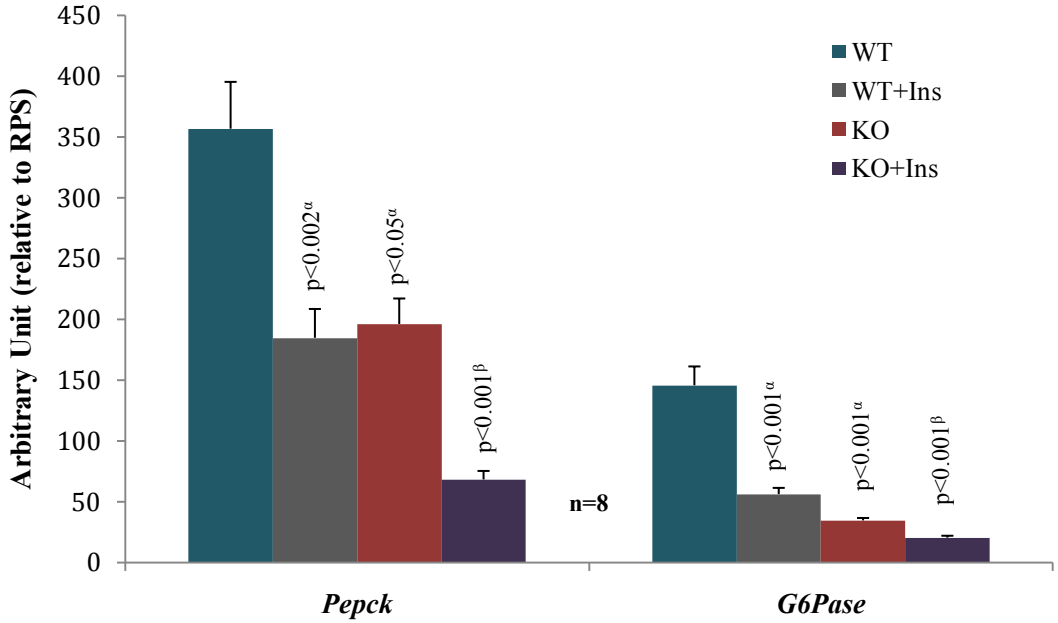

FIGURE 4. **Increased insulin sensitivity in KO mice.** mRNA expression levels of gluconeogenic genes (*Pepck* and *G6pase)* in WT and KO before and after treatment of insulin (INS) in hepatocytes. Real time PCR analysis used to measure gene expression levels. *α*, *versus* WT; *β*, KO+PST *versus* WT+INS and KO. Values are expressed as mean + S.E.

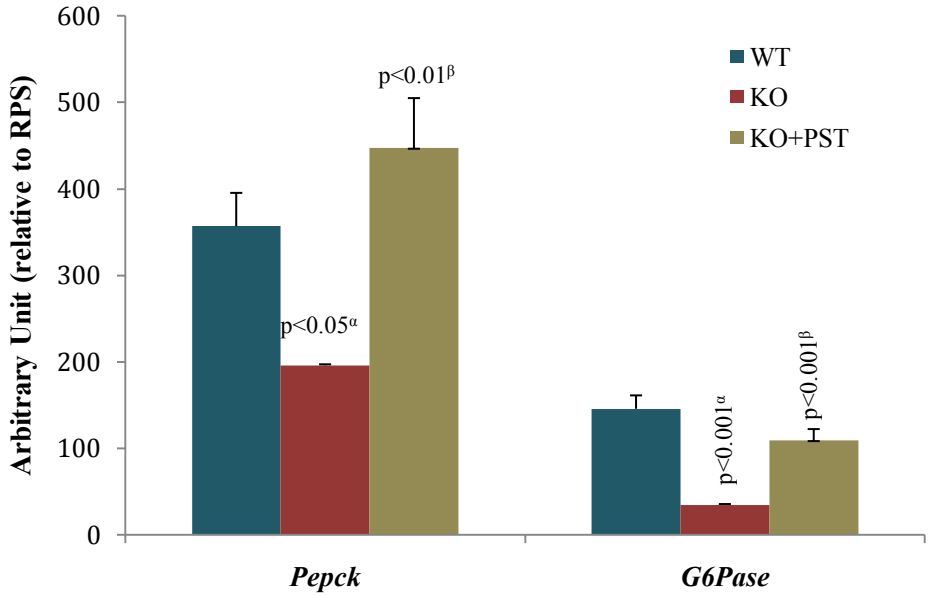

FIGURE 5. **Gluconeogenic gene expression levels in hepatocytes treated with Pancreastatin.** mRNA expression levels of gluconeogenic genes (*Pepck* and *G6pase)* in KO mice before and after treatment of pancreastatin (PST) in liver cells. Real time PCR analysis used to measure gene expression levels. *α*, KO *versus* WT; *β*, KO+PST *versus* KO. Values are expressed as mean + S.E.

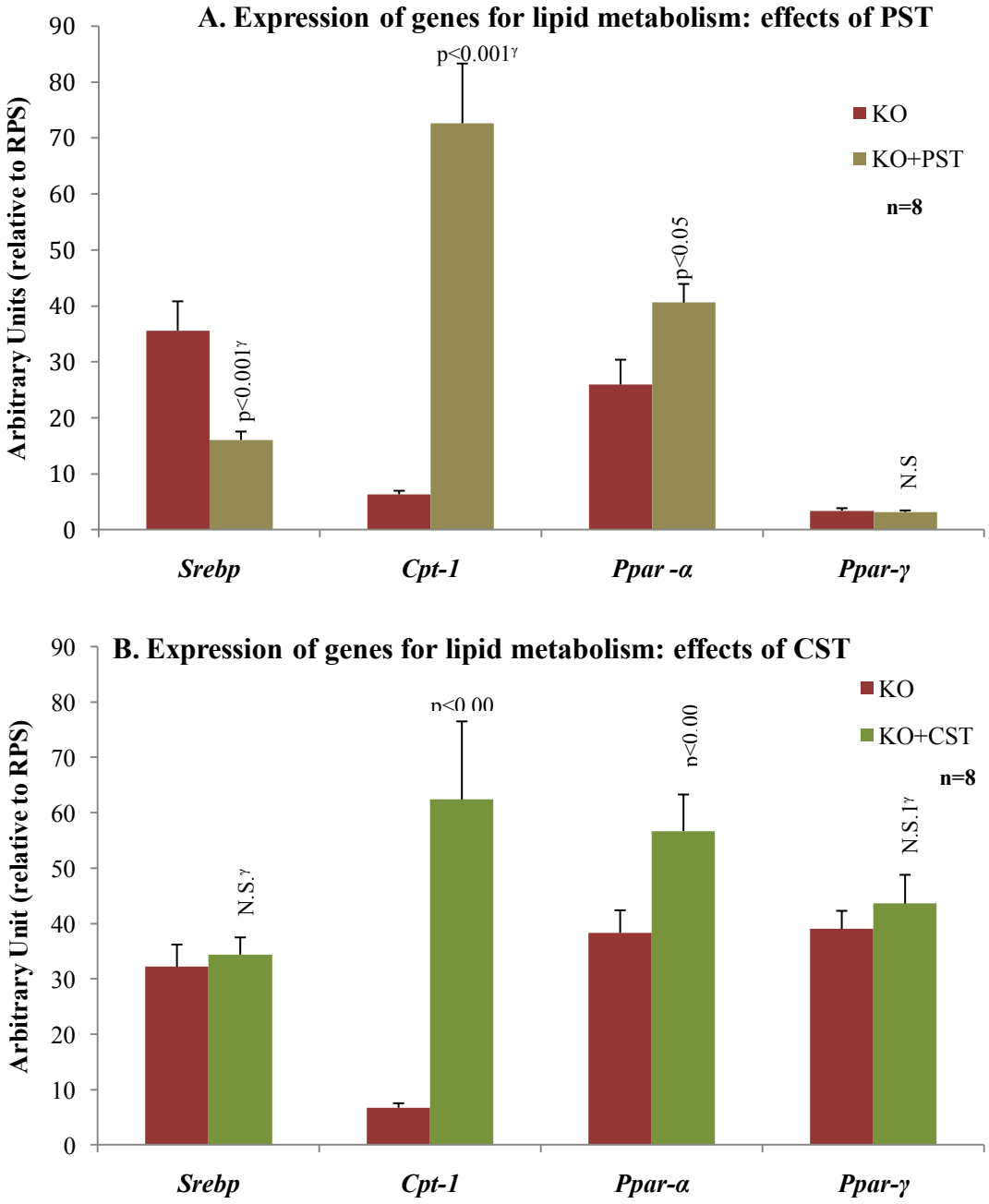

FIGURE 6. **Comparison of effects of PST (A) and CST (B) on gene expression.** *A,* mRNA expression levels of lipogenic genes (*Srepb* and *Ppar-γ)* and β-oxidation genes (*Cpt-1* , and *Ppar-α)* in KO before and after PST treatment in liver tissue. *B,* mRNA expression levels of lipogenic genes (*Srepb* and *Ppar-γ)* and β-oxidation genes (*Cpt-1* and *Ppar-α)* before and after CST treatment in liver tissue. Real time PCR analysis used to measure gene expression levels. γ, KO+PST or CST vs. KO. Values are expressed as mean + S.E.

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