IMPAIRED ENDOCRINE-METABOLIC HOMEOSTASIS: UNDERLYING MECHANISM OF ITS INDUCTION BY UNBALANCED DIET

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ABSTRACT

Aim: to characterize the intrinsic mechanism by which sucrose induces β-cell dysfunction.

Methods: Normal rats received for 3 weeks a standard diet supplemented with 10% sucrose in the drinking water (HS) with/out an antioxidant agent (R/S α-lipoic acid). We measured plasma glucose, insulin, triglyceride, leptin and lipid peroxidation levels; homeostasis model assessment-insulin resistance (HOMA-IR) and HOMA-β indexes were also determined. Insulin secretion, β-cell apoptosis, intracellular insulin and leptin mediators, and oxidative stress (OS) markers were also measured in islets isolated from each experimental group.

Results: HS rats had increased plasma triglyceride, insulin, leptin, and lipid peroxidation (OS marker) levels associated with an insulin resistant state. Their islets developed an initial compensatory increase in glucose-induced insulin secretion and mRNA and protein levels of β-cell apoptotic markers. They also showed a significant decrease in mRNA and protein levels of insulin and leptin signalling pathway mediators. Uncoupling protein 2, Peroxisome proliferator-activated receptor-α and -δ mRNA and protein levels were increased whereas mRNA levels of Sirtuin-1, Glutathione peroxidase and Catalase were significantly lower in these animals. Development of all these endocrine-metabolic abnormalities was prevented by co-administration of R/S α-lipoic acid together with sucrose.

Conclusions: OS may be actively involved in the mechanism by which unbalanced/unhealthy diets induce β-cell dysfunction. Since metabolic-endocrine dysfunctions recorded in HS rats resembled those measured in human pre-diabetes, knowledge of its molecular mechanism could help to develop appropriate strategies to prevent the progression of this metabolic state towards Type 2 Diabetes.

Clinical Perspectives

- Sucrose consumption induces metabolic and endocrine dysfunction in normal rats, characterized by hypertriglyceridemia, hyperleptinemia, insulin- and leptin-resistance, an initial compensatory increase in glucose-stimulated insulin secretion and an increased β-cell apoptosis.
- All the endocrine-metabolic abnormalities induced by sucrose were prevented by co-administration of α-lipoic acid, demonstrating that oxidative stress may be involved in the mechanism by which this unbalanced/unhealthy diet impairs the metabolic-endocrine homeostasis and pancreatic β-cell function.
- All the endocrine-metabolic dysfunctions and enhanced oxidative stress resemble those reported in human pre-diabetes state, thus, the deep knowledge of the mechanisms underlying their development could help to design appropriate strategies to prevent its progression towards Type 2 Diabetes.

Keywords: unbalanced diets, oxidative stress, leptin resistance.

Abbreviations list: T2D, Type 2 diabetes; IR, insulin resistance; OS, oxidative stress; UCPs, mitochondrial uncoupling proteins; TBARS, thiobarbituric acid reactive substances; HOMA, homoeostasis model assessment; HOMA-β, HOMA for β-cell function; HOMA-IR, HOMA for insulin resistance; GSIS, glucose stimulated insulin secretion; ERS, endoplasmic reticulum stress; LR leptin resistance; PPAR, Peroxisome proliferator-activated receptor; Sirt, Sirtuin .

INTRODUCTION

Increased consumption of unhealthy/unbalanced diets and sedentary behavior have actively contributed to the development of the current epidemics of obesity, type 2 diabetes (T2D) and metabolic syndrome [1,2]. In that context, it has already been reported that consumption of sucrose-rich diets results in elevated levels of plasma triglyceride in both humans and experimental animals plus multiple abnormalities in different organs that control glucose metabolism such as adipose tissue, liver, and pancreatic islets [3,4].

The sucrose-induced abnormalities depend on the length of the administration-period [5,6] presenting three different stages: 1) *induction period*: at an early stage (3–5 weeks), the rats develop high levels of serum triglyceride, free fatty acid (FFA), and insulin together with insulin resistance (IR), hypertension, and increased ectopic fat storage in liver and muscle. Despite their hyperinsulinemia, these rats display impaired glucose tolerance, which demonstrates that their β-cells fail to respond appropriately to the increased insulin demand [4,5,7-9]; 2) *adaptation period*: after 8 weeks of this diet all those abnormal parameters spontaneously return to normal values; and 3) *hypertriglyceridemic and hyperglycemic period*: when high sucrose is administered chronically (15-40 weeks), serum triglyceride and glucose levels become permanently elevated (T2D), together with overweight, increased visceral adiposity, and general IR [5,7,10-13].

Although multiple factors are involved in the development of all these endocrine-metabolic dysfunctions, no conclusive evidence exists on the precise mechanism responsible for β-cell failure.

Rats consuming high amounts of sucrose present alterations in plasma adipokine concentration, suggesting their possible pathogenic role in the development of pre-diabetes and its transition to T2D [14]. In fact, leptin modulates glucose homeostasis, insulin gene expression and secretion, as well as β-cell mass and function [15]. The high circulating

level of leptin (indicating a leptin resistant [LR] state) recorded in these animals could be responsible for the appearance of lipid ectopic deposition and tissue damage [16,17]. Such resistance could potentiate its negative metabolic effects due to the concomitant IR state present in these rats particularly at islet level [18].

We previously postulated that oxidative stress (OS) may be a common underlying mechanism for unbalanced diet-induced dysfunction in pancreatic islet, liver, and adipose tissue [19-23].

OS is characterized by excessive production of reactive oxygen species (ROS) [24] while mitochondrial uncoupling proteins (UCPs) play a key role in the antioxidant defense mechanism [25]. Moreover, UCP2 is a key component of β-cell glucose sensing which links obesity, β-cell dysfunction, and T2D [26].

In view of this situation, our current study attempts to further characterize the intrinsic mechanism by which high consumption of sucrose induces β-cell dysfunction. For that purpose we focus our study on the first period of sucrose-induced abnormalities in which rats display β-cell dysfunction associated with other metabolic-endocrine alterations that resemble those recorded for people with pre-diabetes [4]. Consequently, we fed normal rats a commercial standard diet supplemented with 10% sucrose in drinking water in the presence/absence of an antioxidant agent (R/S α-lipoic acid), and measured insulin secretion, β-cell apoptosis, and OS markers, as well as insulin and leptin intracellular signaling pathways.

MATERIAL AND METHODS

Chemicals and drugs

Collagenase was obtained from Serva Feinbiochemica (Heidelberg, Germany). Primary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, California,

USA). BSA (bovine serum albumin) fraction V, rabbit anti-caspase-3 antibody, mouse monoclonal anti β-actin antibody, and other reagents were from Sigma-Aldrich.

Animals

Normal male Wistar rats (180–200 g body weight) were kept at 23° C on a fixed 12-h light–dark cycle (06:00–18:00 h), and divided into 3 different experimental groups: Control (C) with free access to a standard commercial diet and water; the same diet plus 10% sucrose (wt/vol) in the drinking water for 3 weeks (High sucrose [HS] group); and HS rats injected with R/S α-lipoic acid (35 mg/kg, i.p.) during the last five days of treatment (HS+L group). Water intake was measured daily while food consumption and individual body weight were recorded weekly. Experiments were performed according to "Ethical principles and guidelines for experimental animals" (3rd. Edition, 2005) by the Swiss Academy of Medical Sciences (http://www.aaalac.org). All the protocols were approved by the Animal Welfare Committee (CICUAL. Comité Institucional para el Cuidado y Uso de Animales de Laboratorio) of La Plata School of Medicine, UNLP. At the time of euthanasia, the whole pancreas from each animal was removed and islets were isolated by collagenase digestion. Each experimental group included 20 animals.

Plasma measurements

At the end of treatment, blood samples from non-fasted animals from all experimental groups were collected (09:00 h) from the retro-orbital plexus under light halothane anesthesia to measure plasma glucose, triglyceride, insulin, lipid peroxidation (thiobarbituric acid reactive substances [TBARS]), and leptin levels.

Glucose was measured with test strips (Accu-Chek Performa Nano System, Roche Diagnostics. Mannheim, Germany) and triglyceride level was determined using commercial kits (BioSystems S.A., Buenos Aires, Argentina) in an automated clinical analyzer. TBARS were measured by fluorimetric assay and results were expressed as pmol

of malondialdehyde (MDA)/mg of plasma protein. Leptin concentrations were determined by validated specific radioimmunoassay (RIA). Plasma insulin was also measured by RIA [27], using a specific antibody against rat insulin (Sigma Chemical Co.), rat insulin standard (Novo Nordisk Pharma Argentina), and highly purified porcine insulin labeled with ^{125}I. IR was determined by homeostasis model assessment-IR (HOMA-IR) using the formula [insulin (μU/L) x glucose (mmol/L)]/22.5. β-cell function was quantified by HOMA-β [insulin (μU/L) x 20/glucose (mmol/L)] – 3.5 [28]. Since these indexes were validated in humans but not in rodents, we compared values measured for C to the other experimental groups instead of using a cut-off threshold value.

Insulin secretion

Isolated islets from each experimental group were incubated for 60 min at 37° C in 0.6 ml Krebs–Ringer bicarbonate (KRB) buffer (118 mM NaCl, 25.96 mM $NaHCO_3$, 4.74 mM KCl, 2.24 mM $CaCl_2$, 1.19 mM $MgSO_4$, 0.91 mM KH_2PO_4), pH 7.4, previously gassed with a mixture of CO_2/O_2 (5/95%) containing 1% (w/v) BSA and 3.3 or 16.7 mM glucose. For each experimental condition, 10 groups of 5 islets each were incubated, measuring insulin released to the medium. At the end of the incubation period, aliquots from the medium were taken for insulin measurement by RIA [27] as described above. Insulin released into the incubation medium was expressed as ng of insulin/islet/hour.

Quantitative Real-Time PCR

Total RNA was obtained from islets isolated from each experimental group using a Rneasy mini kit (Qiagen), its integrity tested by agarose-formaldehyde gel electrophoresis. Possible contamination with protein or phenol was controlled by measuring the 260:280 nm absorbance ratio, whereas DNA contamination was avoided by treating the sample with DNase I (Invitrogen); 1μg of total RNA was used for reverse transcription with SuperScript III Reverse Transcriptase (Invitrogen) and oligo-dT. Real-time PCRs were run in triplicate

using FastStart SYBR Green Master (Roche) in the iCycler 5 (BioRad). The cycling profile used was: 1 cycle of 1 minute at 95° C (DNA denaturation), 40 cycles of 30 seconds at 95° C, 30 seconds at 60° C and 30 seconds at 72° C followed by a melting curve from 55° C to 90° C.

Sequences of oligonucleotide primers (Invitrogen) used in the study are listed in Table 1. Amplicons were designed in a size range of 90 to 250 bp. Quantified values were normalized against housekeeping gene β actin, using the individual efficiency calculated with a standard curve for each gene.

Western Blotting

Islets were homogenized in 80 mM Tris (pH 6.8), 5mM EDTA, 5% sodium dodecyl sulfate (SDS), 5% dithiothreitol, 10% glycerol, and protease inhibitors (1mM phenyl-methylsulfonyl-fluoride and 4 mg aprotinin). Samples were then fractionated under reducing conditions by SDS/PAGE (polyacrylamide gel electrophoresis) and electroblotted onto polyvinylidene difluoride transfer membrane (Amersham Hybond-P, GE Healthcare, UK). The amount of protein loaded onto the gel was quantified by Bio-Rad protein assay. Nonspecific binding sites were blocked with non-fat milk solution at 4º C for 90 minutes for all antibodies except β-actin which was blocked overnight.

The membranes were then incubated with specific antibodies against Caspase-8 (1:200 dilution), Caspase-9 (1:100 dilution), Caspase-3 (1:1,000 dilution), Bad (1:100 dilution), Bcl-2 (1:200 dilution), Insulin receptor (1:2,000 dilution), Insulin receptor substrate-1 (IRS-1; 1:1,000 dilution), IRS-2 (1:500 dilution), Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K; 1:6,000 dilution), SOCS2 (1:1,000 dilution), JAK2 (1:1,000 dilution), STAT5b (1:1,000 dilution), UCP2 (1:500 dilution), PPAR-α (1:100 dilution) and PPAR-δ (1:100 dilution). β-actin (1:10,000 dilution) was used as internal standard. After rinsing with Tween-tris-buffered saline (T-TBS), blots were incubated with anti-rabbit IgG-HRP

for 1 hour at room temperature. For β-actin, horseradish-peroxidase-conjugated anti-mouse IgG-HRP was used as secondary antibody. Proteins were revealed by an enhanced chemiluminescence detection system (ECL Prime, Amersham, GE Healthcare, UK). Finally, bands were quantified by Image Studio Digits 3.1 software.

Statistical data analysis

Experimental data were statistically analysed using SPSS program (15.0 version, SPSS, Inc, 25 Chicago, IL); ANOVA was applied for independent samples with normal distribution, followed by Tukey's or Tamhane test for similar variance samples. Results are expressed as mean ± standard error of the mean (SEM). Differences between groups were considered significant when p values were <0.05.

RESULTS

Body weight, food intake, and serum parameters

HS and HS+L animals consumed a significantly higher volume of water than C rats (58.80±5.14 and 46.70±6.92 *vs.* 26.97±1.85 ml/rat/day, respectively; $p<0.05$). Conversely, solid food intake was significantly greater in C than in HS and HS+L rats (19.33±0.70 *vs.* 13.93±0.27 and 13.13±1.03 g/rat/day, respectively; $p<0.05$). This fact resulted in a different percentage daily intake of nutrients in C compared to HS and HS+L (carbohydrates:proteins:lipids; C: 45:43:12; HS: 61:30:9 and HS+L: 59:32:9 respectively). Despite these differences, their caloric intake was comparable without significant differences (C: 55.8±2.04; HS: 63.77±2.84; HS+L: 56.62±1.57 Kcal/rat/day). Concordantly, no significant differences were recorded in body weight gain among experimental groups over the 3-week study period (Table 2).

Although no significant differences were recorded in plasma glucose levels among the groups, HS rats had significantly higher levels of serum triglyceride, insulin, leptin, and TBARS, as well as higher HOMA-IR and HOMA-β values than C animals (Table 2). These

data show that HS animals developed dyslipidemia together with insulin and LR, plus an increased general OS rate. Co-administration of R/S α-lipoic acid to these rats prevented the development of all these metabolic-endocrine changes.

Insulin secretion

Islets isolated from animals of all experimental groups increased glucose stimulated insulin secretion (GSIS) as a function of glucose concentration in the incubation media (Figure 1). Although no differences were recorded among the experimental groups at basal glucose concentration, islets from HS rats released significantly larger amounts of insulin than C in response to 16.7 mM glucose. This increased GSIS was not observed in islets isolated from HS rats treated with R/S α-lipoic acid ($p<0.05$ *vs*. HS; Figure 1).

Gene expression (mRNA and protein levels) of pro- and anti- apoptotic markers

Whereas anti-apoptotic protein Bcl-2 gene expression was similar in all experimental groups, islets isolated from HS animals showed a significant increase in mRNA and protein levels of Caspase-8, Caspase-9, Caspase-3, and the pro-apoptotic protein Bad compared to C rats. This stimulatory effect of HS on gene and protein expression of all pro-apoptotic markers was prevented by co-administration of R/S α-lipoic acid to these rats (Figure 2.A and B).

Intracellular insulin mediators

Islets isolated from HS rats showed a significant and coincident decrease in mRNA (Figure 3.A) and protein levels (Figure 3.B) of insulin receptor and PI3K ($p<0.05$ in both cases). R/S α-lipoic acid co-administration to these animals prevented this decreasing effect. Conversely, no significant differences were found in IRS-1 and IRS-2 mRNA and protein levels among groups.

Intracellular leptin mediators

mRNA levels of some of the leptin intracellular mediators (JAK2, STAT5b and SOCS2) showed no differences between C and HS groups. However, islets from HS rats showed a significant decrease of mRNA levels of leptin receptor (OBR-b). Co-administration of the antioxidant agent to these animals prevented this inhibition ($p<0.05$; Figure 4.A).

HS animals also presented a significant decrease in STAT5b protein levels which partly recovered – though not significantly- in HS+L rats.

Concomitantly, protein level of the negative regulator SOCS2 was significantly higher in these rats, an increase prevented by R/S α-lipoic acid co-administration ($p<0.05$; Figure 4.B).

Oxidative stress markers

Concomitant with the increased general OS previously described in HS rats (enhanced plasma TBARS levels), islets isolated from these animals showed a significant reduction of mRNA levels of enzymes involved in the antioxidant system (Glutathione peroxidase and Catalase, $p<0.05$). Co-administration of the antioxidant agent with sucrose significantly increased these mRNA levels ($p<0.05$; Figure 5.A).

Although no significant differences were recorded in mRNA levels of both superoxide dismutase (SOD) enzymes, CuZn-SOD and Mn-SOD, in islets from C and HS animals, Mn-SOD mRNA showed a significant increase in islets isolated from HS+L rats ($p<0.05$; Figure 5.A).

Islets isolated from HS rats showed a significant increase in UCP2 mRNA levels compared to C group, which was prevented by R/S α-lipoic acid co-administration ($p<0.05$; Figure 5.B).

mRNA levels of transcription factors PPAR-α and PPAR-δ, which positively modulate UCP2 expression, were also higher in HS compared to C group. Complementarily, mRNA levels of Sirtuin-1 (Sirt-1; a negative modulator of UCP2 expression) were significantly

lower in these animals. Development of all these abnormalities was also prevented by R/S α-lipoic acid co-administration ($p<0.05$; Figure 5.B).

We also found that UCP2, PPAR-α, and PPAR-δ protein levels were increased in HS islets compared to C. Antioxidant agent co-administration to these animals also restored these high levels to values comparable to those measured in C rats ($p<0.05$; Figure 5.C).

DISCUSSION

The current data confirmed our previous reports: administration of an unbalanced diet (HS) to normal rats for 3 weeks induces a significant increase in serum triglyceride and leptin levels, an IR state (hyperinsulinemia, increased HOMA-IR and HOMA-β indexes) associated with impaired glucose tolerance and increased GSIS *in vitro* [21,29]. These metabolic alterations developed within a framework of increased rate of OS evidenced by higher serum TBARS levels [20,30-32] and decreased gene expression of islet antioxidants enzymes (Glutathione peroxidase and Catalase). All these metabolic abnormalities were prevented by co-administration of an antioxidant agent (R/S α-lipoic acid) with sucrose, thus suggesting that OS plays an active role in their pathogenesis.

The properties of R/S α-lipoic acid antioxidant and other insulin-sensitizing actions have been largely described [33-36], and it has also been used to treat people with T2D (37). It scavenges ROS, potentiates the action of other antioxidants such as vitamins E and C, chelates metals, repairs oxidized proteins, reduces inflammation, and acts as a cofactor for mitochondrial enzymes responsible for glucose oxidation [34,35]. R/S α-lipoic acid administration also improves insulin sensitivity in rodent models [36] and in obese and diabetic people [37]. Further, our group previously reported that its administration to normal rats does not impair metabolic-endocrine homeostasis, suggesting that it does not itself have an impact [19].

Enhanced GSIS recorded in islet from HS animals was associated with a significant decrease in β-cell mass, mainly ascribed to enhanced apoptosis rate [21,29]. These effects result from a combination of enhanced endoplasmic reticulum stress (ERS), OS, mitochondrial dysfunction, and glyco-lipotoxicity [38-40]. The high release of saturated FFA by adipose tissue reported in these rats also contributes to this high β-cell apoptosis rate [13,23]. All these alterations were associated with increased mRNA and protein levels of Bad, Caspase-8, 9, and 3, active players in the last step of the β-cell apoptosis process. The significant prevention of these abnormalities by administration of R/S α-lipoic acid reinforces the assumption that OS might be actively involved in the mechanism by which high sucrose consumption reduces β-cell mass. Other authors´ reports lend further support to our assumption; namely, that: a) fructose administration to rats for 10 weeks induced an increase of pancreatic Caspase-3 expression, prevented by co-administration of α-lipoic acid [41] and b) α-lipoic acid ameliorated ERS-induced cell death in FRTL5 thyroid cells by activating PI3K/Akt signal pathway and modulating cell death-related protein levels (decreasing CHOP and Bax and increasing Bcl-2; [42]).

Leptin plays an important role in regulation of metabolism and energy homeostasis by acting at various peripheral tissues including the pancreas: physiological concentrations of leptin decrease insulin secretion and gene expression as well as glucose transport in β-cells [43,44]. Also, acute and chronic studies have shown a greater leptin-induced reduction in plasma insulin in obese than in lean animals [45]. The fact that overexpression of leptin receptors in diabetic rats lacking functional leptin receptors, was associated with a reduction in triglyceride ectopic stores [46,47] and restoration of GSIS suggests that triglycerides participate in the mechanism by which leptin modulates insulin secretion [48]. Our HS rats showed a significant decrease in leptin receptor (OBR-b) and STAT5b (one of the leptin signaling pathway mediators [49]) together with increased SOCS2 (a negative

regulator of leptin pathway). All together, these changes result in decreased leptin sensitivity with the consequent loss of its β-cell protective effect.

High sucrose consumption also induced an IR state at islet level (decreased gene expression of insulin receptor and PI3K cascade components) which could explain the alterations of the autocrine effect of insulin on islet glucose metabolism previously described in insulin-resistant animals [18,50]. All together, these effects could contribute to impairment of the insulin secretion mechanism observed in HS animals.

Administration of R/S α-lipoic acid to rats consuming high sucrose prevented insulin signaling cascade alterations, increasing insulin receptor and PI3K gene expression. These results, together with the fact that α-lipoic acid acutely stimulates the intracellular insulin pathway [51,52], support the conclusion that the sucrose-induced insulin resistant state could be due to a combined increased of OS and inflammatory process [22].

In that context, antioxidant enzymes gene expression was decreased in our HS rats. Mitochondrial ROS production is one of the major processes involved in OS generation, with active participation of their uncoupling proteins. UCP2 expression is stimulated by high glucose and/or high free FFA levels in both *in vivo* and *in vitro* conditions and is increased in animal models of T2D [53]. Peroxisome proliferator-activated receptors are pivotal actors in transcriptional control of UCP gene expression. Concomitantly, pancreatic PPAR-α is activated by elevated FFA levels, as occurs in obesity, and may contribute to the currently recorded increase in UCP2 expression. In our study, PPAR-α and PPAR-δ (transcription factors positively regulating UCP2 gene expression) were increased whereas Sirt-1 (the main negative regulator of UCP2 expression; [54]) was decreased. Sirt-1 is a factor whose activation improves insulin sensitivity of liver, skeletal muscle, and adipose tissue, and protects pancreatic β-cells mass and function [55]. Co-administration of the

antioxidant agent to HS animals restored gene expression levels of antioxidant enzymes, UCP2 and its modulators to values measured in C rats.

In conclusion, administration of high amounts of sucrose to normal rats induces metabolic-endocrine dysfunction with hypertriglyceridemia and hyperleptinemia associated with IR and LR. These alterations trigger an initial compensatory increase in GSIS but also an increased rate of β-cell apoptosis, perhaps following a combination of β-cell ERS, OS, and high saturated serum FFA levels leading to a decrease in β-cell mass. Since development of all these endocrine-metabolic abnormalities was prevented by co-administration R/S α-lipoic acid and sucrose, OS may be actively involved in the mechanism by which sucrose induces impairment of metabolic-endocrine homeostasis and pancreatic β-cell dysfunction. Although results obtained in animal models may not necessarily be reflected in human beings, since all the endocrine-metabolic dysfunctions and enhanced OS recorded in rats fed an excess of sucrose (summarized in Figure 6) resemble those reported in human pre-diabetes, this knowledge could help to develop appropriate strategies to prevent the progression of this metabolic state towards T2D.

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Declarations of interest

The authors declare that there is no conflict of interest.

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Author contribution statement

LEF, JJG and BM conceived and designed the study and drafted the manuscript; CLR, BM, and LEF carried out the experiments and statistical analyses. All authors read and approved the final manuscript. BM, LEF, and JJG are members of the research career of CONICET and CLR is a fellow of CONICET.

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1 **TABLES AND FIGURES**

2 **Table 1: Primer sequences.** Fw, forward primer and Rv, reverse primer

Gene	Gene Bank	Sequences
Caspase-8	NM_022277.1	Fw 5′-TAAAAAGCAGCCCAGAGGAA-3′ Rv 5′-ATCAAGCAGGCTCGAGTTGT-3′
Caspase-9	NM_031632.1	Fw 5′-CCAGATGCTGTCCCATACC-3′ Rv 5′-ATTGGCGACCCTGAGAAG-3′
Caspase-3	NM_012922.2	Fw 5′-CAAGTCGATGGACTCTGGAA-3′ Rv 5′-GTACCATTGCGAGCTGACAT-3′
Bad	NM_022698.1	Up 5′-CAGGCAGCCAATAACAGTCA-3′ Dw 5′-CCCTCAAATTCATCGCTCAT-3′
Bcl-2	L14680	Fw 5′-CGGGAGAACAGGGTATGA-3′ Rv 5′-CAGGCTGGAAGGAGAAGAT-3′
Insulin receptor	NM_017071	Fw 5′-ATATTGACCCGCCCCAGAGG-3′ Rv 5′-TAGGTCCGGCGTTCATCAGA-3′
IRS-1	NM_012969	Fw 5`-TGTGCCAAGCAACAAGAAAG-3` Rv 5'-ACGGTTTCAGAGCAGAGGAA-3'
IRS-2	NM_001168633.1	Fw 5'-CTACCCACTGAGCCCAAGAG-3′ Rv 5'-CCAGGGATGAAGCAGGACTA-3'
PI3K	NM_053481	Fw 5′-GGTTGTTGTTGCCCCAGAC-3′ Rv 5′-GGTTGTTGTTGCCCCAGAC-3′
OBR-b	NM_012596	Fw 5′-CTGCCCCCACTGAAAGACA-3′ Rv 5′-GGGCTGCAGTGACATTAGAG-3′
SOCS2	NM_058208	Fw 5′-TAAGCAGTTTGACAGCGTGG-3′ RV 5′-AATGCTGAGTCGGCAGAAGT-3′
JAK2	NM_031514	Fw 5′-TCCGTGATCTGAACAGCCTG-3′ Rv 5′-ACATCTCCACACTCCCAAAG-3′
STAT5b	NM_017064	Fw 5′-TTTCTCCATTCGGTCCCTGG-3′ Rv 5′-TGCTTGATCTGTGGCTTCAC-3′
Sirtuin-1	XM_017588053	Fw 5'-CCTGTGGGATACCTGAC -3' Rv 5'-AGAGATGGCTGGAACTG -3'
UCP2	NM_019354	Fw 5′-GGCXTGGCGGTGGTCGGAGATAC-3′ Rv 5′-CATTTCGGGCAACATTGGGAGAGG-3′
PPAR-α	NM_013196	Fw 5'-TTCCAGCCCCTCCTCAGTCA-3' Rv 5'-CGCCAGCTTTAGCCGAATAG -3'
PPAR-δ	NM_013141	Fw 5'-GCGAGGGCGATCTTGACAG -3' Rv 5'-GATGGCCACCTCTTTGCTCT -3'
Mn SOD	NM_017051.2	Fw 5'- ACCGAGGAGAAGTACCACGA-3' Rv 5'-TAGGGCTCAGGTTTGTCCAG-3'
CuZn SOD	NM_017050.1	Fw 5′-GTGCAGGGCGTCATTCACTTC-3′ Rv 5′-YGCCTCTCTTCATCCGCTGGA-3′
Catalase	NM_012520.1	Fw 5'-CCTCAGAAACCCGATGTCCTG -3' Rv 5'-GTCAAAGTGTGCCATCTCGTCG -3'
GPx	NM_030826.3	Fw 5′-TGAGAAGGCTCACCCGCTCT-3′ Rv 5′-GCACTGGAACACCGTCTGGA-3′
β-actin	NM_031144.3	Fw 5'- AGAGGGAAATCGTGCGTGAC-3' Rv 5'-CGATAGTGATGACCTGACCGT-3'

Table 2: Body weight and serum measurements. Values are expressed as means ± SEM (n = 20 rats per group). *p<0.05 *vs*. C; ^p<0.05 *vs*. HS.

Parameter	Control	HS	HS + L
Body weight gain (g)	89.17±6.56	89.50±5.74	75.83±4.31
Glucose (mg/dl)	119.67±4.84	114.33±4.12	111.00±7.45
Insulin (ng/ml)	0.68±0.05	0.93±0.07*	0.66±0.02^
Triglyceride (mg/dl)	96.51±4.85	157.84±4.96*	131.94±6.27^*
Leptin (ng/ml)	5.62±0.70	11.91±1.92*	6.39±1.43^
TBARS (nmol/mg prot)	96.9±9.9	138.9±7.7*	102.7±11.4^
HOMA-IR	4.94±0.34	6.70±0.50*	4.87±0.26^
HOMA-β	48.18±5.40	68.60±6.38*	46.94±2.36^

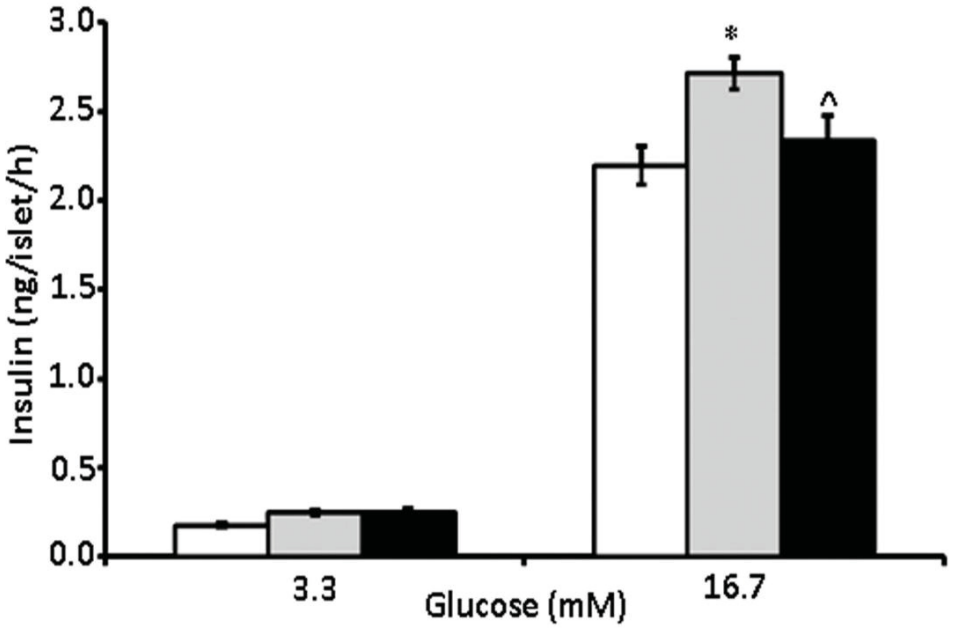
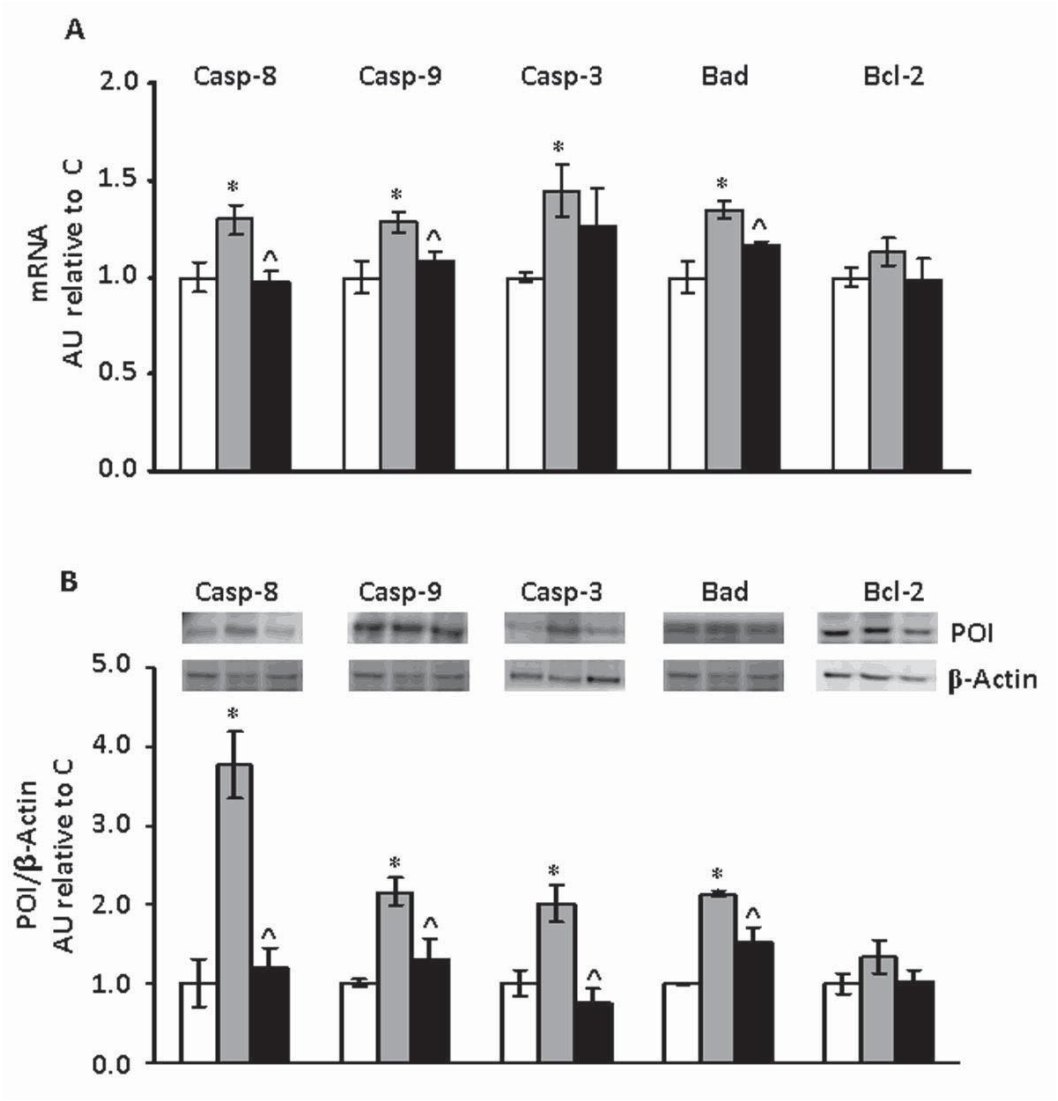


Figure 1: Glucose-induced insulin secretion.

Insulin secretion in response to 3.3 and 16.7 mM glucose by islets isolated from C (white bars), HS (grey bars) and HS+L (black bars) rats. Insulin released into incubation media was expressed as ng of insulin per islet/1 h.

Bars represent means ± SEM from three independent experiments. *$p<0.05$ *vs.* C; ^ $p<0.05$ *vs.* HS.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Casp-9
Casp-3
Bad
Bcl-2
mRNA
AU relative to C
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1.5
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B
Casp-8
Casp-9
Casp-3
Bad
Bcl-2
POI
β-Actin
POI/β-Actin
AU relative to C
5.0
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Figure 2. Apoptotic marker gene expression (mRNA and protein levels).

A. mRNA relative expression (RT qPCR) in islets isolated from C (white bars), HS (grey bars), and HS+L (black bars) rats. β-actin was used as internal standard. Values were expressed as arbitrary units (AU) compared to mRNA level determined in C islets. Bars represent means ± SEM from three independent experiments.

B. Protein levels measured by Western Blot in islet homogenates from the different experimental groups. A representative blot is shown in each case.

Bars represent means ± SEM expressed in arbitrary units (AU) as the ratio between the protein of interest (POI) and β-actin band intensity. *p<0.05 *vs.* C; ^ p<0.05 *vs.* HS.

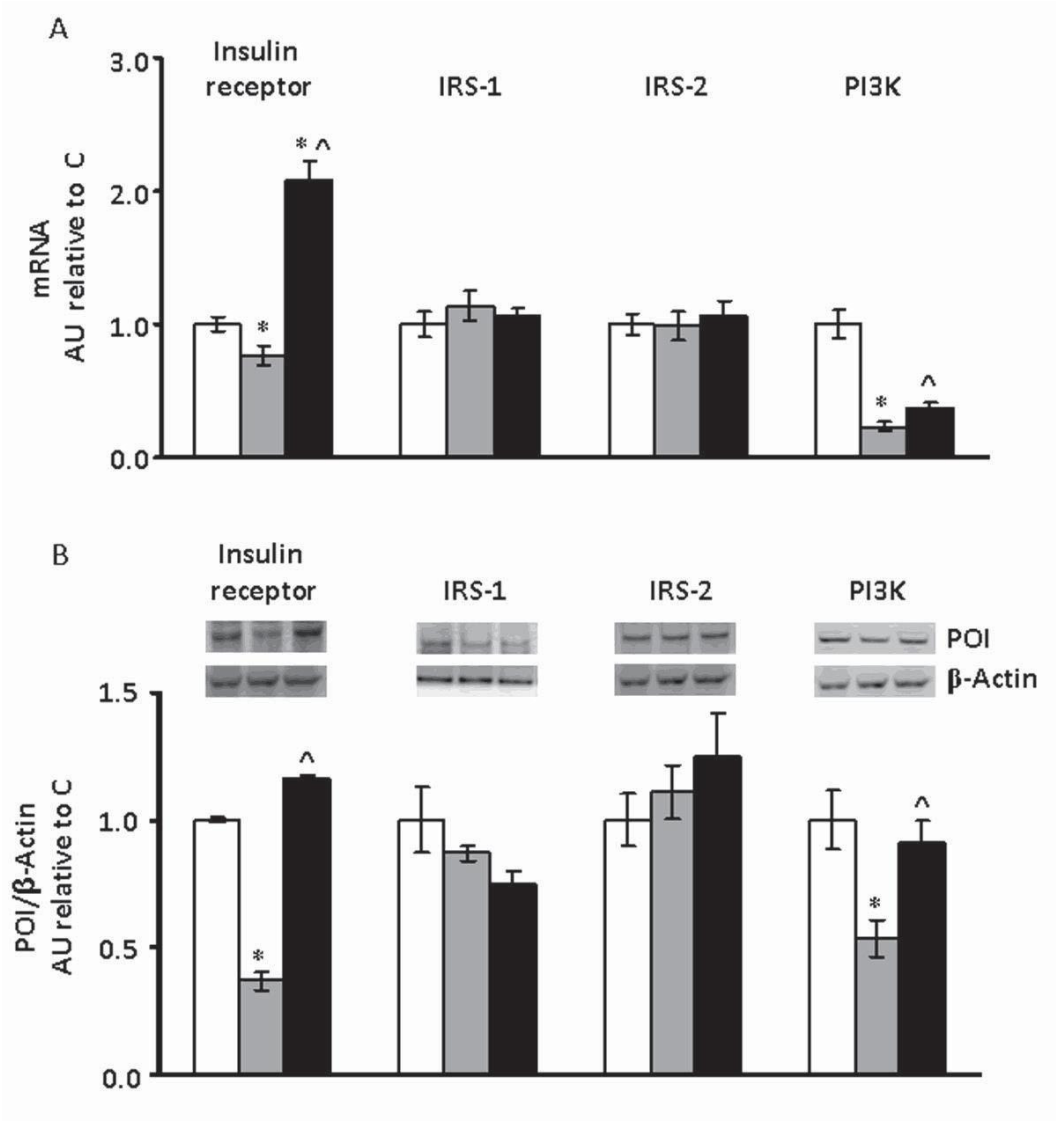

Figure 3: Intracellular insulin mediators: Gene expression (mRNA and protein levels).

A. mRNA relative expression (RT qPCR) in islets isolated from C (white bars), HS (grey bars), and HS+L (black bars) rats. β-actin was used as internal standard. Values were expressed in arbitrary units (AU) with respect to mRNA level determined in C islets. Bars represent means ± SEM from three independent experiments.

B. Protein levels measured by Western Blot in islet homogenates from the different experimental groups. A representative blot is shown in each case.

Bars represent means ± SEM expressed in arbitrary units (AU) as the ratio between the protein of interest (POI) and β-actin band intensity. *p<0.05 *vs.* C; ^ p<0.05 *vs.* HS.

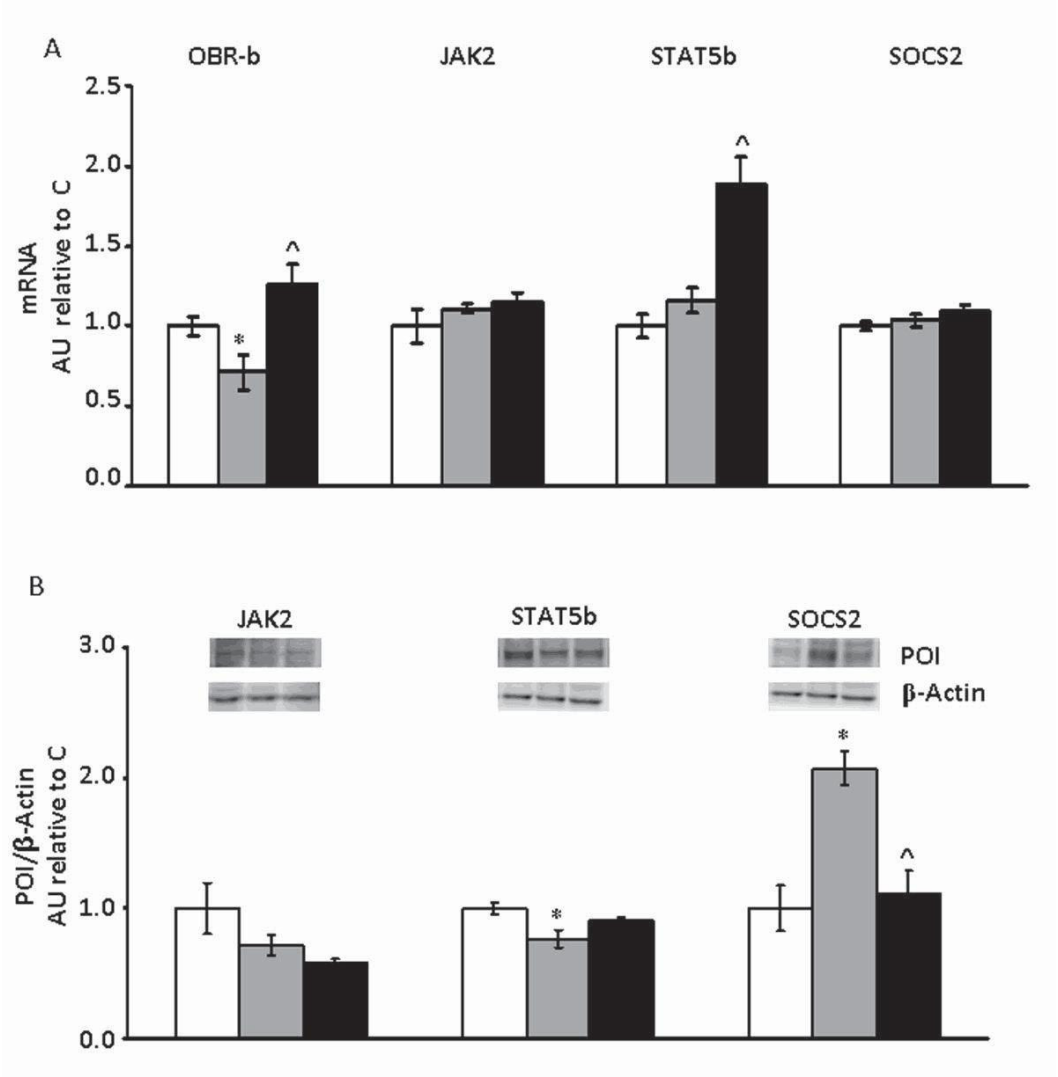

Figure 4: Intracellular leptin mediators: Gene expression (mRNA and protein levels).

A. mRNA relative expression (RT qPCR) in islets isolated from C (white bars), HS (grey bars), and HS+L (black bars) rats. β-actin was used as internal standard. Values were expressed in arbitrary units (AU) with respect to mRNA level determined in C islets. Bars represent means ± SEM from three independent experiments.

B. Protein levels measured by Western Blot in islet homogenates from the different experimental groups. A representative blot is shown in each case.

Bars represent means ± SEM expressed in arbitrary units (AU) as the ratio between the protein of interest (POI) and β-actin band intensity. *$p<0.05$ *vs.* C; ^ $p<0.05$ *vs.* HS.

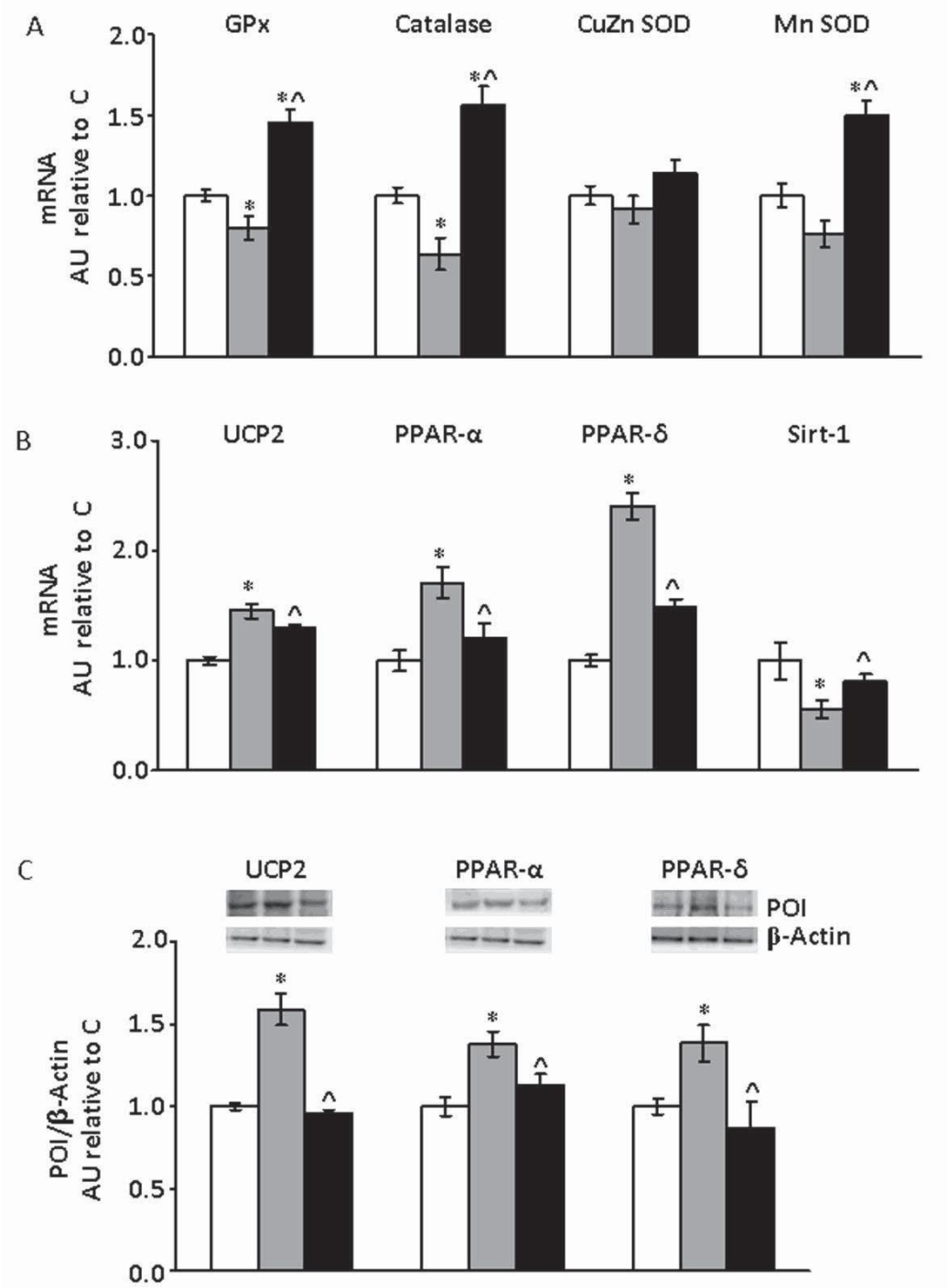

Figure 5: Gene expression of antioxidant enzymes, UCP2 and its modulators (PPARs and Sirtuin-1).

A. mRNA relative expression (RT qPCR) of antioxidant enzymes (Glutathione peroxidase-GPx-; Catalase; Cu Zn Superoxide Dismutase -SOD-; and Mn SOD) in islets isolated from C (white bars), HS (grey bars), and HS+L (black bars) rats. β-actin was used as internal

standard. Values were expressed in arbitrary units (AU) with respect to mRNA level determined in C islets. Bars represent means ± SEM from three independent experiments.

B. mRNA relative expression (RT qPCR) of Uncoupling Protein 2 (UCP2), PPAR factors, and Sirtuin-1 (Sirt-1) in islets isolated from C (white bars), HS (grey bars), and HS+L (black bars) rats. β-actin was used as internal standard. Values were expressed in arbitrary units (AU) with respect to mRNA level determined in C islets. Bars represent means ± SEM from three independent experiments.

C. Protein levels measured by Western Blot in islet homogenates from the different experimental groups. A representative blot is shown in each case.

Bars represent means ± SEM expressed in arbitrary units (AU) as the ratio between the protein of interest (POI) and β-actin band intensity. *$p<0.05$ *vs.* C; ^ $p<0.05$ *vs.* HS.

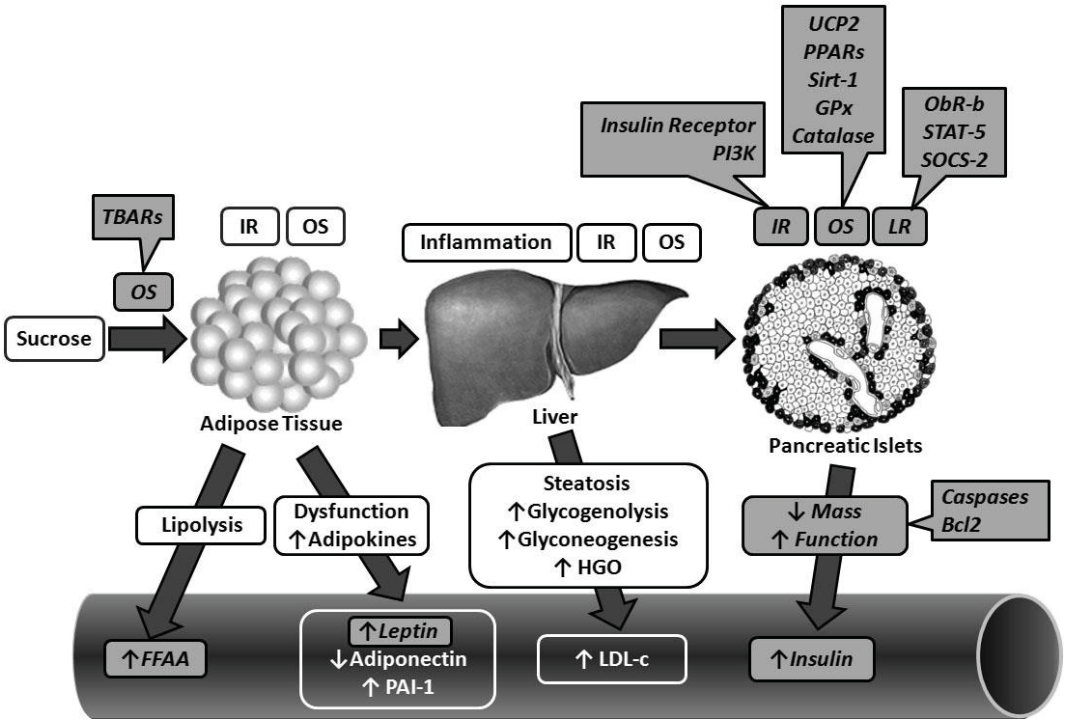

Figure 6: Schematic diagram of sequential events triggered by unbalanced diet. Based on the current results (italic font on gray background) together with those previously reported by our group (clear background), we proposed that HS induces an increased OS state promoting dysfunction of adipose tissue [20,31] and liver [19,22] followed by an initial β-cell compensatory response that results in decreased β-cell mass and function [21,29].

HGO: hepatic glucose output.

Gene	Gene Bank	Sequences
Caspase-8	NM_022277.1	Fw 5′-TAAAAAGCAGCCCAGAGGAA-3′ Rv 5′-ATCAAGCAGGCTCGAGTTGT-3′
Caspase-9	NM_031632.1	Fw 5′-CCAGATGCTGTCCCATACC-3′ Rv 5′-ATTGGCGACCCTGAGAAG-3′
Caspase-3	NM_012922.2	Fw 5′-CAAGTCGATGGACTCTGGAA-3′ Rv 5′-GTACCATTGCGAGCTGACAT-3′
Bad	NM_022698.1	Up 5′-CAGGCAGCCAATAACAGTCA-3′ Dw 5′-CCCTCAAATTCATCGCTCAT-3′
Bcl-2	L14680	Fw 5′-CGGGAGAACAGGGTATGA-3′ Rv 5′-CAGGCTGGAAGGAGAAGAT-3′
Insulin receptor	NM_017071	Fw 5′-ATATTGACCCGCCCCAGAGG-3′ Rv 5′-TAGGTCCGGCGTTCATCAGA-3′
IRS-1	NM_012969	Fw 5`-TGTGCCAAGCAACAAGAAAG-3` Rv 5’-ACGGTTTCAGAGCAGAGGAA-3’
IRS-2	NM_001168633.1	Fw 5’-CTACCCACTGAGCCCAAGAG-3′ Rv 5’-CCAGGGATGAAGCAGGACTA-3’
PI3K	NM_053481	Fw 5′-GGTTGTTGTTGCCCCAGAC-3′ Rv 5′-GGTTGTTGTTGCCCCAGAC-3′
OBR-b	NM_012596	Fw 5′-CTGCCCCCACTGAAAGACA-3′ Rv 5′-GGGCTGCAGTGACATTAGAG-3′
SOCS2	NM_058208	Fw 5′-TAAGCAGTTTGACAGCGTGG-3′ RV 5′-AATGCTGAGTCGGCAGAAGT-3′
JAK2	NM_031514	Fw 5′-TCCGTGATCTGAACAGCCTG-3′ Rv 5′-ACATCTCCACACTCCCAAAG-3′
STAT5b	NM_017064	Fw 5′-TTTCTCCATTCGGTCCCTGG-3′ Rv 5′-TGCTTGATCTGTGGCTTCAC-3′
Sirtuin-1	XM_017588053	Fw 5’-CCTGTGGGATACCTGAC -3’ Rv 5’-AGAGATGGCTGGAACTG -3’
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PPAR-α	NM_013196	Fw 5’-TTCCAGCCCCTCCTCAGTCA-3’ Rv 5’-CGCCAGCTTTAGCCGAATAG -3’
PPAR-δ	NM_013141	Fw 5’-GCGAGGGCGATCTTGACAG -3’ Rv 5’-GATGGCCACCTCTTTGCTCT -3’
Mn SOD	NM_017051.2	Fw 5′- ACCGAGGAGAAGTACCACGA-3′ Rv 5′-TAGGGCTCAGGTTTGTCCAG-3′
CuZn SOD	NM_017050.1	Fw 5′-GTGCAGGGCGTCATTCACTTC-3′ Rv 5′-YGCCTCTCTTCATCCGCTGGA-3′
Catalase	NM_012520.1	Fw 5’-CCTCAGAAACCCGATGTCCTG -3’ Rv 5’-GTCAAAGTGTGCCATCTCGTCG -3’
GPx	NM_030826.3	Fw 5′-TGAGAAGGCTCACCCGCTCT-3′ Rv 5′-GCACTGGAACACCGTCTGGA-3′
β-actin	NM_031144.3	Fw 5′- AGAGGGAAATCGTGCGTGAC-3′ Rv 5′-CGATAGTGATGACCTGACCGT-3′

Parameter	Control	HS	HS + L
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Glucose (mg/dl)	119.67±4.84	114.33±4.12	111.00±7.45
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Triglyceride (mg/dl)	96.51±4.85	157.84±4.96*	131.94±6.27^*
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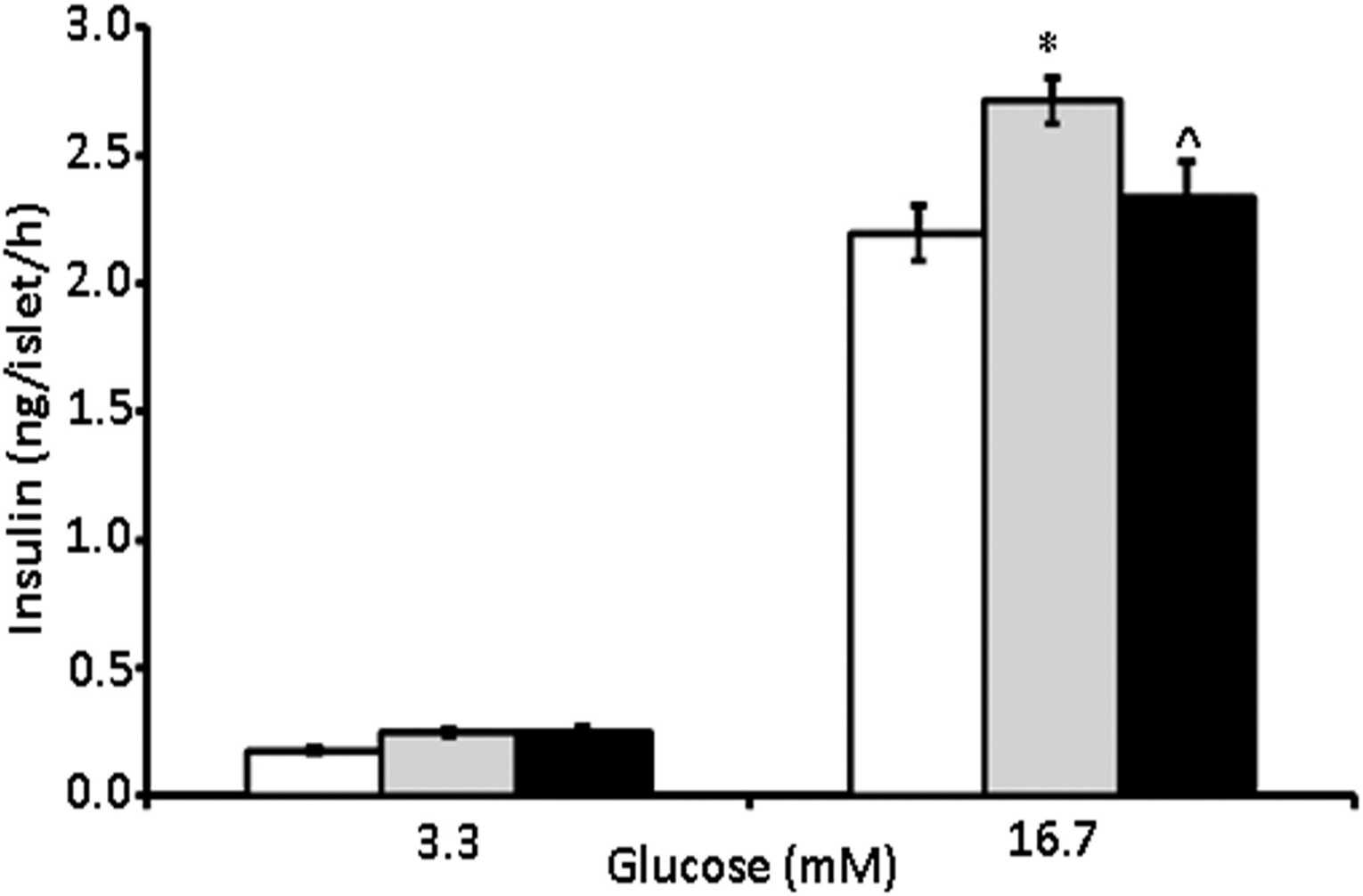
HOMA-β	48.18±5.40	68.60±6.38*	46.94±2.36^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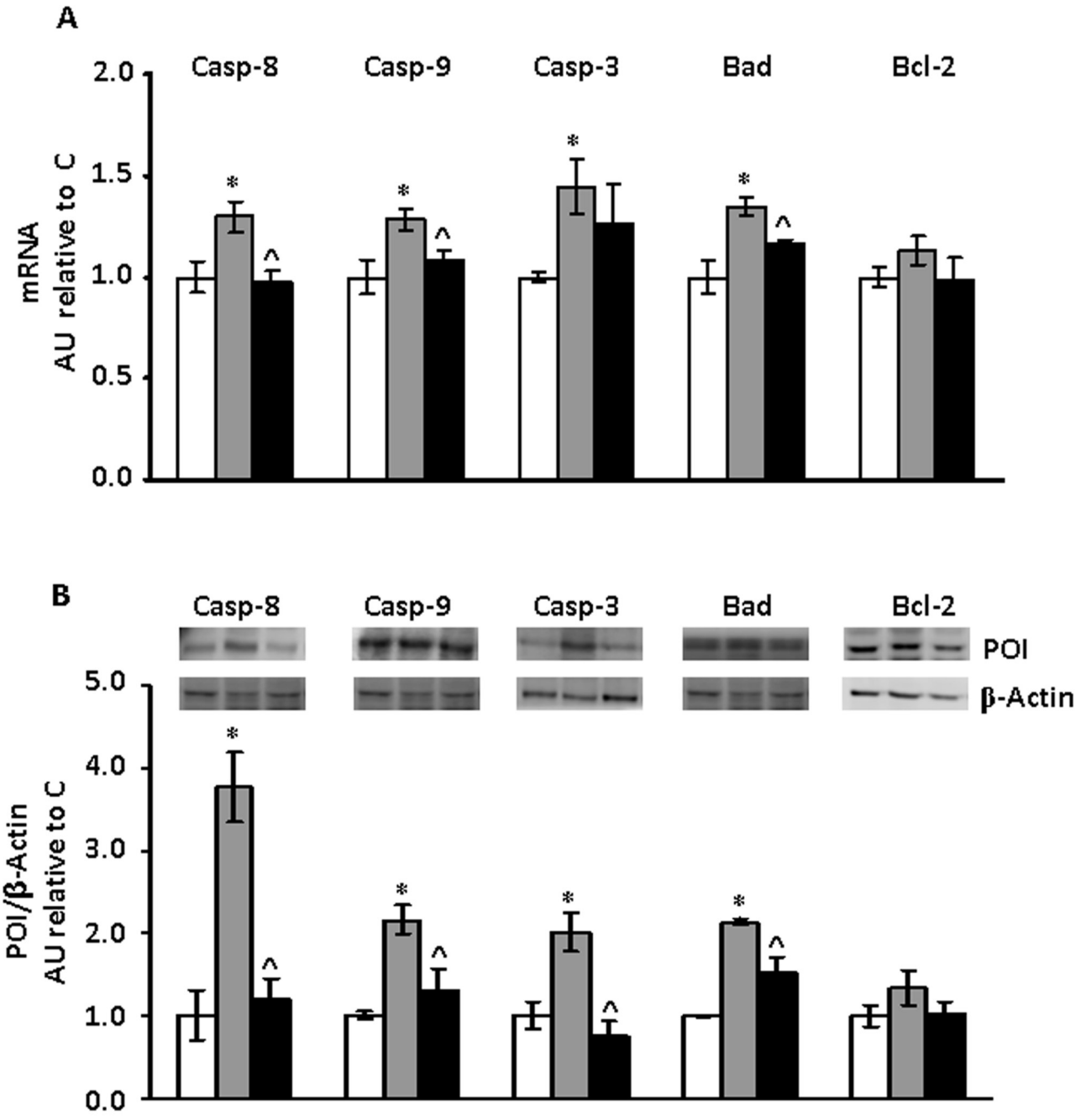
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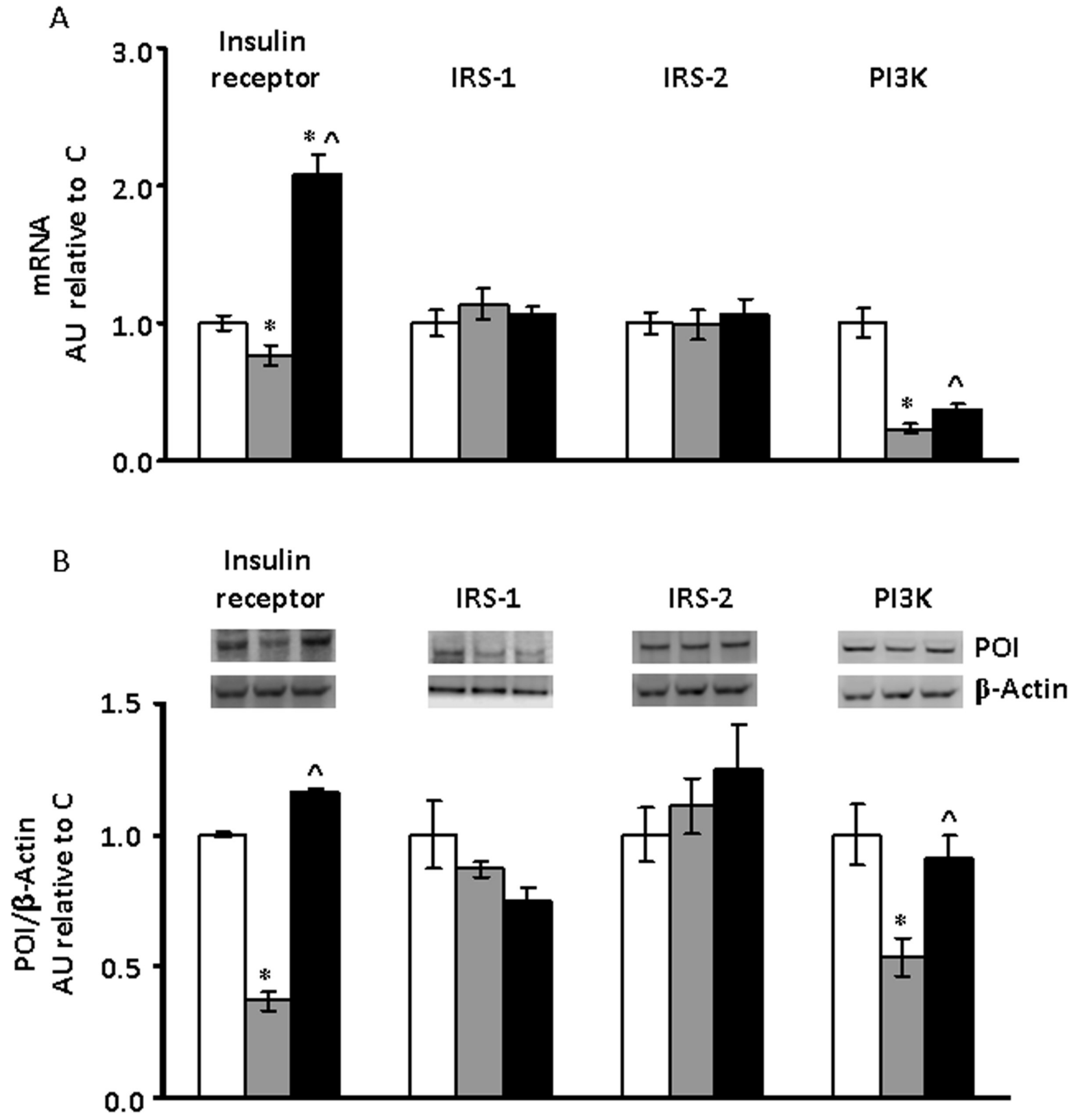
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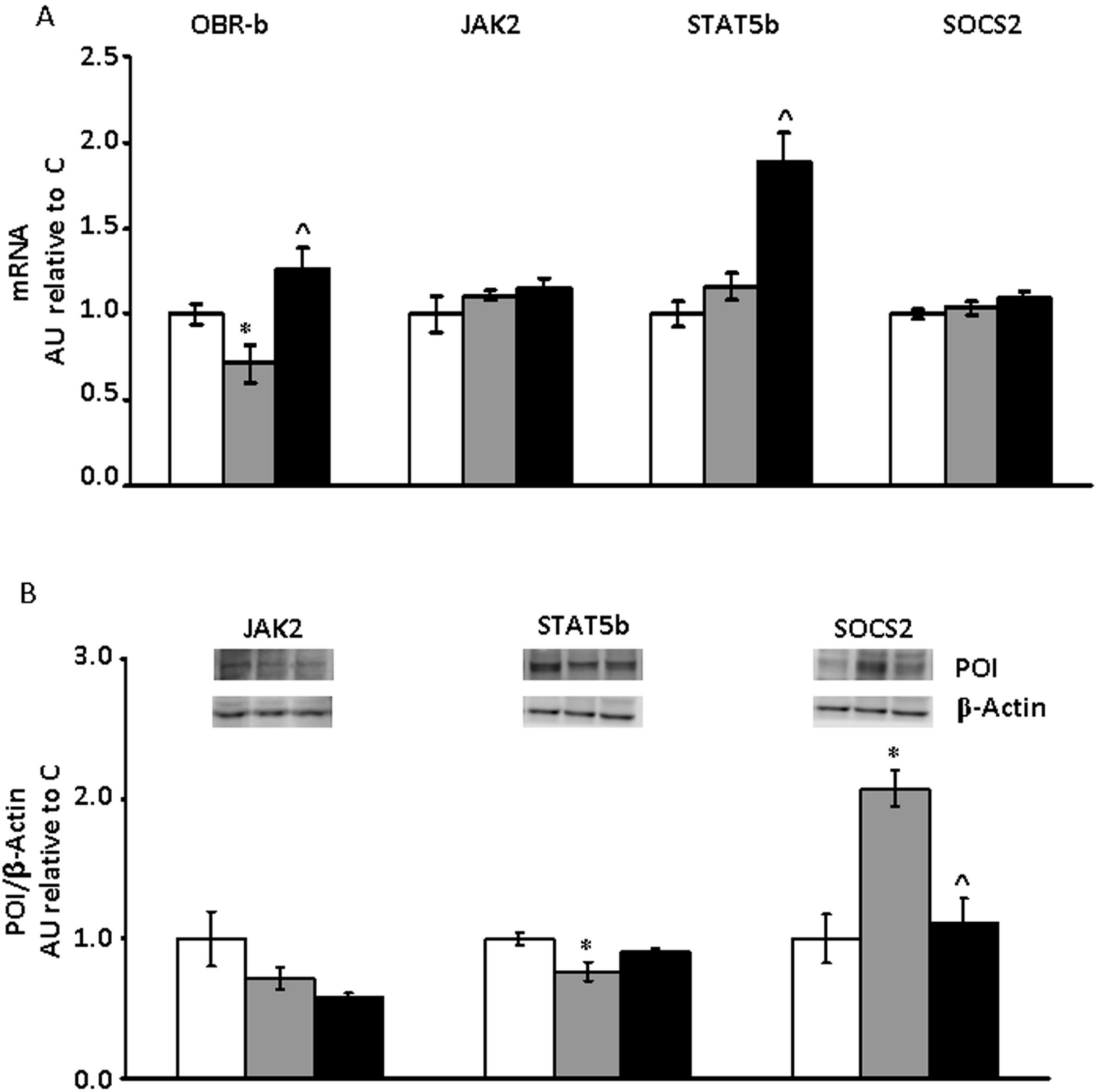
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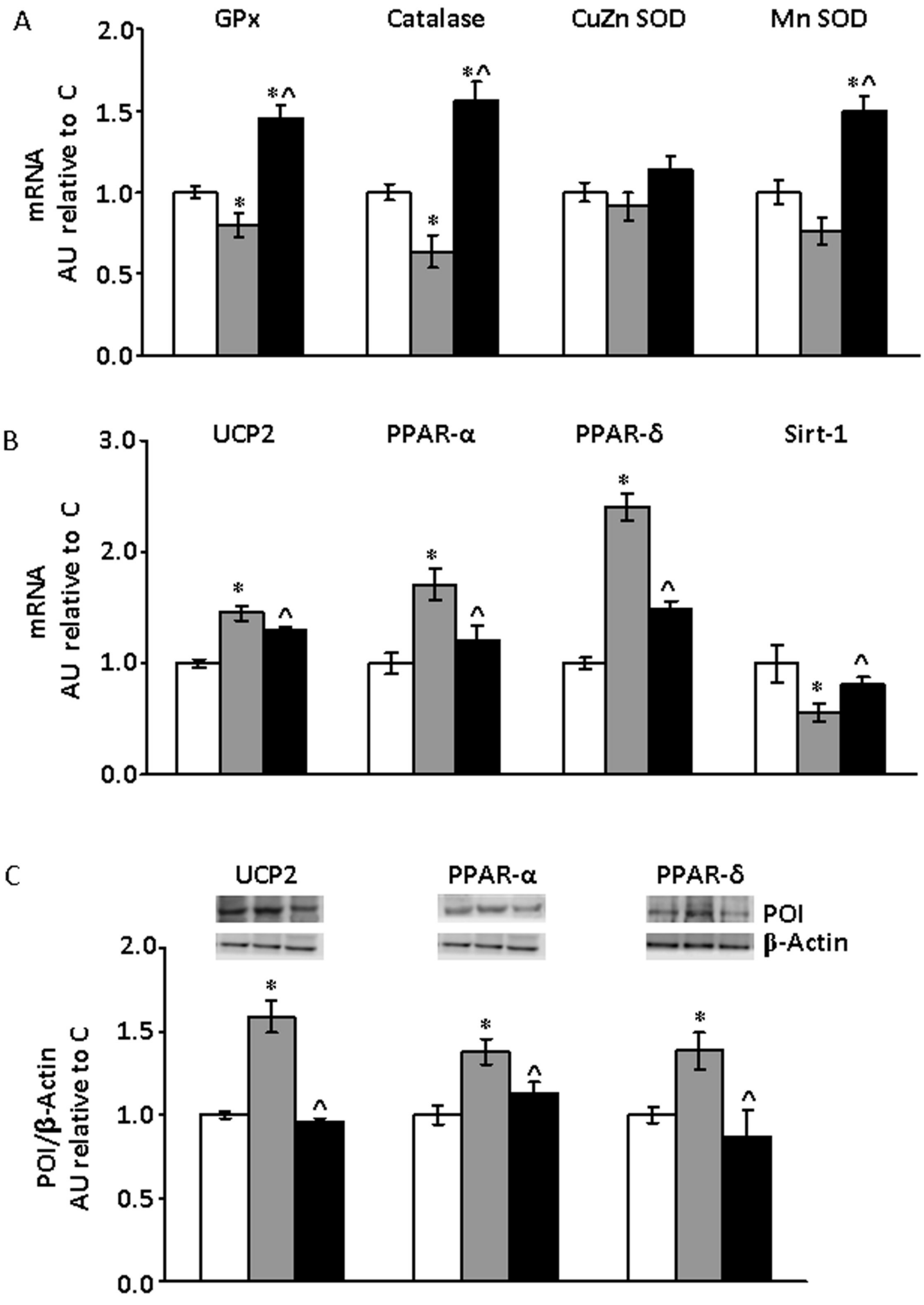
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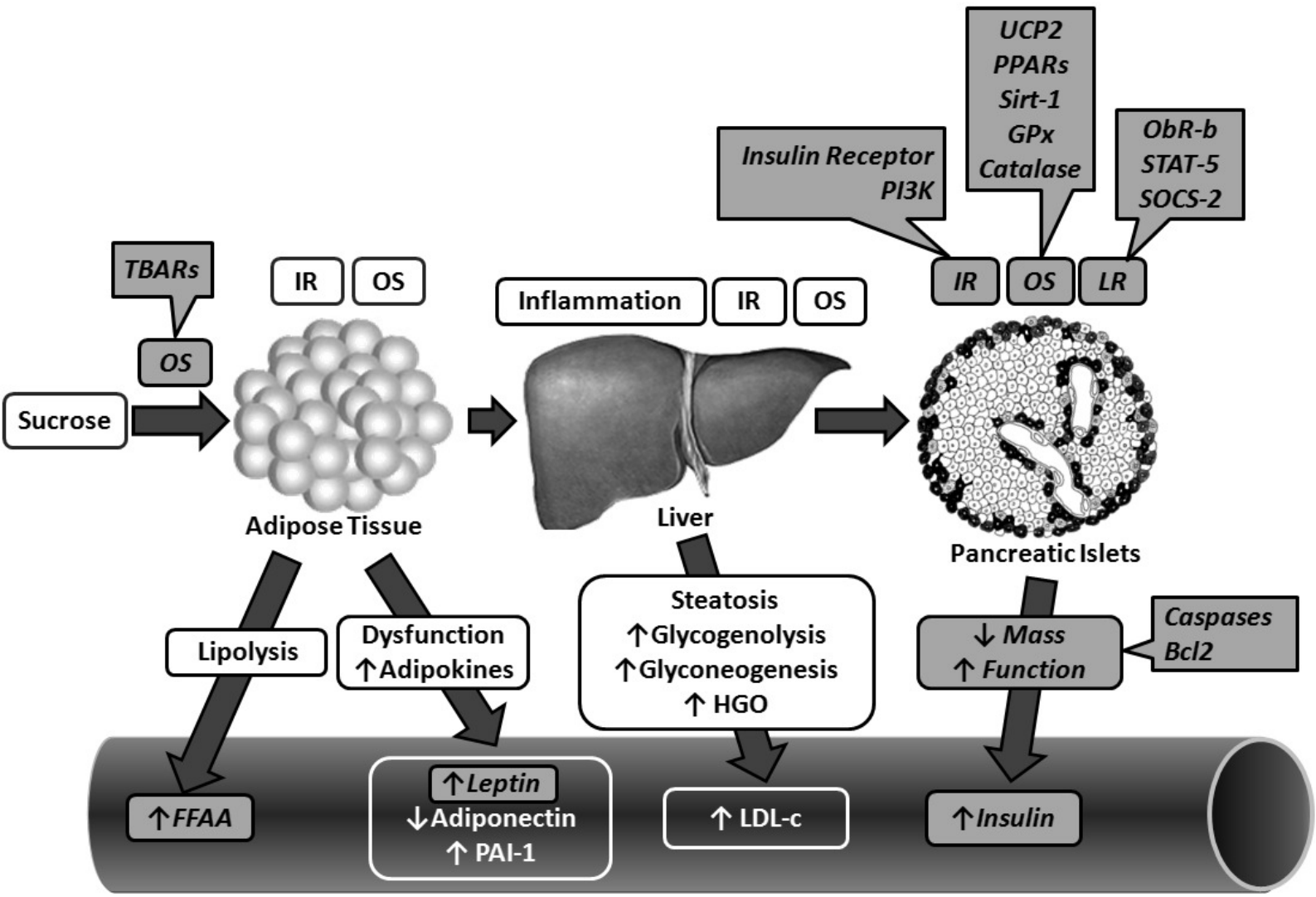
0.0


UCP2
PPARs
Sirt-1
GPx
Catalase
Insulin Receptor
PI3K
ObR-b
STAT-5
SOCS-2
TBARs
IR
OS
Inflammation
IR
OS
IR
OS
LR
OS
Sucrose
Adipose Tissue
Liver
Pancreatic Islets
Lipolysis
Dysfunction
↑Adipokines
Steatosis
↑Glycogenolysis
↑Glyconeogenesis
↑ HGO
↓ Mass
↑ Function
Caspases
Bcl2
↑FFAA
↑Leptin
↓Adiponectin
↑ PAI-1
↑ LDL-c
↑Insulin