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Age and Tissue Specific Differences in the Development of Acute Insulin Resistance Following Injury

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Abstract

Injuries, hemorrhage, sepsis, burn, and critical illnesses all induce insulin resistance, and insulin resistance is strongly associated with advancing age. However, the effect of age on injury-induced insulin resistance is not well studied. We performed surgical trauma in male rats of three different ages (3-, 6- and 10-weeks old). Rats were either hemorrhaged to a mean arterial pressure of 35–40 mmHg and subsequently maintained at that pressure for up to 90 minutes, or maintained without hemorrhage as controls. Results indicate that insulin-induced intracellular signaling was diminished in liver and skeletal muscle of 6- and 10-week old rats following trauma and hemorrhage. In even younger rats, immediately post-weaning (approximately 3 weeks of age), insulin signaling was lost in liver, but not in skeletal muscle. Glucocorticoids can play a role in the chronic development of insulin resistance. Our results demonstrate that corticosterone levels were increased in 6- and 10-week old animals following hemorrhage, but little change was measured in 3-week old animals. Blockade of glucocorticoid synthesis prevented the development of insulin resistance in skeletal muscle, but not in liver of 6- and 10-week old rats. Moreover, skeletal muscle glucocorticoid receptor levels increased dramatically between 3 and 6 weeks of age. These results indicate that trauma and hemorrhage-induced hepatic insulin resistance occurs at all ages tested. However, there is no development of insulin resistance following trauma and hemorrhage in skeletal muscle of post-weaning rats. In skeletal muscle of 6- and 10-week old rats, inhibition of glucocorticoid levels prevents the development of insulin resistance.

Keywords

insulin resistance; hemorrhage; glucocorticoid receptor; liver; skeletal muscle

Introduction

Acute insulin resistance is common following trauma, hemorrhage, sepsis, burn, and critical illness (Cree & Wolfe 2008; Ikezu *et al.* 1997; Carter 1998; Van den Berghe *et al.* 2001). Chronic insulin resistant states, such as Type 2 diabetes, hypertension, cardiovascular disease, and the metabolic syndrome are all associated with advancing age (Alexander 2003; Meneilly & Tessier 1995; McEniery *et al.* 2007). However, little is known about the effects of age on the development of insulin resistance following injuries or critical illness, and whether the degree of insulin resistance varies with age. Injury and critical illness-induced hyperglycemia,

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sometimes referred to as “critical illness diabetes”, may increase mortality and morbidity (Langouche *et al.* 2005; Van den Berghe *et al.* 2001; Bochicchio *et al.* 2005). Hyperglycemia occurs frequently in critically ill children and adults (Klein *et al.* 2007; Garcia *et al.* 2007), and can be readily explained by a decrease of peripheral glucose disposal in muscle, or increased hepatic glucose production, often coincident with decreased responses to insulin (insulin resistance). Intensive insulin therapy of critically ill adult patients in the surgical intensive care unit (ICU) may reduce morbidity and mortality (Van den Berghe *et al.* 2001; Hansen *et al.* 2003). However, there is some controversy since recent studies suggest that intensive insulin therapy may not be as beneficial (Braithwaite 2009; Arabi *et al.* 2008). In addition, intensive insulin therapy increases the incidence of hypoglycemic episodes with the potential for negative outcomes (Finfer *et al.* 2009). Thus, an understanding of the molecular mechanisms of tissue-specific acute insulin resistance may provide new therapeutic approaches in treating the critically ill following injury.

Insulin regulates glucose metabolism by binding to the insulin receptor (IR) on the surface of target cells. Binding of insulin to the IR leads to activation of its tyrosine kinase activity which promotes association and phosphorylation of intracellular insulin receptor substrate proteins (IRSs), which act as docking proteins in IR/phosphatidylinositol 3 kinase (PI3K)/Akt pathway (Nakae & Accili 1999; White 1997). Reduced signaling through this pathway can cause insulin resistance. Previous data from our laboratory indicate that trauma and hemorrhage induces the rapid development of insulin resistance in the liver and skeletal muscle of adult rats (Ma *et al.* 2003; Ma *et al.* 2004; Xu *et al.* 2008; Li *et al.* 2009; Thompson *et al.* 2008), but it is not known whether there is a difference in the development of acute insulin resistance at different ages following injury.

Glucocorticoids are a potential causative factor in chronic and acute insulin resistance (Li *et al.* 2009; Vegiopoulos & Herzig 2007; Qi & Rodrigues 2007; Witchel & DeFranco 2006) by interfering with components of the insulin signaling cascade (Giorgino *et al.* 1993; Saad *et al.* 1993). For the present work, we hypothesized that glucocorticoids play a role in the development of acute insulin resistance in rats following trauma and hemorrhage, and that this role may differ at different ages since the hypothalamic/pituitary/adrenal axis changes with age. We found that trauma and hemorrhage induced insulin resistance in the liver and skeletal muscle of both 6- and 10-week old rats. However, in 3-week old rats, trauma and hemorrhage induced insulin resistance in the liver, but not in skeletal muscle. Glucocorticoids may play an important role in the hemorrhage-induced insulin resistance in skeletal muscle of 6- and 10-week old animals.

Materials and Methods

Animal model of trauma and hemorrhage

All procedures were performed in accordance with the guidelines set forth in the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals by 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. Three different ages of male Sprague-Dawley rats (approximately 3-, 6- and 10- weeks old; 3–5 animals/group) were fasted 18–20 hrs prior to surgery, but were allowed water *ad libitum*. Rats of approximately 10–12 weeks old are most common in studies of the rat trauma and hemorrhage model (Ma *et al.* 2003; Ma *et al.* 2004; Xu *et al.* 2008; Thompson *et al.* 2008). Rats of 6–8 weeks old are most common in the study of insulin sensitivity, since as rats age past this point, they become less responsive to insulin. Our previous work (Li *et al.* 2009) indicates that glucocorticoids play a key role in skeletal muscle insulin resistance around 10–11 week old rats following trauma and hemorrhage. Numerous studies indicate changes in the levels of glucocorticoids in response to stress, but this may not be true in very young rats, prior to weaning. Thus, we chose the three

age groups to study the development of insulin resistance following injury. A rat model of injury, labeled trauma and hemorrhage, as previously described (Ma *et al.* 2003; Ma *et al.* 2004; Xu *et al.* 2008; Li *et al.* 2009; Thompson *et al.* 2008), was used in this study. Briefly, at room temperature, anesthesia by isoflurane inhalation and a ventral midline laparotomy (3, 4, or 5 cm, based on the age of the rat) was performed representing soft tissue trauma, and the abdomen was closed. Polyethylene-50 catheters (Becton Dickinson Intramedic, Sparks, MD) were placed in the right and left femoral arteries and the right femoral vein were for bleeding and monitoring of mean arterial pressure. Rats were bled rapidly to a mean arterial pressure of 35–40 mm Hg within 10 min. Once mean arterial pressure reached 40 mm Hg, the timing of the hemorrhage period began. Small amounts of additional blood was withdrawn or saline was supplied to each animal to maintain a mean arterial pressure of 35–40 mm Hg for the 90 min (TH90') hemorrhage period.

Study design

Due to the considerable trauma incurred during anesthesia, catheterization and opening of the abdominal cavity to perform the insulin injections (see below), it was impossible to have a completely trauma-free control group. Thus, the baseline animals chosen in these experiments were the trauma alone rats (T0') that were subjected to anesthesia, laparotomy, catheterization, and then sacrificed immediately following saline or insulin injection. Other trauma alone groups were subjected to the same procedures but sacrificed at 90 min (T90') after catheterization. The trauma and hemorrhage groups were subjected to the same procedures as the trauma groups, but followed by hemorrhage and subsequent sacrifice at 90 min, the end of the hemorrhage period (TH90').

Drug administration

Metyrapone was purchased from Sigma (St. Louis, MO) and was dissolved in propylene glycol. For metyrapone pretreatment, rats received one injection of metyrapone (150 mg/kg, i.p.) or vehicle 3 hours prior to the surgery, a time and dose known to inhibit the stress-induced increase in plasma corticosterone levels (Li *et al.* 2009).

Tissue and blood harvesting procedures

At the 0' or 90' time points, the abdominal cavity of the animal was opened, the portal vein was exposed, and insulin (5 U) or saline was injected into the portal vein. One minute after the injection, liver, triceps, and extensor digitorum longus (EDL) were removed and snap frozen in liquid nitrogen for future analysis. Immediately before insulin or saline injection, blood was drawn from the inferior vena cava and centrifuged at 5,000 x g for 10 min. The plasma was stored at -80 C for future use. Corticosterone levels were determined using a RIA kit (MP Biomedicals, Orangeburg, NY) according to the manufacturer's instructions by a core facility of the UAB Clinical Nutrition Research Center.

Western immunoblot analysis

Liver and muscle tissues from each animal (0.2 g) were homogenized in extraction buffer, as described previously, and stored at -80°C until use (Ma *et al.* 2003; Ma *et al.* 2004; Xu *et al.* 2008). The protein concentration of tissue lysates were determined by the BCA method (Pierce, Rockford, IL). For immunoblotting, 30 µg of protein per lane was resolved by 10% SDS-PAGE and transferred to nitrocellulose paper. The Western transfers were immunoblotted with the following primary antibodies: anti-phospho-Y972-IR, anti-phospho-Y612-IRS-1 (Invitrogen Biosource International, Carlsbad, CA), anti-total IRβ (Santa Cruz Biotechnology, Santa Cruz, CA), anti-total glucocorticoid receptor, anti-phospho-S473-Akt, anti-total Akt and anti-total ERK (Cell Signaling Technology, Danvers, MA). Horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA) was then added for detection

of bound antibody by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) or Supersignal Femto Maximum Sensitivity Substrate reagent (Pierce Biotechnology, Rockford, IL). The blots were stripped using Reblot reagent (CHEMICON International, Inc., Temecula, CA) for 15 min, and then re-probed with a different antibody.

Densitometric and statistical analysis

Enhanced chemiluminescent images of immunoblots were scanned and quantified using Zero D-Scan (Scanalytics Corp., Fairfax, VA). All data were analyzed by ANOVA by Tukey-Kramer Multiple Comparisons Test or Student's t-test using the InStat statistical program by GraphPad Software, Inc. (San Diego, CA)

Results

Trauma and hemorrhage-induced decreases in insulin signaling (P-Akt) – differences evident in triceps of 3-week old rats

We previously found that insulin signaling in liver and skeletal muscle was only minimally effected by trauma alone, but the combination of trauma and hemorrhage abolished insulin signaling in both liver and skeletal muscle in adult rats (Ma *et al.* 2003; Ma *et al.* 2004; Xu *et al.* 2008; Li *et al.* 2009; Thompson *et al.* 2008). In the present study, we first examined insulin signaling following trauma and hemorrhage in animals at different ages (3-, 6-, 10-weeks old) by determining insulin-induced serine phosphorylation of Akt [P-Akt (S473)], a process that is required for Akt activation (Hill *et al.* 2002). We found that insulin-induced hepatic P-Akt was completely lost in animals at all three ages studied (3-, 6-, and 10-weeks old) 90 min following trauma and hemorrhage (TH90'; Fig. 1A, B). This indicates that trauma and hemorrhage abolish hepatic insulin signaling independent of age.

We next examined insulin-induced P-Akt in skeletal muscle following trauma and hemorrhage at different ages and consistent with our previous findings in adult animals, in 10-week old rats (Thompson *et al.* 2008), insulin-induced P-Akt (S473) was severely diminished in triceps following trauma and hemorrhage (TH90') compared with trauma alone (T90'; Fig. 1C, D). The large decrease of insulin-induced P-Akt (S473) was also observed at 6 weeks of age. However, there was little change in insulin-induced P-Akt (S473) in triceps following trauma and hemorrhage from 3-week old rats (Fig. 1C, D). This suggests something different about the response of skeletal muscle in the youngest rats studied. Although there were an observed decrease in the levels of P-Akt (S473) induced by insulin in trauma only animals (T90') in the liver and triceps from the oldest animals (Fig. 1B, D), the fold-induction by insulin did not reach statistical significance by ANOVA. Moreover, there was no significant difference in the total amounts of the Akt or ERK1/2 proteins in the three age groups (Fig. 1A, C).

Trauma and hemorrhage-induced decreases in insulin signaling (P-IR and P-IRS1) – differences evident in triceps of 3-week old rats

Alterations of P-Akt (S473) may be due to changes in activation of upstream components of the insulin signaling pathway. To test this possibility, experiments were performed to determine the changes of activating tyrosine phosphorylation of insulin receptor (IR) and insulin receptor substrate1 (IRS1) in liver and skeletal muscle. Phosphorylation of tyrosine 972 of the IR (P-IR [Y972]) is known to be essential for the phosphorylation and binding of IRS1 (Backer *et al.* 1990; Biddinger & Kahn 2006). IRS1 is necessary for transduction of the insulin signal, and phosphorylation of tyrosine 612 of IRS1 (P-IRS1 [Y612]) is critical for activation of the PI3K/ Akt pathway (Myers, Jr. *et al.* 1992; Esposito *et al.* 2001). We therefore examined P-IR (Y972) and P-IRS1 (Y612) in both liver and triceps following trauma and hemorrhage in all age groups.

Our results indicate that insulin-induced P-IR (Y972) and P-IRS1 (Y612) were abolished in the liver by hemorrhage (TH90') for all three ages examined (Fig. 2A, B, C). Similar to liver, phosphorylation of IR (Y972) and IRS1 (Y612) in skeletal muscle (triceps) was significantly decreased following trauma and hemorrhage in 6- and 10-week old rats. However, this did not occur in the 3-week old group where insulin-induced P-IR and P-IRS was approximately the same following either trauma alone or following trauma and hemorrhage (Fig. 2D, E, F). Since the total amount of IR in liver and triceps was approximately the same in the three age groups (Fig. 2A, D), these results suggest that in skeletal muscle of 3-week old rats there is some protection from the trauma and hemorrhage-induced inhibition of insulin receptor signaling.

Trauma and hemorrhage did not reduce P-Akt in extensor digitorum longus (EDL) of 3-week old rats

Previous studies in our lab reported that hemorrhage-induced acute insulin resistance was independent of muscle fiber type (Thompson *et al.* 2008). Since we have examined the effect of age on trauma and hemorrhage-induced insulin resistance in triceps, which is a type 1 (slow twitch) skeletal muscle, we next determined whether there was some protection against the development of hemorrhage-reduced insulin signaling in extensor digitorum longus (EDL), a type 2 (fast-twitch) skeletal muscle of 3-week old rats. Consistent with our findings in triceps, in 6- and 10- week old rats, insulin-induced P-Akt (S473) was greatly reduced following the combination of trauma and hemorrhage (TH90') in EDL (data not shown). However, as in triceps, insulin was still fully capable of inducing P-Akt (S473) in the EDL of 3-week old rats (data not shown).

Taken together, it is clear that at 3 weeks of age there is little or no development of acute skeletal muscle insulin resistance, in either slow-twitch (triceps) and fast-twitch (EDL) muscles following trauma and hemorrhage, whereas the acute development of insulin signaling defects still occurs in the liver.

Corticosterone and glucocorticoid receptor levels at different ages and following trauma and hemorrhage

Our previous studies indicate that glucocorticoids play a major role in skeletal muscle insulin resistance following trauma and hemorrhage in adult rats (Li *et al.* 2009). To determine the role of glucocorticoids in hemorrhage-induced skeletal muscle insulin resistance at different ages, we first examined the level of plasma corticosterone, the main plasma glucocorticoid in rodents. Corticosterone levels were not significantly altered 90 min following trauma (T90') compared with trauma alone (T0') in 3-, 6- and 10-week groups, whereas corticosterone levels were significantly increased 90 min following trauma and hemorrhage (TH90') at 6 and 10 weeks of age. There was a greater increase in 10-week old rats, due mainly to the lesser basal level of corticosterone. The higher basal corticosterone concentration of 6-week old rats was also evident in 3-week old rats. However, unlike the 6-week old rats, in the 3-week old rats there was no increase in corticosterone levels following the combination of trauma and hemorrhage (TH90'; Fig. 3A).

The amount of glucocorticoid receptor (GR) protein in liver and skeletal muscle was examined by Western blotting and the levels were significantly higher in the liver of animals at 10 weeks of age than those at 3 and 6 weeks of age (Fig. 3B, C). In skeletal muscle (triceps), there was no difference in the total amount of GR between 6- and 10-week old rats which were both significantly higher than in 3-week old rats (Fig. 3B, D).

Inhibition of corticosterone synthesis did not rescue hepatic insulin signaling following trauma and hemorrhage

To further investigate the role of glucocorticoids in trauma and hemorrhage-induced insulin resistance, we pretreated animals with metyrapone an inhibitor of corticosterone synthesis, followed by determination of insulin resistance following trauma and hemorrhage. Previous work from our lab demonstrated that insulin signaling in control rats was not affected by metyrapone treatment (Li *et al.* 2009). And, as expected, pretreatment with metyrapone, reduced the hemorrhage-induced increase of corticosterone levels in 10- and 6-week old rats (Fig. 3A). Since there was no increase of corticosterone following hemorrhage in 3-week old rats, metyrapone was not administered to these rats. Our previous data indicated that treatment with metyrapone did not change hepatic insulin resistance 60 min following hemorrhage in adult rats (Li *et al.* 2009). Similarly, we found that inhibition of corticosterone synthesis had no effect to protect the liver from developing insulin resistance following trauma and hemorrhage in 6- and 10-week old rats (Fig. 4A, B). In addition, the decrease of insulin-induced phosphorylation of IR (Y972) and IRS1 (Y612), the upstream insulin signaling molecules, were not prevented in the liver by pre-treatment with metyrapone (Fig. 4C, D). Therefore, these data further support our contention that glucocorticoids do not play an important role in the acute development of hepatic insulin resistance following hemorrhage.

Inhibition of corticosterone synthesis prevented skeletal muscle insulin resistance following trauma and hemorrhage

To determine whether glucocorticoids play a role in trauma and hemorrhage-induced insulin resistance in skeletal muscle, we measured phosphorylation of Akt (S473) following trauma and hemorrhage in rats pretreated with metyrapone. The ability of insulin to induce phosphorylation of Akt in triceps was significantly increased, such that the levels of P-Akt (S473) in response to insulin were almost completely normalized by pre-treatment with metyrapone in 10-week old rats (Fig. 5A, B). In addition, the ability of insulin to induce phosphorylation of IR (Y972) and IRS1 (Y612), which were negligible following trauma and hemorrhage in triceps, were also almost completely normalized by pretreatment with metyrapone (Fig. 5C, D). Pretreatment of 6-week old rats with metyrapone also partially restored restored phosphorylation of Akt (S473; Fig. 6A, B) in skeletal muscle. Together, these data demonstrate that the acute development of insulin resistance in skeletal muscle following trauma and hemorrhage can be inhibited by blocking glucocorticoid synthesis. Again, since there was no increase of corticosterone following hemorrhage in 3-week old rats, and since there was no development of insulin resistance in 3-week old rats, the effects of metyrapone were not studied.

Discussion

Hyperglycemia is a risk factor and associated with increased morbidity and mortality following injury, infection and critical illness in children and adults (Garcia *et al.* 2007; Cochran *et al.* 2003; Klein *et al.* 2007; Branco *et al.* 2005; Krinsley 2004). This hyperglycemia is associated with insulin resistance, but little is known about whether this insulin resistant state develops at different ages. Since glucose production by the liver and glucose uptake by skeletal muscle are important in regulating glucose levels, interruption of insulin signaling in liver and skeletal muscle can cause hyperglycemia. Our previous findings indicate that acute insulin resistance in liver and skeletal muscle occurs in adult rats following trauma and hemorrhage (Ma *et al.* 2003; Ma *et al.* 2004; Xu *et al.* 2008; Li *et al.* 2009; Thompson *et al.* 2008). In the present study, we used rats of various ages to determine if hepatic and skeletal muscle insulin resistance occurs at younger ages.

The rapid development of a defect of insulin signaling via the IR/IRS-1/PI3K/Akt pathway in liver and skeletal muscle, what we call acute insulin resistance, was observed following trauma and hemorrhage in liver 3-, 6- and 10-week old rats, and in skeletal muscle from 6- and 10-week old rats. However, we did not observe the development of acute insulin resistance in skeletal muscle of 3-week old rats following trauma and hemorrhage. Measuring alteration of insulin-induced Akt serine phosphorylation as well as tyrosine phosphorylation of IR and IRS1 was completely consistent. Thus, phosphorylation of IR, IRS1 and Akt was decreased in liver and skeletal muscle were decreased rapidly in 6- and 10-week old rats and in the liver of 3-week old rats, following trauma and hemorrhage. However, there was no change in the skeletal muscle of 3-week old rats in response to trauma and hemorrhage. In human studies, insulin resistance in critically ill children may be due to down regulation of insulin receptors (Xu *et al.* 1996). However, due to the rapidity of the response to trauma and hemorrhage in the present studies, the content of insulin receptors did not change in either liver or skeletal muscle.

Skeletal muscle is a mixture of two fiber types, type 1 (slow-twitch) and type 2 (fast-twitch) fibers (Schiaffino & Serrano 2002). The response of slow-twitch skeletal muscle to insulin is greater than fast-twitch skeletal muscle (James *et al.* 1985). We found that trauma and hemorrhage-induced acute insulin resistance did not occur in triceps (slow-twitch) muscle or in extensor digitorum longus (fast-twitch) at 3 weeks, but did at 6 and 10 weeks of age in both muscle types. These results indicate that there is no muscle type specificity in the age-related acute development of insulin resistance following trauma and hemorrhage in skeletal muscles [also see (Thompson *et al.* 2008)].

Adrenal glucocorticoid content differs during the development in the rat (Morii & Huggins 1962), and glucocorticoid receptors levels differ among tissues (Chrousos & Kino 2005). Glucocorticoids levels increase dramatically following injury or infection (Li *et al.* 2009; Vaughan *et al.* 1982). Our earlier work implicates glucocorticoids as a causative factor in skeletal muscle insulin resistance following trauma and hemorrhage in adult rats (Li *et al.* 2009). Therefore, increased glucocorticoid synthesis or differences in glucocorticoid receptor levels in the liver and skeletal muscle could play a role in the acute development of insulin resistance following trauma and hemorrhage. Corticosterone levels increased rapidly following hemorrhage in 10-week old rats. Basal glucocorticoid levels in 3- and 6-week old rats were already as high as the concentration attained in the 10-week old rats following trauma and hemorrhage. However, corticosterone levels increased further only in the 6-week old rats, not changing at all in the 3-week old rats. This indicates that the 3-week old rats do not respond to the stress of trauma and hemorrhage as in 6- and 10-week old rats, but are capable of secreting high basal levels of corticosterone. A likely reason for the lack of an increase in corticosterone following trauma and hemorrhage in 3-week old rats is the lesser sensitivity of the hypothalamic-pituitary-adrenal axis to stress in young rats (Leal *et al.* 1999; Yoshimura *et al.* 2003). The response of 6-week old rats was more modest than in 10-week old rats, but there was an increase of corticosterone in response to trauma and hemorrhage.

We also observed that skeletal muscle glucocorticoid receptor levels in 3-week old rats were one-fourth or lower than in triceps of 6- and 10-week old rats. Thus, with little or no increase of corticosterone levels following trauma and hemorrhage, and with the low levels of triceps glucocorticoid receptors, there is a much lower potential for a response of skeletal muscle of 3-week old rats to glucocorticoids as compared to adult rats (Li *et al.* 2009). Metyrapone decreases corticosterone synthesis levels by inhibiting 11- β hydroxylation of desoxyxoticosterone (DOC) (Schoneshofer *et al.* 1979; Metcalf & Beaven 1968), and can lead to increased adrenal levels of corticosterone and aldosterone precursors (Metcalf & Beaven 1968; Taylor & Fishman 1988; Aupetit-Faisant 1990) which may present a problem if used chronically. In skeletal muscle of 6- and 10-week old rats, which express much higher levels of glucocorticoid receptors, the acute inhibition of glucocorticoid synthesis by metyrapone

resulted in at least a partial rescue of insulin signaling following trauma and hemorrhage. Thus, we hypothesize that the large increase in skeletal muscle glucocorticoid receptor levels between 3 weeks and 6 or 10 weeks of age results in the ability of the skeletal muscle to respond to the increased glucocorticoid levels following trauma and hemorrhage, resulting in the acute development of insulin resistance. However, it is not known whether glucocorticoids play a similar role in the defects of muscle insulin signaling induced by the other stresses (Lang & Frost 2007; Wu *et al.* 2004). In the liver, the level of glucocorticoid receptors is irrelevant since development of acute hepatic insulin resistance is not dependent upon glucocorticoids (Xu *et al.* 2008; Li *et al.* 2009; Thompson *et al.* 2008)

In summary, hepatic insulin resistance following trauma and hemorrhage occurs at all age rats tested, even just after weaning and involves multiple components in the insulin signaling pathway. Insulin resistance does not develop in either slow-twitch (triceps) or fast-twitch (extensor digitorum longus) skeletal muscle following trauma and hemorrhage in 3-week old rats, and the lack of the response to trauma and hemorrhage may be due to the low levels of glucocorticoid receptor in the skeletal muscle of young rats. As the rats age, they develop the ability to respond to trauma and hemorrhage with acute skeletal muscle insulin resistance that is glucocorticoid-dependent.

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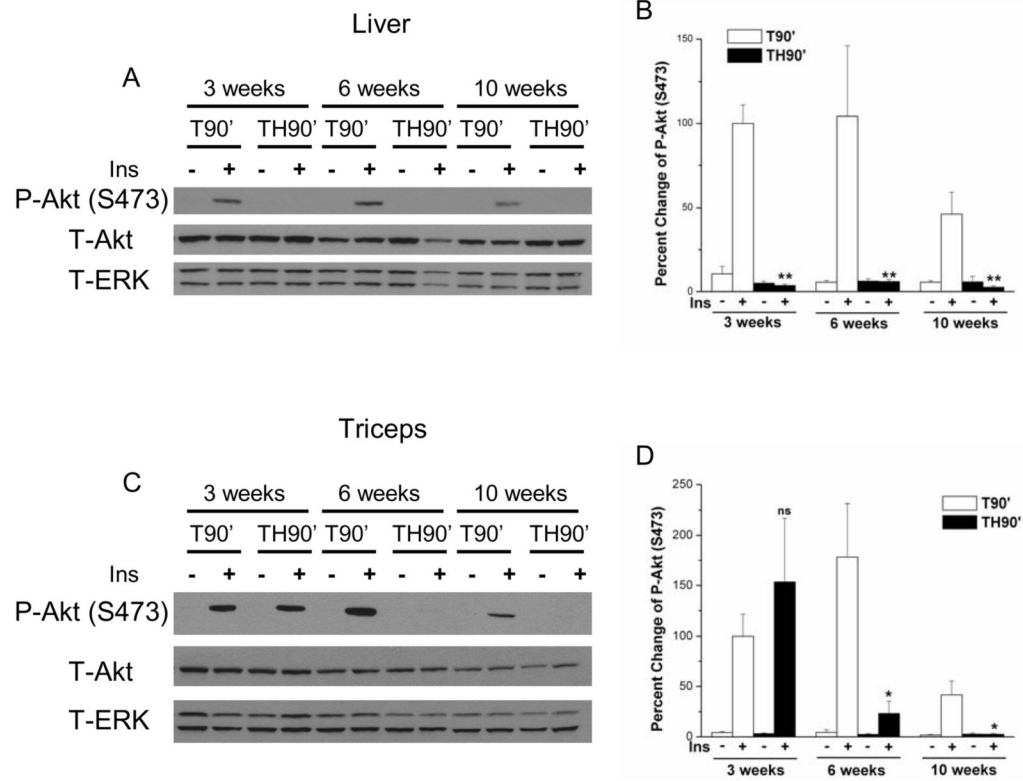


Figure 1. Hepatic and skeletal muscle insulin signaling via serine phosphorylation of Akt at different ages following trauma and hemorrhage

Rats were subjected to trauma alone (T90') or trauma and hemorrhage (TH90'). At 90' (with or without hemorrhage) either saline (-) or 5U insulin (Ins; +) was injected via the portal vein. The liver and triceps were removed after 1 min and frozen in liquid nitrogen. A and C, tissue lysates from liver and triceps were subjected to Western blotting with antibodies specific for phospho-Akt [P-Akt (S473)], total Akt (T-Akt), and total ERK (T-ERK). B and D, autoradiographs were quantified by scanning densitometry. Data are presented as the mean \pm SEM of the percentage change of P-Akt (S473) by insulin of three rats in each group. The phosphorylated protein levels in T90' (3 weeks) with insulin treatment were arbitrarily set to 100 percent. In this and all the following figures, *, $p < 0.05$; **, $p < 0.01$; ***, $P < 0.001$, ns = not significant, compared with T90' with insulin injection of the same age group.

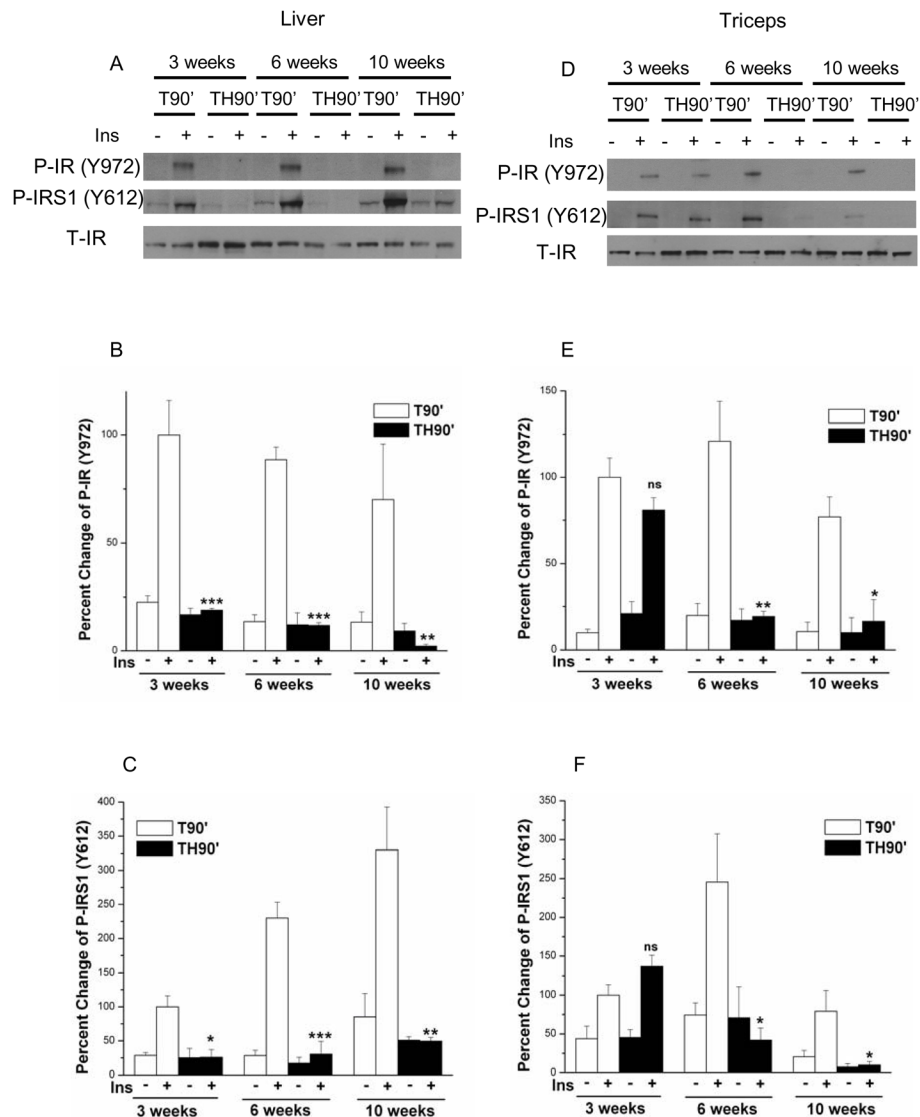


Figure 2. Hepatic and skeletal muscle insulin signaling via tyrosine phosphorylation of IRS-1 and IR at different ages following trauma and hemorrhage

Following the same treatment procedure and time points described in Fig. 1, liver and triceps were removed and frozen in liquid nitrogen. A and D, tissue lysates from liver and triceps were subjected to Western blotting with antibodies specific for phospho-insulin receptor [P-IR (Y972)], phospho-IRS1 [P-IRS1 (Y612)], and total insulin receptor (T-IR). B, C, E, and F, autoradiographs were quantified by scanning densitometry. Data are presented as the mean \pm SEM of three rats in each group.

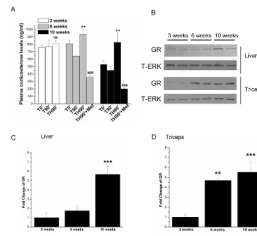


Figure 3. Corticosterone levels and expression of glucocorticoid receptor at different ages
 A, Rats were sacrificed at the end of surgery (T0') in addition to the T90' and TH90' time points and blood was collected for the determination of plasma corticosterone levels. Results are expressed as ng corticosterone/ml plasma. In separate groups, metyrapone (Met) was administered as described in Materials and Methods. Each value represents the mean \pm SEM of 5–6 rats per group. **, $p < 0.01$, and ns = not significant, compared with T90' in the same age group. ###, $p < 0.001$, compared with TH90' in the same age group. B, Following the same treatment procedure and time points described in Fig. 1, liver and triceps were removed and frozen in liquid nitrogen. Tissue lysates were subjected to Western blotting with total glucocorticoid receptor (GR) and T-ERK antibody in liver and triceps. C and D, Data are presented as the mean \pm SEM of samples from 4–6 rats in each group with 1 set at the GR level in 3-week old rats in liver (C) and triceps (D). **, $p < 0.01$, ***, $p < 0.001$, and ns = not significant, compared with 3 weeks group.

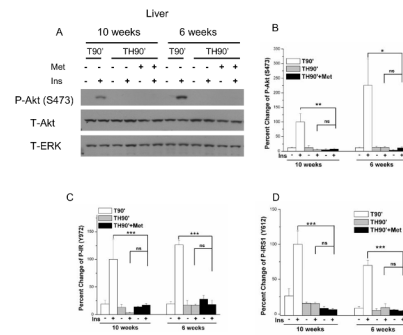


Figure 4. Decreasing corticosterone levels did not affect trauma and hemorrhage-induced insulin resistance in liver

Metyrapone (Met) was administered as described in Materials and Methods to 6- or 10-week old rats which were then subjected to trauma, or trauma and hemorrhage. Liver tissue lysates were subjected to Western blotting with antibodies specific for P-Akt (S473), P-IR (Y972), P-IRS1 (Y612), T-Akt, and T-ERK. A, a representative Western blot. B, C, D, autoradiographs were quantified by scanning densitometry and presented as the mean \pm SEM of three rats in each group and presented as the percentage change by insulin with the 10-week old rats arbitrarily set to 100%.

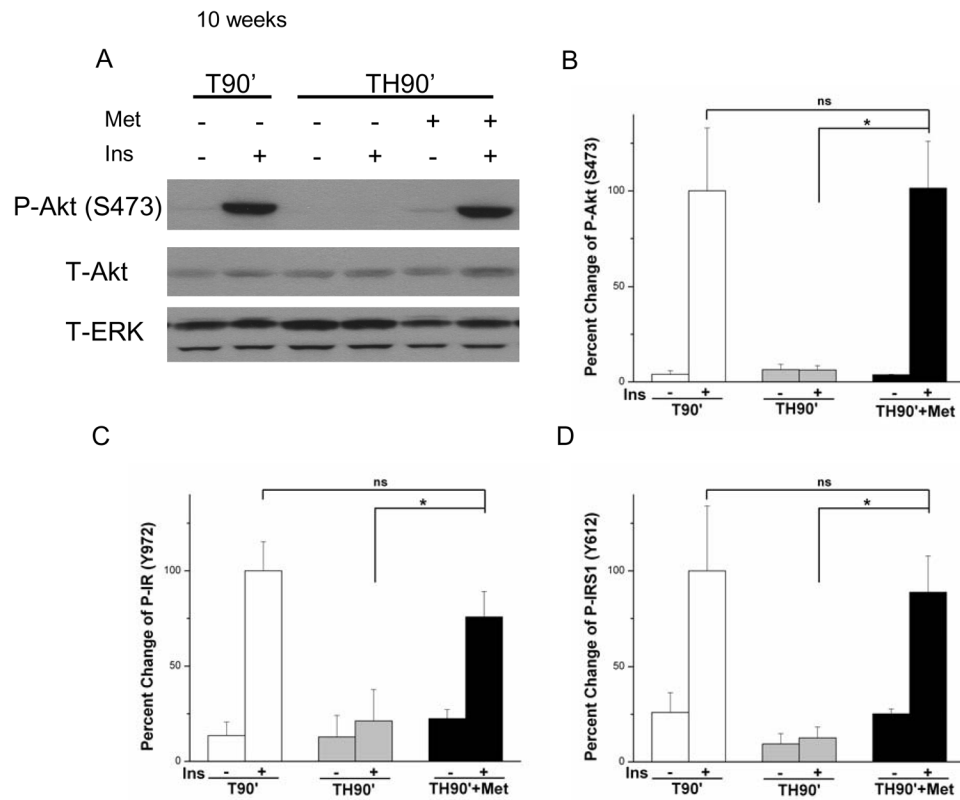


Figure 5. Effects of metyrapone on the impaired skeletal muscle insulin signaling in 10-week old rats

Following the same treatment procedures, time points and antibodies described in Fig. 5, triceps tissue lysates were subjected to Western blotting. A, a representative Western blot. B, C, D, autoradiographs were quantified by scanning densitometry and presented as the mean \pm SEM of 3–5 rats in each group and presented as the percentage change by insulin in the T90' group arbitrarily set to 100%.

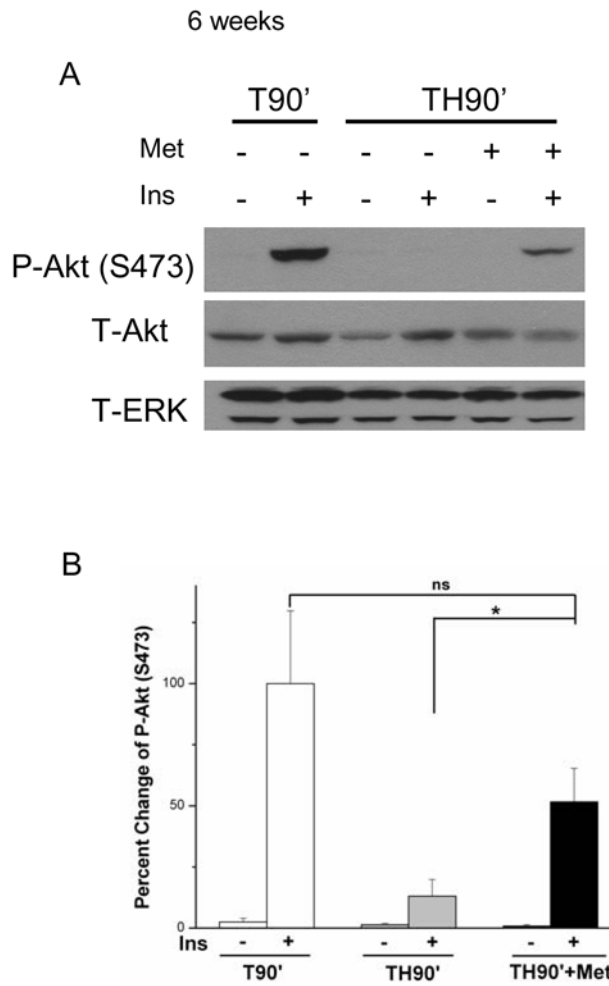


Figure 6. Effects of metyrapone on the impaired skeletal muscle insulin signaling in 6-week old rats
 Following the same treatment procedures, time points and the P-Akt antibody described in Fig. 5, triceps tissue lysates were subjected to Western blotting. A, a representative Western blot. B, autoradiographs were quantified by scanning densitometry and presented as the mean \pm SEM of 3 rats in each group and presented as the percentage change by insulin in the T90' group arbitrarily set to 100%.