

# Positive energy balance is associated with accelerated muscle atrophy and increased erythrocyte glutathione turnover during 5 wk of bed rest<sup>1-3</sup>

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## ABSTRACT

**Background:** Physical inactivity is often associated with positive energy balance and fat gain.

**Objective:** We aimed to assess whether energy intake in excess of requirement activates systemic inflammation and antioxidant defenses and accelerates muscle atrophy induced by inactivity.

**Design:** Nineteen healthy male volunteers were studied before and at the end of 5 wk of bed rest. Subjects were allowed to spontaneously adapt to decreased energy requirement (study A,  $n = 10$ ) or were provided with an activity-matched diet (study B,  $n = 9$ ). Groups with higher (HEB) or lower (LEB) energy balance were identified according to median values of inactivity-induced changes in fat mass ( $\Delta$ FM, assessed by bioelectrical impedance analysis).

**Results:** In pooled subjects ( $n = 19$ ; median  $\Delta$ FM: 1.4 kg), bed rest-mediated decreases in fat-free mass (bioelectrical impedance analysis) and vastus lateralis thickness (ultrasound imaging) were significantly greater ( $P < 0.03$ ) in HEB<sub>AB</sub> ( $-3.8 \pm 0.4$  kg and  $-0.32 \pm 0.04$  cm, respectively) than in LEB<sub>AB</sub> ( $-2.3 \pm 0.5$  kg and  $-0.09 \pm 0.04$  cm, respectively) subjects. In study A (median  $\Delta$ FM: 1.8 kg), bed rest-mediated increases in plasma leptin, C-reactive protein, and myeloperoxidase were greater ( $P < 0.04$ ) in HEB<sub>A</sub> than in LEB<sub>A</sub> subjects. Bed rest-mediated changes of glutathione synthesis rate in erythrocytes (L-[3,3-<sup>2</sup>H<sub>2</sub>]cysteine incorporation) were greater ( $P = 0.03$ ) in HEB<sub>A</sub> (from  $70 \pm 19$  to  $164 \pm 29\%/d$ ) than in LEB<sub>A</sub> (from  $103 \pm 23$  to  $84 \pm 27\%/d$ ) subjects.

**Conclusions:** Positive energy balance during inactivity is associated with greater muscle atrophy and with activation of systemic inflammation and of antioxidant defenses. Optimizing caloric intake may be a useful strategy for mitigating muscle loss during period of chronic inactivity. *Am J Clin Nutr* 2008;88:950–8.

## INTRODUCTION

Fiber atrophy and changes in contractile properties are involved in the adaptation of skeletal muscle to unloading (1, 2). Physical inactivity also is associated with resistance to insulin glucoregulatory action (3) and lower energy requirements (4). Energy intake (EI) should therefore be adapted to avoid positive balance and the preferential deposition of fat tissue in visceral compartments (5). Experimental bed rest in healthy volunteers is a suitable model in which to investigate physiologic adaptation to inactivity. Most previous studies have shown that, when EI was

not strictly controlled, fat mass tended to increase in parallel to lean body mass catabolism (6–14).

Overfeeding and excess body fat increase leptin secretion and are associated with low-grade inflammatory response and greater oxidative stress (15, 16). Proinflammatory mediators and redox unbalance potentially play a causative role in lowering muscle protein synthesis and in accelerating proteolysis (17, 18). Overfeeding led to a significant reduction in the protein fractional synthesis rate (FSR) in muscle of rats (19). Systemic inflammation upregulates cellular antioxidant defenses, including the thiol (functional group composed of a sulfur atom and a hydrogen atom; -SH) tripeptide (L- $\gamma$ -glutamyl-L-cysteinylglycine) glutathione (20), which is highly concentrated in erythrocytes and which provides local and systemic antioxidant protection (21).

The current study was conducted to test the hypothesis that positive energy balance (ie, EI in excess of requirements) leading to fat deposition could accelerate inactivity-induced loss of lean mass and could activate systemic inflammation, free radical production, and antioxidant defenses. Energy balance was determined from the overall 35-d change in body fat mass, as determined by bioelectrical impedance analysis [BIA (22)], and from

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changes in leptin concentration (23). Evaluation of antioxidant defenses included determination of erythrocyte glutathione turnover by the incorporation of stable isotope (24).

## SUBJECTS AND METHODS

### Subjects and experimental design

Nineteen healthy male subjects [ $\bar{x} \pm \text{SEM}$  age:  $24 \pm 1$  y; body mass index (in  $\text{kg}/\text{m}^2$ ):  $23.5 \pm 0.6$ ] were selected to investigate the effects of experimental bed rest at different EIs. Ten subjects were studied in July and August 2006 (study A), and 9 subjects were studied in July and August 2007 (study B). All subjects were physically active before admission to the Valdoltra Hospital, University of Primorska (Ankaran-Capodistria, Slovenia). Their body mass was stable for  $\geq 3$  mo before the studies.

Routine medical and laboratory analyses were performed to exclude chronic diseases. None of the subjects were regularly taking any medication. The experiments were part of comprehensive, international collaborative bed-rest studies. The bed-rest periods were preceded by a 1-wk ambulatory adaptation period, in which each subject received a weight-maintaining diet containing 1.4 times his resting energy expenditure, calculated by using the FAO/WHO equations (25). The diet contained  $\approx 60\%$  of energy as carbohydrate, 25% as fat, and 15% as protein. Six meals were administered daily: 3 main meals (breakfast, lunch, and dinner) and 3 snacks. During the 35-d bed-rest period of study A, a diet with the same energy, frequency, and relative macronutrient content of that planned in the ambulatory adaptation period was offered to subjects. Subjects were not required to consume all served food, but they were allowed to adapt spontaneously to decreased energy requirements during inactivity. Leftover food was monitored semiquantitatively by an expert dietitian to assess the relative macronutrient intake. During the 35-d bed-rest period of study B, each subject received an activity-adjusted diet containing 1.2 times his resting energy expenditure (25) and with the same meal frequency and relative macronutrient content of that planned in study A. Subjects were required to consume all food served.

The body composition of all subjects was measured by using BIA [Human IM Plus; DS Dietosystem, Milan, Italy (22)] at the end of both the ambulatory adaptation period and the bed-rest period of both studies. During studies A and B, the thickness of the vastus lateralis muscle was measured while the subject was in the supine position, by using ultrasound imaging on a portable ultrasound device (MyLab25; ESAOTE, Genoa, Italy) fitted with a 10–15-MHz linear probe (26). Sagittal ultrasound images were obtained at 50% of muscle length measured along the mid-sagittal axis, after identification of the proximal and medial bone insertions of muscle. Muscle thickness was measured, and expressed in cm, as the vertical distance between muscle superficial and deep aponeuroses at an equidistant point from the right and left borders of the image. Only in study A, in addition to the thickness of the vastus lateralis, the thicknesses of the gastrocnemius medialis, tibialis anterior, and biceps brachii muscles were measured. Then, a mean value of the thickness of representative postural and nonpostural muscles was calculated.

Markers of inflammatory response and erythrocyte glutathione turnover rates were determined during the postabsorptive state at the end of both the ambulatory adaptation and the bed-rest periods in study A. After background blood sampling, a primed

continuous infusion of L-[3,3- $^2\text{H}_2$ ]cysteine (Cambridge Isotope Laboratories, Andover, MA) (priming dose:  $150 \mu\text{mol}$ ; continuous infusion rate:  $150 \mu\text{mol}/\text{h}$ ) was started at 0700 or 0800 and continued for 5 h. A total of 10 mL arterialized venous blood was obtained at times 3, 4, and 5 h to measure hematocrit and the concentration and  $^2\text{H}_2$  enrichment of erythrocyte-free cysteine and glutathione (24). Whole blood was immediately centrifuged, and plasma and leukocytes were removed and replaced with an equal volume of cold distilled water. Both plasma and erythrocyte solutions were frozen at  $-80^\circ\text{C}$  for later analysis. Plasma and erythrocyte concentrations of glutathione and of glutathione amino acid precursors were measured in the background sample. At time 5 h, selected plasma hormone, mediator, and metabolite concentrations were measured in plasma, and glutathione peroxidase activity and protein concentrations in the catalytic and modulatory subunits of glutamate cysteine ligase were measured in erythrocytes. At 1200 or 1300, isotope infusion was discontinued.

All volunteers provided written informed consent. The experimental protocols were approved by the Ethics Committee of the University of Ljubljana (Ljubljana, Slovenia), and they conformed to the standards set by the Declaration of Helsinki (2002).

### Analyses

The procedure for analysis of erythrocyte glutathione and cysteine isotopic enrichments was adapted from Lyons et al (24). Erythrocyte suspension obtained from  $400 \mu\text{L}$  whole blood was placed into already-chilled tubes containing 1 mL ice-cold dithiothreitol (20 mmol/L in 1 mol acetic acid/L). Proteins were precipitated with  $400 \mu\text{L}$  sulfosalicylic acid 30% and centrifuged for 15 min at  $10\,000 \times g$  at  $4^\circ\text{C}$ . The supernatant fluid was transferred to a column containing 2 mL of a cation-exchange resin (AG 50W-X8; Bio-Rad, Hercules, CA). After washing with Milli-Q water [ $5 \text{ mL} \times 2$ ; Millipore, Bedford, MA), glutathione was eluted from the column by using  $\text{NH}_4\text{OH}$  (3 mol/L) and collected into derivatization tubes (10 mL). Ammonia was eliminated under gentle nitrogen flow at room temperature. Samples were frozen, lyophilized, and reacted with  $500 \mu\text{L}$  dithiothreitol solution (20 mmol/L in 0.5 mol acetic acid/L) at  $100^\circ\text{C}$  for 1 h to reduce any dimerized glutathione and then dried again in nitrogen flow. Each sample was reacted with  $300 \mu\text{L}$  HCl/methanol solution (250  $\mu\text{L}$  36% HCl in 7.5 mL methanol), incubated for 30 min at  $80^\circ\text{C}$ , and dried in nitrogen flow at  $65^\circ\text{C}$  before being further reacted with 50  $\mu\text{L}$  *N*-methyl-*N*-*tert*-butyldimethylsilyl-trifluoroacetamide and 50  $\mu\text{L}$  acetonitrile for 40 min at  $90^\circ\text{C}$  before injection into a gas chromatograph–mass spectrometer [(GC-MS) HP 5890; Agilent Technologies, Santa Clara, CA]. The GC-MS analyses of glutathione and cysteine were carried out by using a fixed silica capillary column (HP-5MS; Agilent Technologies: 25-mm internal diameter and 0.25- $\mu\text{m}$  film thickness). Column temperature was programmed to rise by  $10^\circ\text{C}/\text{min}$  from 160 to  $300^\circ\text{C}$  (detector temperatures were  $260^\circ\text{C}$ ) with the use of helium as the carrier gas. The derivative was measured under electron-impact ionization by selective ion monitoring at a nominal mass-to-charge ratio ( $m/z$ ) of 363/365 for glutathione enrichment and of 406/408 for cysteine enrichment.

Total glutathione concentrations in plasma and erythrocytes obtained from background samples were measured by using GC-MS and the internal standard technique. Briefly, known amounts of [glycine- $^{13}\text{C}_2$  $^{15}\text{N}$ ]glutathione (Cambridge Isotope

Laboratories, Andover, MA) were added as internal standard to erythrocyte suspension obtained from 400  $\mu\text{L}$  whole blood and to 200  $\mu\text{L}$  plasma. Samples were processed as described above. The derivative was measured under electron-impact ionization by selective ion monitoring at a nominal  $m/z$  of 363/366. Plasma and erythrocyte concentrations of glutathione precursor amino acids (glycine, glutamine, glutamate, methionine, homocysteine, and cysteine in plasma; glycine, glutamine, glutamate, methionine, and cysteine in erythrocytes) were measured in background samples by using GC-MS and the internal standard technique according to Valerio et al (27). Briefly, known amounts of  $L$ - $[^{15}\text{N}]$ glycine,  $L$ - $[^{15}\text{N}]$ glutamate,  $L$ - $[^{15}\text{N}]$ glutamine,  $\text{DL}$ - $[3,3,3',3',4,4,4',4'-^2\text{H}_8]$ -homocysteine,  $L$ - $[1-^{13}\text{C}, \text{methyl-}^2\text{H}_3]$  methionine, and  $L$ - $[3,3-^2\text{H}_2]$ cysteine (Cambridge Isotope Laboratories) were added as internal standards to an erythrocyte suspension obtained from 400  $\mu\text{L}$  whole blood and to 200  $\mu\text{L}$  plasma. Samples were processed as described (27). The derivative was measured under electron-impact ionization by selective ion monitoring at a nominal  $m/z$  of 218/219 for glycine, 432/433 for glutamate, 431/432 for glutamine, 496/500 for homocysteine, 320/324 for methionine, and 406/408 for cysteine.

Plasma leptin concentrations were measured by using an enzyme-linked immunosorbent assay (human Leptin Quantikine kit, catalog #DLP00; R&D Systems, Minneapolis, MN). Plasma C-reactive protein (CRP) concentrations were measured by using a high-sensitivity enzyme-linked immunosorbent assay kit (Diagnostics Biochem, London, Canada). Plasma insulin concentrations were measured by using a radioimmunoassay (Adaltis insulin kit; Adaltis Inc, Montreal, Canada). Total plasma ghrelin concentrations were measured by using a radioimmunoassay (Total Human Ghrelin, GHRT-89HK; Linco, St Charles, MO). Plasma glucose, total cholesterol, HDL-cholesterol, and triglyceride concentrations were measured by using commercially available kits (Olympus System Reagents; Olympus Diagnostica GmbH, Hamburg, Germany) by using an autoanalyzer (Olympus AU400 System; Olympus, Tokyo, Japan). LDL cholesterol was calculated by using the Friedewald equation:  $\text{LDL cholesterol} = \text{total cholesterol} - \text{HDL cholesterol} - (\text{triglycerides} \times 5)$  (28).

With antibodies obtained from rabbits and guinea pigs, neutrophil myeloperoxidase plasma concentrations were measured with the use of an enzyme-linked immunosorbent assay (29). Serum immunoglobulin G was isolated by affinity chromatography after immunization with purified human neutrophil myeloperoxidase. A reference curve was performed with purified human myeloperoxidase. Total -SH (thiol) functions (free -SH or bound to proteins) were measured in plasma by using spectrophotometry (at 412 nm) after the reaction of -SH with 5,5'-dithiobis-2-nitrobenzoic acid (30). A reference curve was performed with reduced glutathione. Total -SH values were expressed as mol/g protein. Total proteins were measured by using the Bio-Rad Protein Assay reagent and expressed in mg/mL. Glutathione peroxidase activity was determined in erythrocytes according to Paglia and Valentine (31) and expressed as  $\mu\text{mol}$  metabolized  $\text{NADPH} \cdot \text{min}^{-1} \cdot \text{g protein}^{-1}$  in the presence of an organic hydroperoxide (cumolhydroperoxide) and of reduced glutathione as enzyme cofactor (31).

Catalytic and modulator subunit expression of glutamate-cysteine ligase in erythrocytes was measured by Western blot analysis (32). Briefly, proteins were extracted from red blood

cells by using a lysis buffer (45 mmol/L Tris-HCl, 0.2% N-laurylsarcosine; Sigma-Aldrich, St Louis, MO) containing protease and phosphatase inhibitors (0.2 mmol phenylmethanesulfonyl fluoride/L, 1 mmol dithiothreitol/L, 2  $\mu\text{g}$  aprotinin/mL, 2  $\mu\text{g}$  pepstatin/mL, 0.1 mmol NaF/mL, and 0.1 mmol  $\text{Na}_3\text{VO}_4$ /L; all: Sigma-Aldrich). After centrifugation (10 min, RT,  $1600 \times g$ ), proteins were separated by sodium dodecyl sulfate-polycrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Protran; Perkin Elmer, Boston, MA). Proteins were recognized by using commercial antibodies raised against the catalytic (GCLc: sc-22755) and modulator (GCLm: sc-22754) (both: Santa Cruz Biotechnology Inc, Santa Cruz, CA) subunits of the glutamate-cysteine ligase. Glyceraldehyde-3-phosphate dehydrogenase was recognized by commercial antibody (sc-25778; Santa Cruz Biotechnology Inc). A goat anti-rabbit horseradish peroxidase-conjugated immunoglobulin G (Sigma-Aldrich) was used as secondary antibody. Protein complexes were detected by enhanced chemiluminescence (Amersham Life Sciences, Arlington Heights, IL) on photographic film (Kodak Biomax Light Film; Sigma-Aldrich). Protein concentrations in the catalytic and modulator subunits of glutamate-cysteine ligase were measured by band densitometry as a ratio with glyceraldehyde-3-phosphate dehydrogenase protein concentration (Model 45-700 Imaging Densitometer; Bio-Rad).

### Calculations

Isotopic enrichments were expressed as tracer-to-tracee ratio (TTR). Erythrocyte cysteine concentrations were measured in separate background samples by dividing internal standard concentration by internal standard enrichment and then normalizing by the erythrocyte volume obtained from hematocrit. Erythrocyte glutathione concentrations were measured in separate background samples by using a standard calibration curve in which fixed amounts of internal standard were added to variable known glutathione concentrations. Values of glutathione concentrations were normalized by erythrocyte volume ( $\mu\text{mol/L}$ ) obtained from hematocrit. Glutathione fractional synthesis rate (FSR; in %/d) was calculated as  $\{[E(^2\text{H}_2\text{glutathione})/t]/E(^2\text{H}_2\text{cysteine})\} \times 24 \times 100$ , where  $E(^2\text{H}_2\text{glutathione})/t$  is the slope (TTR/h) of the regression line describing the rise in erythrocyte  $^2\text{H}_2$ -glutathione enrichment (TTR) as a function of time (hours) over the last 2 h of isotope infusion, and  $E(^2\text{H}_2\text{cysteine})$  is the mean steady-state  $^2\text{H}_2$ -cysteine enrichment (TTR) in erythrocytes over the last 2 h of isotope infusion. Glutathione absolute synthesis rate ( $\mu\text{mol} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$ ) was calculated by multiplying glutathione FSR by erythrocyte glutathione concentration.

### Statistical analysis

All data were presented as means  $\pm$  SEMs. Baseline data on body weight, fat mass, fat-free mass, and vastus lateralis thickness of subjects from study A ( $n = 10$ ) and study B ( $n = 9$ ) were compared by unpaired  $t$  test. There were no significant differences in baseline data of subjects from study A and study B. Results of subjects from studies A and B, evaluated in ambulatory and bed-rest conditions, were analyzed with a repeated-measures analysis of variance (ANOVA), with activity (ambulatory and bed rest) as within-subject factor and group (studies A and B) as between-subject factor. There was no significant group (study A or study B)  $\times$  bed rest interaction for investigated variables.



To investigate the influence of energy balance on fat-free mass and muscle atrophy progression during 35 d of bed rest, pooled subjects from studies A and B ( $n = 19$ ) were divided in 2 groups by using the median value of individual changes in fat mass as threshold. The 10 subjects with fat mass gain greater than the median value were assigned to a group defined as having a higher energy balance (HEB<sub>AB</sub>; subscript letters indicate the study from which subjects were taken, with “<sub>AB</sub>” indicating that subjects were taken from both studies). Conversely, the 9 subjects with fat mass changes lower than the median value were assigned to a group defined as having a lower energy balance (LEB<sub>AB</sub>). There were no significant differences (unpaired *t* test) in baseline data (ie, body weight, fat mass, fat-free mass, and vastus lateralis thickness) in subjects from the HEB<sub>AB</sub> and LEB<sub>AB</sub> groups. Results from the 2 groups, studied in ambulatory and bed-rest conditions, were analyzed with a repeated-measures ANOVA, with activity (ambulatory and bed rest) as within-subject factor and group (higher and lower energy balance) as between-subject factor. Post hoc analysis was performed, when appropriate (ie, significant bed-rest effect and group  $\times$  bed rest interaction), by *t* test with Bonferroni’s adjustment to assess the effects of bed rest in either group.

To investigate the influence of energy balance on bed rest-mediated changes in inflammatory markers and erythrocyte glutathione turnover, subjects from study A ( $n = 10$ ) were divided in 2 groups by using the median value of individual changes in fat mass as threshold. Values of hormones, mediators, and substrates concentrations were included in the analysis after log transformation. Baseline data on subjects from higher (HEB<sub>A</sub>;  $n = 5$ ) or lower (LEB<sub>A</sub>;  $n = 5$ ) energy-balance groups were compared by using an unpaired *t* test. Results from the 2 groups, studied in ambulatory and bed-rest conditions, were analyzed by using a repeated-measures ANOVA, with activity (ambulatory or bed rest) as within-subject factor and group (higher or lower energy balance) as between-subject factor. Post hoc analysis was performed, when appropriate (ie, significant bed-rest effect and group  $\times$  bed rest interaction), by *t* test with Bonferroni’s adjustment to assess the effects of bed rest in either group. All comparisons were considered significant at the conventional  $P < 0.05$ . The relations between variables were analyzed by bivariate correlation using the Pearson’s coefficient. Statistical analysis was conducted with SPSS statistical software (version 12; SPSS Inc, Chicago, IL).

## RESULTS

### Body composition and muscle thickness (study A and study B)

Baseline values of body weight, fat mass, fat-free mass, and vastus lateralis thickness did not differ significantly (unpaired *t* test) between subjects from study A and those from study B. During 5 wk of bed rest, body weight decreased ( $P < 0.001$ , repeated-measures ANOVA) from  $76.5 \pm 2.9$  to  $75.4 \pm 2.8$  kg in study A and from  $76.3 \pm 3.7$  to  $73.9 \pm 3.5$  kg in study B; fat mass increased ( $P = 0.02$ , repeated-measures ANOVA) from  $10.2 \pm 1.6$  to  $11.9 \pm 1.6$  kg in study A and from  $13.6 \pm 2.1$  to  $14.4 \pm 2.3$  in study B; fat-free mass decreased ( $P < 0.001$ , RM-ANOVA) from  $66.3 \pm 2.1$  to  $63.4 \pm 1.9$  kg in study A and from  $62.7 \pm 2.2$  to  $59.4 \pm 1.9$  kg in study B; and thickness of vastus lateralis decreased ( $P = 0.02$ , RM-ANOVA) from  $2.39 \pm 0.09$  to  $2.20 \pm 0.09$  cm in study A and from  $2.09 \pm 0.19$  to  $1.84 \pm 0.17$  cm in study B. There were no significant group (study A or study B)  $\times$  bed rest interactions for body weight, fat mass, fat-free mass, or vastus lateralis thickness. In subjects from study A, the average thickness of the vastus lateralis, gastrocnemius medialis, tibialis anterior, and biceps brachii decreased significantly after bed rest (from  $2.39 \pm 0.09$  to  $2.20 \pm 0.09$  cm;  $P < 0.001$ , paired *t* test), and the decrease correlated directly with fat-free mass both before ( $r = 0.67$ ,  $P < 0.05$ ;  $n = 10$ ) and at the end of ( $r = 0.63$ ,  $P < 0.05$ ;  $n = 10$ ) the bed-rest period. Bed rest-induced percentage changes in fat-free mass and average muscle thickness were directly correlated ( $r = 0.63$ ,  $P < 0.05$ ;  $n = 10$ ).

To investigate the influence of energy balance on fat-free mass and muscle atrophy progression during 35 d of bed rest, pooled subjects from studies A and B were divided into 2 groups by using the median value of individual changes in fat mass (ie, 1.4 kg) as threshold. The 10 subjects (7 from study A and 3 from study B) with fat mass gain greater than the median value were assigned to a group defined as higher energy balance (HEB<sub>AB</sub>). Conversely, the 9 subjects (3 from study A and 6 from study B) with fat mass changes lower than the median value were assigned to a group defined as lower energy balance (LEB<sub>AB</sub>) (Table 1). Baseline body weight, fat mass, fat-free mass, and vastus lateralis thickness did not differ significantly between the 2 groups (Table 1). There

**TABLE 1**

Effects of bed rest at different energy intakes on body weight, fat-free mass, and vastus lateralis thickness<sup>1</sup>

|                       | HEB <sub>AB</sub> ( $n = 10$ ) <sup>2</sup> |                   | LEB <sub>AB</sub> ( $n = 9$ ) |                  | <i>P</i> <sup>3</sup> |                 |             |
|-----------------------|---|-------------------|-------------------------------|------------------|-----------------------|-----------------|-------------|
|                       | Ambulatory                                  | Bed rest          | Ambulatory                    | Bed rest         | Group effect          | Activity effect | Interaction |
| Body weight (kg)      | $80.0 \pm 3.5^4$                            | $78.4 \pm 3.3$    | $72.4 \pm 2.3$                | $70.5 \pm 2.2$   | 0.08                  | <0.01           | 0.66        |
| Fat-free mass (kg)    | $67.1 \pm 1.8$                              | $63.4 \pm 1.8^5$  | $61.8 \pm 2.3$                | $59.5 \pm 2.1^5$ | 0.11                  | <0.01           | 0.03        |
| Vastus lateralis (cm) | $2.43 \pm 0.13$                             | $2.10 \pm 0.13^5$ | $2.04 \pm 0.15$               | $1.95 \pm 0.15$  | 0.19                  | <0.001          | 0.01        |

<sup>1</sup> HEB<sub>AB</sub>, higher energy balance; LEB<sub>AB</sub>, lower energy balance. Fat-free mass and vastus lateralis thickness were measured by using bioelectrical impedance analysis and ultrasound imaging, respectively.

<sup>2</sup> Subjects with HEB<sub>AB</sub> or LEB<sub>AB</sub> were identified according to the median value of inactivity-induced fat mass (bioimpedance) changes (increase of 1.4 kg) after bed-rest study A (10 subjects were allowed to spontaneously adapt to decreased energy requirement) was combined with bed-rest study B (9 subjects were provided with an activity-adjusted diet). Baseline values of fat mass did not differ significantly between HEB<sub>AB</sub> ( $12.9 \pm 2.3$  kg) and LEB<sub>AB</sub> ( $10.6 \pm 1.2$  kg) groups. Changes in body fat in HEB<sub>AB</sub> and LEB<sub>AB</sub> groups averaged  $2.1 \pm 0.4$  and  $0.4 \pm 0.3$  kg, respectively.

<sup>3</sup> Data were analyzed with the use of a 2-factor (group  $\times$  activity) ANOVA.

<sup>4</sup>  $\bar{x} \pm$  SEM (all such values).

<sup>5</sup> Significantly different from the ambulatory adaptation condition,  $P < 0.025$  (Bonferroni’s post hoc analysis).

were significant bed-rest effects and group  $\times$  bed rest interactions for fat-free mass and vastus lateralis thickness. Bed rest-induced decreases in fat-free mass were greater in HEB<sub>AB</sub> ( $3.8 \pm 0.4$  kg) than in LEB<sub>AB</sub> ( $2.3 \pm 0.5$  kg). Bed rest-induced decreases in thickness of vastus lateralis were greater in HEB<sub>AB</sub> ( $0.32 \pm 0.04$  cm; ie,  $14 \pm 2\%$ ) than in LEB<sub>AB</sub> ( $0.09 \pm 0.04$  cm; ie,  $6 \pm 5\%$ ). There were significant bed-rest effects ( $P < 0.001$ ) and group  $\times$  bed rest interactions ( $P < 0.01$ ) for intracellular water, as determined by multifrequency BIA. Intracellular water decreased in HEB<sub>AB</sub> from  $32.4 \pm 1.0$  to  $30.6 \pm 1.0$  kg and in LEB<sub>AB</sub> from  $30.6 \pm 1.0$  to  $29.6 \pm 0.9$  kg. Bed rest-induced decreases in intracellular water were greater in HEB<sub>AB</sub> ( $1.9 \pm 0.2$  kg) than in LEB<sub>AB</sub> ( $1.0 \pm 0.2$  kg).

### Inflammatory markers and erythrocyte glutathione (study A)

To compare the influence of bed rest at different levels of energy balance on inflammatory markers and antioxidant defenses, subjects from study A were divided into 2 groups by using the median value of individual changes in fat mass as threshold (ie, 1.8 kg). The 5 subjects with fat mass gain greater than the median value were assigned to a group defined as HEB<sub>A</sub>. Conversely, the 5 subjects with fat mass changes lower than the median value were assigned to a group defined as LEB<sub>A</sub>. Baseline values of fat mass were not significantly different in the HEB<sub>A</sub> and LEB<sub>A</sub> groups. Before bed rest, absolute values of fat mass directly correlated with plasma leptin concentrations ( $r = 0.72$ ,  $P < 0.05$ ;  $n = 10$ ). There were significant bed-rest effects and group  $\times$  bed rest interactions for plasma leptin concentration. Leptin significantly increased after bed rest in the HEB group ( $105 \pm 19\%$ ) but did not change significantly in the LEB group (Table 2). Bed rest significantly increased plasma ghrelin and insulin concentrations, but there were no group  $\times$  bed rest interactions for ghrelin and insulin. Plasma glucose did not change significantly after bed rest in either group. There were significant group  $\times$  bed rest interactions for plasma CRP and myeloperoxidase concentrations,

as markers of systemic inflammation. Bed rest-mediated changes in CRP and myeloperoxidase concentrations in pooled subjects were directly correlated ( $r = 0.94$ ,  $P < 0.001$ ,  $n = 10$ ). Plasma total thiol group concentrations did not change significantly after bed rest in either group. Finally, there was a significant bed-rest effect in increasing triglyceride and LDL-cholesterol concentrations.

Bed-rest effects at different EIs on plasma and erythrocyte glutathione and glutathione precursor amino acid concentrations are shown in Table 3. Plasma glutathione and glutathione precursor amino acid concentrations did not change significantly after bed rest in the HEB or LEB group. We found a tendency ( $P = 0.06$ ) toward an effect of bed rest on glutathione and glycine concentrations in erythrocytes, but no significant changes were observed in the other amino acids. There was a significant ( $P < 0.01$ ) effect of bed rest in increasing hematocrit both in the HEB (from  $48 \pm 2\%$  to  $50 \pm 2\%$ ) and LEB (from  $48 \pm 2\%$  to  $51 \pm 2\%$ ) groups. However, there was no group  $\times$  bed rest interaction for hematocrit. Precursor  $^2\text{H}_2$ -cysteine enrichment was measured in erythrocytes and reached steady state in all groups and conditions by the end of the third hour of isotope infusion, whereas the  $^2\text{H}_2$  enrichment in erythrocyte glutathione increased linearly with time (Figure 1).

The effects of bed rest at different EIs on the erythrocyte glutathione system are shown in Table 4. Before bed rest, glutathione absolute synthesis rates directly correlated with cysteine concentrations in erythrocytes of pooled subjects ( $r = 0.74$ ,  $P < 0.05$ ;  $n = 10$ ). No significant correlations were found between absolute glutathione synthesis rates and glutamate ( $r = -0.13$ ;  $n = 10$ ) or glycine ( $r = 0.50$ ;  $n = 10$ ) concentrations. Bed rest tended to increase glutathione fractional and absolute synthesis rates in erythrocytes. There were significant group  $\times$  bed rest interactions for glutathione fractional and absolute synthesis rates in erythrocytes. Bed rest-mediated changes in glutathione fractional and absolute synthesis rates in erythrocytes were greater in the HEB<sub>A</sub> group ( $94 \pm 18\%$  and  $215 \pm 42\%$ , respectively) than in the LEB<sub>A</sub> group ( $-28 \pm 37\%$  and  $-40 \pm 88\%$ ,

**TABLE 2**  
Effects of bed rest at different energy intakes on plasma hormone, mediator, and substrate concentrations<sup>1</sup>

|                                     | HEB <sub>A</sub> <sup>2</sup> (n = 5) |                 | LEB <sub>A</sub> <sup>2</sup> (n = 5) |                | P <sup>3</sup> |                 |             |
|-------------------------------------|---------------------------------------|-----------------|---------------------------------------|----------------|----------------|-----------------|-------------|
|                                     | Ambulatory                            | Bed rest        | Ambulatory                            | Bed rest       | Group effect   | Activity effect | Interaction |
| Leptin (ng/mL)                      | $3.4 \pm 1.2^4$                       | $6.9 \pm 2.6^5$ | $2.1 \pm 0.6$                         | $3.2 \pm 1.2$  | 0.51           | 0.01            | 0.04        |
| Ghrelin (ng/mL)                     | $986 \pm 79$                          | $766 \pm 39$    | $1083 \pm 212$                        | $929 \pm 119$  | 0.52           | 0.004           | 0.21        |
| Insulin ( $\mu\text{U}/\text{mL}$ ) | $8.1 \pm 1.2$                         | $12.0 \pm 1.7$  | $8.1 \pm 0.8$                         | $11.2 \pm 2.3$ | 0.77           | 0.05            | 0.65        |
| Glucose (mg/dL)                     | $88 \pm 1.2$                          | $89 \pm 2$      | $92 \pm 3$                            | $87 \pm 3$     | 0.59           | 0.41            | 0.32        |
| CRP ( $\mu\text{g}/\text{mL}$ )     | $0.4 \pm 0.2$                         | $0.8 \pm 0.5$   | $1.5 \pm 0.6$                         | $0.7 \pm 0.2$  | 0.32           | 0.35            | 0.02        |
| Myeloperoxidase (ng/mL)             | $327 \pm 18$                          | $381 \pm 25$    | $345 \pm 25$                          | $326 \pm 22$   | 0.54           | 0.21            | 0.02        |
| Total -SH (pmol/g protein)          | $3.7 \pm 1.0$                         | $3.2 \pm 0.5$   | $4.1 \pm 0.7$                         | $4.3 \pm 0.8$  | 0.41           | 0.79            | 0.60        |
| Triglycerides (mg/dL)               | $71 \pm 10$                           | $100 \pm 37$    | $98 \pm 18$                           | $158 \pm 30$   | 0.15           | 0.01            | 0.27        |
| HDL cholesterol (mg/dL)             | $43 \pm 2$                            | $40 \pm 2$      | $45 \pm 3$                            | $40 \pm 2$     | 0.78           | 0.09            | 0.58        |
| LDL cholesterol (mg/dL)             | $94 \pm 6$                            | $120 \pm 7$     | $104 \pm 7$                           | $126 \pm 8$    | 0.27           | 0.007           | 0.61        |

<sup>1</sup> HEB<sub>A</sub>, higher energy balance; LEB<sub>A</sub>, lower energy balance; CRP, C-reactive protein; -SH, thiol function.

<sup>2</sup> Subjects with HEB<sub>A</sub> or LEB<sub>A</sub> were identified according to the median value of inactivity-induced fat mass (bioimpedance) changes (increase of 1.8 kg) in bed-rest study A (10 subjects were allowed to spontaneously adapt to decreased energy requirement). Baseline values of fat mass did not differ significantly between HEB<sub>A</sub> ( $11.6 \pm 2.6$  kg) and LEB<sub>A</sub> ( $8.8 \pm 1.6$  kg) groups. Changes in body fat in the HEB<sub>A</sub> and LEB<sub>A</sub> groups averaged  $2.6 \pm 0.3$  and  $1.0 \pm 0.5$  kg, respectively.

<sup>3</sup> Data were analyzed after log-transformation by using a 2-factor (group  $\times$  activity) ANOVA with interaction.

<sup>4</sup>  $\bar{x} \pm \text{SEM}$  (all such values).

<sup>5</sup> Significantly different from the ambulatory adaptation condition,  $P < 0.025$  (Bonferroni's post hoc analysis).

TABLE 3

Effects of bed rest at different energy intakes on plasma and erythrocyte glutathione and glutathione precursor amino acid concentrations<sup>1</sup>

|                      | HEB <sub>A</sub> <sup>2</sup> (n = 5) |            | LEB <sub>A</sub> <sup>2</sup> (n = 5) |            | P <sup>3</sup> |                 |             |
|----------------------|---------------------------------------|------------|---------------------------------------|------------|----------------|-----------------|-------------|
|                      | Ambulatory                            | Bed rest   | Ambulatory                            | Bed rest   | Group effect   | Activity effect | Interaction |
| Plasma (μmol/L)      |                                       |            |                                       |            |                |                 |             |
| Glutathione          | 24 ± 3 <sup>4</sup>                   | 23 ± 4     | 29 ± 2                                | 27 ± 1     | 0.16           | 0.61            | 0.76        |
| Glycine              | 240 ± 23                              | 240 ± 18   | 242 ± 16                              | 217 ± 12   | 0.63           | 0.40            | 0.42        |
| Glutamine            | 501 ± 33                              | 562 ± 17   | 514 ± 35                              | 487 ± 31   | 0.25           | 0.64            | 0.24        |
| Glutamate            | 88 ± 6                                | 86 ± 9     | 82 ± 4                                | 85 ± 15    | 0.75           | 0.99            | 0.80        |
| Methionine           | 25 ± 2                                | 27 ± 2     | 27 ± 2                                | 25 ± 2     | 0.93           | 0.90            | 0.15        |
| Homocysteine         | 18 ± 2                                | 18 ± 1     | 13 ± 2                                | 12 ± 2     | 0.06           | 0.17            | 0.69        |
| Cysteine             | 27 ± 1                                | 26 ± 1     | 27 ± 2                                | 25 ± 2     | 0.96           | 0.22            | 0.47        |
| Erythrocyte (μmol/L) |                                       |            |                                       |            |                |                 |             |
| Glutathione          | 2152 ± 209                            | 2254 ± 115 | 2160 ± 85                             | 2394 ± 131 | 0.70           | 0.06            | 0.42        |
| Glycine              | 404 ± 22                              | 396 ± 16   | 451 ± 29                              | 363 ± 23   | 0.78           | 0.06            | 0.11        |
| Glutamine            | 475 ± 33                              | 483 ± 10   | 496 ± 22                              | 451 ± 32   | 0.88           | 0.23            | 0.09        |
| Glutamate            | 458 ± 74                              | 443 ± 75   | 420 ± 17                              | 462 ± 57   | 0.90           | 0.67            | 0.37        |
| Methionine           | 25 ± 3                                | 29 ± 3     | 28 ± 2                                | 29 ± 2     | 0.62           | 0.24            | 0.52        |
| Cysteine             | 67 ± 13                               | 75 ± 13    | 69 ± 16                               | 66 ± 11    | 0.82           | 0.80            | 0.64        |

<sup>1</sup> HEB<sub>A</sub>, higher energy balance; LEB<sub>A</sub>, lower energy balance.<sup>2</sup> Subjects with HEB<sub>A</sub> or LEB<sub>A</sub> were identified according to the median value of inactivity-induced fat mass (bioimpedance) changes (increase in 1.8 kg) in bed-rest study A (10 subjects were allowed to spontaneously adapt to decreased energy requirement). Baseline values of fat mass did not differ significantly between HEB<sub>A</sub> (11.6 ± 2.6 kg) and LEB<sub>A</sub> (8.8 ± 1.6 kg) groups. Changes in body fat in HEB<sub>A</sub> and LEB<sub>A</sub> groups averaged 2.6 ± 0.3 and 1.0 ± 0.5 kg, respectively.<sup>3</sup> Data were analyzed by using a 2-factor (group × diet) ANOVA with interaction.<sup>4</sup>  $\bar{x} \pm \text{SEM}$  (all such values).

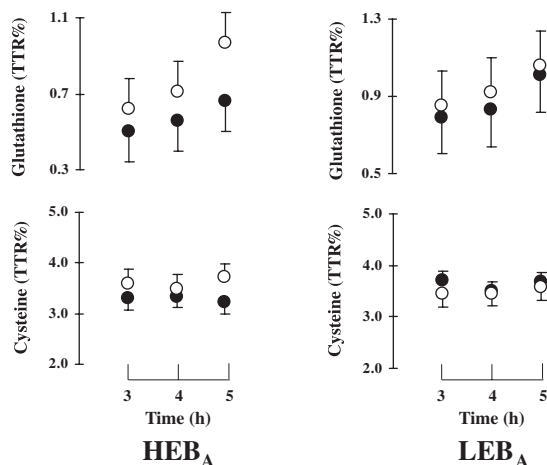
respectively). There was a tendency toward a group × bed rest interaction for the effects of bed rest at different EIs on the catalytic subunit for glutamate cysteine ligase, the key enzyme for glutathione synthesis; however, this trend was not statistically significant. Bed rest-mediated changes in the catalytic subunit for glutamate cysteine ligase tended to be greater in the HEB<sub>A</sub> group (96 ± 65%) than in the LEB<sub>A</sub> group (−10 ± 35%). Bed rest at different EIs did not significantly affect glutathione peroxidase activity.

## DISCUSSION

In healthy humans, physical inactivity is frequently associated with overfeeding and with increased fat mass. We monitored body composition and thickness of the vastus lateralis muscle in healthy volunteers during 35 d of bed rest at different EIs. Most of the subjects accumulated body fat to various degrees. Those who gained more fat suffered the greatest loss of skeletal muscle and fat-free mass. Positive energy balance and muscle atrophy were associated with activation of the systemic inflammatory response and antioxidant defenses.

We used 2 independent methods to assess the effects of bed rest at various levels of energy balance on skeletal muscle and fat-free mass. BIA is a validated method for assessing fat mass and fat-free mass in healthy persons; the latter measure is a close marker of whole-body muscle mass (22). Muscle atrophy assessment was directly performed by ultrasonography measurement of muscle thickness. This is a validated technique for assessing muscle size (26, 33). Thickness of the vastus lateralis, a key postural and locomotor muscle, and total-body fat-free mass decreased after bed rest in subjects with greater increases in body fat more than it decreased in those with smaller changes in body fat. The average thickness of representative postural (vastus lateralis, gastrocnemius medialis, and tibialis anterior) and nonpostural (biceps brachii) muscles correlated directly with whole-body fat-free mass, as determined by BIA, both before and at the end of the bed-rest period.

Glutathione plays a major role in cellular defenses against oxidative stress (20). Higher concentrations are present in erythrocytes, providing both local and systemic antioxidant protection (21). Different components of glutathione system in erythrocytes were considered. Glutathione synthetic capacity was assessed by



**FIGURE 1.** Mean ± SEM precursor <sup>2</sup>H<sub>2</sub>-cysteine and product <sup>2</sup>H<sub>2</sub>-glutathione tracer-to-tracee ratios (TTRs) in erythrocytes during primed continuous infusion of <sup>2</sup>H<sub>2</sub>-cysteine, before (●) and at the end of (○) 35-d bed rest, in the higher-energy-balance (HEB<sub>A</sub>, n = 5) and lower-energy-balance (LEB<sub>A</sub>, n = 5) groups.

**TABLE 4**Effects of bed rest at different energy intakes on glutathione status in erythrocytes<sup>1</sup>

|  | HEB <sub>A</sub> <sup>2</sup> (n = 5) |                       | LEB <sub>A</sub> <sup>2</sup> (n = 5) |             | P <sup>3</sup> |                 |             |
|--|---------------------------------------|-----------------------|---------------------------------------|-------------|----------------|-----------------|-------------|
|  | Ambulatory                            | Bed rest              | Ambulatory                            | Bed rest    | Group effect   | Activity effect | Interaction |
| Glutathione FSR (%/d)  | 70 ± 19 <sup>4</sup>                  | 164 ± 29 <sup>5</sup> | 103 ± 23                              | 84 ± 27     | 0.43           | 0.13            | 0.03        |
| Glutathione ASR (mmol · L <sup>-1</sup> · d <sup>-1</sup> )        | 160 ± 58                              | 376 ± 75 <sup>5</sup> | 217 ± 43                              | 197 ± 62    | 0.40           | 0.09            | 0.05        |
| GCL-C (fraction of GAPDH)  | 0.95 ± 0.23                           | 1.36 ± 0.15           | 1.73 ± 0.57                           | 1.01 ± 0.28 | 0.58           | 0.63            | 0.10        |
| GCL-M (fraction of GAPDH)  | 0.42 ± 0.11                           | 0.48 ± 0.13           | 0.57 ± 0.10                           | 0.63 ± 0.21 | 0.36           | 0.68            | 0.99        |
| GPX activity (μmol · min <sup>-1</sup> · g protein <sup>-1</sup> ) | 1.04 ± 0.13                           | 1.01 ± 0.31           | 1.18 ± 0.54                           | 1.46 ± 0.32 | 0.48           | 0.71            | 0.65        |

<sup>1</sup> HEB<sub>A</sub>, higher energy balance; LEB<sub>A</sub>, lower energy balance; FSR, fractional synthesis rate; ASR, absolute synthesis rate; GCL-C, glutamate cysteine ligase–catalytic subunit; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCL-M, glutamate cysteine ligase–modulator subunit; GPX, glutathione peroxidase.

<sup>2</sup> Subjects with HEB<sub>A</sub> or LEB<sub>A</sub> were identified according to median value of inactivity-induced fat mass (bioimpedance) changes (increase of 1.8 kg) in bed-rest study A (10 subjects were allowed to spontaneously adapt to decreased energy requirement). Baseline values of fat mass did not differ significantly between HEB<sub>A</sub> (11.6 ± 2.6 kg) and LEB<sub>A</sub> (8.8 ± 1.6 kg) groups. Changes in body fat in HEB<sub>A</sub> and LEB<sub>A</sub> groups averaged 2.6 ± 0.3 and 1.0 ± 0.5 kg, respectively.

<sup>3</sup> Data were analyzed by using a 2-factor (group × activity) ANOVA with interaction.

<sup>4</sup>  $\bar{x} \pm \text{SEM}$  (all such values).

<sup>5</sup> Significantly different from the ambulatory condition,  $P < 0.025$  (Bonferroni's post hoc analysis).

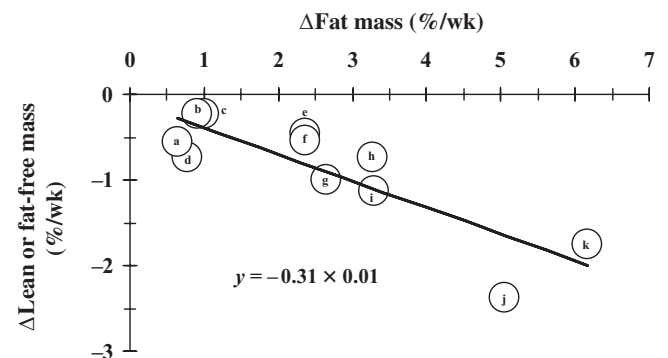
Western blot determination of expression levels of catalytic and modulatory subunits of the glutamate-cysteine ligase enzyme, which catalyzes the rate-limiting step in glutathione biosynthesis (32). The actual rate of glutathione turnover was determined by using a primed continuous infusion of deuterated cysteine and by monitoring incorporation of the labeled precursor into glutathione by MS analysis (24), whereas glutathione function was evaluated through measurement of glutathione peroxidase activity (31). We found group × bed rest interactions for the catalytic subunit of the glutamate-cysteine ligase enzyme and the rate of glutathione turnover leading to greater bed rest-mediated increases in glutathione synthesis in the HEB than in the LEB group. Glutathione peroxidase activity did not change in the 2 groups. Our results indicate that the erythrocyte glutathione system is greatly activated during bed rest at positive energy balance, whereas bed rest at near-neutral balance is not associated with significant changes in glutathione synthesis.

Evidence indicates that glutathione synthesis is up-regulated by proinflammatory mediators and oxidative stress (20). We found significant group × bed rest interactions for plasma CRP and myeloperoxidase concentrations, which led to greater bed rest-mediated increases of these mediators in the HEB group than in the LEB group. Plasma CRP and myeloperoxidase are suitable markers of activation of systemic inflammation (34). These results strongly suggest that an activation of the erythrocyte glutathione system is part of the systemic inflammatory and stress reaction to bed rest at positive energy balance. Inflammatory response and redox unbalance could mediate lean body mass catabolism during bed rest at positive energy balance (17–19). In addition, an increased ghrelin response after a period of over-feeding (35) could contribute to an enhancement of muscle catabolism (36).

One limitation of the present study is that, typically, only a small number of persons can be enrolled in bed-rest studies. The effect of positive energy balance on inactivity-induced muscle atrophy was assessed by combining the results of 2 bed-rest studies, conducted under the same experimental conditions, to achieve a sample size of 19 subjects. In contrast, energy level × bed rest interactions for inflammatory markers and glutathione kinetics were assessed in 2 groups of only 5 subjects. Although

extending these observations in a larger population would reinforce the present findings, but we were able to detect significant differences in key markers of inflammatory response and glutathione kinetics.

Positive energy balance (ie, EI in excess of requirements) leads to fat deposition, and changes in body fat mass closely reflect energy balance over the long term, even in the presence of atrophying muscles. In fact, energy density of fat tissue is 9 times that of muscle and fat-free mass (37). Our study A subjects were allowed to spontaneously adapt to decreased energy requirement during bed rest, whereas study B subjects were provided an activity-adjusted diet (1.2 times their calculated resting energy expenditure). At the end of the bed-rest periods, 9 of 10 study A subjects and 5 of 9 study B participants had gained >0.5 kg fat mass. Thus, study A subjects failed to spontaneously adapt to a lower energy requirement, whereas, in study B, an activity-adjusted diet based on traditional predictive equations (22, 38)



**FIGURE 2.** Relation between average values of absolute changes in fat mass and lean mass (by dual-energy X-ray absorptiometry) or fat-free mass (by bioelectrical impedance analysis) in previous studies and in the present study. Letters inside (or just outside of) circles represent values from these studies: a, Barbe et al (10); b, Scheld et al (13); c, Stein et al (12); d, present study, lower-energy-balance group; e, Blanc et al (6); f, Krebs et al (7); g, Lovejoy et al (11); h, Gretebeck et al (8); i, present study, higher-energy-balance group; j, Ferrando et al (9); and k, Olsen et al (5).  $r = -0.85$ ,  $P = 0.001$ ;  $n = 11$ .



overestimated the true requirement for most subjects. Achievement of energy balance during experimental bed rest is a difficult task that involves constant monitoring of individual energy requirements and intakes, and that goal has rarely been attained in previous investigations. Results obtained in the present study and in 9 previously reported bed-rest studies that found positive mean changes in fat mass (6–14) are shown in **Figure 2**. Changes in fat mass are related to changes in lean body mass (by dual-energy X-ray absorptiometry) or fat-free mass (by BIA). A gain of body fat and a loss of lean (fat-free) mass are expressed as weekly percentage changes from baseline. Linear regression analysis indicates an indirect relation between fat gain and lean (fat-free) mass loss, regardless of the duration of inactivity (Figure 2). Similar results are obtained by excluding the present study from the analysis ( $r = -0.97$ ). Thus, there is excellent agreement between the retrospective analysis (6–14; Figure 2) and the results from the present study, which suggest that, when EI is not strictly controlled during bed rest, fat mass tends to increase in parallel with lean body mass catabolism.

The present study shows that excess fat deposition during physical inactivity is associated with greater muscle loss and greater activation of systemic inflammation and antioxidant defenses. These mechanisms potentially contribute to long-term changes in body composition and to the cardiometabolic risk observed in sedentary persons (39, 40).

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The authors' responsibilities were as follows—GB: design of the experiment, collection and analysis of data, and writing of the manuscript; FA: design of the experiment, collection and analysis of data, and writing of the manuscript; BS: design of the experiment and collection of data; MS: collection and analysis of data; LT: design of the experiment and statistical analysis of data; JCP: collection and analysis of data; GD-D: collection and analysis of data; PM, analysis of data; FS: collection and analysis of data; PdP: design of the experiment; GG: design of the experiment; IBM: design of the experiment and collection and analysis of data; MVN: design of the experiment and collection and analysis of data; and RP: design of the experiment, collection and analysis of data, and writing of the manuscript. None of the authors had a personal or financial conflict of interest.

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