Large-scale mRNA analysis of female skeletal muscles during 60 days of bed rest with and without exercise or dietary protein supplementation as countermeasures

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Submitted 20 February 2009; accepted in final form 21 May 2009

Chopard A, Lecunff M, Danger R, Lamirault G, Bihouee A, Teusan R, Jasmin BJ, Marini JF, Leger JJ. Large-scale mRNA analysis of female skeletal muscles during 60 days of bed rest on the transcriptome of soleus (SOL) and vastus lateralis (VL) muscles in healthy women (BRC group, n = 8), and the potential beneficial impact of protein supplementation (BRN group, n = 8) and of a combined resistance and aerobic training (BRE group, n = 8). Gene expression profiles were obtained using a customized microarray containing 6,681 muscles-relevant genes. A two-class statistical analysis was applied on 2,103 genes with consolidated expression in BRC, BRN, and BRE groups. We identified 472 and 207 mRNAs whose expression was modified in SOL and VL from BRC group, respectively. Further clustering analysis, identifying relevant biological mechanisms and pathways, reported five main subclusters. Three are composed of upregulated mRNAs involved mainly in nucleic acid and protein metabolism, and two made up of downregulated transcripts encoding components involved in energy metabolism. Exercise countermeasure demonstrated drastic compensatory effects, decreasing the number of differentially expressed mRNAs by 89 and 96% in SOL and VL, respectively. In contrast, nutrition countermeasure had moderate effects and decreased the number of differentially-expressed transcripts by 40 and 25% in SOL and VL. Together, these data present a systematic, global and comprehensive view of the adaptive response of female muscle to long-term atrophy.

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Using microarray analysis, the first aim of the present study was to define global changes that occur in the muscle transcriptome during a simulated space mission by 60 days of antithorostatic bed rest with healthy female volunteers. The second objective was to evaluate the potential impact of two types of countermeasures: a combined aerobic and maximal resistance exercise program and a dietary protein supplement.

MATERIALS AND METHODS

Subjects. Twenty-four healthy young European women voluntarily participated in the long-term bed rest (LTBR) study named WISE 2005 (Women International Space Simulation for Exploration). The subjects had no medical history or physical signs of neuromuscular disorder and did not regularly participate in training exercise. Eight subjects were randomly assigned to each of 3 experimental groups: those who performed BR only (BRC), those who performed BR and exercise countermeasure (BRE), and those who performed BR and nutritional countermeasure (BRN). Slight statistical differences were observed in subject age, height, and weight between BRC and BRN groups (Table 1). All subjects gave informed consent to the procedures of the experimentation, which was approved by the local Ethics Committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale, Toulouse).

Long-term bed rest. Two sessions of LTBR experiments were successively conducted in the Medes-IMPS (Space Clinic, Rangueil Hospital, Toulouse, France) under the aegis of the European Space Agency (ESA), the National Aeronautics and Space Administration of the USA (NASA), the Canadian Space Agency (CSA), and France’s Centre National d’Etudes Spatiales (CNES). All subjects completed a period of BR for 60 days with a 20 day ambulatory control period before BR and a 20 day recovery period after BR. Subjects remained in a head-down tilt (–6°) position, even during the exercise training, and were instructed to not produce any unnecessary movements with their limbs. They were supervised by the Medes nursing staff throughout the study, and nutritional care was taken to avoid significant changes in body mass.

Exercise as countermeasure (BRE group). Subjects from the BRE group performed two types of exercise: 1) 40 min of aerobic exercise at 40–80% pre-BR VO2 peak, using the lower body negative pressure (85); and 2) 19 sessions of resistance exercise, using supine squat and calf press exercises on an inertial ergometer (3). These two types of exercise were never performed in the same day. Detailed conditions and timing are described in Supplemental Method S1.

Nutrition as countermeasure (BRN group). Protein content in the diet was 1 g per kg body wt per day in the BRC and BRE groups. In the BRN group, dietary protein content was increased to 1.45 g per kg body wt per day. Free branched chain amino acids (3.6 g/day free leucine, 1.8 g/day free isoleucine, and 1.8 g/day free valine) were added as a supplement (Frliver, Bracco, Italy) and given during main meals to attenuate nitrogen loss during BR (73). Leucine content in the diet was hereby increased from the natural level of 8–20% of total amino acid content in dietary protein. Finally, total daily energy was achieved by proportionally decreasing carbohydrate and fat intake.

Tissue sampling procedures. Eight days before the BR period, biopsies were obtained from the right vastus lateralis muscle (VL), with the subjects in a supine position, and from the right soleus muscle (SOL), with the subjects in a prone position, under local anesthesia using the procedure described by Bergstrom (13). Post-BR biopsies were obtained from the same leg, in a location as close as possible to the pre-BR biopsies, 2 days before reembalization i.e., on day 59 of the BR period. All available muscle biopsies were placed on saline-soaked gauze and divided into several portions. A muscle portion (~20 mg) was rapidly frozen in liquid nitrogen and stored at −80°C until future use in microarray experiments.

Twenty-four pre-BR SOL and 24 pre-BR VL muscle biopsies could be obtained from the eight subjects present in each group from both sessions (see Supplemental Method S2 for experimental design). Due to technical problems related to the biopsy procedure (insufficient tissue sampling, low RNA quality, and subject biopsy refusal), a few biopsies were not available for microarray hybridizations. In total, 18 and 23 pre-BR biopsies, in SOL and VL, respectively, were processed for microarray hybridizations. Six post-BR biopsies of each muscle were obtained for the BRC group, while seven post-BR biopsies were obtained for the BRN group, and five SOL and seven VL post-BR biopsies for the BRE group. Post-BR biopsies were issued from both sessions in the same proportion and represented 80% of the theoretical available biopsies (38 out 48).

Microarrays. The 6,681 human genes represented by 50-mer oligonucleotide probes spotted in triplicates on the microarrays had been selected for involvement in skeletal muscle and/or cardiovascular normal and pathological functioning (see for details on “myochips”: http://cardioserve.nantes.inserm.fr/ptf-puce/spip.php?article59/). Gene selection was based on 1) subtractive hybridization experiment (71), 2) literature data, and later 3) genome-wide microarray screening (see Supplemental Method S3). Microarray preparations and hybridizations, expression data acquisitions and initial processing, and PCR controls are described in Supplemental Method S3. Each pre- and post-BR biopsy was compared with a common reference sample consisting of a pool of equal quantities of mRNA either from the 18 pre-BR SOL or from the 23 pre-BR VL biopsies. These mRNA pools were used as a common point of measurement that enabled the comparison of individual mRNA samples within one muscle type.

Selection of genes of interest: a combined statistical and clustering approach. All pre-BR biopsies, if correctly documented, were used as reference in any statistical procedures. MAD (median absolute deviation) (18) was used to estimate the relative within group data spread consisting of a pool of equal quantities of mRNA either from the 18 pre-BR SOL or from the 23 pre-BR VL biopsies. These mRNA pools were used as a common point of measurement that enabled the comparison of individual mRNA samples within one muscle type.

Significance analysis of microarrays (SAM) (82) was applied to identify transcripts differentially expressed before and after BR, with and without countermeasure. With this method, each gene is first assigned a score on the basis of a modified t-test, and genes with scores greater than a user-defined threshold are selected. Repeated random sample permutations are used to estimate the percentage of...
genes identified by chance (false discovery rate). For each analysis, we arbitrarily fixed the threshold of statistical significance so that the false-discovery rate was <0.5% (corresponding to less than one falsely discovered gene out of 200 selected genes). A score of 0.2, positive if upregulated and negative if downregulated, was arbitrarily given to each transcript differentially selected in one of the six SAM procedures.

Unsupervised hierarchical clustering was applied to the different median-centered gene data sets using the Pearson correlation as a similarity metric and average linkage clustering. Gene clusters were selected using 10 and 0.6 as minimal gene number and minimal correlation respectively. Clustering obtained with genes from pre- and post-BR biopsies in BRC SOL was arbitrarily applied as a reference classification. Each gene data set was independently median-centered in a gene-by-gene fashion, enabling the comparison of any gene subclusters from SOL and VL of BRC, BRN, and BRE groups. Clustering results were subsequently visualized using TreeView (30).

Each of the 6,681 genes spotted on the microarrays has its particular GO definition (19), indicating its potential functional connection with other genes. GoMiner software (92) identifies functional categories that were over- or underrepresented in specific gene subclusters. The cut-off was defined as $P < 0.05$ after correction for multiple hypothesis testing using the false discovery rate. These GoMiner selected genes finally appeared to be implied in some particular GO clusters, concomitantly with other genes identified within the same expression subcluster. A score of 0.2, positive if upregulated and negative if downregulated, was arbitrarily given to each gene significantly selected as a result of the GoMiner procedure.

To finely characterize subclusters of genes of interest, smoothing procedures were independently applied to $P$ values from the classical $t$-tests, and each score obtained from SAM and GoMiner analysis. For each gene, an average value of the $P$ value and of the SAM and GoMiner scores was calculated using a sliding window (±15 proximal genes) all along the gene classification of SOL BRC group. It allowed us to design the local density of genes of interest all along the hierarchical classification. A cumulative representation of the average density values following the three smoothing procedures was used to visualize the coincidence between the three approaches for selecting and characterizing genes of interest (Fig. 1).

Ingenuity Pathways Analysis software (IPA: http://www.ingenuity.com/), which builds dynamic networks and pathway models, was applied to a few gene subclusters. Thus enabling the delivery of a rapid assessment of the signaling and metabolic pathways, molecular networks, and biological processes that are most significantly perturbed in the dataset of interest (Fig. 2).
Network 1: Protein synthesis, RNA damage and repair.

Network 2: Fatty acid metabolism, protein degradation.

Network 3: Cell death, cytoskeleton and ECM remodeling.
RESULTS

Experimental design and global expression profiles. Biopsies of female SOL and VL muscles were obtained before and after LTBR allowing us to study the global changes in mRNA expression during 60 days of hypokinesia and hypodynamia and evaluate the effect of countermeasures. SOL and VL mRNAs from muscle biopsies were analyzed by hybridization against mRNAs pooled from pre-BR SOL and VL, respectively, on replicate microarrays containing triplicate 50-mer oligonucleotides corresponding to 6,681 striated muscle-related genes. According to the experimental protocol (see Supplemental Method S2), gene expression profiles should have been obtained for 96 distinct muscle biopsies corresponding to pre- and post-BR SOL and VL biopsies from BRC, BRN, and BRE groups. Because of ethical or technical problems, 79 biopsies (18 and 23 pre-BR biopsies, and 18 and 20 post-BR biopsies, from each SOL and VL, respectively) were analyzed.

Six distinct data sets of gene expression values were obtained from SOL and VL in BRC, BRE, and BRN groups. This resulted in consolidated gene expression values for 2,103 genes in the six situations (Linking to GEO accessions: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14798).

 Means of the gene expression MADs, within each of the pre-BR SOL and VL, and each of the six post-BR data sets, ranged from 0.10 to 0.12 in terms of expression variations between individuals. We considered 95% of the genes in each population, the expression variation of which was within one standard deviation of their MAD average, as stable. The 5% remaining genes were annotated as within group “variant” genes. “Stable & variant” genes will be found distributed within or out of expression clusters (see statistical landmark A in Supplemental Method S3 and in Supplemental Table S1).

Alterations in mRNA expression in SOL and VL after 60 days of bed rest. Unsupervised hierarchical clustering was independently applied to each data set median-centered on 2,103 genes expressed in all available biopsies from the BRC group. For both muscle types, the clustering revealed a clear-cut partitioning between pre- and post-BR biopsies (Fig. 1, top), indicating drastic changes in the transcript profile after 60 days of BR in SOL and VL. It was also observed that biopsies from both sessions and evaluated independently mixed well in both branches of the biopsy clusters (details not shown). This validated the experimental procedures and highlights the reproducibility of this type of experiments involving human biopsies. Figure 1 shows a comparative representation of gene clusters obtained for SOL and VL in BRC group. This gene classification was deduced from the clustering obtained with the gene data set from pre- and post-BR SOL biopsies from BRC group, and was arbitrarily taken as a reference of gene classification for any other clusters (Fig. 1, left). This parallel representation of identical gene clusters in the BRC groups clearly showed that the two main subject subclusters seen in SOL and VL resulted from two main gene subclusters, composed of up- (in red) or downregulated (in green) transcripts (Fig. 1).

Statistical comparative methods, followed by analysis of ontology and/or function of groups of genes, were applied on SOL and VL data sets to identify and characterize genes of interest, i.e., mRNAs whose expression is modified by LTBR. From the 2,103 genes selected with consolidated expression values, 472 transcripts were modified in SOL, compared with 207 in VL (Table 2A). The quantitative difference between SOL and VL was further shown by the three different smoothing procedures (Fig. 1). Many more peaks and a greater global area below the curve of smoothed P values obtained gene-by-gene (in blue in Fig. 1) are clearly visible in SOL compared with VL. Among these 472 and 207 modified transcripts, 124 are modified in SOL and VL (Table 2A). Such genes of interest, common to SOL and VL, are constitutive of the few peaks that are similarly located in BRC group, all along the cluster classification (Fig. 1).

Parallel to the higher number of modified transcripts in SOL compared with VL, the global subject and gene clustering for SOL and VL in the BRC group showed similar tendencies in terms of global changes in mRNA expression (Fig. 1). Red and black peaks in the same graphs indicate local densities of SAM-detected differentially-expressed transcripts and GoMiner-detected functionally related genes, which often coincided with previous P value peaks (blue peaks). This global analysis allowed the association of different subsets of clustered genes with specific functions or pathways.

Main modified functions following 60 days of bed rest. According to the gene expression clustering, GO, and Ingenuity ontological analyses, five main subclusters (Fig. 1, right: A, B, C, D, and E) of 10 to 50 modified transcripts were identified following BR, either commonly or specific to SOL and VL (Fig. 1). These expression subclusters include transcripts with fine regulation ranging within two- to fivefold. This moderate influence in mRNA expression is consistent with the fact that the muscle phenotype is indeed modified but that essential muscle functions remain intact during atrophy. Some of these fine changes in mRNA expression were verified by quantitative PCR (Supplemental Method S3). All observed changes in mRNA expression in SOL and VL following 60 days of bed rest and effects of countermeasures (see later), actually involved several muscle functions. Supplemental Table S1 summarizes several modified function clusters, including examples of up- or downregulated genes and the relative P values, the

Fig. 2. Examples of 3 functional networks arising from the main subclusters shown in Fig. 1. Modified transcripts following 60 days of bed rest (BRC group) and effects of nutrition (BRN) and exercise (BRE) countermeasures are illustrated. In red, upregulated mRNAs; in green, downregulated mRNAs. Network 1: transcripts mainly upregulated in BRC group, partially corrected with nutrition (BRN), and well corrected with exercise (BRE) countermeasures. Modified transcripts involved in protein synthesis and degradation pathways, RNA damage and repair (From Fig. 1, subcluster B). For example: few mRNAs encoding for proteasome system (e.g., PSMD1, PSMD5), several mRNAs encoding 60S ribosomal proteins (e.g., RPL), and 40S ribosomal proteins (e.g., RPS), as well as NCOA1 (nuclear receptor coactivator 1), HRNP2 (heterogeneous nuclear ribonucleoprotein D/AU-rich element RNA binding protein 1/AUF1), Network 2: transcripts mainly downregulated in BRC group and well corrected in either BRN or BRE groups. Modified transcripts involved in fatty acid metabolism (from Fig. 1, subcluster E), e.g., acyl-CoA dehydrogenases (ACADM, ACADL, ACADVL) and protein degradation, e.g., caspase 3 (CASP3). Network 3: transcripts mainly upregulated in SOL from BRC group, and non- or partially corrected in BRN or in BRE. Modified transcripts involved in cell death, cytoskeleton, and extracellular matrix remodeling (from Fig. 1, subcluster C), for example, the increased expression of tubulin (TUB) and collagen type I (COL1A1, COL1A2) and III (COL3A1) mRNAs exclusively in postural soleus, and not corrected by any countermeasures.
Table 2. Two-class SAM analysis of post-BR versus pre-BR biopsies in the six experimental situations

<table>
<thead>
<tr>
<th>Subcluster</th>
<th>Specific</th>
<th>Differential</th>
<th>Total</th>
<th>Common</th>
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<tbody>
<tr>
<td>SOL</td>
<td>348</td>
<td>472</td>
<td>124</td>
<td></td>
</tr>
<tr>
<td>VL</td>
<td>83</td>
<td>207</td>
<td>124</td>
<td></td>
</tr>
</tbody>
</table>

B. Effects of countermeasures

<table>
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<tr>
<th>Subcluster</th>
<th>Corrected</th>
<th>% Corrected</th>
<th>Maintained</th>
<th>New Differential</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOL</td>
<td>186</td>
<td>39.4</td>
<td>286</td>
<td>73</td>
</tr>
<tr>
<td>VL</td>
<td>52</td>
<td>25.1</td>
<td>155</td>
<td>25</td>
</tr>
</tbody>
</table>

Exercise group (BRE)

<table>
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<tr>
<th>Subcluster</th>
<th>Corrected</th>
<th>% Corrected</th>
<th>Maintained</th>
<th>New Differential</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOL</td>
<td>420</td>
<td>89.0</td>
<td>52</td>
<td>44</td>
</tr>
<tr>
<td>VL</td>
<td>199</td>
<td>96.1</td>
<td>8</td>
<td>3</td>
</tr>
</tbody>
</table>

Result synthesis expressed in number of differential transcripts after 60 days of bed rest and the effects of countermeasures (false discovery rate < 0.5%), and percentage of differential transcripts in countermeasure groups compared with BRC group (%). A: effects of 60 days of bed rest on soleus (SOL) and vastus lateralis (VL). B: effects of nutrition and exercise as countermeasure. Differential, transcripts with differential expression according to SAM analysis; Corrected, transcripts with no more differential expression with bed rest and countermeasure; Maintained, transcripts with still differential expression with bed rest and countermeasure; New Differential, transcripts with differential expression only in countermeasure groups.

Effects of exercise and nutritional countermeasures during LTBR. A comparative overview of the effects of exercise and nutritional countermeasures on mRNA expression changes during BR is shown in Fig. 3. Comparative heat maps and the three curves of smoothed P values, obtained gene-by-gene, in BRC (black line), BRN (red line), and BRE (blue line) groups, clearly indicate that most peaks of modified transcripts following BR in BRC group drastically became flat when exercise was used as a countermeasure (Fig. 3). The effect is much more evident in VL, where 199 out of 207 BRC modified transcripts were “corrected” by exercise (96.1%, Table 2B; examples in Supplemental Table S1), compared with 420 out of 472 BRC modified transcripts in SOL (89.0%, Table 2B; examples in Supplemental Table S1). Most of the subclusters previously observed in BRC group disappeared in BRE group (Fig. 3). In contrast, nutritional countermeasures exhibited a more moderate effect with ~40 and 25% of the BRC transcripts that were corrected in SOL and VL, respectively (Table 2B; examples in Supplemental Table S1).

Ingenuity Pathways Analysis enabled a comprehensive and global view to complement the analysis and characterization of the five identified subclusters. Figure 2 reports examples of functional networks or pathways arising from the five subclusters and illustrates the effects of countermeasures. Networks 1 and 2 show partial or globally positive effect of either nutrition or exercise on the changes observed following bed rest. Conversely, network 3 shows the changes in several expressed transcripts almost exclusively in the postural SOL and demonstrates that neither nutrition nor exercise was effective in counteracting several of these changes. mRNAs encoding for collagen type I and III exhibited large changes. Furthermore, these changes, related to ECM remodeling, were not counteracted by the countermeasures. Others examples, given in Supplemental Table S1, show the respective effects of both countermeasures on each reported changes.

A possible question was to know if the same individuals subjected either to bed rest or to any of the countermeasures, showed less or more pronounced effects in SOL and VL. Because of the low number of subjects available per group and large differences in the number of modified genes within each muscle type, statistical analysis could hardly be made. Calculations of correlation coefficients between expression values in SOL and VL of the same individuals, when available, gave variable results (see statistical landmark B in Supplemental Method S3). From this, we observed that while some individuals presented similar gene variations in both muscles, others did not.

Finally, a category of “new differential” mRNAs appeared in SOL and VL from BRN and BRE groups (Table 2B). By definition, the expression of these transcripts did not change following 60 days of BR, i.e., in the BRC group, but their expression significantly changes when a countermeasure is added during LTBR, i.e., in the BRN and BRE groups. The number of these new differential mRNAs, while limited, was larger in SOL than VL, and larger in the BRN group than in the
BRE group. Most of the transcripts in this category were mixed within previous subclusters found in BRC group. Since these were not associated with new gene subclusters, they did not represent new main functional modifications.

DISCUSSION

This international study gave us the opportunity to investigate the effects of a 60 day LTBR on the transcriptome of different muscle types in 24 healthy women. Our goal is to contribute to the development of optimized countermeasure protocols that are adapted to suit the needs of both sexes to prevent or to slow muscle wasting and to optimize recovery in reambulation or rehabilitation periods. In this context, we separately evaluated the potential beneficial impact of two types of countermeasures: protein supplementation and a combined resistance and aerobic training program. The clustering approaches employed allowed us to ascertain a clear overview of the drastic changes in the transcript profile after 60 days of BR, thereby providing important information on the physiological changes observed in both muscles types. In addition to the 472 mRNAs in SOL and 207 in VL whose expression was affected, five main subclusters of genes involved in integrated functions or pathways (mainly in protein synthesis and degradation, cytoskeletal and ECM remodeling, oxidative phosphorylation, and fatty acid metabolism) appeared to be modified following 60 days of BR. While most peaks corresponding to changes in mRNA expression following BR became massively flat in BRE group, more moderate effects were seen for BRN group.

Since our study was conducted with healthy females, our results should contribute to improving the health and safety of female astronauts and for women subjected to prolonged BR caused by illnesses or trauma or as advocated during pregnancy. Sex differences in skeletal muscle plasticity are often an overlooked aspect. Given that most ground-based studies aimed at simulating the effects of spaceflight have been conducted on male volunteers, our study also represents one of the largest focusing specifically on women.

The present study enabled the identification of several subclusters of modified transcripts related to protein metabolism that may be explained by the muscle remodeling and atrophy following 60 days of BR. The whole muscle atrophy related to this experimentation has been previously reported by another group that demonstrated that the quadriceps femoris and triceps surae muscle volumes from women similarly decrease by ~25% (81). Among the different proteolytic pathways, Ca\(^{2+}\)-activated proteases (calpains) and the ubiquitin-proteasome pathway are known to be major contributors to the catabolic state during skeletal muscle atrophy (43, 84). Our results agree with these previous observations since we observed an induction of calpain 3 and MAFbx mRNAs in VL of BRC and BRN groups (Supplemental Table S1II). The involvement of the...
calpain system in muscle atrophy is well established and may be a prerequisite for the ubiquitination and targeting of myofibrils to the proteasome (78). Increases in MAFbx/atrogin-1 and MURF1, the two main markers of atrophy, and various components of the ubiquitin-proteasome pathway have been reported in several types of atrophy (44), either in animal (16, 65, 74) or human models (45, 58). However, there remains a discrepancy in the observed time point upregulation during the atrophy process. Thus, there is a need for further studies on human biopsies to explore the activity of ubiquitin-proteasome pathway components. In fact, while several studies on animals have shown an upregulation in the expression of these two transcripts, we noted an increase only in MAFbx/atrogin-1 and not MURF1 following 60 days of BR (45, 58).

Subclusters of upregulated mRNAs revealed a marked increase in ribosomal protein RNAs. These types of changes have been reported in other unloading studies and could be in contradiction with the known decrease of protein synthesis that occurs during atrophy (29). In this case, the decrease in protein synthesis is caused by a decrease in the phosphorylation of Akt, which in turn reduces phosphorylation of both mTOR (61), and p70S6 kinase (S6K1) (15), thereby affecting the phosphorylation status of 4E-BP1. Unphosphorylated 4E-BP1 in turn permits the binding of 4E-BP1 to eIF4E and the interaction of the eIF4E mRNA complex to 40S and thus inhibits protein synthesis. While muscle hypokinesia and hypodynamia have been shown to be associated with a rapid decrease in the rate of protein synthesis in short-term disuse studies (31, 75), the present LTBR exhibits the contrary. Thus, in the present study the increase in RNAs encoding ribosomal proteins and several translation initiation and elongation factors remains unclear but may indicate that muscles, and mainly postural muscles, maintain or compensate disuse atrophy by increasing the expression of several proteins involved in stress or remodeling during long-term disuse situations (11, 29).

Very few studies, due to practical and ethical considerations, have directly examined differences in protein metabolism among males and females. There is a sexual dimorphism of skeletal muscle, and the larger muscle mass of men appears as the most important point. Female hormones, estrogen in particular, appears as a potential factor differentiating men and women. The facts also strongly suggest that-testosterone plays a role in the obvious differences in muscle between males and females especially in regard to protein metabolism, but little direct evidence is available (77). The larger muscle mass of men theoretically affects oxygen demand and perfusion during muscle contraction, inducing a clear difference in muscle fatigability between males and females (41). Furthermore, the larger initial muscle volume in men compared with women could be one factor enhancing the degree of atrophy for men when compared with women (54, 90). At the molecular level, a recent study using large-scale microarray analysis of male and female skeletal muscle reported an elevated expression of GRB10 in women (a transcript encoding for a protein that may restrain IGF-1 signaling) and a higher level of ACVR2B, a myostatin receptor gene (87). No changes in myostatin or ACVR2B mRNA expression were observed in the present study or following 2 wk of human immobilization (45). However, our results did show an increase in follistatin (FST), an extracellular factor that binds and modulates activin and myostatin activity (Fig. 2, network 3, and Supplemental Table S1II). FST interacts directly with myostatin to inhibit its binding and growth-preventing activity, thereby promoting increases in muscle mass (42, 51). Of relevance is the finding that FST mRNA is also highly induced during both unloading-induced atrophy (74) and during sarcopenia (59, 86).

In parallel with the changes seen in mRNAs coding for proteins involved in protein metabolism, the present study also shows that muscles are subjected to a marked structural remodeling following LTBR, which is manifested by inductions in several mRNAs encoding cytoskeletal and ECM proteins. One of the most dramatic changes observed was the SOL specific induction of collagen type I and III mRNAs (Fig. 2, network 3, and Supplemental Table S1III). Although there are few data available to compare our results, increases in collagen concentration have been reported in SOL muscle following 5 wk of denervation in animals (7) or following 90 days of BR in humans (39). In addition, ultrastructural studies have clearly shown that membrane folding and the length of the membrane interface between muscle fiber and tendon are largely increased after hindlimb suspension (91) and spaceflight (63), which might confirm the increase in collagen content in disuse situations. Our previous work conducted with muscle unloading (in both humans and animals) also reported structural changes affecting the expression of several cytoskeletal and associated proteins (22–24). Together, these results show that collagen and ECM components play a major role in remodeling of skeletal muscle during disuse-induced atrophy.

Our study also showed that remodeling of skeletal muscle fiber is accompanied by changes in expression of several mRNAs encoding proteins involved in Ca2+ signaling (examples in Supplemental Table S1III). Myoferlin (MYOF) shares the highest sequence homology to dysferlin whose expression increased in human following LTBR (24) and which is a membrane-associated protein that is defective in Miyoshi myopathy and limb-girdle muscular dystrophy type 2B (LGMD2B) (52). Multiple cellular processes are associated with Ca2+ signaling regulated by the sarcoplasmic reticulum (SR) (14). SERCA1, protein and mRNA, have previously been shown to be upregulated following short-term unloading in soleus (55, 60), but little is known about the transcriptional regulation of this gene. The upregulation of other calcium handling mRNAs, such as the ryanodine receptor (RYR1), involved in calcium release, and calsequestrin (CS), involved in calcium storage, has been previously reported following unloading (10, 29). Together, these changes may be linked to the known shift in myofiber types that accompanies muscle atrophy (46).

In addition to the atrophy induced by hypokinesia and hypodynamia, our work conducted on female muscle biopsies showed a marked downregulation of the pathways involved in fatty acid oxidation and oxidative phosphorylation. For example, several transcripts involved in the Krebs cycle and respiratory chain, such as cytochrome c oxidase and those from the ATP synthase complex (Fig. 1, subclusters D and E, and examples in Supplemental Table S1IV). Conversely, our results revealed an increase in several mRNAs encoding proteins involved in the preservation of glucose storage and in the cytoplasmic conversion of glycogen to pyruvate. The shift in pathways involved in muscle fuel utilization during disuse is in agreement with the shift in fiber type seen here (66) and already observed following space-flown rats (8), hindlimb suspension (50), and BR in humans (12, 62). These shifts
suggest that atrophied muscles may compensate for a reduced aerobic capacity via an increase in glycolytic production of ATP, thereby also explaining the decrease in fatigue resistance, one of the hallmarks of muscle disuse atrophy (34, 40). Moreover, the homogenous 60% decreased expression of several mRNAs encoding components of fatty acid oxidation appears larger than that observed either in VL of males after 42 days of BR (33), in VL of rats after 9 days of spaceflight (8), or following hindlimb suspension in rat SOL (88). In control conditions, a greater percentage of type I fibers in female compared with male has been associated with a greater resistance to fatigue (20). The higher relative proportion of type I fibers in females may also explain the higher lipid content and mRNA levels of several proteins related to muscle lipid metabolism (48). In this context, studies have also shown a greater impairment in neuromuscular activation in males compared with females after fatiguing exercise (38) and an enhanced fatigue resistance in females following 4 wk of immobilization (67). It has been shown for example that FAT/CD36 (a plasma membrane fatty acid transporter) protein content was higher in muscles from women than from men (48). The functional consequence of this sex difference in skeletal muscle FAT/CD36 content may involve a higher trans-sarcolemmal transport of long chain fatty acid and storage in women skeletal muscle. Such differences should be taken into account in defining countermeasures during long-term disuse.

To date, considerable efforts are made toward gaining an understanding of the cellular and molecular events involved in skeletal muscle disuse atrophy. However, it appears that there is a great need for additional research focusing specifically on the effects of countermeasures. Our results showed that a combined exercise program had a drastic effect on mRNA expression in SOL and VL during LTBR (higher in VL than in SOL). By contrast, the nutrition countermeasure only had a moderate effect (higher in SOL than in VL).

Our study focused on the combined effects of maximal resistance exercise and endurance training. Maximal resistance exercise appears as a very positive countermeasure to counteract the deficits in muscle structure and function. At the protein level, our group and others demonstrated that resistance exercise could compensate in a large part for the negative effects of 90 days of BR in male on cytoskeletal and associated proteins (24, 64). On the one hand, resistance exercise is able to maintain muscle mass by maintaining an elevated rate of muscle protein synthesis during disuse situations (32, 37). On the other hand, it can also prevent the increase in protein degradation (28) demonstrated, for example in our study, by the abrogation in the increase of MAFbx and calpain 3/p94 expression in VL from BRE group (Supplemental Table S1II).

Aerobic training was the second component of the exercise countermeasure. Characterized by its moderate intensity, this type of training is well known to induce an increase in muscle respiratory capacity: i.e., an increase in components of the mitochondrial respiratory chain, ATP synthase, enzymes of the citrate cycle, enzymes involved in fatty acid and ketone oxidation, and fast-to-slow fiber-type transformation (2). In our study, aerobic exercise significantly contributed to counteracting the effects of LTBR on muscle metabolism (see examples in Supplemental Table S1IV). Thus, changes of mRNAs encoding proteins involved in glucose storage and in different glycolysis steps were almost completely compensated in the BRE group. Furthermore, while both muscle types exhibited a homogeneous downregulation of several mRNAs involved in oxidative phosphorylation during LTBR, these changes did not appear in BRE group (Fig. 3 and Supplemental Table S1IV). Importantly, our results showed a lack of counteracting effect of either exercise or nutrition on the downregulation of several mRNAs encoding proteins of ß-oxidation and transport of fatty acid in SOL and VL (examples in Supplemental Table S1IV), highlighting the crucial negative effect of unloading on muscle fat accumulation and metabolism (see also Ref. 12). Moreover, the absence of a counteracting effect of exercise on the induction of collagen in SOL should stimulate further investigations.

Concerning our findings on “new differential genes” (i.e., transcripts with modified expression exclusively in BRE group and not in BRC group, see Table 2), we show that FLNC mRNA, encoding filamin C that is restricted to striated muscle (76), is one of the transcripts highly upregulated in SOL from the BRE group. Several studies have reported an abnormal localization of filamin C in a number of muscle diseases (17, 76), and its mutation has been associated with a novel form of myofibrillar myopathy (26). Despite its reported role in muscle differentiation and maintenance of elongated muscle fiber structure (26), the precise function of filamin C in skeletal muscle plasticity remains unclear.

Nutritional countermeasures, and especially essential amino acid supplementation like leucine, are well recognized for their anabolic effects. To date, these have been intensively studied to find alternate or complementary interventions to prevent or slow skeletal muscle catabolic process (89). At the same time, essential amino acids are substrates for synthesis of new proteins and act as a nutritional signaling molecules by inducing changes in phosphorylation of several proteins involved in the initiation of mRNA translation (5, 49). In our study, the counteracting effect of protein supplementation with leucine enrichment appears limited and uniformly distributed in all previous subclusters of the BRC group (Fig. 2 and examples in Supplemental Table S1). Oral administration of leucine was shown previously to stimulate protein synthesis in skeletal muscle following exercise (4), food deprivation (5), and glucocorticoid treatment (69), but the mechanisms remain unclear. Insulin and leucine, two effectors with anabolic actions, appear to activate independent intracellular signaling pathways that may commonly converge on mTOR (6, 49, 57). There is evidence that leucine oxidation is higher in males than females, especially during exercise (77). Thus we would expect that combined exercise and protein supplementation as countermeasure would be more beneficial for males than females during LTBR. This point should be further examined.

Our results in the new differential genes category (i.e., transcripts with modified expression exclusively in the BRN, and not in the BRC, group; see Table 2), demonstrated an induction of metallothioneins in SOL and VL. Metallothioneins are induced by oxidative stress, which can protect cells against DNA damage from reactive oxygen species (9). As shown in previous studies that reported oxidative stress induction together with an antioxidant response during disuse (53, 68), we also noted an upregulation of several mRNAs encoding proteins or enzymes involved in the antioxidant stress response in BRC groups (examples in Supplemental Table S1II). Even if the counteracting effects appear moderate, protein supplementation and leucine enrichment have been shown to preferen-
tially target postural muscles and thus may contribute to slow atrophy of the more vulnerable postural muscles.

Conclusions

Our transcriptomics approach allowed us to obtain a comprehensive overview of the signaling pathways and functional networks involved in skeletal muscle remodeling during LTBR with and without countermeasures. It responds specifically to a lack of data on human muscle subjected to long-term disuse and to a lack of knowledge on sex differences. Independent assessment of nutritional and physical countermeasures is necessary for their characterization, but it appears necessary to also obtain large scale transcriptomics and proteomics data on their combined effects, which may be important for designing appropriate and effective countermeasures.

ACKNOWLEDGMENTS

We thank OUEST Genopole and IFR 140 for transcriptome core facilities. As well, special thanks to the “Association Française contre les Myopathies” (AFM) for its constant support. We want to thank in particular the MEDES staff as well as Professor Jacques Mercier for his excellent biopsy procedures.

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