HAL
open science

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

Susana Sangiao-Alvarellos, Steffen Helmling, María J. Vázquez, Sven Klussmann, & Fernando Cordido

To cite this version:

Susana Sangiao-Alvarellos, Steffen Helmling, María J. Vázquez, Sven Klussmann, & Fernando Cordido. Ghrelin neutralization during fasting-refeeding cycle impairs the recuperation of body weight and alters hepatic energy metabolism.. Molecular and Cellular Endocrinology, 2011, 335 (2), pp.177. 10.1016/j.mce.2011.01.010 . hal-00672296

HAL Id: hal-00672296

https://hal.science/hal-00672296v1

Submitted on 21 Feb 2012

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

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

Authors: Susana Sangiao-Alvarellos, Steffen Helmling, María J. Vázquez, Sven Klussmann, & Fernando Cordido

PII: S0303-7207(11)00032-3
DOI: doi:10.1016/j.mce.2011.01.010
Reference: MCE 7750

To appear in: *Molecular and Cellular Endocrinology*

Received date: 26-9-2010
Revised date: 9-1-2011
Accepted date: 11-1-2011

Please cite this article as: Sangiao-Alvarellos, S., Helmling, S., Vázquez, M.J., Klussmann, S., Cordido, F., Ghrelin neutralization during fasting-refeeding cycle impairs the recuperation of body weight and alters hepatic energy metabolism., *Molecular and Cellular Endocrinology* (2010), doi:10.1016/j.mce.2011.01.010

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

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

Susana Sangiao-Alvarellos [1,2], Steffen Helmling [3], María J. Vázquez [4], Sven Klussmann [3] & Fernando Cordido [1, 2,5]

[1] Department of Medicine, School of Health Science, University of A Coruña. Campus de Oza. s/n. 15006 A Coruña. Spain.
[2] INIBIC. Instituto de Investigación Biomédica de A Coruña. Xubias de Arriba, 84. 15006. A Coruña. Spain
[3] NOXXON Pharma AG, Max-Dohrn Str. 8-10, 10589 Berlin, Germany.
[4] Department of Physiology, School of Medicine, University of Santiago de Compostela, Santiago de Compostela, 157852 Spain
[5] Endocrine Department, Complexo 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

Accepted Manuscript

ACCEPTED MANUSCRIPT

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.

Accepted Manuscript

ACCEPTED MANUSCRIPT

Abbreviations:

6PGDH: 6-phosphogluconate dehydrogenase
ACCα: acetyl-CoA carboxylase alpha
ACL: ATP citrate lyase
AKT: total serine/threonine kinase protein kinase B
AMPKα: 5'AMP-activated protein kinase alpha
CPT1A: liver isoform of carnitine palmitoyltransferase1
FAS: fatty acid synthase
FBase: fructose bisphosphatase
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

Accepted Manuscript

ACCEPTED MANUSCRIPT

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 (Broglio et al. 2001, 2003; Vestergaard et al. 2008a, 2008b; Zhao et al. 2010).

Accepted Manuscript

ACCEPTED MANUSCRIPT

Several studies show that treatment with ghrelin induces hyperglycemia and reduces insulin secretion (Broglio 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 (ACCα), 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

Accepted Manuscript

ACCEPTED MANUSCRIPT

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.

Accepted Manuscript

ACCEPTED MANUSCRIPT

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 ± 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 µg/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 refed 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

Accepted Manuscript

ACCEPTED MANUSCRIPT

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 ºC 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 ACCα, 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 ºC for 2 min, 95 ºC for 10 min followed by 40 cycles of 95 ºC for 15 sec and 60 ºC 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 µg) were subjected to SDS-PAGE,

ACCEPTED MANUSCRIPT

electrotransferred on a polyvinylidene difluoride membrane and probed with the indicated antibodies: ACCα and phosphoACCα-Ser79 (pACCα); 5'AMP-activated protein kinase alpha (AMPKα) and phosphoAMPKα-Thr172 (pAMPKα) (*Upstate*); total serine/threonine kinase protein kinase B (AKT) and phospho AKT (pAKT) (*Cell Signaling*); β-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 β-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 µl) and substrates (20 µl, omitted in controls) to the reaction mixture (final volume 250 µl), 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 ± 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.

Accepted Manuscript

ACCEPTED MANUSCRIPT

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 refed 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

Accepted Manuscript

ACCEPTED MANUSCRIPT

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

Accepted Manuscript

ACCEPTED MANUSCRIPT

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. FBase 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 FBase 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 hof 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.

Accepted Manuscript

ACCEPTED MANUSCRIPT

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

In order 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 ACCα 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, ACCα and pACCα (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 ACCα 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 pAMPKα and AMPKα (Fig. 7G-I) were significantly diminished in both groups. The decrease in AMPKα was lower in animals treated with NOX-B11(2) compared with the control group. 48 h after refeeding, protein levels of total AMPKα 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 β-oxidation

ACCEPTED MANUSCRIPT

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.

Accepted Manuscript

ACCEPTED MANUSCRIPT

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 aboved, 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

ACCEPTED MANUSCRIPT

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 AMPKα 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 AMPKα 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 AMPKα activity, although this effect is tissue-specific. In the hypothalamus, central and peripheral ghrelin treatments enhance protein levels of AMPKα and this activation and alterations on lipid metabolism mediate the

ACCEPTED MANUSCRIPT

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 AMPKα 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 AMPKα 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 pAMPKα and AMPKα 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

Accepted Manuscript

ACCEPTED MANUSCRIPT

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 this 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 calorie restriction, are necessaries for survival and maintenance of glucose levels of calorie restricted mice (Zhao et al. 2010). They observed that GH levels were significantly raised by prolonged caloric restriction however this increased 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 calorie 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 appropiate 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 down-stream 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

Accepted Manuscript

ACCEPTED MANUSCRIPT

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.

Accepted Manuscript

ACCEPTED MANUSCRIPT

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.

Accepted Manuscript

ACCEPTED MANUSCRIPT

ACKNOWLEDGEMENTS

We thank Dr. Klaus Buchner for invaluable help with the studies about the capacity of the spiegelmer for neutralize acyl-ghrelin. This work has been supported in part by: FIS del Instituto de Salud Carlos III PI070413 and Xunta de Galicia PS07/12, PGIDT05PXIC91605PN, INCITE08ENA916110ES and Redes 2006/27, Spain.

Accepted Manuscript

ACCEPTED MANUSCRIPT

REFERENCES

Ames, B.N., 2006. Low micronutrient intake may accelerate the degenerative diseases of aging through allocation of scarce micronutrients by triage. Proc Natl Acad Sci U S A 103, 17589-17594.

Andersson, U., Filipsson, K., Abbott, C.R., Woods, A., Smith, K., Bloom, S.R., Carling, D.Small, C.J., 2004. AMP-activated protein kinase plays a role in the control of food intake. J Biol Chem 279, 12005-12008.

Ariyasu, H., Takaya, K., Iwakura, H., Hosoda, H., Akamizu, T., Arai, Y., Kangawa, K.Nakao, K., 2005. Transgenic mice overexpressing des-acyl ghrelin show small phenotype. Endocrinology 146, 355-364.

Asakawa, A., Inui, A., Fujimiya, M., Sakamaki, R., Shinfuku, N., Ueta, Y., Meguid, M.M.Kasuga, M., 2005. Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. Gut 54, 18-24.

Assifi, M.M., Suchankova, G., Constant, S., Prentki, M., Saha, A.K.Ruderman, N.B., 2005. AMP-activated protein kinase and coordination of hepatic fatty acid metabolism of starved/carbohydrate-refed rats. Am J Physiol Endocrinol Metab 289, E794-800.

Baldanzi, G., Filigheddu, N., Cutrupi, S., Catapano, F., Bonissoni, S., Fubini, A., Malan, D., Baj, G., Granata, R., Broglio, F., Papotti, M., Surico, N., Bussolino, F., Isgaard, J., Deghenghi, R., Sinigaglia, F., Prat, M., Muccioli, G., Ghigo, E.Graziani, A., 2002. Ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/AKT. J Cell Biol 159, 1029-1037.

Barazzoni, R., Bosutti, A., Stebel, M., Cattin, M.R., Roder, E., Visintin, L., Cattin, L., Biolo, G., Zanetti, M.Guarnieri, G., 2005. Ghrelin regulates mitochondrial-lipid metabolism gene expression and tissue fat distribution in liver and skeletal muscle. Am J Physiol Endocrinol Metab 288, E228-235.

Barazzoni, R., Zanetti, M., Cattin, M.R., Visintin, L., Vinci, P., Cattin, L., Stebel, M.Guarnieri, G., 2007. Ghrelin enhances in vivo skeletal muscle but not liver AKT signaling in rats. Obesity (Silver Spring) 15, 2614-2623.

Broglio, F., Arvat, E., Benso, A., Gottero, C., Muccioli, G., Papotti, M., van der Lely, A.J., Deghenghi, R.Ghigo, E., 2001. Ghrelin, a natural GH secretagogue produced by the stomach, induces hyperglycemia and reduces insulin secretion in humans. J Clin Endocrinol Metab 86, 5083-5086.

Broglio, F., Gottero, C., Benso, A., Prodam, F., Destefanis, S., Gauna, C., Maccario, M., Deghenghi, R., van der Lely, A.J.Ghigo, E., 2003. Effects of ghrelin on the insulin and glycemic responses to glucose, arginine, or free fatty acids load in humans. J Clin Endocrinol Metab 88, 4268-4272.

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J.Greenberg, M.E., 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 96, 857-868.

Chang, L., Chiang, S.H.Saltiel, A.R., 2004. Insulin signaling and the regulation of glucose transport. Mol Med 10, 65-71.

Chiba, T., Komatsu, T., Nakayama, M., Adachi, T., Tamashiro, Y., Hayashi, H., Yamaza, H., Higami, Y.Shimokawa, I., 2009. Similar metabolic responses to calorie restriction in lean and obese Zucker rats. Mol Cell Endocrinol 309, 17-25.

Chung, H., Seo, S., Moon, M.Park, S., 2008. Phosphatidylinositol-3-kinase/Akt/glycogen synthase kinase-3 beta and ERK1/2 pathways mediate protective effects of acylated and unacylated ghrelin against oxygen-glucose deprivation-induced apoptosis in primary rat cortical neuronal cells. J Endocrinol 198, 511-521.

Corrigan, A.P.Rider, C.C., 1983. Multiple chromatographic forms of ATP citrate lyase from rat liver. Biochem J 214, 299-307.

ACCEPTED MANUSCRIPT

Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M.Hemmings, B.A., 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378, 785-789.

Cui, C., Ohnuma, H., Daimon, M., Susa, S., Yamaguchi, H., Kameda, W., Jimbu, Y., Oizumi, T.Kato, T., 2008. Ghrelin infused into the portal vein inhibits glucose-stimulated insulin secretion in Wistar rats. Peptides 29, 1241-1246.

Cummings, D.E., Purnell, J.Q., Frayo, R.S., Schmidova, K., Wisse, B.E.Weigle, D.S., 2001. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes 50, 1714-1719.

Cummings, D.E., Weigle, D.S., Frayo, R.S., Breen, P.A., Ma, M.K., Dellinger, E.P.Purnell, J.Q., 2002. Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. N Engl J Med 346, 1623-1630.

Davidson, A.L.Arion, W.J., 1987. Factors underlying significant underestimations of glucokinase activity in crude liver extracts: physiological implications of higher cellular activity. Arch Biochem Biophys 253, 156-167.

Denton, R.M., Edgell, N.J., Bridges, B.J.Poole, G.P., 1979. Acute regulation of pyruvate kinase activity in rat epididymal adipose tissue by insulin. Biochem J 180, 523-531.

Foufelle, F.Ferre, P., 2002. New perspectives in the regulation of hepatic glycolytic and lipogenic genes by insulin and glucose: a role for the transcription factor sterol regulatory element binding protein-1c. Biochem J 366, 377-391.

Gauna, C., Meyler, F.M., Janssen, J.A., Delhanty, P.J., Abribat, T., van Koetsveld, P., Hofland, L.J., Broglio, F., Ghigo, E.van der Lely, A.J., 2004. Administration of acylated ghrelin reduces insulin sensitivity, whereas the combination of acylated plus unacylated ghrelin strongly improves insulin sensitivity. J Clin Endocrinol Metab 89, 5035-5042.

Gutierrez, J.A., Solenberg, P.J., Perkins, D.R., Willency, J.A., Knierman, M.D., Jin, Z., Witcher, D.R., Luo, S., Onyia, J.E.Hale, J.E., 2008. Ghrelin octanoylation mediated by an orphan lipid transferase. Proc Natl Acad Sci U S A 105, 6320-6325.

Hayashi, H., Yamaza, H., Komatsu, T., Park, S., Chiba, T., Higami, Y., Nagayasu, T.Shimokawa, I., 2008. Calorie restriction minimizes activation of insulin signaling in response to glucose: potential involvement of the growth hormone-insulin-like growth factor 1 axis. Exp Gerontol 43, 827-832.

Helmling, S., Maasch, C., Eulberg, D., Buchner, K., Schroder, W., Lange, C., Vonhoff, S., Wlotzka, B., Tschop, M.H., Rosewicz, S.Klussmann, S., 2004. Inhibition of ghrelin action in vitro and in vivo by an RNA-Spiegelmer. Proc Natl Acad Sci U S A 101, 13174-13179.

Hofbauer, K.G., Nicholson, J.R.Boss, O., 2007. The obesity epidemic: current and future pharmacological treatments. Annu Rev Pharmacol Toxicol 47, 565-592.

Howard, A.D., Feighner, S.D., Cully, D.F., Arena, J.P., Liberator, P.A., Rosenblum, C.I., Hamelin, M., Hreniuk, D.L., Palyha, O.C., Anderson, J., Paress, P.S., Diaz, C., Chou, M., Liu, K.K., McKee, K.K., Pong, S.S., Chaung, L.Y., Elbrecht, A., Dashkevicz, M., Heavens, R., Rigby, M., Sirinathsinghji, D.J., Dean, D.C., Melillo, D.G., Patchett, A.A., Nargund, R., Griffin, P.R., DeMartino, J.A., Gupta, S.K., Schaeffer, J.M., Smith, R.G.Van der Ploeg, L.H., 1996. A receptor in pituitary and hypothalamus that functions in growth hormone release. Science 273, 974-977.

Ikezaki, A., Hosoda, H., Ito, K., Iwama, S., Miura, N., Matsuoka, H., Kondo, C., Kojima, M., Kangawa, K.Sugihara, S., 2002. Fasting plasma ghrelin levels are negatively correlated with insulin resistance and PAI-1, but not with leptin, in obese children and adolescents. Diabetes 51, 3408-3411.

Iwakura, H., Hosoda, K., Son, C., Fujikura, J., Tomita, T., Noguchi, M., Ariyasu, H., Takaya, K., Masuzaki, H., Ogawa, Y., Hayashi, T., Inoue, G., Akamizu, T., Hosoda, H., Kojima, M., Itoh, H., Toyokuni, S., Kangawa, K.Nakao, K., 2005. Analysis of rat

ACCEPTED MANUSCRIPT

insulin II promoter-ghrelin transgenic mice and rat glucagon promoter-ghrelin transgenic mice. J Biol Chem 280, 15247-15256.

Jaszberenyi, M., Bujdoso, E., Bagosi, Z.Telegdy, G., 2006. Mediation of the behavioral, endocrine and thermoregulatory actions of ghrelin. Horm Behav 50, 266-273.

Karlic, H., Lohninger, S., Koeck, T.Lohninger, A., 2002. Dietary l-carnitine stimulates carnitine acyltransferases in the liver of aged rats. J Histochem Cytochem 50, 205-212.

Keppler, D.Decker, K., 1974. Glycogen determination with amyloglucosidase

Bergmeyer, H.U., Methods of Enzymatic Analysis. Academic Press, New York. 3**.** 1127-1131.

Kersten, S., 2001. Mechanisms of nutritional and hormonal regulation of lipogenesis. EMBO Rep 2, 282-286.

Kirchner, H., Gutierrez, J.A., Solenberg, P.J., Pfluger, P.T., Czyzyk, T.A., Willency, J.A., Schurmann, A., Joost, H.G., Jandacek, R.J., Hale, J.E., Heiman, M.L.Tschop, M.H., 2009. GOAT links dietary lipids with the endocrine control of energy balance. Nat Med 15, 741-745.

Kobelt, P., Helmling, S., Stengel, A., Wlotzka, B., Andresen, V., Klapp, B.F., Wiedenmann, B., Klussmann, S.Monnikes, H., 2006. Anti-ghrelin Spiegelmer NOX-B11 inhibits neurostimulatory and orexigenic effects of peripheral ghrelin in rats. Gut 55, 788-792.

Kohno, D., Sone, H., Minokoshi, Y.Yada, T., 2008. Ghrelin raises [Ca2+]i via AMPK in hypothalamic arcuate nucleus NPY neurons. Biochem Biophys Res Commun 366, 388-392.

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H.Kangawa, K., 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402, 656-660.

Kojima, M., Hosoda, H.Kangawa, K., 2001a. Purification and distribution of ghrelin: the natural endogenous ligand for the growth hormone secretagogue receptor. Horm Res 56 Suppl 1, 93-97.

Kojima, M., Hosoda, H., Matsuo, H.Kangawa, K., 2001b. Ghrelin: discovery of the natural endogenous ligand for the growth hormone secretagogue receptor. Trends Endocrinol Metab 12, 118-122.

Kola, B., Farkas, I., Christ-Crain, M., Wittmann, G., Lolli, F., Amin, F., Harvey-White, J., Liposits, Z., Kunos, G., Grossman, A.B., Fekete, C.Korbonits, M., 2008. The orexigenic effect of ghrelin is mediated through central activation of the endogenous cannabinoid system. PLoS ONE 3, e1797.

Kola, B., Hubina, E., Tucci, S.A., Kirkham, T.C., Garcia, E.A., Mitchell, S.E., Williams, L.M., Hawley, S.A., Hardie, D.G., Grossman, A.B.Korbonits, M., 2005. Cannabinoids and ghrelin have both central and peripheral metabolic and cardiac effects via AMP-activated protein kinase. J Biol Chem 280, 25196-25201.

Korbonits, M., Blaine, D., Elia, M.Powell-Tuck, J., 2007. Metabolic and hormonal changes during the refeeding period of prolonged fasting. Eur J Endocrinol 157, 157-166.

Lage, R., Dieguez, C., Vidal-Puig, A.Lopez, M., 2008. AMPK: a metabolic gauge regulating whole-body energy homeostasis. Trends Mol Med 14, 539-549.

Liu, J., Prudom, C.E., Nass, R., Pezzoli, S.S., Oliveri, M.C., Johnson, M.L., Veldhuis, P., Gordon, D.A., Howard, A.D., Witcher, D.R., Geysen, H.M., Gaylinn, B.D.Thorner, M.O., 2008. Novel ghrelin assays provide evidence for independent regulation of ghrelin acylation and secretion in healthy young men. J Clin Endocrinol Metab 93, 1980-1987.

Longo, K.A., Charoenthongtrakul, S., Giuliana, D.J., Govek, E.K., McDonagh, T., Qi, Y., Distefano, P.S.Geddes, B.J., 2008. Improved insulin sensitivity and metabolic flexibility in ghrelin receptor knockout mice. Regul Pept 150, 55-61.

Lopez, M., Lage, R., Saha, A.K., Perez-Tilve, D., Vazquez, M.J., Varela, L., Sangiao-Alvarellos, S., Tovar, S., Raghay, K., Rodriguez-Cuenca, S., Deoliveira, R.M.,

ACCEPTED MANUSCRIPT

Castaneda, T., Datta, R., Dong, J.Z., Culler, M., Sleeman, M.W., Alvarez, C.V., Gallego, R., Lelliott, C.J., Carling, D., Tschop, M.H., Dieguez, C.Vidal-Puig, A., 2008. Hypothalamic fatty acid metabolism mediates the orexigenic action of ghrelin. Cell Metab 7, 389-399.

Mayorov, A.V., Amara, N., Chang, J.Y., Moss, J.A., Hixon, M.S., Ruiz, D.I., Meijler, M.M., Zorrilla, E.P.Janda, K.D., 2008. Catalytic antibody degradation of ghrelin increases whole-body metabolic rate and reduces refeeding in fasting mice. Proc Natl Acad Sci U S A 105, 17487-17492.

Muller, E.E., Locatelli, V.Cocchi, D., 1999. Neuroendocrine control of growth hormone secretion. Physiol Rev 79, 511-607.

Nakazato, M., Murakami, N., Date, Y., Kojima, M., Matsuo, H., Kangawa, K.Matsukura, S., 2001. A role for ghrelin in the central regulation of feeding. Nature 409, 194-198.

Pacifico, L., Poggiogalle, E., Costantino, F., Anania, C., Ferraro, F., Chiarelli, F.Chiesa, C., 2009. Acylated and nonacylated ghrelin levels and their associations with insulin resistance in obese and normal weight children with metabolic syndrome. Eur J Endocrinol 161, 861-870.

Pfluger, P.T., Kirchner, H., Gunnel, S., Schrott, B., Perez-Tilve, D., Fu, S., Benoit, S.C., Horvath, T., Joost, H.G., Wortley, K.E., Sleeman, M.W.Tschop, M.H., 2008. Simultaneous deletion of ghrelin and its receptor increases motor activity and energy expenditure. Am J Physiol Gastrointest Liver Physiol 294, G610-618.

Pilkis, S.J.Granner, D.K., 1992. Molecular physiology of the regulation of hepatic gluconeogenesis and glycolysis. Annu Rev Physiol 54, 885-909.

Rossi, F., Castelli, A., Bianco, M.J., Bertone, C., Brama, M.Santiemma, V., 2008. Ghrelin induces proliferation in human aortic endothelial cells via ERK1/2 and PI3K/Akt activation. Peptides 29, 2046-2051.

Saggerson, E.D.Greenbaum, A.L., 1970. The regulation of triglyceride synthesis and fatty acid synthesis in rat epididymal adipose tissue. Effects of altered dietary and hormonal conditions. Biochem J 119, 221-242.

Salati, L.M.Amir-Ahmady, B., 2001. Dietary regulation of expression of glucose-6-phosphate dehydrogenase. Annu Rev Nutr 21, 121-140.

Saltiel, A.R.Kahn, C.R., 2001. Insulin signalling and the regulation of glucose and lipid metabolism. Nature 414, 799-806.

Sangiao-Alvarellos, S., Vazquez, M.J., Varela, L., Nogueiras, R., Saha, A.K., Cordido, F., Lopez, M.Dieguez, C., 2009. Central ghrelin regulates peripheral lipid metabolism in a growth hormone-independent fashion. Endocrinology 150, 4562-4574.

Seoane, L.M., Tovar, S.A., Perez, D., Mallo, F., Lopez, M., Senaris, R., Casanueva, F.F.Dieguez, C., 2004. Orexin A suppresses in vivo GH secretion. Eur J Endocrinol 150, 731-736.

Shearman, L.P., Wang, S.P., Helmling, S., Stribling, D.S., Mazur, P., Ge, L., Wang, L., Klussmann, S., Macintyre, D.E., Howard, A.D.Strack, A.M., 2006. Ghrelin neutralization by a ribonucleic acid-SPM ameliorates obesity in diet-induced obese mice. Endocrinology 147, 1517-1526.

Shiiya, T., Nakazato, M., Mizuta, M., Date, Y., Mondal, M.S., Tanaka, M., Nozoe, S., Hosoda, H., Kangawa, K.Matsukura, S., 2002. Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. J Clin Endocrinol Metab 87, 240-244.

Shin, E.S., Cho, S.Y., Lee, E.H., Lee, S.J., Chang, I.S.Lee, T.R., 2006. Positive regulation of hepatic carnitine palmitoyl transferase 1A (CPT1A) activities by soy isoflavones and L-carnitine. Eur J Nutr 45, 159-164.

St-Pierre, D.H., Karelis, A.D., Coderre, L., Malita, F., Fontaine, J., Mignault, D., Brochu, M., Bastard, J.P., Cianflone, K., Doucet, E., Imbeault, P.Rabasa-Lhoret, R., 2007.

Accepted Manuscript

ACCEPTED MANUSCRIPT

Association of acylated and nonacylated ghrelin with insulin sensitivity in overweight and obese postmenopausal women. J Clin Endocrinol Metab 92, 264-269.

Sun, Y., Ahmed, S.Smith, R.G., 2003. Deletion of ghrelin impairs neither growth nor appetite. Mol Cell Biol 23, 7973-7981.

Sun, Y., Asnicar, M., Saha, P.K., Chan, L.Smith, R.G., 2006. Ablation of ghrelin improves the diabetic but not obese phenotype of ob/ob mice. Cell Metab 3, 379-386.

Sun, Y., Butte, N.F., Garcia, J.M.Smith, R.G., 2008. Characterization of adult ghrelin and ghrelin receptor knockout mice under positive and negative energy balance. Endocrinology 149, 843-850.

Tang-Christensen, M., Vrang, N., Ortmann, S., Bidlingmaier, M., Horvath, T.L.Tschop, M., 2004. Central administration of ghrelin and agouti-related protein (83-132) increases food intake and decreases spontaneous locomotor activity in rats. Endocrinology 145, 4645-4652.

Theander-Carrillo, C., Wiedmer, P., Cettour-Rose, P., Nogueiras, R., Perez-Tilve, D., Pfluger, P., Castaneda, T.R., Muzzin, P., Schurmann, A., Szanto, I., Tschop, M.H.Rohner-Jeanrenaud, F., 2006. Ghrelin action in the brain controls adipocyte metabolism. J Clin Invest 116, 1983-1993.

Tian, W.N., Braunstein, L.D., Apse, K., Pang, J., Rose, M., Tian, X.Stanton, R.C., 1999. Importance of glucose-6-phosphate dehydrogenase activity in cell death. Am J Physiol 276, C1121-1131.

Tian, W.N., Braunstein, L.D., Pang, J., Stuhlmeier, K.M., Xi, Q.C., Tian, X.Stanton, R.C., 1998. Importance of glucose-6-phosphate dehydrogenase activity for cell growth. J Biol Chem 273, 10609-10617.

Tschop, M., Smiley, D.L.Heiman, M.L., 2000. Ghrelin induces adiposity in rodents. Nature 407, 908-913.

Tschop, M., Wawarta, R., Riepl, R.L., Friedrich, S., Bidlingmaier, M., Landgraf, R.Folwaczny, C., 2001a. Post-prandial decrease of circulating human ghrelin levels. J Endocrinol Invest 24, RC19-21.

Tschop, M., Weyer, C., Tataranni, P.A., Devanarayan, V., Ravussin, E.Heiman, M.L., 2001b. Circulating ghrelin levels are decreased in human obesity. Diabetes 50, 707-709.

Van der Lely, A.J., Tschop, M., Heiman, M.L.Ghigo, E., 2004. Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. Endocr Rev 25, 426-457.

Vestergaard, E.T., Djurhuus, C.B., Gjedsted, J., Nielsen, S., Moller, N., Holst, J.J., Jorgensen, J.O.Schmitz, O., 2008a. Acute effects of ghrelin administration on glucose and lipid metabolism. J Clin Endocrinol Metab 93, 438-444.

Vestergaard, E.T., Gormsen, L.C., Jessen, N., Lund, S., Hansen, T.K., Moller, N.Jorgensen, J.O., 2008b. Ghrelin infusion in humans induces acute insulin resistance and lipolysis independent of growth hormone signaling. Diabetes 57, 3205-3210.

Westbury, K.Hahn, P., 1984. Fructose-1,6-biphosphatase activity in the intestinal mucosa of developing rats. Am J Physiol 246, G683-686.

Wren, A.M., Seal, L.J., Cohen, M.A., Brynes, A.E., Frost, G.S., Murphy, K.G., Dhillo, W.S., Ghatei, M.A.Bloom, S.R., 2001a. Ghrelin enhances appetite and increases food intake in humans. J Clin Endocrinol Metab 86, 5992.

Wren, A.M., Small, C.J., Abbott, C.R., Dhillo, W.S., Seal, L.J., Cohen, M.A., Batterham, R.L., Taheri, S., Stanley, S.A., Ghatei, M.A.Bloom, S.R., 2001b. Ghrelin causes hyperphagia and obesity in rats. Diabetes 50, 2540-2547.

Yada, T., Dezaki, K., Sone, H., Koizumi, M., Damdindorj, B., Nakata, M.Kakei, M., 2008. Ghrelin regulates insulin release and glycemia: physiological role and therapeutic potential. Curr Diabetes Rev 4, 18-23.

Yang, H., Youm, Y.H., Nakata, C.Dixit, V.D., 2007. Chronic caloric restriction induces forestomach hypertrophy with enhanced ghrelin levels during aging. Peptides 28, 1931-1936.

Accepted Manuscript

ACCEPTED MANUSCRIPT

Yang, J., Brown, M.S., Liang, G., Grishin, N.V.Goldstein, J.L., 2008. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. Cell 132, 387-396.

Zhang, X., Gan, L., Pan, H., Guo, S., He, X., Olson, S.T., Mesecar, A., Adam, S.Unterman, T.G., 2002. Phosphorylation of serine 256 suppresses transactivation by FKHR (FOXO1) by multiple mechanisms. Direct and indirect effects on nuclear/cytoplasmic shuttling and DNA binding. J Biol Chem 277, 45276-45284.

Zhao, T.J., Liang, G., Li, R.L., Xie, X., Sleeman, M.W., Murphy, A.J., Valenzuela, D.M., Yancopoulos, G.D., Goldstein, J.L.Brown, M.S., 2010. Ghrelin O-acyltransferase (GOAT) is essential for growth hormone-mediated survival of calorie-restricted mice. Proc Natl Acad Sci U S A 107, 7467-7472.

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 ± 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 ± 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 ± SEM of eight animals per group. Different letters indicate statistical significance ($p < 0.05$).

Figure 5

Accepted Manuscript

ACCEPTED MANUSCRIPT

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 ± 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 ± 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 ± 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, β-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 ± SEM of eight animals per group. Different letters indicate statistical significance ($p < 0.05$).

Figure 9.

Glycolytic and lipogenic pathways in the liver.

Accepted Manuscript

ACCEPTED MANUSCRIPT

Table 1

Gene	Accession number		
ACCα	NM_022193	Forward	5'-TGGGCGGGATGGTCTCTTT-3'
		Reverse	5'-AGTCGCAGAAGCAGCCCATT-3'
		Probe	FAM-5'-ACCTTTGAAGATTTCGTCAGGATCTTTGATGA-3'-TAMRA
FAS	NM_017332	Forward	5'-GACATTTCATCAGGCCACC-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.

Accepted Manuscript

Figure

Figure 1

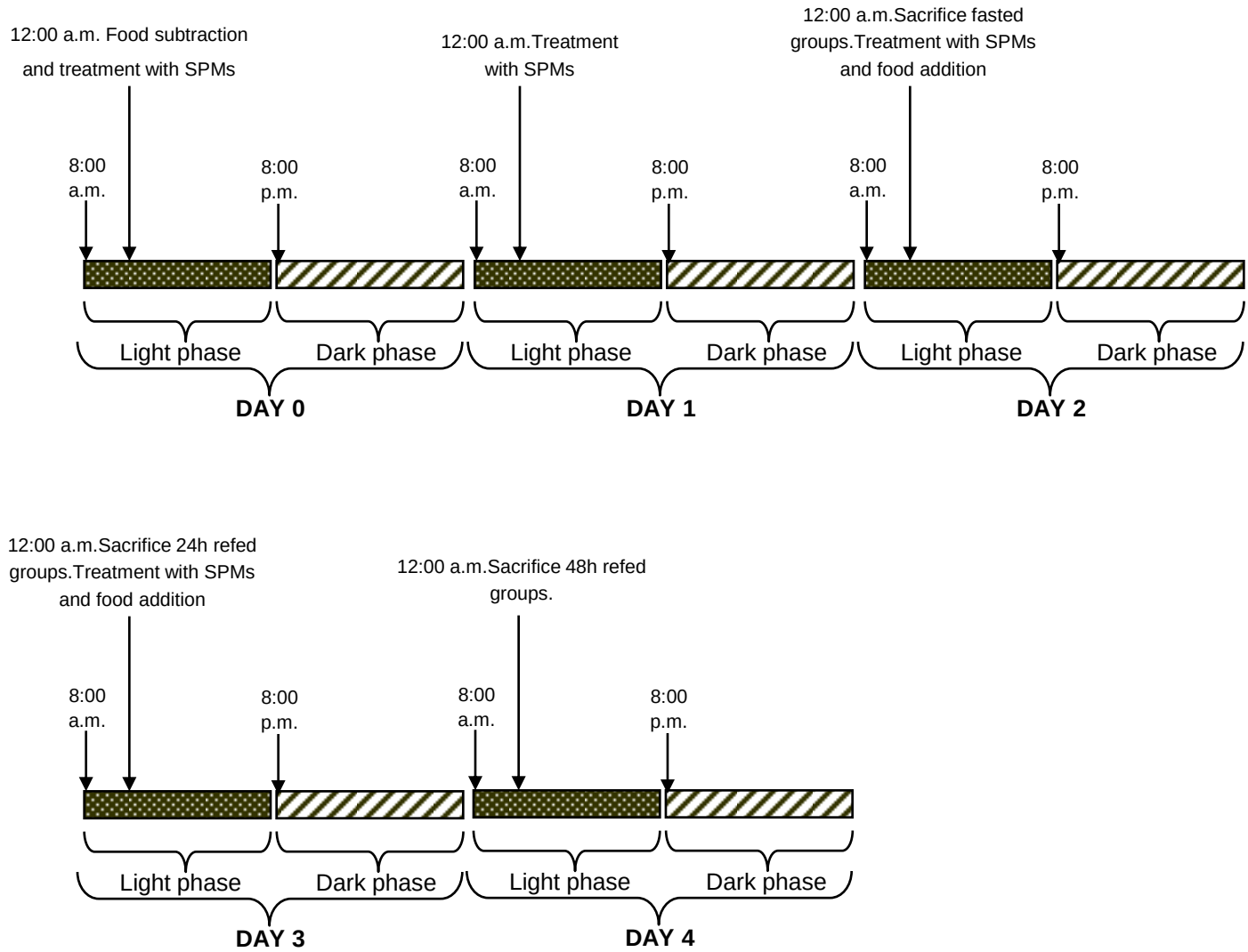


Figure 2

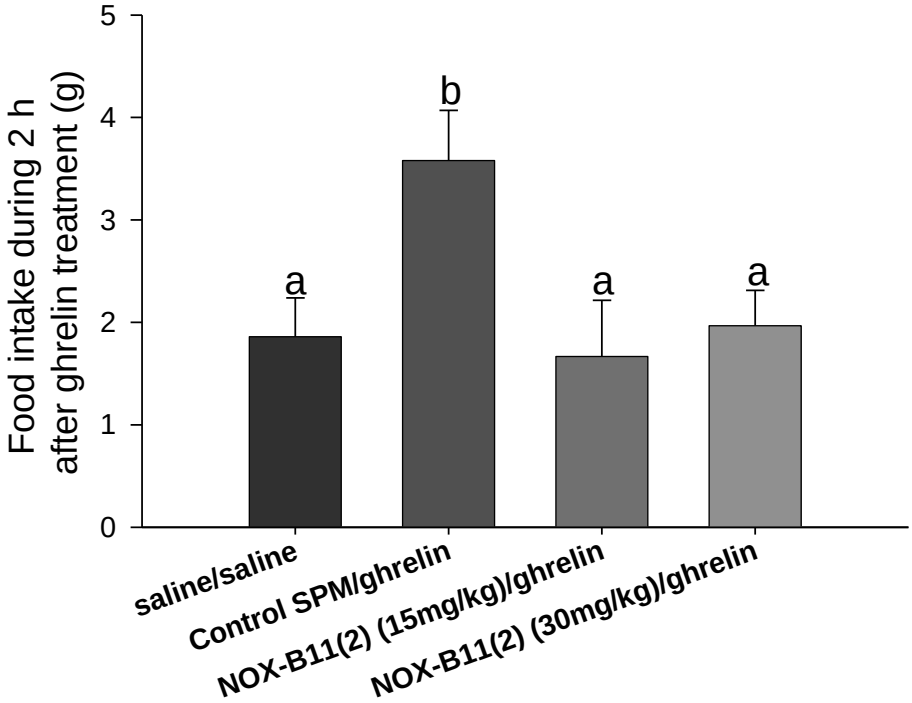


Figure 3

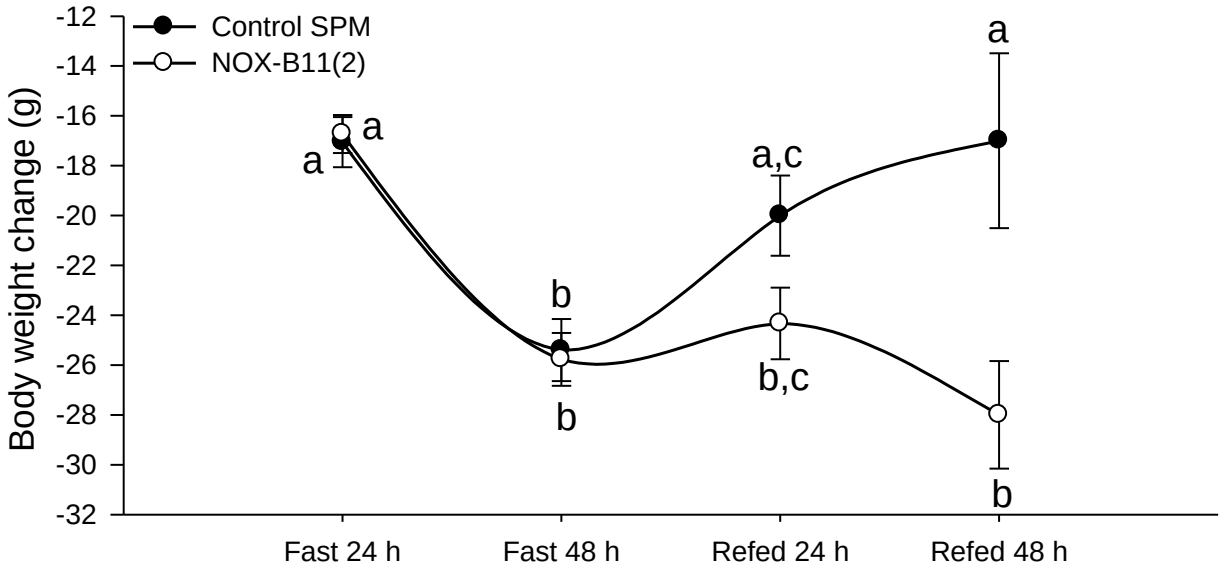


Figure 4

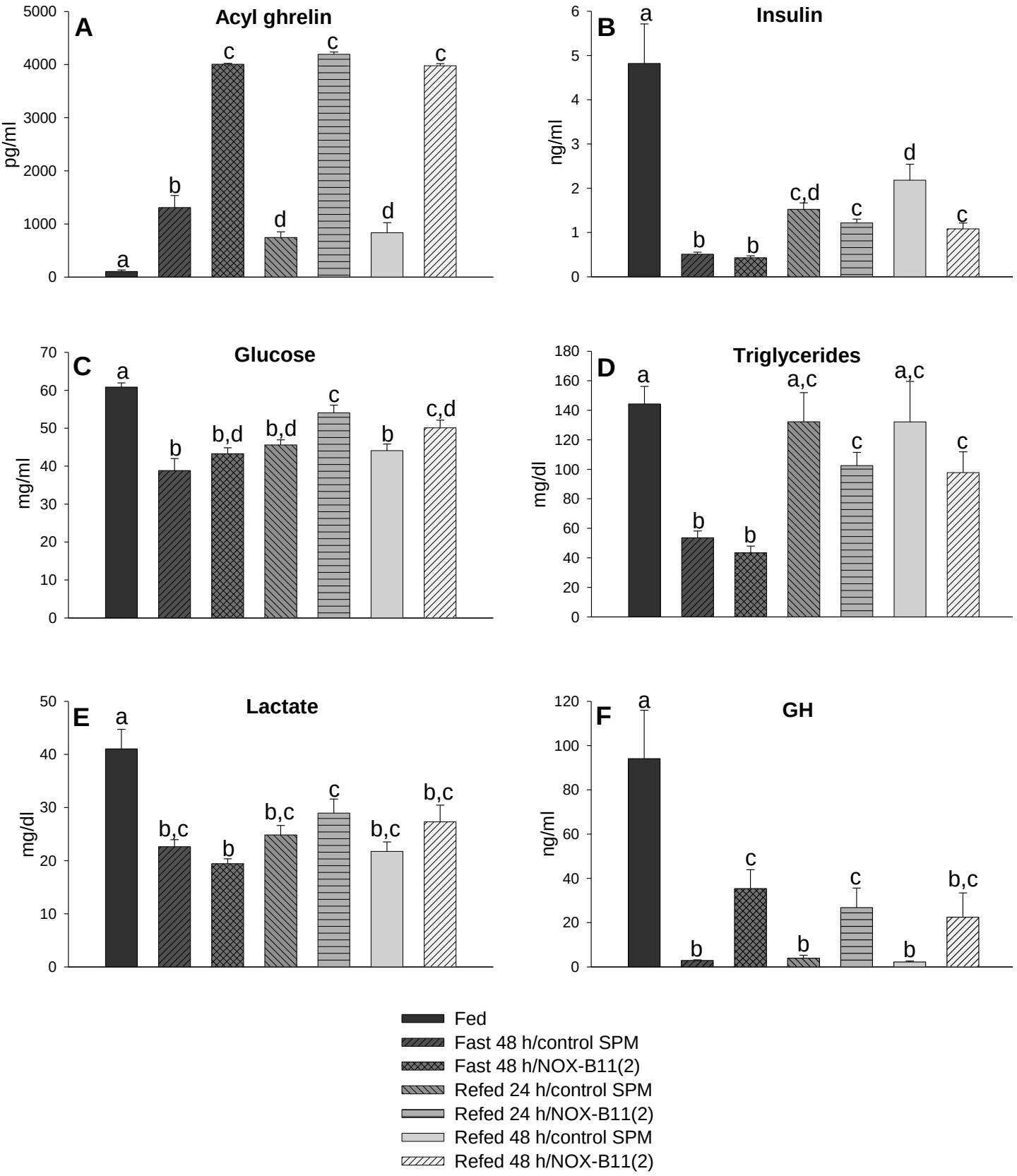

A
Acyl ghrelin
pg/ml
5000
4000
3000
2000
1000
0
a
b
c
d
c
d
c
B
Insulin
ng/ml
6
5
4
3
2
1
0
a
b
b
c,d
c
d
c
C
Glucose
mg/ml
70
60
50
40
30
20
10
0
a
b
b,d
b,d
c
b
c,d
D
Triglycerides
mg/dl
180
160
140
120
100
80
60
40
20
0
a
b
b
a,c
c
a,c
c
E
Lactate
mg/dl
50
40
30
20
10
0
a
b,c
b
b,c
c
b,c
b,c
F
GH
ng/ml
120
100
80
60
40
20
0
a
b
c
b
c
b
b,c
Fed
Fast 48 h/control SPM
Fast 48 h/NOX-B11(2)
Refed 24 h/control SPM
Refed 24 h/NOX-B11(2)
Refed 48 h/control SPM
Refed 48 h/NOX-B11(2)

Figure 5

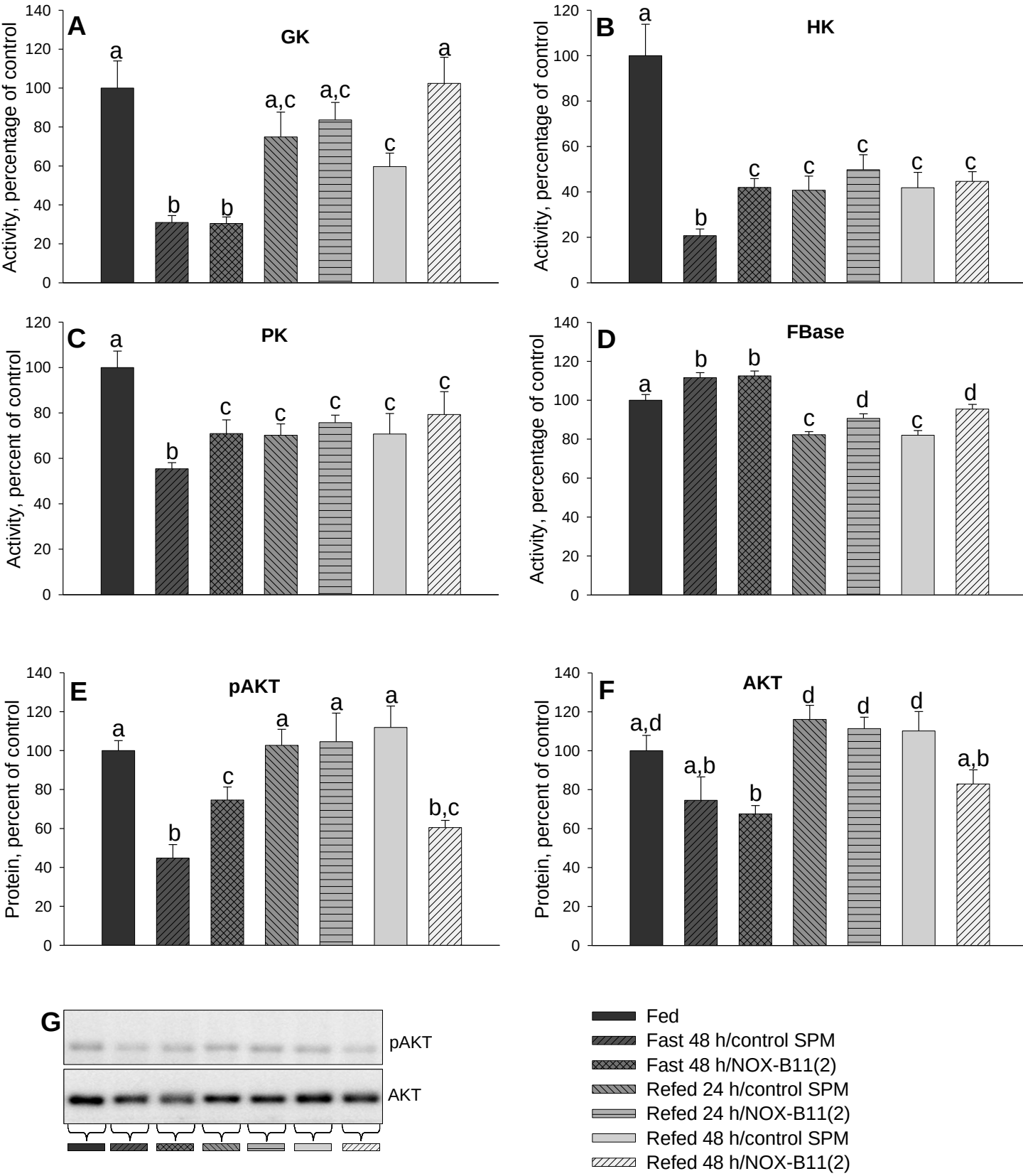

A
GK
Activity, percentage of control
a
b
b
a,c
a,c
c
a
B
HK
Activity, percentage of control
a
b
c
c
c
c
c
C
PK
Activity, percent of control
a
b
c
c
c
c
c
D
FBase
Activity, percentage of control
a
b
b
c
d
c
d
E
pAKT
Protein, percent of control
a
b
c
a
a
a
b,c
F
AKT
Protein, percent of control
a,d
a,b
b
d
d
d
a,b
G
pAKT
AKT
Fed
Fast 48 h/control SPM
Fast 48 h/NOX-B11(2)
Refed 24 h/control SPM
Refed 24 h/NOX-B11(2)
Refed 48 h/control SPM
Refed 48 h/NOX-B11(2)

Figure 6

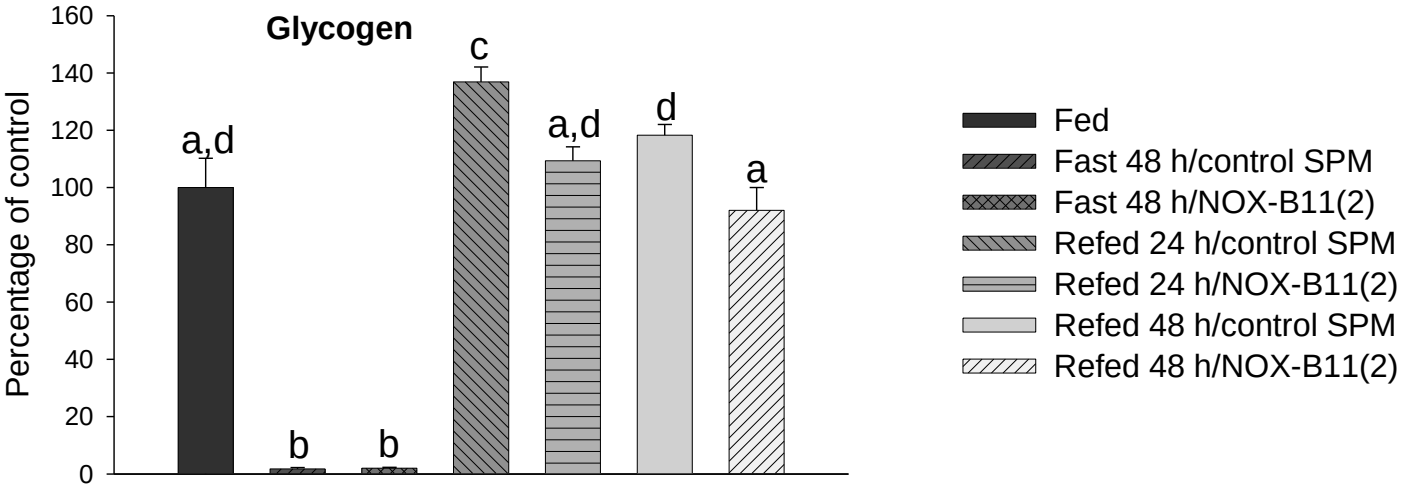

Glycogen
Percentage of control
160
140
120
100
80
60
40
20
0
a,d
b
b
c
a,d
d
a
Fed
Fast 48 h/control SPM
Fast 48 h/NOX-B11(2)
Refed 24 h/control SPM
Refed 24 h/NOX-B11(2)
Refed 48 h/control SPM
Refed 48 h/NOX-B11(2)

Figure 7

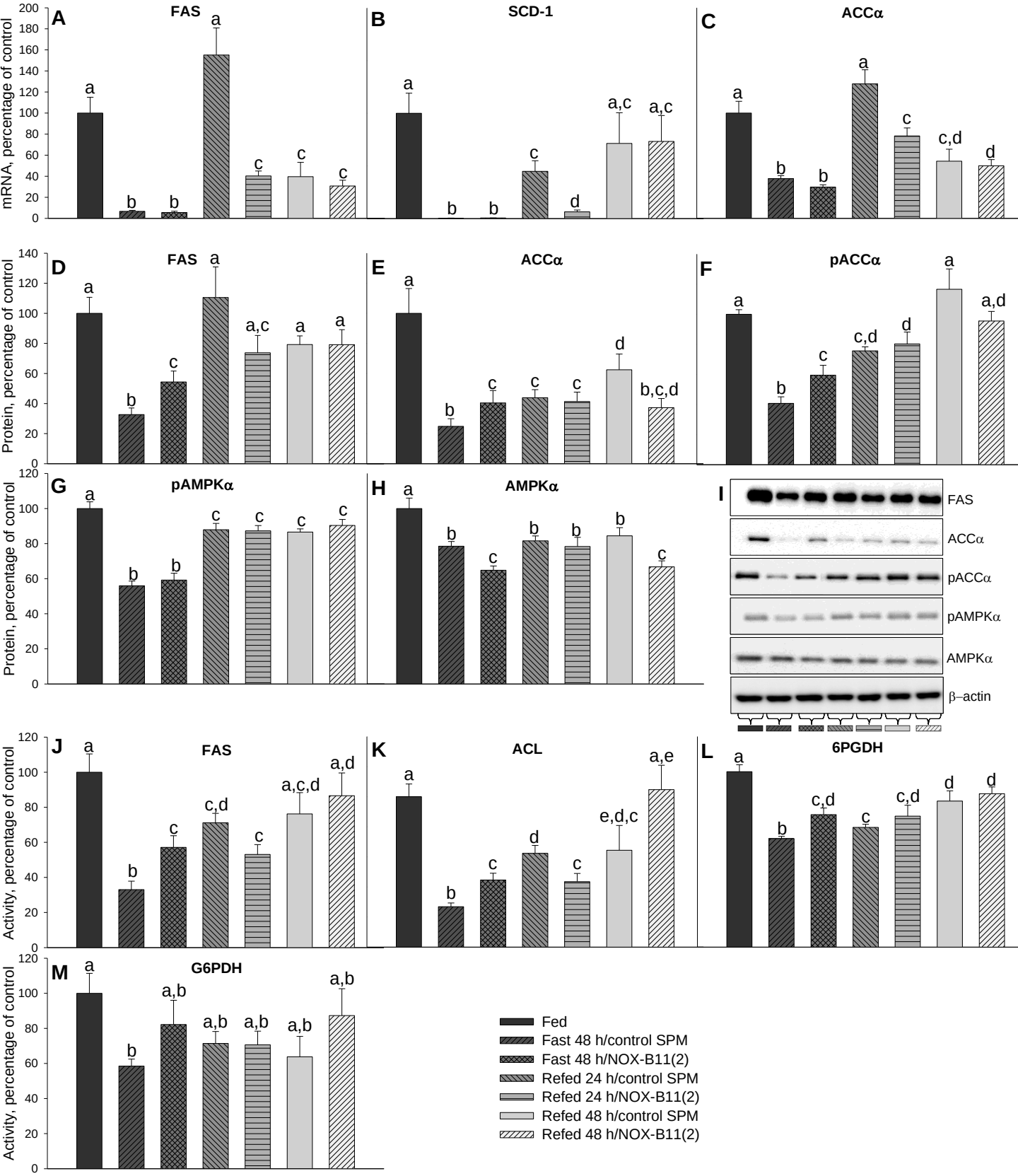

A
FAS
B
SCD-1
C
ACCα
mRNA, percentage of control
D
FAS
E
ACCα
F
pACCα
Protein, percentage of control
G
pAMPKα
H
AMPKα
I
FAS
ACCα
pACCα
pAMPKα
AMPKα
β–actin
Protein, percentage of control
J
FAS
K
ACL
L
6PGDH
Activity, percentage of control
M
G6PDH
Activity, percentage of control
Fed
Fast 48 h/control SPM
Fast 48 h/NOX-B11(2)
Refed 24 h/control SPM
Refed 24 h/NOX-B11(2)
Refed 48 h/control SPM
Refed 48 h/NOX-B11(2)

Figure 8

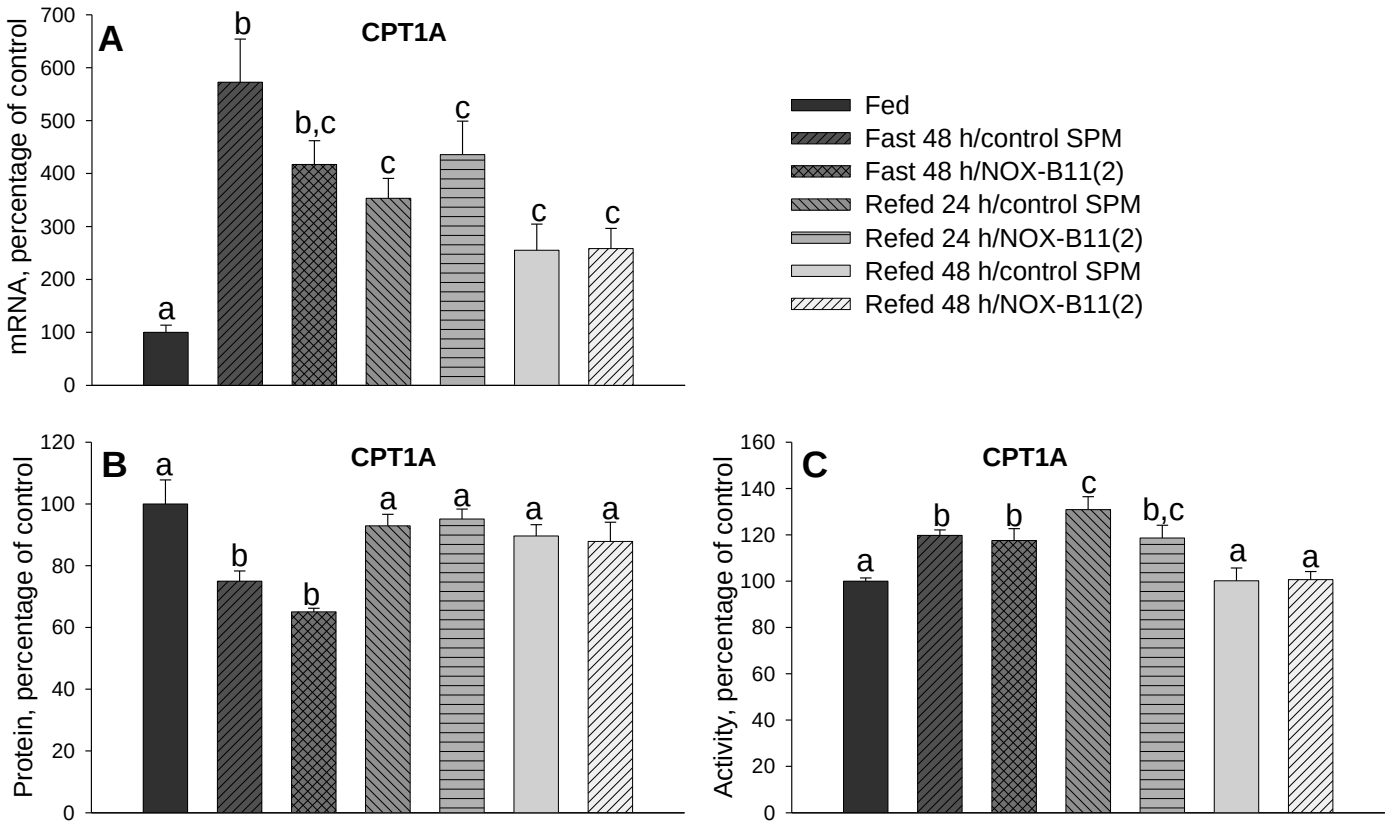
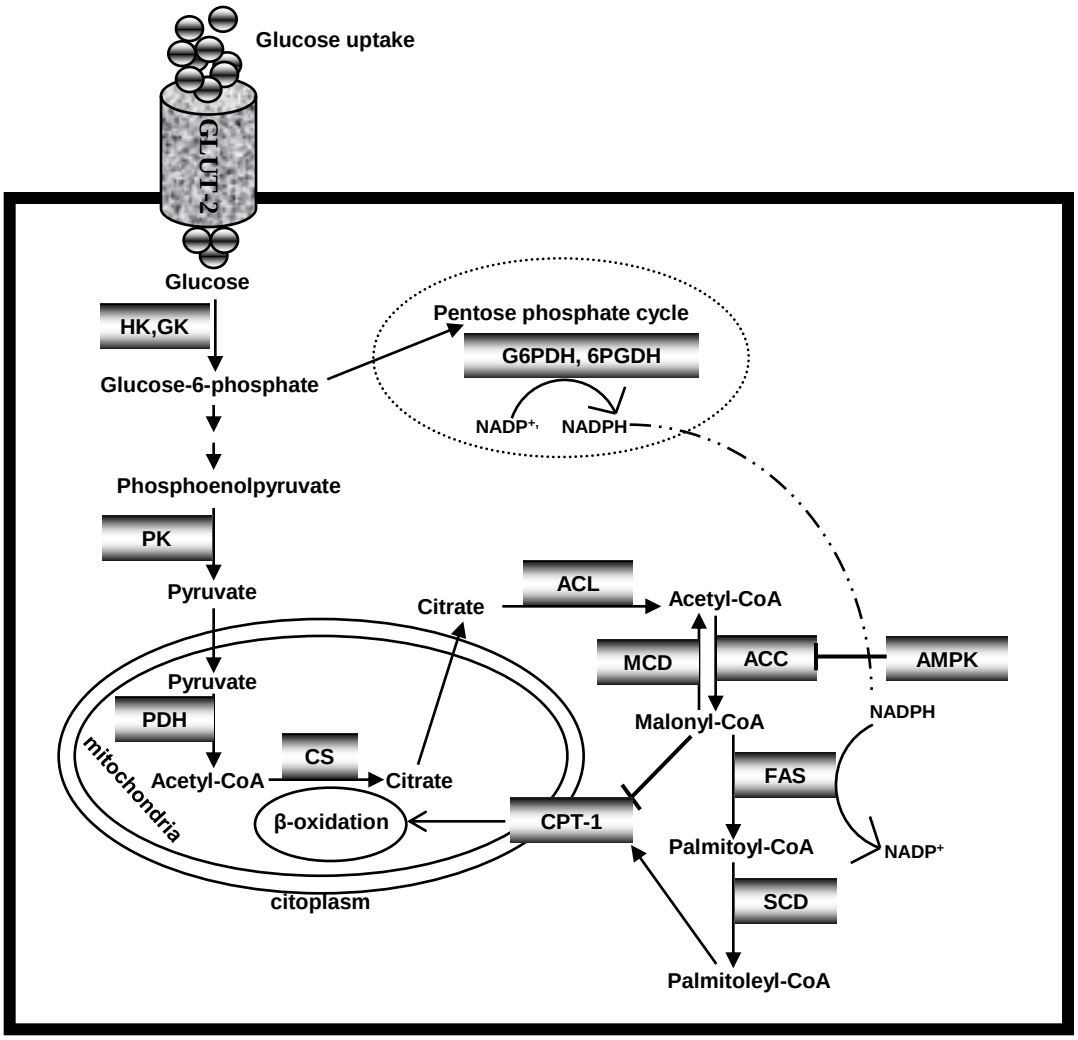

A
CPT1A
mRNA, percentage of control
700
600
500
400
300
200
100
0
a
b
b,c
c
c
c
c
Fed
Fast 48 h/control SPM
Fast 48 h/NOX-B11(2)
Refed 24 h/control SPM
Refed 24 h/NOX-B11(2)
Refed 48 h/control SPM
Refed 48 h/NOX-B11(2)
B
CPT1A
Protein, percentage of control
120
100
80
60
40
20
0
a
b
b
a
a
a
a
C
CPT1A
Activity, percentage of control
160
140
120
100
80
60
40
20
0
a
b
b
c
b,c
a
a

Figure 9


Glucose uptake
GLUT-2
Glucose
HK,GK
Glucose-6-phosphate
Pentose phosphate cycle
G6PDH, 6PGDH
NADP+
NADPH
Phosphoenolpyruvate
PK
Pyruvate
Citrate
ACL
Acetyl-CoA
MCD
ACC
AMPK
Pyruvate
PDH
Malonyl-CoA
NADPH
mitochondria
CS
Acetyl-CoA
Citrate
FAS
β-oxidation
CPT-1
Palmitoyl-CoA
NADP+
citoplasm
SCD
Palmitoleyl-CoA