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## **GHRELIN TREATMENT PROTECTS LACTOTROPHS FROM APOPTOSIS IN THE PITUITARY OF DIABETIC RATS**

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## **ABSTRACT**

Poorly controlled diabetes is associated with hormonal imbalances, including decreased prolactin production partially due to increased lactotroph apoptosis. In addition to its metabolic actions, ghrelin inhibits apoptosis in several cell types. Thus, we analyzed ghrelin's effects on diabetes-induced pituitary cell death and hormonal changes. Six weeks after onset of diabetes in male Wistar rats (streptozotocin 70 mg/kg), mini-pumps infusing saline or 24 nmol ghrelin/day were implanted (jugular). Rats were killed two weeks later. Ghrelin did not modify body weight or serum glucose, leptin or adiponectin, but increased total ghrelin ( $p<0.05$ ), IGF-I ( $p<0.01$ ) and prolactin ( $p<0.01$ ) levels. Ghrelin decreased cell death, iNOS and active caspase-8 ( $p<0.05$ ) and increased prolactin ( $p<0.05$ ), Bcl-2 ( $p<0.01$ ) and Hsp70 ( $p<0.05$ ) content in the pituitary. In conclusion, ghrelin prevents diabetes-induced death of lactotrophs, decreasing caspase-8 activation and iNOS content and increasing anti-apoptotic pathways such as pituitary Bcl-2 and Hsp70 and serum IGF-I concentrations.

## INTRODUCTION

In poorly controlled diabetic animals or humans increased cell death occurs in different tissues and organs (Arroba et al., 2003; Barber et al., 1998; Cai et al., 2002; García-Cáceres et al., 2008; Li et al., 2002; Pesce et al., 2002), with this cell loss being involved in many of the secondary complications of diabetes. In the anterior pituitary, lactotrophs appear to be more susceptible to diabetes induced cell death than other cell types (Arroba et al., 2003, 2005, 2006; Yamauchi and Shiino, 1986). We previously demonstrated that in poorly controlled diabetes caspase 8, the prototypical initiator caspase of the extrinsic cell death pathway (Boldin et al., 1996, Wajant, 2002), is activated and colocalizes with prolactin (PRL) producing cells (Arroba et al., 2005), possibly explaining their increased death rate. However, pituitary levels of proteins involved in the intrinsic cell death pathway, including members of the Bcl-2 family, X-linked inhibitor of apoptosis (XIAP), and the effector caspases 3 and 6, are either unchanged or balanced towards cell survival (Arroba et al., 2005), with these changes being cell-type specific (Arroba et al., 2007).

Increased apoptosis of lactotrophs may underlie, at least in part, the reduction in circulating PRL concentrations in poorly controlled diabetic patients or animals (Arroba et al., 2003; Boujon et al., 1995; Ikawa et al., 1992; Iranmanesh et al., 1990; Mera et al., 2007; Steger et al., 1989; Ostrom et al., 1993). This decrease in PRL could result in the reduced or delayed milk production observed in some diabetic women (Hartmann and Cregan, 2001; Neubauer et al. 1990, 1993; Neville et al., 1988) and streptozotocin-induced diabetic rats (Ikawa et al., 1992; Lau et al., 1993). In diabetic males, decreased prolactin secretion has also been associated with sexual dysfunction (Sudha et al., 1999).

Ghrelin has both endocrine and non-endocrine activities including regulation of food intake and energy homeostasis (Wren et al., 2001), induction of adiposity (Tschop et al., 2000), actions on insulin secretion and glucose metabolism (Egido et al., 2002; Dezaki et al., 2006; Reimer et al., 2003) and stimulation of pituitary hormones including GH, prolactin and ACTH (Kojima et al., 1999; Arvat et al., 2001; Cheng et al., 1993; Broglio et al., 2003; Tassone et al., 2003). In addition, anti-inflammatory and anti-apoptotic actions of ghrelin have been reported in different organs and tissues (Baldanzi et al., 2002; Dixit et al., 2004; Granado et al., 2005; Granata et al., 2006).

Ghrelin has also been shown to have beneficial effects in diseases associated with catabolism and body weight loss (DeBoer, 2008), including cancer, diabetes or sepsis (Kamiji and Inui, 2008). Indeed, ghrelin treatment could be beneficial in the treatment of diabetes as both acylated (AG) and unacylated ghrelin (UAG) increase glucose-induced  $\beta$ -cell insulin secretion and, stimulate proliferation and prevent apoptosis of pancreatic  $\beta$ -cells (Granata et al., 2006, 2007). However, how this hormone affects metabolic parameters, such as circulating IGF-I, leptin, insulin, adiponectin or ghrelin concentrations, as well as pituitary hormone concentrations and pituitary cell apoptosis, in diabetic rats has not yet been investigated. Hence, the aim of this study was to analyze the effect of ghrelin treatment on the metabolic state

of streptozotocin-induced diabetic rats, focusing on its possible beneficial effect on apoptosis in the pituitary and prolactin levels.

## **MATERIAL AND METHODS**

### **Animals and experimental design**

Adult male Wistar rats (250 g) were used. The procedures followed the guidelines recommended by the European Union for the care and use of laboratory animals, and the animal protocols were approved by the University Animal Care Committee. Rats were housed three or four per cage with free access to food and water, under constant conditions of temperature (20–22°C) and light (lights on from 07:30 to 19:30). Before diabetes induction rats were adapted for one week to the new environment and diet.

Rats were injected (i.p.) with 70 mg/kg streptozotocin (STZ; Sigma). Control rats received vehicle only. Blood glucose concentrations were measured the morning following STZ injection via tail puncture (Glucocard Memory 2; Menarini Diagnostic, Florence, Italy) and animals were considered to be diabetic if they had glucose levels superior to 300 mg/dl. Six weeks after diabetes onset a catheter was placed in the jugular vein and this was connected to a mini-pump (Alzet, Durect Co., Canada) that was placed subcutaneously. Control rats were infused with 12 µl/day of saline and diabetic rats were divided into two groups: One group received 12 µl/day of saline and the other 24 nmol/day of n-Octanoyl-ghrelin (ANASPEC, San Jose, CA). Eight weeks after diabetes induction all rats were killed by decapitation. Trunk blood was collected in cooled tubes, allowed to clot, and centrifuged. Serum was stored at -80°C until hormone levels were measured. Pituitary glands were removed, weighed and stored at -80° C until processed. Three to six rats per group were used for each analysis.

### **Pituitary homogenization and protein quantification**

Tissue was homogenized on ice in 200 µl of radioimmunoprecipitation assay lysis buffer with an EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). After homogenization, samples were centrifuged at 14,000 rpm for 20 min at 4°C. Supernatants were transferred to a new tube and protein concentration was estimated by Bradford protein assay.

### **ELISA cell death detection**

This photometric enzyme immunoassay for the *in vitro* quantification of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) was performed according to the instructions of the manufacturer (Roche Diagnostics). The same amount of total protein was loaded in all wells and each sample was measured in duplicate (Tecan Infinite M200, Grödig, Austria). The background value was subtracted from the mean value of each sample and all values are referred to the mean value of the control group.

### **Terminal dUTP nick-end labelling (TUNEL) plus immunohistochemistry**

Immunohistochemistry/TUNEL assays were performed on frozen 12 µm cryostat pituitary mounted on positively charged slides as previously

described (Arroba et al., 2003, 2005, 2006, 2007). The TUNEL assays were performed following the manufacturer's instructions (Roche). Briefly, after fixation in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), pituitary were washed three times in buffer and incubated for 30 min with a 0.1% sodium citrate, 0.1% Triton X-100 solution to increase tissue permeability. Slides were again washed three times with buffer, and incubated with TUNEL solution for 90 min at 37 °C in a humid chamber in the dark. After washing, the slides were incubated with antibody towards prolactin (1:2000), in TBS containing 3% BSA and 1% Triton X-100 and left for 48 h at 4°C. The slides were incubated with Alexa Fluor antfluorescein-488 and -633-conjugated goat anti-guinea pig IgG (Molecular Probes) in blocking buffer both at a dilution of 1:1000. Finally, the slides were again washed three times before mounting in glycerol. Results were visualized with a confocal microscope (Leica model DMIRB; Leica, Wetzlar, Germany).

### **Immunoblotting**

In each assay the same amount of protein was loaded in all wells (1–60 µg depending on the protein to be detected) and resolved by using 12% SDS-acrylamide gels. After electrophoresis proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) and transfer efficiency was determined by Ponceau red dyeing. Filters were then blocked with Tris-buffered saline (TBS) containing 5% (wt/vol) non-fat dried milk and incubated with the appropriate primary antibody (for details, see Table 1). Membranes were subsequently washed and incubated with the corresponding secondary antibody conjugated with peroxidase (1:2000; Pierce, Rockford, IL). Bound peroxidase activity was visualized by chemiluminescence and quantified by densitometry using a Kodak Gel Logic 1500 Image Analysis system and Molecular Imaging Software, version 4.0 (Rochester NY, USA). All blots were rehybridized with actin to normalize each sample for gel-loading variability. All data are normalized to control values on each gel.

### **Determinations of serum levels of total and acylated ghrelin, leptin, insulin, adiponectin and IGF-I**

Total and acylated ghrelin were measured by radioimmunoassay following the manufacturer's instructions (Linco Research, St Charles, MI, USA). The sensitivity of the method was 93 pg/ml for total ghrelin and 7.8 pg/ml for acylated ghrelin and the intra-assay variation was 6.4% for total ghrelin and 7.4% for acylated ghrelin.

Serum insulin, leptin and adiponectin levels were measured with commercial ELISA kits from Linco Research (St Charles, MI, USA) following the procedure indicated by the manufacturer. The sensitivity was 0.2 ng/ml for insulin and leptin, and 50 pg/ml for adiponectin. The intra- assay variation was 1.9% for insulin, 2.1% for leptin and 8.1% for adiponectin.

Serum IGF-I concentrations were measured by a double-antibody RIA as previously described (Granado et al., 2008).

All samples were run in duplicate and within the same assay for all analyses.

### **Determination of pituitary levels of ACTH, TSH, LH, GH and serum PRL by Multiplex bead array assays**

Pituitary levels of ACTH, TSH, LH, GH and PRL and serum PRL levels were measured by using a rat pituitary panel LINCoplex kit (Linco Research, St Charles, MI, USA) following the guidelines of the manufacturer. Briefly, pituitary lysates and serum samples (25  $\mu$ l) were incubated for 18 hours at 4°C with polystyrene beads labeled with a specific ratio of two different fluorescent dyes and conjugated with a capture antibody specific for the antigen to be detected. After incubation, wells were washed using a vacuum manifold and antibody conjugated to biotin (25  $\mu$ l) was added. After an incubation period of 30 minutes at RT, beads were incubated for 30 minutes with 50  $\mu$ l of streptavidin conjugated to phycoerythrin. Beads (a minimum of 100 beads per parameter) were analyzed in a Bio-Plex suspension array system 200 (Bio-Rad Laboratories, Hercules, CA, USA). Raw data (mean fluorescence intensity) were analyzed by using Bio-Plex Manager 4.1. software. For appropriate quantification, serum samples were diluted 1:3 in serum matrix and pituitary lysates were diluted 1:50000 in assay buffer for GH determination and 1:1000 for ACTH, TSH, LH and PRL determination.

### **Statistical analysis**

Statistics were performed using the statistics program GraphPad Prism 4.0. Data are presented as mean  $\pm$  SEM and differences among experimental groups were analyzed by one-way analysis of variance. Post-hoc comparisons were made using subsequent Bonferroni multiple range tests. The values were considered significantly different when the p value was lower than 0.05.

## **RESULTS**

### **Body weight gain, glycemia and serum insulin levels**

Body weight gain of control and diabetic rats from the time of minipump implantation until termination of the animals is shown in Table 2. There was no significant effect of ghrelin treatment on weight gain in diabetic rats.

Glycemia was increased in diabetic rats treated with saline or ghrelin compared to control rats ( $P < 0.001$ ), with ghrelin having no significant impact on blood glucose levels in diabetic rats. In addition, serum insulin levels were undetectable in all diabetic animals ( $P < 0.05$ ; Table 2).

### **Serum leptin, ghrelin, adiponectin and IGF-I levels.**

All diabetic animals had lower serum leptin ( $P < 0.001$ ) and adiponectin ( $P < 0.001$ ) concentrations than control rats, regardless of whether they had been treated with saline or ghrelin (Table 2). However, although acylated ghrelin did not differ between groups, ghrelin administration to diabetic rats resulted in significantly higher total ghrelin levels compared to control and diabetic rats administered saline ( $P < 0.001$ ; Table 2).

Serum IGF-I concentrations were decreased in diabetic rats infused with saline ( $P < 0.05$ ; Table 2), while serum IGF-I levels in diabetic rats treated with ghrelin were not significantly different from control values.

### **Pituitary hormones**

Table 3 shows ACTH, TSH, LH, GH and PRL levels in the pituitary of control and diabetic rats. Neither diabetes nor ghrelin treatment modified total ACTH or TSH content in the pituitary. Pituitary LH content was significantly increased in diabetic rats treated with saline ( $P < 0.05$ ), and ghrelin treatment had no effect. In contrast, total pituitary GH content was reduced in all diabetic rats, regardless if they had been infused with saline or ghrelin ( $P < 0.01$ ).

In accordance with our previous studies (Arroba et al., 2003), serum PRL levels (control:  $2415.6 \pm 370.9$ , diabetic + saline:  $440.0 \pm 114.2$ , diabetic + ghrelin  $1571.5 \pm 83.8$ , ANOVA  $p < 0.001$ ) and total pituitary PRL content were decreased in diabetic rats infused with saline ( $P < 0.05$ ). In this study we demonstrate that treatment of diabetic rats with ghrelin for two weeks increased both pituitary and serum PRL to levels that were not statistically different from control values. To verify these observations pituitary PRL levels were also measured by western blot, obtaining the same results (Figure 1).

### **Pituitary apoptosis markers**

In order to elucidate the mechanisms by which ghrelin is able to prevent the decreased PRL content in the pituitary of diabetic rats, cell death and different proteins related to apoptosis previously found to be altered in the pituitary of diabetic rats (Arroba et al., 2005) were measured in this tissue.

#### ***Total cell death in the anterior pituitary***

Total cell death in the pituitary, as determined by ELISA for histones, was increased in diabetic rats infused with saline, while treatment of diabetic rats with ghrelin returned these values to control levels ( $100 \pm 9.7$ ;  $2248 \pm 709$ ;  $146 \pm 9.4$ ). Immunohistochemistry in combination with TUNEL (Figure 2) indicated that his cell death corresponded primarily to lactotrophs as previously demonstrated (Arroba et al., 2007). Very few TUNEL labeled cells were found in pituitaries of control rats or diabetic rats treated with ghrelin.

#### ***Caspases 3, 6 and 8***

As previously reported (Arroba et al., 2005), the increase in pituitary cell death was associated with caspase-8 activation, as shown in Figure 3. Both the proform (55 kDa) and cleaved caspase-8 (42-44 kDa) were increased in the pituitaries of diabetic rats treated with saline, and ghrelin administration returned these parameters to control levels ( $P < 0.001$  and  $P < 0.05$ , respectively).

Pituitary caspase-3 content was not modified by diabetes, and ghrelin treatment had no effect on this parameter (Figure 4A & B). On the contrary, procaspase 6 levels were up-regulated in diabetic rats treated with saline, but not in diabetic animals treated with ghrelin ( $P < 0.001$ ; Figure 4C), whereas the fragmented form of this caspase did not differ between experimental groups (Figure 4D).

#### ***XIAP***

There was no difference between experimental groups in the intact form of XIAP (Figure 5A). However, the fragmented form of the anti-apoptotic protein XIAP was up-regulated in the pituitaries of diabetic animals treated

with saline (Figure 5B,  $P < 0.01$ ), and treatment of diabetic rats with ghrelin returned the pituitary content of this protein to control values.

#### ***Heat shock protein (Hsp) 70 and Bcl-2***

The anti-apoptotic proteins Hsp 70 and Bcl-2 were unchanged in diabetic rats treated with saline (Figure 6A & B, respectively). However, treatment of diabetic rats with ghrelin resulted in a significant increase in the pituitary content of these proteins ( $P < 0.05$  and  $P < 0.01$ , respectively).

#### ***Inducible nitric oxide synthase (iNOS)***

Streptozotocin-induced diabetes increased inducible nitric oxide synthase (iNOS) content in the pituitary ( $P < 0.05$ ) and ghrelin treatment blocked this effect (Figure 7).

## **DISCUSSION**

In this study we have investigated the effects of ghrelin treatment on different metabolic parameters and pituitary hormone levels in streptozotocin-induced-diabetes, focusing on ghrelin's protective effect in diabetes-induced apoptosis of lactotrophs. To our understanding, this is the first study showing the beneficial effect of ghrelin treatment on pituitary cell apoptosis in an experimental model of diabetes mellitus.

Ghrelin modulates insulin secretion and glucose metabolism (Reimer et al., 2003), augmenting glycemia by decreasing glucose-induced insulin release from pancreatic islets (Dezaki et al., 2006). However, we found no effect of ghrelin treatment on glycemia or serum insulin levels in diabetic rats. This suggests that not only was ghrelin unable to stimulate insulin secretion, most likely due to the complete destruction of  $\beta$ -pancreatic cells by the streptozotocin, but it was also unable to stimulate  $\beta$ -cell proliferation in this experimental model, possibly due to the destruction of precursor cells.

Ghrelin and GH-secretagogues are reported to increase body weight gain both in humans and animals (DeBoer, 2008). In our experimental model, diabetic rats treated with ghrelin did not gain significantly more weight than diabetic rats administered saline. In agreement with our data, although a marked increase in body weight gain is reported in control rats treated with ghrelin (Dembinski et al., 2005; Nakazato et al., 2001; Strassburg et al., 2008), this effect has not always been found in animals suffering from catabolic diseases such as cancer, colitis or diabetes (de Smet et al., 2009; Irako et al., 2006). Thus, the lack of significant weight gain in response to ghrelin could be due to the state of catabolism and extreme weight loss of the diabetic rats. It should also be noted that although circulating levels of total ghrelin increased significantly with the infusion of ghrelin, acylated ghrelin levels did not. Hence, the lack of effect on weight gain could be the result of no change in acylated ghrelin levels. Whether this lack of increase in acylated ghrelin levels is due to the instability of the compound in the minipump, or to active deacylation in the blood remains to be determined. However, it is clear that the results reported in these studies may be due mainly to an increase in circulating unacylated ghrelin levels.

As previously described, all diabetic rats had lower serum leptin levels than control rats as a result of hyperglycemia and their extreme catabolic state

(Havel et al., 1998; Sivitz et al., 1998). Ghrelin treatment had no effect on this parameter, coincident with no significant effect on body weight. Similarly, serum adiponectin concentrations were also reduced in all diabetic rats, regardless if they had been treated with saline or ghrelin. However, ghrelin-treated rats had higher serum IGF-I and total ghrelin concentrations than diabetic rats infused with saline, suggesting that these rats may be in a better metabolic state than those without treatment (Froesch and Hussain, 1994). In agreement with our data, ghrelin has been previously reported to increase serum IGF-I levels (Dembinski et al., 2005). However, the increase in serum IGF-I levels was not associated with an increase in pituitary GH levels, even though ghrelin was originally reported to stimulate both GH production and secretion (Kojima et al., 1999). No change in pituitary GH content does not necessarily indicate that production and secretion are not modified, as both could be increased resulting in unchanged static content. However, it has been reported that whereas intermittent ghrelin treatment increases GH secretion, chronic ghrelin administration does not (Thompson et al., 2003). The stimulatory action of ghrelin on serum IGF-I could be a direct effect of this hormone in the liver, as GHRP-2, another ghrelin analogue, directly increases IGF-I mRNA in cultured hepatic cells (Granado et al., 2008). Although it has been reported that the liver does not express GHSR1a (Gnanapavan et al., 2002), others have shown that hepatic non-parenchyma cells, but not hepatocytes, do express this receptor at a low level (Granado et al., 2008). Furthermore, ghrelin could be acting through a GHSR1a-independent mechanism to stimulate IGF-I production (Filigheddu et al., 2007, Granata et al., 2007).

Apart from GH inhibition, poorly controlled diabetes is also associated with alterations in the concentrations of other pituitary hormones (Valimaki et al., 1991). However, we previously reported that no changes in cell turnover of thyrotrophs, corticotrophs or gonadotrophs occur in poorly controlled diabetic rats (Arroba et al., 2006; Arroba et al., 2007), suggesting that changes in the synthesis and secretion by individual cells could explain the observed changes in circulating hormones. In our study pituitary TSH and ACTH content was not modified by either diabetes or ghrelin treatment to diabetic rats. As diabetes significantly decreases circulating ACTH (Revsin et al., 2008) and TSH levels (Chamras and Hershman, 1990), no effect on pituitary hormone content, suggests a down regulation of both synthesis and secretion. As circulating LH levels have been previously reported to decrease in streptozotocin-induced diabetic rats (Dong et al., 1991), an increase in pituitary content suggests a decrease in secretion. Indeed, recent indicates that in this experimental model the anterior pituitary conserves its capacity to secrete LH in response to physiological stimulation (Castellano et al., 2009).

Prolactin levels in both serum and pituitary were significantly decreased in diabetic rats and increased in response to ghrelin. Indeed, ghrelin has been reported to stimulate PRL synthesis and secretion both in animals (Kaiya et al., 2003; Riley et al., 2002) and in humans (Arvat et al., 2001; Baldelli et al., 2004; Broglio et al., 2003; Rubinfeld et al., 2004). It is also possible that ghrelin inhibits the increase in lactotroph cell death seen in poorly controlled diabetes. Indeed, ghrelin treatment decreases cell death and activation of caspase 8 in the anterior pituitary. Lactotrophs are the pituitary cell type most affected by diabetes-induced apoptosis (Arroba et al., 2003) and this involves

caspase-8 activation (Arroba et al., 2005), and this in conjunction with increased pituitary PRL content, suggests that ghrelin may decrease lactotroph cell loss in diabetic animals. As previously described, no activation of the intrinsic cell death pathway in the pituitary, only an increase in procaspase-6 levels, was found (Arroba et al., 2005). Although ghrelin had no effect on the activation of caspase 6 or 3, it inhibited the rise in the proform of caspase 6, suggesting that this hormone reverted the various processes previously described to occur in both the intrinsic and extrinsic cell death pathways.

Although the precise mechanism of action cannot be deduced from these studies, ghrelin's anti-apoptotic effects could be mediated, at least in part, by the increase in serum IGF-I levels. Both circulating and locally produced IGF-I have cell-proliferative and anti-apoptotic properties (Kooijman, 2006; Kurmasheva and Houghton, 2006) by acting directly through its receptor, which is present in most organs and tissues (Werner and LeRoith, 2000), or indirectly by inhibiting inflammatory mediators such as TNF- $\alpha$  or nitric oxide (Hijikawa et al., 2008; Hill et al., 1999). Accordingly, it has been recently reported that IGF-I treatment prevents liver injury through the inhibition of TNF- $\alpha$  and iNOS induction in D-galactosamine and LPS-treated rats (Hijikawa et al., 2008) and that GHRP-2 treatment, a ghrelin analogue, prevents LPS-induced liver injury by increasing hepatic IGF-I and decreasing nitric oxide concentrations and TNF- $\alpha$  gene expression (Granado et al., 2008).

The pituitary content of iNOS was upregulated in diabetic rats infused with saline, but not in ghrelin treated animals, suggesting a ghrelin-induced decrease in the generation of free radicals. Similarly, ghrelin has been reported to decrease nitric oxide production by peritoneal macrophages (Granado et al., 2005) and to decrease high glucose-induced apoptosis of endothelial cells and apoptosis induced by oxygen-glucose deprivation in hypothalamic neuronal cells through reactive oxygen species inhibition (Chung et al., 2007; Zhao et al., 2007). Moreover, it has recently been reported that UCP2 mediates ghrelin's orexigenic effect on NPY/AgRP neurons by lowering free radicals (Andrews et al., 2008). The diabetes-induced increase of iNOS could be due to the increase in TNF- $\alpha$  pituitary as TNF- $\alpha$  up-regulates iNOS gene expression in anterior pituitary cells (Candolfi et al., 2004) and TNF- $\alpha$  is increased in the pituitary of diabetic rats (Arroba et al., 2006). Moreover, nitric oxide (NO) mediates the inhibitory effect of TNF- $\alpha$  on PRL release (Candolfi et al., 2004) and has been reported to induce apoptosis through caspase-8 activation (Du et al., 2006); thus, iNOS inhibition could possibly be one of the mechanisms of decreased-apoptosis in ghrelin-treated rats.

Levels of the anti-apoptotic proteins Bcl-2 and Hsp 70 were higher in the pituitaries of diabetic animals without treatment than in control animals, suggesting that there is a balance between apoptosis activation and apoptosis inhibition. Ghrelin further increased the levels of these proteins in diabetic rats, suggesting that it may decrease apoptosis due to both inhibition of pro-apoptotic and activation of anti-apoptotic pathways in the pituitary. In agreement with our data, ghrelin promotes cell survival by increasing Bcl-2 and Hsp 70 levels in hypothalamic and cortical neurons in an experimental model of focal ischemia/reperfusion (Chung et al., 2007; Miao et al., 2007; Yang et al., 2007).

The fragmented form of XIAP, another anti-apoptotic protein that decreases caspase activation, was up-regulated in the pituitaries of diabetic rats treated with saline, as previously reported (Arroba et al., 2005), but not in ghrelin-treated diabetic rats. Although XIAP is increased in the anterior pituitary of diabetic rats, this protein is expressed in less than 1% of lactotrophs and gonadotrophs, and in a higher proportion in somatotrophs (50%), corticotrophs (90%) and thyrotrophs (90%) (Arroba et al., 2007). This indicates that XIAP protects pituitary cell populations other than lactotrophs. The observation that XIAP returned to control levels when cell death was no longer induced, suggests that the increase in this apoptosis inhibitor may be in response to the increased stress of the neighboring lactotrophs.

The fact that only half of the somatotrophs were found to express XIAP suggests that these cells may be protected from cell death by another mechanism, as only a small number of apoptotic somatotrophs were found (Arroba et al., 2005). Whether the apoptotic GH-expressing cells also express PRL remains to be determined. Indeed, pituitary somatomammotrophs express both GH and PRL, and early lactotrophs are reported to also produce GH, suggesting that most lactotrophs arise from GH producing cells (Hoeffler et al., 1985), although this dogma has recently been challenged (Luque et al., 2007). It has also been suggested that in the adult animal these cell types may inter-change their hormonal identity depending upon their surrounding environment (Porter et al., 1990). However, in response to diabetes this does not appear to be the case as the number of PRL producing cells decreases with no corresponding increase in GH producing cells (Arroba et al., 2003). Pituitary cell proliferation is also increased in response to poorly controlled diabetes, with lactotrophs again being the most affected cell type (Arroba et al., 2006). This increase in proliferation temporally follows the increase in cell death, suggesting that it may be in response to the increased death. Indeed, in the ghrelin treated diabetic rats where pituitary cell death was decreased, markers of proliferation were also normalized (data not shown).

In conclusion, ghrelin treatment reduces diabetes-induced cell death in the anterior pituitary decreasing pro-apoptotic pathways such as caspase-8 activation and iNOS pituitary content and increasing anti-apoptotic factors such as serum IGF-I concentrations and pituitary Bcl-2 and Hsp70 levels. Furthermore, these anti-apoptotic effects of ghrelin could be involved in the observed normalization of prolactin levels through the reduction in lactotroph cell turnover.

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## FIGURE LEGENDS

### Figure 1. Pituitary prolactin levels

Total pituitary prolactin content in diabetic rats administered saline was significantly lower than in control rats ( $P < 0.01$ ). However, prolactin content in the pituitary of diabetic rats treated with ghrelin was not statistically different from control rats. Data are represented as mean  $\pm$  SEM and referred to 100% of control values ( $n = 3-6$  rats).

### Figure 2. Terminal dUTP nick-end labelling (TUNEL) plus immunohistochemistry for prolactin

Representative TUNEL labeling (green) is shown in the pituitary of a control (A), diabetic rat (B) and diabetic rat treated with ghrelin (C). The majority of TUNEL labeled cells (green) colocalized in cells that were positive for prolactin (red) immunostaining. Double labeling for prolactin (D) and TUNEL labeling (E) is shown to colocalize (F) in the pituitary of a diabetic rat. The scale bar in A-C represents 250  $\mu\text{m}$  and in D-F 50  $\mu\text{m}$  ( $n = 3$  rats).

### Figure 3. Pituitary caspase-8 levels

(A) The proform of caspase-8 (44kDa) was increased in diabetic rats treated with saline and ghrelin treatment decreased this effect ( $P < 0.001$ ). (B) The active form of caspase-8 (25kDa) was also significantly up-regulated in diabetic rats administered saline ( $P < 0.05$ ), whereas there were no statistical differences between control and diabetic rats treated with ghrelin. Data are represented as mean  $\pm$  SEM and referred to 100% of control values ( $n = 3-6$  rats).

### Figure 4. Pituitary caspase-3 and caspase-6 levels

There was no significant difference between groups in levels of the proform of caspase-3 (32kDa) (A) or in the active fragmented form of caspase-3 (17kDa) (B). The proform of caspase-6 was increased in diabetic rats administered saline ( $P < 0.001$ ) and ghrelin treatment blunted this up-regulation (C). There was no significant difference between groups in fragmented caspase-6 (30kDa) (D). Data are represented as mean  $\pm$  SEM and referred to 100% of control values ( $n = 3-6$  rats).

### Figure 5. Pituitary content of X-linked inducer of apoptosis (XIAP)

(A) There were no statistical differences between groups in the 57kDa form of XIAP. (B) Ghrelin treatment prevented the diabetes-induced increase in the 30kDa fragment of XIAP ( $P < 0.05$ ). Data are represented as mean  $\pm$  SEM and referred to 100% of control values ( $n = 3-6$  rats).

### Figure 6. Pituitary Hsp70 and Bcl-2 levels

Pituitary Hsp 70 (A) and Bcl-2 (B) content were increased in diabetic rats, but this rise was only significant in those diabetic rats treated with ghrelin ( $P < 0.05$  and  $P < 0.01$ , respectively). Data are represented as mean  $\pm$  SEM and referred to 100% of control values ( $n = 3-6$  rats).

**Figure 7. Pituitary iNOS content**

There was an up-regulation of pituitary iNOS content in diabetic rats infused with saline ( $P < 0.05$ ), whereas pituitary iNOS levels of diabetic rats treated with ghrelin were not significantly different from those of control rats. Data are represented as mean  $\pm$  SEM and referred to 100% of control values ( $n = 3-6$  rats).

**Table 1. Antibodies used in Western blot analyses****Table 2. Body weight gain, glycemia and serum insulin, leptin, adiponectin acylated ghrelin, total ghrelin and IGF-I levels in control and diabetic rats**

There were no significant differences in body weight gain between groups. Glycemia was increased in all diabetic rats ( $P < 0.001$ ) and serum leptin, insulin and adiponectin levels were decreased in diabetic rats regardless if they had been infused with saline or ghrelin ( $P < 0.001$ ,  $P < 0.05$  and  $P < 0.001$ , respectively). There were no significant differences in serum acylated ghrelin levels, whereas ghrelin treated rats had significantly higher total ghrelin serum concentrations ( $P < 0.001$ ). Serum IGF-I levels were decreased in diabetic rats treated with saline ( $P < 0.001$ ) and ghrelin treatment returned them to control levels ( $P < 0.05$ ) ( $n = 5-7$  rats).

**Table 3. Pituitary ACTH, TSH, LH, GH and PRL levels**

There were no significant differences in pituitary ACTH or TSH content between groups. On the contrary, diabetes increased pituitary LH ( $P < 0.05$ ) and decreased GH content ( $P < 0.01$ ). Ghrelin treatment had no effect on LH or GH levels. Pituitary prolactin levels were decreased in diabetic rats infused with saline ( $P < 0.01$ ), whereas prolactin levels in diabetic rats treated with ghrelin were not different from control rats ( $n = 5-7$  rats).

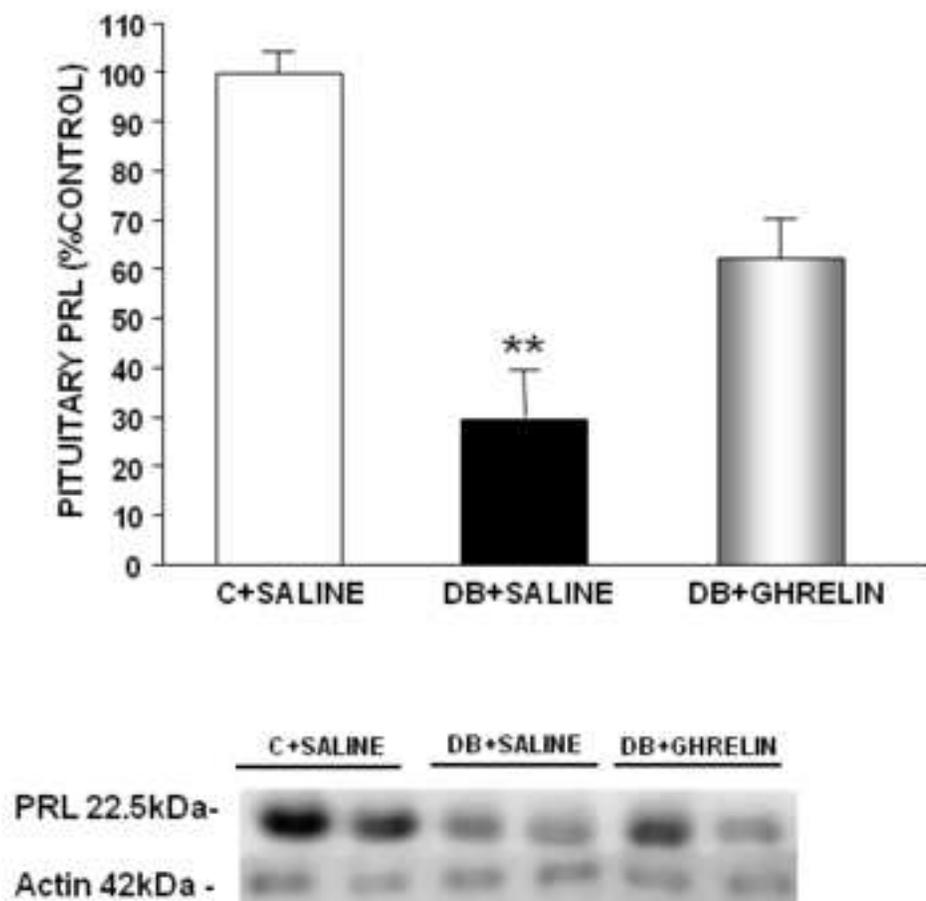


Figure 1

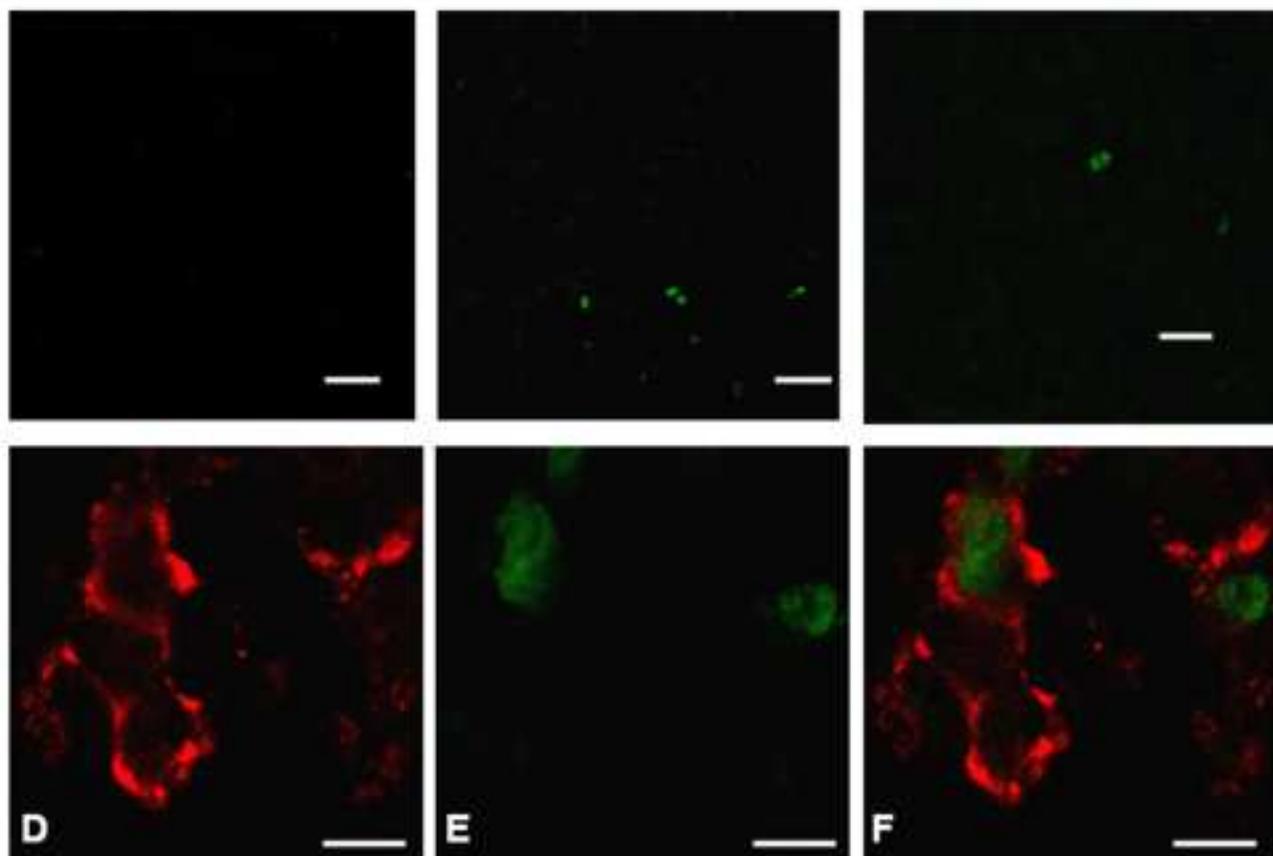


Figure 2

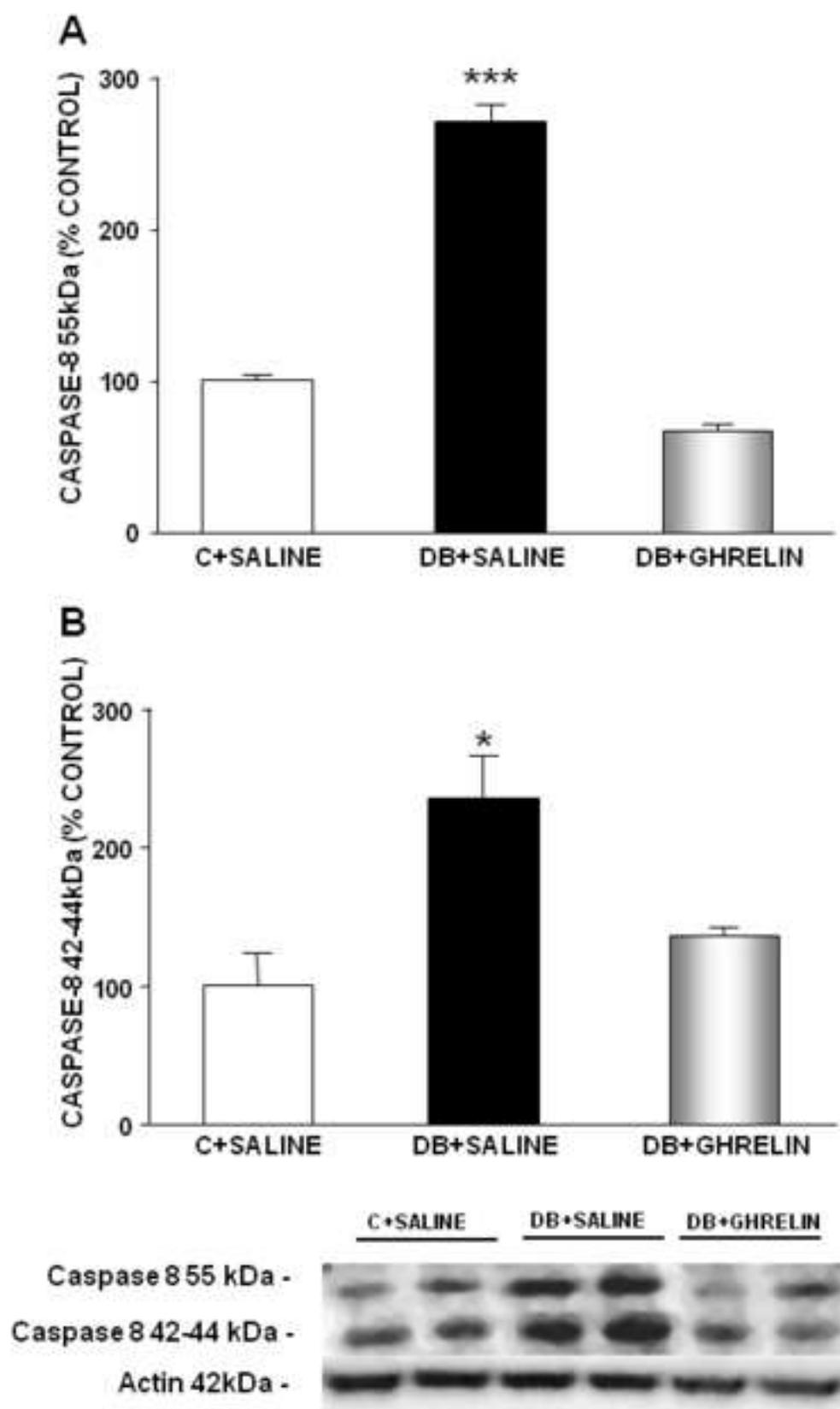


Figure 3

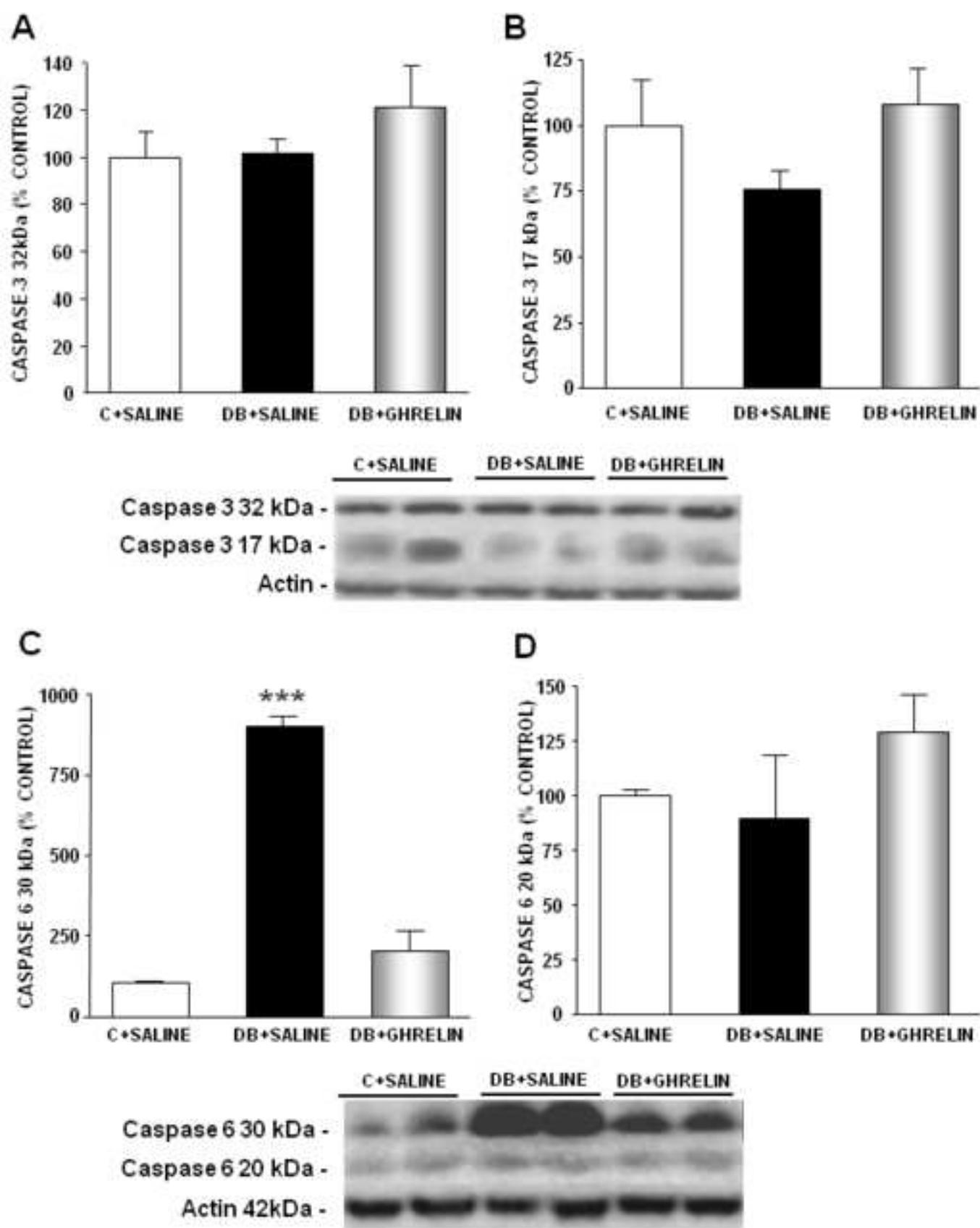


Figure 4

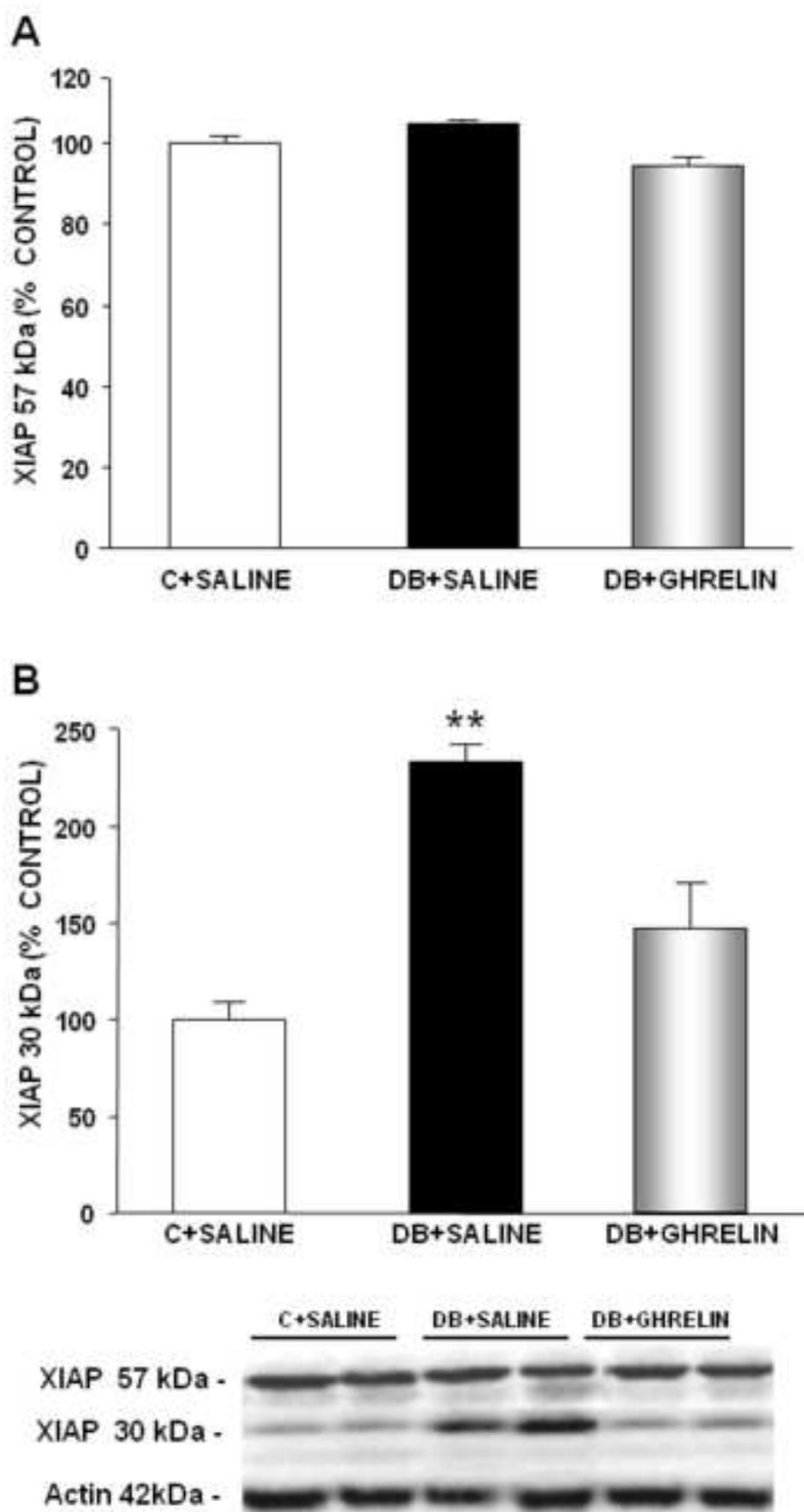


Figure 5

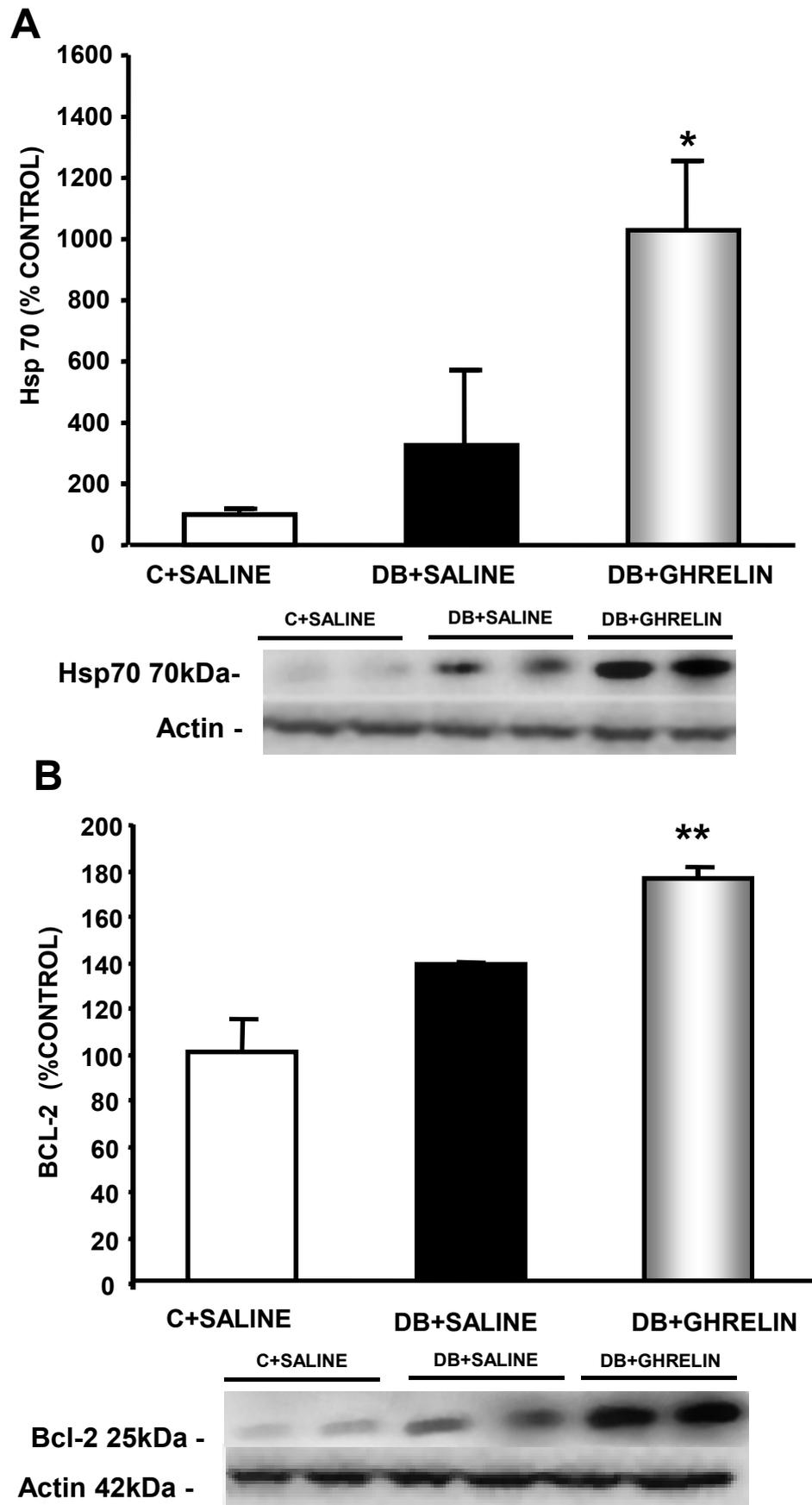


Figure 6

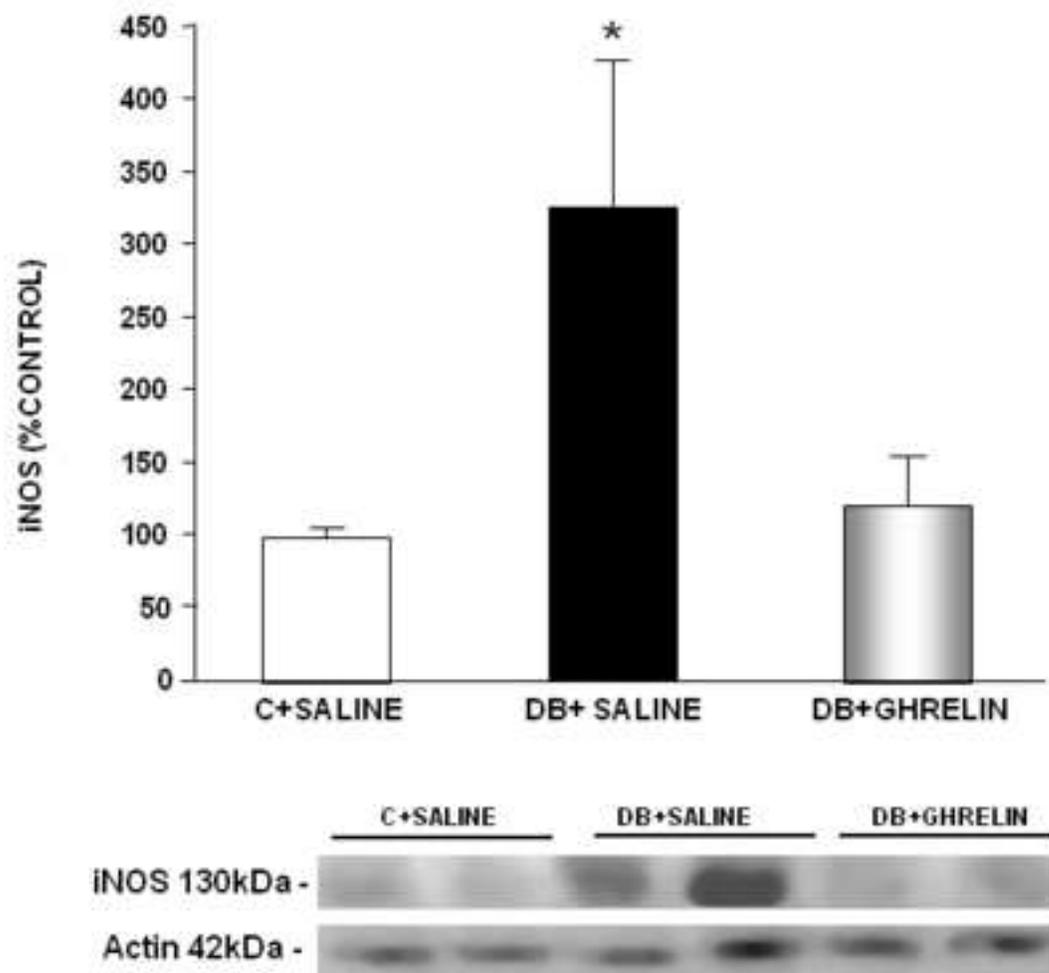


Figure 7

**Table 1. Antibodies used for Western Blot**

<b>ANTIBODY</b>	<b>SOURCE</b>	<b>WESTERN BLOT CONCENTRATION</b>
Prolactin	National Hormone and Peptide Program (Torrance, CA)	1:10000
Caspase 8	Neomarkers (Fremont, CA)	1:1000
XIAP	Becton-Dickinson Biosciences	1:1000
Caspase 3	Becton-Dickinson Biosciences (Franklin Lakes, NJ)	1:1000
Cleaved caspase 3	Cell Signaling Technology (Beverly, MA)	1:1000
Caspase 6	Medical Biological Laboratories	1:1000
iNOS	BD Biosciences	1:500
Hsp70	Stressgen Bioreagents (Ann Arbor, MI, USA)	1:1000
Actin	Santa Cruz Biotechnology	1:1000

**Table 2. Body weight gain, glycemia, and serum insulin, leptin, adiponectin, acylated ghrelin, total ghrelin and IGF-I levels. (\* P<0.05 vs CONTROL+SALINE, \*\*\* P<0.001 vs CONTROL+SALINE).**

	<b>CONTROL+SALINE</b>	<b>DIABETES+SALINE</b>	<b>DIABETES+GHRELIN</b>
Body weight gain (g)	6.62 ± 3.2	-4.2 ± 6.2	2.8 ± 7.4
Glycemia (mg/dl)	84.6 ± 1.7	600 ± 0.0 ***	550.4 ± 24.7 ***
Insulin (ng/ml)	1.48 ± 0.54	Undetectable *	Undetectable *
Leptin (ng/ml)	11.13 ± 2.09	0.26 ± 0.16 ***	0.18 ± 0.08 ***
Adiponectin (ng/ml)	31 ± 2	5 ± 0.9 *	5.9 ± 0.3 *
Acylated ghrelin (pg/ml)	461.6 ± 167.9	505.0 ± 144.6	829.3 ± 331.2
Total ghrelin (pg/ml)	1840.75 ± 839.39	1776.47 ± 380.24	13470 ± 1530 ***
IGF-I (ng/ml)	1309 ± 154	428 ± 137 ***	895 ± 37

Table 3. Pituitary content of ACTH, TSH, LH, GH and PRL (ng/ $\mu$ g protein). (\* P<0.05 vs CONTROL+SALINE, \*\* P<0.01 vs CONTROL+SALINE).

	CONTROL+SALINE	DIABETES+SALINE	DIABETES+GHRELIN
ACTH	2.97 $\pm$ 0.42	2.52 $\pm$ 0.59	2.96 $\pm$ 1.2
TSH	207.5 $\pm$ 34.5	181.5 $\pm$ 24.2	156.3 $\pm$ 25.5
LH	147.7 $\pm$ 6.03	354.5 $\pm$ 29.8 *	324.1 $\pm$ 38.0 *
GH	4836.2 $\pm$ 309.4	2131.1 $\pm$ 686.3 **	2848.3 $\pm$ 137.5 **
PRL	287.2 $\pm$ 41.6	115.6 $\pm$ 45.5*	274.2 $\pm$ 65.6