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**Ghrelin neutralization during fasting-refeeding cycle impairs the recuperation of body weight and alters hepatic energy metabolism.**

Susana Sangiao-Alvarellos<sup>1,2</sup>, Steffen Helmling<sup>3</sup>, María J. Vázquez<sup>4</sup>, Sven Klussmann<sup>3</sup> & Fernando Cordido<sup>1,2,5</sup>

<sup>1</sup> Department of Medicine, School of Health Science, University of A Coruña. Campus de Oza. s/n. 15006 A Coruña. Spain.

<sup>2</sup> INIBIC. Instituto de Investigación Biomédica de A Coruña. Xubias de Arriba, 84. 15006. A Coruña. Spain

<sup>3</sup> NOXXON Pharma AG, Max-Dohrn Str. 8-10, 10589 Berlin, Germany.

<sup>4</sup> Department of Physiology, School of Medicine, University of Santiago de Compostela, Santiago de Compostela, 157852 Spain

<sup>5</sup> Endocrine Department, Complejo Hospitalario Universitario A Coruña (CHUAC). 15006. A Coruña, Spain.

**\* Address correspondence to:**

Susana Sangiao-Alvarellos, PhD

Department of Medicine, School of Health Science, University of A Coruña. Campus de Oza. s/n. 15006 A Coruña. Spain

Tel: +34 981 178000

Fax: +34 981 178273

E-mail: susana.sangiao@udc.es

**ABSTRACT**

Ghrelin, a hormone whose levels increase during food deprivation, plays a pivotal role in the regulation of food intake, energy metabolism and storage, as well as in insulin sensitivity. Here, we investigated the effects of acyl-ghrelin neutralization with the acyl-ghrelin-binding compound NOX-B11(2) during the fasting-refeeding cycle. Our data demonstrate that ghrelin neutralization with NOX-B11(2) impairs recuperation of lost body weight after food deprivation. Analysis of enzymes involved in glucose and lipid metabolism in liver of fed, fasted and refed rats revealed that neutralization of acyl-ghrelin resulted in minor decreases in the enzymes of glycolytic and lipogenic pathways during fasting. However, during refeeding these enzymes as well as glycogen levels recovered more slowly when acyl-ghrelin was blocked. The high levels of ghrelin in response to food deprivation may contribute to an adequate decrease in hepatic glycolytic and lipogenic enzymes and aid in the recovery of body weight and energetic reserves once food becomes available after the fasting period.

**Key words:** ghrelin, liver, fasting, refeeding, lipid and glucose metabolism.

**Abbreviations:**

6PGDH: 6-phosphogluconate dehydrogenase  
ACCA: acetyl-CoA carboxylase alpha  
ACL: ATP citrate lyase  
AKT: total serine/threonine kinase protein kinase B  
AMPK $\alpha$ : 5'AMP-activated protein kinase alpha  
CPT1A: liver isoform of carnitine palmitoyltransferase1  
FAS: fatty acid synthase  
FBase: fructose biphosphatase  
G6PDH: glucose-6-phosphate dehydrogenase  
GK: glucokinase  
GOAT: ghrelin O-Acyltransferase  
HK: hexokinase  
IR: insulin receptor  
LPL: lipoprotein lipase  
PK: pyruvate kinase  
SCD-1: stearoyl-CoA desaturase-1

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## 1. INTRODUCTION

Energy homeostasis is a complex and tightly regulated process under control of neural and endocrine inputs. The survival of animals is intimately linked with the drive to find and accumulate food. It has been hypothesized that the scarcity of food throughout evolution favored dominant genetic pathways leading to increased caloric intake over mechanisms that cause reduction in energy intake (Ames 2006). However, currently in Western societies obesity and its co-morbidities are the fastest growing health problem. Available pharmaceuticals have poor efficacy and are associated with undesirable side effects, emphasizing the need for new therapies (Hofbauer et al. 2007).

In the last decade, ghrelin, a hormone secreted predominantly from the stomach, has attracted much attention due to its orexigenic effects as well as its function in mediating body weight gain and adiposity, demonstrated both in humans and rodents (Nakazato et al. 2001; Sangiao-Alvarellos et al. 2009; Theander-Carrillo et al. 2006; Tschop et al. 2000; Wren et al. 2001a, 2001b). Ghrelin circulates in the bloodstream in two different forms: acylated (AG) and unacylated (UAG). AG has a unique feature: a posttranslational esterification of a fatty acid (n-octanoic or, to a lesser extent, n-decanoic) on serine at position three. It acts as the endogenous ligand of the growth hormone secretagogue receptor (GHSR) (Kojima et al. 1999, 2001a, 2001b; Nakazato et al. 2001) and its acylation is considered necessary for its actions via GHSR1a (the functional variant of GHSR) (Howard et al. 1996; Kojima et al. 1999). Recently, several groups identified ghrelin O-Acyltransferase (GOAT), a membrane-bound enzyme that transfers octanoate from octanoyl CoA to serine-3 of ghrelin, forming an acyl ester (Gutierrez et al. 2008; Yang et al. 2008) and a recent study observed that GOAT links dietary lipids with the endocrine control of energy balance (Kirchner et al. 2009).

Despite much pharmacological data, the evidence for a physiological role of ghrelin in the control of appetite is still less clear. Mice with targeted deletions in either the gene for ghrelin or the GHSR as well as transgenic models exhibit an essentially normal metabolic phenotype when fed a regular chow diet, suggesting ghrelin may have a redundant role in the regulation of food intake (Ariyasu et al. 2005; Asakawa et al. 2005; Iwakura et al. 2005; Longo et al. 2008; Pfluger et al. 2008; Sun et al. 2003, 2006, 2008). Nevertheless, the current body of evidence implicates ghrelin to play an important role in the control of glucose and lipid homeostasis (Broglia et al. 2001, 2003; Vestergaard et al. 2008a, 2008b; Zhao et al. 2010).

Several studies show that treatment with ghrelin induces hyperglycemia and reduces insulin secretion (Broglia et al. 2001; Cui et al. 2008). Moreover, it was found that a relation between ghrelin levels and insulin resistance exists (Gauna et al. 2004; Pacifico et al. 2009; Vestergaard et al. 2008b). Altered plasma ghrelin levels are associated with insulin resistance in healthy, overweight and obese humans, and are thought to contribute to the association of insulin resistance and metabolic syndrome (Pacifico et al. 2009; St-Pierre et al. 2007; Yada et al. 2008).

Furthermore, ghrelin administration induces lipid storage and synthesis by promoting mRNA expression of fat storage-promoting enzymes in white adipose tissue (WAT) and liver, such as lipoprotein lipase (LPL), acetyl-CoA carboxylase alpha (ACC $\alpha$ ), fatty acid synthase (FAS), and stearoyl-CoA desaturase-1 (SCD-1). In contrast, ghrelin inhibits lipid oxidation, decreasing activity and gene expression of the liver isoform of carnitine palmitoyltransferase 1 (CPT1A) (Barazzoni et al. 2005; Sangiao-Alvarellos et al. 2009; Theander-Carrillo et al. 2006).

These findings and the observations that circulating ghrelin levels are increased prior to a meal and during food deprivation and caloric restriction, as well as in disease states such as anorexia and cachexia, but reduced after a meal and in obesity (Ikezaki et al. 2002; Liu et al. 2008; Shiiya et al. 2002; Tschop et al. 2001a, 2001b; Van der Lely et al. 2004; Yang et al. 2007), strongly implicate ghrelin being involved in the regulatory processes controlling meal initiation, priming metabolic pathways and leading to more efficient calorie storage.

The aim of this study was to block acyl-ghrelin in order to investigate the metabolic adjustments during the fasting-refeeding cycle on glucose and lipid metabolism in the liver, the principal tissue of intermediary metabolism. Protein and mRNA levels as well as enzyme activities of the main enzymes involved in glucose and lipid metabolism were assessed. The neutralization of AG was accomplished by using the compound NOX-B11(2), an L-RNA-based antagonist (also called a Spiegelmer) of ghrelin. NOX-B11(2) does not interact with the ghrelin receptor, but is highly specifically binding the bioactive n-octanoylated form of ghrelin itself. Moreover this compound neutralizes other types of acylated ghrelin like decanoyl-ghrelin (data not show, decenoyl-ghrelin was not tested). It blocks the biological effect by preventing ghrelin's activation of the endogenous receptor (Helmling et al. 2004). The effects of ghrelin neutralization with NOX-B11(2) and related compounds on growth

hormone secretion, food intake and body weight gain in diet-induced obese mice were previously demonstrated (Helmling et al. 2004; Kobelt et al. 2006; Shearman et al. 2006).

Our data suggest that ghrelin has an essential role in body weight recovery after prolonged fasting. Moreover, neutralization of ghrelin impairs the normal decrease in lipogenic and glycolytic enzymes in the liver during fasting. Upon availability of food following a fasting period, the recovery of energetic reserves and metabolic pathways is compromised when ghrelin is neutralized.

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## 2. MATERIALS AND METHODS

### 2.1. Animals and compounds

All experiments and animal protocols involved in this study were reviewed and approved by the *Ethics Committees* of the UDC and CHUAC, in accordance with EU Normative for the use of experimental animals. Male Wistar rats (2-3 months old; body weight: 378 g  $\pm$  5 g) were housed in a temperature controlled room, with a 12 h light, 12 h dark cycle (lights from 08:00 to 20:00 h). All rats were provided with *ad libitum* access to water.

Acyl-ghrelin was purchased from Bachem (Bachem, Switzerland). Spiegelmer (SPM) NOX-B11(2) and the non-functional control SPM were synthesized at NOXXON Pharma AG using standard phosphoramidite chemistry. NOX-B11(2) is a derivative of SPM NOX-B11 whose selection has been described previously (Helmling et al. 2004). It was designed to bind specifically to acylated (bioactive) ghrelin, thus preventing the hormone from activating the GHS-R1a (Helmling et al. 2004; Shearman et al. 2006).

### 2.2. Effects of acute ghrelin treatment on food intake in animals treated with anti ghrelin SPM NOX-B11(2)

To study the efficacy of NOX-B11(2) counteracting acyl-ghrelin, male Wistar rats *fed ad libitum* were intraperitoneally (IP) injected with vehicle (saline, n = 7), NOX-B11(2) (15 or 30 mg/kg, n = 7), or control SPM (30 mg/kg, n = 7) at 9:00 a.m.. Two hours later, at 11:00 a.m. the animals were injected with saline or ghrelin (25  $\mu$ g/rat, IP). Cumulative food intake was measured 2 h after treatment at 1:00 p.m..

### 2.3. Fasting and fasting-refeeding trials

In order to clarify the influence of acyl-ghrelin neutralization on hepatic glucose and lipid metabolism during the fasting-refeeding cycle, rats were fasted for 48 h or fasted and refeed for 24 h or 48 h. Every 24 h animals (eight rats per group) were injected intraperitoneally with NOX-B11(2) (30 mg/kg, IP) or control SPM (30 mg/kg, IP). The first injection was given once food had been removed. During the refeeding period, food was restricted to 80% of the daily amount of food ingested by the animals when given *ad libitum* access. Food was provided daily and both groups ate all the food offered. This

refeeding regimen was chosen as it was expected to lead to a more pronounced effect, if ghrelin neutralization affects the utilization of nutrients. The regimen of treatment is illustrated in Fig. 1.

Rats were sacrificed by cervical dislocation and trunk blood was extracted. Livers were dissected, divided in parts, and stored at  $-80^{\circ}\text{C}$  until further processing or homogenized with a motor pestle in ice-cold buffer containing 50 mM Tris-HCl (pH 7.4), 250 mM sucrose, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 100 mM NaF and protease inhibitor cocktail (Roche). Subsequently, homogenates were centrifuged for 10 min at 3000 r.p.m. and the supernatant was extracted and used for studies of CPT1 activity.

#### 2.4. Plasma measurements

Insulin levels were measured by a commercially available radioimmunoassay (*Linco Research Inc.*) according to the manufacturer's specifications. Plasma acyl-ghrelin levels were measured by ELISA according to the manufacturer's instructions (*Millipore*). Plasma glucose, lactate and triglyceride levels were assessed using a commercial kit based on a colorimetric method (*Glucose, lactate and Triglyceride Spinreact*). Plasma GH concentrations were determined by double antibody RIA using materials supplied by the National Hormone Pituitary Program, as described previously (Seoane et al. 2004)

#### 2.5. Real-time quantitative PCR

mRNA levels of ACC $\alpha$ , FAS, SCD-1 and CPT1A were studied by real-time PCR (*Roche*) using specific primers and probes (Table 1). All reactions were carried out using the following cycling parameters:  $50^{\circ}\text{C}$  for 2 min,  $95^{\circ}\text{C}$  for 10 min followed by 40 cycles of  $95^{\circ}\text{C}$  for 15 sec and  $60^{\circ}\text{C}$  for 1 min. For data analysis, the input value of the target gene was standardized to the 18S value for each sample. Data were expressed in comparison with the average value for the fed rats.

#### 2.6. Western blotting

Liver samples were homogenized in 10 volumes ice-cold buffer containing 50 mM Tris-HCl (pH 7.4), 250 mM sucrose, 1 mM EDTA, 1mM DTT, 100 mM NaF and protease inhibitor cocktail (*Roche*). For each sample, total protein lysates from liver (20  $\mu\text{g}$ ) were subjected to SDS-PAGE,

electrotransferred on a polyvinylidene difluoride membrane and probed with the indicated antibodies: ACC $\alpha$  and phosphoACC $\alpha$ -Ser79 (pACC $\alpha$ ); 5'AMP-activated protein kinase alpha (AMPK $\alpha$ ) and phosphoAMPK $\alpha$ -Thr172 (pAMPK $\alpha$ ) (*Upstate*); total serine/threonine kinase protein kinase B (AKT) and phospho AKT (pAKT) (*Cell Signaling*);  $\beta$ -actin (*Sigma*); FAS and CPT1A (*Santa Cruz Biotechnology*). For protein detection we used horseradish peroxidase-conjugated secondary antibodies and bands were visualized by enhanced chemiluminescence as instructed by the manufacturer (*Amersham Biosciences*). For quantification we applied ImageJ software based analysis (<http://rsb.info.nih.gov/ij/>). Protein levels were normalized to  $\beta$ -actin for each sample.

### 2.7. Enzyme and glycogen assays

With the exception of CPT1 activity, which was analyzed in fresh tissue, all enzymatic assays were carried out with liver supernatants that were prepared as described above for Western blot assays. The reactions were started by the addition of homogenates (30  $\mu$ l) and substrates (20  $\mu$ l, omitted in controls) to the reaction mixture (final volume 250  $\mu$ l), and the reactions were incubated at 37 °C for pre-established times (5–15 min). Enzyme activities were determined by spectrophotometry using a microplate reader (*Tecan*). FAS (Saggerson and Greenbaum 1970), glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH) (Tian et al. 1998, 1999), CPT1 (Karlic et al. 2002; Shin et al. 2006), ATP citrate lyase (ACL) (Corrigan and Rider 1983), glucokinase (GK), hexokinase (HK) (Davidson and Arion 1987), fructose bisphosphatase (FBase) (Westbury and Hahn 1984) and pyruvate kinase (PK) (Denton et al. 1979) activities were measured using methods previously described. Glycogen levels in the liver extracts were determined as described by Keppler and Decker (Keppler and Decker 1974).

### 2.8. Statistical analysis

Data are expressed as percentage of fed rats (control group) and presented as mean  $\pm$  SEM and analyzed using SigmaStat 3.1 (Systat software, Inc.). Statistical significance was determined by one way ANOVA with *post hoc* Student Newman Keuls test.  $P < 0.05$  was considered significant. Different letters above bars indicate statistical significance.

### 3. RESULTS

#### *3.1. SPM NOX-B11(2) inhibits orexigenic effects of ghrelin.*

In order to test the efficacy of NOX-B11(2) in neutralizing ghrelin's effect on food intake, we treated rats with Spiegelmer and AG as described under Material and Methods. As expected, pre-treatment with NOX-B11(2) blocked ghrelin-stimulated food intake, whereas the non-binding control SPM did not affect food intake induced by ghrelin (Fig. 2). Neither NOX-B11(2) nor control SPM altered food intake compared with animals treated with saline when administered without exogenous AG (data not shown).

#### *3.2. Effect of NOX-B11(2) on body weight during fasting-refeeding cycle*

To determine the effects of ghrelin neutralization during refeeding rats were fasted and re-fed with or without treatment with NOX-B11(2) as described in Material and Methods and body weight changes were recorded (Fig. 3). Body weight (BW) loss induced by fasting was unaffected by the neutralization of ghrelin. In contrast, treatment with NOX-B11(2) exerted a strong effect on the recovery of weight loss during the refeeding period. Animals treated with control Spiegelmer began to regain body weight 24 hours after refeeding and this process continued until 48 h. In contrast, rats treated with NOX-B11(2) did not recover corporal weight after the refeeding until 48 h. This effect was not related to an inhibition of appetite as both treatment groups ingested all the food offered during the refeeding period. Thus, the inhibitory effect of NOX-B11(2) on body weight recuperation during refeeding following a 48 h period of food deprivation appears to be independent of food intake.

#### *3.3. Effects of ghrelin neutralization during fasting-refeeding cycle on plasma parameters*

In order to further analyze the effects of ghrelin neutralization, various plasma parameters were examined (Fig. 4). As expected, 48 h after fasting plasma AG levels had increased significantly in rats treated with control SPM compared to the fed group. The AG levels decreased during refeeding, but did not return to the initial levels during the 48 h refeeding period. In rats treated with NOX-B11(2), fasting led to an even larger increase of AG levels and the increase did not diminish during the refeeding period (Fig. 4A). The tremendous increase in AG levels in animals treated with NOX-B11(2) is likely due to the presence of circulating AG-Spiegelmer complexes that are cleared

more slowly, a finding that was reported previously (Shearman et al. 2006) and is typical for a drug binding to a ligand.

Plasma insulin levels decreased equally in both groups following fasting. However, after refeeding insulin levels in animals treated with NOX-B11(2) were lower than in animals injected with control SPM (Fig. 4B). Similarly, plasma glucose levels were decreased after fasting in both Spiegelmer treatment groups. During refeeding, only in the animals treated with NOX-B11(2) a significant increase in plasma glucose levels was observed (Fig. 4C). In both groups glucose and insulin levels remained significantly below the levels of fed animals and we believe that this fact is for the feeding regimen, since after 48 hours of fasting the animals were subjected to caloric restriction. After 48 h of fasting, triglyceride (Fig. 4D) and lactate levels (Fig. 4E) were decreased in both experimental groups. During the refeeding period, plasma triglyceride levels in animals treated with control SPM recovered the values of fed rats within 24 h, while those treated with NOX-B11(2) remained lower even after 48 hours of refeeding (Fig. 4D). Lactate levels increased slightly during the refeeding period in rats treated with NOX-B11(2) only (Fig. 4E), but the fasting-induced decline in lactate levels was not reversed in either treatment group during the refeeding period. As expected, 48 h after fasting plasma GH levels had decreased significantly in rats treated with control SPM and NOX-B11(2) compared to the fed group, however the decrease was lower in animals treated with NOX-B11(2). During the refeeding period, either group recovered the values of fed rats but those treated with NOX-B11(2) remained higher compared to control group even after 48 hours of refeeding (Fig. 4F).

#### ***3.4. Effects of ghrelin neutralization during fasting and refeeding cycle on liver glucose metabolism***

To assess the effect of AG neutralization during the fasting-refeeding cycle on glucose metabolism we analyzed the activity levels of enzymes involved in glucose metabolism. The results for several key enzymes of the glycolytic pathway are shown in Fig. 5A-C. Food deprivation clearly diminished activity levels of GK (Fig. 5A), HK (Fig. 5B) and PK (Fig. 5C). Activity levels of GK were strongly reduced following fasting in both experimental groups. After 24 h of refeeding the recovery of GK activity was similar in animals treated with NOX-B11(2) and control Spiegelmer. However, while GK activity in the NOX-B11(2) group returned to the level of fed animals after 48 h

of refeeding, it did not recover in the control group (Fig. 5A). Activity levels of HK (Fig. 5B) and PK (Fig. 5C) were reduced following fasting in both animals treated with NOX-B11(2) and animals treated with control SPM. Following fasting there was significantly higher HK and PK activity for the NOX-B11(2) treatment group than the control Spiegelmer group, suggesting that ghrelin neutralization may have some protective effect on the activity of these enzymes during fasting. This effect disappeared during refeeding. No differences in HK and PK activities between control and NOX-B11(2) groups were observed following refeeding and the activity levels remained significantly below the levels of fed animals.

Activity levels of FBpase (Fig. 5D), a key enzyme of gluconeogenesis, increased significantly during fasting in both experimental groups and diminished during refeeding. FBpase activity during refeeding was moderately (but significantly) higher in animals in which ghrelin was neutralized. This suggests that ghrelin neutralization impairs the normal response of FBpase activity during refeeding following fasting (Fig. 5D).

Several studies relate the effects of ghrelin to changes in protein levels of serine/threonine kinase protein kinase B (AKT) and pAKT (Fig. 5E-G) (Baldanzi et al. 2002; Barazzoni et al. 2007; Chung et al. 2008; Rossi et al. 2008). Moreover, in the liver, insulin controls both lipid and glucose metabolism through its cell surface receptor and intracellular mediators such as phosphatidylinositol 3-kinase and serine-threonine kinase AKT. We observed that fasting caused a decrease in protein levels of phospho-(active) and total-AKT (Figures 5E-G). Interestingly, after 48 h fasting the levels of pAKT were significantly less reduced in rats treated with NOX-B11(2), whereas reduction of total AKT levels was the same. In animals treated with the inactive control Spiegelmer both pAKT- and AKT-levels recovered to reach the levels of fed animals after 24 h of refeeding and these values did not change after 48 h of refeeding. In animals treated with NOX-B11(2) pAKT- and AKT-levels recovered to those of fed animals after 24 h of refeeding, but they decreased again after 48h refeeding.

To further investigate the role of AG during fasting and refeeding on hepatic glycemic reserves, glycogen levels were assessed (Fig. 6). The neutralization of AG did not affect the decrease in hepatic glycogen levels during fasting. However, after 24 and 48 h of refeeding, glycogen levels were lower in NOX-B11(2) treated animals compared with their controls.

### ***3.5. Effects of ghrelin neutralization during fasting-refeeding cycle on the hepatic lipogenic pathway and the pentose phosphate pathway.***

In order to examine ghrelin function on hepatic lipogenesis during a fed-fasted-refed cycle, mRNA, protein and activity levels of enzymes involved in lipid synthesis were assessed in the different treatment groups. The levels of mRNA, protein and enzymatic activity of key enzymes involved in the regulation of lipid metabolism in liver are shown in Fig. 7. As expected, fasting markedly diminished mRNA levels of the fat storage promoting enzymes such as FAS, SCD-1, and ACC $\alpha$  in all animals, whether treated with NOX-B11(2) or not. However, after 24 h of refeeding, these enzymes' expression increased to a greater extent in animals in which AG was not neutralized (Fig. 7A-C). Similarly, protein levels of FAS, ACC $\alpha$  and pACC $\alpha$  (Fig. 7D,F, I), and activity levels of FAS (Fig. 7J) and ACL (Fig. 7K) also decreased during fasting, but this decrease was less pronounced in animals treated with NOX-B11(2). Moreover, after 24 h of refeeding protein levels of FAS and ACC $\alpha$  and activity levels of FAS and ACL significantly increased in the control group over fasted animals, but not in the NOX-B11(2) group. However these enzymes (mRNA, protein or activity) did not show any differences between control group and animals treated with NOX-B11(2) after 48 h of refeeding.

After 48 h of fasting, protein levels of pAMPK $\alpha$  and AMPK $\alpha$  (Fig. 7G-I) were significantly diminished in both groups. The decrease in AMPK $\alpha$  was lower in animals treated with NOX-B11(2) compared with the control group. 48 h after refeeding, protein levels of total AMPK $\alpha$  were further decreased in NOX-B11(2) treated animals.

Fatty acid synthesis requires NADPH. It is produced by malic enzyme (ME) and *via* the pentose phosphate pathway by activation of G6PDH and 6PGDH. Interestingly, after 48 h of fasting, the enzymatic activities of G6PDH and 6PGDH exhibited a greater decrease in the animals treated with SPM control compared with those treated with NOX-B11(2). After 24 h of refeeding only the activity of 6PGDH in control animals increased significantly (Fig. 7L-M).

Overall, these data suggest a role of AG in hepatic inhibition/restoration of lipogenesis during fasting and refeeding cycle.

### ***3.6. Effects of ghrelin neutralization during fasting and refeeding cycle on liver $\beta$ -oxidation***

We next examined levels of mRNA, protein and activity of CPT1A, an enzyme involved in lipid oxidation. The levels of mRNA for CPT1A increased during fasting and decreased with refeeding in both treatment groups. In animals treated with NOX-B11(2) the decrease of CPT1A mRNA levels appeared to recover more slowly during the first 24 h of refeeding compared to the control group, but both treatment groups exhibited the same levels after 48 h of refeeding (Fig. 8A). Protein levels of CPT1A decreased in fasted animals and recovered the values of the fed state after 24 h of refeeding in both treatment groups (Fig. 8B). Activity levels of CPT1A increased during fasting, independent of treatment. This increase persisted during the first 24 h of refeeding, but returned to the activity level of fed animals after 48 h of refeeding.



#### 4. DISCUSSION

Our results provide a clear indication that ghrelin neutralization during the fasting-refeeding cycle impairs body weight recuperation and alters hepatic glucose and lipid metabolism.

Since its identification ghrelin has received much attention due to its orexigenic and adipogenic effects, both in rodents and humans (Tschop et al. 2000; Wren et al. 2001a). However, the observations that transgenic models and ghrelin knockout animals exhibit normal metabolic phenotypes make it difficult to pinpoint ghrelin's exact role in the control of appetite and body weight (Ariyasu et al. 2005; Asakawa et al. 2005; Iwakura et al. 2005; Longo et al. 2008; Pfluger et al. 2008; Sun et al. 2003, 2006, 2008). The findings that ghrelin levels are increased before food intake, during caloric restriction, body weight loss or anorexia, but decreased after food intake and obesity (Cummings et al. 2001, 2002; Shiiya et al. 2002) are in agreement with the hypothesis for ghrelin being part of a mechanism by which the organism senses changes in nutrient availability and triggers biological responses that modulate the efficiency of energy storage during periods of overflow or scarcity of nutrients. Such a model is supported by the observation that treatment with exogenous ghrelin increases expression and protein levels of lipogenic enzymes independently of any orexigenic effects (Theander-Carrillo et al. 2006).

To test the hypothesis mentioned above, we analyzed if ghrelin neutralization during the fasting-refeeding cycle alters metabolic pathways and energy storage in the liver. The main form of energy storage in liver is represented by glycogen and lipids. The origin of the stored lipids can be either the diet or *de novo* synthesis from carbohydrate through lipogenesis. Therefore, the major function of glycolysis in the liver is to provide the necessary glucose metabolites for *de novo* lipid synthesis (Foufelle and Ferre 2002). The effects of ghrelin neutralization on enzymes involved in glycolysis and lipogenesis (summarized in Figure 9) were analyzed in this study.

Here, we demonstrated that ghrelin blockade during the fasting-refeeding cycle with a previously described AG-specific Spiegelmer compound (Shearman et al. 2006), impairs BW recuperation during refeeding. This result suggests a role for AG in the regulation of energy storage following a period of fasting. This finding is in agreement with previous studies in which on the one hand ghrelin neutralization was shown to lead to reduced energy efficiency and to ameliorate obesity in diet-induced obese mice (Shearman et al. 2006) and on the other hand it was demonstrated that

treatment with ghrelin induces improved efficiency of food processing (Sangiao-Alvarellos et al. 2009; Theander-Carrillo et al. 2006). Ghrelin-mediated conservation of energy was shown to be mediated by down-regulation of thermogenesis, energy expenditure and spontaneous locomotor activity and these processes could help to explain the absence of body weight gain during refeeding when ghrelin was neutralized (Jaszberenyi et al. 2006; Tang-Christensen et al. 2004; Theander-Carrillo et al. 2006).

Another line of evidence in support of ghrelin's regulatory role in response to nutrient availability was provided in a study by Mayorov *et al.* in which mice treated with antibodies that hydrolyze the octanoyl moiety of ghrelin to form des-acyl ghrelin showed greater whole body energy expenditure during fasting than untreated animals (Mayorov et al. 2008), suggesting that AG restrains energy expenditure during fasting.

Our results indicate that the down-regulation of HK and PK activities, observed as a response to fasting, is mitigated by ghrelin neutralization. In addition, the protein and activity levels of enzymes related to lipid synthesis such as FAS, ACC and ATP citrate-lyase also exhibited higher levels during fasting in rats treated with NOX-B11(2) compared to control animals. Similarly, the reduction of 6PDGH and G6PDH activity, two important enzymes for the supply of NADPH for *de novo* lipogenesis, was also less pronounced by neutralization of AG during 24 h fasting (Kersten 2001; Salati and Amir-Ahmady 2001). Overall, these results suggest that blockade of ghrelin during prolonged fasting interferes with the normal down-regulation of the hepatic glycolytic pathway and ultimately of lipogenesis. Our findings illustrate ghrelin's importance during fasting as a key factor to promote the use of fat over glucose in all tissues where possible, and to provide glucose in sufficient amounts for glucose-dependent tissues such as the brain and erythrocytes.

Many studies link the response to food deprivation/restriction to AMPK $\alpha$  as it is an energy sensing kinase which, once activated, promotes energy production and limits energy utilisation to ensure cellular survival (Lage et al. 2008). These studies point to the possibility that AMPK $\alpha$  coordinates the changes in enzymes of lipid metabolism during the starved-refed transition (Assifi et al. 2005). It has been demonstrated that administration of ghrelin to rats affects AMPK $\alpha$  activity, although this effect is tissue-specific. In the hypothalamus, central and peripheral ghrelin treatments enhance protein levels of AMPK $\alpha$  and this activation and alterations on lipid metabolism mediate the

orexigenic effects of ghrelin (Andersson et al. 2004; Kohno et al. 2008; Kola et al. 2005, 2008; Lopez et al. 2008). In fed animals peripheral chronic and acute ghrelin treatments provoked AMPK $\alpha$  activation in the heart, while in liver and adipose tissue it was inhibited and no effect was detected on skeletal muscle (Barazzoni et al. 2005; Kola et al. 2005). In this work we showed that ghrelin neutralization caused lower AMPK $\alpha$  total protein levels after 48 hours of refeeding compared with their controls, just like during 48 hours of fasting. These results suggest that ghrelin's effects are dependent on the energetic status and that its neutralization during fasting and caloric restriction after fasting alters liver metabolism. The data demonstrate the importance of ghrelin in correct nutrient partitioning during negative energy balance. We show that the hepatic levels of pAMPK $\alpha$  and AMPK $\alpha$  are decreased after food deprivation in control SPM and NOX-B11(2) treated rats. However, it should be noted that the ratio of pAMPK to AMPK increased during fasting and decreased after refeeding in both groups (data not shown).

During starvation, the central mechanism to provide the organism with glucose is de novo synthesis in the liver from precursors such as lactate, gluconeogenic amino acids, and glycerol. Likewise, when glucose is directly available from external sources, gluconeogenesis is dispensable and consequently needs to be shut off (Pilkis and Granner 1992). Insulin is an anabolic hormone that in liver suppresses gluconeogenesis and activates lipogenesis during times of nutritional abundance through the activation of the insulin receptor (IR). Our data indicates that the drop in plasma insulin elicited by fasting is only partially restored following 24 h and 48 h of refeeding. Neutralization of ghrelin appears to have a significant inhibitory effect on the recovery of insulin levels during 48 h of refeeding. Moreover, FBPase levels increase during fasting but are suppressed during refeeding and the finding that FBPase levels are suppressed less in NOX-B11(2) treated animals is consistent with the effect of ghrelin neutralization on insulin levels during refeeding.

When ghrelin was blocked, pAKT, a key protein kinase downstream of IR (Brunet et al. 1999), exhibited a weaker down-regulation in response to fasting. The results could suggest that ghrelin neutralization during fasting enhances insulin sensitivity in liver, causing a lesser decrease in glucose uptake and consequently in glycolytic and glucogenic pathways. Previously, several works reported differences on insulin sensitivity in animals with deletions in the ghrelin and/or ghrelin receptor genes. Double knockout mice showed a trend towards improved glucose tolerance and insulin

sensitivity after an overnight fast (Pfluger et al. 2008). Although these differences were not statistically significant, the findings are supported by independent studies that demonstrate that GHSR and ghrelin null mice display enhanced insulin sensitivity when subjected to caloric restriction or fasting (Sun et al. 2008). Recently Zhao *et al.* generated GOAT KO mice and they found that when these animals were subject to severe prolonged restriction caloric showed hypoglycemia compared with wild-type, contrary to results observed in this work. However, their caloric restriction was more severe (60%) and they only observed differences after 3 days of caloric restriction and when the blood was collected one-half hour before feeding. The authors suggested that GOAT activity and hence acyl-ghrelin levels, that enhanced in wild-type mice during caloric restriction, are necessary for survival and maintenance of glucose levels of caloric restricted mice (Zhao et al. 2010). They observed that GH levels were significantly raised by prolonged caloric restriction however this increase is impaired in GOAT KO mice (Zhao et al. 2010). Their data reveal one essential relation between ghrelin and plasma GH levels during periods of severe caloric restriction, thereby assuring sufficient blood glucose to permit survival. In rats, food deprivation inhibits GH secretion (Muller et al. 1999) and in this study we observed that this decrease was lesser when acyl-ghrelin was neutralized. Different works suggested that food deprivation in rats primarily reduces the activation of insulin signaling for glucose metabolism through reduction of GH and/or subsequent IGF-1 signaling (Hayashi et al. 2008). The data obtained in our study and GOAT KO mice (Zhao et al. 2010) would reveal one function of ghrelin in maintenance of appropriate plasma GH levels during periods of fasting or food restriction. Recently Chiba *et al.* suggested a model where NPY and ghrelin play a crucial role during food deprivation in rats (Chiba et al. 2009). They proposed that caloric restriction decreased plasma insulin and leptin levels, whereas it increased ghrelin levels and that these hormonal changes result in an increased NPY expression in the arcuate nucleus. NPY/AgRP neurons project their axons to downstream neurons such as GHRH neurons suppressing them. They also suggested that NPY up-regulation could also enhance oxidative stress resistance and hepatic glucose production (Chiba et al. 2009). In addition, intraventricular injection of NPY inhibited GH secretion, and a NPY antiserum led to elevated plasma GH levels and partially restored GH secretory pulses in food-deprived rats (Muller et al. 1999). Acyl-ghrelin neutralization during food deprivation might do that the increase of the NPY was minor, which might explain a minor decrease in plasma GH levels and some of the changes

observed in the hepatic metabolism, nevertheless further studies are necessary to demonstrate this theory.

We did not observed changes in glucose levels during fasting in animals treated with NOX-B11(2) compared with their controls, although our results showed that neutralization of ghrelin during fasting contributes to incorrect inhibition of hepatic glycolysis and lipogenesis. Other studies with more prolonged fasting and caloric restrictions could to help to clarify these results.

Insulin promotes the storage and synthesis of lipids, protein, and carbohydrates (Saltiel and Kahn 2001). The first step by which insulin increases energy storage or utilization involves the regulated transport of glucose into the cell (Chang et al. 2004) hence, lower insulin levels observed during refeeding in NOX-B11(2) treated rats could help explain lower level of hepatic glycogen and slightly higher glycemia that showed these animals. On the other hand, the insulin receptor substrate (IRS) proteins, initiates activation of the phosphatidylinositol 3-kinase pathway, resulting in stimulation of protein kinases such as AKT (Chang et al. 2004) that suppresses gluconeogenesis and activates glycogen synthesis via phosphorylation of FOXO1 (Zhang et al. 2002) and GSK-3 (Cross et al. 1995) respectively. Phosphorylation of GSK-3 inactivates GSK-3 kinase activity and leads to increased activity of glycogen synthetase (GS), resulting in enhanced glycogen deposition (Cross et al. 1995). For these reasons, the decrease in insulin levels and AKT phosphorylation after 48 hours refeeding when ghrelin was blocked, could elucidate the decrease in glycogen levels present in the animals. However, further studies are necessary to clarify this hypothesis.

In summary, our study delineates several lines of evidence that implicate ghrelin as an important regulator of the response to changes in energy availability: First, ghrelin neutralization during the fasting-refeeding cycle interferes with normal recovery of body weight; second, the fasting-induced rise in ghrelin levels contributes to inhibition of hepatic glycolysis and lipogenesis, which appears to be linked to insulin sensitivity; and third, once food becomes available following the fasting, ghrelin promotes lipogenesis recovery and favors glycogen deposition in order to minimize negative effects from periods of food scarcity.

The results point to ghrelin as a key player in regulating the enzymatic activities involved in restoring body weight following diet-induced weight loss. Ghrelin may thus be a relevant target in controlling the well-known phenomenon of patients quickly regaining weight that was lost in the course of a diet.

Moreover, ghrelin manipulation could be therapeutically relevant to avoid refeeding syndrome, a potentially fatal clinical syndrome related to the treatment of malnutrition, which is associated with very low ghrelin levels (Korbonits et al. 2007). The understanding of ghrelin's role in the molecular mechanisms underlying the events that occur during fasting and refeeding will hopefully lead to new strategies for the design and development of suitable drugs for the treatment of pathologies of obesity, its co-morbidities, and other endocrine disorders affecting energy metabolism.

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## FIGURE LEGENDS

### Figure 1

Schematic representation of treatments of the animals.

### Figure 2

Effect of an IP ghrelin treatment on cumulative food intake during 2 h following injection in rats (seven animals per group) treated previously with non-functional control SPM or NOX-B11(2). Values are expressed as mean  $\pm$  SEM. Different letters indicate statistical significance ( $p < 0.05$ ).

### Figure 3

Effect of ghrelin neutralization on body weight change in fasted (48 h) and fasted (48 h)-refed (24 or 48 h) rats. Values are means  $\pm$  SEM of eight animals per group. Different letters indicate statistical significance ( $p < 0.05$ ).

### Figure 4

Plasma levels of acyl-ghrelin, insulin, glucose, triglycerides, lactate and GH in fed, fasted (48 h) and fasted (48 h)-refed (24 or 48 h) rats treated with NOX-B11(2) or non-functional control SPM. Values are means  $\pm$  SEM of eight animals per group. Different letters indicate statistical significance ( $p < 0.05$ ).

### Figure 5

Hepatic activity levels of glucokinase (GK), hexokinase (HK), pyruvate kinase (PK), fructose bisphosphatase (Fbase) (A-D) and protein levels of total serine/threonine kinase protein kinase B AKT and pAKT (E-G) in fed, fasted (48 h) and fasted (48 h)-refed (24 or 48 h) rats treated with NOX-B11(2) or control non-functional SPM. Values are means  $\pm$  SEM of eight animals per group. Different letters indicate statistical significance ( $p < 0.05$ ).

**Figure 6**

Hepatic glycogen levels in fed, fasted (48h) and fasted (48h)-refed (24 or 48h) rats treated with NOX-B11(2) or non-functional control SPM. Values are means  $\pm$  SEM of eight animals per group. Different letters indicate statistical significance ( $p < 0.05$ ).

**Figure 7**

Hepatic mRNA (A-C), protein (D-I) and activity levels (K-M) of lipid metabolism-related enzymes in fed, fasted (48 h) and fasted (48 h)-refed (24 or 48 h) rats treated with NOX-B11(2) or non-functional control SPM. Values are means  $\pm$  SEM of eight animals per group. Different letters indicate statistical significance ( $p < 0.05$ ).

**Figure 8**

Hepatic mRNA (A), protein (B) and activity levels (E) of CPT1A,  $\beta$ -oxidation-related enzyme in fed, fasted (48 h) and fasted (48 h)-refed (24 or 48 h) rats treated with NOX-B11(2) or non-functional control SPM. Values are means  $\pm$  SEM of eight animals per group. Different letters indicate statistical significance ( $p < 0.05$ ).

**Figure 9.**

Glycolytic and lipogenic pathways in the liver.

**Table 1**

Gene	Accession number		
ACC $\alpha$	NM_022193	Forward	5'-TGGGCGGGATGGTCTCTTT-3'
		Reverse	5'-AGTCGCAGAAGCAGCCCATT-3'
		Probe	FAM-5'-ACCTTGAAGATTCGTCAGGATCTTTGATGA-3'-TAMRA
FAS	NM_017332	Forward	5'-GACATTCATCAGGCCACC-3'
		Reverse	5'-CCTCTAGCAGCCGCACCTC-3'
		Probe	FAM-5'-CTGCCCAGGACAGGAACCG-3'-TAMRA
SCD-1	NM_139192.2	Forward	5'-TGCCAGAGGGAATAGGGAAA-3'
		Reverse	5'-CTCTCCCATCCTTACTTACAAACCA-3'
		Probe	FAM-5'-TCACCTTGAGAGAAGAATTAGCACGCACGG-3'-TAMRA
CPT1A	NM_031559	Forward	5'-ATGACGGCTATGGTGTCTCC-3'
		Reverse	5'-TCATGGCTTGTCTCAAGTGC-3'
		Probe	FAM-5'-TGAGACAGACTCAGACCGCT-3'-TAMRA
18S	V01270.1	Forward	5'-CGGCTACCACATCCAAGGAA-3'
		Reverse	5'-GCTGGAATTACCGCGGCT-3'
		Probe	FAM-5'-GACGGCAAGTCTGGTGCCAGCA-3'-TAMRA

Primer sequences used for real time RT-PCR.

Figure

Figure 1

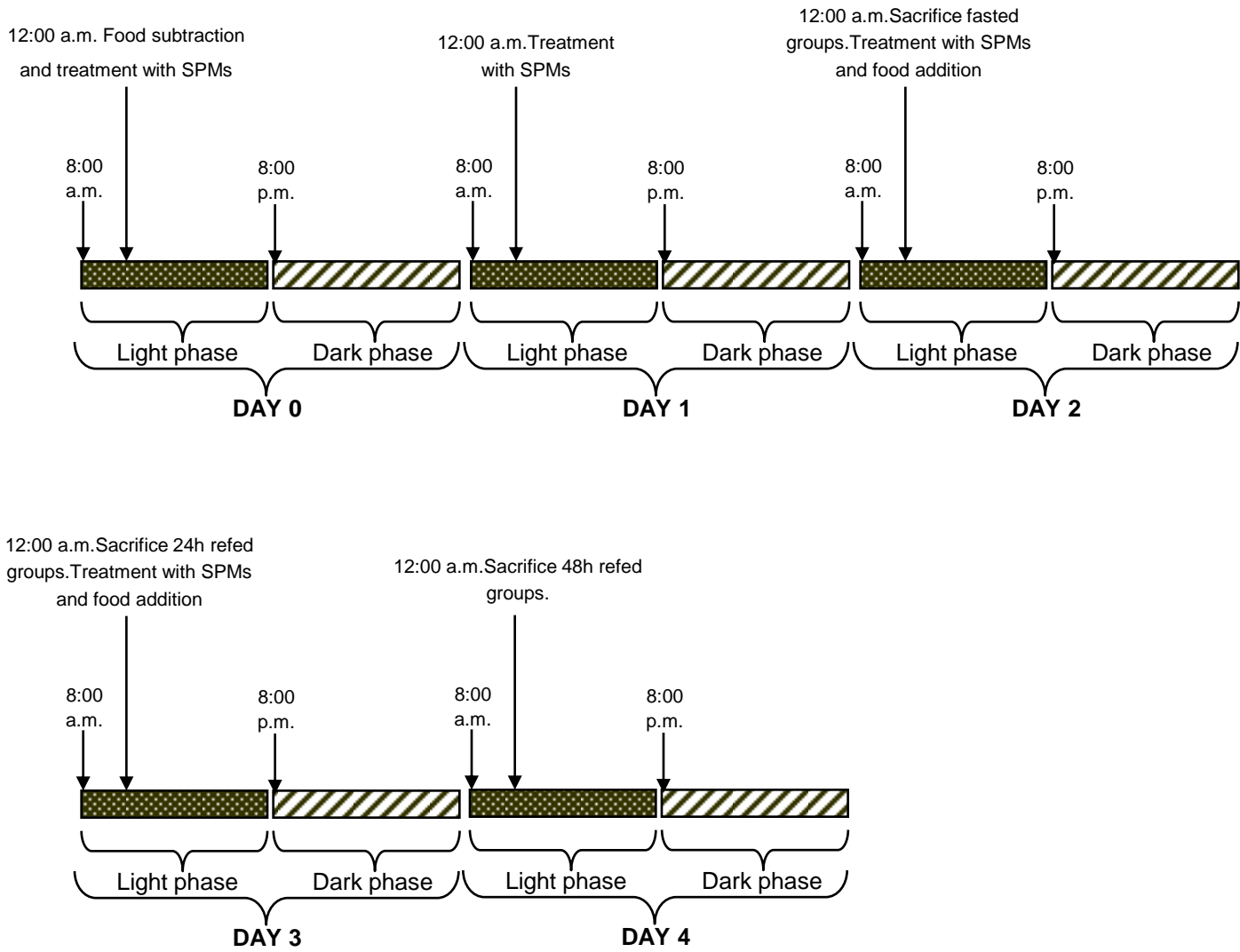




Figure 2

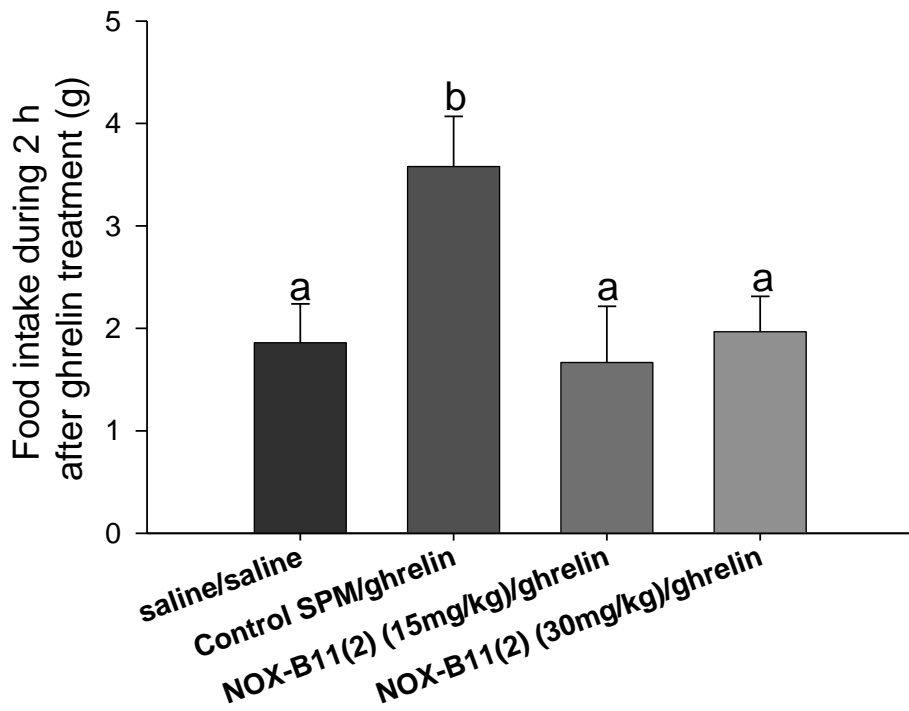
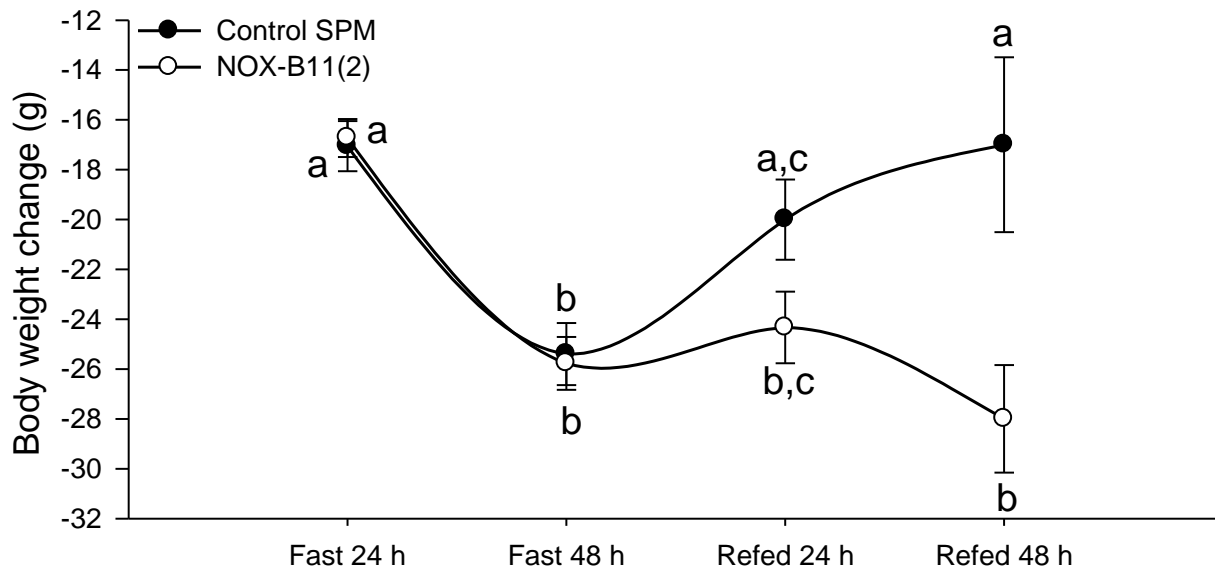
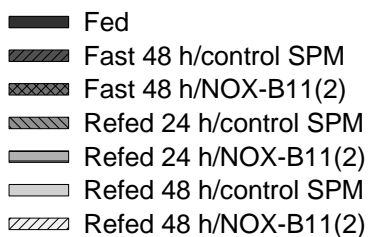
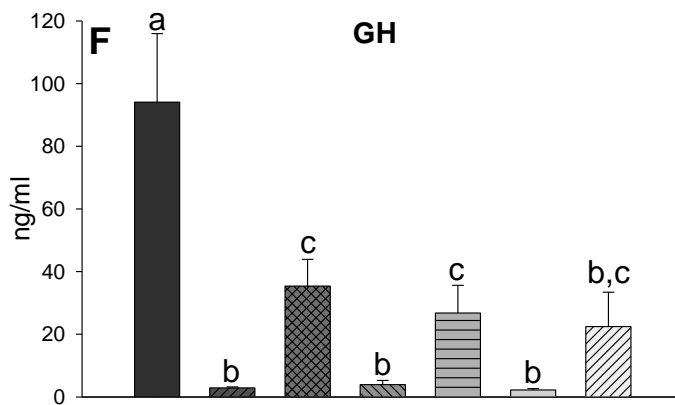
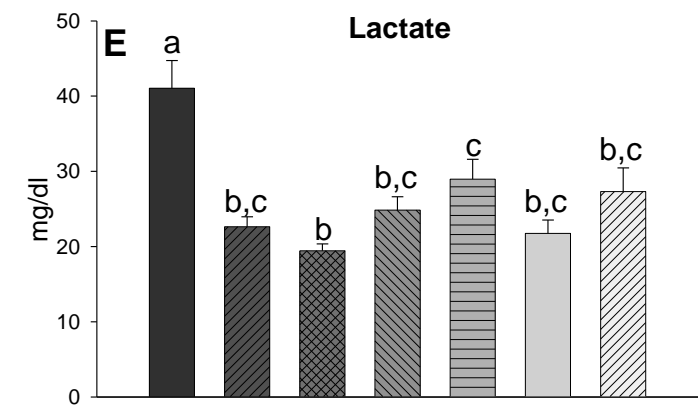
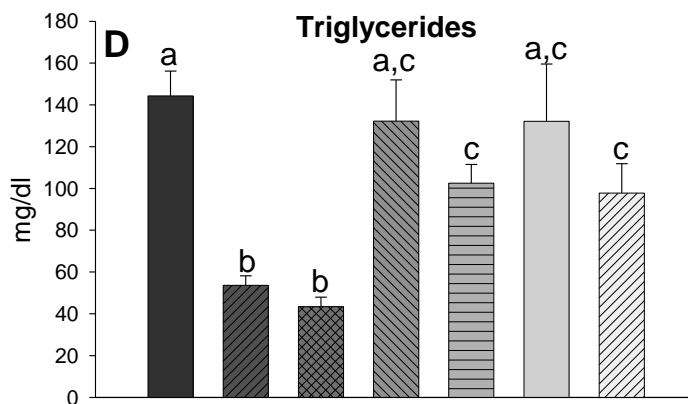
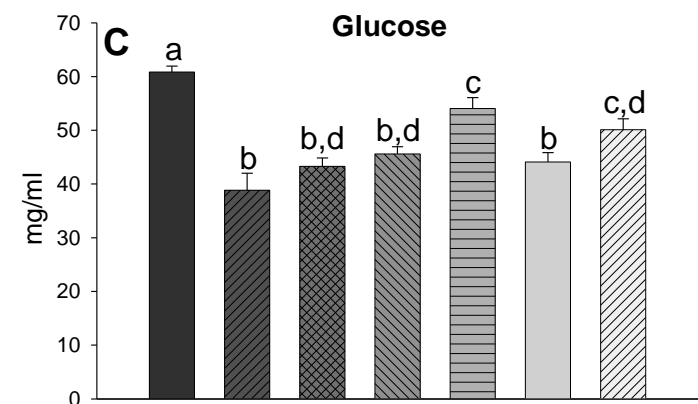
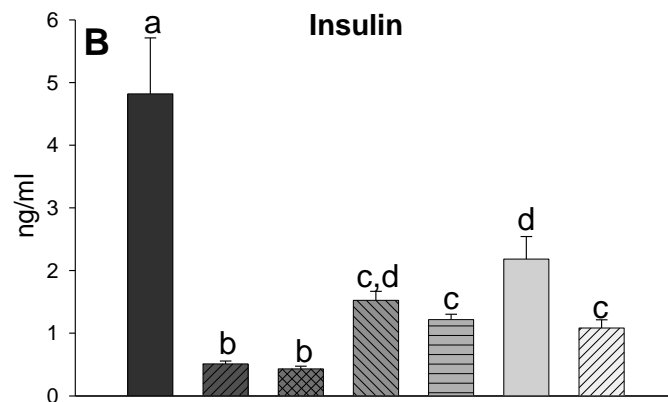
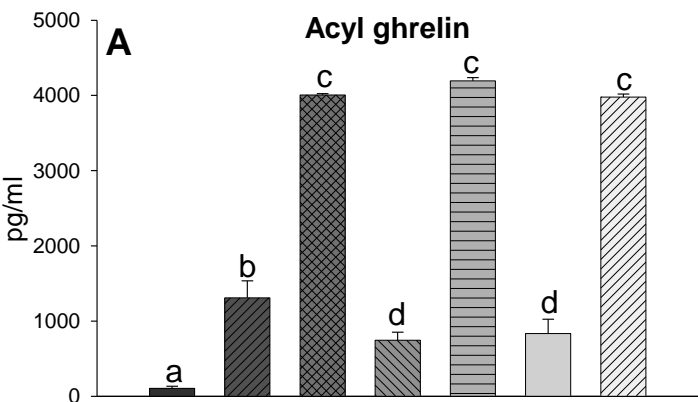


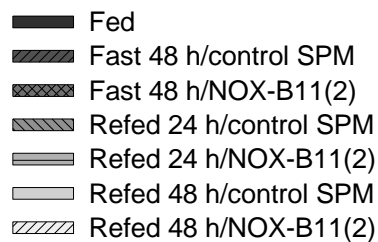
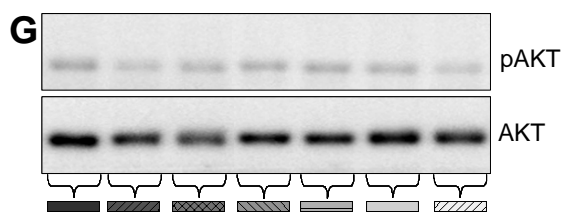
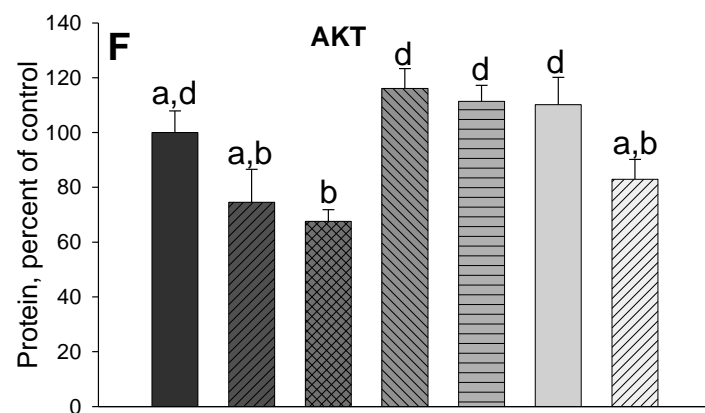
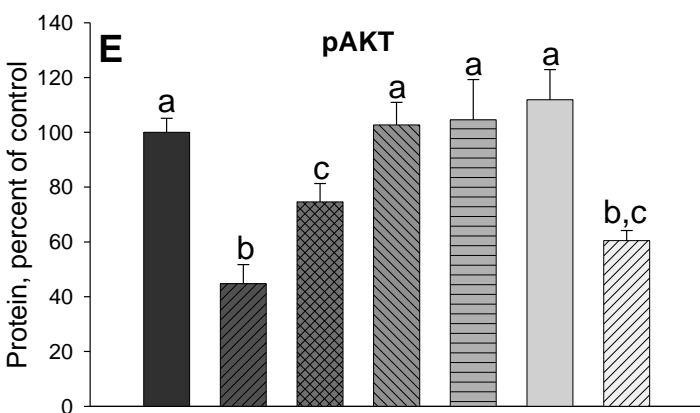
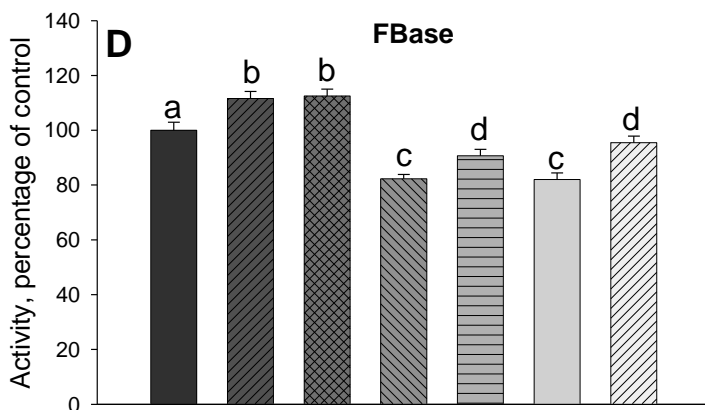
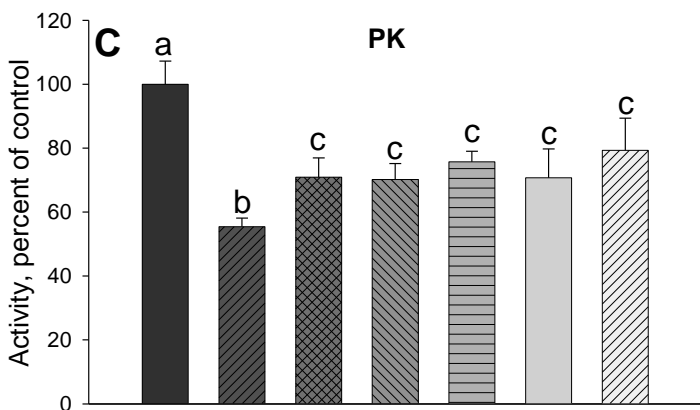
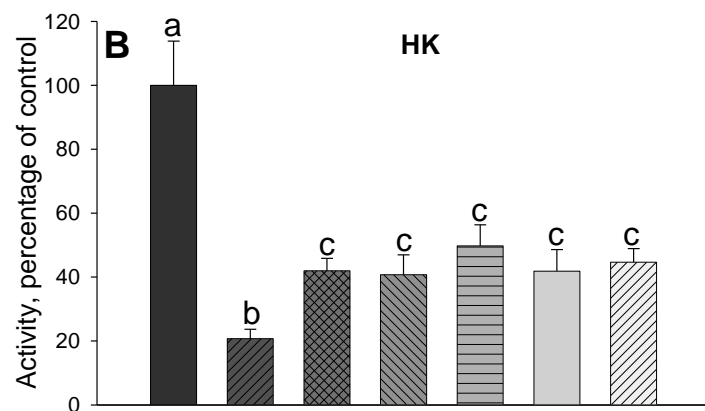
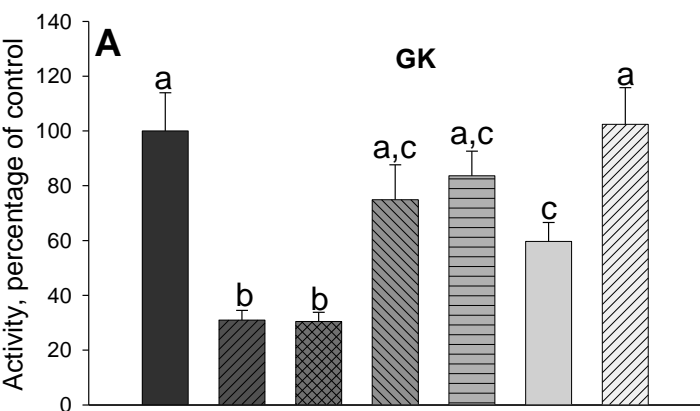
Figure 3



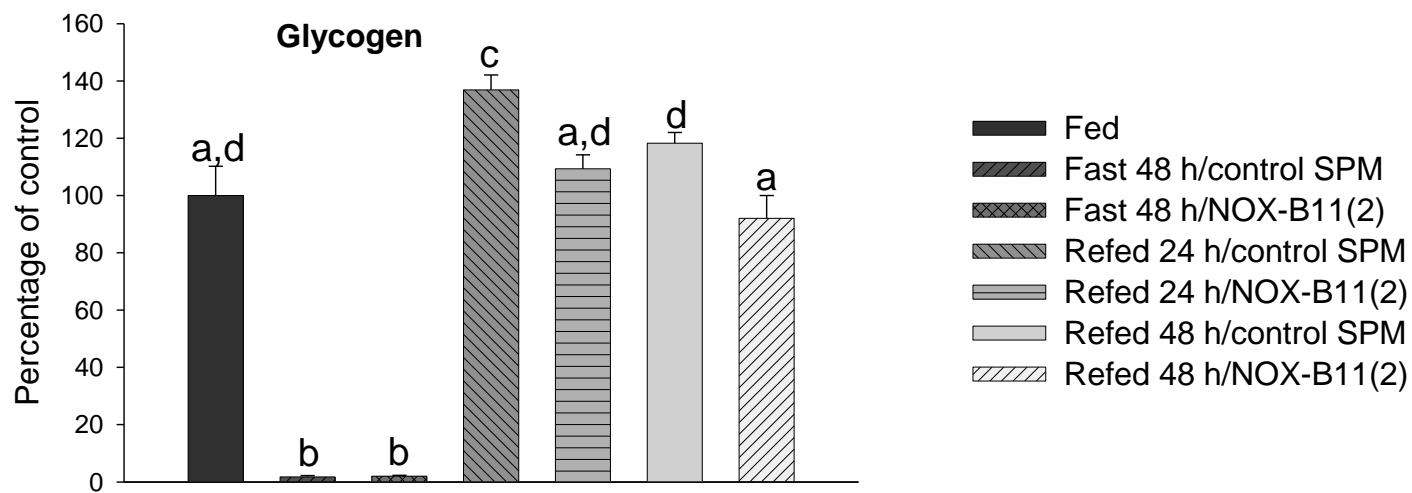
**Figure 4**



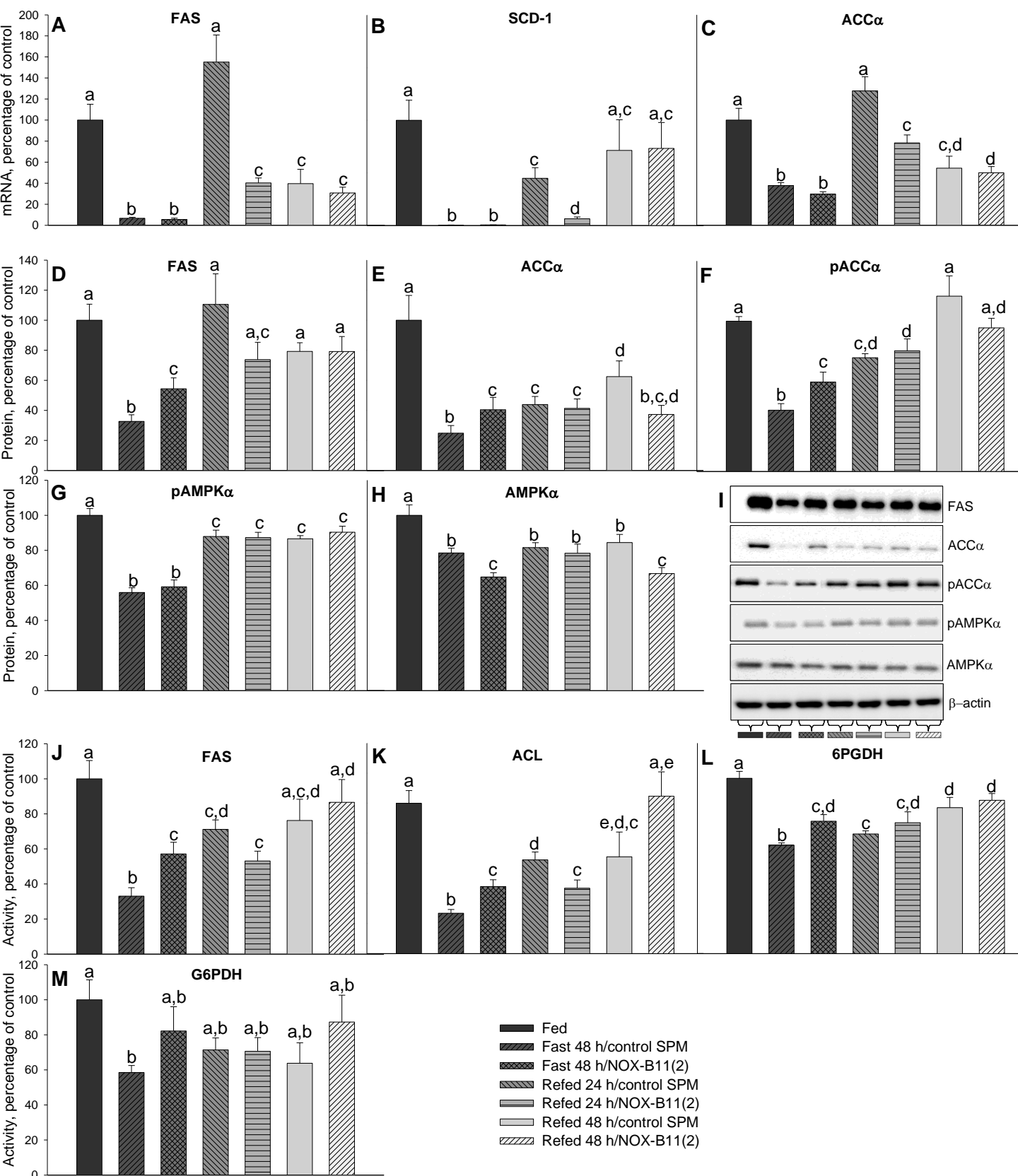
**Figure 5**



**Figure 6**



# Figure 7



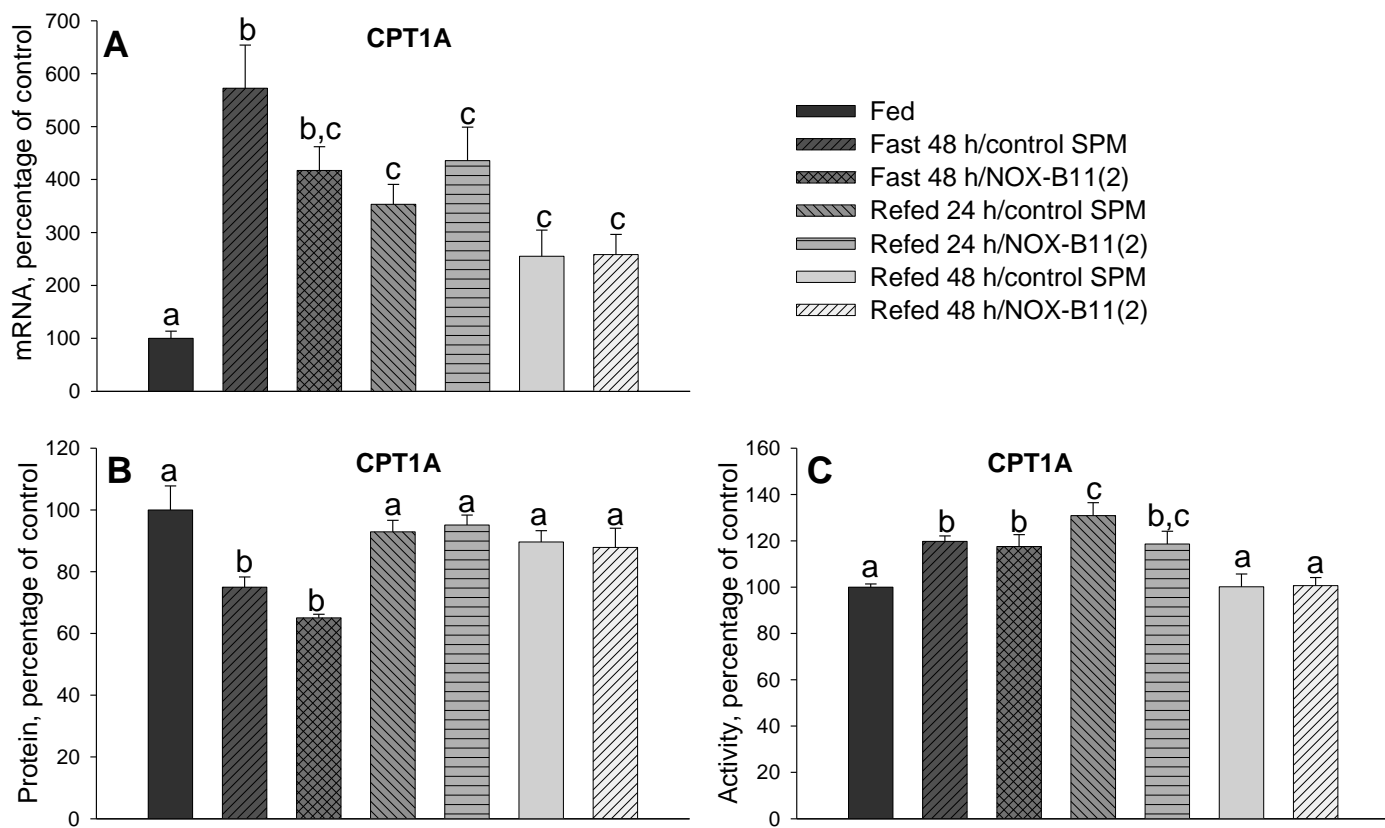
**Figure 8**

Figure 9

