Regulation of hepatic insulin receptor activity following injury

Shaoning Jiang,1 Tatiana A. Gavrikova,1 and Joseph L. Messina1,2

1Department of Pathology, Division of Molecular and Cellular Pathology, University of Alabama at Birmingham, Birmingham, Alabama; and 2Veterans Affairs Medical Center, Birmingham, Alabama

Submitted 25 April 2013; accepted in final form 27 March 2014

Jiang S, Gavrikova TA, Messina JL. Regulation of hepatic insulin receptor activity following injury. Am J Physiol Gastrointest Liver Physiol 306: G886–G892, 2014. First published April 3, 2014; doi:10.1152/ajpgi.00128.2013.—Impaired insulin receptor (IR) activity has been found in various models of insulin resistance, including models of injury or critical illness and Type 2 diabetes. However, mechanisms that modulate IR function remain unclear. With an animal model of critical-illness diabetes, we found insulin-induced IR tyrosine phosphorylation in the liver was impaired as early as 15 min following trauma and hemorrhage. Possible mechanisms for this defect were examined, including IR protein levels and IR posttranslational modifications. The total amounts of hepatic IRα and IRβ subunits and the membrane localization of the IR were not altered by trauma and hemorrhage, and, likewise, no change in IR tyrosine nitration was found in the liver. However, there was a decrease in the level of protein O-linked β-N-acetylglucosamine (O-GlcNac) modification on Ser/Thr in the liver following trauma and hemorrhage. Inhibition of JNK increased IR O-GlcNac modification, implicating an involvement of JNK. These findings suggest that a balance between O-GlcNac modification and JNK-induced phosphorylation may exist, with decreased Ser/Thr O-GlcNac modification following trauma and hemorrhage, allowing JNK to phosphorylate the IR on neighboring Ser/Thr residues, which subsequently inhibits IR activity. The present studies suggest potential mechanisms of hemorrhage-induced defects in IR activity and a potential role for acutely decreased O-GlcNac and increased serine phosphorylation of the IR.

O-linked β-N-acetylglucosamine; c-Jun NH2-terminal kinase; insulin resistance; liver; injury

The Insulin Receptor (IR) consists of two extracellular α subunits and two transmembrane β subunits, disulfide linked into an α2β2 heterotetrameric complex. Binding of insulin to the α subunit results in a conformational change and subsequent activation of the receptor’s protein tyrosine kinase activity, which, in turn, activates downstream insulin signaling (14, 23). Defects in IR activity have been found in patients with Type 2 diabetes (23) and in various animal models of insulin resistance, including after sepsis (17) and viral infections (11). With animal models of surgical trauma and hemorrhage, we previously observed impaired IR activity following trauma and hemorrhage, which is characterized by defects in insulin-induced IR phosphorylation on multiple tyrosine residues (12, 13, 15, 16, 21, 22, 25, 26). Although the evidence suggests that acute and chronic regulation of IR contributes to impaired insulin sensitivity and glucose homeostasis, the molecular mechanisms underlying the impaired IR activation after injuries remain largely unclear.

Possible mechanisms accounting for impaired insulin signaling at the level of IR include cell surface IR content or modification of IR enzymatic activity. Reduced IR cell surface content and insulin binding can be caused by increased IR degradation, decreased IR transcription or translation, or increased IR intracellular localization. Reduced IR kinase activity can also result from posttranslational modifications, which include tyrosine nitration, Ser/Thr phosphorylation, and glycosylation (23). Tyrosine nitration of IR is involved in lipid-induced insulin resistance in both liver and muscle (4). Serine (S944) phosphorylation of the IR likely contributes to insulin resistance in the liver of obese rats (18), and multiple serine kinases have been implicated in IR serine phosphorylation (3, 18). JNK, a serine kinase, is widely known to be involved in both the acute and chronic development of insulin resistance through inducing phosphorylation of insulin receptor substrate 1 (IRS1) on serine residues (6, 10, 13, 16, 22, 24, 25, 27, 28). Hepatic activation of JNK activity occurs rapidly following trauma and hemorrhage, and inhibition of JNK1 results in improved IR activity and downstream insulin signaling (13, 22, 25). Blocking JNK1 expression also inhibits the decrease of IR tyrosine phosphorylation in 3T3 adipocytes by free fatty acids (19), suggesting that JNK1 can also interfere with insulin signaling directly at the IR, although this has not been clearly demonstrated.

Another modification that can occur on serine residues is O-linked β-N-acetylglucosamine (O-GlcNac) catalyzed by OGT (O-GlcNac transferase) (7). Analogous to phosphorylation, O-GlcNac modification is a highly dynamic posttranslational modification, which has an important role in a number of intracellular processes, including signal transduction and metabolism (8). Evidence suggests extensive cross talk between O-GlcNacylation and phosphorylation. OGT-catalyzed O-GlcNac modification and kinase-induced serine phosphorylation can occur at the same or adjacent serine residues and can compete with each other (7, 8). However, the regulation of IR O-GlcNacylation following injuries, and its relationship with JNK kinase-induced IR phosphorylation has not been studied.

In the present work, possible mechanisms for the defect in insulin-induced IR activation following trauma and hemorrhage were examined. The presented data suggest that the IR is a target for OGT catalyzed O-GlcNac modification and that injury resulted in decreased IR O-GlcNac modification coincident with decreased IR activation. Inhibition of JNK1 kinase increased IR tyrosine phosphorylation/activation and insulin signaling, and an increase of IR O-GlcNac modification. These findings suggest O-GlcNac modification of the IR is likely to be involved in JNK activation-induced impairment of IR activity and development of acute hepatic insulin resistance following trauma and hemorrhage.
MATERIALS AND METHODS

Animal model of trauma and hemorrhage. All experimental procedures were carried out in accordance with the guidelines of the Care and Use of Laboratory Animals by the National Institutes of Health, and the experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham (UAB). Male Sprague-Dawley rats (200–250 g, Harlan) were housed in the animal facilities for at least 1 wk before experiments. Surgical trauma and hemorrhage were performed as described previously (12, 13, 16, 20–22). Rats were fasted 18–20 h before the experiments. Rats were anesthetized by inhalation of 1.5% isoflurane, and a 5-cm midline laparotomy was performed. PE-50 catheters were placed in both femoral arteries and the right femoral vein for bleeding, monitoring of blood pressure, and resuscitation, respectively. Rats were bled rapidly to reach a mean arterial pressure (MAP) of 35–40 mmHg within 10 min and were maintained at 35–40 mmHg for 15 min (TH15'), 30 min (TH30'), 60 min (TH60'), or 90 min (TH90'). For the longer time point, 210 min (TH210'), the animals were resuscitated following the 90-min hemorrhage with four times the withdrawn blood volume of Ringer’s lactate at a constant infusion rate over 60 min and allowed to recover for 60 min. The trauma-alone (T) groups were subjected to the exact same anesthesia and surgical procedures as the trauma and hemorrhage groups, but were not hemorrhaged. In all groups of rats, 5 U of insulin in saline or saline alone were injected into the hepatic portal vein, and tissues were harvested 1 min after injection and quickly frozen in liquid nitrogen. Because of the considerable trauma incurred during catheterization and opening of the abdominal cavity to perform the insulin/saline injections, it was impossible to have a completely naive control group. Thus, another control group was used in some experiments, and referred to as Normal, in which the rats were anesthetized, the abdominal cavity was quickly opened, and insulin or saline injections immediately administered.

Western blot analysis. Liver tissues from each animal were homogenized in lysis buffer, as described previously (11, 13, 16, 22). The tissue lysates were centrifuged, and the supernatants were stored at −80°C until use. The concentrations of the protein lysates were assayed by BCA assay following the manufacturer’s instructions (Pierce, Rockford, IL), and the lysates (30 μg/lane) were separated on SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were immunoblotted with anti-PY972 (IR tyrosine-972)-IR (Invitrogen, Carlsbad, CA), anti-Pi3K p85 (Upstate Biotechnology, Lake Placid, NY), anti-total-IRα, anti-total-IRβ (Santa Cruz Biotechnology, Santa Cruz, CA), anti-O-GlcNac (kindly provided by Dr. John C. Chatham, UAB), anti-total-ERK, and anti-OGT (Sigma-Aldrich, St. Louis, MO) antibodies.

Immunofluorescence staining. The liver tissues were rinsed in PBS and fixed in 4% paraformaldehyde for 24 h and subjected to paraffin embedding. The liver tissues were sectioned (10 μm) and subjected to immunofluorescence staining with anti-IR antibody. The liver sections were blocked with 0.1% sodium azide and 0.5% H2O2 in PBS for 15 min. Overnight incubation with the primary antibody (anti-IR, 1:50 in 1% BSA) was followed by green fluorescence-conjugated secondary antibody. The images were obtained using a confocal microscope (Zeiss 170).

Immunoprecipitation. Liver extract (1 mg) was incubated with an antibody specific for IRβ or anti-JNK1 (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Protein A-beads were then added and incubated for 4 h at 4°C. Immunoprecipitated proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with anti-Pi3K p85, anti-PY (Upstate Biotechnology), anti-nitrotyrosine (Cell Signaling, Danvers, MA), and anti-O-GlcNac and OGT antibodies.

Recombinant adenoviral vectors. The recombinant adenovirus vector expressing a dominant negative mutant (T183A/Y185F) of JNK1 (Ad-DN-JNK1) was purchased from Cell Biolabs (San Diego; ADV-115) and was rendered kinase dead by two mutations within the JNK kinase domain. The Ad vectors were amplified in 293A cells and purified twice by cesium chloride gradient ultracentrifugation followed by dialysis (11, 13). The biological titer [plaque forming units (pfu)/ml] was determined by the TCID50 (tissue culture infectious dose 50) method (Adenovector Vector System) based on the development of a cytopathic effect in 293 cells. Replication-competent adenovirus contamination was not detected in purified virus stocks as screened by PCR with the primers flanking the E1 region (11, 13). Rats were injected with the purified Ad vectors (1010 pfu/rat) via the tail vein. At 4 days postinjection, surgical trauma and hemorrhage for 60 min were performed, and tissues were harvested for analyzing insulin signaling and kinase activity.

Densitometric and statistical analysis. Enhanced chemiluminescence images of immunoblots were scanned and quantified using Zero D-Scan (Scantecanlytics, Fairfax, VA). Data are presented as the means ± SE. The statistical differences were analyzed by ANOVA followed by Student-Newman-Keuls post hoc test for comparison among multiple groups or Student’s t-test for comparison between two groups. \( P < 0.05 \) was considered to be significantly different.

Fig. 1. Levels of insulin receptor (IR) tyrosine phosphorylation and IR total protein levels in the liver following trauma and hemorrhage. The rats were used prior to surgery (normals, N), following surgical trauma (T), or trauma and hemorrhage (TH) for 15', 30', 60', 90' or 210' (min). Livers were harvested, and tissue lysates were subjected to Western blot analysis with 1) phosphorylated IR tyrosine-972 (PY-972)-IR and T-ERK or 2) IRα and IRβ antibodies. Multiple Western blots were performed, and representative Western blots are presented for each antibody. The Western blots were scanned, and the fold-change in tyrosine phosphorylation of the insulin receptor is presented as means ± SE (\( n = 4 \) rats in each group).
Insulin resistance commonly occurs following injuries. We previously demonstrated that hepatic insulin-induced Akt phosphorylation was rapidly impaired after surgical trauma and hemorrhage (13, 16). Phosphorylation of the IR at tyrosine-972 serves as a binding site for the phosphotyrosine-binding domains of IRS1, facilitating subsequent insulin signal transduction. As presented in Fig. 1A, hepatic insulin-induced IR tyrosine-972 (Y972) phosphorylation was impaired as early as 15 min following the initiation of hemorrhage.

The total amounts of IR α and IRβ subunits were next determined by Western blot analysis, and there were no changes in the levels of the IRα and IRβ subunits in the liver of rats following trauma and hemorrhage (Fig. 1B). Therefore, the acute trauma and hemorrhage-induced impairment in insulin signaling is likely not due to a rapid decrease in IRα or IRβ subunit protein levels.

No decrease in plasma membrane localization of IR in liver by trauma and hemorrhage. Because insulin exerts its effect through binding to cell surface IRs, the plasma membrane level of the IR is determinant for insulin and activation of insulin signaling pathways. To determine whether trauma and hemorrhage alters the intracellular translocation of IR, the cellular location of IR on liver cells was determined by immunohistochemical staining. There was no noticeable change in the membrane-localized IR following trauma and hemorrhage for 60 min (Fig. 2). Similar results were found following trauma and hemorrhage for 15 min (not shown). Thus, trauma and hemorrhage did not appreciably alter IR translocation, so membrane localization did not likely account for the impaired hepatic IR activity.

Tyrosine nitration of IR likely does not account for impaired IR activity in the liver following trauma and hemorrhage. Activity of the IR can be affected by posttranslational modifications, and increased tyrosine nitration of the IR has been suggested to be involved in the development of diet-induced insulin resistance (4). We determined whether trauma or the combination of trauma and hemorrhage affected IR nitration in the liver of rats by immunoprecipitation of the IR and use of nitrotyrosine-specific antibodies. There was no obvious change in hepatic IR tyrosine nitration following trauma and hemorrhage for 90 min or 110 min (Fig. 3), trauma alone, or trauma and hemorrhage for 15 min (not shown). Therefore, IR tyrosine nitration did not likely account for the impaired hepatic IR activity.

Decreased O-GlcNac modification of IR following trauma and hemorrhage. Protein modifications, such as O-GlcNac and phosphorylation, interact in regulating protein activity, and O-GlcNac addition can interfere with kinase-induced phosphorylation on the same or proximal serine or threonine residues (7, 8). Measurable O-GlcNac modification of the IRβ subunit was significantly decreased following trauma and hemorrhage for 90 and 110 min (Fig. 4A). The level of IR O-GlcNac modification was also decreased following trauma and hemorrhage for 15 min (Fig. 4B), which was associated with the rapid impairment of insulin-induced IR phosphorylation.

**Results**

Rapid impairment of IR tyrosine phosphorylation in the liver following trauma and hemorrhage is not due to decreased total IR. Liver tissues were harvested from normal rats (N) or rats following trauma and hemorrhage for 90 min (TH90) or 110 min (TH110). The liver proteins were immunoprecipitated with an IRβ antibody and probed with antibodies specific for nitrotyrosine and T-IRβ. The (-) lane is the negative control with no IP antibody added. The next to last lane is a skipped lane, and the last lane was loaded with nitrated BSA as a positive control for the nitrotyrosine-specific antibody. Representative Western blots from three separate experiments are presented.
Hemorrhage resulted in reduced cellular levels of the p78 O-GlcNac transferase isoform and reduced binding of p78 with the IR. O-GlcNac transferase (OGT) catalyzes the addition of O-GlcNac to Ser/Thr residues of proteins. In the liver, OGT has two isoforms, a 110-kDa and a 78-kDa isoform. The abundant p110 isoform of OGT was not altered by trauma alone (not shown) or by the combination of trauma and hemorrhage (Fig. 5A). However, the level of the p78 OGT isoform significantly decreased following trauma and hemorrhage for 90 min (Fig. 5A). Further, the OGT p78, but not the p110 isoform, coimmunoprecipitated, with the hepatic IR, suggesting protein-protein interaction (Fig. 5B, left two lanes of the

Fig. 4. Decreased O-GlcNAC modification of the IR following trauma and hemorrhage. Liver tissues were harvested from normal rats (N) or rats following trauma and hemorrhage for 90 min (TH90') or 210 min (TH210') or trauma and hemorrhage (TH15') or trauma alone for 15 min (T15'). Liver protein lysates were immunoprecipitated with an IRβ-specific antibody and probed with antibodies specific for O-linked beta-N-acetylglucosamine (O-GlcNAC) and T-IRβ. A: representative Western blots are presented, as is a bar graph of the scanned Western blots, presenting data as means ± SE from n = 4 rats in each group. B: representative Western blots from three rats in each group are presented.

Fig. 5. Decreased level of IR-associated O-linked beta-N-acetylglucosamine (O-GlcNAC) transferase (OGT) p78 isoform following trauma and hemorrhage. Liver tissues were harvested from normal rats (N) or rats following trauma and hemorrhage for 90 min (TH90') or 210 min (TH210'). A: liver protein lysates were subjected to Western blot analysis with specific antibodies for OGT and PI3K p85; right panels are scans of these representative Western blots that were repeated in four separate experiments. A: lanes are from a single exposure of a single scanned image of the same gel/Western blot. Additional lanes not relevant to this figure were cropped out for clarity. Representative Westerns blots are presented, as is a bar graph, with the means ± SE presented from n = 4 rats in each group. B: liver proteins were immunoprecipitated with IRβ antibody and probed with antibodies specific for OGT and T-IRβ. Representative Western blots are presented, as is a bar graph of the scanned Western blots, with the mean ± SE presented from n = 4 rats in each group.
Western blot). In addition, the p78 isoform was significantly decreased in IR coimmunoprecipitates following trauma and hemorrhage (Fig. 5B). These data indicate that the IRβ subunit is a target for O-GlcNac modification (Fig. 4) and that the p78 isoform of OGT is likely responsible for IR O-GlcNAC modification (Fig. 5). Following trauma and hemorrhage, the decreases of whole cell p78 OGT and IR-associated p78 OGT (Fig. 5) coincide with decreased IR O-GlcNac modification (Fig. 4).

Potential involvement of JNK activation in O-GlcNAC modification of the IR. We have previously demonstrated the rapid activation of JNK in response to trauma and hemorrhage (13, 22) at the same time that IR Y972 phosphorylation is decreased (Fig. 1). Inhibition of JNK1 kinase by hepatic overexpression...
of a dominant-negative JNK1 resulted in improved insulin-induced IR tyrosine phosphorylation following trauma and hemorrhage (Fig. 6A). In addition, a direct interaction of JNK1 with the IR was indicated by their coimmunoprecipitation (Fig. 6B), which implicates the IR as a direct target for the JNK1. In Ad-LacZ-injected control rats, trauma and hemorrhage dramatically decreased hepatic IR O-GlcNac modification (Fig. 6, C and D), similar to what occurred in non-AdLacZ-injected, normal rats following trauma and hemorrhage (Fig. 4). Inhibition of JNK1 by expression of a dominant-negative JNK resulted in a small increase of IR O-GlcNac modification (Fig. 6, C and D, which approached significance), coincident with the improvement of insulin-induced IR tyrosine phosphorylation (insulin signaling; 6A). In addition, the trauma and hemorrhage-induced decrease in total cellular levels of the OGT p78 isoform was largely prevented by inhibition of JNK activation (Fig. 6, E and F). Together, these data suggest an important role for JNK activation in inhibiting O-GlcNac modification of the IR, and in inhibiting IR activation following trauma and hemorrhage.

DISCUSSION

Insulin receptor activation/tyrosine phosphorylation is an initial cellular response to insulin, and hepatic IR activation is acutely impaired following trauma and hemorrhage. As the initial molecule of the signaling cascade, moderate changes in IR content or function may not impact insulin action, and studies focusing on the regulation of the IR have produced inconsistent results (5, 19, 23). However, defects in the ability of insulin to induce IR tyrosine phosphorylation have been found in various chronic models of insulin resistance, including models of Type 2 diabetes and obesity (5, 19). Studies with heterozygous IR knockout mice indicated that a reduction of the number of IRs can have a significant physiological impact, decreasing insulin signal transduction and insulin action (2). In response to trauma and hemorrhage, we demonstrated that the cellular amounts of the IRα and IRβ subunits were not appreciably altered in the liver within 210 min following trauma and hemorrhage. Since an impaired ability of insulin to induce IR tyrosine phosphorylation following trauma and hemorrhage occurs within 15 min, the loss of insulin signaling is unlikely due to a reduced hepatic content of IR.

Intracellular translocation of the IR could occur within minutes, reducing cell surface IR and the insulin signaling cascade. However, the presented data imply that hepatic plasma membrane IR levels were not dramatically altered following trauma and hemorrhage compared with trauma only or normal rats, suggesting that these injuries do not increase the intracellular translocation of the IR. Thus, regulation of IR’s tyrosine kinase activity most likely accounts for the decreased response to insulin.

Increased tyrosine nitration of IR, as a result of oxidative stress, is involved in lipid-induced insulin resistance in both liver and muscle (4). There was little change in the level of tyrosine-nitrated IR following trauma and hemorrhage, suggesting IR nitrotyrosine modification is unlikely involved in trauma and hemorrhage-induced hepatic insulin resistance. O-GlcNac modification of serine/threonine residues is known to affect protein function, to regulate intracellular signal transduction, and to compete with phosphorylation by protein kinases at the same or adjacent sites (7). In the present study, we demonstrate that the IR is a target for O-GlcNac modification and that the rapid decrease in insulin-induced IR activation was associated with reduced IR O-GlcNac modification. Two OGT isoforms, p78 and p110 are present in liver. Trauma and hemorrhage reduced the protein levels of the p78, but not the p110 isoform, and decreased the association of OGT p78 with the IR. These studies suggest a role of O-GlcNac modification in regulating IR activity and that the p78 isoform is likely responsible for O-GlcNac modification of the IR (Fig. 7).

JNK1 is one of the serine kinases known to phosphorylate IRS1, inhibiting insulin signal transduction. In previous work, we have found a rapid increase in inhibitory serine phosphorylation of IRS1 (13, 16, 22, 25), which contributes to impaired IR activity, since inhibition of JNK resulted in improvement of insulin-induced IR tyrosine phosphorylation (1). Although several studies have suggested that activation of JNK1 inhibits IR activation by insulin (9, 19), the role of JNK1 directly on the IR has not been extensively investigated. In the present work, inhibition of JNK1 resulted in partial recovery of IR O-GlcNac modification, suggesting that JNK has a role in inhibiting O-GlcNac modification of the IR. Since the same residues can be targets for both phosphorylation and O-GlcNacylation (8), there is clearly the potential for direct interactions between JNK-induced phosphorylation and OGT-induced O-GlcNac modification of the identical or adjacent serine/threonine residues of IR. Therefore, a balance between O-GlcNac modification and JNK-induced phosphorylation may exist, with decreased Ser/Thr O-GlcNac modification following trauma and hemorrhage, concomitant with increased JNK1 phosphorylation of the IR, which inhibits IR activity (Fig. 7).

ACKNOWLEDGMENT

We would like to thank John L. Franklin for technical assistance, Dr. John Chatham for suggestions, and Luyun Zou for technical help and for providing the O-GlcNac antibody. We acknowledge University of Alabama at Birmingham (UAB) Comprehensive Cancer Center tissue procurement core facility for tissue paraffin embedding and UAB Comparative Pathology Laboratory for tissue sectioning.

GRANTS

This research is supported by grants from the National Institutes of Health (DK-62071) and the Veteran’s Administration Merit Review to J. L. Messina.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: S.J. and J.L.M. conception and design of research; S.J. and T.A.G. performed experiments; S.J. and J.L.M. analyzed data; S.J. and T.A.G. interpreted results of experiments; S.J. drafted manuscript; J.L.M. edited and revised manuscript; J.L.M. approved final version of manuscript.

REFERENCES

3. Bollag GE, Roth RA, Beaudoin J, Mochly-Rosen D, Koshland DE Jr. Protein kinase C directly phosphorylates the insulin receptor in vitro and...