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Role of Tissue Macrophages in the Development of Critical Illness Diabetes

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Abstract

Macrophage-derived factors, including TNF α , are known as important inducers of insulin resistance. However, the role of macrophages in insulin resistance in the liver is unclear. Hyperglycemia and insulin resistance commonly occur following acute injuries or critical illness, referred to as “critical illness diabetes”. In the present studies, the roles of macrophages in hepatic insulin resistance following surgical trauma and hemorrhage were investigated. Intravenous administration of Gadolinium chloride (GdCl₃) or Clodronate-liposome (CLD-lipo) resulted in depletion of macrophages in both liver and spleen of rats. Macrophage depletion by either GdCl₃ or CLD-lipo did not prevent the development of trauma and hemorrhage-induced insulin resistance in the liver of rats, as indicated by impaired hepatic insulin signaling following a 90 minute-hemorrhage period. Similarly, hepatic insulin resistance still developed in rats after removal of the spleen (splenectomy). In contrast, macrophage depletion significantly reversed the hepatic insulin resistance several hours later, following resuscitation. As a comparison, splenectomy resulted in improvement in hepatic insulin signaling following resuscitation, but to a lesser extent, suggesting both liver and spleen resident macrophages have a role in the continuation of hepatic insulin resistance following resuscitation. These studies demonstrated that the initial development of insulin resistance in liver is macrophage-independent in a rodent model of critical illness diabetes, whereas both liver and spleen macrophages have a role in the later maintenance of the insulin resistant state, following resuscitation.

Keywords

Insulin resistance; liver; spleen; trauma; hemorrhage

INTRODUCTION

Insulin regulates glucose homeostasis through suppressing glucose production in liver and promoting glucose disposal in muscle and adipose tissue. Hyperglycemia and insulin resistance commonly occur in patients after acute injuries and critical illness, which has been referred to “diabetes of injury” or “critical illness diabetes” (1–6).

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Hyperglycemia after injuries or critical illness is an acute metabolic response to stress, which has been linked to increased mortality and morbidity in critically ill patients (7–9). Hemorrhage is a common complication of severe injuries or complex surgeries. A rodent surgical trauma and hemorrhage model was used to investigate the mechanisms of acute insulin resistance. Previous studies indicate that trauma and hemorrhage results in rapid impairment of hepatic insulin signaling, which results in hyperglycemia and hyperinsulinemia as well as impaired glucose and insulin tolerance (10–14). The acute onset of hemorrhage-induced insulin resistance in liver has been attributed, in part, to excess production of reactive oxygen species (ROS) and activation of c-Jun N terminal kinase (JNK) and Inhibitory κ B kinase (IKK) kinases (15; 16). As opposed to the initial onset of hepatic insulin resistance following trauma and hemorrhage, TNF α , plays a dominant role in continuation of the insulin resistant state later following resuscitation (12). However, the specific cellular sources of ROS or TNF α and the cell types involved in the development of hepatic insulin resistance following trauma and hemorrhage remain unclear.

Tissue macrophages play an important role in initiating an immune response and promoting inflammation and injury (17; 18). Splenic macrophages are known as an important source of inflammatory factors and splenectomy has been shown to reduce serum TNF α levels in sepsis and liver injury models in rats (19; 20). Compared with the other organs, the liver has the largest resident populations of macrophages, Kupffer cells, which are known as a major source of TNF α . Numerous studies have demonstrated the involvement of macrophages in insulin resistance as a source of proinflammatory cytokines and ROS (21–23). In the present studies, we hypothesized that macrophages, especially Kupffer cells, are a major source of ROS or TNF α after rapid blood loss, which stimulate the activation of hepatic JNK and IKK kinases and induce hepatic insulin resistance.

To directly determine the contribution of tissue macrophages in the acute development of hepatic insulin resistance following trauma and hemorrhage, two approaches of macrophage depletion were used: gadolinium chloride (GdCl₃) and Clodronate-liposome (CLD-lipo). Gadolinium chloride, a rare earth metal, can form an insoluble gadolinium hydroxide colloid at neutral pH, which can be taken up by macrophages and cause their selective elimination (24). Clodronate, a non-toxic bisphosphonate, when embedded in liposomes, is taken up by macrophages, followed by intracellular release and accumulation of clodronate, which induces macrophage apoptosis (25; 26).

In the current studies, depletion of macrophages in both liver and spleen was achieved after intravenous administration of GdCl₃ or CLD-lipo. Macrophage depletion did not prevent the development of insulin resistance in liver following trauma and hemorrhage, but completely reversed the hepatic insulin resistance several hours later, following resuscitation, suggesting that macrophages are not necessary for the initial onset of hemorrhage-induced hepatic insulin resistance, but have a role in the later maintenance of the insulin resistant state. As a comparison, splenectomy resulted in a lesser improvement in hepatic insulin signaling following resuscitation, suggesting both liver and spleen resident macrophages have a role in the recovery of hepatic insulin resistance following resuscitation.

RESEARCH DESIGN 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 (220–270g, Harlan) were housed in animal facilities for at least one week before experiments. Surgical trauma and

hemorrhage was performed as described previously (10–12; 14). Rats were fasted for 18~20 hrs and anesthetized by 1.5% isoflurane and a 5 cm midline laparotomy was performed. Catheters (PE-50) 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 maintained at 35~40 mmHg for 60min (TH60') or 90 min (TH90'). The trauma alone (T) groups were subjected to the same surgical procedures as the trauma and hemorrhage groups, but without hemorrhage. For later time points, rats were resuscitated over 60 min after the 90 min-hemorrhage period with Ringer's lactate ($4 \times$ the withdraw blood volume), and killed at 60 min after resuscitation (TH210'). At 1 min before harvesting, 5 U of insulin (Bovine, Sigma) or saline (500 μ l) was injected into the portal vein. Body temperature of the rats were not maintained during surgery or during trauma and hemorrhage.

Experimental design and administration of GdCl₃ and CLD-lipo

Clodronate was a gift of Roche Diagnostics GmbH, Mannheim, Germany. The PBS containing liposome (PBS-lipo) and clodronate containing liposome (CLD-lipo) were prepared as described (27). The PBS-lipo (200 μ l) or CLD-lipo (200 μ l) was injected into rats via the tail vein. Another group of rats were injected with GdCl₃ (Sigma, 10 mg/kg body weight) diluted in 500 μ l saline. At 48 hours post-injection, surgical trauma and hemorrhage for 90 min or 210 min were performed, and tissues were harvested for analyzing insulin signaling and kinase activity.

Procedure of Splenectomy

Splenectomy was performed either during the trauma procedure (immediately after laparotomy) or 10 days prior to the trauma and hemorrhage experiments. The rats were anesthetized and a short (~2cm) left-sided laparotomy was performed. Splenic artery, vein and nerves were tightly ligated by a silk suture to prevent any blood flow and nerve signaling ("functional splenectomy"). The spleen was then removed and the abdominal incision was sutured and bathed with 1% lidocaine to reduce postoperative pain. In sham control animals, only laparotomy was performed.

Western Blotting analysis

Liver tissue from each animal was homogenized in lysis buffer as described previously (10; 14). 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 μ g/lane) were subjected to SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were immunoblotted with anti-PY972-IR, anti-PY612-IRS1, anti-PT183/Y185-JNK1/2, (Invitrogen, Carlsbad, CA), anti-total-IR, anti-total-IRS1 (Santa Cruz Biochnology, Santa Cruz, CA), anti-pan-Actin, anti-total-ERK, anti-P473-Akt, anti-total-Akt, anti-PS180/S181-IKK α/β (Cell Signaling, Danvers, MA) antibodies. The bands were analyzed by imaging densitometry.

Immunohistochemistry

Liver or spleen tissue was rinsed in PBS and embedded in HistoPrep Frozen Tissue Embedding Media media (Fisher Scientific Inc., MA) and snap frozen in liquid nitrogen. Frozen livers were cryosectioned (10 μ m) and subjected to immunohistochemical staining with anti-CD68 antibody (AbD serotec, NC). The tissue sections were fixed in 70% ethanol overnight and blocked with 0.1% sodium azide and 0.5% H₂O₂ in PBS for 10 minutes. Overnight incubation with the primary antibody (Anti-CD68, 1:200 in 1% BSA) was followed by HRP-conjugated secondary antibody and DAB substrate. The sections were

counterstained briefly with hematoxin and images were obtained using a Leica microscope (Leica Microsystems Inc. IL).

Plasma TNF α level measurement

Just before insulin or saline injection, blood was withdrawn from the inferior vena cava in the presence of heparin, and then centrifuged at 10000 rpm for 10min. The plasma was stored at -80°C . Plasma TNF α levels were measured by the ELISA and MSD assay (Meso Scale Discovery, Gaithersburg, MD).

Densitometry and Statistical analysis

Enhanced chemiluminescence images of immunoblots were scanned and quantified using Zero D-Scan (Scanalytics Corp., Fairfax, VA). Data is presented as the mean $\pm$ SEM. The statistical differences were analyzed by ANOVA followed by Student-Newman-Keuls post test for comparison among multiple groups or Student's t-test for comparison between two groups. Mann-Whitney test was used when one of the data sets was not a Gaussian distribution. $P \geq 0.05$ was considered not statistically significant.

RESULTS

Depletion of macrophages in liver and spleen following intravenous administration of GdCl $_3$

To directly determine the role of macrophages in hepatic insulin resistance following hemorrhage, GdCl $_3$ were injected into rats via the tail vein. At 48 hours post-injection, liver and spleen tissues were frozen and sectioned and subjected to immunohisto-staining using a CD68 (a macrophage marker) antibody. Macrophages in both liver and spleen were largely ablated after administration of GdCl $_3$ (Fig. 1).

Hemorrhage-induced hepatic insulin resistance still developed after macrophage depletion by GdCl $_3$

We previously reported that hepatic insulin resistance occurs in rats 90 min following trauma and hemorrhage (10–12; 14; 28). The rats were injected with GdCl $_3$ and 48 hours post-injection, surgical trauma and hemorrhage for 90 min was performed. At the end of the 90 min hemorrhage period, prior to resuscitation, insulin was injected into the rats via the hepatic portal vein to determine the hepatic insulin signaling. Consistent with previous findings in our laboratory, insulin-induced S473-Akt phosphorylation was completely abolished after trauma and hemorrhage for 90 min in the non-injected control groups, indicating development of hemorrhage-induced hepatic insulin resistance (Fig. 2A, B). The ability of insulin to induce Akt phosphorylation was still impaired following the 90 min hemorrhage period (TH90') after macrophage depletion by GdCl $_3$ pretreatment (Fig. 2A, B). The expression of total Akt protein was not changed, whether the animal was treated with GdCl $_3$ or not (Fig. 2A).

Insulin signaling upstream of Akt was examined next. The expression of total IR and IRS1 proteins were not changed by GdCl $_3$ treatment (Fig. 2C). Similar to what occurred with P-Akt, insulin induced tyrosine phosphorylation of the insulin receptor and IRS1 (Y972-IR and Y612-IRS1, respectively) after trauma and hemorrhage for 90 min were not improved with GdCl $_3$ pretreatment compared to control group (Fig. 2C, D, E). These data suggest that liver and spleen macrophages are not important in mediating the initial development of hemorrhage-induced hepatic insulin resistance, since the insulin resistance developed whether or not macrophages had been depleted.

No effects of macrophage depletion on activation of stress signaling pathways in liver following trauma and hemorrhage

Our previous studies demonstrated activation of stress signaling pathways in liver, including the JNK and IKK pathways, which contribute to the onset of hepatic insulin resistance after trauma and hemorrhage (16). The effects of macrophage depletion on hemorrhage-induced activation of hepatic JNK and IKK kinases were examined. Consistent with our previous studies, in the control groups without GdCl₃, hemorrhage resulted in increased activation/phosphorylation of JNK1/2 (Fig. 3A, B), IKK α (Fig. 3A, C) and IKK β (Fig. 3A, D). After pretreatment with GdCl₃, activation of JNK1/2, IKK α , and IKK β were not altered after trauma and hemorrhage for 90 min compared to non-treated control groups (Fig. 3A, B, C, D). This suggests that activation of JNK1 and IKK kinases following trauma and hemorrhage are macrophage-independent.

No effects of macrophage depletion by CLD-lipo on the onset of hemorrhage-induced hepatic insulin resistance

To confirm the findings with GdCl₃, another approach of macrophage depletion was used, where CLD-lipo was injected into the rats via tail vein at 48 hours before surgical procedures, and either the trauma alone or the trauma and hemorrhage protocols were performed. Similar to what was found with GdCl₃, CLD-lipo pretreatment did not improve insulin signaling in liver after trauma and hemorrhage for 90 min, as determined by the levels of insulin-induced S473-Akt, Y972-IR, Y612-IRS1 phosphorylation (Fig. 4A, B, C, D, E). This further confirmed that the development of hepatic insulin resistance following trauma and hemorrhage is macrophage independent. Similar to what was found with GdCl₃, activation of JNK, IKK α , and IKK β were not affected by CLD-lipo (data not shown).

No effects of splenectomy on the onset of hemorrhage-induced hepatic insulin resistance

To determine a potential role of the spleen in the acute development of hepatic insulin resistance following trauma and hemorrhage, splenectomy was performed at either 10 days before or immediately before hemorrhage. Hepatic insulin signaling following trauma and hemorrhage for 60 min (TH60') was not improved after splenectomy, as insulin signaling determined by insulin-induced phosphorylation of Y972-IR, Y612-IRS1 and S473-Akt (Fig. 5A, B). This suggests that the spleen is not necessary for the acute development of hemorrhage-induced hepatic insulin resistance.

Recovery of hepatic insulin signaling following macrophage depletion

As a dominant role of TNF α in the later maintenance of insulin resistance has been indicated in our previous studies, the effect of macrophage depletion on hepatic insulin signaling later, following resuscitation, was next determined. Unlike what was found at the earlier time point (TH90'), the ability of insulin to induce phosphorylation of Akt, IR and IRS1 in the liver of rats several hours following hemorrhage and resuscitation (TH210') was significantly increased after macrophage depletion by GdCl₃ (Fig. 6 A, B, C, D). This suggests that macrophages have an important role in maintaining the insulin resistant state later, despite not being necessary for the onset of insulin resistance.

Expression of IGFBP-1 is normally inhibited by insulin via activation of the PI3K/Akt pathway (10). Similar to our previous studies measuring IGFBP-1 mRNA by Northern blots (10; 12), there was an increase in IGFBP-1 by trauma alone, and a further 1.5-fold induction by the combination of trauma and hemorrhage. Treatment of rats with GdCl₃ completely abolished the hemorrhage-induced increase in IGFBP-1, significantly ($p < 0.05$) reducing the level to that of the trauma-only control animal. This suggests that macrophage depletion could also restore insulin's action downstream of Akt activation.

Partial recovery of hepatic insulin signaling following splenectomy

To better understand whether the later maintenance of hepatic insulin resistance is mainly attributed to local liver macrophages or systemic factors derived from the spleen, insulin signaling in the liver of rats after spleen removal was examined. As opposed to almost complete recovery in insulin signaling with macrophage depletion of both liver and spleen, splenectomy at either 10 days before or right before hemorrhage resulted in a partial improvement in insulin induced PY972-IR, PY612-IRS1 and PS473-Akt at 210 min following trauma and hemorrhage after resuscitation (Fig. 7A, B, C, D). This suggests that both liver and spleen have a role in maintaining the hepatic insulin resistant state later, following resuscitation.

Macrophage depletion or splenectomy resulted in decreased circulating TNF α level following resuscitation

We next determined the effects of macrophage depletion or splenectomy on the levels of circulating TNF α in rats following trauma and hemorrhage and resuscitation. Consistent with previous data (11), at 210 min following trauma, hemorrhage and resuscitation, circulating TNF α increased to an average of 108 pg/ml compared to 16 pg/ml following trauma alone. After depletion of Kupffer cells and splenic macrophages by GdCl₃, the level of circulating TNF α decreased significantly to an average of 18 pg/ml (Fig. 8) at 210 min following trauma and hemorrhage, similar to the value in the absence of hemorrhage. Circulating TNF α also decreased in rats after removal of spleen at 10 days before surgery and 210 min after trauma and hemorrhage (27.3pg/ml, Fig. 8), approaching the values in the absence of hemorrhage or following GdCl₃, and significantly less than trauma and hemorrhage without splenectomy. This data confirms that macrophages in general, and the spleen in particular, are important sources of circulating TNF α following trauma, hemorrhage and resuscitation, and this TNF α has important role in the maintenance of the insulin resistant state in the liver of rats later following resuscitation (12).

DISCUSSION

With a surgical trauma and hemorrhage model of critical illness diabetes, we have previously demonstrated a causative role of ROS in the rapid development of hemorrhage-induced hepatic insulin resistance and a dominant role of TNF α in the later maintenance of the insulin resistant state (11; 12; 14). The aim of the present work is to understand the specific cellular sources of ROS or TNF α and the type of cells involved in the pathogenesis of acute insulin resistance. Our current data demonstrated that macrophages, in both liver and spleen, are not necessary for the acute onset of insulin resistance following trauma and hemorrhage, but contribute to the maintenance of the insulin resistant state later after resuscitation.

Following hemorrhage or experimental liver ischemia, most studies have focused on the activity and function of macrophages after resuscitation or reperfusion, in which macrophages are classically activated and produce a number of pro-inflammatory factors and ROS (29; 30). However, not much is known about the activity and role of macrophages during the hemorrhage or ischemia period, prior to resuscitation or reperfusion. We previously found that increased production of ROS and activation of the cell stress (JNK) and inflammatory (IKK/NF κ B) pathways occur within minutes following hemorrhage, which play a key role in the rapid development of hepatic insulin resistance, partly through inducing inhibitory IRS-1 serine phosphorylation (15; 16).. CLD-lipo and GdCl₃ have been widely used experimentally in elucidating the role of macrophages and are able to deplete macrophages in both the liver and spleen (25; 26; 31–34). In the present studies, both approaches, GdCl₃, and CLD-lipo, were used, and demonstrated that after depletion of

macrophages, hepatic insulin resistance still developed after the combination of trauma and hemorrhage. In addition, the development of hepatic insulin resistance cannot be prevented by splenectomy. Therefore the initial development of hepatic insulin resistance, during the hemorrhage period, was macrophage-independent. In the absence of macrophages in the liver and spleen, the observed activation of JNK and IKK kinases in liver after trauma and hemorrhage likely occurs within hepatocytes, in which they directly interact with IR or IRS proteins and inhibit insulin signal transduction.

Our previous studies demonstrated that inhibition of ROS by NAC resulted in decreased activation of JNK and IKK kinases in liver (15), suggesting ROS is the upstream inducer of JNK and IKK activation in liver following trauma and hemorrhage. The unaltered activation of JNK and IKK following macrophage depletion suggests that macrophages are not a major source of ROS during the hemorrhage period. Thus, other cells, such as hepatocytes or endothelial cells, may be the source of ROS in response to trauma and hemorrhage. When macrophages are eliminated prior to trauma and hemorrhage, other cells are capable of providing sufficient ROS which results in activation of JNK and IKK, and, therefore, insulin resistance can still develop in the absence of Kupffer cells and splenic macrophages.

The recovery of hepatic insulin signaling, later, following resuscitation, by depletion of macrophages suggests that macrophages are important for maintenance of the insulin resistant state following its initial development. Our previous findings indicate that increased circulating TNF α has a role in hepatic insulin resistance following resuscitation (12), but TNF α does not increase rapidly enough to explain the initial development of insulin resistance (manuscript submitted). In the present studies, the circulating level of TNF α following resuscitation, several hours after the initial trauma and hemorrhage, was largely decreased after macrophage depletion, that is depletion of Kupffer cells, splenic macrophages, and other macrophage populations. In addition, the circulating level of TNF α was also significantly decreased by removal of the spleen, suggesting that much of the increased TNF α following trauma and hemorrhage was from the spleen. Thus, tissue macrophages in the liver or spleen, are likely activated in response to blood loss and resuscitation to release sufficient inflammatory factors, such as TNF α , which acts systemically or locally on hepatocytes and inhibits hepatic insulin signaling.

The spleen is reported as one of the main sites producing inflammatory mediators, including TNF α , in response to ischemia reperfusion injury (19; 20). Compared to GdCl₃ treatment, which deplete macrophages in both liver and spleen with nearly complete reversal of insulin resistance following resuscitation, removal of the spleen also resulted in improvement in hepatic insulin signaling, but to a lesser extent. This suggests that the spleen has a role in maintaining the insulin resistant state following resuscitation, at least in part by inhibiting hepatic insulin signaling, and is an important source of circulating TNF α .

In summary, the present studies demonstrated differential roles of macrophages between the initial development and later maintenance of hemorrhage-induced hepatic insulin resistance. Our findings suggest that the initial onset of insulin resistance is macrophage independent, whereas following resuscitation macrophages in both liver and spleen are activated and release factors, such as TNF α locally and into the circulation, which result in the maintenance of insulin resistance.

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ABBREVIATION

CLD-lipo	Clodronate-liposome
GdCl₃	Gadolinium chloride
IR	insulin receptor
IRS	insulin receptor substrate
PI3K	phosphoinositide 3-kinase
IKK	Inhibitory κ B kinase
JNK	c-Jun N-terminal kinase
TH	trauma and hemorrhage
T	trauma alone
ERK	extracellular signal-regulated kinase

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Fig. 1. Macrophage depletion after GdCl₃ administration

Rats were injected with GdCl₃ (10 mg/kg) via the tail vein. At 48 hours post-injection, surgical trauma and hemorrhage for 210 min were performed and liver and spleen tissues were harvested. Immunohistochemistry was performed on frozen liver sections with anti-CD68 antibody, followed by HRP-conjugated secondary antibody and DAB substrate. Magnification: 10×. Images are representatives of repeated experiments; the microscopic fields were randomly selected and the analysis was performed in a blinded manner.

Fig. 2. Insulin signaling in liver following trauma and hemorrhage for 90 min with macrophage depletion by GdCl₃

Rats were injected with GdCl₃ (10 mg/kg) via the tail vein, at 48 hours post-injection, surgical trauma and hemorrhage for 90 min was performed and liver tissues were harvested. (A). Liver protein extracts were subjected to Western Blot analysis with anti-PS473-Akt and total-Akt antibodies. (B) The level of PS473-Akt was presented as the mean $\pm$ SEM in the graph (n=3 for each group); (C) Liver protein extracts were subjected to Western Blot analysis with anti-PY972-IR, PY612-IRS1, total-IRS1 and total-IR antibodies. (TH90': trauma and hemorrhage for 90 min; T90': trauma alone for 90 min). The levels of PY972-IR (D) and PY612-IRS1 (E) was presented as the mean $\pm$ SEM in the graphs (n=3–5 for each group).

Fig. 3. Effect of macrophage depletion by GdCl₃ on the activation of JNK and IKK kinases in liver following trauma and hemorrhage

Rats were injected with GdCl₃ (10 mg/kg) via the tail vein, at 48 hours post-injection, surgical trauma and hemorrhage for 90 min was performed and liver tissues were harvested. (A). Liver protein extracts were subjected to Western Blot analysis with anti-PT181/Y183-JNK1/2, PS181-IKK β /PS180-IKK α , and total-ERK antibodies. (B, C, D). The levels of P-JNK1 (B), P-IKK α (C) and P-IKK β (D) in liver was presented as the mean $\pm$ SEM in the graph (n=4-9 for each group; TH90': trauma and hemorrhage for 90 min; T90': trauma alone for 90 min).

Fig. 4. Insulin signaling in liver following trauma and hemorrhage for 90 min with macrophage depletion by CLD-lipo

Rats were injected with PBS-lipo (200 μ l) or CLD-lipo (200 μ l) via the tail vein, at 48 hours post-injection, surgical trauma and hemorrhage for 90 min was performed and liver tissues were harvested. (A). Liver protein extracts were subjected to Western Blot analysis with anti-PS473-Akt and total-Akt antibodies; (B) The level of PS473-Akt was presented as the mean $\pm$ SEM in the graph (n=3 for each group); (C). Liver protein extracts were subjected to Western Blot analysis with anti-PY972-IR, PY612-IRS1 and total-IR antibodies. The levels of PY972-IR (D) and PY612-IRS1 (E) was presented as the mean $\pm$ SEM in the graphs. (n=3–5 for each group. TH90': trauma and hemorrhage for 90 min; T90': trauma alone for 90 min).

Fig. 5. Hepatic insulin signaling following trauma and hemorrhage for 60 min after splenectomy Splenectomy was performed on rats at 10 days before or immediately prior to the surgical trauma and hemorrhage procedure. (A). Liver protein extracts were subjected to Western Blot analysis with anti-PS473-Akt, PY612-IRS1, PY972-IR, and total-Akt antibodies. (B). The level of PS473-Akt is presented as the mean $\pm$ SEM in the graph. (ins: insulin; $n=3$ rats for each group; TH60': trauma and hemorrhage for 60 min; T60': trauma alone for 60 min).

Fig. 6. Insulin signaling in liver following trauma and hemorrhage for 210 min after resuscitation with macrophage depletion by GdCl₃

Rats were injected with GdCl₃ (10 mg/kg) via the tail vein, at 48 hours post-injection, surgical trauma and hemorrhage for 90 min were performed and after resuscitation over 60 min and recovery in the cage for 60 min, liver tissues were harvested. (A). Liver protein extracts were subjected to Western Blot analysis with anti-PS473-Akt, PY972-IR, PY612-IRS1, and total-Akt antibodies. The levels of PY972-IR (B), PY612-IRS1 (C), and PS473-Akt (D) are presented as the mean $\pm$ SEM in the graph (n=2-4 for each group; TH210': trauma and hemorrhage for 90 min + resuscitation for 60 min + recovery for 60 min; T210': trauma alone for 210 min).

Fig. 7. Partial recovery of hepatic insulin signaling following trauma and hemorrhage for 210 min after splenectomy

Splenectomy was performed on rats at 10 days before or immediately prior to the surgical trauma and hemorrhage procedure. After a hemorrhage period of 90 min, resuscitation over 60 min and recovery in the cage for 60 min, liver tissues were harvested. (A). Liver protein extracts were subjected to Western Blot analysis with anti-PS473-Akt, PY972-IR, PY612-IRS1, and total-Akt antibodies. The levels of PY972-IR (B), PY612-IRS1 (C), and PS473-Akt (D) are presented as the mean $\pm$ SEM in the graph ($n=2-4$ for each group; TH210': trauma and hemorrhage for 90 min + resuscitation for 60 min + recovery for 60 min; T210': trauma alone for 210 min; #: $P<0.05$ vs T210'+ins in control or splenectomized groups respectively; *: $P<0.05$ vs TH210' +ins in non-splenectomized control group; &: $P=0.064$ vs TH210' +ins in non-splenectomized control group; \$: $P=0.055$ vs TH210' +ins in non-splenectomized control group).

Fig. 8. Levels of circulating TNFα following trauma and hemorrhage for 210 min after resuscitation with macrophage depletion or splenectomy

Rats were injected with GdCl₃ (2 days before surgical procedures) or subjected to splenectomy (10 days before surgical procedures) as previously described. Surgical trauma and hemorrhage for 90 min were performed and after resuscitation over 60 min and recovery in the cage for 60 min, liver tissues were harvested. The level of plasma TNFα are presented as the mean $\pm$ SEM in the graph (n=4–10 for each group; TH210': trauma and hemorrhage for 90 min + resuscitation for 60 min + recovery for 60 min; T210': trauma alone for 210 min).