Diaphragm Unloading via Controlled Mechanical Ventilation Alters the Gene Expression Profile

Authors:
Keith C. DeRuisseau, **R. Andrew Shanely**, Nagabhavani Akunuri, Marc T. Hamilton, Darin Van Gammeren, A. Murat Zergeroglu, Michael McKenzie, and Scott K. Powers

Abstract

Rationale: Prolonged controlled mechanical ventilation results in diaphragmatic inactivity and promotes oxidative injury, atrophy, and contractile dysfunction in this important inspiratory muscle. However, the impact of controlled mechanical ventilation on global mRNA alterations in the diaphragm remains unknown.

Objectives: In these experiments, we used an Affymetrix oligonucleo-tide array to identify the temporal changes in diaphragmatic gene expression during controlled mechanical ventilation in the rat.

Methods: Adult Sprague-Dawley rats were assigned to either control or mechanical ventilation groups (n =5/group). Mechanically venti-lated animals were anesthetized, tracheostomized, and ventilated with room air for 6 or 18 h. Animals in the control group were acutely anesthetized but not exposed to mechanical ventilation.

Measurements and Main Results: Compared with control diaphragms, microarray analysis identified 354 differentially expressed, unique gene products after 6 and 18 h of mechanical ventilation. In general, genes in the cell growth/cell maintenance, stress response, and nucleic acid metabolism categories showed predominant upregula-tion, whereas genes in the structural protein and energy metabo-lism categories were predominantly downregulated.

Conclusions: We conclude that mechanical ventilation results in rapid changes in diaphragmatic gene expression, and subsequently, many of these changes may contribute to atrophy and muscle fiber remodeling associated with unloading this primary inspiratory mus-cle. Importantly, this study also provides new insights into why the diaphragm, after the onset of contractile inactivity, atrophies more rapidly than locomotor skeletal muscles and also highlights unique differences that exist between these muscles in the mRNA response to inactivity.

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Keywords: gene expression; microarray; muscle atrophy; respiratory muscle; weaning from mechanical ventilation

Mechanical ventilation (MV) is used clinically to maintain blood gas homeostasis in patients who are incapable of maintaining adequate alveolar ventilation on their own. For many of these patients, an unfortunate consequence of prolonged MV is the ensuing development of respiratory muscle weakness (1). It is now clear that prolonged MV is associated with significant atrophy and contractile dysfunction of the diaphragm (2–6). Recent animal studies indicate that controlled MV leads to significantly greater respiratory muscle weakness than assisted MV (7) because controlled MV results in complete loss of neural activation and mechanical activity of the diaphragm (3, 7, 8). Therefore, controlled MV is a useful physiologic model to investigate the effects of prolonged inactivity of the diaphragm (2, 3).

The diaphragm experiences remarkable physiologic and biochemical alterations during periods of unloading. The time frame of phenotypic changes that the diaphragm undergoes during MV occurs on the order of hours (3, 9), whereas diaphragmatic inactivity imposed by phrenic nerve denervation and cervical spinal cord hemisection occurs over the course of days (10–12). Work by our laboratory has demonstrated increased oxidative stress (6, 9), decreased rates of protein synthesis (13), and enhanced rates of proteolysis (6, 14), in addition to impaired diaphragm contractility after MV. Importantly, a remarkable distinction between atrophy of the diaphragm induced by MV and that of locomotor muscle after disuse is the time course of the atrophy process. The rapid rate of MV-induced diaphragmatic atrophy greatly exceeds the rate of atrophy observed in locomotor muscles during periods of disuse (i.e., immobilization or hindlimb suspension). For example, using the rat as an experimental model, the onset of disuse muscle atrophy occurs approximately eight times faster in the diaphragm compared with locomotor skeletal muscle (6, 15). Therefore, the current study investigated the gene expression responses underlying the processes associated with the prompt onset of MV-induced diaphragmatic atrophy and muscle fiber remodeling.

To date, the mechanisms responsible for MV-induced diaphragmatic atrophy, contractile dysfunction, and oxidative injury have been studied primarily at the protein level. Only a limited number of genes (7, 13, 16–19) have been studied to provide insight into the molecular events that occur in the diaphragm as a result of MV. Nonetheless, the observed physiologic and biochemical diaphragmatic alterations induced by MV are consistent with the notion that MV results in altered levels of diaphragmatic gene expression.

Historically, the study of transcriptional changes required the analysis of individual mRNA transcripts. However, recent advances in biotechnology, including microarray analysis, permit the simultaneous measurement of large numbers of gene transcripts. For example, recent studies have investigated skeletal muscle gene expression in response to both hind-limb immobilization and hind-limb unloading (20–25). However, at present, the mRNA alterations that contribute to MV-induced changes in the diaphragm are unknown; this forms the rationale for the current experiments. The current study used the Affymetrix RG-U34A (Affymetrix, Santa Clara, CA) array to investigate the effects of controlled MV on gene expression in the diaphragm. On the basis of previous studies from our laboratory, we hypothesized that, compared with control diaphragms, diaphragms

exposed to MV would exhibit altered transcript levels for genes linked to specific cellular functions associated with the stress response, protein metabolism, and calcium regulation. Some of the results from these studies have been previously reported in the form of an abstract (26).

METHODS

Animals and Experimental Design

These experiments were approved by the University of Florida Animal Use Committee. Adult (4-mo-old) female Sprague-Dawley rats were randomly assigned to either control, 6 h MV (MV6), or 18 h MV (MV18; n = 5/group). Animals were killed around the same time (± 2 h) of the day to minimize possible influences of circadian rhythm on gene expression levels (27). A brief outline of the experimental methods follows, with methodologic details for each procedure provided in the online supplement.

Protocol for Control Animals

Control animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg bodyweight). The diaphragm was quickly removed and segments from the medial costal diaphragm were frozen in liquid nitrogen and stored at –80˚C.

Protocol for MV Animals

All surgical procedures were performed using aseptic techniques. MV animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg bodyweight), tracheostomized, and mechanically ventilated using a volume-driven small-animal ventilator (Harvard Apparatus, Cambridge, MA). An arterial catheter was inserted into the carotid artery for constant measurement of systolic blood pressure, periodic blood sampling, and infusion of isotonic saline. A venous catheter was inserted into the jugular vein for continuous infusion of sodium pentobarbital (rv 10 mg/kg/h). Body temperature was maintained at approximately 37˚C by use of a recirculating heating blanket. Heart rate was monitored via a lead II ECG using subcutaneous needle electrodes. Animals were administered enteral nutrition using the AIN-76 rodent diet (Research Diets Inc., New Brunswick, NJ) and received continuous care and monitoring. On completion of MV, the diaphragm was quickly removed and segments from the medial costal diaphragm were frozen in liquid nitrogen and stored at –80˚C.

Isolation of Total RNA

A portion of the medial costal diaphragm was homogenized in Trizol reagent (Invitrogen, Carlsbad, CA) and processed according to the manufacturer's instructions.

Microarray Processing

Total RNA was prepared for hybridization according to the protocols in the GeneChip expression analysis technical manual (Affymetrix).

Reverse Transcription and cDNA Quantification for Real-Time Polymerase Chain Reaction

Reverse transcription was performed using the Superscript III First-Strand Synthesis System for reverse transcriptase–polymerase chain reaction (PCR; Life Technologies, Carlsbad, CA) followed by cDNA quantification (Molecular Probes, Eugene, OR).

Quantitative Real-Time PCR

Quantitative real-time PCR for hypoxanthine guanine phosphoribosyltransferase, growth arrest and DNA damage–inducible 45 a (Gadd45a), and thioredoxin reductase-1 (TxnRd1) was performed using the ABI Prism 7000 Sequence Detection System (ABI, Foster City, CA).

Data Analysis

Microarray images were analyzed using statistically based Affymetrix Microarray Suite 5.0 (Affymetrix). In reporting differentially expressed genes, we used a conservative approach to the data analysis using the following criteria: (*1*) a transcript was required to be present on all five chips within any one of the three experimental groups, (*2*) genes needed to be significant at $p < 0.001$, and (*3*) transcripts must have displayed at least a ± 1.5-fold change. Changes in mRNA expression measured by real-time PCR were assessed with an unpaired Student's *t* test, with a Bonferonni correction on normalized (hypoxanthine guanine phosphoribosyltransferase) input values. A p values less than 0.05 was considered significant.

RESULTS

Systemic and Biological Response to MV

Total RNA was obtained from diaphragms of animals included in a previously reported study (9). As reported previously, animal bodyweights did not differ ($p > 0.05$) between the control (296 ± 5 g), MV6 (pre-MV = 277 ± 6 g; post-MV = 276 ± 5 g), or MV18 (pre-MV = 296 ± 15 g; post-MV = 293 ± 14 g) groups. These results confirm that our program of enteral nutrition and rehydration was adequate.

To determine if our MV protocol was successful in maintaining homeostasis, we measured arterial blood pressure, pH, and the partial pressures of both CO_2 and O_2 in all animals within the first and final hour of MV. Although variations in arterial blood pressure, blood gases, and pH existed between animals during MV, our data indicate that adequate arterial blood pressure and blood gas/pH homeostasis were maintained in all animals during MV. As reported previously, systolic blood pressure and arterial pH ranged from 95 to 115 mm Hg and 7.42 to 7.45, respectively (9).

Because sepsis is associated with diaphragmatic contractile dysfunction, strict aseptic techniques were followed throughout the experiments. Our results indicate that the animals included in our analysis did not develop infections during MV. This statement is supported by the observation that microscopic examination of blood revealed no detectable bacteria and postmortem examination of the lungs (visual) and peritoneal cavity (visual) displayed no detectable abnormalities. Furthermore, our MV animals were afebrile during the investigation. Collectively, these results indicate that the MV animals used in our analysis were free of significant infection.

Overview of Microarray Findings

In these experiments we used the Affymetrix U34A rat chip containing 8,799 probe sets that represent unique genes and expressed sequence tags. In our data analysis, after the removal of probe sets not receiving at least one present call, the number of expressed transcripts in the diaphragm was 4,405. Under the present criteria, which require five present calls within any one of the three experimental groups, the number of expressed transcripts was reduced to 3,303. Data analysis identified 508 differentially expressed gene transcripts at the $p < 0.001$ level. From this list of candidate genes, we further identified a subclass of 420 differentially expressed transcripts (i.e., 354 unique gene products) that demonstrated at least a ± 1.5-fold difference in expression. Our use of stringent criteria in the data analysis provides a high level of confidence in the reported list of differentially expressed genes. With our analysis, we opted to use an approach that would limit the number of reported false-positives (type I error).

Quantitative real-time PCR data for two genes (Gadd45a and TxnRd1) exhibiting disparate expression patterns are presented in Table 1. Importantly, the PCR results for these genes showed excellent agreement with the microarray analysis with respect to direction and magnitude of change.

The list of differentially expressed genes was grouped into 19 functional categories. Because of the number of differentially expressed genes, we elected to narrow our discussion to selected categories (Tables 2–5). A comparison of differentially expressed genes in the inactive diaphragm and the inactive soleus muscle

TABLE 1. QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION CONFIRMATION OF MICROARRAY DATA

	Microarray				Real-Time PCR			
mRNA	Fold* MV6	p Value	Fold MV18	p Value	Fold MV6	p Value†	Fold MV18	p Value†
TxnRd1	↑ 1.27	8.38E-02	↑ 3.51	5.42E-06	↑ 1.22	3.32E-01	↑ 3.85	1.09E-03
Gadd45α	↑ 8.28	1.32E-04	↑ 16.67	4.62E-08	↑ 6.02	5.15E-03	↑ 20.30	1.06E-04
	↑ 8.01	6.50E-04	↑ 15.49	5.85E-05				
	↑ 6.68	1.56E-03	↑ 13.20	1.94E-05				
	↑ 6.66	1.76E-03	↑ 13.14	1.63E-05				

Definition of abbreviations: Gadd45α = growth arrest and DNA damage–inducible 45α; PCR = polymerase chain reaction; TxnRd1 = thioredoxin reductase-1.

* Fold indicates the fold change from control values.

† p Value calculated by Student's *t* test on linear, normalized (hypoxanthine guanine phosphoribosyltransferase) input values.

is also made in Table 6. Remaining genes not presented in the discussion together with the list of expressed sequence tags are contained within Tables E1–E8 on the online supplement.

For nearly all probe sets, a more pronounced fold change was observed after 18 h than after 6 h of MV. Only seven genes demonstrated a greater fold change at 6 compared with 18 h of MV; however, the greatest difference between the two time points was only 0.3-fold. In addition, only one gene (peripheral myelin protein 22) was differentially expressed ± 1.5-fold at 6 h but not at 18 h of MV.

The list of differentially expressed genes in the following discussion was categorized into stress response, protein metabolism, calcium regulation, or energy metabolism categories. As reported previously by our lab group and others, MV leads to diaphragm contractile dysfunction, oxidative stress, and atrophy. Therefore, we chose to limit our discussion to categories of genes with particular relevance to these outcomes.

DISCUSSION

Our results support the hypothesis that, compared with control diaphragms from spontaneously breathing animals, diaphragms exposed to MV exhibit altered transcript levels for numerous genes linked with the stress response, protein metabolism, and calcium regulation. In addition, we observed significant changes in diaphragmatic transcript levels for genes involved in energy metabolism. A discussion of our major findings follows.

Stress-responsive Genes

Oxidative stress has been implicated as a contributing factor to the progression of skeletal muscle atrophy during disuse (14, 28–31). Importantly, recent work in our laboratory has also revealed that oxidative stress in the diaphragm develops within the first 6 h of the onset of MV (6, 9). Oxidative stress is likely preceded by a disruption in the intracellular redox balance, which

TABLE 2. DIFFERENTIAL EXPRESSION OF STRESS-RESPONSE GENES IN THE DIAPHRAGM AFTER 6 AND 18 H OF MECHANICAL VENTILATION

	MV6/Control		MV18/Control			
Affymetrix Probe	p Value*	Fold†	p Value*	Fold†	Gene	Description
L19998_g_at	3.20E-01	-0.9	6.12E-04	-2.8	Sulfotransferase family 1A, phenol-preferring, member 1	Toxilogic response
M11942_s_at	2.83E-01	0.9	6.26E-04	1.5	Heat shock protein 8	Chaperone
rc_AI176546_at	1.74E-01	0.9	3.82E-04	2.0	Heat shock protein 86	Chaperone
M86389cds_s_at	4.09E-02	1.7	3.16E-04	2.6	Heat shock 27-kD protein 1	Chaperone
rc_AA998683_g_at	3.01E-03	1.8	1.21E-05	2.1	Heat shock 27-kD protein 1	Chaperone
rc_AI176658_s_at	2.37E-03	1.9	4.70E-04	2.2	Heat shock 27-kD protein 1	Chaperone
rc_AA998683_at	4.37E-04	2.4	3.29E-04	3.4	Heat shock 27-kD protein 1	Chaperone
L33869_at	9.20E-02	1.3	4.14E-07	2.4	Ceruloplasmin	Trace metal metabolism
E01415cds_s_at	2.11E-01	-1.9	5.63E-06	-2.7	Glutathione S transferase	Toxilogic response
rc_AI233261_i_at	2.02E-01	1.4	3.85E-04	2.5	Glutamate cysteine ligase, modifier subunit	Glutathione synthesis
AF037072_at	9.37E-02	-1.3	2.91E-04	-2.4	Carbonic anhydrase 3	CO_2 disposal
rc_AI179610_at	3.40E-03	7.6	2.31E-04	18.8	Heme oxygenase 1	Trace metal metabolism
J02722cds_at	1.09E-02	10.7	9.62E-05	26.5	Heme oxygenase	Trace metal metabolism
rc_AA799678_s_at	2.90E-03	-1.7	6.44E-05	-2.0	EGL nine homolog 3 (*C. elegans*)	Oxygen sensor
U06713_at	2.54E-04	-1.5	2.93E-05	-2.0	EGL nine homolog 3 (*C. elegans*)	Oxygen sensor
rc_AI070295_g_at	1.76E-03	6.7	1.63E-05	13.1	Growth arrest and DNA damage–inducible 45a	Response to DNA damage stimulus
L32591mRNA_g_at	1.56E-03	6.7	1.94E-05	13.2	Growth arrest and DNA damage–inducible 45a	Response to DNA damage stimulus
rc_AI070295_at	6.50E-04	8.0	5.85E-05	15.5	Growth arrest and DNA damage–inducible 45a	Response to DNA damage stimulus
L32591mRNA_at	1.32E-04	8.3	4.62E-08	16.7	Growth arrest and DNA damage–inducible 45a	Response to DNA damage stimulus
rc_AI102562_at	7.36E-05	12.6	1.63E-04	21.8	Metallothionein	Trace metal metabolism
M11794cds#2_f_at	6.56E-06	20.9	2.10E-04	25.9	Metallothionein-2 and metallothionein-1	Trace metal metabolism
Z24721_at	3.33E-02	-1.6	2.36E-05	-3.7	Superoxide dismutase 3	Redox regulation
rc_AA891286_at	8.38E-02	1.3	5.42E-06	3.5	Thioredoxin reductase-1	Redox regulation
rc_AI230247_s_at	9.72E-03	-1.4	3.02E-04	-2.3	Selenoprotein P, plasma, 1	Redox regulation
rc_AA799650_at	1.77E-01	-0.8	9.60E-04	-1.6	Peroxiredoxin 3	Redox regulation

Definition of abbreviation: EGL = egg-laying defective.

* p Values calculated by Wilcoxon's signed rank test.

† Fold indicates the fold change from control values.

TABLE 3. DIFFERENTIAL EXPRESSION OF PROTEIN METABOLISM GENES IN THE DIAPHRAGM AFTER 6 AND 18 h OF MECHANICAL VENTILATION

	MV6/Control		MV18/Control			
Affymetrix Probe	pValue*	Fold†	pValue*	Fold†	Gene	Description
S61960_s_at	6.95E-02	-0.4	4.02E-04	-1.7	Cysteine conjugate [3-lyase	Amino acid derivative metabolism
J04171_at	4.16E-01	-0.2	1.04E-05	-1.7	Glutamate oxaloacetate transaminase 1	Amino acid metabolism
rc_AA800318_at	1.28E-01	1.9	4.27E-04	3.0	Serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1	Complement activation
D87515_at	1.29E-03	-1.9	1.87E-05	-2.5	Aminopeptidase B	Membrane alanyl aminopeptidase
L07281_at	2.81E-01	-0.2	3.55E-05	-1.8	Carboxypeptidase E	Peptide hormone processing
rc_AI231547_at	5.80E-01	-0.7	3.67E-04	-1.9	FK506 binding protein 4 (59 kD)	Protein folding
U68544_at	4.71E-01	-0.5	3.83E-04	-2.3	Peptidylprolyl isomerase F	Protein folding
M19533mRNA_i_at	7.91E-01	-0.3	9.37E-04	2.1	Peptidylprolyl isomerase A	Protein folding
rc_AA818858_s_at	4.13E-01	0.5	8.74E-05	2.1	Peptidylprolyl isomerase A (cyclophilin A)	Protein folding
rc_AA818487_s_at	4.40E-01	0.6	5.08E-04	1.6	Cyclophilin B	Protein folding
rc_AA859942_at	1.24E-01	1.3	5.93E-05	1.7	N-myristoyltransferase 1	Protein modification
M26686_g_at	3.96E-02	-1.1	9.72E-04	-2.1	Protein-L-isoaspartate (D-aspartate) O-methyltransferase1	Protein modification
rc_AI178828_i_at	7.09E-01	2.1	5.61E-05	4.0	Eukaryotic translation initiation factor 4E binding protein 1	Protein synthesis
rc_AI639441_at	3.11E-01	0.2	4.03E-04	1.6	Eukaryotic translation initiation factor 2B, subunit 3	Protein synthesis
U38253_at	1.54E-01	0.7	4.15E-05	1.8	Eukaryotic translation initiation factor 2B, subunit 3	Protein synthesis
rc_AA892367_i_at	3.39E-01	0.9	2.81E-04	3.0	Ribosomal protein L3	Protein synthesis
rc_AI013194_at	2.22E-01	0.9	8.02E-04	2.9	Eukaryotic initiation factor 5 (eIF-5)	Protein synthesis
U38253_g_at	5.76E-02	1.1	9.89E-05	1.6	Eukaryotic translation initiation factor 2B, subunit 3 (γ, 58 kD)	Protein synthesis
J02646_at	6.53E-02	1.3	1.34E-04	1.5	Eukaryotic translation initiation factor 2, subunit 1 (α)	Protein synthesis
X62166cds_s_at	1.45E-01	1.3	3.68E-05	2.3	Ribosomal protein L3	Protein synthesis
rc_AI012604_at	2.46E-03	1.3	7.89E-05	1.9	Eukaryotic initiation factor 5 (eIF-5)	Protein synthesis
U05014_g_at	3.74E-03	2.5	2.40E-07	4.0	Eukaryotic translation initiation factor 4E binding protein 1	Protein synthesis
U05014_at	2.84E-02	2.9	8.42E-05	4.8	Eukaryotic translation initiation factor 4E binding protein 1	Protein synthesis
AF061726_s_at	3.77E-02	-1.3	8.70E-05	-2.4	Calpain 3	Protein degradation
M38135_at	2.72E-05	-1.3	8.67E-04	-1.5	Cathepsin H	Protein degradation
AF052540_s_at	1.41E-01	-1.2	6.02E-05	-2.3	Calpain 3	Protein degradation
J05121_s_at	5.70E-01	-0.5	3.41E-05	-1.9	Calpain 3	Protein degradation
X82396_at	2.24E-01	1.7	2.57E-04	3.1	Cathepsin B	Protein degradation
S85184_g_at	1.47E-01	2.0	3.55E-08	4.2	Cathepsin L proenzyme	Protein degradation
rc_AI176595_s_at	1.78E-01	2.5	8.06E-05	5.0	Cathepsin L	Protein degradation
S85184_at	1.03E-01	2.8	4.25E-07	6.5	Cathepsin L proenzyme	Protein degradation
D90211_s_at	6.90E-02	1.1	2.99E-04	2.0	Lysosomal membrane glycoprotein 2	Intrinsic lysosomal membrane protein
X83537_at	2.79E-01	0.5	3.12E-04	2.2	Matrix metalloproteinase 14, membrane-inserted	Protein degradation
rc_AI169327_g_at	1.19E-01	18.0	6.52E-04	66.2	Tissue inhibitor of metalloproteinase 1	Protein degradation

* p Value calculated by Wilcoxon's signed rank test.
† Fold indicates the fold change from control values.

leads to the altered expression of proteins involved in redox homeostasis. In the present investigation, we observed several alterations in gene expression that are consistent with the notion that controlled MV results in redox disturbances in the diaphragm.

Superoxide dismutase-3 and selenoprotein P, an extracellular glycoprotein containing several selenocysteine residues, progressively declined over the course of MV. In addition, we observed a decrease in peroxiredoxin-3 (Prdx3) mRNA in the diaphragm during MV. Prdx3 is a mitochondrial member of the Prdx proteins, which is induced by oxidative stress and possesses antioxidant properties (32). In contrast, we observed an increase in TxnRd1 in the diaphragm during MV. TxnRd1 is a cytosolic selenoenzyme that, together with its substrate, thioredoxin, can regenerate dehydroascorbate and various proteins inactivated by oxidative stress. Moreover, the observed increases in the modifier subunit of glutamate cysteine ligase may indicate increased glutathione synthesis, the principal intracellular nonprotein thiol and an important antioxidant.

TABLE 4. DIFFERENTIAL EXPRESSION OF CALCIUM REGULATION GENES IN THE DIAPHRAGM AFTER 6 AND 18 h OF MECHANICAL VENTILATION

	MV6/Control		MV18/Control			
Affymetrix Probe	pValue*	Fold†	pValue*	Fold†	Gene	Description
M17069_at	9.52E-03	-1.2	4.86E-04	-1.6	Calmodulin 2	Calcium effector
X13933_s_at	4.10E-01	0.6	5.08E-04	2.0	Calmodulin 1	Calcium effector
Y09453cds_at	9.69E-03	-1.5	1.11E-04	-2.5	Calcium channel, voltage-dependent, γ subunit 1	Calcium ion transport
M30581_at	1.06E-03	-1.3	1.69E-05	-1.8	ATPase, Ca^{++} transporting, ubiquitous (SERCA3)	Calcium ion transport
L04684_at	1.39E-01	-0.6	5.64E-04	-1.6	Calcium channel, voltage-dependent, L type, α1S subunit	Calcium ion transport
U33287_at	7.87E-02	-1.3	1.62E-04	-3.7	Calsequestrin 2	Calcium storage

* p Value calculated by Wilcoxon's signed rank test.
† Fold indicates the fold change from control values.

TABLE 5. DIFFERENTIAL EXPRESSION OF ENERGY METABOLISM GENES IN THE DIAPHRAGM AFTER 6 AND 18 h OF MECHANICAL VENTILATION

	MV6/Control		MV18/Control			
Affymetrix Probe	p Value*	Fold†	p Value*	Fold†	Gene	Description
U00926_g_at	6.30E-01	-0.5	2.82E-04	-1.7	ATP synthase, H+ transporting, mitochondrial F1 complex, o	ATP biosynthesis
U00926_at	8.31E-01	-0.1	9.40E-05	-1.7	ATP synthase, H+ transporting, mitochondrial F1 complex, delta	ATP biosynthesis
D13123_s_at	7.63E-01	0.3	4.83E-05	-1.6	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c, isoform 1	ATP biosynthesis
rc_AA801286_at	3.22E-02	-0.9	9.43E-04	-1.6	Inositol (myo)-1(or 4)-monophosphatase 1	Carbohydrate metabolism
AB015433_s_at	3.93E-03	1.7	3.46E-06	2.4	Solute carrier family 3, member 2	Carbohydrate transport
AF093773_s_at	4.12E-01	-0.1	3.22E-07	-1.7	Malate dehydrogenase 1	Citric acid cycle
rc_AA892485_at	7.85E-02	-1.0	3.03E-06	-2.3	Dihydrolipoamide acetyltransferase	Conversion of pyruvate to acetyl-CoA
rc_AI170911_at	1.92E-01	1.1	1.53E-04	1.9	Pyruvate dehydrogenase E1 a-like	Conversion of pyruvate to acetyl-CoA
U83880UTR#1_g_at	3.80E-01	-0.5	1.53E-05	-2.4	Glycerol-3-phosphate dehydrogenase 2	Electron transport
rc_AA875269_at	5.36E-04	-2.1	4.57E-05	-2.0	Stearoyl-coenzyme A desaturase 2	Fatty acid metabolism
rc_AI044900_s_at	7.02E-02	-1.8	2.21E-04	-3.2	Fatty acid coenzyme A ligase, long chain 2	Fatty acid metabolism
D90109_at	1.27E-01	-1.4	6.66E-05	-2.8	Fatty acid coenzyme A ligase, long chain 2	Fatty acid metabolism
S56508_s_at	3.95E-01	-0.7	9.29E-05	-2.5	Fatty acid coenzyme A ligase, long chain 6	Fatty acid metabolism
U26033_at	1.20E-01	-0.4	2.39E-04	-2.3	Carnitine O-octanoyltransferase	Fatty acid metabolism
J02773_at	2.35E-01	-0.4	1.67E-04	-1.9	Fatty acid binding protein 3	Fatty acid metabolism
D85189_at	1.52E-01	1.3	3.23E-05	3.3	Fatty acid coenzyme A ligase, long chain 4	Fatty acid metabolism
rc_AI236284_s_at	3.85E-02	1.3	3.85E-06	2.1	Fatty acid coenzyme A ligase, long chain 4	Fatty acid metabolism
AJ005046_g_at	2.84E-01	-0.7	8.45E-05	-2.9	Fructose-1,6-bisphosphatase	Gluconeogenesis
X07320_at	2.62E-03	-1.4	2.13E-05	-2.0	Phosphorylase kinase γ1	Glycogen degradation
X15580complete_seq_s_at	9.06E-01	-1.1	2.27E-04	-5.3	6-phosphofructo-2 kinase/fructose 2, 6-biphosphatase 1	Glycolysis
S56464mRNA_g_at	1.93E-02	1.4	9.27E-05	1.8	Hexokinase II	Glycolysis
rc_AI169417_s_at	8.69E-02	1.4	6.04E-04	2.5	Phosphoglycerate mutase 1	Glycolysis
S63233_g_at	4.23E-03	1.4	2.13E-04	2.3	Phosphoglycerate mutase type B	Glycolysis
L11694_at	8.81E-01	0.0	1.25E-04	-1.8	Phosphoglucomutase 1	Glycogen metabolism
S81497_i_at	7.31E-01	-0.3	7.06E-04	-1.9	Lipase A, lysosomal acid	Lipid catabolism
D88666_at	1.77E-03	2.9	5.84E-05	4.7	Phosphatidylserine-specific phospholipase A1	Lipid catabolism
rc_AA874813_s_at	1.82E-01	-0.9	9.31E-04	-2.3	mitofusin 2	Mitochondrial fusion
rc_AI102505_g_at	2.10E-01	-0.8	1.58E-04	-1.8	Cytochrome C oxidase, subunit VIIIa	Mitochondrial oxidative phosphorylation
L48209_s_at	9.83E-01	-0.7	7.14E-05	-2.1	Cytochrome C oxidase, subunit VIIIa	Mitochondrial oxidative phosphorylation
rc_AA799466_at	6.75E-02	-0.6	8.41E-04	-1.5	Adenylate kinase 2	Regulation of adenine nucleotide composition
U42413_at	3.33E-02	-0.8	4.13E-04	-1.7	5-AMP-activated protein kinase, γ1 noncatalytic subunit	Energy balance
L20427_at	4.06E-01	-0.4	2.09E-05	-2.1	Coenzyme q (ubiquinone) biosynthetic enzyme 3	Ubiquinone synthesis
D10655_g_at	6.87E-01	-0.7	4.57E-06	-2.0	Dihydrolipoamide acetyltransferase	Pyruvate metabolism
D10655_at	4.87E-01	-0.3	3.94E-04	-1.6	Dihydrolipoamide acetyltransferase	Pyruvate metabolism
D28561_s_at	7.06E-01	-0.1	5.19E-05	-2.3	GLUT4	Glucose transporter
S68135_s_at	2.35E-02	2.9	7.31E-04	10.4	GLUT1	Glucose transporter

* p Value calculated by Wilcoxon's signed rank test.
† Fold indicates the fold change from control values.

Another protein that exhibits antioxidant properties is carbonic anhydrase III, a soluble protein present in high levels in slow-twitch skeletal muscle (33). Carbonic anhydrase III overexpression enhances resistance against H_2O_2-mediated oxidation and apoptosis in skeletal muscle (34). A decrease in carbonic anhydrase III mRNA was detected after 18 h of MV. It is possible that downregulated carbonic anhydrase III mRNA levels during MV may compromise the intracellular antioxidant buffering capacity.

Heme oxygenase-1 (Hmox1, also known as HSP32) and metallothionein were among the 10 most responsive genes to MV. Both genes are upregulated by oxidative stress and both proteins possess antioxidant properties. Indeed, Hmox1-mediated iron release may lead to increased ferritin synthesis and protection against iron-mediated oxidative damage (35). Metallothionein may act as an antioxidant via the ability to form complexes with metals in addition to acting directly as a free radical scavenger (36). Several genes of the heat shock protein (HSP) family were upregulated in the diaphragm in response to MV. Of these, Hmox1 displayed the greatest response followed by HSP27, HSP86 (HSP90 family), and HSP8 (HSP70 family). HSPs are induced in response to various stressors to help maintain homeostasis and protect against cell injury and death.

In summary, our findings are consistent with the concept that controlled MV leads to oxidative stress in the diaphragm (6, 9). The expression pattern of stress-responsive genes is consistent with the notion that nonmitochondrial, intracellular oxidant production is elevated in the diaphragm during MV.

Protein Metabolism

Skeletal muscle disuse accelerates protein degradation and thus the rate of muscle atrophy. Work from our laboratory has demonstrated elevated proteolysis in the diaphragm after MV (6, 14). Importantly, changes in the mRNA expression patterns

TABLE 6. COMPARISON OF mRNA ALTERATIONS IN THE DIAPHRAGM AFTER 6 AND 18 h MECHANICAL VENTILATION AND LOCOMOTOR SKELETAL MUSCLE (SOLEUS) AFTER 12 h HIND-LIMB UNLOADING

	Diaphragm				Locomotor Muscle		
	MV6/Control		MV18/Control		Inactivity/Control		
GenBank Accession	pValue*	Fold†	pValue*	Fold†	pValue*	Fold†	Description
J03969	3.23E-01	0.6	1.02E-04	1.8	5.6E-06	2.0	Nucleophosmin 1
S74351	2.63E-05	-7.2	2.06E-03	-5.5	5.6E-07	-2.3	Protein tyrosine phosphatase, nonreceptor type 16
AI639233	5.54E-01	0.6	6.98E-05	2.0			Decorin
Z12298					6.7E-07	6.1	Decorin
X59859					4.0E-06	3.2	Decorin
X59859					3.2E-05	2.7	Decorin
L23148	1.04E-04	-2.5	2.15E-03	-2.6	8.9E-07	-2.4	Inhibitor of DNA binding 1, helix-loop-helix protein (splice variation)
U02553	5.73E-04	-3.3	6.87E-03	-3.1	4.5E-06	-2.5	Protein tyrosine phosphatase, nonreceptor type 16
L32591	1.56E-03	6.7	1.94E-05	13.2	< 1E-08	2.4	Growth arrest and DNA damage-inducible 45a
L32591	1.32E-04	8.3	4.62E-08	16.7	2.4E-06	2.0	Growth arrest and DNA damage-inducible 45a
AF039583	3.12E-04	-5.7	1.07E-04	-18.8	1.3E-06	-2.8	Decay-accelerating factor
M32167					9.3E-06	-2.0	VEGF
L20913	1.04E-01	-0.9	3.34E-04	-1.6			VEGF
S85184	1.47E-01	2.0	3.55E-08	4.2	4.4E-07	4.7	Cathepsin L proenzyme
S85184	1.03E-01	2.8	4.25E-07	6.5	1.5E-05	2.7	Cathepsin L proenzyme
U77829	2.24E-02	2.0	1.97E-05	3.9	4.2E-04	2.5	Rattus norvegicus gas-5 growth arrest homolog nontranslated mRNA sequence
U77829	3.51E-02	2.4	3.36E-05	5.3			Rattus norvegicus gas-5 growth arrest homolog nontranslated mRNA sequence
D42137	2.83E-02	-1.1	9.35E-04	-1.7	1.3E-05	-2.1	Annexin V
U50736	6.65E-02	16.8	1.91E-06	56.5	2.2E-07	-2.7	Ankyrin-like repeat protein
AI169370	2.89E-01	0.9	9.12E-04	2.2			a-Tubulin
V01227					3.3E-07	-2.4	a-Tubulin
M86564	9.04E-01	-0.4	3.99E-04	1.9	1.9E-06	-1.6	a-Prothymosin
X15580	9.06E-01	-1.1	2.27E-04	-5.3	2.6E-06	3.4	6-phosphofructo-2 kinase/fructose 2,6-biphosphatase 1
S68135	2.35E-02	2.9	7.31E-04	10.4	3.3E-05	-2.1	GLUT1
S63233	4.23E-03	1.4	2.13E-04	2.3	1.6E-06	-2.1	Phosphoglycerate mutase type B
L19998	3.20E-01	-0.9	6.12E-04	-2.8	3.3E-07	2.8	Sulfotransferase family 1A, phenol-preferring, member 1
L19998					1.9E-06	2.5	Sulfotransferase family 1A, phenol-preferring, member 1
U12187	1.02E-04	12.8	1.52E-05	17.1	3.3E-07	-8.4	ras-related protein

Definition of abbreviation: VEGF = vascular endothelial growth factor.

Note that the locomotor skeletal muscle mRNA data are from a previous study (21) using similar methods to the current study. Genes that responded in a similar fashion to mechanical ventilation and hind-limb unloading are above the horizontal line and those that responded antithetically are below the line.

* p Value calculated by Wilcoxon's signed rank test.

† Fold indicates the fold change from control values.

of several proteolytic enzymes in response to MV are consistent with these previous observations. Several members of the cathepsin family of proteases were upregulated in response to disuse; however, the contribution of this system to overall proteolysis is considered to be low (37). Calpain-3 (i.e., muscle-specific calpain) mRNA decreased in response to MV. It has been speculated that decreased calpain-3 activity observed during muscle disuse may impact skeletal muscle apoptosis and decrease nuclear factor-KB activation (38). In addition, matrix metalloproteinase-14 mRNA increased in response to 18 h of MV. Degradation of the extracellular matrix as a consequence of muscle disuse may further compromise myofiber integrity through alterations in membrane permeability (39). The largest fold change indicated on the array (+ 66.2-fold) was for tissue inhibitor of metalloproteinase-1 (TIMP-1) mRNA, an inhibitor of several matrix metalloproteinases. Increased TIMP-1 protein may indicate the occurrence of enhanced extracellular matrix remodeling (39).

It is well documented that a contributing factor to skeletal muscle atrophy during disuse is the rapid decrease in protein synthesis rates (40). In accordance with these data, we have recently reported that MV results in decreased protein synthesis in the diaphragm after only 6 h that persists through at least 18 h of MV (13). Several mRNAs encoding eukaryotic translation initiation factors (eIFs) were upregulated in the diaphragm in response to MV, including eIF-5, eIF-2a, and eIF-2B. It is interesting to note that, despite a decreased rate of protein synthesis during MV, an increased expression of eIF mRNAs was observed. In addition, MV was associated with increased ribosomal protein L3 mRNA, a 40S ribosomal subunit. Conversely, increased eIF-4E binding protein 1 was upregulated in response to MV, a finding that is consistent with the observed decrease in the rate of protein synthesis in the diaphragm during MV (41). Overall, the diaphragm may upregulate the protein translation capacity after MV. However, increased expression of eIF-4E may contribute to the decreased protein synthesis that is actually observed after 6 to 18 h of MV (13).

Calcium Regulation

Dysregulation of intracellular calcium is considered to be a key factor in the progression of skeletal muscle atrophy during disuse (28, 42, 43). Intracellular calcium signaling is conveyed largely through calmodulin (CALM), a ubiquitous intracellular calcium receptor that regulates numerous cellular processes. Five transcripts, specified by three calmodulin genes (CALM1, CALM2, CALM3), encode a single CALM protein in mammals (44). In response to MV, differential expression was observed for CALM1, which increased, whereas CALM2 decreased. Because

CALM transcripts may exhibit differences in their subcellular localization, differential expression of the CALM genes may reflect a mechanism for the cell to adjust the subcellular redistribution and thus local regulation of the CALM protein (44). MV decreased transcript levels of calsequestrin 2, the cardiac isoform of calsequestrin expressed in cardiac and slow-twitch skeletal muscle (45). A decreased expression of sarco-endoplasmic reticulum calcium ATPase-3 mRNA was observed in response to 18 h of MV. A decreased ability of the cell to control cytoplasmic calcium may contribute to elevations in free calcium levels in the cytosol. In addition, mRNA encoding two subunits (α1S and γ1) of the L-type voltage-dependent calcium channel was decreased in response to MV. Although the α1S subunit forms the calcium conduction pore and detects voltage changes, the role of the γ1 subunit is not well understood (46). Decreased expression of the L-type calcium channel, particularly of the α1S subunit, could adversely affect the transmission of surface depolarization, thus leading to impaired excitation-contraction coupling and diaphragmatic contractile dysfunction. Also, MV decreased the expression of troponin I slow isoform mRNA after 18 h, potentially impacting the calcium sensitivity of the contractile apparatus (*see* online supplement).

MV Alters Energy Metabolism Pathways

Controlled MV results in complete diaphragmatic inactivity (3, 7, 8). Under such conditions, the metabolic requirements of the diaphragm are decreased, which likely impacts the rate of cellular energy production. Decreased mRNA of cytochrome C oxidase and subunits of the F0 and F1 ATP synthase complex were detected after MV. In addition, decreases in glycerol-3-phosphate dehydrogenase-2 mRNA levels, a mitochondrial enzyme responsible for the transfer of reducing equivalents to the mitochondria, were also observed. Lack of diaphragmatic contractile activity may reduce ATP utilization and explain the decreased expression of AMP protein kinase. In addition, MV was associated with decreased dihydroxypolyprenylbenzoate methyltransferase mRNA, an enzyme involved in ubiquinone synthesis. An additional regulator of mitochondrial ATP production decreased by MV was mitofusin 2 (Mfn2). Repression of Mfn2 in cultured myotubes leads to morphologic fragmentation of the mitochondrial network and reductions in O_2 consumption and glucose oxidation (47). Malate dehydrogenase 1, a citric acid cycle component, decreased in response to 18 h of MV. Thus, diaphragm unloading during MV downregulates mRNA expression of several mitochondrial components, possibly in response to a decreased energy demand.

Several genes encoding enzymes of fat metabolism were affected during MV, including long-chain fatty acid coenzyme A ligase (Facl), carnitine O-octanoyltransferase (COT), fatty acid binding protein 3 (FABP3), and stearoyl-CoA desaturase 2. Five isoforms of Facl have been identified in rats that demonstrate substrate specificity, differences in tissue distribution, and cellular location (48). Isoform 4 (Facl4) demonstrated elevated transcript levels, as opposed to isoforms 2 (Facl2) and 6 (Facl6), which demonstrated decreased levels after MV. Changes in the isoform composition with diaphragm unloading could lead to alterations in the regulation of fatty acid entry into synthetic or oxidative pathways (49). After 18 h of MV, COT decreased and this may result in a reduced ability to oxidize fatty acids in the mitochondria. Also, MV was associated with decreased levels of FABP3 mRNA, which may affect fatty acid uptake and utilization. Downregulation of stearoyl-CoA desaturase-2 after MV may reflect a decrease in lipid synthesis. Collectively, MV appears to downregulate lipid uptake and transport and possibly redirect fatty acid metabolism from the oxidative to the synthetic pathways.

Altered mRNA expression of proteins involved in the metabolism of glucose may indicate alterations in the use of this substrate for energy metabolism. Although GLUT4 mRNA was decreased after MV, elevations in the mRNA expression of GLUT1 and hexokinase II were observed. In addition, MV increased mRNA encoding phosphoglycerate mutase, which may result in an enhanced ability to derive reducing equivalents from glucose. Conversely, phosphoglucomutase-1 and phosphorylase kinase, proteins involved in glycogen turnover, were downregulated after MV. It is interesting to note that MV downregulated two genes involved in a key regulatory step of the glycolysis/gluconeogenesis pathway (fructose-1,6-bisphosphatase and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-1), especially since skeletal muscle is not considered to be a major site of glucose synthesis. Overall, MV may decrease the energy demand of the diaphragm and thus alter mRNA expression patterns of many genes encoding key energy metabolism enzymes.

Disuse-induced mRNA Alterations in the Diaphragm versus Locomotor Skeletal Muscle

Although several recent studies have used microarray analysis to explore alterations in the gene expression profile of atrophying skeletal muscles, a meaningful comparison between many prior studies and the current investigation is not possible due to experimental differences in animal models, differing durations of muscle unloading, and variance in the microarray methods. However, a direct comparison between the global mRNA responses of soleus and diaphragm muscle to mechanical unloading is now possible because we recently completed a locomotor muscle study using a very similar experimental design to the current study (Table 6) (21). More specifically, the two experiments used a similar analysis of Affymetrix microarrays, young adult, female Sprague-Dawley rats, and a comparable duration of muscle unloading. Our locomotor muscle study used the hind-limb suspension model to mechanically unload locomotor skeletal muscles. Hind-limb suspension is a well established model to reduce energy demand and contractile activity in hind-limb skeletal muscles. The present study, coupled with our previous investigation, is important because it provides a direct comparison at the genomic level of differentially expressed genes between the rat diaphragm and soleus muscle during similar durations of mechanical unloading.

Differentially expressed genes common to both the unloaded diaphragm and soleus muscles include genes that are not only related to atrophy but other functional categories, including cell growth and/or maintenance, signal transduction, extracellular matrix, energy metabolism, and structural proteins. Several genes common to both the unloaded diaphragm and soleus muscles demonstrated similar changes in both magnitude and direction. Most notably, cathepsin L mRNA was augmented in both muscles and has been shown to be upregulated in several models of muscle atrophy. Also upregulated was decorin, a highly abundant proteoglycan found in the extracellular matrix of skeletal muscle, which appears to be regulated by motor neuron–stimulated muscle activity (50). Growth arrest–specific 5 is a transcript expressed during the growth arrest phase of the cell cycle that was upregulated to a similar degree in both the diaphragm and soleus. An additional gene also involved in cell cycle arrest is growth arrest and Gadd45α, although more dramatic increases were detected in the unloaded diaphragm compared with the soleus. In addition, similar changes between the unloaded diaphragm and soleus were also noted for vascular endothelial growth factor, a mitogen that acts on endothelial cells to promote the formation of new blood vessels. Similarly, downregulation was also observed with protein tyrosine phosphatase, nonreceptor type 16 (dual specificity), in both muscles. Protein

tyrosine phosphatases inactivate mitogen-activated protein kinase signaling by hydrolyzing the phosphate monoesters of tyrosine residues of p38, c-Jun N-terminal kinase, and, in some instances, extracellular signal-regulated kinase (51).

Several genes differentially expressed in both the unloaded diaphragm and soleus responded in a reciprocal fashion. For example, six of the eight common genes demonstrated downregulated expression in the soleus compared with upregulation in the diaphragm. Included in this list was ankyrin-like repeat protein, which was the most responsive gene in the list of 354 differentially expressed transcripts in the unloaded diaphragm. Interestingly, this gene is also expressed in cardiomyocytes and acts as a negative regulator of cardiac-specific gene expression. Additional transcripts demonstrating reciprocal expression were genes associated with energy metabolism (6-phosphofructo-2 kinase/fructose 2,6-biphosphatase 1 and phosphoglycerate mutase type B), structural proteins (a-tubulin), apoptosis (a-prothymosin), stress response (sulfotransferase family 1A), and signal transduction (ras-related protein).

Collectively, the observed differences in global gene expression between the unloaded diaphragm and soleus muscles are consistent with the reported differences in the rate of disuse muscle atrophy between these two muscles. Furthermore, also highlighted by comparisons made between the unloaded diaphragm and soleus are the similar as well as dissimilar alterations in gene expression. These comparisons reflect not only how these two muscles respond in a similar fashion but also how they respond differently to mechanical unloading. Indeed, an assessment of these two studies provides for the first time a direct comparison at the genomic level of differentially expressed genes between the rat diaphragm and soleus muscle during similar durations of mechanical unloading.

Critique of Experimental Model

Many adult patients requiring MV are maintained on some form of pressure-assist MV. To investigate the effects of MV on diaphragmatic gene expression, we chose to use controlled MV because this mode of MV results in no diaphragmatic contractile activity and because controlled MV has relevance in several clinical situations. For example, controlled MV is used in adult patients in numerous circumstances (e.g., drug overdose, spinal cord injury, and surgery) and is also commonly used in pediatric patients (52). In addition, we chose sodium pentobarbital as the general anesthetic in these experiments because of direct evidence that this anesthetic does not negatively impact diaphragmatic atrophy or contractile dysfunction and does not promote oxidative injury (3, 5, 6).

An important consideration in the present experiments is the potential impact of the anesthetic on gene expression levels. Our decision to use acutely anesthetized animals as controls instead of spontaneously breathing animals was based on two main factors. First, anesthetized, spontaneously breathing animals can become hypoxic and hypercapnic, and develop arterial (respiratory-induced) acidosis. Although these factors do not result in impaired diaphragm contractile properties *in vitro*, there is the potential for these factors to alter gene expression. Second, sodium pentobarbital suppresses respiratory drive and decreases alveolar ventilation, resulting in hypoventilation. It follows that the hypoventilation results in a significant reduction in the work of breathing. Hence, a "perfect" MV control group does not exist. Therefore, by comparing MV to acutely anesthetized control animals, we are, in effect, reporting the combined effects of the anesthetic and diaphragm unloading that are experienced during MV. Nonetheless, we strongly believe that this investigation provides new and unique insight into the influence of controlled MV on global gene expression patterns in the diaphragm.

Conclusions

This study identified the differential expression of mRNA transcripts in the diaphragm in response to 6 and 18 h of MV. We identified 420 probe sets (i.e., 354 unique gene products) displaying at least a ± 1.5-fold change with respect to control animals at the $p < 0.001$ level. Genes could be categorized into 19 functional categories, of which we chose to focus on five categories with relevance to MV-induced diaphragm dysfunction. Stress-response genes demonstrated the largest changes, which likely illustrate protective adaptations in response to stressors, including increased oxidant production. Clearly, the data contained in these experiments provide further insight into molecular adaptations that occur in the diaphragm during controlled MV, which ultimately may assist scientists and clinicians in the development of countermeasures to retard MV-induced diaphragmatic atrophy and contractile dysfunction.

Conflict of Interest Statement: None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

1. Chevrolet JC. Difficult weaning from mechanical ventilation. *Lung* 1990;168:829–832.
2. Le Bourdelles G, Viires N, Boczkowski J, Seta N, Pavlovic D, Aubier M. Effects of mechanical ventilation on diaphragmatic contractile properties in rats. *Am J Respir Crit Care Med* 1994;149:1539–1544.
3. Powers SK, Shanely RA, Coombes JS, Koesterer TJ, McKenzie M, Van Gammeren D, Cicale M, Dodd SL. Mechanical ventilation results in progressive contractile dysfunction in the diaphragm. *J Appl Physiol* 2002;92:1851–1858.
4. Sassoon CS, Caiozzo VJ, Manka A, Sieck GC. Altered diaphragm contractile properties with controlled mechanical ventilation. *J Appl Physiol* 2002;92:2585–2595.
5. Shanely RA, Coombes JS, Zergeroglu AM, Webb AI, Powers SK. Short-duration mechanical ventilation enhances diaphragmatic fatigue resistance but impairs force production. *Chest* 2003;123:195–201.
6. Shanely RA, Zergeroglu MA, Lennon SL, Sugiura T, Yimlamai T, Enns D, Belcastro A, Powers SK. Mechanical ventilation-induced diaphragmatic atrophy is associated with oxidative injury and increased proteolytic activity. *Am J Respir Crit Care Med* 2002;166:1369–1374.
7. Sassoon CS, Zhu E, Caiozzo VJ. Assist-control mechanical ventilation attenuates ventilator-induced diaphragmatic dysfunction. Am J Respir Crit Care Med 2004.
8. Tobin MJ, Jubran A, Hines E Jr. Pathophysiology of failure to wean from mechanical ventilation. *Schweiz Med Wochenschr* 1994;124:2139–2145.
9. Zergeroglu MA, McKenzie MJ, Shanely RA, Van Gammeren D, DeRuisseau KC, Powers SK. Mechanical ventilation-induced oxidative stress in the diaphragm. *J Appl Physiol* 2003;95:1116–1124.
10. Zhan WZ, Miyata H, Prakash YS, Sieck GC. Metabolic and phenotypic adaptations of diaphragm muscle fibers with inactivation. *J Appl Physiol* 1997;82:1145–1153.
11. Geiger PC, Bailey JP, Zhan WZ, Mantilla CB, Sieck GC. Denervation-induced changes in myosin heavy chain expression in the rat diaphragm muscle. *J Appl Physiol* 2003;95:611–619.
12. Geiger PC, Cody MJ, Macken RL, Bayrd ME, Sieck GC. Effect of unilateral denervation on maximum specific force in rat diaphragm muscle fibers. *J Appl Physiol* 2001;90:1196–1204.
13. Shanely RA, Van Gammeren D, DeRuisseau KC, Zergeroglu AM, McKenzie MJ, Yarasheski KE, Powers SK. Mechanical ventilation depresses protein synthesis in the rat diaphragm. *Am J Respir Crit Care Med* 2004;170:994–999.
14. Betters JL, Criswell DS, Shanely RA, Van Gammeren D, Falk D, Deruisseau KC, Deering M, Yimlamai T, Powers SK. Trolox attenuates mechanical ventilation-induced diaphragmatic dysfunction and proteolysis. *Am J Respir Crit Care Med* 2004;170:1179–1184.
15. Thomason DB, Biggs RB, Booth FW. Protein metabolism and beta-myosin heavy-chain mRNA in unweighted soleus muscle. *Am J Physiol* 1989;257:R300–R305.
16. Yang L, Luo J, Bourdon J, Lin MC, Gottfried SB, Petrof BJ. Controlled mechanical ventilation leads to remodeling of the rat diaphragm. *Am J Respir Crit Care Med* 2002;166:1135–1140.

17. Gayan-Ramirez G, de Paepe K, Cadot P, Decramer M. Detrimental effects of short-term mechanical ventilation on diaphragm function and IGF-I mRNA in rats. *Intensive Care Med* 2003;29:825–833.
18. DeRuisseau KC, Kavazis AN, Deering MA, Falk DJ, Van Gammeren D, Yimlamai T, Ordway GA, Powers SK. Mechanical ventilation induces alterations of the ubiquitin-proteasome pathway in the diaphragm. *J Appl Physiol* 2005;98:1314–1321.
19. Zhu E, Sassoon CSH, Nelson R, Pham HT, Zhu L, Baker MJ, Caiozzo VJ. The early effects of mechanical ventilation on isotonic contractile properties and MAF-box gene expression in the diaphragm. *J Appl Physiol* 2005;99:747–756.
20. Stevenson EJ, Giresi PG, Koncarevic A, Kandarian SC. Global analysis of gene expression patterns during disuse atrophy in rat skeletal muscle. *J Physiol* 2003;551:33–48.
21. Bey L, Akunuri N, Zhao P, Hoffman EP, Hamilton DG, Hamilton MT. Patterns of global gene expression in rat skeletal muscle during unloading and low-intensity ambulatory activity. *Physiol Genomics* 2003;13:157–167.
22. Cros N, Tkatchenko AV, Pisani DF, Leclerc L, Leger JJ, Marini JF, Dechesne CA. Analysis of altered gene expression in rat soleus muscle atrophied by disuse. *J Cell Biochem* 2001;83:508–519.
23. Wittwer M, Fluck M, Hoppeler H, Muller S, Desplanches D, Billeter R. Prolonged unloading of rat soleus muscle causes distinct adaptations of the gene profile. *FASEB J* 2002;16:884–886.
24. Pattison JS, Folk LC, Madsen RW, Childs TE, Spangenburg EE, Booth FW. Expression profiling identifies dysregulation of myosin heavy chains IIb and IIx during limb immobilization in the soleus muscles of old rats. *J Physiol* 2003;553:357–368.
25. Pattison JS, Folk LC, Madsen RW, Booth FW. Selected contribution: identification of differentially expressed genes between young and old rat soleus muscle during recovery from immobilization-induced atrophy. *J Appl Physiol* 2003;95:2171–2179.
26. Shanely RA, Van Gammeren D, DeRuisseau KC, Zergeroglu AM, McKenzie M, Popp M, Powers SK. Mechanical ventilation-induced transcriptional alterations in the diaphragm [abstract]. *Am J Respir Crit Care Med* 2003;167:A417.
27. Holzberg D, Albrecht U. The circadian clock: a manager of biochemical processes within the organism. *J Neuroendocrinol* 2003;15:339–343.
28. Kondo H, Miura M, Nakagaki I, Sasaki S, Itokawa Y. Trace element movement and oxidative stress in skeletal muscle atrophied by immobilization. *Am J Physiol Endocrinol Metab* 1992;262:E583–E590.
29. Appell HJ, Duarte JA, Soares JM. Supplementation of vitamin E may attenuate skeletal muscle immobilization atrophy. *Int J Sports Med* 1997;18:157–160.
30. Powers SK, Kavazis AN, DeRuisseau KC. Mechanisms of disuse muscle atrophy: role of oxidative stress. *Am J Physiol Regul Integr Comp Physiol* 2005;288:R337–R344.
31. Ikemoto M, Nikawa T, Kano M, Hirasaka K, Kitano T, Watanabe C, Tanaka R, Yamamoto T, Kamada M, Kishi K. Cysteine supplementation prevents unweighting-induced ubiquitination in association with redox regulation in rat skeletal muscle. *Biol Chem* 2002;383:715–721.
32. Fujii J, Ikeda Y. Advances in our understanding of peroxiredoxin, a multifunctional, mammalian redox protein. *Redox Rep* 2002;7:123–130.
33. Brownson C, Loughna P. Alterations in the mRNA levels of two metabolic enzymes in rat skeletal muscle during stretch-induced hypertrophy and disuse atrophy. *Pflugers Arch* 1996;431:990–992.
34. Raisanen SR, Lehenkari P, Tasanen M, Rahkila P, Harkonen PL, Vaananen HK. Carbonic anhydrase III protects cells from hydrogen peroxide-induced apoptosis. *FASEB J* 1999;13:513–522.
35. Vile G, Basu-Modak S, Waltner C, Tyrrell R. Heme oxygenase 1 mediates an adaptive response to oxidative stress in human skin fibroblasts. *Proc Natl Acad Sci USA* 1994;91:2607–2610.
36. Hussain S, Slikker W Jr, Ali SF. Role of Metallothionein and other antioxidants in scavenging superoxide radicals and their possible role in neuroprotection. *Neurochem Int* 1996;29:145–152.
37. Taillandier D, Aurousseau E, Meynial-Denis D, Bechet D, Ferrara M, Cottin P, Ducastaing A, Bigard X, Guezennec CY, Schmid HP, *et al.* Coordinate activation of lysosomal, Ca 2+-activated and ATP-ubiquitin-dependent proteinases in the unweighted rat soleus muscle. *Biochem J* 1996;316:65–72.
38. Jones SW, Hill RJ, Krasney PA, O'Conner B, Peirce N, Greenhaff PL. Disuse atrophy and exercise rehabilitation in humans profoundly affects the expression of genes associated with the regulation of skeletal muscle mass. *FASEB J* 2004;18:1025–1027.
39. Reznick AZ, Menashe O, Bar-Shai M, Coleman R, Carmeli E. Expression of matrix metalloproteinases, inhibitor, and acid phosphatase in muscles of immobilized hindlimbs of rats. *Muscle Nerve* 2003;27:51–59.
40. Booth FW, Seider MJ. Early change in skeletal muscle protein synthesis after limb immobilization of rats. *J Appl Physiol* 1979;47:974–977.
41. Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ, *et al.* Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat Cell Biol* 2001;3:1014–1019.
42. Ingalls CP, Warren GL, Armstrong RB. Intracellular Ca2+ transients in mouse soleus muscle after hindlimb unloading and reloading. *J Appl Physiol* 1999;87:386–390.
43. Soares JM, Duarte JA, Carvalho J, Appell HJ. The possible role of intracellular Ca2+ accumulation for the development of immobilization atrophy. *Int J Sports Med* 1993;14:437–439.
44. Toutenhoofd SL, Strehler EE. The calmodulin multigene family as a unique case of genetic redundancy: multiple levels of regulation to provide spatial and temporal control of calmodulin pools? *Cell Calcium* 2000;28:83–96.
45. Arai M, Alpert NR, Periasamy M. Cloning and characterization of the gene encoding rabbit cardiac calsequestrin. *Gene* 1991;109:275–279.
46. Wang MC, Dolphin A, Kitmitto A. L-type voltage-gated calcium channels: understanding function through structure. *FEBS Lett* 2004;564:245–250.
47. Bach D, Pich S, Soriano FX, Vega N, Baumgartner B, Oriola J, Daugaard JR, Lloberas J, Camps M, Zierath JR, *et al.* Mitofusin-2 determines mitochondrial network architecture and mitochondrial metabolism: a novel regulatory mechanism altered in obesity. *J Biol Chem* 2003;278:17190–17197.
48. Lewin TM, Kim JH, Granger DA, Vance JE, Coleman RA. Acyl-CoA synthetase isoforms 1, 4, and 5 are present in different subcellular membranes in rat liver and can be inhibited independently. *J Biol Chem* 2001;276:24674–24679.
49. Coleman RA, Lewin TM, Muoio DM. Physiological and nutritional regulation of enzymes of triacylglycerol synthesis. *Annu Rev Nutr* 2000;20:77–103.
50. Brandan E, Fuentes ME, Andrade W. Decorin, a chondroitin/dermatan sulfate proteoglycan is under neural control in rat skeletal muscle. *J Neurosci Res* 1992;32:51–59.
51. Clark AR. MAP kinase phosphatase 1: a novel mediator of biological effects of glucocorticoids? *J Endocrinol* 2003;178:5–12.
52. Hess D, Kacmarek R. Essentials of mechanical ventilation. New York: McGraw-Hill; 1996.