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

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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 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 TNFα and TNFα-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 after hemorrhage. Hepatic levels of TNFα mRNA and protein were increased as was phosphorylation of IRS-1 serine 307 after 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 TNFα may play a role in inducing this hepatic insulin resistance after trauma and hemorrhage. (Endocrinology 145: 5168–5176, 2004)

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 TNFα, 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 after trauma and infection. In recent work, intensive insulin therapy has been used 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 auto-phosphorylation 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-phosphatidylinositol 3-kinase (PI3K)-Akt pathway and the Ras-MAPK (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 TNFα 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 after 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. TNFα, free fatty acids, and other factors can induce insulin resistance by activating serine/threonine phosphorylation that then inhibits insulin-stimulated tyrosine (Tyr) phosphorylation of IRS proteins (25, 31–33).

There is consistent evidence of muscle insulin resistance after 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

Abbreviations: IR, Insulin receptor; IRS, IR substrate; JNK, Jun N-terminal kinase; PI3K, phosphatidylinositol-3 kinase; Ser307, serine 307; Ser612, serine 612; T, trauma alone; TH, trauma and hemorrhage; Tyr, tyrosine.

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becomes resistant to insulin, increased hepatic gluconeogenesis can contribute to the hyperglycemia and hyperinsulinemia that 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 or 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 increased as was the phosphorylation at the serine 307 (Ser307) of IRS-1 after trauma and hemorrhage. These data suggest that after 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 TNFα and serine phosphorylation of IRS-1.

Materials and Methods

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 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, MA) 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 Guide 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) that 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 that 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 after completion of the 60 min resuscitation period (TH 210’ = 90 min hemorrhage + 60 min resuscitation + 60 min recovery).

Measurement of plasma TNFα levels

Immediately before insulin or saline injection, blood was withdrawn from the right femoral artery for TNFα measurement. TNFα levels were measured by a rat TNFα ELISA kit (BioSource, Camarillo, CA).

Immunoprecipitation and immunoblots protocol

Liver tissue from each animal (approximately 0.2 g) was homogenized in 1 ml lysis buffer containing 20 mM HEPES (pH 7.9), 1.5 mM MgCl2, 20 mM KCl, 20% glycerol, 0.2 mM EDTA, 2 mM Na3VO4, 10 mM NaF, 1% Triton X-100, 0.2 mM PMSF, 10 mg/ml aprotinin, and 10 mg/ml leupeptin. Tissue lysates were centrifuged at 10,000 × g for 10 min, and the supernatants were stored at −80 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 Biotechnology, Santa Cruz, CA) or IRS-1 or IRS-2 (Upstate Biotechnology, Lake Placid, NY) overnight at 4 C. Protein A-agarose (fast flow, Pharmacia Biotech, Providence, RI) was then added, and incubations continued for 2 h at 4 C. Immunoprecipitated proteins were resolved by sodium dodecyl sulfate, 10% PAGE, and transferred to nitrocellulose paper. The Western transfers were immunoblotted with anti-IR (Santa Cruz Biotechnology), anti-IRS-1, anti-IRS-2, anti-phospho-Akt, and anti-phospho-subunit of PI3K antibodies (Upstate Biotechnology).

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-Ser307, and anti-phospho-Ser612 of IRS-1 and anti-TNFα antibodies (Biosource) followed by addition of horseradish peroxidase-conjugated secondary antibody for detection of bound antibody by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). Each blot was stripped 30 min at 50 C in stripping buffer [100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mM Tris-HCl (pH 6.7)] 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 C for 50 min. Of this cDNA, 2 μl were added to the PCR. Each PCR 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 C for 30 sec, annealing at 55 C for 30 sec, and extension at 72 C for 1 min. β-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 TNFα (411 bp) were 5 ’-TCCCAACAAAGGAGGAGGAAATT-3 ’ and 5 ’-TCATACCAGGGCTTGAGCTCAG-3 ’. For Western blotting, 15 μg protein per lane was resolved by 10% PAGE and transferred to nitrocellulose paper. The Western transfers were immunoblotted with anti-IR, anti-IRS-1, anti-IRS-2, anti-phospho-Ser307, and anti-phospho-Ser612 of IRS-1 and anti-TNFα antibodies (Biosource) followed by addition of horseradish peroxidase-conjugated secondary antibody for detection of bound antibody by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). Each blot was stripped 30 min at 50 C in stripping buffer [100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mM Tris-HCl (pH 6.7)] and then reprobed with a different antibody (36).

Densitometric and statistical analysis

Enhanced chemiluminescence images of immunoblots were scanned and quantified using Zero D-Scan (Scanalytics Corp., Fairfax, VA). All data were analyzed by one-way ANOVA using the InStat statistical program by GraphPad Software, Inc. (San Diego, CA).

Results

IR and IRS-1 do not change after trauma and hemorrhage

Previously we found that after trauma and hemorrhage, the IR/IRS/PI3K/Akt signaling pathway was compromised...
(36). After trauma and hemorrhage, insulin-induced phosphorylation of Akt was completely lost (Fig. 1, lanes 8 and 10, upper panel). Because reduced insulin signaling may be due to a decrease in IR or IRS protein levels, experiments were performed to determine whether such changes occur after trauma and hemorrhage. After trauma alone, or trauma and hemorrhage, total protein levels of insulin receptor and IRS-1 did not change (Fig. 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.

**IR autophosphorylation does not change after trauma and hemorrhage**

Because the total cellular IR content was not decreased after trauma and hemorrhage, insulin-induced IR phosphorylation was investigated. After trauma alone, or trauma and hemorrhage, insulin-induced total IR tyrosine phosphorylation was not changed (Fig. 2A). Although IR total tyrosine phosphorylation was not altered, there was still the possibility of alteration of phosphorylation in specific tyrosines in the IR. Thus, phosphorylation of four tyrosine sites in the IR, 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 IR β-subunit, and their phosphorylation is correlated with full activation of the IR tyrosine kinase (21, 38–41). There was no measurable change in phosphorylation of these sites after either trauma or trauma and hemorrhage (Fig. 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 after either trauma or trauma and hemorrhage (Fig. 2B, bottom panel). Together, these data suggest that insulin signaling after trauma and hemorrhage is not compromised at the level of the IR.

**Tyrosine phosphorylation of IRS-1 decreases after trauma and hemorrhage**

Next, any changes in tyrosine phosphorylation of IRS-1 were examined. After 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 after trauma alone (T 0', T 90', and T 210', Fig. 3A, middle panel). Data from multiple animals in each group were quantified and presented as fold induction by insulin (+) compared with no insulin (−) injection at the same time points after 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 (Fig. 3B, hatched bars). This is indicative of a significant loss of insulin signaling via the IR-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 after fluid resuscitation (TH 210').
Association of IRS-1 with PI3K decreases after trauma and hemorrhage

Because association of IRS-1 protein with PI3K depends on 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 after trauma and hemorrhage (Fig. 3A, lower panel). In the trauma-alone group (T 0/H11032), there was a 15-fold increase in association of IRS-1 and PI3K 1 min after insulin injection. There was little change in this insulin-induced association at the T 90/H11032 and T 210/H11032 time points (13- and 16-fold, respectively). However, this association was abolished in both the TH 90/H11032 and TH 210/H11032 groups (Fig. 3B, solid bars).

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

In experiments similar to those with IRS-1, after 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/H11032 and TH 210/H11032). There was no change of IRS-2 phosphorylation after trauma alone (T 0/H11032, T 90/H11032, and T 210/H11032; Fig. 4A, middle panel).

Data from multiple animals in each group were quantified and presented as fold induction by insulin (+) compared with no insulin (−) injection at the same time points after 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 after insulin injection at the T 0/H11032, T 90/H11032, and T 210/H11032 time points, respectively. However, after trauma and hemorrhage, there was a large decrease of insulin’s ability to induce tyrosine phosphorylation of IRS-2 (at both TH 90/H11032 and TH 210/H11032 time points; Fig. 4B, hatched bars). This is indicative of a significant loss of insulin signaling via the IR-IRS-PI3K pathway, resulting in a loss of IRS-2 phosphorylation.

Similar to the loss of insulin-induced association of IRS-1 with PI3K after trauma and hemorrhage, there was also a complete loss of insulin-induced PI3K association with IRS-2. In the trauma alone group (T 0/H11032), there was a 20-fold increase in association of IRS-1 and PI3K 1 min after insulin injection. There was little change in this insulin-induced association at the T 90/H11032 and T 210/H11032 time points (16- and 18-fold, respec-
Serum TNFα concentrations are elevated after trauma and hemorrhage

TNFα is thought to contribute to insulin resistance, and proinflammatory cytokines are induced after trauma and hemorrhage. However, it was unknown whether TNFα would be increased rapidly enough to contribute to the insulin resistance that occurred within the 90-min hemorrhage period. Thus, we next measured serum TNFα levels. Circulating TNFα levels after trauma alone increased slightly at both 90 and 210 min, compared with T 0’ animals, from 10 to 28 and 32 pg/ml, respectively. This increase in serum TNFα 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 TNFα levels to 200 pg/ml and a further increase to 220 pg/ml at 60 min after the completion of fluid resuscitation (TH 210’; Fig. 5).

Expression of TNFα is increased in the liver after trauma and hemorrhage

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

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

Serine phosphorylation of IRS-1 is increased after trauma and hemorrhage

Recent evidence suggests that TNFα 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 after trauma and hemorrhage was investigated. Basal levels of phosphorylation of IRS-1 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 with T 0’. In contrast, there were significant increases in IRS-1 Ser307 phosphorylation after trauma and hemorrhage, at both TH 90’ (2.02-fold) and TH 210’ (2.21-fold) time points (Figs. 8A, upper panel, and 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 (Fig. 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 (Fig. 8A, lower panel).

Phosphorylation/activation of ERK and p38, but not c-Jun N-terminal kinase (JNK), is increased after trauma and hemorrhage

Because previous studies indicate that activation of p38, ERK, or JNK may be involved in the phosphorylation of Ser307 of IRS-1, we next examined whether there were any changes in the phosphorylation/activation state of these MAPKs. After 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’) after trauma and hemorrhage (Fig. 9, A and B). However, there were no significant changes in phosphorylation of ERK1/2 or p38 after trauma alone. Unlike ERK and p38, there were no measurable changes in phosphorylation/activation of JNK after trauma and hemorrhage (Fig. 9). Because increased activation of ERK and p38 are associated with increased Ser307 phosphorylation of IRS-1, phosphorylation/activation of ERK and/or p38 may contribute to increased Ser307 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 after 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 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 after injury and critical illness.

It is well established that insulin resistance develops in muscle and adipose tissue after 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-P13K-Akt signaling and increased IGF binding protein-1 expression (an insulin-inhibited, P13K-Akt-dependent gene), after 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 P13K, or numerous other postreceptor defects.

After binding of insulin to the IR and activation of the cytoplasmic protein-tyrosine kinase domain (42, 58), there is a rapid phosphorylation of multiple tyrosine residues of the IR β-subunit. The catalytic loop within the 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, to achieve full activation of the IR tyrosine kinase activity (21, 40, 41). Tyrosine 960 in rat, corresponding to 972 in human, is part of the juxtamembrane Asn-Pro-Glu-Tyr 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 IR protein levels and insulin-induced total tyrosine phosphorylation of the IR after trauma and hemorrhage. Although binding of insulin was not measured, no changes in insulin-induced total tyrosine phosphorylation of the IR after trauma and hemorrhage suggests little or no change in insulin binding and activation of the IR tyrosine kinase activity. In addition, after trauma and hemorrhage, there was no change in tyrosine phosphorylation of the IR at Tyr960, which would suggest little or no defect in the potential ability of the IR to associate with IRS proteins. Lastly, using a specific antibody that recognizes the IR only after IR is phosphorylated at all three tyrosines, 1146, 1150, and 1151, there were no changes in tyrosine phosphorylation, which implies no defect in IR tyrosine kinase activity. Thus, the IR seemed to be functioning relatively normally, and hemorrhage-induced hepatic insulin resistance was likely not an IR defect, but occurred at a level downstream of the initial activation of the IR 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-P13K 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, hemorrhage-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 P13K 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 P13K and resulting in a loss of insulin signal transduction downstream of IRS-1 and IRS-2.

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

Due to the increase in circulating TNFα, 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 any of the three branches of the MAPK signaling pathway. There were significant increases in phosphorylation/activation of ERK1/2 and p38 (see Ref. 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 TNFα 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 protein kinase C (32, 65). To answer whether the increase in local TNFα 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 after trauma and hemorrhage, with no change in phosphor-
ylation of IRS-1 Ser612. Because Ser612 of IRS-1 was not phosphorylated after trauma and hemorrhage, it suggests that activation of protein kinase C is not involved in the phosphorylation of IRS-1 serine residues and that TNFα is a prime candidate as a causative factor in the IR/IRS-1/P3K signaling defect. However, the mechanisms by which TNFα 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, i.e., 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 after 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 with 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- to 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 after 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 after trauma and hemorrhage. However, other factors are likely also involved, possibly including other proinflammatory cytokines and catecholamines that rapidly increase after trauma and hemorrhage. Thus, further studies are necessary to determine the exact role of TNFα and other factors in the acute development of hepatic insulin resistance after trauma and hemorrhage.

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