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Autophagy-mediated Insulin Receptor Down-regulation Contributes to ER Stress-induced Insulin Resistance

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Running title: Down-regulation of insulin receptor by ER stress

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Abbreviations: HFD, high fat diet; TG, thapsigargin; TUDCA, tauroursodeoxycholic acid; 3-MA, 3-methyladenine (3-MA); CHOP, C/EBP homologous protein; WAT, white adipose tissue; UPR, unfolded protein response; IRE1, inositol requiring ER-to-nucleus signal kinase-1; JNK, c-Jun N-terminal protein kinase.

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Abstract

Endoplasmic reticulum (ER) stress is associated with obesity-induced insulin resistance, yet the underlying mechanisms remain to be fully elucidated. Here we show that ER stress-induced insulin receptor (IR) down-regulation may play a critical role in obesity-induced insulin resistance. The expression levels of IR are negatively associated with the ER stress marker CHOP in insulin target tissues of *db/db* and high fat diet-fed mice. Significant IR down-regulation was also observed in fat tissue of obese human subjects and in 3T3-L1 adipocytes treated with ER stress inducers. ER stress had little effect on IR tyrosine phosphorylation *per se* but greatly reduced IR downstream signaling. The ER stress-induced reduction in IR cellular levels was greatly alleviated by the autophagy inhibitor 3-methyladenine but not by the proteasome inhibitor MG132. Inhibition of autophagy prevented IR degradation but did not rescue IR downstream signaling, consistent with an adaptive role of autophagy in response to ER stress-induced insulin resistance. Finally, chemical chaperone treatment protects cells from ER stress-induced IR degradation *in vitro* and obesity-induced down-regulation of IR and insulin action *in vivo*. Our results uncover a new mechanism underlying obesity-induced insulin resistance and shed light on potential targets for the prevention and treatment of obesity-induced insulin resistance and type 2 diabetes.

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Introduction

Obesity is associated with various metabolic diseases and has currently reached epidemic proportions in the western industrialized world (Kahn et al., 2006). While the mechanisms by which obesity leads to insulin resistance remains a subject of current intensive investigation, recent studies suggest that endoplasmic reticulum (ER) stress plays a critical role in mediating obesity-induced insulin resistance (Nakatani et al., 2005; Ozcan et al., 2004). Consistent with this view, chemical chaperones have been found to alleviate insulin resistance in cell or animal models of obesity (Ozcan et al., 2006). In addition, overexpression of ER-chaperone ORP150 in obese mice improved insulin tolerance (Ozawa et al., 2005).

ER is the place responsible for the synthesis, assembly, and/or modification of transmembrane and secreted proteins. Numerous protein chaperones are present in the ER lumen that yield an oxidizing environment necessary for correct folding and assembly of various membrane and secretive proteins (Eizirik et al., 2008). Under pathophysiological nutrient excess conditions such as obesity, ER stress may be induced due to accumulation and aggregation of misfolded proteins in the ER lumen, leading to activation of a signal response termed the unfolded protein response (UPR), a coping system that alleviates ER stress and restores ER homeostasis. Incorrectly folded proteins can be exported from the ER and degraded by the proteasome- or autophagy-associated protein degradation pathway (Ogata et al., 2006; Yorimitsu et al., 2006). If UPR fails to restore ER homeostasis, the apoptosis pathway will be activated by ER stress (Szegezdi et al., 2006).

Insulin activates cellular events by binding to its membrane receptor, which leads to insulin receptor (IR) tyrosine kinase activation and subsequent tyrosine phosphorylation of downstream signaling molecules such as IRS-1 and IRS-2 (Withers and White, 2000). Tyrosine phosphorylation of IRS proteins activates the phosphatidylinositol 3-kinase (PI3K) signaling pathway, which stimulates a variety of cellular responses including glucose uptake, cell growth and proliferation. Insulin signaling is inhibited by ER stress, yet the underlying mechanisms remain elusive. There is some data suggesting that serine phosphorylation of

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IRS-1/2 by c-Jun N-terminal protein kinase (JNK) plays a critical role in ER stress-induced insulin resistance (Ozcan et al., 2004; Ozcan et al., 2006). However, it remains unclear whether additional mechanisms are involved in obesity-induced insulin resistance.

In the present study, we show that the cellular levels of IR are down-regulated in animal models of obesity as well as in fat tissues of obese human subjects. In addition, we have found that ER stress is sufficient to down-regulate IR levels in 3T3-L1 adipocytes via the autophagy pathway. Furthermore, reducing ER stress by chemical chaperones rescued obesity-induced IR down-regulation and insulin resistance, both *in vitro* and *in vivo*. Our results elucidate a mechanism by which ER stress induces insulin resistance and identify potential new targets for the prevention and treatment of obesity-induced insulin resistance and type 2 diabetes.

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Materials and methods

Chemicals and antibodies- The following chemicals were used: thapsigargin (350-004, Alexis Biochemicals, San Diego, CA), tunicamycin (T7765, Sigma-Aldrich Inc., St. Louis, MO), 3-methyladenine (3-MA) (M9281, Sigma-Aldrich Inc.), tauroursodeoxycholic acid (TUDCA) (580549, Calbiochem, Gibbstown, NJ), and sp600125 (420119, Calbiochem). The following primary antibodies were used: Polyclonal anti-IRβ (sc-711, Santa Cruz Biotechnology, Santa Cruz, CA) anti-IRS-1 (sc-559, Santa Cruz Biotechnology), monoclonal anti-CHOP (sc-7351, Santa Cruz Biotechnology), anti-phosphor-Tyrosine 4G10 (05-1050, Upstate Biotechnology, Lake Placid, NY), anti-Akt (17-186, Upstate Biotechnology), anti-phosphor-Thr^{308}-Akt (9275, Cell Signaling Technology, Danvers, MA), anti-LC3 (2775, Cell Signaling Technology), anti-phosphor-Ser^{52}-eIF2α (9721, Cell Signaling Technology), and eIF2α (9722, Cell Signaling Technology).

Cell culture- 3T3-L1 cell differentiation- Cell culture and 3T3-L1 cell differentiation were performed as described in our previous studies (Wick et al., 2003).

Quantitative RT-PCR- Total mRNAs were purified from cells according to a similar protocol as described in our previous study (Ramos et al., 2006). cDNAs were reverse transcribed using superscript III (11752-050, Invitrogen). Quantitative real-time (RT)-PCR was performed on an ABI 7900HT system (Applied Biosystems, Foster City, CA), using the primers specific for mouse IR, 5'-GTTGACGATTTCCAGGAAG-3' and 5'-CTTCTTCGAGAGCTGGGGC-3' and the primers for glyceraldehydes-3-phosphate dehydrogenase (GAPDH), 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'. Thapsigargin treated samples were normalized relative to controls, where control samples were set at a value of 1.

Glucose uptake- 2-deoxylglucose uptake measurements were carried out essentially as described previously (Mao et al., 2006). In brief, 3T3-L1 cells were seeded in 6-well plates and differentiated. The cells were treated with 0.01 μM thapsigargin (TG) with or without 3-MA for 36 hrs. The cells were rinsed with serum-free medium, serum starved for 2 hrs, and

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then stimulated with 100 nM insulin for 10 min. 2-deoxy-D-2-^{3}H-glucose (0.5 μCi ml^{-1}, American Radiolabeled Chemicals) and 10 μM 2-deoxyglucose (Sigma) were added to the cells. After 10 min-incubation, cells were washed with cold PBS and lysed in 0.5 ml of 0.1 M NaOH. The non-specific uptake was measured in the presence of 10 μM cytochalasin B (Sigma).

Animal study- Male *db/db* mice and their lean controls (stock# 000642, 3-5 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were group-housed in a specific pathogen–free facility at 22–24°C on a 12-h light/12-h dark cycle with the lights on at 0800 h. C57/B6 mice were fed with standard rodent chow (7012, Harlan Teklad) or high fat diet (60 Kcal% of mostly saturated fats, D12492, Research Diets, Inc., New Brunswick, NJ) for 16 weeks. *db/db* mice and their lean controls were fed with standard rodent chow only. All animals had access to water ad libitum. Food was only withdrawn if required for an experiment. At 8-weeks of age, the mice were i.p. injected with TUDCA (250 mg/kg) or equal volumes of vehicle twice daily (0800h and 2000h) for 26 days. Mice were then sacrificed and mouse tissues were isolated according to the procedure as described in our recent studies (Wang et al., 2007). All animal procedures were approved by the University of Texas Health Science Center Animal Care and Use Committee.

Human subjects- Sixteen Chinese subjects (8 lean and 8 overweight/obese individuals) undergoing abdominal surgery for cholecystitis or cholelithiasis at the Department of surgery, Shanghai Jiaotong University Affiliated Sixth People's Hospital, were recruited (Table 1). The study was approved by the human research ethics committee of the hospital, and informed consent was obtained from all subjects. Characteristics of the study subjects were determined and presented in Table 1. During the operation, abdominal visceral adipose tissues (about 2cc each) were collected, snap-frozen and stored at -70°C prior to immunoblot analysis.

Insulin tolerance test (ITT)- Male db/db mice and lean control mice (8-10 mice/group; 4-week-old) were fasted for 6 hrs followed by i.p. injection with insulin (2 IU/kg animal body weight). Blood was obtained from the tail vein before (0 min) and after insulin injection (30,

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60, and 90 min). Glucose levels were measured by using an automatic glucometer (Rightest GM300; Bionime Corp, San Diego, CA).

Immunofluorescence and H&E staining studies

Cell image studies were performed as previously described (Lim et al., 2003). The localization and expression of IR were visualized with an Olympus IX 81 confocal microscope with a 40X, 1.35 NA oil immersion objective. For H&E staining, isolated mouse organs were fixed overnight in 10% formalin at 4 ºC. The fixed organs were washed with 30% ethanol for 30 minutes and stored in 70% ethanol. Organs were embedded in paraffin, sectioned, and stained with H&E.

Western blot and statistical analyses- The expression and phosphorylation levels of various proteins were detected by Western blot of cell lysates, tissue homogenates, or immunoprecipitates with specific antibodies. Quantification of the relative increase in protein phosphorylation (expressed as percentage of basal phosphorylation (arbitrarily set as 1.0)) was performed by analyzing Western blots using the Scion Image Alpha 4.0.3.2 program (Scion Corporation) and was normalized for the amount of protein expression in each experiment. The relative expression of IRβ was normalized to tubulin. Statistical evaluation of the data was done using student's t-test.

HOMA and QUICKI analysis- Insulin sensitivity was assessed by homeostasis model assessment (HOMA) according to the formula: [fasting insulin (μU/ml) × fasting glucose (mmol/l)]/22.5 (Matthews et al., 1985) and quantitative insulin sensitivity check index (QUICKI) according to the formula: 1/[log fasting insulin (μU/mL) + log fasting glucose (mg/dL)] (Katz et al., 2000).

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Results

Obesity induces ER stress and IR down-regulation in insulin target tissues. Obesity is well established as a key factor in causing insulin resistance, yet the underlying mechanisms remain to be fully characterized. We found that the expression levels of IR, but not the signaling molecules downstream of IR such as IR substrate-1 (IRS-1), PI3K, PDK1, and Akt, were significantly down-regulated in the white adipose tissue (WAT) of the *db/db* and high fat diet (HFD)-fed mice (Fig. 1A and data not shown). A significant decrease in IR expression was also observed in visceral adipose tissue isolated from obese human subjects (Fig. 1B) as well as liver and skeletal muscle of db/db mice and HFD-fed mice (data not shown). Since down-regulation of IR levels may decrease insulin signaling and action, these results suggest a potential mechanism by which obesity induces insulin resistance *in vivo*.

ER stress impaired insulin signaling and down-regulated IR levels in cell. ER stress has previously been shown to be induced in animal models of obesity such as the *db/db* mice and HFD-fed mice (Ozcan et al., 2004). Since IR is a transmembrane protein that is synthesized and processed in the ER (Olson et al., 1988), it is possible that obesity induced ER stress plays a role in IR down-regulation. Consistent with these results, we found that the expression levels of C/EBP homologous protein (CHOP), a ER stress marker (Wang et al., 1996), are greatly increased in WAT of HFD-fed mice and db/db mice (data not shown). To determine whether ER stress is sufficient to cause IR down-regulation, we treated mouse hepatocytes and 3T3-L1 adipocytes with thapsigargin (TG), a chemical known to induce ER stress by inhibiting ER Ca^{2+} ATPase (Ozcan et al., 2004). In agreement with the findings of others (Kouroku et al., 2007; Ozcan et al., 2004), we found that TG treatment promoted CHOP expression and JNK phosphorylation in both of these cells (Figure 2A and data not shown). Concurrent with increased ER stress, the cellular levels of IR were markedly reduced in TG-treated 3T3-L1 adipocytes (Figure 2A) and mouse hepatocytes (data not shown). Consistent with impaired insulin signaling, insulin-stimulated tyrosine phosphorylation of IRS-1 was greatly inhibited in TG-treated 3T3-L1 adipocytes (Figure 2B).

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ER stress down-regulated IR protein levels but not IR tyrosine phosphorylation per se. Activation of JNK has previously been shown to mediate ER stress-induced insulin resistance. To characterize the role of JNK activation in ER stress-induced IR down-regulation and insulin resistance, we asked whether inhibition of JNK is able to restore insulin signaling in TG-treated cells. Treating 3T3-L1 adipocytes with the JNK specific inhibitor SP600125, which greatly suppressed JNK1 phosphorylation, did not prevent TG-induced down-regulation of IR and insulin-stimulated Akt phosphorylation (Fig. 2C).

To determine if reducing IR expression levels is responsible for impaired insulin signaling caused by ER stress, we examined the effect of TG treatment on IR tyrosine phosphorylation. While treating 3T3-L1 adipocytes with TG led to a time-dependent decrease in IRβ tyrosine phosphorylation, a similar reduction in the IRβ protein levels was also observed (Fig. 2D). Interestingly, when the same amount of the IR β-subunits from the TG-treated and vehicle-treated cells were compared, no significant difference in IRβ tyrosine phosphorylation was detected (Fig. 2E). Similar results were also observed in mouse hepatocytes (data not shown). These results indicate that the reduced IRβ protein levels, rather than the tyrosine phosphorylation of the receptor *per se*, are involved in ER stress-induced insulin resistance.

ER stress down-regulates IR by autophagy dependent ER associated degradation (ERAD). ER stress is known to regulate cellular protein levels by regulating gene transcription (Yoshida, 2007) and protein degradation (Ogata et al., 2006; Werner et al., 1996). To determine the mechanism underlying ER stress-induced down-regulation of IR, we investigated the effect of ER stress on the mRNA levels of IR. TG treatment had little effect on IR mRNA levels in 3T3L1 adipocytes (data not shown), suggesting that protein degradation may play a major role in ER stress-induced IR down-regulation. One of the mechanisms by which ER stress induces protein degradation is via the proteasome-dependent pathway (Yorimitsu et al., 2006). To determine whether proteasome is involved in ER stress-induced IR down-regulation, we treated 3T3-L1 adipocytes with MG132, a proteasome

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specific inhibitor. MG132 treatment did not protect IRβ from TG-induced degradation, even with a low dose of TG treatment (0.01μM) (data not shown), suggesting that other mechanisms, rather than proteasome-dependent protein degradation, were involved in ER stress-induced IR degradation. To explore this possibility, we asked whether autophagy, which has recently been found to be induced by ER stress in SK-N-SH neuroblastoma cells and in yeast (Ogata et al., 2006; Yorimitsu et al., 2006), is involved in ER stress-induced IR down-regulation. Treating 3T3-L1 adipocytes with 3-methyladenine (3-MA), a compound known to inhibit autophagy by blocking class III PI3K (Petiot et al., 2000), suppressed TG-induced autophagy, as demonstrated by the inhibition of the conversion of microtubule-associated protein 1 light chain 3-I (LC3-I) to LC3-II (Fig. 3A, panel 4), a hallmark of autophagy induction (Kabeya et al., 2004; Mizushima and Yoshimori, 2007). 3-MA treatment also greatly protected IR from ER stress induced degradation (Figs. 3A top panel and B). Interestingly, we found that 3-MA was unable to restore insulin-stimulated Akt phosphorylation in the TG-treated cells (Fig. 3A, the second panel and Fig. 3C third panel). These results suggest that while blocking autophagy prevents ER stress-induced degradation of IR, most of the "rescued" IR may already be defective, probably due to ER stress-induced misfolding. Consistent with this view, the tyrosine phosphorylation of the "rescued" IR was greatly reduced compared to the IR from the control cells (Fig. 3C top panel).

To determine if ER stress-induced IR down-regulation affects insulin action, we examined insulin-stimulated glucose uptake in 3T3-L1 adipocytes treated with or without the ER stress inducer TG. We found that insulin-stimulated glucose uptake was significantly reduced in cells treated with TG (Fig. 3D). The TG-induced suppression of glucose uptake was not restored by treating cells with 3-MA (Fig 3D), suggesting that the rescued IR was functionally defective.

Chemical chaperone treatment protects cells from ER stress induced IR degradation and insulin resistance in vitro. Chemical chaperones can stabilize ER-synthesized proteins to improve ER-loading. Tauroursodeoxycholic acid (TUDCA) is one of the chemical chaperones

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that has previously been shown to effectively alleviate both chemical treatment- or obesity-induced ER stress *in vitro* and *in vivo* (Ozcan et al., 2006). To characterize the role of ER stress in IR down-regulation, 3T3-L1 adipocytes were pretreated with or without TUDCA for 1 hr and then with the ER stress inducer TG. TUDCA treatment significantly protected the cells from TG-induced IR degradation and Akt de-phosphorylation (Figs. 4A and 4B). Interestingly, while inhibition of IR degradation by 3-MA failed to prevent ER stress-induced down-regulation of IRβ tyrosine phosphorylation (Fig. 3C) and its mis-targeting to the plasma membrane (Fig. 4D), the cellular levels and tyrosine phosphorylation of IRβ (Fig 4C) and IR membrane targeting (Fig. 4D) in the TG-treated cells were greatly improved by treating cells with TUDCA. These results provided evidence that the alleviation of ER stress may promote IR correct folding and membrane targeting, leading to improved insulin signaling.

Chemical chaperone treatment improved obesity-induced insulin resistance in vivo. To determine if reducing ER stress is able to protect IR degradation *in vivo*, we examined the effect of TUDCA on insulin signaling in *db/db* mice. Treating the db/db mice with TUDCA led to decreased fasting blood glucose levels (Fig 5A) and improved insulin sensitivity (Fig. 5B). The IRβ levels and Akt phosphorylation at Thr^{308} were significantly lower in WAT of saline-treated *db/db* mice compared to the lean control mice (Figs. 5C and D). TUCDA treatment also greatly reduced eIF2α phosphorylation (another widely used ER stress marker (Ozcan et al., 2004) in white adipose tissue, which is associated with a significant increase in IRβ protein levels (Fig 5D). Similar beneficial effects of TUCDA on both the protein levels and tyrosine phosphorylation of IRβ were also observed in liver and muscle of the mice (data not shown). Consistent with the findings observed in 3T3-L1 adipocytes (Fig. 3C), the ratio of LC3-II to LC3-I was notably induced in adipose tissue of db/db mice compared to lean mice (Fig. 5C, panel 4), suggesting an increased autophagy in the *db/db* mice. Treating the *db/db* mice with TUDCA greatly suppressed the conversion of LC3-I to LC3-II (Fig. 5C, panel 4), suggesting that the increased autophagy in adipose tissues of the db/db mice is caused by ER

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stress. Together, these results provide evidence that ER stress-induced IR dysfunction plays a key role in obesity-induced insulin resistance.

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DISCUSSION

ER stress has been recognized as an important mechanism for obesity-related insulin resistance and type 2 diabetes. Recent studies showed that ER stress leads to activation of JNK, which inhibits insulin signaling by inducing serine phosphorylation of IRS-1 (Ozcan et al., 2004). However, it is unclear whether additional mechanisms are involved in obesity-induced insulin resistance.

In the present study, we show that obesity induces insulin resistance by ER stress-dependent down-regulation of the IR. In addition, we show that ER stress-stimulated IR degradation is mediated by the autophagy-dependent process. IR is one of the most important molecules involved in insulin signaling and cell metabolism. Ubiquitous deletion of the IR gene in mice led to marked hyperglycemia and the mice died within the first week of life (Joshi et al., 1996). Point mutations that either prevent IR processing or affect IR autophosphorylation have been shown to cause insulin resistance and diabetes in human (Formisano et al., 1993; Haruta et al., 1993; Iwanishi et al., 1993; Yoshimasa et al., 1988). Consistent with these findings, we found that the expression levels of IR are significantly reduced in insulin resistant obese animal models (Fig. 1) and cells with chemical-induced ER stress (Fig 2). In addition, we found that reducing ER stress by chemical chaperons prevents IR from degradation, which is associated with improved insulin signaling and insulin sensitivity (Figs. 4 and 5). Together, these results indicate that ER stress-induced IR down-regulation play an important role in obesity-induced insulin resistance. Thus, reducing ER stress-induced IR misfolding and subsequent degradation could be a promising therapeutic target for the treatment of insulin resistance and type 2 diabetes.

Recent studies have demonstrated that autophagy, a catabolic process that is responsible for the degrading and recycling of cellular organelles and proteins through the liposomal system, could be induced by ER stress (Kouroku et al., 2007). Autophagy has been considered to play an important role in recycling substance and energy for cell survival (Wang and Klionsky, 2003). However, the role of autophagy in insulin resistance and type 2

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diabetes remains largely unknown. Our studies have provided evidence that autophagy is induced in fat, liver and skeletal muscle of db/db mice high-fat diet-fed mice (Figs. 1 and 5C and data not shown). In addition, the ER stress-induced IR degradation was blocked by the autophagy inhibitor 3-MA (Fig. 3A). Taken together, these results show that autophagy is induced in the peripheral insulin-sensitive tissues in respond to obesity/ER stress-induced insulin resistance. However, blocking autophagy failed to improve ER stress-induced down-regulation of insulin signaling in 3T3L1 adipocytes (Fig. 3A), suggesting that under the ER stress condition, the rescued IR is already impaired in function. Thus, autophagy-dependent clearance of these potentially toxic IR aggregates may provide a mechanism for maintaining normal cell function in response to obesity-induced insulin resistance and ER stress. Our results are consistent with recent findings that autophagy plays an important role in β cell homeostasis in response to HFD (Ebato et al., 2008; Jung et al., 2008).

Down-regulation of the IR protein levels in insulin-target tissues such as liver, skeletal muscle, and adipose tissue has been shown to correlate with insulin resistance (Boden et al., 1994; Garvey et al., 1986; Venkatesan and Davidson, 1995). However, whether reducing IR level is sufficient to cause insulin resistance remains to be established. The $IR^{+/-}$ heterozygous knockout mice showed normal glucose tolerance and normal phenotype with less insulin receptor protein levels (Joshi et al., 1996), suggesting the presence of "spare" IR for normal cell function. In the current study, we found that ER stress leads to a marked reduction in the IR protein levels in 3T3-L1 adipocytes, which is correlated with reduced IR downstream signaling (Figs. 1-3). In addition, we found that the insulin-stimulated glucose uptake is suppressed by ER stress in 3T3-L1 adipocytes (Fig. 3D), suggesting ER stress-induced IR down-regulation may play a contributing role in insulin resistance in 3T3-L1 adipocytes. Consistent with this view, we found that inhibition of JNK, a kinase which has been shown to cause insulin resistance by serine phosphorylation of IRS-1 at Ser^{307} (Aguirre et al., 2000; Bandyopadhyay et al., 2005; Gao et al., 2004; Hiratani et al., 2005; Ozcan et al., 2004), did not rescue ER stressed-induced suppression of Akt phosphorylation (Fig. 2C). Thus, obesity

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may lead to insulin resistance by JNK-dependent and JNK-independent mechanisms and the later could be mediated by down-regulation of insulin signaling molecules such as IR.

In summary, our study reveals that ER stress-induced IR dysfunction and down-regulation in peripheral insulin target tissues contributes to obesity-induced insulin resistance. These findings further strengthen the role of ER stress in the development of insulin resistance and type 2 diabetes and suggest that chemicals preventing ER stress should be a promising therapy for type 2 diabetes and related metabolic disorders.

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FIGURE LEGENDS

Figure.1. Obesity induces ER stress and IR down-regulation.

(A). The expression levels of IRβ in white adipose tissue (WAT) of 4 months old db/db mice or lean control mice fed with normal chow diet were determined by Western blot and quantified and normalized to β-tubulin. (B). Proteins from visceral adipose tissue of normal weight (NW) and obese patients (OB) (Table 1) were determined by Western blot with anti-IRβ antibody. The expression levels of IRβ were quantified and normalized to β-tubulin. Data represent the mean ± SEM. Single asterisks indicates *P <0.05.

Figure. 2. ER stress down-regulates IR protein levels and impairs insulin signaling.

(A). Differentiated 3T3-L1 cells were treated with thapsigargin (TG, 1μM) for different times as indicated, followed with or without 10 nM insulin for 5 mins as indicated. The expression of proteins in cell lysates was determined by Western blot with specific antibodies as indicated. (B). Differentiated 3T3-L1 adipocytes were treated with or without TG (1 μM) for 36 hrs followed by 10 nM insulin for 5 mins. Cells were lysed and IRS-1 in cell lysates was immunoprecipitated using an antibody to IRS-1. The tyrosine phosphorylation and protein expression of immunoprecipitated IRS-1 were determined by Western blot analysis with specific antibodies as indicated. (C) Differentiated 3T3-L1 adipocytes were pretreated with or without SP600125 (10 μM) for 1 hr and then with vehicle or TG (1μM) for 36 hrs. Cells were then stimulated with 10 nM insulin for 5 mins. Cell lysates were resolved by SDS-PAGE and the

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phosphorylation and/or expression of IRβ, JNK1, Akt, and CHOP were determined by Western blot analysis with specific antibodies as indicated. (D)**.** Differentiated 3T3L1 adipocytes were treated with 1 μM TG for different times as indicated, followed with or without 10 nM insulin stimulation for 5 mins as indicated. IRβ was immunoprecipitated from cell lysates and the tyrosine phosphorylation and protein expression were determined by Western blot analysis with an antibody to phosphor-Tyr (upper panel) and to IRβ (lower panel), respectively. (E). Differentiated 3T3-L1 adipocytes were treated with or without TG (1 μM) for 36 hrs, flowed by 10 nM insulin for 5 mins and then lysed. IRβ was immunoprecipitated from cell lysates. Same amount of IRβ was separated by SDS-PAGE and analyzed by Western blot using an antibody to phosphor-Tyr (upper panel) or IRβ (lower panel), respectively. Data are representatives of 3 independent experiments with similar results.

Figure. 3. ER stress reduces IR cellular levels by an autophagy-dependent mechanism.

(A). Differentiated 3T3-L1 adipocytes were pre-treated with or without 3-MA (5 μM) for 1 hr followed by TG (0.01 μM) for different times as indicated. The cells were then treated with 10 nM insulin for 5 mins and lysed. The phosphorylation and/or protein levels of IRβ, Akt, LC3 and β-tubulin were determined by Western blot with specific antibodies as indicated. Data are representatives of 4 independent experiments with similar results. (B). The relative expression levels of IRβ were quantified and normalized to β-tubulin. Data represent the mean ± SEM. **P <0.01. (C). 3T3-L1 adipocytes were pre-treated with or without 3-MA (5μM) for 1 hr followed with TG (0.01 μM) for 36 hrs. Cells were then

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treated with insulin (10 nM) for 5 mins and lysed. IRβ was immunoprecipitated from cell lysates and its tyrosine phosphorylation and protein levels were determined by Western blot using an antibody to phosphotyrosine and IRβ, respectively. Akt phosphorylation and the cellular levels of β-tubulin in cell lysates were determined by Western blot using specific antibodies. β-tubulin was shown as a loading control. Data are representatives of 3 independent experiments with similar results. (D) 3T3L1 adipocytes were pre-treated with or without 3-MA (5μM) for 1 hr followed with TG (0.01 μM) for 36 hrs. Cells were then serum starved for two hrs and treated with insulin (100 nM) or PBS for 10 mins. 2-deoxy-D-2-^{3}H-glucose (0.5 μCi/ ml) and 10 M 2-deoxyglucose were added to the cells. Uptake was allowed at 37 °C for 10 min. After intense washing, cells were lysed, followed by measurements of 2-deoxy-D-2-^{3}H-glucose radioactivity. Data represent the mean ± SEM. ** P <0.01; n=4.

Figure. 4. Chemical chaperone (TUDCA) prevents ER stress-induced IR down-regulation in 3T3L1 adipocytes.

(A) Differentiated 3T3-L1 adipocytes were pre-treated with or without TUDCA (1 mM) for 1 hr followed with TG (0.01 μM) for different times as indicated. The cells were stimulated with 10 nM insulin for 5 mins and lysed. The expression levels of IRβ and phosphorylation of Akt (B.) were determined by Western blot and quantified. Data represent the mean ± SEM. *P <0.05. (C). 3T3-L1 adipocytes were pre-treated with or without TUDCA for 1 hr followed with or without TG for 36 hrs. Cells were then treated with 10 nM insulin for 5 mins and lysed. IRβ was immunoprecipitated from cell lysates and its tyrosine

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phosphorylation and protein levels were determined by Western blot using an antibody to phosphor-Tyr (top panel) or to IRβ (second panel), respectively. Akt phosphorylation and expression in cell lysates were determined by Western blot with antibodies to phospho-Akt (T308) and to Akt, respectively (the third and fourth panels). Tubulin is shown as a loading control. Data are representatives of 3 independent experiments with similar results. (D). Differentiated 3T3-L1 adipocytes were pre-treated with or without TUDCA (1 mM) or 3-MA (5 μM) for 1 hr, followed with or without 0.01 μM TG for 36 hrs. The cells were then fixed and the cellular localization of IRβ was detected by immunofluorescence studies. Scale Bar, 20 μm. Data are representatives of 3 independent experiments with similar results.

Figure. 5. **Chemical chaperone (TUDCA) prevents obesity-induced IRβ down-regulation and improves insulin sensitivity *in vivo*.**

(A). Fasting blood glucose levels were measured after treating the lean mice and *db/db* mice with saline or TUDCA for 18 days. Data represent the mean ± SEM. *p<0.05. (B) Insulin (2 IU/kg) tolerance tests (ITT) of lean control mice and db/db mice treated with vehicle (saline) or TUDCA for 22 days. Data represent the mean ± SEM. n=8. **p <0.01, ***p< 0.001 (TUDCA vs vehicle-treated mice). (C) Lean mice and *db/db* mice treated with saline or TUDCA for 26 days followed by stimulation of insulin (2IU/kg, 10mins) before termination as indicated. The expression levels of IRβ, LC3-I, and LC3-II as well phosphorylation of Akt (Thr^{308}) or eIF2α (Ser^{52}) were determined by Western blot with specific antibodies as indicated. β-actin was used as a loading control. The relative expression levels of IRβ (D)

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and phosphorylation of Akt (E) or eIF2α (F) were quantified. Data represent the mean ± SEM. **P <0.05. n=8.

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Table 1: Characteristics of the human subjects selected for analysis of IR protein levels.

	normal-weight (n=8)	overweight/obese (n=8)
Age (years)	53.38±7.17	60.75±3.83
BMI (kg/m^2)	20.84±0.49*	27.88±0.89
Fasting plasma glucose (mmol/L)	5.21±0.28	5.35±0.29
Fasting insulin (mIU/L)	12.60±1.92	18.93±3.01
HOMA-IR	2.68 (1.89-3.80)	4.15 (2.97-5.81)
QUICKI	0.318 (0.302-0.341)	0.314 (0.285-0.348)

1 means±SE, these variables were tested by t-test

2 median value (25% Q1-75% Q3), and these variables were Logarithmic transformed before *t*-test

*P<0.05 for normal-weight group vs. overweight/obese group

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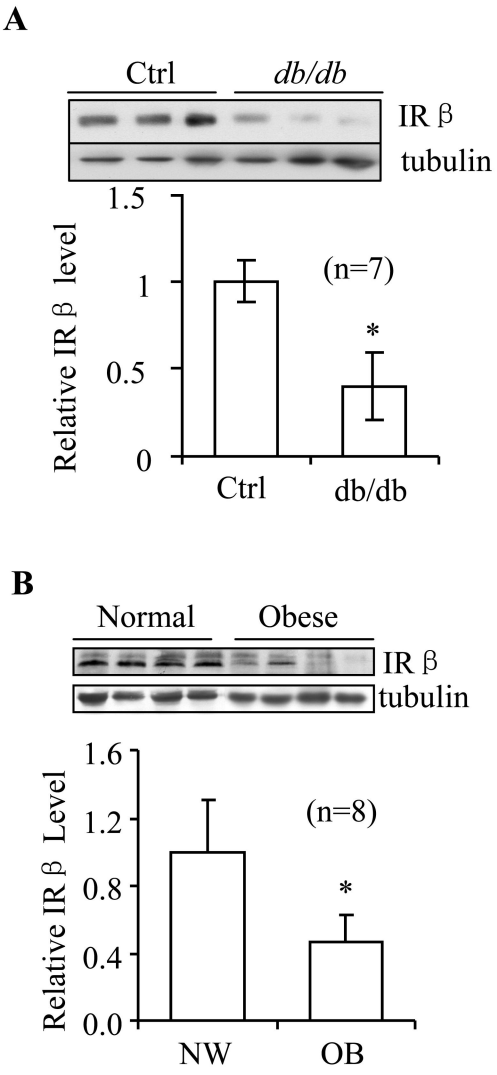

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Figure 1

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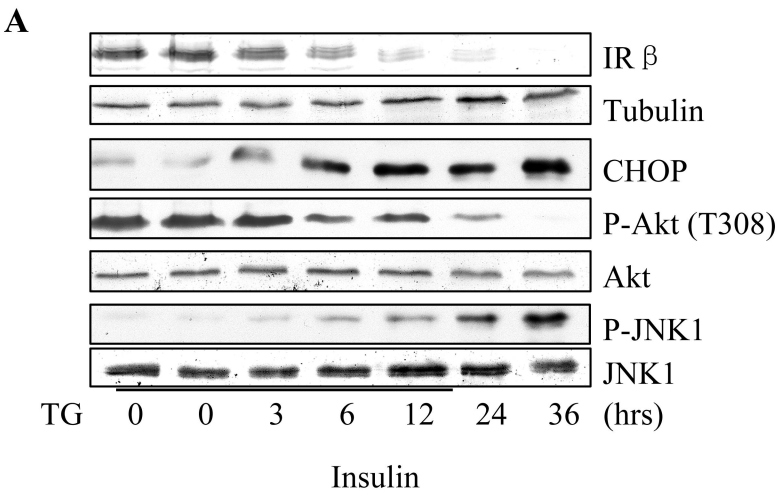

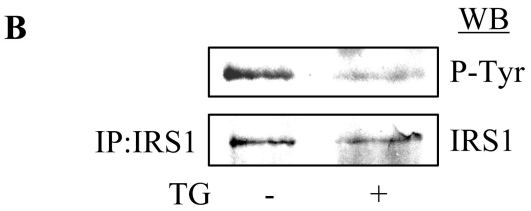

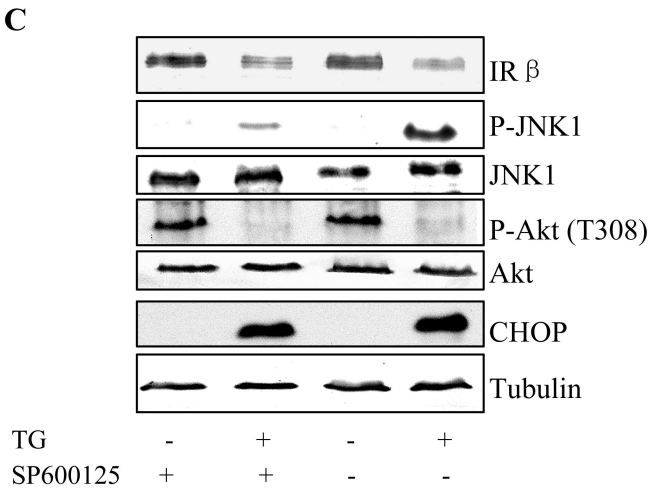

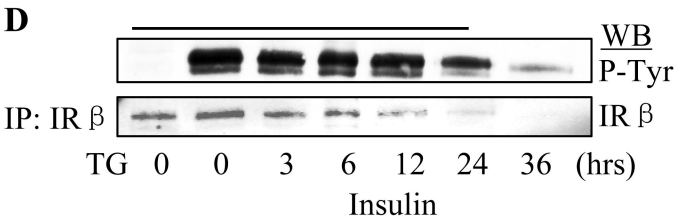

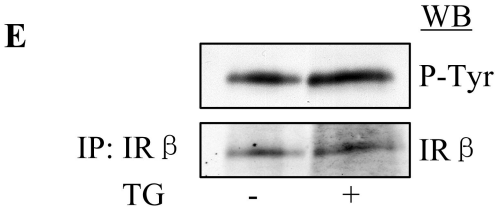

Figure 2

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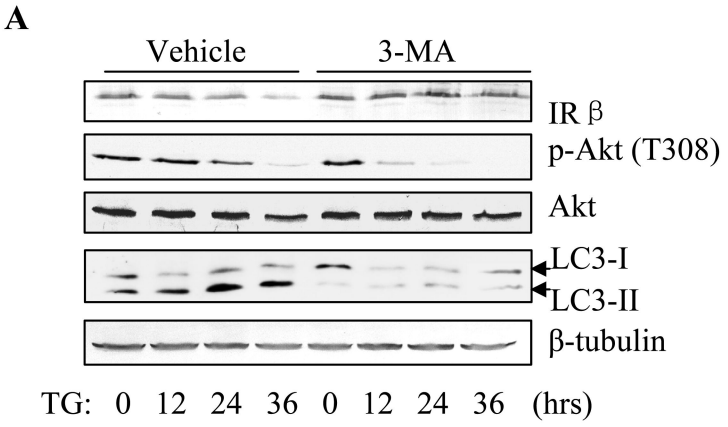

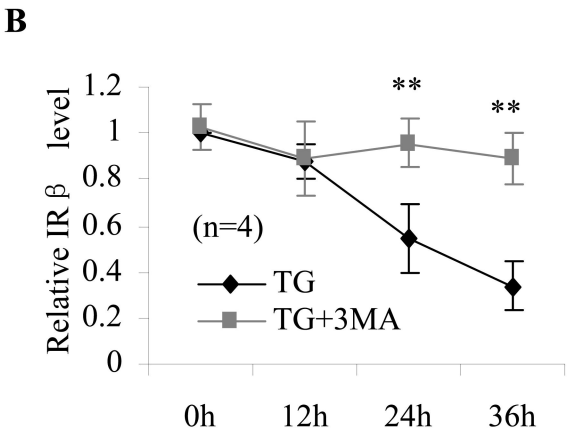

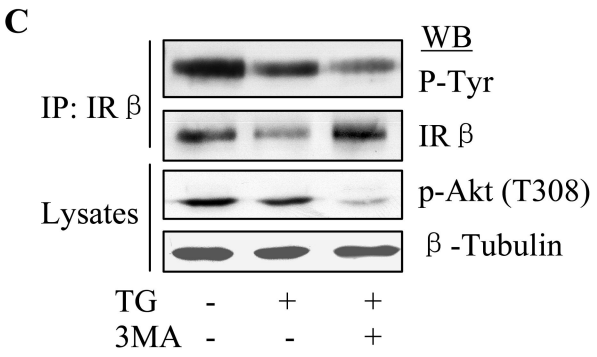

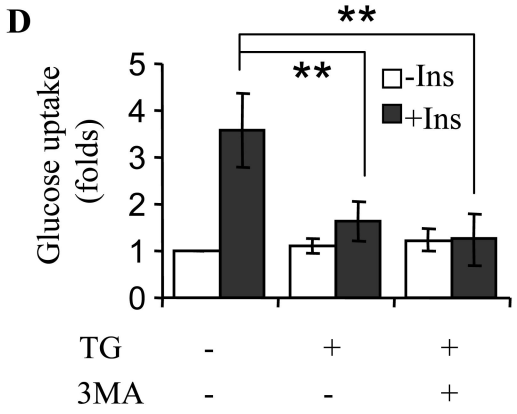

Figure 3

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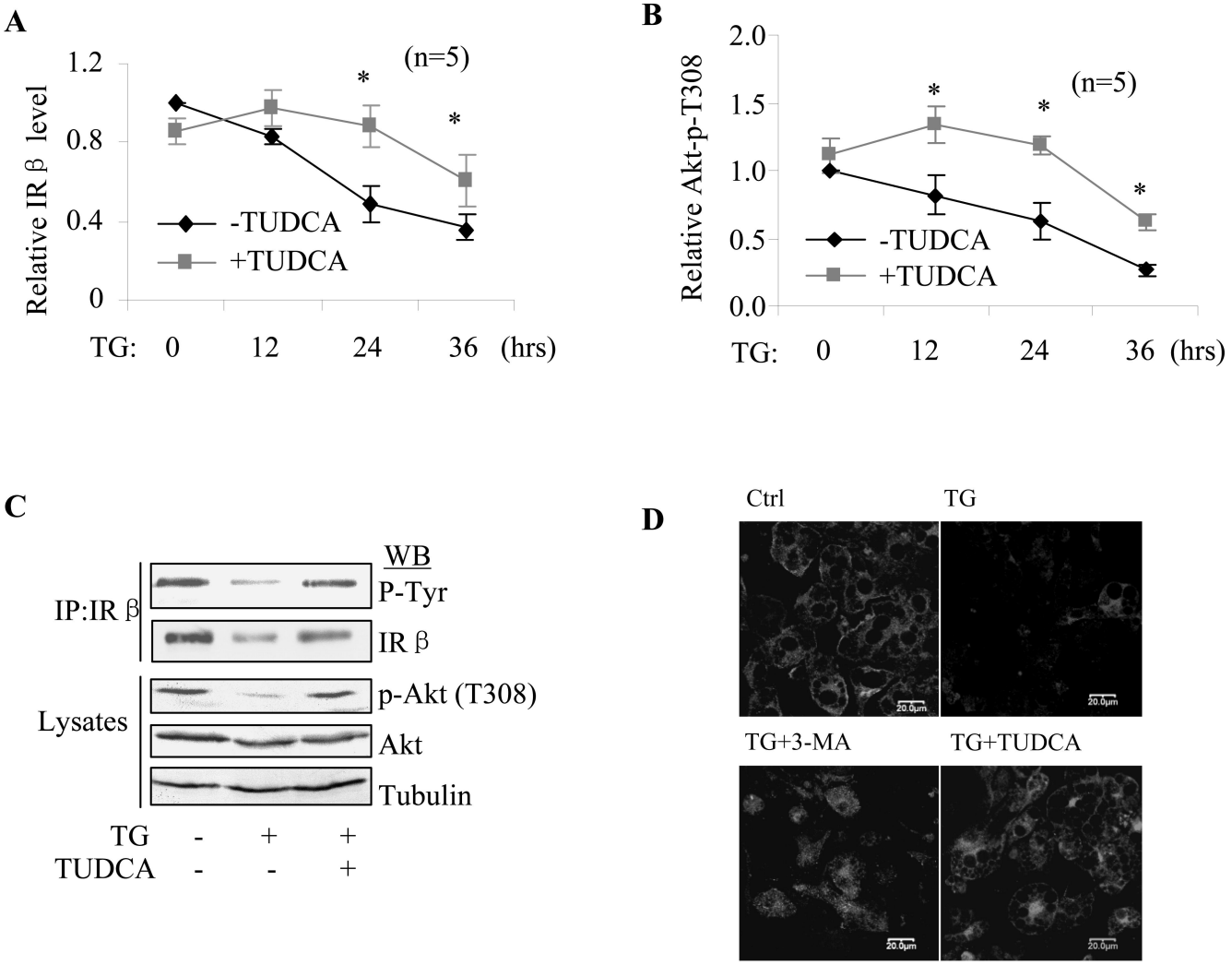

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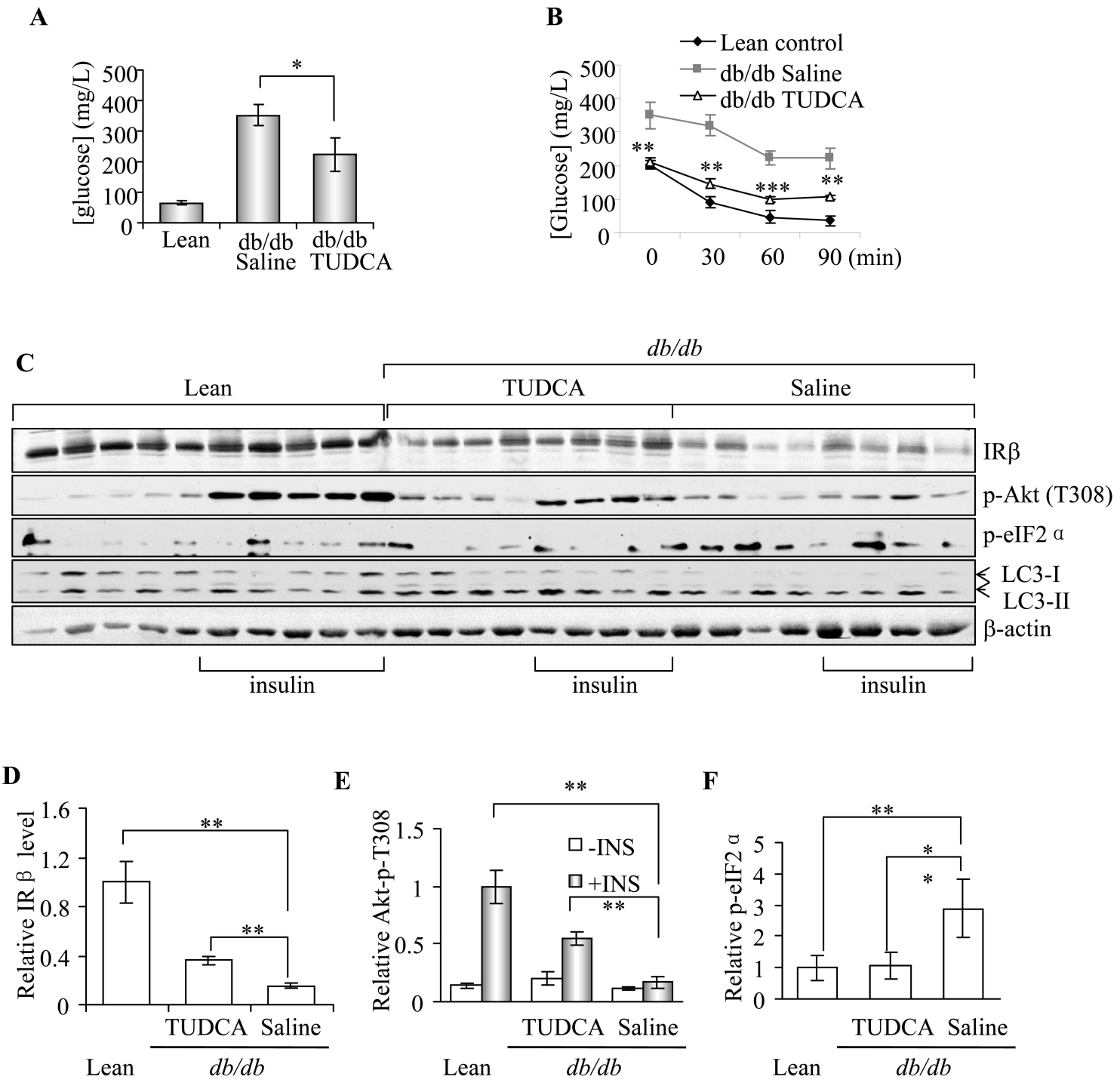

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Figure 5