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Mechanisms of Hemorrhage-induced Hepatic Insulin Resistance: Role of Tumor Necrosis Factor-α

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Running Title: Hepatic insulin resistance in trauma and hemorrhage

Abstract

Hemorrhage, sepsis, burn injury, surgical trauma and critical illness all induce insulin resistance. Recently, we found that trauma and hemorrhage acutely induced hepatic insulin resistance in the rat. However, the mechanisms of this hemorrhage-induced acute hepatic insulin resistance are unknown. Here we report on the mechanisms of this hepatic insulin resistance. Protein levels and phosphorylation of the insulin receptor and of insulin receptor substrate-1/2 (IRS-1/2) were measured, as was the association between IRS-1/2 and phosphatidylinositol 3-kinase (PI3K). Also examined were the hepatic expression of tumor necrosis factor alpha (TNF-α) and TNF-α-induced serine phosphorylation of IRS-1. Insulin receptor and IRS-1/2 protein levels, and insulin-induced tyrosine phosphorylation of the insulin receptor were unaltered. In contrast, insulin-induced tyrosine phosphorylation of IRS-1/2 and association between IRS-1/2 and PI3K were dramatically reduced following hemorrhage. Hepatic levels of TNF-α mRNA and protein were increased as was phosphorylation of IRS-1 serine 307 following hemorrhage. Our data provide the first evidence that compromised IRS-1/2 tyrosine phosphorylation and their association with PI3K contribute to hemorrhage-induced acute hepatic insulin resistance. Increased local TNF-α may play a role in inducing this hepatic insulin resistance following trauma and hemorrhage.

Introduction

Injuries, such as accidental and surgical trauma and burn, as well as hemorrhage and sepsis, often induce hyperglycemia and insulin resistance (1-5). These same injuries and infections are associated with a proinflammatory response and increases in the proinflammatory cytokines TNF-α, IL-6 and IL-1β (6-11). Insulin resistance is also common in critically ill patients, even those who have not previously had diabetes (12-14). This injury/infection-induced insulin resistance results in hyperglycemia, enhancing the concentration gradient-dependent facilitative glucose transport into injured tissues and organs involved in the immunologic response to stress (5,15,16). Thus, acute insulin resistance and hyperglycemia may be important in the immediate response to injury, but extended periods of insulin resistance are not conducive to recovery following trauma and infection. In recent work, intensive insulin therapy has been utilized to overcome this insulin resistance and to restore normoglycemia in critically ill individuals. Intensive insulin therapy resulted in 34 - 50% reductions in septicemia, renal failure, transfusions, polyneuropathy, and mortality. With intensive therapy, patients are less likely to require mechanical ventilation and antibiotics and inflammatory markers are reduced (12,17-20). Thus, treatment to overcome the insulin resistance associated with critical illness is important for recovery.

Insulin exerts its biological effects by binding to its specific tyrosine kinase receptor on the surface of target cells (21,22). Activation of the receptor tyrosine kinase leads to its autophosphorylation and further phosphorylation of insulin receptor substrates (IRS) and Shc, which serve as “docking” molecules, favoring the generation

of intracellular signals (23,24). There are two main insulin intracellular signaling pathways: IRS-phophatidylinositol 3-kinase (PI3K)-Akt pathway and the Ras-mitogen-activated protein kinase (MEK-ERK) pathway (25-27). Insulin resistance refers to the failure to respond to normal circulating concentrations of insulin due to impairment of one or more signaling pathways (28). Molecular mechanisms of insulin resistance are complicated and may differ in different conditions and tissues. There is evidence that TNF-α plays a role in the development of chronic insulin resistance in Type 2 diabetes and obesity (29,30), but little is known about its role in acute insulin resistance following injury. Recent work suggests that induced insulin resistance may in part be due to phosphorylation-based negative-feedback, which may uncouple the insulin receptor or insulin receptor docking proteins from its downstream signaling pathway, altering insulin action (23,31). The IRS proteins are major targets for this phosphorylation-based, negative-feedback control of insulin signaling. TNF-α, free fatty acids and other factors can induce insulin resistance by activating serine/threonine (Ser/Thr) phosphorylation which then inhibits insulin-stimulated tyrosine (Tyr) phosphorylation of IRS proteins (25,31-33).

There is consistent evidence of muscle insulin resistance following injury, illness or infection, but it was not known whether the liver also becomes insulin resistant (2,3). The liver is the main site of gluconeogenesis, and insulin is a primary suppressor of hepatic glucose output. If the liver becomes resistant to insulin, increased hepatic gluconeogenesis can contribute to the hyperglycemia and hyperinsulinemia which are correlated with increased mortality of critically ill patients (12). Hepatic insulin resistance may also result in dysregulation of a large number of liver-expressed, insulin-regulated

genes, thereby compromising insulin actions on metabolism and multiple other hepatic functions (34-36). We recently demonstrated the rapid development of hyperglycemia and hyperinsulinemia in a rat model of trauma and hemorrhage (36). Hepatic insulin resistance developed within 90 min, with defective insulin-induced phosphorylation of Akt and at least partially competent insulin-induced MEK-ERK signaling. In the present study, this rat model of injury and hemorrhage is used to delineate the causes and mechanisms of hemorrhage-induced acute insulin resistance. The compromised insulin signaling was not due to acute changes in insulin receptor or IRS-1/2 protein levels, nor in insulin-induced tyrosine phosphorylation of the insulin receptor. Insulin-induced tyrosine phosphorylation of IRS-1/2 and association between IRS-1/2 and PI3K were rapidly and dramatically decreased following trauma and hemorrhage. Circulating and local hepatic levels of TNF-α were rapidly increased as was the phosphorylation at the serine 307 (Ser307) of IRS-1 following trauma and hemorrhage. These data suggest that following trauma and hemorrhage, compromised IRS-1 tyrosine phosphorylation and its association with PI3K contribute to hemorrhage-induced hepatic insulin resistance, possibly due to increased local TNF-α and serine phosphorylation of IRS-1.

Experimental Procedure

Animal Model of Trauma and Hemorrhage. A model of trauma and hemorrhage in the rat, as previously described (36,37), was used in this study with minor modifications. Briefly, male Sprague-Dawley rats were anesthetized, a 5-cm ventral midline laparotomy was performed representing soft-tissue trauma and the abdomen was closed. Polyethylene-50 catheters (Clay-Adams, Parsippany, NJ) were placed in the right and left femoral arteries and the right femoral vein for bleeding, monitoring of mean arterial pressure, and fluid resuscitation, respectively. The rats were allowed to awaken after which they were bled rapidly to a mean arterial pressure (MAP) of 35-40 mm Hg within 10 min. Once mean arterial pressure reached 40 mm Hg, the timing of the hemorrhage period began and was maintained for 90 min. At the end of the hemorrhage period, the rats were resuscitated with four times the withdrawn blood volume using Ringer's lactate infused by syringe pump (Harvard Apparatus, South Natick, Mass) at a constant rate over 60 min. Sham-operated rats underwent the same surgical procedure (laparotomy and catheterization) but neither hemorrhage nor resuscitation was carried out. All procedures were carried out in accordance with the guidelines set forth in the Animal Welfare Act and the Guild for the Care and Use of laboratory Animal by the National Institutes of Health, and the experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Study Design. Due to the considerable trauma incurred during anesthesia and opening

of the abdominal cavity to perform the insulin injections (see next section), it was impossible to have a completely untreated control group. Thus, the 'baseline' animal was selected in these experiments to be the trauma alone rats (T 0') which were subjected to anesthesia, laparotomy and catheterization, and then killed immediately. Additional trauma alone groups were subjected to these same procedures and then killed at 90' (T 90') or 210' (T 210') after catheterization. Matched to these groups were the trauma plus hemorrhage (TH) groups which were subjected to the same procedures as the T groups, but also subjected to hemorrhage and then killed at 90 min, the end of the hemorrhage period (TH 90'), or 60 min following completion of the 60 min resuscitation period (TH 210' = 90 min hemorrhage + 60 min resuscitation + 60 min recovery).

Measurement of plasma TNF-α Levels. Immediately prior to insulin or saline injection, blood was withdrawn from the right femoral artery for TNF-α measurement. TNF-α levels were measured by a rat TNF-α ELISA kit (BioSource, Camarillo, CA).

Immunoprecipitation and Immunoblots protocol. Liver tissue from each animal (approximately 0.2 gram) was homogenized in 1 ml of lysis buffer containing 20 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 20 mM KCl, 20% glycerol, 0.2 mM EDTA, 2 mM Na_3Vo_4, 10 mM NaF, 1% Triton X-100, 0.2 mM PMSF, 10mg/ml aprotinin and 10 mg/ml leupeptin. Tissue lysates were centrifuged at 10,000 x g for 10 min and the supernatants were stored at –80 ^{0}C until use (36). Tissue lysate protein concentrations were assayed (Bio-Rad Laboratories, Hercules, CA).

For immunoprecipitation, 300 µg protein from each liver sample in lysis buffer was incubated with antibody against the insulin receptor (IR; Santa Cruz Biotech, Santa Cruz, CA) or IRS-1or IRS-2 (Upstate Biotechnology, Lake Placid, NY) overnight at 4 ^{0}C. Protein A-agarose (fast flow, Pharmacia Biotech, Providence, RI) was then added and incubations continued for 2 h at 4 ^{0}C. Immunoprecipitated proteins were resolved by sodium dodecyl sulfate (SDS), 10% polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose paper. The Western transfers were immunoblotted with anti-IR (Santa Cruz Biotech, Santa Cruz, CA), anti-IRS-1, anti-IRS-2, anti-phospho-tyrosine and anti-p85 subunit of PI3-kinase antibodies (Upstate Biotechnology, Lake Placid, NY).

For Western blotting, 15 µg protein per lane was resolved by 10% SDS-PAGE and transferred to nitrocellulose paper. The Western transfers were immunoblotted with anti-IR, anti-IRS-1, anti-IRS-2, anti-phospho-serine 307 and anti-phospho-serine 612 of IRS-1 and anti-TNF-α antibodies (Biosource, Camarillo, CA) followed by addition of horseradish peroxidase-conjugated secondary antibody for detection of bound antibody by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Piscataway, NJ). Each blot was stripped 30 min at 50 ^{0}C in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH6.7), and then reprobed with a different antibody (36).

RT-PCR protocol: Total RNA (2 µg) from liver tissue was reverse transcribed in a 20 µl reaction using a random hexamer primer and ThermoScript RT (Invitrogen, Carlsbad, CA) at 55 ^{0}C for 50 min. Of this cDNA, 2 µl was added to the PCR reaction. Each PCR reaction was conducted in a total volume of 25 µl with Platinum Taq DNA polymerase. The conditions for PCR were 35 cycles of PCR amplification with the denaturing at 94 ^{0}C

for 30 sec, annealing at 55 ^{0}C for 30 sec and extension at 72 ^{0}C for 1 min. ß-actin was used as a control to monitor RT-PCR amplification. PCR products were separated by electrophoresis in a 1.5 % agarose gel and visualized by ethidium bromide staining and UV illumination. The primers used for PCR of TNF-α (411 bp) were 5'-TCCCAACAAGGAGGAGAAATT-3' and 5'-TCATACCAGGGCTTGAGCTCAG-3', β-actin (765 bp) were 5'-TTGTAACCAACTGGGACGATATGG-3' and 5'-GATCTTGATCTTCATGGTGCTAGG-3'.

Densitometric and Statistical Analysis. ECL images of immunoblots were scanned and quantified using Zero D-Scan (Scanalytics Corp., Fairfax, VA). All data was analyzed by One-way ANOVA using the InStat Statistical program by GraphPad Software, Inc. (San Diego, CA).

Results

Insulin receptor and IRS-1 do not change following trauma and hemorrhage.

Previously we have found that following trauma and hemorrhage, the insulin receptor/IRS/PI3K/Akt signaling pathway was compromised (36). Following trauma and hemorrhage, insulin-induced phosphorylation of Akt was completely lost (Lane 8, 10 in Figure 1, upper panel). Since reduced insulin signaling may be due to a decrease in insulin receptor or IRS protein levels, experiments were performed to determine if such changes occur following trauma and hemorrhage. Following trauma alone, or trauma and hemorrhage, total protein levels of insulin receptor and IRS-1 did not change (Figure 1, middle and lower panels) and injection of insulin (1 min.) via the portal vein, had no effect on total insulin receptor and IRS-1 protein levels.

Insulin receptor autophosphorylation do not change following trauma and hemorrhage.

Since the total cellular insulin receptor content was not decreased following trauma and hemorrhage, insulin-induced insulin receptor phosphorylation was investigated. Following trauma alone, or trauma and hemorrhage, insulin-induced total insulin receptor tyrosine phosphorylation was not changed (Figure 2A). Although insulin receptor total tyrosine phosphorylation was not altered, there was still the possibility of alteration of phosphorylation in specific tyrosines in the insulin receptor. Thus, phosphorylation of four tyrosine sites in the insulin receptor, tyrosine 960, 1146, 1150 and 1151 was measured. The three tyrosines, 1146, 1150, 1151 (corresponding to Tyr 1158, 1162, 1163 in human) were studied using a single antibody that interacts only when all three tyrosines are

phosphorylated. These three tyrosine are within the catalytic loop of the insulin receptor β-subunit and their phosphorylation is correlated with full activation of the insulin receptor tyrosine kinase (21,38-41). There was no measurable change in phosphorylation of these sites following either trauma or trauma and hemorrhage (Figure 2B, top panel). Next, phosphorylation of tyrosine 960 (in rat; corresponding to 972 in human), was measured. This juxtamembrane tyrosine may be required for the binding or phosphorylation of the adapter protein IRS-1 (41-43). Like the triple tyrosine phosphorylation site there were no consistent changes in phosphorylation of this site following either trauma or trauma and hemorrhage (Figure 2B bottom panel). Together, these data suggest that insulin signaling following trauma and hemorrhage is not compromised at the level of the insulin receptor.

Tyrosine phosphorylation of IRS-1 decreases following trauma and hemorrhage.

Next, any changes in tyrosine phosphorylation of IRS-1 were examined. Following immunoprecipitation with an anti-IRS-1 antibody, there was a complete loss of immunoreactive insulin-induced tyrosine phosphorylation of IRS-1 in the trauma and hemorrhage groups (TH 90' and TH 210'), with no change of IRS-1 phosphorylation following trauma alone (T 0', T 90' and T 210'; Figure 3A, middle panel). Data from multiple animals in each group was quantified and presented as fold induction by insulin (+) compared to no insulin (-) injection at the same time points following trauma or both trauma and hemorrhage. In the trauma alone group (T 0'), there was an 18-fold induction of tyrosine phosphorylation of IRS-1 1 min after insulin injection. At the T 90' and T 210' time points there were 16-fold (T90') and 19-fold (T210') induction of tyrosine

phosphorylation of IRS-1. However, there was a reproducible, large decrease of insulin's ability to induce tyrosine phosphorylation of IRS-1 in both the TH 90' and TH 210' groups (Figure 3B, hatched bars). This is indicative of a significant loss of insulin signaling via the insulin receptor-IRS-PI3K pathway clearly indicated by the loss of IRS-1 phosphorylation within the 90 min hemorrhage period (TH 90') which persists even 60 min following fluid resuscitation (TH 210').

Association of IRS-1 with PI3K decreases following trauma and hemorrhage.

Because association of IRS-1 protein with PI3K depends upon tyrosine phosphorylation of IRS proteins, we next studied whether association of IRS-1 with PI3K was decreased. Like tyrosine phosphorylation of IRS-1, insulin-induced association of IRS-1 with PI3K was also completely lost following trauma and hemorrhage (Figure 3A, lower panel). In the trauma alone group (T 0'), there was a 15-fold increase in association of IRS-1 and PI3K 1 min following insulin injection. There was little change in this insulin-induced association at the T 90' and T 210' time points (13-fold and 16-fold, respectively). However, this association was abolished in both the TH 90' and TH 210' groups (Figure 3B, solid bars).

Tyrosine phosphorylation of IRS-2 and association of IRS-2 with PI3K decrease following trauma and hemorrhage.

In experiments similar to those with IRS-1, following immunoprecipitation with an anti-IRS-2 antibody, there was a complete loss of insulin-induced tyrosine phosphorylation of IRS-2 in the trauma and hemorrhage groups (TH 90' and TH 210'). There was no change

of IRS-2 phosphorylation following trauma alone (T 0', T 90' and T 210'; Figure 4A, middle panel). Data from multiple animals in each group was quantified and presented as fold induction by insulin (+) compared to no insulin (-) injection at the same time points following either trauma or both trauma and hemorrhage. In the trauma alone groups there were 22-, 24- and 20-fold increases of IRS-2 tyrosine phosphorylation 1 min following insulin injection at the T 0', T 90', and T 210' time points, respectively. However, following trauma and hemorrhage, there was a large decrease of insulin's ability to induce tyrosine phosphorylation of IRS-2 (at both TH 90' and TH 210' time points; Figure 4B, hatched bars). This is indicative of a significant loss of insulin signaling via the insulin receptor-IRS-PI3K pathway, resulting in a loss of IRS-2 phosphorylation.

Similar to the loss of insulin-induced association of IRS-1 with PI3K following trauma and hemorrhage, there was also a complete loss of insulin-induced PI3K association with IRS-2. In the trauma alone group (T 0'), there was a 20-fold increase in association of IRS-1 and PI3K 1 min following insulin injection. There was little change in this insulin-induced association at the T 90' and T 210' time points (16-fold and 18-fold, respectively). However, this association was abolished in both the TH 90' and TH 210' groups (Figure 4B, solid bars).

Serum TNF-α concentrations are elevated following trauma and hemorrhage.

TNF-α is thought to contribute to insulin resistance and proinflammatory cytokines are induced following trauma hemorrhage. However, it was unknown whether TNF-α would be increased rapidly enough to contribute to the insulin resistance that occurred within the 90 min hemorrhage period. Thus, we next measured serum TNF-α levels. Circulating

TNF-α levels following trauma alone increased slightly at both 90 and 210 min compared to T 0' animals, from 10 to 28 and 32 pg/ml, respectively. This increase in serum TNF-α was likely due to the stress of continued anesthesia and/or the surgical procedures. However, trauma followed by hemorrhage for 90 min (TH 90') resulted in a large and significant increase in serum TNF-α levels to 200 pg/ml and a further increase to 220 pg/ml at 60 min after the completion of fluid resuscitation (TH 210'; Figure 5).

Expression of TNF-α is increased in the liver following trauma and hemorrhage.

Since increased serum TNF-α might be due to TNF-α production by other tissues as well as the liver, it was determined whether hepatic TNF-α expression was rapidly increased following trauma and hemorrhage. By Western blot analysis of total hepatic protein, liver TNF-α levels were also found to increase dramatically in the trauma and hemorrhage groups [TH 90' (8-fold) and TH 210' (11-fold), respectively; Figure 6] but not following trauma alone (T 90' and T 210').

When hepatic TNF-α mRNA was measured by RT-PCR, it was found to dramatically increase in the liver in the trauma and hemorrhage groups, and only slightly following trauma alone (Figure 7, upper panel). Data were presented as the ratio of TNF-α to β-actin. The ratios increased slightly at T 90' (0.063) and T 210' (0.065) compared to T 0' and to a much greater extent at TH 90' (0.533) and TH 210' (0.462), respectively (Figure 7, lower panel). Thus, the increased local hepatic TNF-α protein probably results from an increase in hepatic TNF-α gene expression and is correlated with compromised insulin signal transduction via the insulin receptor/IRS/PI3K pathway.

Serine phosphorylation of IRS-1 is increased following trauma and hemorrhage.

Recent evidence suggests that TNF-α may inhibit insulin signaling by promoting increased phosphorylation of specific serine sites of the IRS-1 protein (31,44). Therefore, whether there was an increase of IRS-1 serine phosphorylation following trauma and hemorrhage was investigated. Basal levels of phosphorylation of IRS-1 serine 307 (Ser307) and serine 612 (Ser612) were detectable in T 0' group. Thus, there might be a rapid increase in phosphorylation of these sites in the T 0' group, possibly due to the anesthesia and surgery. Alternatively, there could be a basal level of phosphorylation of these sites in vivo which has been observed by other investigators (31,33,44). There was no significant change in IRS-1 phosphorylation at Ser307 at the T 90' and T 210' time points compared to T 0'. In contrast, there were significant increases in IRS-1 Ser307 phosphorylation following trauma and hemorrhage, at both TH 90' (2.02-fold) and TH 210' (2.21-fold) time points (Figure 8A, upper panel and Figure 8B). As a control for specificity, there were no significant changes in IRS-1 phosphorylation of Ser612 in any group or at any of the time points measured (Figure 8A, middle panel). As a further control, total IRS-1 was also probed, and as described previously, there was no change in the total cellular content of IRS-1 (Figure 8A, lower panel).

Phosphorylation/activation of ERK and p38, but not JNK, is increased following trauma and hemorrhage.

Since previous studies indicate that activation of p38, ERK or JNK may be involved in the phosphorylation of Ser 307 of IRS-1, we next examined whether there were any changes in the phosphorylation/activation state of these MAPK's. Following trauma and

hemorrhage a 10.6-fold (TH 90') and 9.4-fold (TH 210') increase in phosphorylation of ERK1/2 was measured. In addition, there was increased phosphorylation of p38, of 4.2-fold (TH 90') and 5-fold (TH 210') following trauma and hemorrhage (Figure 9A,B) . However, there were no significant changes in phosphorylation of ERK1/2 or p38 following trauma alone. Unlike ERK and p38, there were no measurable changes in phosphorylation/activation of JNK following trauma and hemorrhage (Fig. 9). Since increased activation of ERK and p38 are associated with increased Ser 307 phosphorylation of IRS-1, phosphorylation/activation of ERK and /or p38 may contribute to increased Ser 307 phosphorylation of IRS-1.

Discussion

Insulin resistance is a common pathological condition in which target cells fail to respond to insulin. Chronic insulin resistance is frequently associated with Type 2 diabetes, obesity and hypertension (45-48). Acute insulin resistance occurs following various stresses such as surgical and accidental trauma, hemorrhage, sepsis, burns and other critical illnesses (5,12,36,49-51). Acute insulin resistance may be important in the immediate response to injury, but extended periods of insulin resistance are correlated with poor patient outcomes. Intensive insulin therapy, to overcome this insulin resistance and to restore normoglycemia in critically ill individuals, resulted in decreased mortality (12,17-20). An understanding of the causes and mechanisms of acute insulin resistance may be important for the development of approaches to aid recovery following injury and critical illness.

It is well established that insulin resistance develops in muscle and adipose tissue following injury (1-3,52). However, much less is known about the development of insulin resistance in the liver which may or may not occur concurrently with insulin resistance in muscle and fat. Insulin regulates the expression of a large number of hepatic genes (53-56). Insulin resistance in the liver may result in dysregulation of these genes, resulting in impaired insulin actions in both inhibiting gluconeogenesis and multiple other hepatic functions (34-36,57). We recently demonstrated the rapid development of hepatic insulin resistance, with compromised IRS-PI3K-Akt signaling and increased IGFBP-1 expression (an insulin-inhibited, PI3K-Akt-dependent gene), following experimental trauma and hemorrhage (36). Insulin was still able to signal via the MEK-ERK pathway. Unlike other

models of insulin resistance, trauma and hemorrhage is an acutely inducible model of insulin resistance in normal rats that occurs within 90 min of the beginning of hemorrhage, resulting in a complete loss of insulin-induced Akt phosphorylation. Development of insulin resistance can be due to impaired insulin binding, decreased receptor number, impaired insulin receptor phosphorylation and/or tyrosine kinase activity, failure of insulin receptor association with its docking proteins, decreased phosphorylation of the IRS proteins, impaired association of IRS with PI3K, or numerous other post-receptor defects.

Following binding of insulin to the insulin receptor and activation of the cytoplasmic protein-tyrosine kinase domain (42,58), there is a rapid phosphorylation of multiple tyrosine residues of the insulin receptor β-subunit. The catalytic loop within β-subunit contains a three tyrosine motif including Tyr1146, Tyr1150 and Tyr1151 [corresponding to Tyr 1158/1162/1163 in the human insulin receptor; (38,39)]. It is believed that Tyr1146 must initially be phosphorylated, followed by phosphorylation of Tyr1150 and Tyr1151, in order to achieve full activation of the insulin receptor tyrosine kinase activity (21,40,41). Tyrosine 960 in rat, corresponding to 972 in human, is part of the juxtamembrane Asn-Pro-Glu-Tyr (NPEY) motif (43) and phosphorylation of Tyr960 is required for the binding and/or phosphorylation of IRS-1 (41,42). In the present study, there were no measurable changes in insulin receptor protein levels and insulin-induced total tyrosine phosphorylation of the insulin receptor following trauma and hemorrhage. Although binding of insulin was not measured, no changes in insulin-induced total tyrosine phosphorylation of the insulin receptor following trauma and hemorrhage suggests little or no change in insulin binding and activation of the insulin receptor tyrosine kinase activity. In addition, following trauma and hemorrhage, there was no change in tyrosine

phosphorylation of the insulin receptor at Tyr960, which would suggest little or no defect in the potential ability of the insulin receptor to associate with IRS proteins. Lastly, using a specific antibody that recognizes the insulin receptor only after insulin receptor is phosphorylated at all three tyrosines, 1146, 1150 and 1151, there were no changes in tyrosine phosphorylation which implies no defect in insulin receptor tyrosine kinase activity. Thus, the insulin receptor seemed to be functioning relatively normally and hemorrhage-induced hepatic insulin resistance was likely not an insulin receptor defect, but occurred at a level downstream of the initial activation of the insulin receptor by insulin.

Previous studies have suggested that IRS proteins are a main target for development of chronic insulin resistance (25), but the role of IRS proteins in acute, injury-associated insulin resistance is unknown. Chronic insulin resistance may result from down-regulated IRS protein levels, decreased IRS tyrosine phosphorylation or defects of IRS-PI3K association. Although decreased IRS levels may occur in Type 2 diabetes (59), compromised IRS tyrosine phosphorylation may be the predominant cause of insulin resistance (60,61). The present work provides the first evidence indicating no changes in IRS-1 or IRS-2 protein levels in this acute, hemorrhaged-induced hepatic insulin resistance, but a complete loss of insulin-induced IRS-1 and IRS-2 tyrosine phosphorylation. Association of IRS-1 or IRS-2 with PI3K was also completely lost, suggesting that hemorrhage-induced acute hepatic insulin resistance may be due to a defect in IRS-1/2 tyrosine phosphorylation, leading to a failure to associate with PI3K, and resulting in a loss of insulin signal transduction downstream of IRS-1 and IRS-2.

Following trauma and hemorrhage, there was an increase in serum TNF-α,

consistent with previous findings with this animal model (10). Although TNF-α concentrations were high in serum, local concentrations are more important than systemic TNF-α in inducing insulin resistance. Following trauma and hemorrhage, there was a dramatic increase of TNF-α protein in the liver. The question was then if this increased local TNF-α level was due to increased local production? Thus, the hepatic expression of TNF-α mRNA was examined by RT-PCR and a rapid increase in total hepatic TNF-α mRNA was found following trauma and hemorrhage. This suggests that following trauma and hemorrhage, an increase in hepatic TNF-α production may contribute to insulin resistance of the liver. Current data indicate that the hepatic Kupffer cells are the main source of TNF-α during an inflammatory response and it is proposed that they are the main source of this trauma and hemorrhage-induced increase in hepatic TNF-α mRNA and protein (62).

Due to the increase in circulating TNF-α, other cytokines and chemokines, and other hormones and growth factors, it was asked whether there were trauma and hemorrhage-induced increases in phosphorylation/activation of either of the three branches of the MAPK signaling pathway. There were significant increases in phosphorylation/activation of ERK1/2 and p38 [and see (36)], but little measurable change in phosphorylation/activation of JNK1/2.

There is mounting evidence that kinase-mediated serine/threonine phosphorylation of IRS proteins can result in insulin resistance by impairing the ability of IRS proteins to associate with the insulin receptor and/or inhibiting insulin-stimulated tyrosine phosphorylation of IRS proteins (25,63,64). Previous studies indicate that TNF-α promotes phosphorylation of IRS-1 at Ser307 by activating one or more MAPK signaling

pathways (31,44), and that Ser307 and Ser612 of IRS-1 can also be phosphorylated in response to activation of PKC (32,65). To answer whether the increase in local TNF-α could play a role in trauma and hemorrhage-induced insulin resistance, the serine phosphorylation of IRS-1 at Ser307 and Ser612 were examined. We found a 2-fold increase in phosphorylation of IRS-1 Ser307 following trauma and hemorrhage, with no change in phosphorylation of IRS-1 Ser612. Since Ser612 of IRS-1 was not phosphorylated following trauma and hemorrhage, it suggests that activation of PKC is not involved in the phosphorylation of IRS-1 serine residues, and that TNF-α is a prime candidate as a causative factor in the insulin receptor/IRS-1/PI3K signaling defect. However, the mechanisms by which TNF-α increases phosphorylation of IRS-1 at Ser307 are unknown. Further work needs to be performed to check the direct and specific role of one or more MAPK pathways, for instance the ERK and p38 pathways found to be activated in the present studies, or the role of other signaling pathways in the trauma and hemorrhage-induced increase of IRS-1 Ser307 phosphorylation.

A question is whether this modest 2-fold increase in serine phosphorylation of IRS-1 at Ser307 can completely explain the total loss of tyrosine phosphorylation of IRS-1 following trauma and hemorrhage. In support of this, insulin-resistant obese mice have an approximate 2.7-fold increase in hepatic IRS-1 Ser307 phosphorylation compared to lean animals (44). In fatty acid infusion-induced insulin resistance, rat soleus muscle IRS-1 Ser307 phosphorylation was increased 1.6-fold (33). This 1.6-2.7 fold increase in IRS-1 Ser307 phosphorylation in conditions of obesity (liver) and lipid (muscle) is similar to our finding, a 2-fold increase in hepatic IRS-1 Ser307 phosphorylation following trauma and hemorrhage. This suggests that the 2-fold increase in serine phosphorylation of IRS-1 at

Ser307 is involved in the development of hepatic insulin resistance following trauma and hemorrhage. However, other factors are likely also involved, possibly including other proinflammatory cytokines and catecholamines that rapidly increase following trauma and hemorrhage. Thus, further studies are necessary to determine the exact role of TNF-α and other factors in the acute development of hepatic insulin resistance following trauma and hemorrhage.

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Abbreviations

extracellular-regulated kinase 1/2	(ERK1/2)
Jun N–terminal kinase 1/2	(JNK1/2)
insulin receptor	(IR)
insulin receptor substrate 1 , 2	(IRS-1, IRS-2)
phosphatidylinositol-3 kinase	(PI3K)
tumor necrosis factor-alpha	(TNF-α)
trauma	(T)
trauma and hemorrhage	(TH)

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Figure Legends

Figure 1. Unchanged total protein levels of the insulin receptor (IR), IRS-1 and decreased insulin-induced P-Akt in rat liver following trauma alone or trauma and hemorrhage. Rats were subjected to trauma alone (T) or trauma and hemorrhage (TH). At 0' (following trauma but before any further treatment), or 90' (without or with hemorrhage) or 210' (without or with 60 min of recovery following 60 min of resuscitation), either saline (-) or 5 U. of insulin (+) was injected into the portal vein and 1 min later the liver was removed and protein extracts were prepared, resolved by SDS-PAGE and subjected to Western blot analysis using specific anti-Phospho-Akt, anti-IR and anti-IRS-1 antibodies. Representative Western blots are presented for phosphorylation of Akt (top panel), total proteins of IR (middle panel) and IRS-1 (lower panel). The data shown are representative of three experiments with similar results (3 rats in each group).

Figure 2. Unchanged insulin-induced tyrosine phosphorylation of insulin receptor in rat liver following trauma alone or trauma and hemorrhage. At the same time points and treatment regimens described in Figure 1, either saline (-) or 5 U. insulin (+) was injected into the portal vein and 1 min later the liver was removed. Representative Western blots are presented: (A) Total tissue lysate was immunoprecipitated with specific anti-IR antibody, then subjected to Western blot analysis by specific anti-IR (upper panel) and anti-phospho-tyrosine antibodies (lower panel). (B) Tissue lysate was subjected to

Western blot analysis using specific anti-IR pY1146/1150/1151 and anti-IR pY960 antibodies. The data shown are representative of three experiments with similar results (3 rats in each group).

Figure 3.Changes in insulin-induced tyrosine phosphorylation of IRS-1 following trauma alone or trauma and hemorrhage. At the same time points and treatment regimens described in Figure 1, either saline (-) or 5 U insulin was injected into the portal vein and 1 min later the liver was removed. (A) Representative Western blots; total tissue lysate was immunoprecipitated with specific anti-IRS-1 antibody, then subjected to Western blot analysis by specific anti-IRS-1 (upper panel), anti-phospho-tyrosine (middle panel) and anti-PI3K (lower panel) antibodies. (B) Autoradiographs were quantified by scanning densitometry and the data (fold induction by insulin) presented as the mean ± SEM of 3 rats in each group. * $p<0.05$ compared with T 0' time point.

Figure 4. Changes in insulin-induced tyrosine phosphorylation of IRS-2 following trauma alone or trauma and hemorrhage. At the same time points and treatment regimens described in Figure 1, either saline (-) or 5 U insulin was injected into the portal vein and 1 min later the liver was removed. (A) Representative Western blots; total tissue lysate was immunoprecipitated with specific anti-IRS-2 antibody, then subjected to Western blot analysis by specific anti-IRS-2 (upper panel), anti-phospho-tyrosine (middle panel) and anti-PI3K (lower panel) antibodies. (B) Autoradiographs were quantified by scanning

densitometry and the data (fold induction by insulin) presented as the mean ± SEM of 3 rats in each group. * $p<0.05$ compared with T 0' time point.

Figure 5. Changes in circulating TNF-α levels following trauma alone or trauma and hemorrhage. At the same time points and treatment regimens described in Figure 1, blood TNF-α concentrations were measured by ELISA. Data are presented as the mean ± SEM of blood samples from 3 rats in each group. * $p<0.05$ compared to the T 0' time point.

Figure 6. Changes in TNF-α protein levels following trauma alone or trauma and hemorrhage. At the same time points and treatment regimens described in Figure 1, total liver protein was subjected to Western blot analysis using specific anti-TNF-α antibody (upper panel is a representative blot). Autoradiographs were quantified by scanning densitometry and the data presented as the mean ± SEM of 3 rats in each group (lower panel). * $p<0.05$ compared with T 0' time point.

Figure 7. Changes in TNF-α mRNA levels in the liver following trauma alone or trauma and hemorrhage. At the same time points and treatment regimens described in Figure 1, liver total RNA was extracted and subjected to RT-PCR analysis using β-actin as an internal control. Upper panel is a representative gel photo. Autoradiographs were

quantified by scanning densitometry and the data presented as the ratio of TNF-α to β-actin of 3 rats in each group (lower panel). * $p<0.05$ compared with T 0' time point.

Figure 8. Changes in serine phosphorylation of IRS-1 following trauma alone or trauma and hemorrhage. At the same time points and treatment regimens described in Figure 1, the liver was removed. (A) Representative Western blots; total tissue lysate was subjected to Western blot analysis with specific anti-IRS-1 pS307, anti-IRS pS612 and anti-total IRS-1 antibodies. (B) Autoradiographs were quantified by scanning densitometry and the data presented as the mean ± SEM of 3 rats in each group. * $p<0.05$ compared with T 0' time point.

Figure 9. Changes in phosphorylation of P-ERK, P-p38 and P-JNK following trauma alone or trauma and hemorrhage. At the same time points and treatment regimens described in Figure 1, the liver was removed. (A) A representative Western blot; total tissue lysate was subjected to Western blot analysis with specific anti-P-ERK1/2, anti-P-p38, anti-P-JNK1/2 and anti-total ERK antibodies. (B) Autoradiographs were quantified by scanning densitometry and the data presented as the mean ± SEM of 3 rats in each group. * $p<0.05$ compared with T 0' time point.

Fig 1

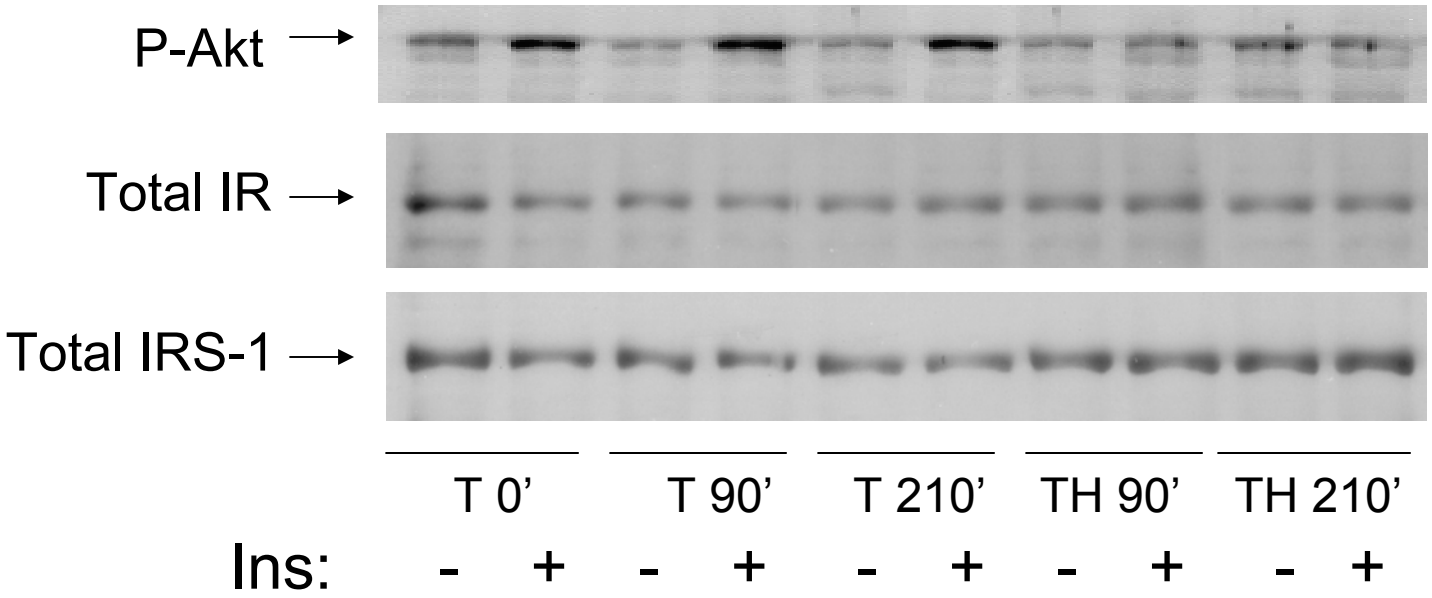


Fig 2A

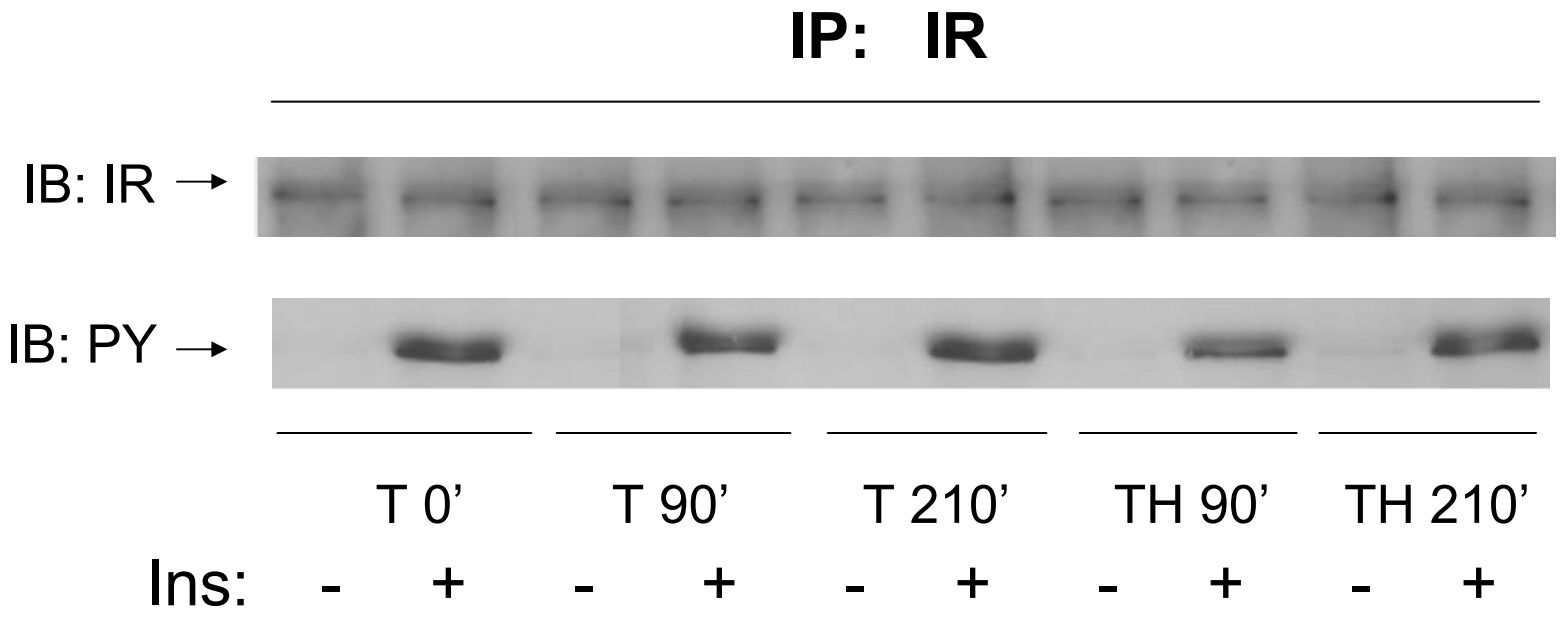


Fig 2B

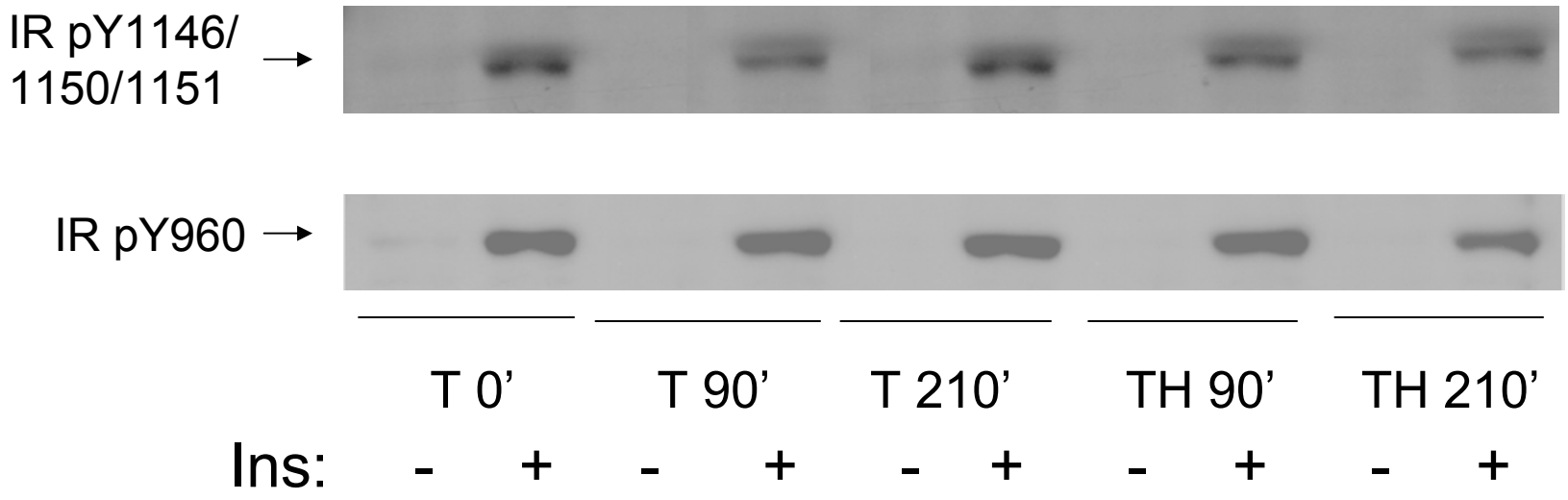


Fig 3A

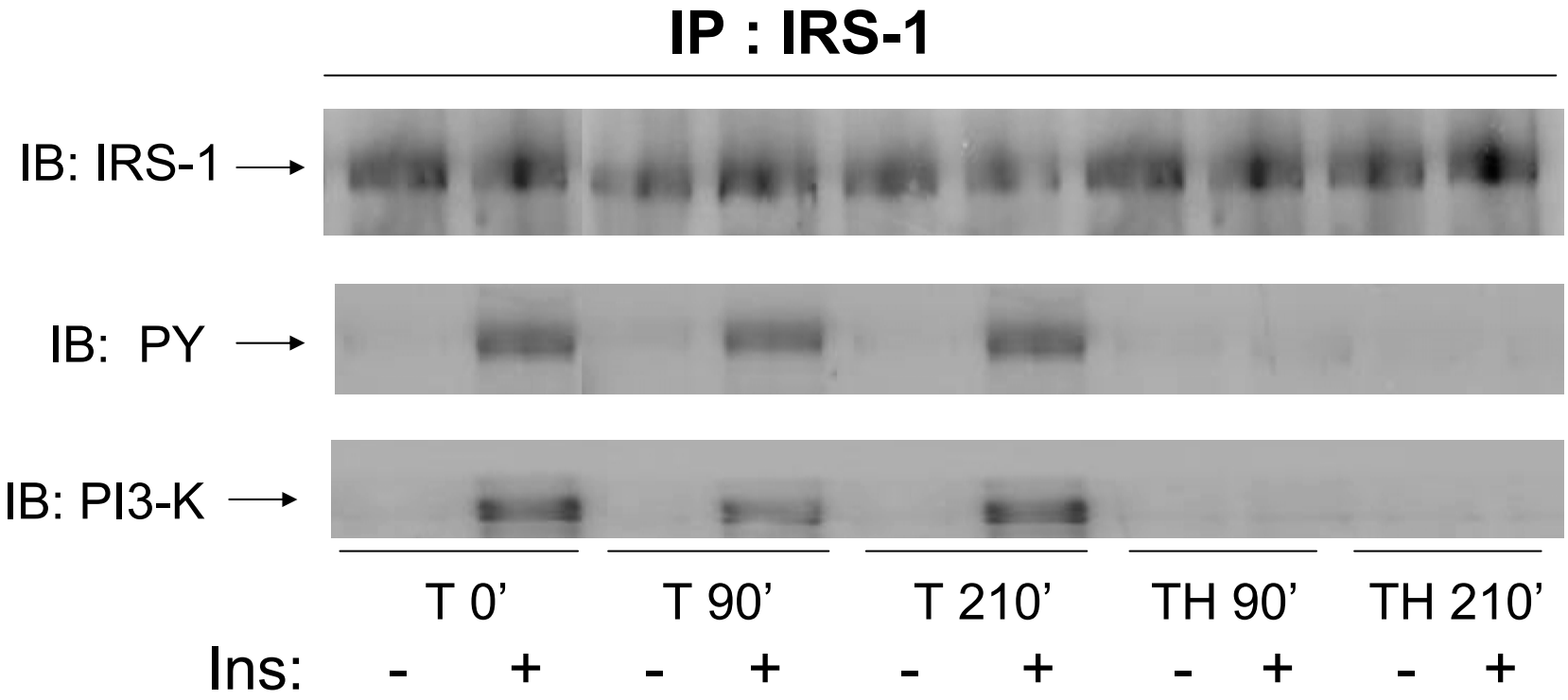


Fig 3B

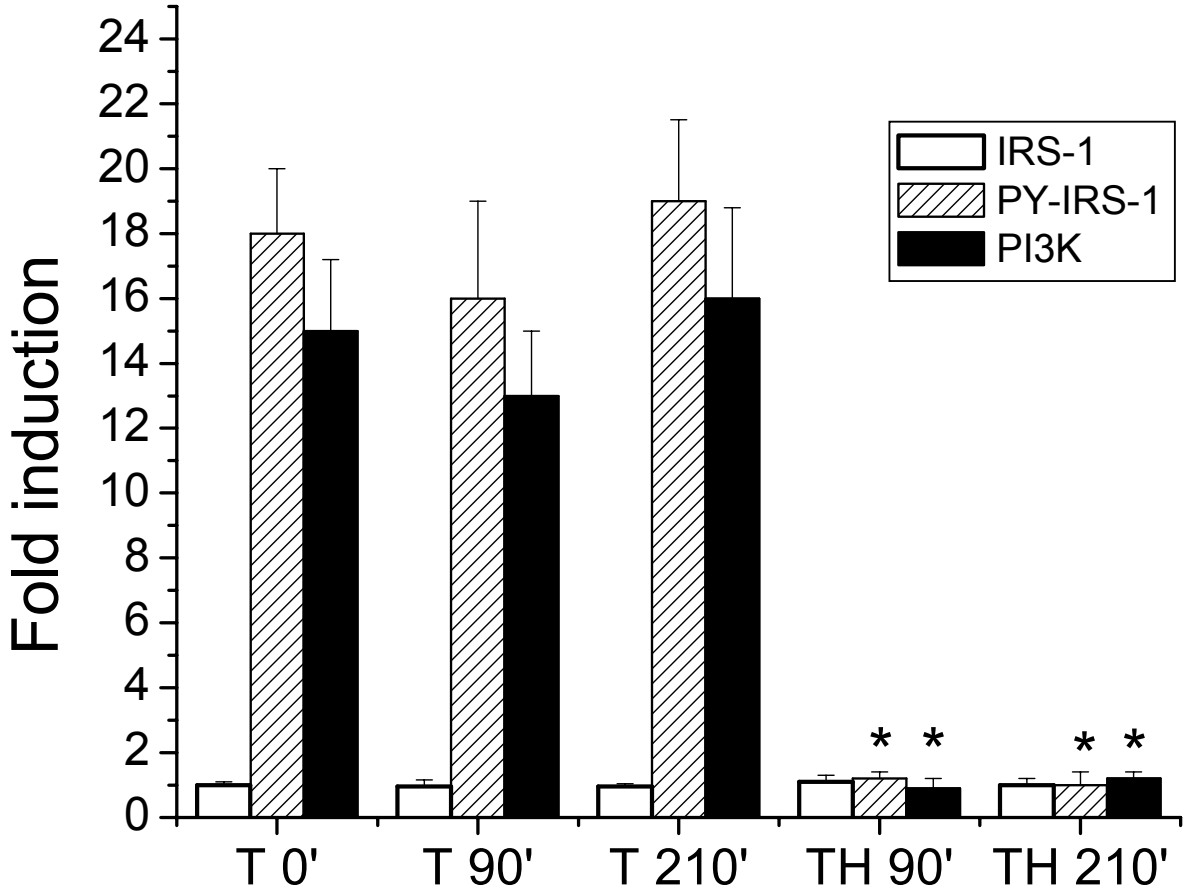

Fold induction
24
22
20
18
16
14
12
10
8
6
4
2
0
IRS-1
PY-IRS-1
PI3K
* *
* *
T 0'
T 90'
T 210'
TH 90'
TH 210'

Fig 4A

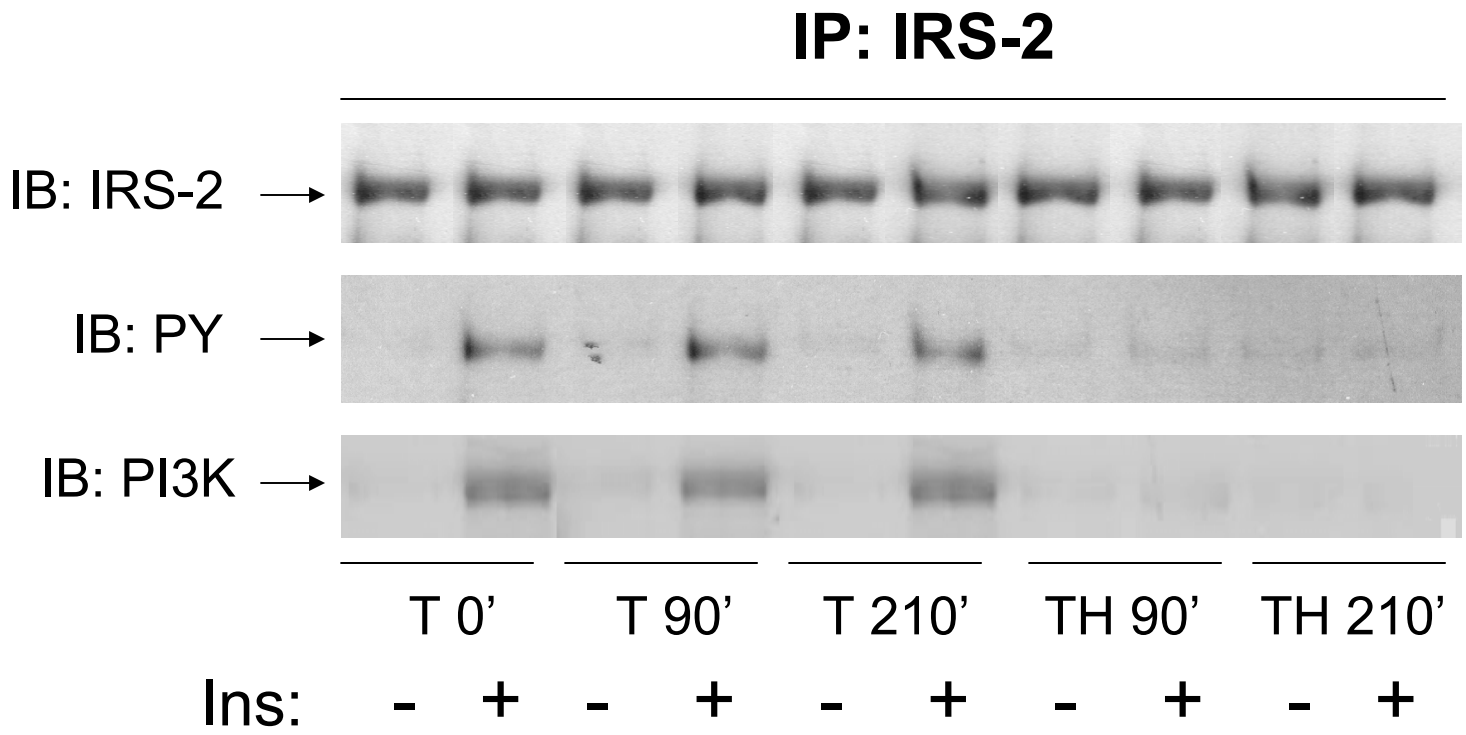


Fig 4B

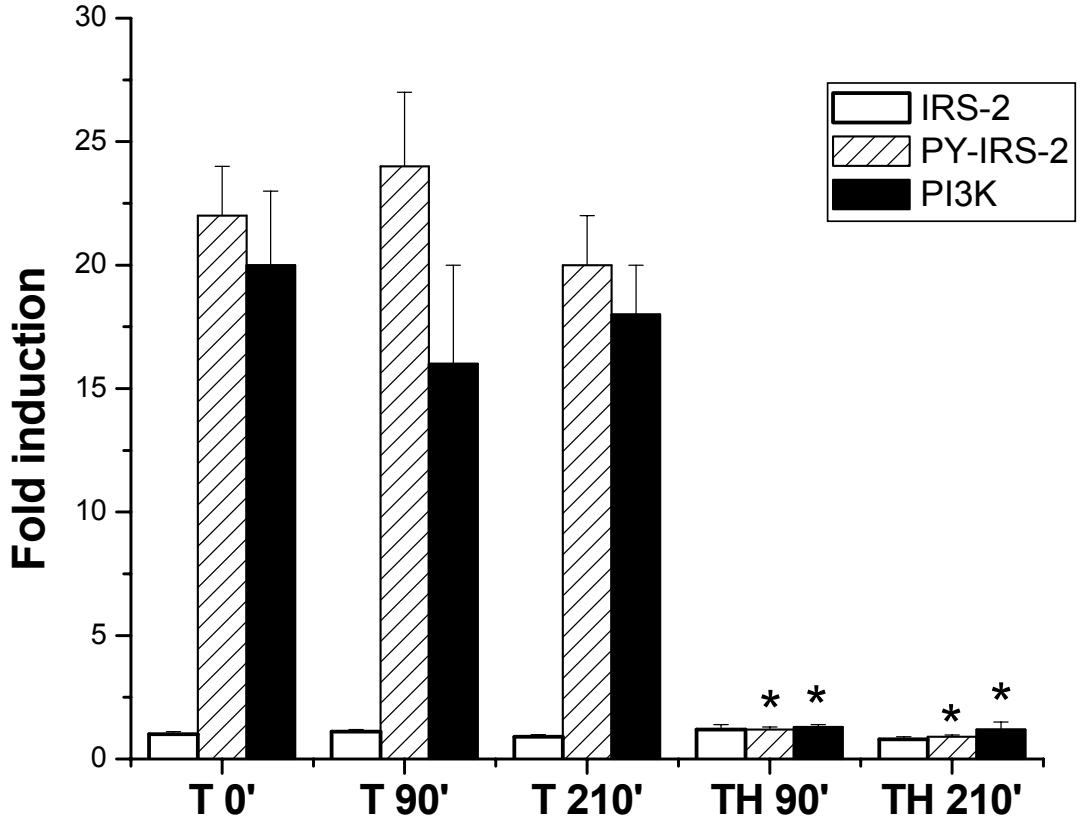

30
25
20
15
10
5
0
Fold induction
IRS-2
PY-IRS-2
PI3K
*
*
*
*
T 0'
T 90'
T 210'
TH 90'
TH 210'

Fig 5

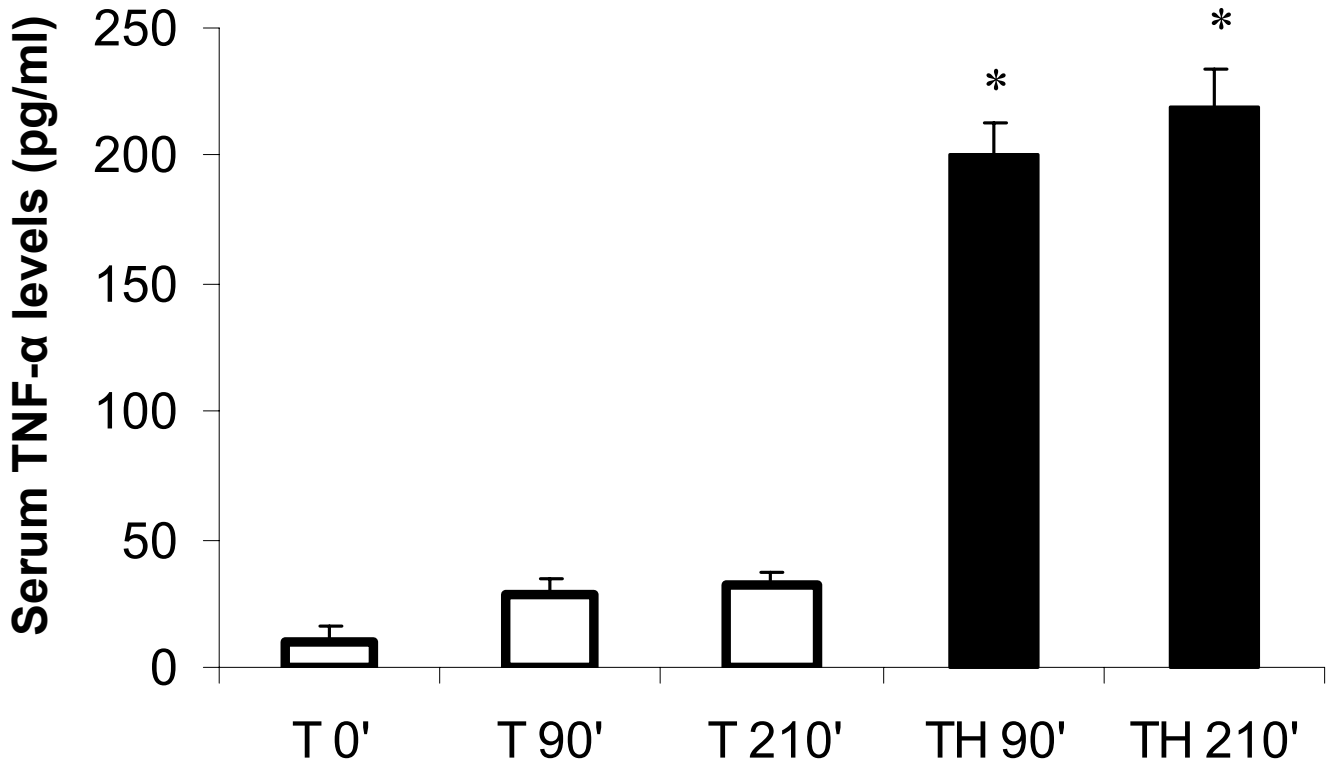

Serum TNF-α levels (pg/ml)
250
200
150
100
50
0
*
*
T 0'
T 90'
T 210'
TH 90'
TH 210'

Fig 6

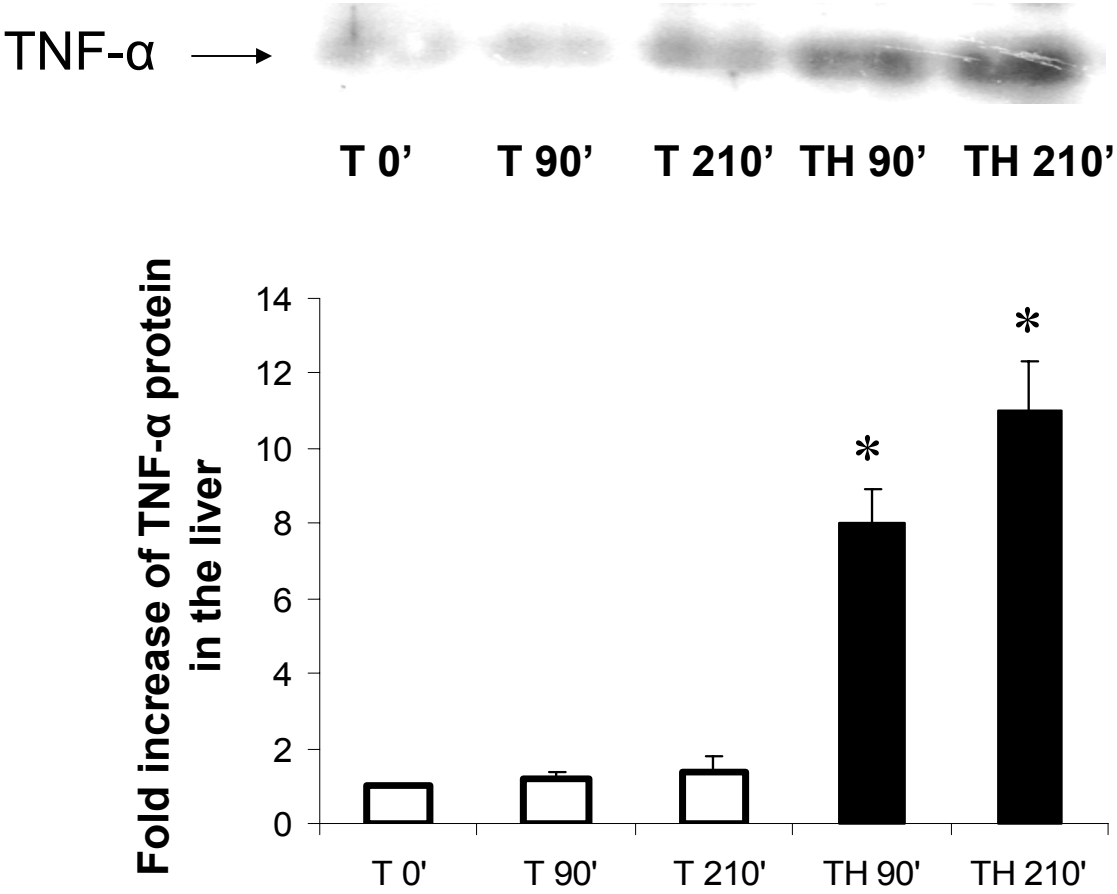

TNF-α
T 0’
T 90’
T 210’
TH 90’
TH 210’
Fold increase of TNF-α protein in the liver
14
12
10
8
6
4
2
0
*
*
T 0'
T 90'
T 210'
TH 90'
TH 210'

Fig 7

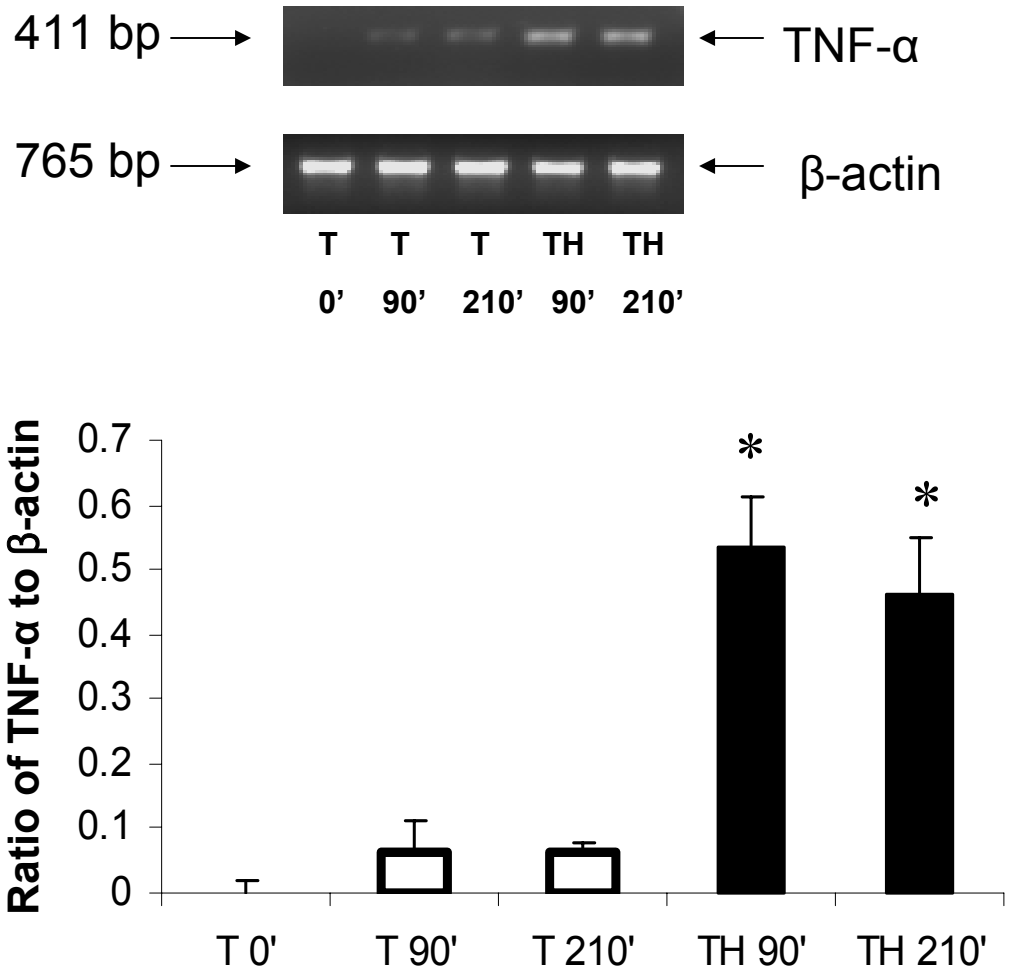

411 bp
TNF-α
765 bp
β-actin
T 0'
T 90'
T 210'
TH 90'
TH 210'
Ratio of TNF-α to β-actin
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
T 0'
T 90'
T 210'
TH 90'
TH 210'

Fig 8A

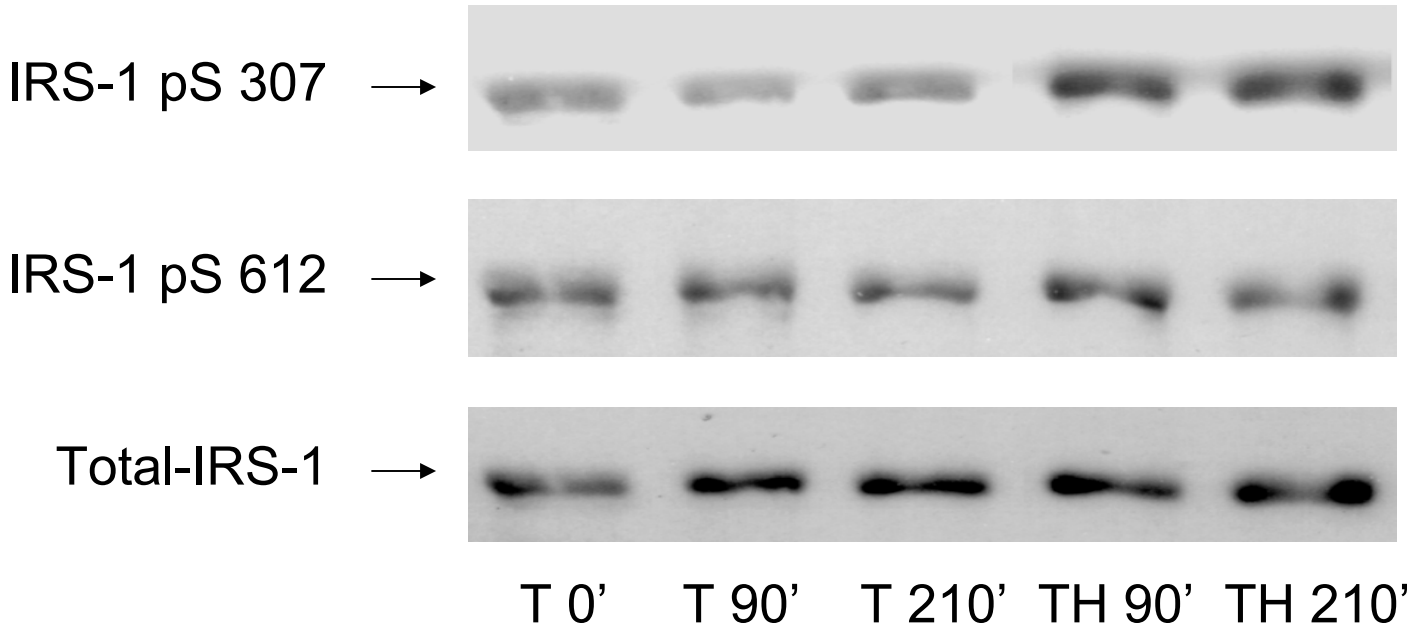


Fig 8B

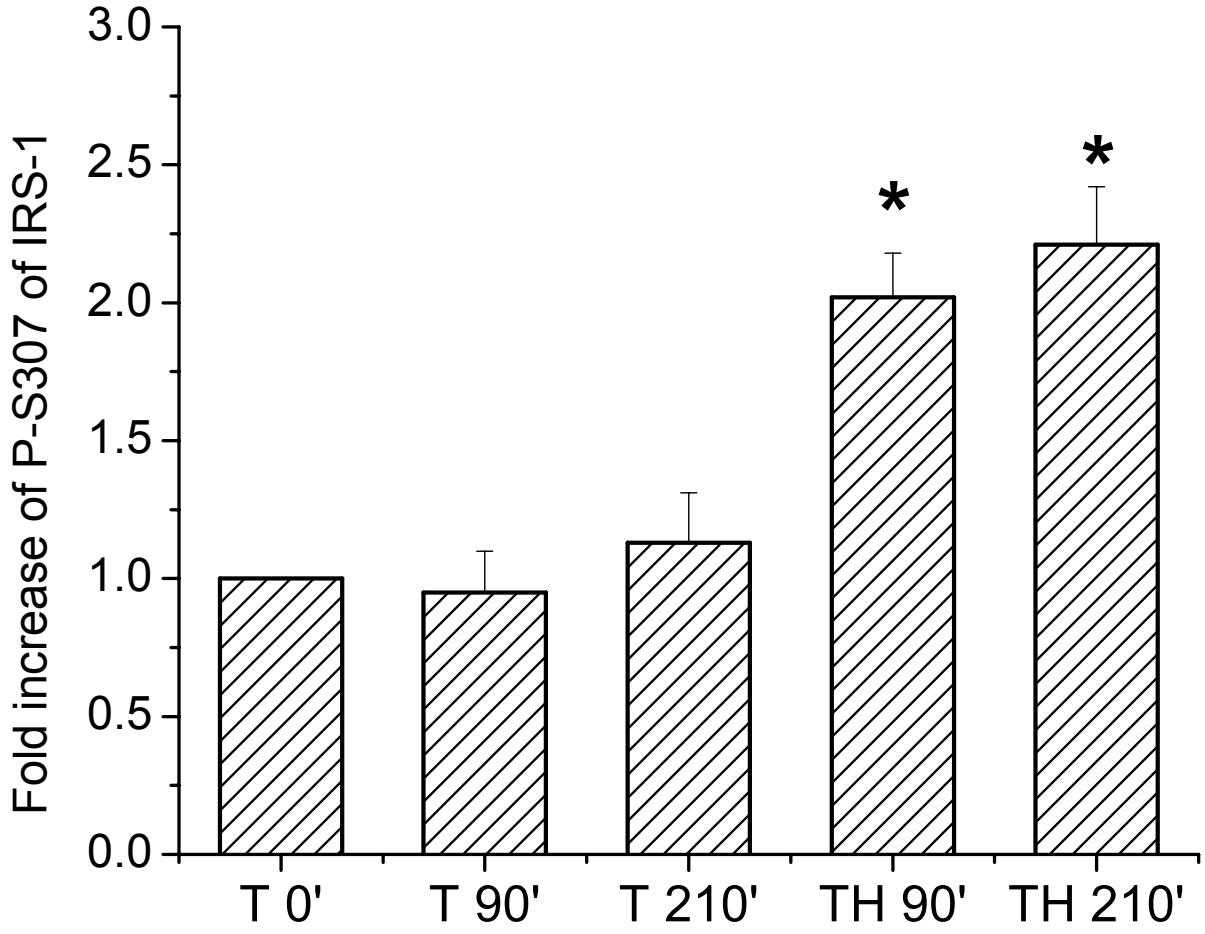

Fold increase of P-S307 of IRS-1
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
T 0'
T 90'
T 210'
TH 90'
TH 210'

Fig 9A

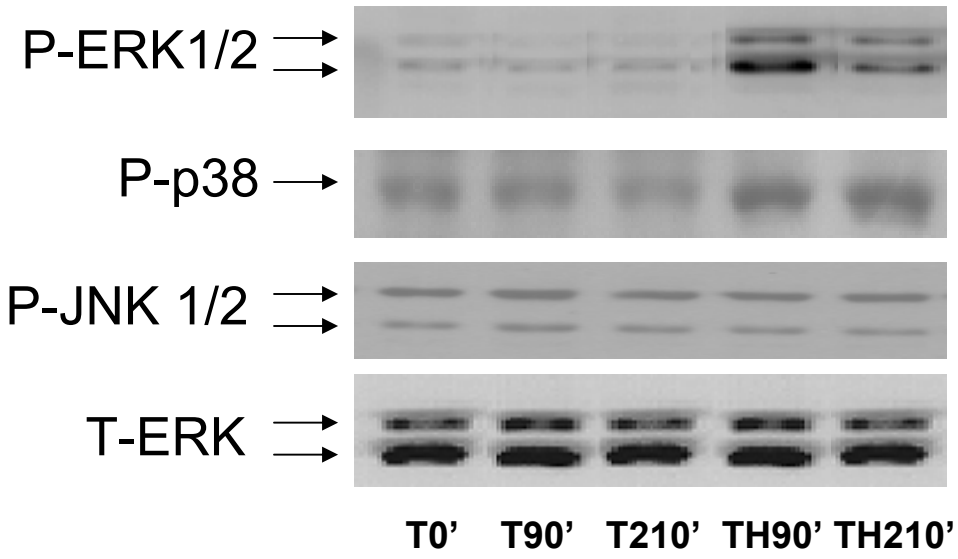
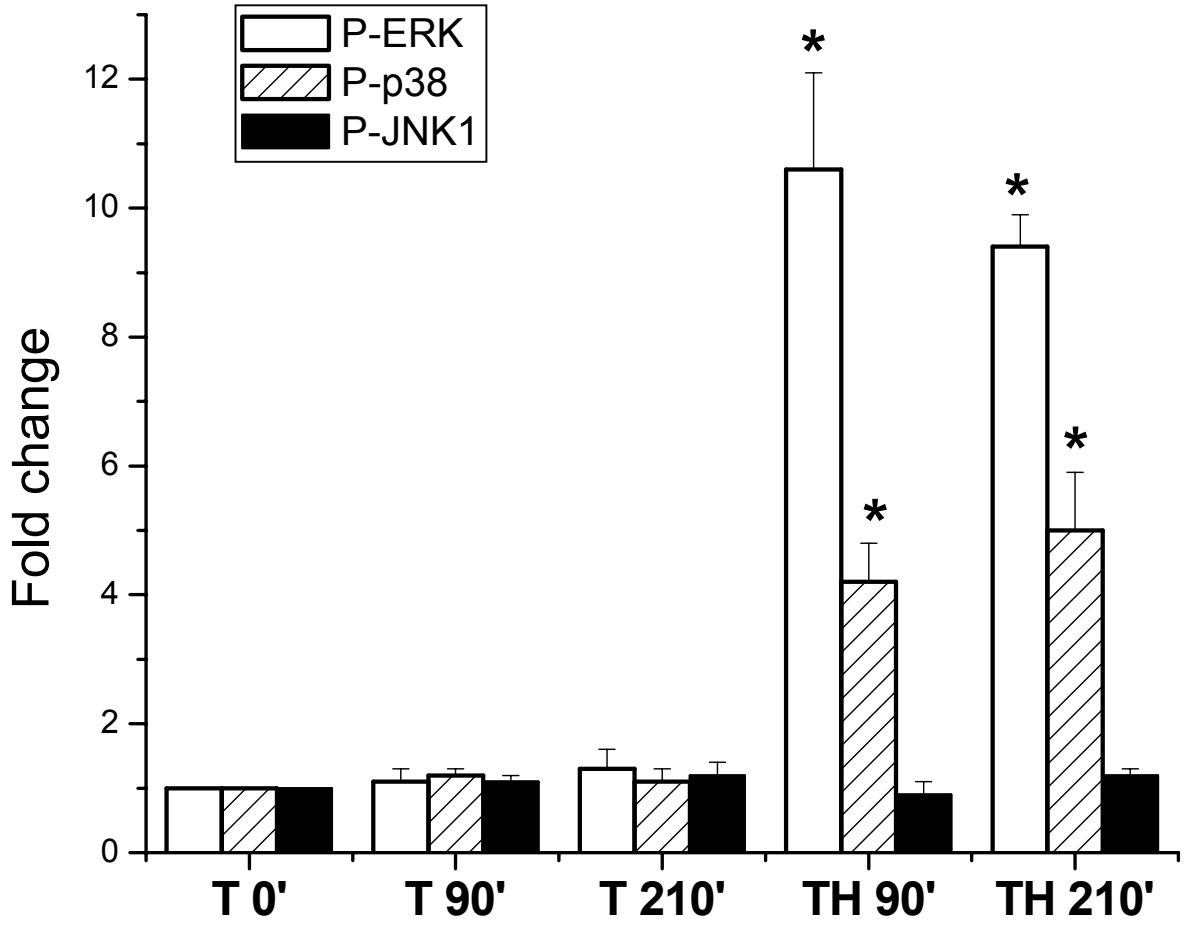


Fig 9B


P-ERK
P-p38
P-JNK1
Fold change
0
2
4
6
8
10
12
*
*
*
*
T 0'
T 90'
T 210'
TH 90'
TH 210'