Short-term bed rest increases TLR4 and IL-6 expression in skeletal muscle of older adults

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ACUTE HOSPITALIZATION because of illness or injury can have devastating consequences on the functional capacity and performance of activities of daily living in older adults (3, 25). Profound inactivity characterizes acute hospitalization in older adults and can significantly and independently contribute to muscle and functional loss (11, 12). Several studies have shown that short-term bed rest leads to rapid deterioration of muscle mass and strength of the lower extremity even in healthy older subjects (7, 24). However, the precise mechanisms are still unclear. A better understanding of the cellular mechanisms leading to muscle atrophy with bed rest will allow us to identify specific targets for treatment to preserve physical function and independence in hospitalized older adults.

Muscle loss following bed rest is largely a result of reduced postabsorptive and feeding-induced muscle protein synthesis rates (7, 9, 24). Protein breakdown may also play a role in muscle loss especially in circumstances of muscle wasting such as in critical ill (5, 22) and septic (41) patients and during cast immobilization (4, 16, 23). Pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), have catabolic effects on skeletal muscle (1, 15, 17–19). Moreover, pro-inflammatory cytokines are commonly elevated in the circulation of critical ill patients (5, 22, 41), during prolonged bed rest (2), and with exercise deconditioning (47), all circumstances associated with rapid muscle loss. Conversely, increased levels of physical activity are associated with a reduction in circulating inflammatory levels (10, 20). Moreover, nonimmune cells, such as skeletal muscle, also express pro-inflammatory cytokines (15, 27, 38, 43), suggesting that cytokines may also have local biological effects on the muscle tissue. In support of this notion, Haddad et al. (19) reported decreased myofibrillar protein content in rodent limb muscle following 14 days of local IL-6 infusion. This effect was independent of changes in circulating IL-6 levels.

The biological mechanisms regulating skeletal muscle tissue inflammation are complex but may originate partly through the toll-like receptor 4 (TLR4). TLR4 is most noted for a critical role in innate immunity as a first line of defense against specific pathogens. TLR4 has a number of endogenous ligands, including heat-shock protein 60 (HSP60). Specifically, HSP60 binds TLR4 in monocytes of diabetic patients and under conditions of cellular stress (6, 28, 30, 35). Activated TLR4 in turn initiates a signaling cascade that operates through the inhibitor of κB (IκB)/nuclear factor-κB (NF-κB) pathway. Upon activation, NF-κB increases the expression of several inflammatory cytokines including IL-6, TNF-α, and interleukin-1β (29, 31). TLR4 can be found in various cell types, including human skeletal muscle (14, 42). This raises the possibility that TLR4 may be involved in initiating events associated with the stimulation of pro-inflammatory cytokine transcription within skeletal muscle.
The purpose of the current investigation was to characterize the mechanisms of bed rest-induced muscle wasting by examining the regulatory pathways of muscle inflammation through TLR4 signaling in the postabsorptive state. A secondary aim was to determine the effect of bed rest on muscle anti-inflammatory mediators (interleukin-10) and cytokines associated with anabolic (interleukin-15) (34, 40). We hypothesized that a 7-day bed-rest protocol, mimicking an acute hospitalization, would increase systemic and local levels of pro-inflammatory cytokines, including TLR4 signaling in skeletal muscle biopsy samples of older adults. Furthermore, we hypothesized that anti-inflammatory and anabolic cytokines would be reduced after short-term bed rest in skeletal muscle samples of these older adults.

METHODS

Participants. Six healthy older adults (5 men, 1 woman; age 67 ± 2 yr; height 174 ± 2 cm; weight 75 ± 4 kg) were recruited from the University of Texas Medical Branch Pepper Center Volunteer Registry. Subjects were community-dwelling, overall healthy, independent older adults as determined by clinical history, mini-mental state exam, physical examination, and laboratory tests. All subjects read and signed a written informed consent before participating in the study. The informed consent and protocol were approved by the Institutional Review Board of the University of Texas Medical Branch. Body composition and protein turnover data from this study have been previously published (7).

Experimental design. Research participants underwent a 7-day bed-rest experiment in the University of Texas Medical Branch Institute for Translational Sciences Clinical Research Center. Bed rest was preceded by a 3-day in-hospital run-in (maintaining the subject’s habitual diet and physical activity) and followed by a 3-day in-hospital rehabilitation period (7). Over the bed-rest period, nonpharmacological deep venous thrombosis prevention was performed, which consisted of intermittent lower leg compression devices, compression stockings, and daily passive range of motion by a physical therapist. Bathing and hygiene activities were performed in the bed with nursing help, while toilet privileges were limited to a bedside commode. Adherence to bed rest was continuously monitored via closed-circuit cameras and reinforced daily by nursing staff and study personnel. A dual-energy X-ray absorptiometry (DXA) scan to measure body tissue composition was conducted before and after bed rest as reported previously (7). DXA scan measurements were carefully controlled between measurements, which entailed measurements taken at the same time of day (8:00 AM) after an overnight fast, participants wearing the same clothing and controlling for fluid shifts with participants laying supine before scans.

After an inpatient stay and an overnight fast, percutaneous muscle biopsies were collected in the morning of the initiation of bed-rest day 1 (pre-bed rest) and at the same time in the morning of bed-rest day 7 (post-bed rest). Muscle biopsies were taken from the vastus lateralis muscle of one leg (pre-bed rest) and the opposite leg (post-bed rest) using aseptic technique, local anesthesia (1% lidocaine), and a 5-mm Bergström biopsy needle with suction. All muscle tissue was immediately blotted and dissected free of visible nonmuscle tissue, flash-frozen in liquid nitrogen, and stored at −80°C until analysis.

Cytokines. Venous blood samples from participants were collected before bed rest and on the second, third, fourth, and seventh day of bed rest. Samples were collected at the same time in the morning, after an overnight fast. Blood samples were allowed to clot at room temperature for 20 min in a serum separator tube, of which the tubes were then centrifuged. Supernatant serum was collected and stored at −80°C until analysis. Cytokines [IL-1β, -2, -4, -5, -6, -7, -8, -10, -12, -13, -17, G-CSF, granulocyte macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), monocyte chemoattractant protein-1 (MCP-1), MIP-1β, and TNF-α] were measured in serum samples using a Bio-Plex Pro Human Cytokine 17-plex Assay (M50-00031Y; Bio-Rad, Hercules, CA) on a Bio-Plex 200 Instrument (Bio-Rad). For each analyte, a standard curve was generated using recombinant proteins to estimate protein concentration in the unknown sample (BioPlex Array Manager).

SDS-PAGE and immunoblotting. Muscle tissue was homogenized using a glass pestle and prechilled tube in a buffer cocktail with protease and phosphatase inhibitors (50 mM Tris-HCl, 250 mM mannitol, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml soybean trypsin inhibitor). Whole muscle homogenates were centrifuged and the supernatant was collected. Total protein concentration for each sample was determined on a SmartSpec (Bio-Rad) using a colorimetric protein assay (Bio-Rad; Bradford) and an albumin standard curve. Whole muscle homogenates were diluted 1:1 in a 2× sample buffer. Homogenates (50 μg of total protein) were loaded on a 7.5% and 15% polyacrylamide gel (Criterion; Bio-Rad), depending on the molecular weight of the protein, and subjected to SDS-PAGE (150 V) for 1 h in running buffer. Each gel contained alternating pre- and post-bed rest samples loaded in duplicate and a molecular weight ladder. An internal control (rodent muscle homogenate) was loaded in duplicate on each gel for band normalization and comparisons across blots. Protein was transferred (50 V, 1 h) to a polyvinylidene fluoride membrane in transfer buffer and then blocked for 1 h at room temperature with 5% non-fat dry milk (NFDM) in Tris-buffered saline in 0.1% Tween-20 (TBST). Membranes were incubated overnight in primary antibody diluted in 5% NFDM or bovine serum albumin in TBST. The next morning, blots were rinsed in TBST for 5 min, rocked in secondary antibody for 1 h at room temperature in 5% NFDM in TBST, and then washed sequentially (15 min, 3 × 5 min) in TBST. Chemiluminescence reagent (ECL Plus, GE Healthcare) was applied to each blot for 5 min. Optical density measurements were obtained with a digital imager (ChemiDoc XRS, Bio-Rad). Membranes containing phospho-specific proteins were stripped in 2% (Bio-Rad) and incubated in 5% (Bio-Rad) NFDM and secondary antibodies then reprobed for the total protein of specific target. Densitometric analysis was performed using Quantity One 4.5.2 software (Bio-Rad). After the background was subtracted out, all Western blot data were normalized to the internal control and replicate samples were averaged. α-Tubulin was used to verify equal loading across lanes.

Antibodies. The following antibodies were used in this experiment: TLR4 (cat. no. sc-10741) from Santa Cruz Biotechnology (Santa Cruz, CA), whereas the following antibodies were purchased from Cell Signaling Technology (Boston, MA): HSP60 (cat. no. 4870), phosphorylated IkBα (S32; cat. no. 2859) and total IkBα (cat. no. 9242), phosphorylated NF-κB p65 (S536; cat. no. 3033) and total NF-κB p65 (cat. no. 8242). α-Tubulin was purchased from Sigma-Aldrich (cat. no. F2168). Donkey anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody was purchased from GE Healthcare-Amersham, whereas a mouse secondary antibody was purchased from Santa Cruz Biotechnology.

RNA extraction, cDNA synthesis, and semiquantitative qPCR. Total RNA, cDNA synthesis, and real-time qPCR were conducted as previously reported (8). Total RNA was isolated by homogenizing 15–20 mg tissue with a hand-held homogenizing dispenser (T10 Basic Ultra Turrax, IKA, Wilmington, NC) in a solution containing 1 ml of Tri reagent. The RNA was separated into an aqueous phase using 0.2 ml of chloroform and precipitated from the aqueous phase using 0.5 ml of isopropanol. Extracted RNA was washed with 1 ml of 75% ethanol, dried, and then suspended in a known amount of nuclease-free water. RNA integrity for these samples (RIN: ~9) have been

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published previously (7). RNA was DNase-treated using a commercially available kit (DNA-free, Ambion, Austin, TX). Afterwards, 1 µg of total RNA was reverse transcribed into cDNA (iScript; Bio-Rad). All isolated RNA and cDNA samples were stored at −80°C until analyzed with qPCR. Real-time qPCR was carried out with an iQ5 Multicolor Real Time PCR cycler (Bio-Rad). Taqman pre-designed primers were purchased from Applied Biosystems (Carlsbad, CA). The thermal cycling conditions were the following: a 10-min initial denaturing step at 95°C followed by 40 cycles consisting of denaturation for 15 s at 95°C and anneal and elongation for 1 min at 60°C. Values were normalized to β2-microglobulin (since Ct values were within range of the standard curve). Significance was set at P ≤ 0.05. All values are presented as means ± SE.

RESULTS

Lean mass. After 7-days of bed rest, older adults lost ~1.6 kg of total body lean mass (Pre: 50.4 ± 2.9 kg; Post: 48.8 ± 2.5 kg; P = 0.03), whereas ~50% (0.8 kg) of this loss came from leg (right + left) lean mass (Pre: 18.3 ± 1.1 kg; Post: 17.5 ± 1.0 kg; P = 0.01).

Serum cytokines. We found no changes across time in 14 of the 17 cytokines. INF-γ (P = 0.05) and MIP-1β (P = 0.02) increased over time. Serum IL-2 was undetectable throughout the experimental period, and IL-17, G-CSF, and GM-CSF were detectable only in n = 4. All detectable serum cytokines were within range of the standard curve.

Skeletal muscle TLR4 and NF-κB signaling. We found that the 7-day bed rest increased the abundance of TLR4 in skeletal muscle by ~70% compared with pre-bed rest values (Figure 1A; P = 0.03). However, heat shock protein 60 (HSP60), a ligand for TLR4, was unaltered following bed rest (Fig. 1B; 

<table>
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<tr>
<th>Cytokine</th>
<th>Baseline</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Main Effect-Time (P Value)</th>
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<td>1.7 ± 0.5</td>
<td>1.4 ± 0.3</td>
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<td>2.6 ± 0.7</td>
<td>2.0 ± 0.5</td>
<td>2.4 ± 0.4</td>
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<tr>
<td>IL-5</td>
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<td>2.6 ± 0.6</td>
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<td>IL-6</td>
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<td>IL-7</td>
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<td>IL-12</td>
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<td>IFN-γ</td>
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<td>MIP-1β</td>
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<td>51.8 ± 23.1</td>
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<td>TNF-α</td>
<td>14.0 ± 3.5</td>
<td>15.8 ± 2.7</td>
<td>18.1 ± 4.4</td>
<td>15.1 ± 3.0</td>
<td>15.1 ± 2.0</td>
<td>0.93</td>
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Values are pg/ml and are means ± SE (n = 6). P value signifies main effect for time (days of bed rest). Values for IL-17, G-CSF, and GM-CSF are n = 4. IL-1β, interleukin-1β; IL-4, interleukin-4; IL-5, interleukin-5; IL-6, interleukin-6; IL-7, interleukin-7; IL-8, interleukin-8; IL-10, interleukin-10; IL-12, interleukin-12; IL-13, interleukin-13; IL-17, interleukin-17; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte macrophage-colony stimulating factor; IFN-γ, interferon-γ; MCP-1, monocyte chemotactic protein-1; MIP-1β, macrophage inflammatory protein-1β; TNF-α, tumor necrosis factor-α.
To evaluate the effect of increased TLR4 protein expression, we measured NF-κB signaling. We found no differences when NF-κB phosphorylation (S536) was expressed relative to total NF-κB p65 protein abundance (Fig. 2A; \( P = 0.37 \)) following 7-days of bed rest in skeletal muscle of healthy older subjects. Similarly, bed rest did not affect the phosphorylation of NF-κB at S536 (Pre: 3.53 ± 0.58; Post: 2.77 ± 0.31 AU; \( P = 0.26 \)) or NF-κB p65 protein levels (Pre: 1.18 ± 0.26; Post: 1.03 ± 0.25 AU; \( P = 0.50 \)) and total IκBα protein levels (Pre: 1.78 ± 0.77; Post: 2.14 ± 0.93 AU; \( P = 0.13 \)).

**Pro-inflammatory mRNA expression.** We found that NF-κB1 mRNA expression in skeletal muscle biopsy samples was increased \( \sim 40\% \) compared with pre-bed rest values (Fig. 3A; \( P = 0.03 \)) but independent of changes in total protein (Fig. 3B). Additionally, IL-6 mRNA expression increased \( \sim 130\% \) after 7-days of bed rest in older adult skeletal muscle (Fig. 3B; \( P = 0.04 \)). Bed rest did not affect TNF-α (Fig. 3C; \( P = 0.64 \)), TNFR1SA1 (Fig. 3D; \( P = 0.42 \)), or IL1β mRNA expression.

**Fig. 2.** Effect of bed rest on nuclear factor-κB (NF-κB) signaling. Data represent NF-κB phosphorylation (S536) relative to NF-κB total protein expression (A) and IκBα phosphorylation (S32) relative to IκBα total protein expression (B) before (light gray bars) and after (dark gray bars) 7 days of bed rest in vastus lateralis skeletal muscle of healthy older adults (n = 6). Insets are representative immunoblot images. Line plots below figures represent individual means for males (M1–5) and female (F1). \( P \) values are indicated on figures. Values are presented as means ± SE.

**Fig. 3.** Effect of bed rest on pro-inflammatory muscle mRNA markers. Data represent NF-κB1 (A), IL-6 (B), tumor necrosis factor-α (TNF-α) (C), TNFR1SA1 (D), and IL-1β mRNA expression (E) before (light gray bars) and after (dark gray bars) 7 days of bed rest in vastus lateralis skeletal muscle of healthy older adults (n = 6). Line plots below figures represent individual means for males (M1–5) and female (F1). Values are reported as fold change from baseline. \( P \) values are indicated on figures. Values are presented as means ± SE.
Relationship between bed rest-induced changes in leg lean mass and TLR4 and IL-6 expression. There was not a statistically significant correlation between changes in TLR4 protein and IL-6 mRNA expression and leg lean mass: TLR4 protein expression and leg (right + left) lean mass (Fig. 5A; $R^2 = 0.31$, $P = 0.25$) and IL-6 mRNA expression and leg lean mass (Fig. 5B; $R^2 = 0.42$, $P = 0.16$).

DISCUSSION

The primary finding of this study was that 7 days of controlled bed rest, mimicking the inactivity of an acute hospital stay, markedly increased TLR4 protein abundance and IL-6 mRNA expression in the skeletal muscle biopsy samples of healthy older adults. Conversely, we did not find significant changes in muscle NF-κB signaling while changes in circulating inflammatory levels were modest and mostly absent. We also found that some, but not all, pro-inflammatory and anabolic cytokine mRNA expression in muscle biopsy samples was increased after bed rest. These data provide preliminary evidence that short-term physical inactivity can locally induce a small, but likely important, pro-inflammatory response in skeletal muscle biopsy samples of healthy older adults in the absence of an obvious increase in systemic inflammation. The elevation in TLR4 with bed rest may suggest an increased muscle capacity to mount a rapid and possibly uncontrolled inflammatory response in the presence of TLR4 ligands, such as during an acute illness or infection, in older adults.

This study is the first demonstrating an elevation in TLR4 expression in skeletal muscle biopsy samples following 7 days of controlled bed rest in healthy older subjects. Previous work has shown that increased physical activity and exercise training can reduce TLR4 mRNA in skeletal muscle of healthy (51) and diabetic rats (36) and in frail obese older adults (26). When interpreted from those results, our novel data support the notion that TLR4 expression is highly sensitive and inversely correlated to the level physical activity in human skeletal muscle.

A signature response of increased TLR4 activity is an upregulation of pro-inflammatory cytokines including TNF-α, IL-6, and IL-1β (27). We found that bed rest increased the skeletal muscle levels of IL-6 mRNA, but TNF-α, TNF-α receptor (TNFR1SA1), and IL-1β mRNA were unchanged. Our findings are consistent with those reported by Reyna and colleagues (42) who found elevated skeletal muscle levels of TLR4 mRNA and protein and IL-6 mRNA in obese adults with Type 2 diabetes compared with lean nondiabetic controls. Other human models of muscle wasting (critically ill) have also been associated with elevated muscle IL-6 mRNA levels, whereas changes in TNF-α have been inconsistent (5, 22). To the best of our knowledge, until now no one had examined muscle tissue levels of inflammatory cytokines as a result of controlled bed rest. While we cannot exclude that macrophages and other immune cells could have contributed to the increased inflammatory state as noted by detectable levels of CD-45 mRNA levels in our muscle homogenate samples, the finding that 15 of the 17 serum cytokines were unaltered following bed rest strongly suggests the lack of a systemic pro-inflammatory

![Fig. 4. Effect of bed rest on the mRNA expression of anti-inflammatory and anabolic cytokines. Data represent SOCS3 (A), IL-10 (B), and IL-15 mRNA expression (C) before (light gray bars) and after (dark gray bars) 7 days of bed rest in vastus lateralis skeletal muscle of healthy older adults ($n = 6$). Line plots below figures represent individual means for males (M1–5) and female (F1). Values are reported as fold change from baseline. $P$ values are indicated on figures. Values are presented as means ± SE.](http://ajpregu.physiology.org/ by 10.220.32.2 on April 3, 2017)
rest, in the absence of disease, increased TLR4 protein levels without activating NF-κB because of lack of TLR4 ligands (i.e., HSP60). If this is the case, then inactivity per se in older adults increases the capacity to mount an elevated, yet abnormal, muscle inflammatory response to acute noxious stimuli (e.g., an infection or a cardiovascular event requiring hospitalization). At best, changes in TLR4 and IL-6 showed a tendency to be related to muscle loss following bed rest, but interpretation must be taken with caution with a small sample size and making correlations with DXA measurements. Perhaps, with longer periods of bed rest-induced inactivity, an oversensitization to infection (i.e., TLR4) may lead to a much larger and potentially catastrophic loss of skeletal muscle mass.

We also found that skeletal muscle IL-10 mRNA expression was upregulated with bed rest in older adults, whereas there were no observed changes in SOCS3 mRNA. IL-10 is well known for its function to suppress inflammatory immune responses by targeting the transcription of pro-inflammatory mediators (33). Since IL-10 mRNA was elevated with bed rest, it is possible that this might be a mechanism to counteract a pro-inflammatory response (21). In fact, IL-6 is known to stimulate IL-10 production, thereby providing a physiological brake on the overall pro-inflammatory status (45). We also found that the anabolic cytokine IL-15 mRNA (34, 40) was increased after bed rest as previously reported in animals (39). Taken together, the increased expression of local anti-inflammatory and anabolic cytokines following acute bed rest in older adults may be a mechanism to counter heightened levels of IL-6.

An interesting observation was that bed rest largely did not affect serum inflammatory markers even in the early period of bed rest (days 2–4). In a previous report, 14 days of bed rest in young volunteers resulted in increased circulating IL-6 levels (2). Perhaps we did not observe changes in serum inflammatory cytokines because cellular sources of inflammatory mediators require a longer period of bed rest to be distributed into the circulation. Although a majority of circulating cytokines were unaltered during (and after) 7 days of bed rest even with a comprehensive cytokine profiling, we did find elevated serum levels of MIP-1β and a tendency for INF-γ to be increased. An increase in MIP-1β may represent an immune response to regenerate new tissue during bed rest (46, 49), whereas elevated INF-γ is consistent with an increased pro-inflammatory response as observed following muscle injury in old rodents (48).

We conclude that short-term bed rest in healthy older adults increased some, but not all, pro-inflammatory mediators, namely TLR4 protein and IL-6 mRNA expression, as well as anti-inflammatory (IL-10) and anabolic (IL-15) cytokine mRNA expression. These changes were largely independent of alterations in local NF-κB signaling and serum markers of inflammation. Increased expression of TLR4 may be an important predisposing factor for catastrophic inflammatory and muscle catabolic responses triggered by a concomitant acute disease and bed rest inactivity in older adults (e.g., inactivity during hospitalization for an acute illness).

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