INSULIN EFFECTS ON GLUCOSE TOLERANCE, HYPERMETABOLIC RESPONSE, AND CIRCADIAN-METABOLIC PROTEIN EXPRESSION IN A RAT BURN AND DISUSE MODEL

Heather F. Pidcoke, Lisa A. Baer; Xiaowu Wu; Steven E. Wolf; James K. Aden; Charles E. Wade

1 US Army Institute of Surgical Research, 3698 Chambers Pass, Fort Sam Houston, TX 78234, USA
2 University of Texas Health Science Center at Houston, 6431 Fannin St., MSB 5.204, Houston, TX 77030, USA
3 University of Texas Southwestern Medical Center, 5323 Harry Hines, Dallas, TX 75390, USA

Author Contributions: HFP, LAB, XW, SEW, CEW Designed the study; HFP, LAB, XW, SEW, CEW Performed animal work and assay measurements; HFP, LAB, CEW, JKA Analyzed the data; All authors Drafted, edited, revised and approved the final version of the manuscript.

Running Title: Effects of insulin in rat model of burn and disuse

Corresponding Author:
Charles E. Wade, PhD
Professor of Surgery
Deputy Director, Center for Translational Injury Research
University of Texas Health Science Center at Houston
6431 Fannin St., MSB 5.204
Houston, TX 77030
713-500-6818-phone
713-500-0685-fax
Charles.E.Wade@uth.tmc.edu

Copyright © 2014 by the American Physiological Society.
INSULIN EFFECTS ON GLUCOSE TOLERANCE, HYPERMETABOLIC RESPONSE, AND CIRCADIAN-METABOLIC PROTEIN EXPRESSION IN A RAT BURN AND DISUSE MODEL

ABSTRACT

Insulin controls hyperglycemia after severe burns, and its use opposes the hypermetabolic response. Underlying molecular mechanisms are poorly understood and previous research in this area has been limited due to the inadequacy of animal models to mimic the physiologic effects seen in humans with burns. Using a recently published rat model that combines both burn and disuse components, we compare the effects of insulin treatment versus vehicle on glucose tolerance, hypermetabolic response, muscle loss, and circadian-metabolic protein expression after burns. Male Sprague Dawley rats were assigned to three groups: cage-controls (n=6); vehicle-treated (VBH; n=11) and insulin-treated (IBH; n=9). With the exception of cage-controls, rats underwent a 40% TBSA burn with hindlimb unloading, then IBH rats received 12 days of subcutaneous insulin injections (5 units/kg/day), and VBH rats received an equivalent dose of vehicle. Glucose tolerance testing was performed on day 14, after which blood and tissues were collected for analysis. Body mass loss was attenuated by insulin treatment (VBH=265±17 g versus IBH=283±14 g, p=0.016) and glucose clearance capacity was increased. Soleus and gastrocnemius muscle loss was decreased in the IBH group. IRS-1, AKT, FOXO-1, Caspase-3, and PER1 phosphorylation was altered by injury and disuse, with levels restored by insulin treatment in almost all cases. Insulin treatment after burn and during disuse attenuated the hypermetabolic response, increased glucose clearance, and normalized circadian-metabolic protein expression patterns. Therapies aimed at targeting downstream effectors may provide the
beneficial effects of insulin without hypoglycemic risk.

KEY WORDS:
Insulin; Glucose tolerance; Hypermetabolism; Circadian rhythm; Burn and disuse
Introduction

Hypermetabolism is a profoundly debilitating consequence of severe burns and is characterized by insulin resistance, hyperglycemia, protein and lipid catabolism, total body protein loss, muscle wasting, elevated temperature, tachycardia, and high energy requirements that last up to a year after injury (23, 27, 34). Insulin attenuates hyperglycemia after severe burn and decreases the hypermetabolic response as evidenced by reduced body mass loss, a marker frequently used to track the progression and resolution of the hypermetabolic condition in clinical and research settings (4, 8, 9, 13, 14, 25, 31, 32, 34-36, 52, 63, 64, 69). Associated benefits of treating hyperglycemia include reduced insulin resistance and fewer adverse outcomes (52, 63, 69). The clinical features of the hypermetabolic response to trauma, burns, and disuse are well characterized, as is the relationship to increased protein turnover, inefficient substrate cycling and body mass loss (15, 16, 34, 37, 56, 60). However, underlying molecular mechanisms are not fully understood and established animal models do not adequately reproduce the combined physiologic effects of burns and bed rest that contribute to the profound body mass losses seen in humans (13, 31, 33, 34). A recently published rat model brings together, for the first time, both burn and disuse components from two well-established, highly cited models (71). The resulting losses in total body mass (TBM) and lean body mass (LBM) were similar to those of hypermetabolic, burned humans, thus can be used as a marker of the hypermetabolic response in this rat model. Hypermetabolism can be inferred if experimental rats lose body mass while the cage control rats gain or maintain their weight (64). It is reasonable to assume that hypermetabolism is the cause of this weight loss as there is a very large body of literature documenting hypermetabolism and associated body mass loss due to burns both in humans and rats (8, 9, 13, 15, 16, 25, 31, 34, 36, 37, 56, 60, 64). Multiple methods have been used to
establish that subjects are hypermetabolic after burns, and that it results in weight loss (9, 13, 24, 26, 31, 36, 43, 67). Unlike its predecessors, the rat model used here recapitulates the clinical features of body mass loss demonstrating a higher degree of hypermetabolism, and therefore is adequate to study changes in glucose and insulin metabolism in response to large burns and the molecular targets involved (71).

Evidence for integration between the peripheral metabolic pathways and circadian biological rhythms has been growing (3, 17, 38, 51). Diabetic models demonstrate that chronic insulin resistance is linked to alterations in diurnal patterns in glucose and insulin metabolism (6). Many of these same regulators are also involved in atrophy which may explain complex clinical manifestations after injury (42, 62). To date, these interactions have not been fully described and remain an area of intense investigation. Here we compare the effects insulin treatment versus vehicle on glucose tolerance, hypermetabolic response, and circadian-metabolic protein expression after burns with hindlimb unloading. We hypothesized that the combined injury and disuse model, previously shown to produce a hypermetabolic insult, also results in decreased glucose clearance increase insulin resistance and muscle loss, and altered circadian-metabolic protein expression. Furthermore, we hypothesized that insulin treatment attenuates the hypermetabolic response, improves glucose tolerance, decreases muscle loss, and normalized dysregulation in circadian-metabolic protein expression.

**Methods**

Regulatory approval was obtained from the US Army Institute for Surgical Research and University of Texas Health Science Center San Antonio Institutional Animal Care and Use
committees. Adult male Sprague Dawley rats (Charles Rivers, Wilmington, MA) were housed in metabolic cages which allowed for accurate quantification of urine and fecal output. They were given water and a certified diet (Harlan Teklad #2018 in powder-form) ad libitum. Room temperature was maintained at 76±2 °F and 30-80% relative humidity. After a week, the rats weighed approximately 300 g and were assigned to one of three groups: cage-controls (CC; n=6); vehicle-treated (VBH; n=11) or insulin-treated (IBH; n=9).

We examined the effects of glucose clearance in the burn and hindlimb unloading (BH) injury model on, and compared findings between animals treated with insulin to those treated with vehicle. Because rat responses to glucose loading vary widely in published literature, the CC group provided baseline reference values for glucose tolerance testing (GTT) in healthy rats from this population. The CC group did not undergo BH procedures or daily injections, but a sham procedure was performed on day 0, and GTT was performed just prior to euthanasia. CC results served as reference laboratory, body mass, GTT, and protein expression values for fasted healthy rats subjected to housing in metabolic cages, the stress of sham and GTT procedures, but not injury or treatment.

Scald Procedure

Rats undergoing the BH procedure were weighed and given a pre-procedure dose of buprenorphine (0.1 mg/kg) subcutaneously (SQ) for analgesia. General anesthesia was induced with inhalation of 2-3 % isoflurane in 100% oxygen. Animals were initially placed in an induction chamber, then transferred to face mask. Scald procedures were performed according to the method described by Walker and Mason (65). Briefly, dorsal and ventral surfaces were shaved and the animals placed in plexiglass molds designed to expose 20% of the total body
surface area. The dorsal surface was exposed to 100° C water for 10 seconds, followed by the ventral surface for 2 seconds, creating a 40% total body surface area injury. Ringer’s lactate (20 ml) was injected into the peritoneal space to be absorbed over time for resuscitation (57). Cage-control animals underwent a sham procedure in which anesthesia was administered and the rats were exposed to water at room temperature.

Post-Injury and Hindlimb Unloading Procedures

Following the burn, BH rats were returned to the metabolic cages where they received 2 doses of buprenorphine (0.05 mg/kg, SQ) for analgesia over the first 24 hours. Hindlimb unloading (HU) was performed according to previously published methods (46-48). Briefly, after the animals regain full consciousness from anesthesia and were able to ambulate, their hindquarters were unloaded using a tail traction system. The device prevented weight-bearing on the hindlimbs while allowing 360º access to the cage environment. The rats were observed for apparent signs of distress and monitored several times a day.

Insulin and Vehicle Administration

Treatment animals were injected daily with SQ long-acting insulin in approximately 0.04 ±0.01 ml of saline (5 units/kg/day, PZI® insulin, BCP Veterinary Pharmacy, Houston, Texas) every morning for 12 days per the dose-response curve published by Jeschke, et al (35, 39). Vehicle animals received an equivalent volume of saline SQ. Dose volumes varied by 0.01 ml because of the variability in animal weights upon which the doses were calculated.

Physiological Data Collection and Analysis
Behavior, food and water intake, and urine output were assessed and recorded twice daily. Total body mass (BM) was measured daily as a marker of hypermetabolism which has been previously reported in this model (64). Measurements were taken in the hindlimb-unloaded position to avoid weight-bearing. Body temperature was measured daily via laser skin thermometer. Urine corticosterone levels were measured for each 24-hour period (2, 64). Plasma analysis was done once on day 14, including glucose, insulin, C-peptide, and free fatty acids (FFA). Exogenous insulin was calculated as the result of subtracting C-peptide from total plasma insulin.

Effect of Injury on GTT with and without Insulin Therapy

The purpose of this experiment was to determine the effect of long-term (14 day) insulin therapy on glucose clearance (GTT) in IBH rats compared to VBH and CC. Insulin was withheld for 24 hours, and rats were fasted approximately 10 hours in preparation for the procedure. Glucose tolerance was tested under general anesthesia (1.5-3% isoflurane in 100% oxygen) at day 7 or 8 for CC rats due to limitations of staff and procedure room availability, and at day 14 for BH rats (Figure 1). The purpose of the CC group was to provide a baseline of reference values for GTT values, post-study laboratory values, normal growth curves in healthy rats in the absence of burn and bedrest, and protein phosphorylation due to the GTT procedure alone. Body mass in the CC group was expected to rise due to normal growth, but be somewhat attenuated due to the stress of being housed in metabolic cages and handled twice daily. Seven to eight days were considered sufficient to allow such stress effects to equilibrate and to establish the trajectory of growth curves for comparison to those from experimental groups. After measurements of total body mass and baseline blood glucose were taken, a 50% glucose infusion
was administered, delivering a 1g/kg intravenous glucose load. Blood glucose was measured at 5, 10, 15, 30, and 60 minutes (Figure 1). Rate of glucose clearance was calculated as the total area under the glucose curve and the slope of the GTT natural log regression curve as a measure of disposal rate. The data from the non-insulin treated VBH rats provided baseline measurements in injured and stressed animals, and data from the cage-controls provided baseline measurements in healthy animals.

Euthanasia and Post Euthanasia Procedures

After GTT, animals were euthanized via terminal exsanguination under anesthesia. Organ tissue (heart, liver, spleen, kidney, testes, adrenals, and dorsal fat pads) and skeletal muscle (bilateral tibialis anterior, extensor digitorum longus, plantaris, soleus, and gastrocnemius) were rapidly dissected and weighed. Total muscle was the sum of bilateral muscle weights. Tissue samples were immediately flash frozen with liquid nitrogen, after which lean body mass was determined with dual-emission X-ray absorptiometry (DEXA) scanning (Lunar Prodigy, GE Healthcare, Madison, WI).

Western Blots

Proteins from soleus muscle were extracted for Western blots via liquid nitrogen biopulverization in ice cold 1X commercial cell lysis buffer (Cell Signaling, Boston, MA) containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and phenylmethylsulfonyl fluoride (Santa Cruz Biotechnology, Santa Cruz, CA) followed by homogenization with a motorized polytetrafluoroethylene pestle. Samples were centrifuged at 14000 rpm for 10 min at 4C, and the supernatant collected for sample analysis. Western blots
were run on graduated 4-12% bis-tris or 4% tris-acetate gels (Invitrogen Corporation, Carlsbad, CA) and transferred onto nitrocellulose or polyvinylidene fluoride membranes (iBlot® Dry Blotting System, Invitrogen Corporation, Carlsbad, CA). The membranes were incubated with blocking buffer (Odyssey blocking buffer, Li-Cor, Lincoln, NE) for an hour, followed by the following primary antibodies: total IRS-1, AKT, phosphorylated AKT (Ser473), AMPK alpha 1, phosphorylated AMPK alpha 1 (Thr172), phosphorylated FOXO-1 (Ser256) FOXO-1 (Cell Signaling Technology, Inc., Danvers, MA); phosphorylated IRS-1 (Serine 312), PPAR β, Caspase-3, CRY1, CRY2, PER1, PER2, and PER3 (Santa Cruz Biotechnology, Inc., Santa Cruz, California); phosphorylated IRS-1 (Tyrosine 612, Abcam, Cambridge, MA). Primary antibodies were incubated at room temperature for 40 minutes followed by overnight at 4°C. After washing steps were completed, bands were detected and quantified by incubation with fluorescent secondary antibodies (species appropriate IRDye™ 680 and IRDye™ 800 secondary antibodies, Li-Cor, Lincoln, NE). Membranes were analyzed with a commercial analyzer (Odyssey Infrared Imager, LI-COR Biosciences, Lincoln, NE). Proteins were normalized to β-Actin levels and, if the phosphorylated form of the protein, to the total abundance. Ratios were calculated for each individual subject and then averaged for the group.

**Data Analysis**

Group size was determined via power analysis with alpha set at 0.05 and beta set at 0.2. Organ and muscle mass was normalized to body mass and analyzed with commercial software (SigmaPlot 11.2, Systat, San Jose, CA). Significance was set at p<0.05. Normally distributed data were compared using two-way repeated measures ANOVA and t-tests. Values greater than two standard deviations from the mean were excluded. Post-hoc analysis was performed with the
Results

Effects of Injury and Disuse on Hypermetabolism and Atrophy

There were no significant differences between groups in baseline BM and food intake (Figure 2). Twenty-four hours after administration, insulin treatment resulted in markedly elevated exogenous plasma insulin levels in both experimental groups (p=0.005; Table 1). The percent contribution of endogenous insulin production was negligible as determine by C-peptide (CC: 0.70±0.26%, n=6; VBH: 1.20±0.50%, n=7; IBH: 0.26±0.26%, n=8). Exogenous insulin typically has a suppressive effect on endogenous insulin production, although the phenomenon is likely an indirect effect of lower plasma glucose rather than direct insulin effects (1). Contrary to expectations; however, C-peptide (as a marker of endogenous insulin production) was not suppressed in the IBH group treated with high-dose insulin (CC: 3.9±1.8 ng/mL; VBH: 3.8±1.5 ng/mL; IBH: 2.9±1.1 ng/mL; p≥0.178). This demonstrates that once-daily subcutaneous dosing resulted in sustained elevations of plasma insulin over the entire period and these elevations did not result in suppression of endogenous insulin production.

Hypermetabolic effects due to injury and disuse were evident in changes in body mass, urine corticosterone, and the organs involved in stress hormone production. The burn and hindlimb unloading procedure resulted in an initial weight gain in the experimental groups due to administration of intraperitoneal fluid resuscitation (Figure 2), however; beginning on Day 2, animals in both experimental groups lost BM over time. Prior to the fasted period, VBH and IBH animals together lost an average of 12.9±3.5% of their BM, in contrast to CC animals, who
gained weight over time ($p \leq 0.001$, Figure 2). All three groups lost approximately 5% of their body mass when fasted in preparation for the GTTs ($p \geq 0.168$, Figure 2).

The presence of a hypermetabolic insult was similarly manifested in average daily urine corticosterone levels, a hormone involved in the stress response. Levels were elevated in the experimental groups compared to CC ($p \leq 0.001$, Table 1). In keeping with this finding, the adrenals, involved in stress hormone production, were hypertrophied ($p \leq 0.001$, Table 2).

Testicles were atrophied, further evidence of chronically high levels of endogenous steroids ($p \leq 0.001$, Table 2). Interestingly, the heart was hypertrophied in both injury/hindlimb suspended groups compared to CC ($p \leq 0.007$).

**Effect of Insulin Treatment on Hypermetabolism and Atrophy**

On day 6, insulin treatment attenuated BM loss compared to injury and disuse alone ($p = 0.046$; Figure 2) and continued through the end of the study (Day 14: $p = 0.016$; Figure 2). While both experimental groups lost weight, insulin-treated animals lost a smaller percentage of their BM on average compared to the VBH group ($11 \pm 2.6\%$ versus $15 \pm 3.3\%$; $p = 0.012$, Figure 2). Lean BM decreased in both groups and differences between experimental groups were not statistically significant (Table 3); however, soleus and gastrocnemius muscle loss were both attenuated by insulin treatment. Total food intake over the study period was increased in the IBH group compared to VBH ($p = 0.002$) but not water consumption ($p = 0.986$).

Hypermetabolic changes in some organ weights were attenuated by insulin treatment. The liver was hypertrophied in both the VBH and IBH groups, however; this effect was partially reversed in the insulin cohort ($p \leq 0.001$, Table 2). The meaning of changes in the spleen and kidney are unclear, particularly as insulin treatment did not affect daily urine output (VBH: $2.9 \pm$
1.3 ml/100g BM; IBH: 3.4 ± 2.5 ml/100g BM, p=0.136). Plasma FFA levels were unchanged by injury/disuse or insulin treatment (Table 1). Rats experienced a significant decrease in fat mass due to hypermetabolism, but the effect was abolished by insulin treatment (CC: 0.88 g/100g BM; VBH: 0.53 g/100g BM, p≤0.001 compared to CC; IBH: 0.82 g/100g BM, p≤0.001 compared to VBH, p=0.396 compared to CC).

Effect of Insulin Treatment on Glucose Clearance

Analysis of the GTT curves showed that burn and disuse decreased glucose clearance in the VBH group compared to CC (p=0.03; Figure 3). Glucose levels rose steeply in both experimental groups after glucose loading (117 to 393±50 versus 38 to 302±56 g/dL, p=0.552). The area under the GTT curve, a measure of total plasma glucose concentration over time, was lower in the insulin-treated group (VBH: 10865±2003 mg*min/dL; IBH: 6985±1260 mg*min/dL; p=0.003). The slope of the natural log regression of GTT curves quantifies the rate of glucose clearance, which was over two times faster in the insulin-treated group (p ≤ 0.001; Figure 3).

Effect of Insulin on Metabolic and Circadian Protein Expression

IRS-1

Relative abundance of total IRS-1 was decreased in VBH soleus muscle compared to CC (p=0.007, Figure 4A) and in IBH samples (p=0.018). Phosphorylation of IRS-1 at serine 312 (Ser312) mediates insulin resistance in diabetic models by inhibiting IRS-1 tyrosine phosphorylation and promoting IRS-1 degradation via an mTOR mediated mechanism (7). After correcting for decreased total IRS-1 levels, we found that Ser312 was increased in VBH samples.
compared to CC (p=0.034, Figure 4B). Insulin treatment reversed this effect and results were similar to those found in the CC group (p≥0.05).

Interestingly, phosphorylation of IRS-1 at tyrosine 612 (Tyr612) corrected for total IRS-1 abundance was also increased, arguing for a compensatory response to overcome insulin resistance (p=0.008, Figure 4C). Increases in Tyr612 propagate the IRS-1 activation signal via the p85 subunit of phosphatidylinositol 3-kinase, promoting insulin-mediated effects such as glucose transport, glycogen and fatty acid synthesis, inhibition of apoptosis, and cellular growth via the AKT pathway (30, 66). Comparing the ratio of stimulatory phosphorylation to inhibitory clarifies the relationship; injury and disuse increased Tyr612 phosphorylation, but not enough to overcome the concomitant increase in Ser312 (p=0.008, Figure 4D). Insulin normalized the balance between the two, and the ratio of Tyr612 to Ser312 were increased to levels similar to those found in the CC samples (p=0.049, Figure 4D).

AKT

Serine 473 (Ser473) phosphorylated AKT, which activates AKT and propagates the insulin signal downstream via phosphorylation of FOXO-1 and other effectors, was decreased in VBH soleus samples compared to CC (p=0.005, Figure 5) but insulin treatment reversed this effect (5). Ser473 phosphorylation is also mediated by mTOR, thus results are consistent with the above finding that insulin treatment decreases inhibitory phosphorylation while increasing the state of activation in the insulin pathway (5). This is in contrast to the effect of insulin in diabetic models, in which inhibitory phosphorylation is increased by chronically elevated insulin levels (6, 7, 21).
PPAR β

PPAR β was not different between groups (CC: 0.022±0.015; VBH: 0.022±0.005; IBH: 0.023±0.015; p≥0.891).

FOXO-1, Caspase-3, AMPK

FOXO-1, Caspase-3, and AMPK contribute to apoptosis and have been implicated in atrophy in a variety of models (22, 45, 49, 55). Here we found no difference between groups in the expression of AMPK phosphorylated at Threonine172 (Thr172), the active form of AMPK, (p≥0.391, data not shown). Conversely, FOXO-1 and Caspase-3 appear to play a role in atrophy due to injury and disuse.

Phosphorylation of FOXO-1 at serine 256 (Ser256) by AKT excludes FOXO-1 from the nucleus, inhibiting the transcription of mediators involved in apoptosis and cell cycle arrest (22). The net effect of Ser256 phosphorylation is to promote cell growth and oppose the cellular processes underlying atrophy. Injury and atrophy caused the Ser256 to total FOXO-1 ratio to decrease relative to cage controls, thereby identifying one pathway to loss of muscle mass in the VBH group (p=0.014, Figure 6A). Insulin treatment reversed this effect; Ser256 levels in the IBH group increased to baseline levels (IBH versus VBH: p=0.001; IBH versus CC: p=NS; Figure 6A). Conversely, Caspase-3 levels were increased in both experimental groups compared to cage controls (p≤0.046, Figure 6B) and this effect did not respond to insulin treatment. Taken together, these data show that insulin effects on FOXO-1 attenuated injury-related soleus muscle loss, while Caspase-3 levels were not affected.

CRY1, CRY2, PER1, PER2
The CRY and PER family of proteins are regulators of the central clock via auto-feedback loops, and they are now understood to have a role integrating peripheral circadian rhythms back to the brain (58). CRY1 (p≥0.429), CRY2 (p≥0.132) and PER2 (p≥0.268) expression were not affected by injury, disuse, or insulin treatment.

Conversely, PER1 was altered in the experimental groups. Nuclear PER1 (MW 45) is the active form responsible for inhibition of CLOCK:BMAL1 transcription, therefore a high nuclear to cytoplasmic ratio is consistent with decreased circadian oscillations and a loss of central to peripheral integration (58). The nuclear to cytoplasmic PER1 ratio was elevated in the VBH group compared to CC (p=0.001, Figure 7), while levels in the insulin-treated group were similar to cage controls (IBH versus CC: p=0.418; IBH versus VBH: p=0.012; Figure 7).

Discussion

Severe injury induces a systemic catabolic response characterized by increased energy expenditure; massive loss of lean body mass and decreased bone mineral content (19, 25, 37). Major burn is perhaps the best model for this, as it causes the greatest increase in metabolism (10). Muscle protein synthesis and breakdown are both accelerated, but breakdown accelerates more than synthesis, resulting in a net loss of muscle protein (11, 14). Burn-induced imbalance of protein synthesis and breakdown is sustained continuously from the time of hospital discharge, when wounds are 95% or more healed, and persists for one year thereafter, resulting in substantial muscle wasting and atrophy during convalescence (23, 25, 40). Molecular mechanisms and optimal treatments have not been fully characterized.

In a previous study using a modified Walker Mason model of severe burn injury, acute phase proteins were significantly increased after burn (65), however they nearly normalized by
day 14 and this rapid onset of recovery did not adequately mimic the clinical situation seen in severely burned patients (33). Humans display sustained catabolism with continuous loss of muscle mass throughout the hospital stay and for months after complete wound healing (19, 25). One possible explanation is that, since the burned rats in the above study were not restricted in movement after injury, the muscles of the hind-limb received intermittent tension from weight loading thereby providing a stimulus for muscle retention after injury. In contrast to rats, patients who have sustained a severe burn usually require long-term bed-rest during hospitalization, and have limited mobility once leaving the hospital (37). This inactivity may contribute to the increase in muscle catabolism seen after severe injury and play a role in prolonging the recovery.

Recently, investigators from our laboratory demonstrated that the addition of a disuse component to an established burn model exacerbates hypermetabolism with a cumulative effect that more closely approximates the severity of the human response (2, 71). The model combines a well-published hindlimb-unloading model (HU) with a highly cited burn model (46-48, 65, 70). Animals exhibit body mass (BM) losses similar to those seen in hypermetabolic humans after burn injury, demonstrating that this model can be used to study the physiologic response to large burns (71). Hindlimb unloading has been commonly used to study muscle metabolism and the physiologic changes due to muscle disuse during micro-gravity aspects of space flight and muscle disuse (46, 47, 50, 59). Adding hindlimb unloading and burns to produce a combined model (BH) more closely mimics physiologic changes seen in severely burned patients under clinically induced long-term bed-rest (71). Burn in combination with hindlimb unloading results in a greater loss of body mass than hindlimb unloading or burn alone, and losses continue through post-injury day 21 (72). BH rats lost both predominantly fast twitch muscle seen in burns alone and the slow twitch muscle fibers associated with hindlimb unloading (71). Total
body mass was significantly decreased in BH rats at day 14 compared to burn ambulatory,
suggesting that muscle disuse plays an additive role in induction and development of muscle
catabolism after burn (71).
Prior to the present study, it was not known whether the above model produces the same
glucose intolerance seen in hypermetabolic human subjects. Continuous daily insulin therapy is
known to prevent lean body mass losses by opposing catabolic processes and has been shown to
have antiproteolytic action in burned rats (14, 20, 61), but not enough is known about the
physiological changes responsible to identify potentially beneficial results or fully characterize
the underlying molecular mechanisms.
Our results showed that injury in combination with disuse causes glucose intolerance and
increased markers of hypermetabolism in rats, and insulin therapy reduced both glycemic
dysregulation and body mass loss. Given these findings, we proceeded to study muscle protein
expression and characterize altered relative abundance of phosphorylated proteins associated
with decreased muscle catabolism and improved glucose control after sustained insulin therapy.
Because the mediators involved in glycemic and insulin pathways have circadian effects, we
wondered whether these proteins would also display altered expression patterns. While our
previous data and that of others demonstrated preserved circadian rhythms in critically injured
subjects treated with insulin, a recent publication reported the opposite finding (12, 41, 53).
Given the mounting evidence for multiple interactions between metabolic and circadian
pathways, we investigated expression patterns for IRS-1, AKT, PPAR β, AMPK, FOXO-1,
Caspase-3, PER2 and CRY1 in two experimental groups, comparing treatment with insulin to
vehicle alone.
As expected, this study confirmed that the BH model elicits a hypermetabolic response
with both total and lean body mass loss sustained over time (71). Furthermore, significant losses were seen in all muscle groups measured. Corticosterone was elevated in both experimental groups compared to cage controls, a finding that is consistent with the other hypermetabolic effects of the model. Fat mass loss was marked, and the pattern of adrenal hypertrophy in combination with testicular atrophy was indicative of a prolonged stress state. Interestingly, there was no difference in plasma free fatty acids between groups, and no difference in PPAR β expression, a key regulator of fat metabolism.

Elevated plasma insulin levels drawn more than a day after the final insulin injection confirmed that once-daily subcutaneous insulin dosing successfully resulted in elevated plasma insulin over the entire 24-hour period. Glucose tolerance tests were further confirmation of the effects of insulin treatment. VBH rats had decreased glucose clearance and a slower rate of change compared to IBH.

Effects of insulin treatment on total IRS-1 expression were equally interesting. Injury and atrophy had a significantly depressive effect on IRS-1 total protein abundance, but this was partially reversed by treatment with insulin. Differences between cage controls and the insulin-treated group did not meet statistical significance. This effect was seen again in two phosphorylated forms of the protein; Tyr612 is associated with an activated IRS-1 state, while Ser312 is an inhibitory phosphorylation site associated with insulin resistance. The abundance ratio of two phosphorylated forms elucidates the relationship; Tyr612 phosphorylation increases, but not enough to overcome the concomitant increase in Ser312 thus the net effect is inhibitory consistent with the development of insulin resistance after injury. Insulin treatment increased the stimulatory phosphorylation more than it affected the inhibitory, changing the balance between Tyr612 and Ser312 IRS-1 phosphorylation. Thus, the relative ratio of stimulatory to inhibitory
IRS-1 phosphorylation in the VBH group was statistically different from both the IBH and CC samples, but IBH and CC ratio levels were similar. As with IRS-1 levels, the ratio of phosphorylated (Ser473) to total AKT provides further evidence of insulin resistance in the VBH group, and insulin treatment normalized the relationship. The rats studied in this model were in the acute ‘flow’ phase after burn and disuse, which in humans, can last up to a year. Evidence is mounting that the hyperglycemia that is characteristic of this phase may be mediated by insulin resistance. Many burn patients experience a prolonged period of hyperglycemia that lasts well into the rehabilitation phase (1 to 3 years), as is evidenced by mild to moderate obesity that can follow when the hypermetabolic phase wanes (37). While publications in the 1970’s pointed to increased hepatic gluconeogenesis as the culprit (68), recent publications have reported insulin resistance in burned rabbits as measured by euglycemic clamp as well as degradation of IRS-1 in a murine model (44, 73). These findings suggest, as does our work, that the prevalence of hyperglycemia after burns likely has an insulin resistance component, and are further supported by observations of abnormal insulin sensitivity in post-burn pediatric patients (18). This phase is usually temporary and eventually resolves for most patients; however, it lasts much longer than the two week duration of these experiments. Our findings suggest that this period of insulin resistance is a product of competing IRS-1 phosphorylation, with inhibitory signaling predominating in the absence of exogenous insulin. Conversely, PPAR β was not altered either by injury and disuse or by insulin treatment.

Results for mediators of atrophy were surprising; AMPK involvement in trauma and disuse has been reported, however; in this study, protein expression of Thr172, the active form of AMPK, was similar between groups (28). This finding is while interesting given the classical role of AMPK in regulating cellular metabolism and as a measure of cellular energy balance.
Phosphorylated AMPK was suppressed after the glucose load, which is expected; however, chronic insulin administration should have resulted in up-regulation in the insulin-treated group. This was not seen; however, for reasons that are not clear, and may be a limitation of the study design in obtaining muscle biopsies after glucose loading. Conversely, Caspase-3 was elevated in both experimental groups compared to cage controls and, as expected, was not modulated by insulin. Attenuation of injury- and disuse-related atrophy was primarily mediated via phosphorylation of FOXO-1 at Ser256, promoting FOXO-1 inactivation, cellular growth and inhibition of apoptosis; however, it was not sufficient to prevent soleus muscle loss. Ser256 was significantly decreased in the VBH group, and insulin treatment increased FOXO-1 inactivation resulting in Ser256 levels that were similar to that found in the CC groups. This provides a potential mechanism for overcoming the effects of Caspase-3 in the setting of injury and disuse; effects in other tissues must be examined.

PER1 was the only circadian modulator with altered expression between groups, a finding that is consistent with its known role of integrating central and peripheral circadian inputs. The elevated ratio of nuclear to cytoplasmic PER1 indicates an increase in negative control, and therefore dampened circadian oscillations (58). It was beyond the scope of this study to determine whether such attenuation exists, however; this is an area ripe for further exploration. We have shown in retrospective and animal studies that there is an associated between circadian rhythm dampening and adverse outcome, and this has also been reported by Palmieri and her colleagues (29, 53, 54, 64). This has not been studied in animal models, in part because eliciting a profound hypermetabolic response has been elusive. The combined burn injury and disuse model more closely resembles clinical scenarios and may allow closer study of the interaction between peripheral and central regulation of glucose in the Burn ICU setting where
hyperglycemia is persistent and zeitgeibers are altered. While such an investigation was beyond the scope of this work, we established that insulin treatment modulated the ratio of nuclear and cytoplasmic PER 1, and our findings suggest that insulin treated rats may have a better outcome in a longer term study. Future work is needed to further elucidate circadian patterns, gene expression, and the link to outcome.

Our study had limitations; aside from the insufficient number of animals to study diurnal patterns, we elected to forego daily blood sampling to avoid attendant additive stress effects that might obscure study findings and prevent a circadian design. While we measured glucose clearance, there was no true insulin clamp to assess insulin resistance. That said, we were able to show that the glucose tolerance test was performed under clamp-like conditions, because the clearance rate of the subcutaneous insulin dose was prolonged to such an extent that the net change over the 60 minute GTT period was presumed to be negligible.

CONCLUSIONS

Insulin treatment attenuates hypermetabolism caused by injury in combination with disuse, and improves both the rate and quantity of glucose clearance. Therapies aimed at targeting downstream effectors may provide the beneficial effects of insulin in opposing body mass loss and inappropriate protein breakdown without hypoglycemic risk. Molecular mechanisms responsible for insulin resistance after injury and disuse appear to be inhibitory phosphorylation of IRS-1 that overcomes the stimulatory pathway, an effect that is reversed with insulin administration. Body mass losses are mediated by Caspase-3 and FOXO-1, while insulin partially opposes these effects via downstream phosphorylation and inactivation of FOXO-1. PER1 protein expression is altered; further research is needed to elucidate how it interacts with
other metabolic proteins and whether this results in altered circadian oscillations.

PERSPECTIVES AND SIGNIFICANCE

This study evaluates, for the first time, the effect of insulin administration on physiologic changes, increases in markers of hypermetabolism, and relative abundance of molecular effectors caused by a rat model of severe injury and disuse. This model, when published in 2010, opened up new avenues of investigation. It provides a test bed for examining the effects of potential therapies in the context of a hypermetabolic insults that more closely approximates the severity seen in humans, particularly in the early phases after burn. Here we demonstrated the feasibility of the strategy by defining important changes induced by insulin administration; however, similar investigations of other mediators known to alter hypermetabolism could be equally fruitful. An exciting area of research involves effects on circadian rhythms, which, because of their function in coordinating central and peripheral changes in metabolism, have the potential to explain some of the global phenomena affecting severely injured populations. This study, while not designed to definitively describe these effects, identified changes in PER1 protein expression suggesting that this may be a productive area of further research.
Disclosures:

This research was funded by the National Institutes of Health (1R01GM063120), the Technologies for Metabolic Monitoring (TMM)/Julia Weaver Fund, a Congressional Directed Program Jointly Managed by the USAMRMC, NIH, NASA and the Juvenile Diabetes Research Foundation, and the Combat Casualty Care Division United States Army Medical Research and Materiel Command. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense. This study has been conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations and in accordance with the principles of the Guide for the Care and Use of Laboratory Animals. No conflicts of interest, financial or otherwise, are declared by the authors.

Acknowledgements:

The authors would like to thank Andrew Cap, MD, PhD, for his support and feedback. The authors would also like to thank the following individuals for their invaluable assistance in the execution of this study: Erica Hagerman; D. Todd Silliman; Krystal Valdez; Alejandra Mora. The authors would like to thank Angela Beeler for her editorial assistance.
LITERATURE CITED


TABLE 1: Plasma exogenous insulin, plasma free fatty acid, and average daily urinary corticosterone levels in response to injury with and without insulin treatment. CC = cage controls; VBH = vehicle-treated; IBH = insulin-treated. Average values with standard deviations are represented. Post-hoc T-Test p-values are given for interactions in which the ANOVA was significant.

TABLE 2. Organ mass on Day 14 expressed as g/100g of Day 14 body mass (BM) in cage-controls (CC, n=6), vehicle-treated (VBH, n=8), and insulin-treated rats (IBH, n=8). Average values with standard deviations are represented. Post-hoc T-Test p-values are given for interactions in which the ANOVA was significant. P-values <0.05 are bolded.

TABLE 3. Muscle mass on Day 14 expressed as g/100g of lean body mass (BM) in cage-controls (CC), vehicle-treated (VBH), and insulin-treated rats (IBH). Average values with standard deviations are represented. Post-hoc T-Test p-values are given for interactions in which the ANOVA was significant. P-values <0.05 are bolded.
FIGURE LEGENDS:

Figure 1: Glucose tolerance test timeline. Animals were fasted for 10 hours prior to testing, which was performed on day 7 or 8 for cage control rats and day 14 for insulin and vehicle hindlimb unloaded rats. Sequential blood glucose measurements were taken at times indicated.

Figure 2: Average daily (A) body mass; (B) food intake; (C) water intake. Initial and post-burn body mass (g), food intake (g/100g BM), and water intake (g/100g BM) were recorded daily until the end of the study on day 14. † = IBH versus CC: p≤0.001; * = IBH versus VBH: p≤0.05.

Figure 3: (A) Glucose tolerance test and (B) rate of glucose clearance as a function of the slope of the natural log of glucose values. Slope of VBH v CC, p=0.03; slope of IBH v VBH, p ≤ 0.001).

Figure 4: (A). Change in total IRS-1 due to burn and disuse. The total IRS-1 data from Figure 4A were used to derive the ratios of phosphorylated to total IRS-1 shown in Figs 4B and C. (B). Ratio of IRS-1 phosphorylated at serine 312 to total IRS-1. (C). Ratio of IRS-1 phosphorylated at tyrosine 612 to total IRS-1. (D). Ratio of IRS-1 phosphorylated at tyrosine 612 to IRS-1 phosphorylated at serine 312. Measurements are in arbitrary units and reported as mean ± SD; bars indicate *p < 0.05.
Figure 5: (A) Ratio of AKT phosphorylated at serine 473 to total AKT and (B) Ratio of Thr 172 to total AMPK. Measurements are in arbitrary units and reported as mean ± SD; bars indicate *p < 0.05.

Figure 6: (A). Ratio of FOXO-1 phosphorylated at serine 256 to total FOXO-1. (B). Change in Caspase-3 due to burn and disuse. Measurements are in arbitrary units and reported as mean ± SD; bars indicate *p < 0.05.

Figure 7: Ratio of nuclear to cytoplasmic PER-1. Measurements are in arbitrary units and reported as mean ± SD; bars indicate *p < 0.05.
Figure 1: Glucose tolerance test timeline.

- Day 0
- Day 14
- Glucose Load
- HU
- Burn
- 0 min: Draw baseline blood sample
- 5 min: Draw blood sample
- 10 min: Draw blood sample
- 15 min: Draw blood sample
- 30 min: Draw blood sample
- 60 min: Draw blood sample, followed by
- Fasted overnight prior to study
- Muscle Excision
- Blood Collection
- Organ Bx
Figure 2A: Average daily body mass.
Figure 2B: Average daily food intake.
Figure 2C: Average daily water intake.
Figure 3A: Glucose tolerance test

Average Glucose (mg/dL)

Time (minutes)
Figure 3B: Rate of glucose disposal

\[
y = -0.0108x + 5.8532 \\
y = -0.0078x + 5.9393 \\
y = -0.0171x + 5.6779
\]
Figure 4A:

A.

IRS-1

CC  VBH  IBH

*
Figure 4B

B.

Ser 312:tot IRS-1

- CC
- VBH
- IBH
Figure 4C

C.

Tyr 612:tot IRS-1

*
Figure 4D

D.

Tyr 612:Ser 312

CC

VBH

IBH
Figure 5:

A. Ser 473:tot AKT

CC

VBH

IBH
Figure 6A:

A. Ser 256:tot FOXO-1

CC VBH IBH

* *
Figure 6B:

B.

Caspase-3

CC  VBH  IBH

*
Figure 7:

NUC PER-1 : CYT PER-1

Comparison between CC, VBH, and IBH conditions.
Table 1: Exogenous insulin, free fatty acid, and average daily corticosterone levels.

<table>
<thead>
<tr>
<th></th>
<th>Exogenous Insulin (pg/ml)</th>
<th>SD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CC</td>
<td>CC v VBH</td>
</tr>
<tr>
<td>CC</td>
<td>606 ± 315</td>
<td>0.036</td>
<td></td>
</tr>
<tr>
<td>VBH</td>
<td>316 ± 70</td>
<td>CC v IBH 0.030</td>
<td></td>
</tr>
<tr>
<td>IBH</td>
<td>1910 ± 1257</td>
<td>VBH v IBH 0.005</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>FFA (nmol/ul)</th>
<th>SD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CC</td>
<td>CC v VBH</td>
</tr>
<tr>
<td>CC</td>
<td>0.12 ± 0.05</td>
<td>0.113</td>
<td></td>
</tr>
<tr>
<td>VBH</td>
<td>0.08 ± 0.02</td>
<td>CC v IBH 0.718</td>
<td></td>
</tr>
<tr>
<td>IBH</td>
<td>0.14 ± 0.09</td>
<td>VBH v IBH 0.172</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Corticosterone (ng/day)</th>
<th>SD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CC</td>
<td>CC v VBH</td>
</tr>
<tr>
<td>CC</td>
<td>494 ± 92</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>VBH</td>
<td>938 ± 460</td>
<td>CC v IBH 0.000</td>
<td></td>
</tr>
<tr>
<td>IBH</td>
<td>1117 ± 632</td>
<td>VBH v IBH 0.079</td>
<td></td>
</tr>
</tbody>
</table>
Table 2: Organ mass on Day 14.

<table>
<thead>
<tr>
<th>Organ</th>
<th>CC</th>
<th>VBH</th>
<th>IBH</th>
<th>p value IBH to CC</th>
<th>p value IBH to VBH</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM</td>
<td>329 ± 6 g</td>
<td>272 ± 12 g</td>
<td>287 ± 11 g</td>
<td>0.001</td>
<td>0.025</td>
</tr>
<tr>
<td>Adrenals</td>
<td>0.016 ± 0.002</td>
<td>0.024 ± 0.003</td>
<td>0.024 ± 0.002</td>
<td>0.000</td>
<td>0.829</td>
</tr>
<tr>
<td>Heart</td>
<td>0.34 ± 0.02</td>
<td>0.38 ± 0.03</td>
<td>0.39 ± 0.04</td>
<td>0.007</td>
<td>0.775</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.31 ± 0.02</td>
<td>0.38 ± 0.03</td>
<td>0.33 ± 0.02</td>
<td>0.094</td>
<td>0.001</td>
</tr>
<tr>
<td>Liver</td>
<td>2.65 ± 0.12</td>
<td>3.00 ± 0.13</td>
<td>2.80 ± 0.04</td>
<td>0.008</td>
<td>0.001</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.19 ± 0.03</td>
<td>0.19 ± 0.02</td>
<td>0.15 ± 0.02</td>
<td>0.002</td>
<td>0.000</td>
</tr>
<tr>
<td>Testes</td>
<td>1.06 ± 0.10</td>
<td>0.44 ± 0.05</td>
<td>0.41 ± 0.05</td>
<td>0.001</td>
<td>0.343</td>
</tr>
</tbody>
</table>
Table 3: Muscle mass on Day 14.

<table>
<thead>
<tr>
<th></th>
<th>CC</th>
<th>VBH</th>
<th>IBH</th>
<th>p value IBH to CC</th>
<th>p value IBH to VBH</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBM</td>
<td>234 ± 5 g</td>
<td>194 ± 12 g</td>
<td>203 ± 12 g</td>
<td>0.000</td>
<td>0.115</td>
</tr>
<tr>
<td>Muscle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plantaris</td>
<td>0.35 ± 0.01</td>
<td>0.28 ± 0.02</td>
<td>0.29 ± 0.01</td>
<td>0.000</td>
<td>0.100</td>
</tr>
<tr>
<td>Soleus</td>
<td>0.13 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.000</td>
<td>0.025</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>1.76 ± 0.06</td>
<td>1.30 ± 0.06</td>
<td>1.37 ± 0.07</td>
<td>0.000</td>
<td>0.028</td>
</tr>
<tr>
<td>Total Muscle</td>
<td>2.98 ± 0.08</td>
<td>2.34 ± 0.10</td>
<td>2.43 ± 0.09</td>
<td>0.000</td>
<td>0.059</td>
</tr>
</tbody>
</table>