Role of Inhibitory κB Kinase and c-Jun N-Terminal Kinase in the Development of Hepatic Insulin Resistance in Critical Illness Diabetes

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ABSTRACT:

Hyperglycemia and insulin resistance induced by acute injuries or critical illness are associated with increased mortality and morbidity, as well as later development of Type 2 diabetes. The molecular mechanisms underlying the acute onset of insulin resistance following critical illness remain poorly understood. In the present studies, the roles of serine kinases, Inhibitory κB kinase (IKK) and c-Jun N-terminal kinase (JNK), in the acute development of hepatic insulin resistance were investigated. In our animal model of critical illness diabetes, activation of hepatic IKK and JNK was observed as early as 15 minutes, concomitant with the rapid impairment of hepatic insulin signaling and increased serine phosphorylation of insulin receptor substrate (IRS) 1. Inhibition of IKKα or β, or both, by adenovirus vector-mediated expression of dominant negative IKKα or β in liver, partially restored insulin signaling. Similarly, inhibition of JNK1 kinase by expression of dominant negative JNK1, also resulted in improved hepatic insulin signaling, indicating both IKK and JNK1 kinases contribute to critical illness-induced insulin resistance in liver.

Key words: Serine kinase, insulin signaling, liver, injury
INTRODUCTION:

Insulin resistance is involved in the pathogenesis of a number of chronic metabolic disorders, such as Type 2 diabetes and obesity, and develops over months or years or decades. However, insulin resistance can also occur extremely rapidly after injuries, infection, or critical illnesses, and is referred to as “diabetes of injury” or, more inclusively, “critical illness diabetes” (3; 8; 29; 44; 46; 47). Critical illness diabetes-induced acute insulin resistance results in elevated blood glucose due to enhanced hepatic glucose production and/or impaired peripheral glucose uptake (29; 44; 46). This acute development of hyperglycemia and insulin resistance predisposes critically ill patients to detrimental outcomes and increased mortality (6; 14; 22; 45; 53). In addition, much like gestational diabetes, recent clinical studies suggest that the acute development of critical illness diabetes after surgery, and the concomitant hyperglycemia, is strongly correlated with the later development of Type 2 diabetes (10). Currently, the molecular mechanisms underlying the development of this acute insulin resistance in critical illness diabetes remain poorly understood. Insights into the pathogenesis of acute insulin resistance may provide novel and specific therapeutic strategies for improving recovery and survival of critically ill patients, and may suggest interventions for the greater likelihood of later development of Type 2 diabetes.

Insulin is a major hormone regulating glucose homeostasis through suppressing glucose production in liver and promoting glucose disposal in muscle and adipose tissue (38; 41). Insulin binds to the insulin receptor (IR) on the surface of target cells, resulting in IR autophosphorylation (39; 41). Activated IR recruits and phosphorylates insulin receptor substrate proteins (IRS) at multiple tyrosine residues which in turn binds to phosphatidylinositol-3-kinase (PI3K) and activates the kinase Akt and its downstream targets, regulating glucose metabolism (39; 41). Injuries or critical illness are associated with increased
intracellular stress and activation of inflammatory pathways (17; 20; 44; 50). Possible contributors to the insulin resistance of critical illness diabetes are serine kinases, such as Inhibitory κB kinase (IKK) β and c-Jun amino-terminal kinase (JNK), which share the ability to directly induce serine phosphorylation of IRS1, decrease insulin-stimulated tyrosine phosphorylation of IRS1, and inhibit insulin action (13; 16; 55; 58; 59).

The IKK complex is central to the inflammatory nuclear factor-kappaB (NF-κB) pathway (15) and consists of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit Nemo. Overexpression of the active form of IKKβ in hepatocytes eventually leads to insulin resistance in both liver and skeletal muscle (7). Deletion or inhibition of the IKKβ in liver (5; 7; 13), but not in skeletal muscle (37), attenuates obesity-related chronic insulin resistance. In addition, Nemo is suggested to be necessary in formation of a IKK-IRS1 complex in TNFα-induced insulin resistance (34).

The JNK kinase may also regulate insulin resistance. Total JNK activity is elevated in both dietary and genetic (ob/ob) models of obesity, and absence of JNK1 results in improved insulin sensitivity and decreased adiposity in obese mice (16). Suppression of the JNK pathway in liver improves insulin resistance and decreases blood glucose level in genetic models of severely obese (db/db) mice (35). However, there is nothing known about the contribution of the IKK and JNK1 kinase pathways to the development of the acute insulin resistance that develops in critical illness diabetes. Unlike high-fat diet or genetic models of obesity or Type 2 diabetes, which are chronic problems that take many weeks, months, years or decades to develop, the development of critical illness diabetes occurs very rapidly after an injury or infection. There is no a priori reason to believe that critical illness diabetes will have the same or different mechanisms of development. Since it can develop in our animal model of critical illness
diabetes within minutes after hemorrhage (23; 24; 27; 28; 43; 52), the mechanisms of development may
be very different than in chronic forms of insulin resistance.

In the present studies, we found that hepatic IKK and JNK were activated within minutes after
trauma and hemorrhage, with a similar time course to the rapid impairment of hepatic insulin signaling
and increased serine phosphorylation of IRS1. Inhibition of IKK and/or JNK1 kinases in liver improved
hepatic insulin signaling and attenuated hemorrhage-induced increase in IRS1 serine phosphorylation.
The present studies suggest both JNK1 and IKK kinases contribute to the acute development of insulin
resistance in critical illness diabetes.
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. Male Sprague Dawley rats (200~250 g, Harlan) were housed in animal facilities for at least one week before experiments. Surgical trauma and hemorrhage was performed as described previously (24; 27; 28; 52). Rats were fasted 18~20 hours before the experiment. 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 minutes and was maintained within 35~40 mmHg for 15 minutes (TH15’), 30 minutes (TH30’), 60 minutes (TH60’), or 90 minutes (TH90’). 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 minute after injection and quickly frozen in liquid nitrogen. This is a high dose of insulin to ensure the maximal possible induction even if the liver is insulin resistant and ensure sufficient delivery to the liver during hemorrhage.

Recombinant adenoviral vectors:

The recombinant adenovirus vectors expressing a dominant negative mutant (K44M) of IKKα
(Ad-DN-HA-IKKα) (9; 56), a dominant negative mutant (K44A) of IKKβ (Ad-DN-HA-IKKβ) (9; 56), and the control adenovirus vector expressing β-galactosidase (Ad5-nt-LacZ) were purchased from the University of Iowa Vector Core (9). The two IKK mutants were rendered incapable of binding ATP and, thus, lost functional kinase capability. The adenovirus vector expressing a dominant negative mutant (T183A/Y185F) of JNK1 (Ad-DN-JNK1) was purchased from Cell Biolabs, Inc. (ADV-115, San Diego) and was rendered kinase dead by these 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. The biological titer [plaque forming units (pfu)/ml] was determined by the TCID_{50} (tissue culture infectious dose 50) method (AdenoVactor™ 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.

Administration of Adenovirus vectors in vivo:

Rats were injected with the purified Ad vectors (10^{10} pfu/rat) via the tail vein. At 4 days post-injection, surgical trauma and hemorrhage for 60 minutes were performed, and tissues were harvested for analyzing insulin signaling and kinase activity.

Western Blotting analysis:

Liver tissues from each animal were homogenized in lysis buffer as described previously (24; 28). 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 as described in the manufacturer’s
instruction (Pierce, Rockford, IL) and the lysates (30 µg/lane) were subjected to SDS-PAGE gel and transferred to nitrocellulose membrane. The membranes were immunoblotted with anti-PY972-IR, anti-PT183/Y185-JNK1/2, and anti-PS312-IRS1 (S307 rat, Invitrogen, Carlsbad, CA), anti-total-IR and anti-JNK1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-pan-Actin, anti-total-ERK, anti-P473-Akt, anti-total-Akt, anti-PS180/S181-IKKα/β, anti-IKKα, and anti-IKKβ (Cell Signaling, Danvers, MA) antibodies.

*Gel-shift assay:*

Nuclear proteins were extracted from frozen liver samples according to the manufacturer’s instruction (Active Motif, Inc. Carlsbad, CA) and stored at -80 °C until use. Protein concentrations were measured by BCA protein assay. Oligonucleotides containing the κB consensus sequence (5'-AGTTGAGGGGACTTTCCCAGGC-3') were labeled with [32P] CTP using Klenow DNA polymerase. The binding reaction was performed as described previously (26). Briefly, nuclear protein (10 µg) was incubated with labeled oligonucleotide probe for 30 minutes on ice and separated on 4-20% polyacrylamide Tris-glycine-EDTA gels. Excess of a specific unlabeled competitor sequence were used to out-compete the specific interactions to indicate specific binding.

*Immunohistochemistry:*

The liver tissues were rinsed in PBS and embedded in Histoprep Frozen Tissue Embedding Media (Fisher Scientific Inc., MA) and snap frozen in liquid nitrogen. Frozen livers were cryosectioned (10 µm) and subjected to immunohistochemical staining with anti-HA antibody (Cell Signaling, Danvers, MA).
The liver sections were fixed in 70% ethanol overnight and blocked with 0.1% sodium azide and 0.5% H$_2$O$_2$ in PBS for 10 minutes. Overnight incubation with the primary antibody (Anti-HA, 1:200 in 1% BSA) was followed by HRP-conjugated secondary antibody and detected using DAB substrate. The sections were counterstained briefly with hematoxin and images were obtained using a Leica microscope (Leica Microsystems Inc. IL).

**Densitometric and statistical analysis:**

Enhanced chemiluminescence images of immunoblots were scanned using a flatbed scanner (HP, Palo Alto, CA) and quantified using Zero D-Scan (Scanalytics Corp., Fairfax, VA). Data are presented as the mean±SEM. The statistical differences were analyzed by ANOVA for comparison among multiple groups or Student’s t-test for comparison between two groups. P ≥ 0.05 was considered not statistically significant different.
RESULTS:

Activation of IKK/NF-κB pathway in liver following trauma and hemorrhage

Critical illnesses and injuries, including hemorrhagic shock, can initiate acute inflammatory responses characterized by increased production of inflammatory factors and activation of inflammatory pathways such as the IKK/NF-κB pathway (4; 49). The activation of the IKK complex depends on the phosphorylation sites within the activation loop of the kinase (15). To determine whether IKK kinases are activated in liver following trauma and hemorrhage, phosphorylation of IKK kinases was examined with an antibody specific for PS181-IKKβ and PS180-IKKα. Increased phosphorylation/activation of both IKKβ and IKKα were detected after trauma and hemorrhage for 90 minutes (TH90’) compared to trauma alone [T90’; (Fig. 1A)]. When measured, total-IKKα and total IKKβ levels were not altered by trauma and hemorrhage (Fig. 1A) at the times that P-IKKβ and P-IKKα were induced.

NF-κB, a downstream target of the IKK complex, is an important transcription factor regulating expression of inflammatory factors (15). Activation of NF-κB in liver after trauma and hemorrhage was examined by gel-shift assay with a radiolabeled NF-κB consensus sequence probe and DNA binding activity of NF-κB was significantly higher at 90 minutes after trauma and hemorrhage (TH90’) compared to the trauma alone group [T90’; (representative blot, Fig. 1B)]. These studies indicate an activation of the IKK/NF-κB pathway in response to hemorrhage. In additional experiments there was a slight increase in DNA binding activity of NF-κB in trauma only animals, when compared to normal (no trauma or hemorrhage) animals. This suggests a minor activation of this pathway by trauma alone, and agrees with our previous work indicating a mild level of insulin resistance following trauma alone (28).
Time-course of IKK and JNK activation

We have found that hepatic insulin resistance developed at 15 minutes following trauma and hemorrhage as indicated by severely decreased insulin-induced Akt phosphorylation [Fig. 2A, B and see (24)]. In parallel experiments, the time-course of IKKβ, JNK1 and S312-IRS1 phosphorylation in liver following trauma and hemorrhage was examined. Significantly elevated P-IKKβ, P-JNK1 and PS312-IRS1 were observed at 15 minutes following trauma and hemorrhage compared to trauma alone groups, with levels continuing to increase through at least 90 minutes (Fig. 2A, B). We demonstrated previously that the total levels of Akt, JNK and IRS1 are not affected by trauma and hemorrhage (28; 52). The induction of IKKβ, JNK1 and S312-IRS1 phosphorylation presented here are not dependent upon the administration, or not, of insulin, but due to hemorrhage. This early activation of IKKβ, JNK1 and S312-IRS1 phosphorylation may allow them to play a role in the rapid development of insulin resistance in liver following trauma and hemorrhage.

Inhibition of IKK kinases resulted in improved hepatic insulin signaling

To directly investigate the role of IKKβ on hepatic insulin signaling following trauma and hemorrhage, rats were injected with adenovirus vectors expressing dominant negative IKKβ (Ad-DN-IKKβ, 10^{10} pfu) via the tail vein. Since IKKα, also a serine kinase, exists in the complex interacting with IRS1 (13), it may also contribute to IRS1 serine phosphorylation and participate in insulin resistance. We therefore included two other groups: an Ad-DN-IKKα (10^{10} pfu) injected group, and a co-injected group: both Ad-DN-IKKβ (5x10^9 pfu) and Ad-DN-IKKα (5x10^9 pfu). At 4 days post-injection, surgical trauma and hemorrhage for 60 minutes (TH60’) was performed and insulin or
saline was injected immediately before tissue harvesting. Expression of dominant negative IKK kinases in liver in all rats was confirmed by Western blot analysis with total IKKα or IKKβ specific antibodies, which recognize both endogenous and the dominant negative mutants of the IKK kinases. The expression of endogenous IKKα or IKKβ was not affected by the control adenovirus vector Ad-nt-LacZ (Fig. 3A), or by injection of the dominant negative of the other IKK kinase, whereas expression of dominant negative IKKα, IKKβ, or both, was achieved by their injection. Immunohistological examination indicated a significant number, but not all, liver cells expressed the HA-tagged dominant negative constructs (IKKβ, a representative liver section shown in Fig. 3B).

As expected, in the trauma alone uninfected and the Ad-nt-LacZ infected groups (T60’), insulin stimulated hepatic S473-Akt phosphorylation. However, induction of P-Akt was almost completely abolished after the combination of trauma and hemorrhage for 60 minutes (TH60’; Fig. 3C, D). Expression of dominant negative IKKβ in liver increased insulin-induced Akt phosphorylation following trauma and hemorrhage compared to the uninfected and the Ad-nt-LacZ control rats (Fig. 3C, D). Significant improvements in insulin-induced Akt phosphorylation following trauma and hemorrhage were also detected following expression of dominant negative IKKα. In addition, there was a significantly greater improvement in animals following co-administration of both dominant negative IKKα and IKKβ (Fig. 3C, D). These results suggest both IKKα and IKKβ contribute to hemorrhage-induced defects in insulin signaling. However, for all groups, insulin-induced Akt phosphorylation following trauma and hemorrhage was still lower than the trauma alone groups, indicating only a partial recovery of insulin signaling by adenovirus vector-mediated expression of dominant negative mutant IKK kinases.

Insulin signaling upstream of Akt was next examined. Tyrosine 972 of the insulin receptor serves as
binding site for IRS1 (54). Insulin-induced PY972-IR was impaired following trauma and hemorrhage in the uninfected and Ad-nt-LacZ control rats (Fig. 4A). Expression of dominant negative IKKβ, dominant negative IKKα, or both resulted in increased Y972-IR phosphorylation in liver compared to uninfected and Ad-nt-LacZ control groups (Fig. 4A, B). These results suggest that hemorrhage-induced defects in hepatic insulin signaling occurs at the level of the insulin receptor, and IKK kinases may inhibit insulin signaling through decreasing insulin receptor tyrosine phosphorylation upstream of PI3k/Akt signaling (Fig. 3).

Inhibition of JNK1 improved hepatic insulin signaling

To directly investigate the role of JNK1 kinase in the acute development of hemorrhage-induced hepatic insulin resistance, rats were injected with adenoviruses vector expressing dominant negative JNK1. At 4 days post-injection, surgical trauma and hemorrhage was performed. Expression of DN-JNK1 was determined using a total-JNK1 specific antibody. Total JNK 1 increased consistently and significantly following administrations of Ad-DN-JNK1 (Fig. 5A, B). In the same animals, JNK1 phosphorylation was decreased by 50% following administration of Ad-DN-JNK1 (Fig. 5A, C). Inhibition of JNK1 by Ad-DN-JNK1 also resulted in increased insulin-induced Akt phosphorylation (Fig. 5D, E) as well as phosphorylation of IR Y972 after trauma and hemorrhage (Fig. 5F, G), indicating a potential role of JNK1 kinase in hemorrhage-induced insulin resistance.

Inhibition of IKKα, IKKβ, or JNK1 results in decreased IRS1 S312 phosphorylation

Both JNK1 and IKKβ have been shown to associate with IRS1 and phosphorylate IRS1 at serine 312
residue in vitro (1; 13), which impairs IR (18) or IRS1 tyrosine phosphorylation (2). The effect of the dominant negative IKKs or dominant negative JNK1 expression on the hemorrhage-induced increase of IRS1 S312 phosphorylation was determined. The level of IRS1 S312 phosphorylation increased following trauma and hemorrhage and administration of Ad-nt-LacZ was ineffective in altering this increase. However, PS312-IRS1 decreased significantly following inhibition of JNK1 or IKKβ, (Fig. 6) and approached significance following inhibition of IKKα (P=0.08). This suggests that following acute hemorrhage, activation of JNK1 and IKKβ, and possibly IKKα, contribute to the increased phosphorylation of IRS1 on serine 312 in the liver of rats, which may then inhibit insulin signaling.

Inhibition of both JNK1 and IKKβ, not JNK1 and IKKα, resulted in an additive effect on hepatic insulin signaling

The effects of inhibition of either IKK kinase in combination with inhibition of JNK1 kinase on acute hepatic insulin resistance was then determined. Rats were co-injected with Ad-DN-IKKβ or Ad-DN-IKKα and Ad-DN-JNK1. Expression of DN-IKKβ or DN-IKKα in the liver of co-injected groups were confirmed by Western blot analysis with total-IKKβ (Fig. 7A) or total-IKKα (Fig. 7B). Expression of DN-IKKβ and DN-JNK1 simultaneously in liver significantly increased insulin-induced PS473-Akt following trauma and hemorrhage, which was higher than the effect of either DN-IKKβ or DN-JNK1 alone (Fig. 7C). In contrast, insulin-induced PS473-Akt after trauma and hemorrhage in the Ad-DN-IKKα and Ad-DN-JNK1 co-injected group was not different from either Ad-DN-IKKα or Ad-DN-JNK1 injected alone (Fig. 7D). This suggests that IKKα and IKKβ may act differently, and IKKα and JNK kinases may act in series on inducing hepatic insulin resistance following trauma and hemorrhage, resulting in no
additive effect. Whereas IKKβ may function in parallel with JNK1, allowing for an additive effect when
both are inhibited.

**Crosstalk between IKK and JNK kinases**

To determine the crosstalk between IKK and JNK kinases, effects of IKK or JNK inhibition on
activation of the other kinase was examined. JNK1 inhibition didn’t alter the phosphorylation of IKKα or
IKKβ (Fig. 8A), whereas expression of dominant negative IKKα in liver resulted in a significant decrease
in JNK1 phosphorylation/activation after trauma and hemorrhage (Fig. 8B). Unlike DN-IKKα, there is no
significant decrease of JNK phosphorylation after expression of DN-IKKβ (data not shown). This
suggests that IKKα may act upstream of JNK kinase following trauma and hemorrhage.
DISCUSSION:

Critical illness diabetes, as evidenced by elevated blood glucose, is common in patients with injuries or critical illness due to increased hepatic glucose production and/or impaired peripheral glucose uptake, independent of previous diabetic status (29; 44; 46). The development of critical illness diabetes and its hyperglycemia in patients after acute injuries is of clinical concern due to the occurrence of adverse outcomes (6; 14; 22; 53). However, deleterious effects can also occur if the hyperglycemia is treated too aggressively by insulin, with increased hypoglycemic incidents (12). To delineate the mechanisms underlying the acute onset of insulin resistance after injuries, we have established an experimental rat model of critical illness diabetes. Our previous studies demonstrate a rapid development of insulin resistance in liver (23; 24; 27; 52). In the present studies, the rapid activation of JNK and of another signaling pathway, the IKK/NF-κB pathway, was established. The causative roles of the IKK and JNK kinases in the acute development of insulin resistance were determined by direct inhibition of the IKK and JNK1 kinases with the adenovirus-mediated expression of dominant negative mutants of IKKs and JNK1 in liver.

The response to injury/hemorrhage involves a complex coordination of the immune, cardiovascular, endocrine, and nervous systems (20), resulting in increased cellular stresses. These include (but are not limited to) oxidative stress and increased production of proinflammatory cytokines, which can then induce the activation of the JNK and IKK/NF-κB pathways (31). In the present studies, the time courses of IKK/NF-κB and JNK activation were examined and found to increase as early as 15 minutes, and continued to increase in liver through the 90 minute hemorrhage period. Thus, activation of the JNK and IKK/NF-κB pathways may represent early and rapid signaling events in liver following...
injury/hemorrhage. Activation of IKK/NF-κB and JNK continued to increase and were maintained throughout the 90 minute hemorrhage period as was the defect in insulin signaling. In separate studies we found that hemorrhage induces a rapid increase of reactive oxygen species (ROS) in liver and inhibition of the increase of ROS significantly decreased the acute development of hepatic insulin resistance. The data to date implies that the rapid activation of JNK and IKKs may be caused by the increase of ROS levels during hemorrhage.

Numerous studies have indicated the important role of IKKβ and JNK1 kinases in chronic insulin resistance in response to high fat diet or obesity (5; 7; 13; 16; 35). However, the role of acute activation of IKK and JNK kinases on the rapid development of insulin resistance is not known. Unlike high-fat diet-induced obesity or Type 2 diabetes, which are chronic problems that take many weeks, months, years or decades to develop, the development of critical illness diabetes occurs very rapidly after an injury or infection. There is no *a priori* reason to believe that critical illness diabetes will have the same or different mechanisms of development. Since the mechanisms of development are unknown, and critical illness diabetes is clearly an important clinical problem, the mechanisms need to be explored. In the present studies, dominant negative IKKβ, IKKα, or JNK1 expression significantly improved insulin signaling, suggesting the early activation of the IKK and JNK kinases contribute to the rapid onset of insulin resistance in critical illness diabetes. Co-injection of Ad-DN-IKKβ and Ad-DN-JNK1 resulted in additive effects on insulin sensitivity, further confirming that they both contribute to the development insulin resistance and may act in parallel. Thus, the acute development of hepatic insulin resistance within critical illness diabetes may have, as partial causative factors, the activation of the IKK and JNK signaling pathway within minutes of hemorrhage.
However, defects in hepatic insulin signaling were only partially recovered by adenovirus-mediated expression of DN-IKks, DN-JNK1 or both. The immunohistochemical staining of liver sections indicated that a large number of liver cells, but certainly not all hepatocytes, expressed dominant negative proteins delivered by the adenovirus vectors. Since the dominant negative protein works intracellularly, only those cells infected and expressing sufficient quantities of the dominant negative protein will be affected. This may, in part, account for the partial blockade of the development of insulin resistance (partial increase of insulin signaling) after DN-IKK and DN-JNK1 expression. In addition, the adenovirus vector itself can initiate host inflammatory responses (19) and activate the JNK and IKK pathways (30; 33; 42), which may inhibit the activity of the dominant negative IKK and JNK1 kinases, and lower their effectiveness in blocking the development of insulin resistance in liver. Moreover, critical illness diabetes is complex, and we assume there are multiple other causative factors, and therefore potentially other signaling pathways in addition to the IKK and JNK kinases, such as mTOR and p38, which are likely involved in inducing the full insulin resistant state. These other contributors remain to be determined in future studies on the development of acute insulin resistance in critical illness diabetes.

Among the three major subunits of the IKK complex, IKKβ is most clearly involved in the development of the chronic insulin resistant state (5; 7; 13), with Nemo also necessary for TNFα-induced insulin resistance (34), whereas the role of IKKα in insulin resistance has not been as well studied. IKKα and IKKβ are catalytic subunits of the IKK complex, and share extensive structural homology (36). Both IKKα and β contribute to Inhibitory-κB phosphorylation and activation of NF-κB, and their similar abilities suggest that their functions are likely to be redundant and overlapping (36). In addition to forming heterodimers with IKKβ, IKKα can also form a homodimer, which may play a distinct role (36). Our data
indicate an additive effect of expression of both DN constructs (DN-IKKβ or DN-IKKα) suggesting they may also have separate actions in the development of acute insulin resistance in our animal model. Since IKKα exists in a complex which interacts with IRS1 (13), IKKα may also participate in inhibiting insulin signaling. Inhibition of IKKα by adenovirus-mediated expression of a dominant negative mutant was found to improve hepatic insulin sensitivity in our model of critical illness diabetes. Thus, unlike chronic forms of insulin resistance, IKKα also likely contributes to acute insulin resistance that develops in critical illness diabetes.

There was an additive effect of expression of the dominant negative forms of IKKβ and JNK1, suggesting that they act separately, potentially in parallel pathways to cause acute insulin resistance. However, unlike IKKβ, no additive effect was detected following expression of both dominant negative IKKα and JNK1, suggesting that IKKα may act in series with JNK kinase on inhibiting hepatic insulin signaling in our model of critical illness diabetes. Further experiments demonstrated that inhibition of JNK kinase had no effect on IKKα or β activation, whereas expression of DN-IKKα in liver resulted in decreased JNK1 activation. Together, these data suggest that IKKα acts upstream of JNK kinase in inducing hepatic insulin resistance. Thus, following activation of IKKα, IKKα can activate JNK kinase. In combination with other possible activators of JNK kinase, and activation of a separate pathway containing activated IKKβ, there is a significant reduction of insulin signaling in our experimental model or critical illness diabetes.

Several studies have demonstrated crosstalk between the IKK and JNK pathways, and IKK can act as a positive or negative regulator of JNK activation depending on the type of stress (25; 40; 51). Phosphorylation of JNK is induced by a series of serine kinases, including MEKK1, MKK4, and MKK7.
Thus, IKKα, itself a serine kinase, could phosphorylate/activate an intermediate protein which is important for JNK activation. Another possibility is that alterations of IKKα activity, an important kinase for both canonical and non-canonical NF-κB activation, could result in altered expression of a number of genes, such as PKC, leading to activation of JNK. However, this is unlikely in our animal model of critical illness since activation of the IKK and JNK pathways occurs so rapidly. Thus, the exact mechanisms of how IKKα involved in hemorrhage-induced JNK activation remain to be determined.

A common feature of the IKK and JNK1 kinases is their ability to directly bind to and phosphorylate IRS1 at serine residue 312, which may inhibit insulin signal transduction (13; 16; 55; 58; 59). Increased IRS1 S312 phosphorylation was observed in liver following the development of critical illness diabetes, and inhibition of JNK1 or IKKs resulted in decreased PS312-IRS1, suggesting that IRS1 could be a target molecule for active IKK and JNK kinases. However, an acute defect in insulin signaling after injury/hemorrhage was also detected at the insulin receptor, upstream of IRS1 and inhibition of IKK or JNK signaling improved insulin-induced insulin receptor tyrosine phosphorylation. A possible explanation is provided by Hotamisligil and colleagues who found that serine phosphorylation of IRS1 allows IRS1 to inhibit insulin-induced insulin receptor tyrosine auto-phosphorylation (18). Thus, increased hepatic IRS1 S312 phosphorylation following could feed back to decrease insulin-induced insulin receptor activation. In addition, although much less extensively studied, serine phosphorylation of the insulin receptor may also be an important mechanism for the development of insulin resistance, which could result in decreased activation of the insulin signaling pathway (32). Thus, activation of serine kinases in the acute development of critical illness diabetes could result in serine phosphorylation of the
insulin receptor, leading to hemorrhage-induced hepatic insulin resistance. This possibility needs to be studied further but at this time no candidate serines of the insulin receptor have been identified.

In summary, we demonstrate that activation of the JNK and IKK/NF-κB pathways, occurs early in the development of critical illness diabetes. Both the JNK1 and IKK kinases contribute to the acute development of critical illness diabetes-associated hepatic insulin resistance. Intensive insulin therapy has proven to be beneficial (47). However, there is some debate as to blood glucose target and therefore the intensity of insulin treatment which is most beneficial (11; 12; 21; 48). Our current studies suggest IKKs or JNK inhibitors as potential, novel therapeutic agents for treating or preventing the development of insulin resistance and hyperglycemia in critical ill patients.
ABBREVIATIONS:

IKK: Inhibitory κB kinase; JNK: c-Jun N-terminal kinase; NF-κB: nuclear factor-kappa B; Ad:
adeno-virus; IR: insulin receptor; IRS: insulin receptor substrate; PI3K: phosphoinositide 3-kinase; DN:
dominant negative; TH: trauma and hemorrhage; T: trauma alone.
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**FIGURE LEGENDS:**

**Fig. 1.** Activation of IKK/NF-κB pathway in liver after trauma and hemorrhage. (A) Representative Western Blot: liver extracts from TH90’ and T90’ rats were subjected to Western analysis with anti-PS181-IKKβ/PS180-IKKα, total-IKKα, total-IKKβ, and total-ERK antibodies; the level of total-ERK was probed as a loading control. (B) Representative Electromobility Shift Assay: liver nuclear proteins from TH90’ and T90’ rats were subjected to gel-shift assay with a 22 nt radiolabeled probe of NF-κB consensus sequence. Lane 1: probe only (control); lane2: T90’; lane3: TH90’; lane4: TH90’+20x-unlabeled competitor. Fig. 1B shows a representative of several repeated experiments. (TH90’: trauma and hemorrhage for 90 minutes; T90’: trauma alone for 90 minutes.)

**Fig. 2.** Time course of IKKβ, JNK1, S312-IRS1 phosphorylation following trauma and hemorrhage. Rats were subjected to trauma and hemorrhage or trauma alone and tissue were harvested at 15, 30, 60 and 90 minutes. (A) Liver protein extracts were subjected to Western analysis with anti-PS473-Akt, PT181/Y183-JNK1/2, PS181-IKKβ/PS180-IKKα, PS312-IRS1 and total-ERK antibodies. (B) The data were presented as the mean +/- SEM in the graph. (n=6 for each group; *: P < 0.05; **: P < 0.01; ***: P < 0.001 vs. T group at the corresponding time point)

**Fig. 3.** Effects of IKK kinase inhibition on hepatic insulin signaling after trauma and hemorrhage. Rats were injected with Ad-DN-IKKα (10^10 pfu), Ad-DN-IKKβ (10^10 pfu), or Ad-nt-LacZ (10^10 pfu) or co-injection of Ad-DN-IKKα (5 x 10^9 pfu) and Ad-DN-IKKβ (5 x 10^9 pfu) through the tail vein. At 4 days post-injection, surgical trauma and hemorrhage were performed and liver tissues were harvested. (A)
Representative Western Blot: liver lysates were subjected to Western analysis with total-IKKα, total-IKKβ and pan-Actin specific antibodies. (B) Immunohistochemistry was performed on frozen liver sections with anti-HA antibody. Magnification: 10 X. (C) Liver lysates were subjected to Western analysis with PS473-Akt and total-Akt antibodies. (D) The bar graph is presented as the mean +/- SEM (n= 3-4 rats/group).

Fig. 4. Effects of IKK kinase inhibition on insulin-induced IR phosphorylation after trauma and hemorrhage. Rats were injected with Ad-DN-IKKα (10^{10} pfu), Ad-DN-IKKβ (10^{10} pfu), or Ad-nt-LacZ (10^{10} pfu) or co-injection of Ad-DN-IKKα (5 x 10^{9} pfu) and Ad-DN-IKKβ (5 x 10^{9} pfu) through the tail vein. At 4 days post-injection, surgical trauma and hemorrhage were performed and liver tissues were harvested. (A) Liver lysates were subjected to Western analysis with PY972-IR and total IR antibodies. (B) Bar graph of PY972-IR presented as the mean +/- SEM (n= 3-4 rats/group).

Fig. 5. Effects of JNK1 kinase inhibition on hepatic insulin signaling after trauma and hemorrhage. Rats were injected with Ad-DN-JNK1 (10^{10} pfu), or Ad-nt-LacZ (10^{10} pfu) through the tail vein. At 4 days post-injection, surgical trauma and hemorrhage for 60 minutes were performed and liver tissues were harvested. (A, B, C) liver lysates were subjected to Western analysis with PT181/Y183-JNK1/2, total-JNK1, and Pan-Actin specific antibodies. (D, E) Liver lysates were subjected to Western analysis with PS473-Akt and total-Akt antibodies. (F, G) Liver lysates were subjected to Western Blot analysis with PY972-IR and Total-IR antibodies. The bar graphs are presented as the mean +/- SEM (n= 3-4 rats/group).
Fig. 6. Level of IRS1 S312 phosphorylation after inhibition of IKKs or JNK1. Rats were injected with Ad-nt-LacZ (10^{10} pfu), Ad-DN-IKKα (10^{10} pfu), Ad-DN-IKKβ (10^{10} pfu), or Ad-DN-JNK1 (10^{10} pfu) through the tail vein. At 4 days post-injection, surgical trauma and hemorrhage for 60 minutes were performed and liver tissues were harvested. Liver lysates were subjected to Western analysis with PS312-IRS1 and total-IRS1 specific antibodies. Bar graph of the ratio of PS312-IRS1 to Total-IRS1 is presented as mean +/- SEM (n= 3-4 rats/group).

Fig. 7. Effects of inhibition of both IKKβ or α and JNK1 kinases on hepatic insulin signaling after trauma and hemorrhage. Rats were injected with Ad-DN-IKKα (10^{10} pfu), Ad-DN-IKKβ (10^{10} pfu), or Ad-nt-LacZ (10^{10} pfu) or co-injection of Ad-DN-IKKα (5 x 10^{9} pfu), or Ad-DN-IKKβ (5 x 10^{9} pfu) with Ad-DN-JNK1 (5 x 10^{9} pfu) through the tail vein. At 4 days post-injection, surgical trauma and hemorrhage were performed and liver tissues were harvested. (A, C) Representative Western Blots: liver lysates were subjected to Western analysis with total-IKKβ (A), total-IKKα (C), Total-JNK1, and pan-Actin specific antibodies. (B, D) Representative Western Blots: Liver lysates were subjected to Western analysis with PS473-Akt and total-Akt antibodies. Bar graphs are presented as the mean +/- SEM (n= 3-4 rats/group).

Fig. 8. Crosstalk between IKK and JNK kinases. (A) Rats were injected with Ad-DN-JNK1 (10^{10} pfu), or Ad-nt-LacZ (10^{10} pfu) through the tail vein. At 4 days post-injection, surgical trauma and hemorrhage for 60 minutes were performed and liver tissues were harvested. Liver lysates were subjected
to Western analysis with PS180IKKα/S181IKKβ and total-Akt specific antibodies. Bar graphs are presented as the mean +/- SEM (n= 4-5 rats/group). (B) Rats were injected with Ad-DN-IKKα (10^10 pfu), or Ad-nt-LacZ (10^10 pfu) through the tail vein. At 4 days post-injection, surgical trauma and hemorrhage for 60 minutes were performed and liver tissues were harvested. Liver lysates were subjected to Western analysis with PT181/Y183-JNK1/2 and total-JNK1 antibodies. Bar graphs are presented as the mean +/- SEM (n= 4-5 rats/group).
Fig. 1.

A.

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<tr>
<th>Protein</th>
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<tr>
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<tr>
<td>T-ERK</td>
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B.

Protein only → 
Probe only → 
TH90' → 
TH90' + Cold competitor →

NF-κB →
Unbound Probe →
Fig. 2.

A. Time after trauma and hemorrhage (minutes)

<table>
<thead>
<tr>
<th>Time after trauma and hemorrhage (minutes)</th>
<th>PS473-Akt</th>
<th>P-JNK p54</th>
<th>P-JNK p46</th>
<th>P-IKKβ</th>
<th>PS312-IRS1</th>
<th>T-ERK2</th>
<th>T-ERK1</th>
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<td>T</td>
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</table>

B. (Fold change)

- PS473-Akt
- P-JNK
- P-IKKbeta
- PS312-IRS1

Time after trauma and hemorrhage (minutes)
Fig. 3.

A. 

B. Ad-nt-LacZ

Ad-DN-IKKβ

10 X

HA

C.

PS473-Akt

Total-Akt

Insulin:

T60'

T60'

T60'

TH60'

Uninfected

Ad-nt-LacZ

Ad-DN-IKKβ

Ad-DN-IKKα

Ad-DN-IKKβ+α

D. 

- Insulin

+ Insulin

PS473-Akt (Fold change)
Fig. 4.

A. 

<table>
<thead>
<tr>
<th>Insulin:</th>
<th>- + - + - + - + - + - + - +</th>
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<tbody>
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PY972-IR

Total-IR

B. 

- Insulin
+ Insulin

P<0.05
P<0.01
P<0.01

PY972-IR/Total-IR

T60' TH60' T60' TH60'

Uninfected Ad-nt-LacZ Ad-DN-IKKβ Ad-DN-IKKα Ad-DN-IKKβ+α

Ad-DN-IKK/g533
Ad-DN-IKK/g302
Ad-DN-IKK/g533+/g302
Fig. 5.

A.

B. [Graph showing comparison among Uninfected, Ad-nt-LacZ, and Ad-DN-JNK1 with bars for Total-JNK1 to Pan-Actin (Ratio).]

C. [Bar graph showing comparison among Uninfected, Ad-nt-LacZ, and Ad-DN-JNK1 with bars for P-JNK (Fold Change).]

D. [Graph showing PS473-Akt and Total-Akt comparison among - + - + - + - + - + Insulin: T60' TH60' T60' TH60'.]

E. [Graph showing comparison among T00', TH60', T00'xAd-nt-LacZ, TH60'xAd-nt-LacZ, TH60'xAd-DN-JNK1 with bars for PS473-Akt (%).]

F. [Graph showing comparison among Uninfected, Ad-nt-LacZ, and Ad-DN-JNK1 with bars for PY972-IR and Total-IR.]

G. [Graph showing comparison among T60', TH60', T60'+Ad-nt-LacZ, T60'+Ad-DN-JNK1 with bars for PY972/Total-IR.]

- P<0.05
- P<0.001
Fig. 6.

A.
Fig. 7. (A) Total-IKK$\beta$ +JNK1

(B) Total-IKK$\alpha$ +JNK1

(C) PS473-Akt

(D) PS473-Akt

<table>
<thead>
<tr>
<th>Uninfected</th>
<th>Ad-nt-LacZ</th>
<th>Ad-DN-IKK$\beta$</th>
<th>Ad-DN-JNK1</th>
<th>Ad-DN-IKK$\beta$+JNK1</th>
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<td>- + - + -</td>
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<table>
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<th>Ad-DN-JNK1</th>
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</table>

- Insulin
- + Insulin

Total-IKK/g533
Pan-Actin

Ad-DN-IKK/g533 +JNK1

Uninfected
Ad-nt-LacZ
Ad-DN-IKK$\beta$
Ad-DN-JNK1
Ad-DN-IKK$\beta$+JNK1

PS473-Akt

Total-Akt

PS473-Akt

Total-Akt

P<0.01
P<0.001
P<0.05
P<0.05
NS

P<0.05
P<0.05
P<0.05

Total-JNK1

Total-JNK1
Fig. 8.

A. P-IKKβ
P-IKKα
Total-IKKα

Uninfected Ad-nt-LacZ Ad-DN-JNK1

T60' TH60' T60' TH60'

P-JNK p54 P-JNK p46

0 10 20 30 40 50 60

P-JNK (Fold change)

T60' TH60'

P<0.05

B. P-IKKα

Uninfected Ad-nt-LacZ Ad-DN-IKKα

Uninfected Ad-nt-LacZ Ad-DN-JNK1

NS

P-IKKα (Fold Change)

T60' TH60'

P<0.05

P-JNK (Fold change)