Acute, Muscle-Type Specific Insulin Resistance Following Injury

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Acute insulin resistance can develop following critical illness and severe injury, and the mortality of critically ill patients can be reduced by intensive insulin therapy. Thus, compensating for the insulin resistance in the clinical care setting is important. However, the molecular mechanisms that lead to the development of acute injury/infection-associated insulin resistance are unknown, and the development of acute insulin resistance is much less studied than chronic disease-associated insulin resistance. An animal model of injury and blood loss was utilized to determine whether acute skeletal muscle insulin resistance develops following injury, and surgical trauma in the absence of hemorrhage had little effect on insulin-mediated signaling. However, following hemorrhage, there was an almost complete loss of insulin-induced Akt phosphorylation in triceps, and severely decreased tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1. The severity of insulin resistance was similar in triceps and extensor digitorum longus muscles, but was more modest in diaphragm, and there was little change in insulin signaling in cardiac muscle following hemorrhage. Since skeletal muscle is an important insulin target tissue and accounts for much of insulin-induced glucose disposal, it is important to determine its role in injury/infection-induced hyperglycemia. This is the first report of an acute development of skeletal muscle insulin signaling defects. The presented data indicates that the defects in insulin signaling occurred rapidly, were reversible and more severe in some skeletal muscles, and did not occur in cardiac muscle.

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with insulin resistance, skeletal muscle, adipose tissue, and liver become insulin resistant. However, it is not known which of these three main insulin target tissues become insulin resistant acutely following injury. Since skeletal muscle is a main insulin target tissue, and accounts for approximately 80% of insulin-induced glucose disposal in the human body (19), it is important to understand its role in the acute development of insulin resistance. In the current study, we utilized a rat model of surgical trauma and hemorrhage to determine the development, timing, and muscle selectivity of hemorrhage-induced skeletal muscle insulin resistance.

**MATERIALS AND METHODS**

**Reagents and Materials**

All reagents and materials were obtained from Fisher Scientific (Pittsburgh, PA, USA) or Sigma-Aldrich (St. Louis, MO, USA), unless otherwise noted.

**Animal Model of Surgical Trauma and Hemorrhage**

All procedures were carried out in accordance with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals and the National Institutes of Health. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. A model of surgical trauma and hemorrhage in the rat, as previously described (6,7), was used with modifications. Briefly, male Sprague-Dawley rats received continuous inhalation of low levels of isoflurane (Mallinckrodt Veterinary, Mundelein, IL, USA) throughout the surgery and hemorrhage periods. A 5-cm ventral midline laparotomy was performed representing soft-tissue trauma, the abdomen was closed in layers, and the wounds were bathed with 1% lidocaine (Elkins-Sinn, Cherry Hill, NJ, USA). The right and left femoral arteries and the right femoral vein were catheterized for bleeding, monitoring of mean arterial pressure and fluid resuscitation, respectively. The rats were bled to a mean arterial pressure (MAP) of 35–40 mmHg within 10 min. Once MAP reached 40 mmHg, the timing of the hemorrhage period began and was maintained for up to 90 min. If the rats were not killed during the hemorrhage period, they were resuscitated with Ringer’s lactate (4 × the withdrawn blood volume) infusion over 60 min. Sham-operated rats underwent the same surgical procedures, but without hemorrhage. To ensure that the effects observed were not due to the initial bleed, following the initial bleed, the animal’s original whole blood (heparinized) was returned, and the animal maintained for 60 min.

**Experimental Design**

Due to the considerable stress incurred by anesthesia and surgical trauma, it was impossible to have a completely untreated control group. Thus, trauma-alone rats (T0′) that were subjected to anesthesia, laparotomy, and catheterization, and then immediately killed were selected as the “baseline” animals in these experiments (6,7). Additional trauma-only groups were subjected to the same procedures and then killed at 30 (T30′), 60 (T60′), and 90 min (T90′), and 5 (T5h) and 24 h (T24h) after catheterization. Trauma plus hemorrhage (TH) groups were subjected to the same procedures as the T groups, but also subjected to hemorrhage (see above) and then killed at 0 (TH0′), 15 (TH15′), 30 (T30′), 60 (TH60′), and 90 min (TH90′), and 5 (TH5h) and 24 h (TH24h) after the initial bleed period.

**Blood and Tissue Harvesting Procedures**

At the appropriate time points, the abdominal cavity was opened again, blood samples were collected, and insulin (5U) or saline was injected into the portal vein. The triceps, extensor digitorum longus, diaphragm, and heart were removed and frozen in liquid nitrogen 2–3 min following the injection.

**Measurement of Fasting Blood Insulin and Glucose Levels**

Serum samples were collected and stored at –80° C until analysis. Insulin levels were determined using a rat insulin radioimmunoassay kit (Linco Research, St. Charles, MO, USA). Glucose levels were measured using a GM7 Analyzer (Analox Instruments, Lunenburg, MA, USA). All analysis was performed by the UAB Clinical Nutrition Research Unit.

**Preparation of Tissue Lysates**

Tissue lysates were prepared as described previously (20). Pulverized muscle tissue (50–60 mg; triceps, extensor digitorum longus, diaphragm, or heart) from each animal was homogenized with a pestle mounted in a motorized homogenizer in ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 0.5% Igepal, 100 μM PMSF, 1 mM Na3VO4, 0.1 μM okadaic acid, and 0.5 × P2714 protease inhibitors. A protease/phosphatase inhibitor cocktail containing 100 μM PMSF, 1 mM Na3VO4, 0.1 μM okadaic acid, and 0.5 × P2714 protease inhibitors was added to each tissue lysate and the lysates were placed on ice for 30 min. Tissue lysates were centrifuged twice at 15,000g and supernatants were stored at –80° C until use.

**Western Blot Analysis**

Protein concentrations were determined (BCA, Pierce, Rockford, IL, USA) and 30 μg/lane of total protein was resolved by SDS-PAGE and transferred to nitrocellulose membranes (6,21,22). The membranes were immunoblotted with anti-phosphoserine-Akt (S473), anti-total Akt, and anti-total ERK1/2 antibodies (Cell Signaling Technology, Beverly, MA, USA); anti-phosphotyrosine-IR (Y972), and anti-phosphotyrosine-IRβ (Y612; Invitrogen, Carlsbad, CA, USA); and anti-total IRβ (Santa Cruz Biotechnology, Santa Cruz, CA, USA), also used. Membranes were developed with ECL (Amersham Biosciences, Piscataway, NJ, USA).
Densitometric and Statistical Analysis

ECL images of immunoblots were scanned and quantified using Flurochem FC digital imaging software (α Innotech, San Leandro, CA, USA; 6,22). All data are presented as means ± SEM. Analysis of variance (ANOVA) and Student t test were performed using GraphPad InStat version 3 software (San Diego, CA, USA).

RESULTS

Insulin-Induced Phosphorylation of Akt Is Abolished in Skeletal Muscle Following Hemorrhage

The initial experiments were to determine whether hemorrhage would lead to the acute development of skeletal muscle insulin resistance. Based on previous studies, immediately following surgery (T0’), 90 min following surgery (T90’), or 90 min following surgery and hemorrhage (TH90’), insulin (+) or saline (−) was injected, and serine phosphorylation of Akt (S473) in skeletal muscle (triceps) was examined first. Phosphorylation of S473 is essential for full activation of Akt (23), and was decreased dramatically following TH90’, compared with either T0’ or T90’ (Figure 1A). In many insulin resistant states, skeletal muscle insulin resistance is an early event and precedes the development of insulin resistance in other target tissues (24). To ensure the consistent development of hemorrhage-induced skeletal muscle insulin resistance, a second time point was selected. Following trauma and hemorrhage for 60 min, there was abolishment of insulin-induced phosphorylation of Akt on S473 (Figure 1B) in triceps, with no change in total Akt protein levels. Skeletal muscle Akt phosphorylation was not altered following trauma only (T60’), indicating that decreased insulin signaling is the result of hemorrhage. Quantified data from multiple animals is presented as the fold-change in P-Akt (S473) in the presence (+) or absence (−) of insulin (Figure 1C). In trauma followed by immediate death (T0’) and 60 min after surgery (T60’), there were increases in P-Akt (S473) in triceps (11.6- and 9.3-fold, respectively) following insulin injection. However, following trauma and hemorrhage for 60 min (TH60’), there was a complete loss of insulin-induced triceps P-Akt (S473). Trauma and hemorrhage for 60 min did result in a small decrease in basal (non-insulin stimulated) Akt phosphorylation, but this decrease did not reach statistical significance compared with trauma-only animals. Thus, the data indicates a rapid, hemorrhage-induced insulin signaling defect in skeletal muscle. Approximately 60% of the total blood volume is removed during the initial bleed. To ensure that the decrease in P-Akt in skeletal muscle is not the result of the stress of the initial bleed step, blood withdrawal followed by immediate replacement of that blood was performed. In these experiments, following the initial 10 min bleed necessary to decrease the MAP to 35–40 mmHg, the rat’s whole blood, with added heparin, was returned (replaced) to that rat (blood withdrawal and replacement: BWR). Following a recovery period of 60 min (BWR60’), there was no change in insulin-induced phosphorylation of Akt (S473; Figure 1D). An additional control (blood with control: BWC) was added to this experiment to ensure that the addition of heparin itself was without effect. This data suggests that the decreases observed following hemorrhage are the result of a sustained decrease in MAP and are not simply due to the initial stress of decreased blood volume.
Fasting Serum Glucose Levels Are Increased Following Trauma and Hemorrhage

Changes in fasting glucose and insulin levels following trauma and hemorrhage were examined and trauma alone for 60 min (T60') had no effect on fasting glucose levels compared with fasting glucose levels in normal rats. However, following trauma and hemorrhage (TH60') there was a significant increase in fasting glucose levels to 161 mg/dL (Figure 2A). This increase in fasting glucose is suggestive of insulin resistance and may, in part, be due to a decrease in insulin action in skeletal muscle. In the current studies, the rise in insulin levels varied from animal to animal, resulting in increased insulin levels early following injury during the “ebb” phase of the injury (27,28).

IRS-1 and IR Tyrosine Phosphorylation in Skeletal Muscle Is Decreased Following Hemorrhage

A reduction in insulin signaling via P-Akt may be due to alterations in phosphorylation of upstream components of the insulin signaling pathway. To address this possibility, experiments were performed to examine the phosphorylation of a specific tyrosine residue of IRS-1, tyrosine 612, that is important for activation of the PI3-kinase/Akt pathway in skeletal muscle. There was an increase in triceps insulin-stimulated P-IRS-1 (Y612) immediately following (T0'; 29-fold) and 60 min (T60'; 19-fold) after the initial trauma (Figure 3). Unlike trauma alone, trauma and hemorrhage caused an increase in basal IRS-1 (Y612) phosphorylation in triceps. However, this increase in triceps basal P-IRS-1 (Y612) was accompanied by a complete lack of any further effect of insulin (Figure 3A,B), indicating a resistance to exogenous insulin, with no change in total protein levels (not shown). We next questioned whether there were changes in phosphorylation of the insulin receptor (IR), upstream of IRS-1, in skeletal muscle. When measured, phosphorylation of tyrosine 972 of the IR (P-IR [Y972]), required for the binding and phosphorylation of IRS-1, was induced by insulin 6.4-fold and 8.0-fold in skeletal muscle following trauma only for 0 min (T0' and T60'), respectively (Figure 3C,D). However, phosphorylation of P-IR (Y972) was decreased significantly to two-fold following trauma and hemorrhage (TH60'), with no change in total IR protein levels.

Time Course of Alterations in Skeletal Muscle Insulin Signaling Following Trauma and Hemorrhage

Similar experiments were performed to determine the timing of the develop-
ment of triceps insulin resistance following trauma and hemorrhage. Immediately following surgery and after the initial bleed (TH0'), insulin-induced skeletal muscle P-Akt is not altered, and insulin-induced P-Akt signaling is still functional in skeletal muscle following 15 and 30 min of hemorrhage (TH15' and TH30'; Figure 4A,B,C).

The time course of changes in skeletal muscle IRS-1 and IR phosphorylation after hemorrhage also was studied and indeed there were similar decreases observed in tyrosine phosphorylation of IRS-1 and IR (Figure 5) in skeletal muscle. Whereas P-IRS-1 (Y612) was decreased significantly 60 and 90 min following trauma and hemorrhage (TH60', TH90'), P-IRS-1 (Y612) was unchanged following only 30 min (TH30'; Figure 5A,B). This also was true of the loss of P-IR (Y972) in skeletal muscle observed at 60 and 90 (TH60' and TH90'), but not 30 min (TH30'; Figure 5C,D) following hemorrhage, and was consistent with a normal-fold induction of P-Akt induction by insulin in triceps at the TH30' time point. Together, these data suggest a distinct time course of the hemorrhage-induced defect of insulin signaling that occurred in skeletal muscle between 30 and 60 min following the initiation of hemorrhage.

**Insulin-Induced Phospho-Akt Is Disrupted in Extensor Digitorum Longus after Trauma and Hemorrhage, but Less So in Diaphragm, and Not in Cardiac Muscle**

To determine whether there are changes in skeletal muscle insulin signaling in other skeletal muscles, changes in insulin-induced P-Akt (S473) in diaphragm and extensor digitorum longus muscles were examined. Insulin-induced phosphorylation of P-Akt was decreased in diaphragm following trauma and hemorrhage, but not trauma alone, at the 60 and 90 min time points (only TH60' shown; Figure 6A), although the decrease was much less severe than in triceps. In contrast, extensor digitorum longus muscle responded to hemorrhage in much the same way as triceps muscle: following 60 (Figure 6B) and 90 min (not shown), there was a complete loss in insulin-induced P-Akt (S473), but no significant change occurred at only 30 min (not shown) following hemorrhage. This is suggestive of hemorrhage inducing severe insulin resistance in a subset (triceps and extensor digitorum longus), but not all, skeletal muscles. The acute development of insulin resistance appears to be independent of muscle fiber type since both slow-twitch (triceps) and fast-twitch (extensor digitorum longus) muscles appear to be affected by hemorrhage.

It was then asked whether the modest (diaphragm) or the more severe effect of trauma and hemorrhage on the development of insulin resistance in the triceps and EDL also occurred in cardiac muscle. Insulin-induced phosphorylation of P-Akt was not altered in cardiac muscle following trauma and hemorrhage at either the 60 or 90 min hemorrhage time points (TH60' and TH90'). There also were no significant changes in basal P-Akt (Figure 6C), and no changes in total Akt levels (not shown), following trauma alone or trauma and hemorrhage in cardiac muscle. This indicates that, unlike skeletal muscle, hemorrhage had little or no effect on insulin signaling in cardiac muscle.
Lastly, it was asked whether there was recovery from the trauma and hemorrhage-induced insulin signaling defect in skeletal muscle. At the end of the 90 min hemorrhage period, the animals were resuscitated and allowed to recover. There was little difference in the ability of a high dose insulin injection to elicit increased phosphorylation of P-Akt following hemorrhage and resuscitation and then 5 (TH5h) or 24 h (TH24h) of recovery, when compared with rats that had undergone trauma alone (Figure 7). Thus, there was a recovery of the response to high dose insulin 5 and 24 h following resuscitation.

**DISCUSSION**

Hyperglycemia is a hallmark of insulin resistance. Since skeletal muscle is a primary site of insulin-regulated glucose disposal, hyperglycemia can be caused by decreased insulin action on skeletal muscle. Skeletal muscle insulin resistance is common in many chronic clinical syndromes, including Type 2 diabetes, obesity, and metabolic syndrome (25,29–32). Common to all of these clinical syndromes is the chronic nature of the syndrome, and a slow development of symptoms, including insulin resistance, prior to the first clinical presentation of the disease. In the present work, a defect in insulin signaling in skeletal muscle was observed as early as 60 min following trauma and hemorrhage.
In the present studies, a rat model of surgical trauma and hemorrhage was used (6,7) to study the acute alterations in insulin signaling that may be involved in hemorrhage-induced skeletal muscle insulin resistance. We demonstrate that skeletal muscle insulin resistance developed quickly (within 60 min) following hemorrhage. This insulin resistance was characterized by a rapid decrease in Akt phosphorylation following the onset of hemorrhage at an important serine residue, S473, which is essential for Akt activity (23,33). The presented data also indicates that the alteration in Akt phosphorylation was due to a sustained decrease in MAP, and not simply due to the stress of a transitory decrease in blood volume. Coincident with the decrease in insulin signaling is a significant rise in blood glucose levels. Because of the decrease in hematocrit due to blood loss, the rise in blood glucose levels might be underestimated, but hyperglycemia is observed clearly after only 60 min following hemorrhage.

Skeletal muscle fibers can be classified broadly into two categories, slow- and fast-twitch. Insulin is known to elicit a greater response in slow-twitch skeletal muscle versus fast-twitch skeletal muscle (34). We observed severe inhibition of insulin signaling both in slow-twitch (triceps) and fast-twitch (extensor digitorum longus) muscles following trauma and hemorrhage. However, the decrease of insulin signaling in diaphragm (slow-twitch) was less extensive. In addition to skeletal muscle, cardiac muscle also was examined and little change in insulin-induced Akt signaling was demonstrated in the heart. Thus, the rapid development of insulin resistance following trauma and hemorrhage is specific to the skeletal muscle and not cardiac muscle, is independent of fiber type, but may be dependent on other factors, such as muscle function. Following hemorrhage, the maintenance of proper insulin signaling in a subset of muscles, such as the diaphragm (essential for proper ventilation) and the heart (already severely affected by the decrease in blood volume and pressure) are critical to ensure oxygenation of the remaining blood and perfusion of the brain and heart, respectively, both necessary for survival. It is not yet known whether the maintenance of blood flow to the heart and diaphragm protect them from developing severe insulin resistance, or whether there is some alternate explanation, such as the differential use of IGF-1/insulin hybrid receptors. This question will require further research. However, it is clear from the present studies that insulin signaling in skeletal muscles involved in locomotion (such as the triceps and extensor digitorum longus) may be compromised rapidly for the overall benefit of the critically injured individual.

In addition to altered Akt phosphorylation, other components of the insulin signaling pathway were altered in skeletal muscle within 60 min following trauma and hemorrhage. We present the first evidence that both insulin-induced IR and IRS-1 tyrosine phosphorylation were decreased rapidly in skeletal muscle following hemorrhage. Previous data from our laboratory suggest that in the liver, IR function (phosphorylation) is not changed by hemorrhage, suggesting post-receptor defects are solely responsible for the hepatic alterations observed (7), but here there is clearly a defect at the level of insulin receptor activation. Together with the current data, it suggests that there are tissue-specific differences in the mechanism underlying hemorrhage-induced insulin resistance. A clearer understanding of this difference will require further examination. However, it is important to note that variability in the phosphorylation state of the IR is not uncommon and has been highly variable when measured by different laboratories (35–42).
In recent years, the importance of IRS-1 in the molecular mechanism underlying insulin resistance has emerged. Reduced tyrosine phosphorylation of IRS-1 may contribute to insulin resistance in human skeletal muscle in chronic diseases such as obesity and Type 2 diabetes. Phosphorylation of a specific tyrosine (Y612) of IRS-1, which may serve as a docking site for PI3-kinase (45), was examined in the present studies and there was an acute decrease of insulin-induced IRS-1 tyrosine 612 phosphorylation in skeletal muscle following trauma and hemorrhage, which likely resulted in reduced association with PI3-kinase. Most relevant is that acute defects of insulin signaling occurred by 60 min, not the weeks, months, or years often necessary in chronic diseases.

There is a significant recovery of insulin signaling that occurs upon resuscitation following hemorrhage. Resuscitation with Ringer’s lactate is similar to the treatment of patients following surgical or accidental blood loss. It is not known how rapidly recovery occurs, but the data indicates that in response to a high dose of insulin, insulin signaling is mostly recovered within 5 h after resuscitation. To determine whether there is complete recovery, more extensive time-course and dose response studies will need to be performed. However, more importantly, further information on the mechanisms by which skeletal muscle becomes insulin resistant is needed. Our preliminary data indicates a complex array of contributing factors that are injury-type and tissue-specific. Once these mechanisms are understood, and this may be as complicated as chronic states of insulin resistance, there will need to be an understanding of the mechanisms by which the insulin signaling defects are reversed.

The rapid recovery of insulin responsiveness in rats following resuscitation indicates no permanent tissue damage and minimal or no loss of cells (apoptosis or necrosis) following hemorrhage for 90 min. However, longer bouts of reduced blood pressure and more extensive blood loss are less conducive to recovery, but the point at which blood loss is too severe for recovery of insulin signaling is not yet known and may be tissue and species specific. In recent work, we found that less blood loss results in more modest forms of insulin resistance in liver (8). In ICU patients, attainment of euglycemia, achieved either on their own or following intensive treatment with insulin, greatly enhances recovery and reduces mortality (17,18,46,47). Therefore the ability to recover insulin responsiveness, and/or euglycemia, may be an important predictor for reduction of morbidity and mortality in the intensive care environment.

In summary, skeletal muscle insulin resistance following hemorrhage occurs quickly and involves multiple alterations in the IR/IRS-1/Akt signaling pathway. This insulin resistance occurs in both fast-twitch (extensor digitorum longus) and slow-twitch (triceps) skeletal muscle, but it is not uniform to all muscles. The insulin signaling defects are not permanent, with recovery by 5 h following fluid resuscitation. There are multiple neural, hormonal, and immune/cytokine responses to hemorrhage that may play a role in the acute development of insulin resistance, and in the recovery of insulin signaling following resuscitation. There are likely different causative factors for the acute development of insulin resistance in different tissues, and additional factors in more severe versus less severe degrees of injury and/or hemorrhage, which will require continued research to delineate. The present work indicates that a rapid defect of insulin signaling can be measured in some rat skeletal muscles, but it is not uniform, and seems to occur less in constantly used muscles (diaphragm) and not at all in cardiac muscle.

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DISCLOSURE

The authors have nothing to disclose.

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