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Energy metabolism pathways in rat muscle under conditions of simulated microgravity

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Abstract

Evidence from rats flown in space suggests that there is a decrease in the ability of the soleus muscle to oxidize long chain fatty acids during space flight. The observation suggests that a shift in the pathways involved in muscle fuel utilization in the absence of load on the muscle has occurred. It is also possible that the reduction is part of a general down-sizing of metabolic capacity since energy needs of inactive muscle are necessarily less. The rodent hind limb suspension model has proved to be a useful ground based model for studying the musculo-skeletal systems changes that occur with space flight. Microarray technology permits the screening of a large number of the enzymes of the relevant pathways thereby permitting a distinction to be made between a shift fuel utilization pattern or a general decrease in metabolic activity. The soleus muscle was isolated from 5 control and 5 hindlimb suspended rats (21 days) and the Affymetrix system for assessing gene expression used to determine the impact of hindlimb unloading on fuel pathways within the muscle of each animal. Results: Suspended rats failed to gain weight at the same rate as the controls (337 ± 5 g vs 318 ± 6 g, $p < 0.05$) and muscle mass from the soleus was reduced (135 ± 3 mg vs 48 ± 4 mg, $p < 0.05$). There was a consistent decrease ($p < 0.05$) in gene expression of proteins involved in fatty acid oxidation in the suspended group whereas glycolytic activity was increased ($p < 0.05$). Gene expressions of individual key regulatory enzymes reflected these changes. Carnitine palmitoyltransferase I and II were decreased ($p < 0.05$) whereas expression of hexokinase, phosphofructokinase and pyruvate kinase were increased ($p < 0.05$). Conclusion: Disuse atrophy is associated with a change in mRNA levels of enzymes involved in fuel metabolism indicative of a shift in substrate utilization away from fat towards glucose.

Keywords: Muscle atrophy; mRNA; Microarray; Space flight; β-oxidation; Glycolysis

1. Introduction

Space flight is associated with profound changes in metabolism. The major changes are associated with the reductive remodeling of the weight bearing components of the musculo-skeletal system. There is also some evidence suggesting that space flight induces changes in intermediary metabolism in the affected muscles. Baldwin et al reported that the ability of the soleus muscle to oxidize long chain fatty acids is decreased in space flown rats [3].

This paper explores the use of microarray gene expression technology as an approach to investigating the reason for this apparent shift in fuel preferences using the ground based rat hind limb suspension (HLS) model. Microarray technology yields large amounts of data from very little material in a single experiment. This is particularly important for studying the response of rodent tissues to space flight because tissue sample availability is very limited and what there is usually has to be divided between several investigator teams. There is therefore a premium on using methods that maximize the return from minimal amounts of material.

In the few studies where lipid metabolism has been investigated in ground models of space flight (bed rest,

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HLS), fatty acid oxidation was reduced [1,3,15,33]. With bed rest, both Hikeda et al. and Ferrerti et al. found 3 hydroxyacyl CoA dehydrogenase, a key enzyme in muscle fatty acid oxidation to be decreased in the vastus lateralis muscle [14,22]. Other studies on rat muscles from hind limb, unloaded (HLS) rats found an increase in the capacity to transport glucose [3,37].

Collectively these observations suggest that there has been a shift in the metabolic pathways associated with fuel metabolism away from lipid and towards glucose. There is no information on whether the decreased ability to oxidize long chain fatty acids is due to a decrease in the activity of a specific rate controlling enzyme or a general reduction in the activity in the enzymes involved in β oxidation [3]. Another possibility is that the reduction is part of a general down-sizing of metabolic capacity since energy needs of inactive muscle are necessarily less. Finally it is also possible that this is an effect of space flight that is not reproduced on the ground by the HLS model [18]. Overall the rodent hind limb suspension model has proved to be a useful ground based model for studying the muscle systems changes that occur with space flight and bed rest [31].

The primary objective of this experiment was to evaluate the utility of the Affymetrix microarray analysis of gene expression for investigating the changes in fuel processing in rat muscle that occur in response to chronic unloading using the number of rats that can be flown on a flight experiment. Microarray technology offers the opportunity to assess gene expression for a large number of genes in a single experiment. The secondary objective was to use this methodology to distinguish between (i) a shift in fuel utilization pattern, (ii) a general decrease in metabolic activity or (iii) no effect in the HLS rat model.

2. Methods of procedure

2.1. Experimental animals

The experiment was conducted using ten male Sprague-Dawley derived albino rats (Simonsen Laboratories, Gilroy, CA). Throughout the study the rats were kept on a reversed 12:12-h light-dark cycle and maintained at 23 ± 1°C. After 8 days to acclimate to the new environment the animals were place in individual metabolic suspension cages [19]. Food (powdered chow, Purina, diet #5012) and distilled water were provided ad libitum. Animals were randomly assigned to control or hindlimb suspension (HLS) treatment groups (n = 5 per group). At the time of assignment to treatment groups the animals weighed 203 ± 1.6 g for controls and 202 ± 1.9 g for HLS**.** The HLS group was suspended using the tail casting procedure of Morey-Holton and Wronski [28]. All animals were monitored daily. On day 21 of the experiment the animals were euthanized by an overdose of anesthetic and the soleus muscles removed, weighed and frozen in liquid nitrogen for later analysis. Tissues were stored at −70°C. Animal care and use were in accord with the Ames Research Center Users Guide (AHB 7180) and the NIH Guide for the Care and Use of Laboratory Animals and were approved by the institutional Animal Care and Use Committee.

2.2. mRNA analysis

The total mRNA was isolated from the soleus muscle using the RNeasy Mini Kit (Qiagen, Vallencia, CA). Double stranded cDNA was synthesized from the total mRNA using the Gibco BRL Super Script Choice System (Life Technologies, Inc., Rockville MD). In vitro transcription (IVT) was performed with the BioArray High Yield RNA transcript Labeling Kit (Enzo Diagnostics, Farmingdale NY) to yield biotinylated cRNA from the synthesized cDNA. The resultant biotinylated RNA was then fragmented and hybridized to the Affymetrix U34A rat genome microarray chip (Affymetrix Inc., Santa Clara, CA).

3. Data analysis

The Affymetrix GeneChip Expression analysis software (version 3.2, Affymetrix Inc., Santa Clara, CA) was used to normalize the data. The process involves dropping the highest and lowest 2% intensities, summing the remainder and normalizing the set such that the mean intensity is 2500. We then manually searched through data set for all probes identifying enzymes in glycolysis, fatty acid oxidation and the TCA cycle.

Two criteria to minimize inclusion of false positives were used. Firstly we eliminated all probes from further consideration where the mean minus 3.54 times the SEM (4 standard deviations) was greater than zero (99.99% confidence limit) for either the cases or the controls. Secondly, we examined the data for outliers, with an outlier being defined as a data point more than 2.5 SD away from the mean. There were none. The remainder of the data analysis was done on this limited data set.

To determine whether the overall pathway was changed we calculated the Z score for each data point from the relationship Z = (individual rat probe intensity − overall mean)/(mean SD) where probe intensity is the intensity value for a specific cell, the overall mean is the mean intensity value for a probe for all ten rats (controls + HLS) and the mean standard deviation. $Z = (SD_{controls} + SD_{HLS})/2$. The mean value of the Z scores for a given pathway for each rat was calculated to give the aggregate mean Z score. t-tests with $\alpha = 0.05$ were then used to determine whether there was a difference in the aggregate pathway mean Z scores between the controls and the HLS rats. If the aggregate means were different, the significance of any differences in the intensity data for the individual probes was determined by two tailed t-tests with $\alpha = 0.05$. The analysis was repeated using the log transformed intensity values.

As an internal control we also examined the shifts in the expression of myofibril isoforms: myosin heavy chain, myosin light chain, tropomyosin, and troponin. Here the direction of change of myofibril expression had been established by other methods beyond any reasonable doubt [5,34].

4. Results

Data was obtained from 5 control and 5 HLS rats. No rats were dropped from the analysis. The suspended rats had a reduced rate of body mass gain (final body weights, 337 ± 5 vs 305 ± 10 g, $p < 0.05$). Soleus muscle mass was decreased with HLS when expressed either as the total weight (135 ± 3 vs 48 ± 4 mg, $p < 0.05$) or relative to 100 g of body weight (40 ± 1 vs 16 ± 2 mg/100 g BW, ($p < 0.05$).

Food intake was not measured in this experiment. However we and others using the NASA Ames research facility have measured food intake and weight gain on rats run on the same protocol [29,38]. Briefly, there is an initial 2–3 day lag during which intake is depressed, thereafter both energy intake (5.4 ± 0.03 vs 5.4 ± 0.04 g. $d^{-1} \cdot 100$ g body wt) and fluid intake (10.3 + 0.24 vs 9.9 + 0.3 ml. $d^{-1} \cdot 100$ g body wt.) are the same for control and HLS rats [38].

Fifty-three probes of interest were identified of which 33 passed the 99% confidence limit test. One probe was excluded because some of the individual values failed to be within 2.5 SD of the mean. This left a set of 32 probes ($n = 18$ for fatty acid oxidation and $n = 14$ for glycolysis). The mean coefficient of variation (100 * SD/mean) for the five control rats was 16 ± 1%, and for the five HLS rats 21 ± 2%. Figs. 1a and 1b show the aggregate mean Z scores for the fatty acid (Fig. 1a) and glycolytic (Fig. 1b) pathways for the 10 rats. It is apparent from both figures that the HLS rats are very different from the controls and that the figure for the fatty acid oxidation is the opposite of the figure for glycolysis. In both cases the aggregate mean Z scores for the HLS rats are significantly different from the control values ($p < 0.05$). The same results are found if the logarithms of the intensity values are used in the Z score calculations.

Tables 1 and 2 give the mean intensity values, the difference in intensity between HLS and controls, their significance and the percentage change. Since both pathways were significantly altered ($p < 0.05$) with unloading, the tables give the individual probe comparisons as calculated by two tailed t-tests.

The results for fatty acid metabolism are consistent. Where change was found, the change was towards a reduction in activity (Table 1, Fig. 1a). The capacity for fatty acid oxidation was decreased at both the level of entry into the mitochondria (decreased carnitine acyl transferase activity) and the expression of all the enzymes in the β oxidation pathway (Table 1). Also decreased is the expression of enzymes involved in the oxidation of unsaturated fatty acids

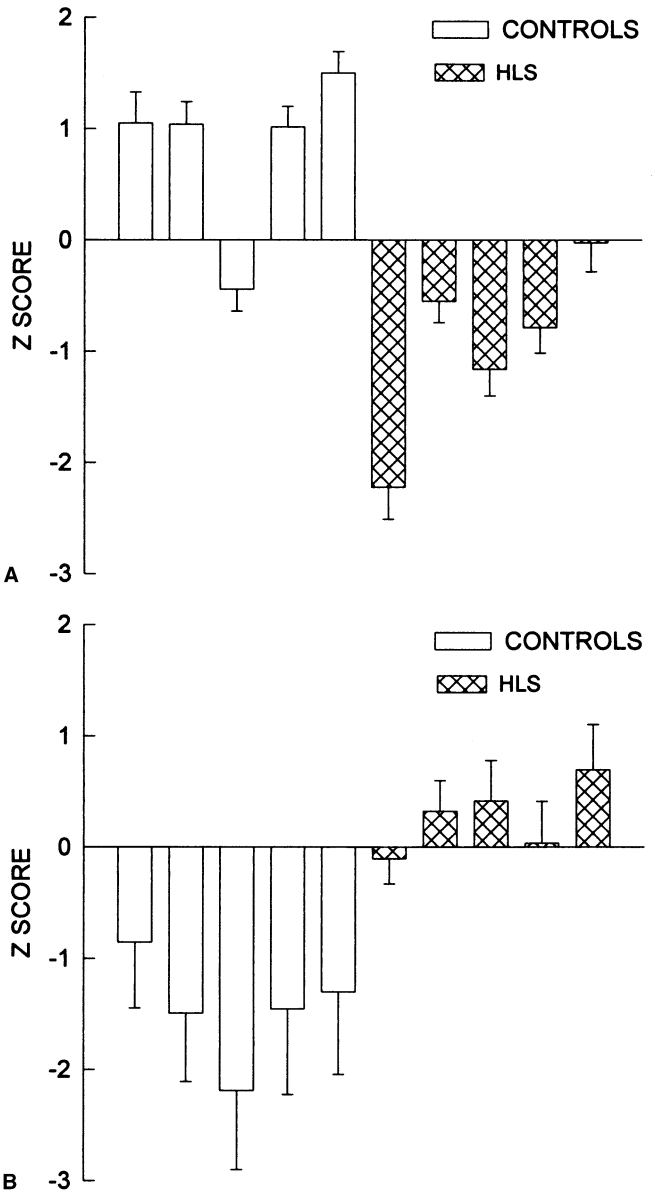

Fig. 1a. (top). Aggregate Z scores for the 10 rats (5 controls, 5 HLS) for genes expressed from the fatty acid oxidation pathway. The difference between the control and HLS rats is statistically significant ($p < 0.05$). Fig. 1b (bottom). Aggregate Z scores for the 10 rats (5 controls, 5 HLS) for genes expressed from glycolytic pathway. The difference between the control and HLS rats is statistically significant ($p < 0.05$). Each bar represents the data from a single rat.

($p < 0.05$, Table 1) and a trend towards a decrease in a minor pathway, the peroxisomal pathway (Table 1).

In contrast to the decrease in lipid oxidative capacity, there was an increase in the expression of genes involved in glycolysis ($p < 0.05$, Table 2, Fig. 1a). In particular, the gene expression of Hexokinase and Phosphofructokinase ($p < 0.05$), key regulatory enzymes for the glycolytic pathway were increased (Table 2). For pyruvate kinase, the chip only detected the M1 and M1 subunit genes. We assumed that this was a surrogate for the pyruvate kinase enzyme as a whole. The expression analysis process did not detect

Table 1
Change in expression of enzymes involved in fatty acid oxidation

Description	NCBI Accession #	Intensity Control	Intensity HLS	Intensity Change	P<	% Change
TRANSPORT						
Carnitine palmitoyltransferase I	D43623(2)	31019 ± 23	19805 ± 26	−11214	0.05#	−36
Carnitine palmitoyltransferase II	J05470	11861 ± 9	6308 ± 9	−5553	0.01#	−47
Mean		214	130	−8384	0.01*	−41
SEM		95	67	2831		5
β OXIDATION, SATURATED FATTY ACIDS						
Long-chain acyl-CoA synthetase	D90109	11200 ± 13	6973 ± 8	−4228	0.05#	−38
Very long chain Acyl-CoA dehydrogenase	D30647	8516 ± 6	5888 ± 6	−2628	0.05#	−31
Long chain acyl-CoA dehydrogenase	J05029	35109 ± 47	21625 ± 30	−13484	0.05#	−38
Medium chain acyl coenzyme A dehydrogenase	J02791	26648 ± 38	19718 ± 33	−6930	ns	−26
Short chain acyl-coenzyme A dehydrogenase	J05030	7184 ± 6	5466 ± 6	−1717	ns	−24
Enoyl-CoA hydratase	X15958	16278 ± 12	14651 ± 24	−1627	ns	−10
Long-chain enoyl-CoA hydratase/	D16478(2)					
3-hydroxyacyl-CoA dehydrogenase α-subunit		29494 ± 30	18112 ± 30	−11382	0.01#	−39
Long-chain 3-ketoacyl-CoA thiolase β-subunit	D16479	29043 ± 34	13681 ± 11	−15362	0.05#	−53
3-oxoacyl-CoA thiolase	X05341	34078 ± 21	23131 ± 28	−10947	0.05#	−32
Mean		219	143	−7590	0.05*	−32
SEM		37	22	1776		4
β OXIDATION, UNSATURATED FATTY ACIDS						
Acetoacetyl-CoA thiolase	D00512	22763 ± 20	22025 ± 38	−738	ns	−3
Delta-3, delta 2-enoyl-CoA isomerase	D00729	31173 ± 15	16416 ± 19	−14757	0.01#	−47
Alpha-methylacyl-CoA racemase	U89905	1336 ± 1	1056 ± 1	−280	ns	−21
Mean		184	131	−5258	ns	−24
SEM		88	62	4751		13
PEROXISOMAL OXIDATION						
3-ketoacyl-CoA thiolase	J02749	1518 ± 1	2157 ± 2	−640	0.05#	42
Acyl-CoA oxidase	J02752	4871 ± 6	4524 ± 5	347	ns	−7
Enoyl hydratase	U08976	15159 ± 10	12108 ± 15	3051	ns	−20
Very-long-chain acyl-CoA thioesterase	Y09333	1874 ± 2	924 ± 1	951	0.01#	−51
Mean		58	49	927	ns	−9
SEM		31	25	780		19
ALL DATA COMBINED						
Mean		177	119	−5809	0.05*	−27
SEM		28	18	1309		5

Explanation of column headings. (1) NCBI accession number is the National Center for Biotechnology Information accession number. (2) The control ± SEM, HLS ± SEM and mean change in intensities are the normalized intensity values for the control rats, HLS rats and the difference between them. (3) The first % change column is the change in intensity from the control expressed as a percentage, the second column is the mean percentage change for that particular enzyme step. (4) The overall statistic for a pathway was calculated from the aggregate Z scores (*), statistical significance for individual probes were calculated by two tailed t-tests (#).

enough of the TCA enzymes (n = 2) that met our stringent criteria to assess TCA cycle capability.

The myofibril expression data is summarized in Table 3. Identification of the myosin isoforms type was made by copying the sequence data from the NCBI data base and then using the NCBI–'BLAST' program to identify the protein. As predicted from the literature there was a shift in myofibril isoforms indicative of a change in muscle fiber type from slow twitch oxidative to fast twitch glycolytic (Table 3, [2,36]).

5. Discussion

5.1. Data interpretation

There is a good connection between a gene product and its expression pattern. Genes are only expressed in cells when the product of the gene expression in some way contributes to the activity of the cell [7]. mRNA expression analysis of metabolic pathways status is particularly useful for studying chronic adapted states since metabolic regulation in the long term is primarily by changes in protein concentration rather than allosteric or endocrine effects [39, 40]

The magnitude of the changes found is reasonable, for the most part less than two fold (Tables 1–2). Unlike studies with bacteria and of cells in tissue culture, where large changes are often found, changes of a similar magnitude are not to be expected with adult rat tissues in a new steady state following acclimation. The manufacturer, Affymetrix suggests that only individual changes greater than two fold are likely to be credible because of the low power due to the very large data base involved. Most adaptive changes in rats are not that large, this decreases the utility of the system for

Table 2
Change in expression of enzymes involved in glycolysis

Description	NCBI Accession #	Intensity Control	Intensity HLS	Intensity Change	P<	% Change	% Change
Hexokinase II	S56464 (2)	3380 ± 214	8463 ± 1637	5083	0.05#	150	
Hexokinase II	M68971	2374 ± 435	3377 ± 503	1003	ns	42	96
Phosphoglucomutase	L11694	11598 ± 584	22303 ± 3769	10705	0.05#	92	92
Phosphofructokinase (muscle)	U25651	41675 ± 1907	59747 ± 5919	18072	0.05#	43	
Phosphofructokinase (muscle)	D21869	3743 ± 923	10169 ± 1871	6426	0.05#	172	108
Aldolase A	M12919 (2)	174586 ± 11283	107721 ± 15610	−66866	0.01#	−38	
Aldolase C	X06984	1748 ± 159	3874 ± 420	2126	0.01#	122	42
Glyceradlehyde-3-phosphate dehydrogenase	M17701	151070 ± 10085	94129 ± 12659	−56941	0.01#	−38	−38
Phosphoglycerate kinase	M31788 (2)	12664 ± 430	13273 ± 833	609	ns	5	5
Phosphoglycerate mutase	S63233 (2)	4416 ± 313	3492 ± 316	−924	0.05#	−21	
Phosphoglycerate mutase	Z17319	15369 ± 1002	26338 ± 3577	10969	0.05#	71	25
Enolase	X02610 (2)	14489 ± 1465	15800 ± 1240	1311	ns	9	9
Pyruvate kinase	AA818951	34315 ± 2156	70820 ± 2990	36505	106	106	106
Mean		36264	33808	−2456	0.01*	55	49
SEM		15996	10134	7847		20	18

Explanation of column headings. (1) NCBI accession number is the National Center for Biotechnology Information accession number. (2) The control ± SEM, HLS ± SEM and mean change in intensities are the normalized intensity values for the control rats, HLS rats and the difference between them. (3) The first % change column is the change in intensity from the control expressed as a percentage, the second column is the mean percentage change for that particular enzyme step. (4) The overall statistic for a pathway was calculated from the aggregate Z scores (*), statistical significance for individual probes were calculated by two tailed t-tests (#).

detecting individual mRNA changes, but does not invalidate its' use for examining expression associated with a defined pathway where a cumulative pattern of change across several linked mRNAs, can be searched for.

Microarray experiments yield huge amounts of data and usually there is not enough statistical power for a rigorous statistical analysis of the data set when treated as a whole. Two factors contributed to simplifying the data analysis in this experiment. Firstly, we were testing a specific hypothesis. Secondly, testing for whether a linked sequence of enzyme mRNA changes for a given pathway moved in the same direction does not require highly complex statistics to prove that the change in direction is statistically significant. It allows the use of Z scores and for overall pathway

Table 3
Changes in fast and slow twitch myofibril isoforms with HLS

Description	NCBI Accession #	Intensity Control	Intensity HLS	Intensity Change	P<	% Change
SLOW TWITCH MYOFIBRIL ISOFORMS						
MYOSIN, LIGHT CHAIN						
Myosin heavy chain (MHC IIa)	L13606	76188 ± 11702	40486 ± 9449	−35702	0.05#	−47
β Cardiac myosin heavy chain (MHC 1β)	X15939	141391 ± 12377	108396 ± 15097	−65435	0.05#	−29
MYOSIN LIGHT CHAINS						
Heart myosin light chain (MLC 2s)	M11851	99791 ± 8757	73832 ± 6065	−26263	0.05#	−26
Ventricular myosin light chain 1 (MLC 1s)	X14812	176659 ± 15730	128938 ± 13875	−47721	0.05#	−27
TROPOMYOSIN						
β-Tropomyosin (TM βs)	L00382	65829 ± 3087	55577 ± 4291	−10252	ns	−16
TROPONIN						
Troponin (Tnl s)	J04993	141210 ± 12137	94188 ± 7502	−47022	0.01#	−33
FAST TWTCH MYOFIBRIL ISOFORMS						
MYOSIN, HEAVY CHAIN						
Myosin heavy chain (MHC IIb)	L24897	916 ± 132	64506 ± 13565	63591	0.01#	6944
Myosin heavy chain (MHC IId(x))	S68736	1451 ± 162	83104 ± 7148	81653	0.01#	5627
MYOSIN LIGHT CHAINS						
Myosin light chain (MLC2f)	XD0975	88522 ± 14857	106244 ± 12433	17692	ns	20
TROPOMYOSIN						
α-Tropomyosin (TM □f)	M15474	35828 ± 7999	141328 ± 10627	105550	0.01#	294
α-Tropomyosin (TM □f)	XD2412	38359 ± 7666	78458 ± 7722	40099	0.01#	105
TROPONIN						
Troponin (Tnl f)	M73701	52810 ± 12157	90121 ± 8477	−37311	0.05#	−71

Data expression is the same as for tables 1 and 2. Significance for individual probes has been estimated by two tailed t-tests.

direction and for evaluation of significance within a pathway, two tailed t-tests.

5.2. Data reliability

As with any new technique it is important to establish how reliable the data is and to validate the approach used to interpret the results. An unquestioned change in muscle metabolism with disuse atrophy is a complex shift in muscle fiber type away from a slow twitch to fast twitch fiber types (Table 3, [34]). The changes in the myosin isoforms expression of the soleus muscle in response to unloading have been well characterized. The profile of myosin heavy chains is a consistent reduction in the level of MHC Iβ, a variable response for MHC IIa and increases in both MHC IId(x) and MHC IIb [2,36]. The response of myosin light chains, troponin, and tropomyosin to unloading is increased expression of fast isoforms [6,13,24]. The changes in mRNA expression in response to HLS are consistent with these observations (Table 3). In addition the magnitude of the changes were similar to those observed previously. The changes in the mRNA profiles have been generally consistent with the pattern observed at the protein level [2,34]. Based on the expression profile for the contractile proteins with HLS there is a change in muscle fiber type with unloading from slow twitch oxidative to a fast twitch glycolytic and this matches the changes in enzyme activity presented in Tables 1 and 2 and Fig. 1. Thus the data found by microarray mRNA analysis is comparable in direction and magnitude with what has been found using more conventional methods for myosin isoform expression analysis.

Finally the statistical significance of the data for the glycolytic and fatty acid oxidation pathways is not materially altered when the logarithms of the individual intensity values are used to calculate either the aggregate Z scores or individual probe statistics.

5.3. Lipid metabolism

The results for fatty acid metabolism are consistent, where change was found, the change was towards a reduction in activity. The data set in Table 1 shows that the capacity to oxidize lipids was reduced at both the level of entry into the mitochondria (decreased carnitine acyl transferase activity) and the absolute gene expression of the key enzymes in the β oxidation pathway. The overall reduction is about 30%, which is similar to that found by Baldwin with the space flown rats [3].

The shift away from fat towards glucose is not limited to rats (space flown or ground based HLS). Several studies in other disuse atrophy situations have reported a shift in metabolic fuel utilization away from fat towards glucose. Decreased lipid oxidation has been found with humans after space flight [25]. bed rest [4,33] and space flown rats [3,10, 30]. The present findings show that disuse atrophy is associated with a down-regulation of the pathways involved in fatty acid oxidation rather than a specific decrease in the activity of one or two enzymes.

The one exception to these reports is an HLS study by Grichko et al. which claimed to have failed to reproduce Baldwin's post flight findings in a HLS model [18]. However their data is not clear-cut, as they admitted in their paper. In Grichko's study palmitate oxidation by soleus muscle homogenates (mean $\pm$ SEM with n in parentheses) were for controls and treatment 539 ± 53 [5] vs 465 ± 29 [5] nmol $\cdot$ g tissue^{-1} $\cdot$ min^{-1} ($t = 1.22$, $p = 0.25$). This is a trend towards a decrease; with more rats, the data might have attained significance. It is not a definitive negative result. Grichko also isolated mitochondria and examined their potential for fatty acid oxidation. A null result was found, but the yield was poor (5%) and they specifically stated 'thus the possibility exists that the mitochondria assayed were not representative of the entire cell population' [18]. They did however find the glycolytic shift. The discrepancy between the studies needs to be resolved experimentally.

5.4. Glucose metabolism

In contrast to the down-regulation of fatty acid oxidation, the capacity to metabolize glucose was increased (Table 2, $p < 0.05$). Three of the principal enzymes involved in the regulation of glycolysis are hexokinase, phosphofructokinase and pyruvate kinase. Gene expression of all of these enzymes were significantly increased with HLS ($p < 0.05$, Table 2).

Other studies of individual enzyme activities from atrophied muscles showed either no change or an increase in glycolytic potential during disuse atrophy. Two single fiber studies showed that glycolytic enzymes were increased after microgravity [9,26]. Hexokinase and pyruvate kinase were increased in rats with space flight [9,26] as was glycerol-3-phosphate dehydrogenase. Phosphoglycerate mutase was increased in HLS rats [23]. In agreement with these studies, we found increases in hexokinase, pyruvate kinase and phosphoglycerate mutase (Table 2). Phosphofructokinase, an important regulatory enzyme of the glycolytic pathway was unchanged with bed rest [22] or space flight [15] but was increased in the present study (Table 2). Overall, our results show an increase in HLS rats in these enzymes as part of an overall increase in glycolytic capacity.

A key point about the results presented here is that the changes found are not just with individual enzymes, they are a description of the entire pathway. With individual enzyme assays a change can reflect altered activity potential reflecting less need for that enzyme because other regulatory mechanisms are operative (substrate regulation, allosteric modification, feed back inhibition etc.) or reduced enzyme availability. Increased/decreased activity of a pathway does not prove that the actual substrate fluxes through a metabolic pathway are increased/decreased but is strong evi-

dence for that being the case because there is no other simple explanation of why pathway processing capabilities would be altered unless there was a need to respond to a change in substrate availability.

There was no overall down regulation of energy substrate metabolism. Were that to be the case, a decrease in glucose oxidative capacity should have been found. Yet a key metabolic process, protein synthesis, which accounts for as much as 20% of the BMR is decreased with atrophy [41]. It is likely that other processes are also reduced in the atrophied muscle cells. The decrease in the ability to process fat is counter-balanced by the increased glycolytic capacity suggesting that more energy is entering the cells than is actually needed. With both space flown rats and bed rest there is an increase in glycogen accumulation within the I bands and glycogen occupies some of the space in the myofibrils vacated by thin filaments lost during fiber atrophy [20,21,27,42].

5.5. Underlying physiology

Unexpectedly for an adaptive response which should be of benefit, the shift away from fat towards glucose leaves the muscle more fatigable even though the capacity to transport glucose is enhanced [3,37]. Indeed, several authors have suggested that these biochemical changes are contributing factors to the muscle fatigue, weakness, poor coordination, and delayed onset muscle soreness that is found in both humans and rats after space flight and bed rest [11,16,17,32,35].

The cause of the fuel shift is not known. One possibility is the transition from slow to fast twitch fibers that occurs with disuse atrophy (Table 3). Fast twitch fibers are more glycolytic in their fuel requirements [12,15]. Another possibility is that the shift is an aberration of the response to exercise. In some ways, space flight is the opposite of an exercise program for muscle. Chronic exercise is associated with a shift in energy utilization away from the limited supplies of glucose available from the small glycogen stores to fat. The shift towards fat even occurs in the basal state following exercise training. A study by Calles-Escandon showed that increasing physical fitness in adult males with an exercise program led to a shift in fuel substrates oxidized in the basal state [8]. In the wild, prolonged inactivity is likely to be associated with immobilization for one reason or another. Immobilization will result in an inability to forage for food and hence glucose supply to the tissues will be limited to that available from gluconeogenesis. The combination of circumstances promoting disuse atrophy and allowing for adequate dietary intake is an anomaly of modern civilization and there is no reason for an adaptive response to have evolved.

5.6. Applicability of microarrays

Literature values for the changes in fuel processing enzymes found with space flight and associated models are for the most part less than two fold (Tables 1 and 2). This weakens the use of the gene chip methodology for examining changes in individual enzyme changes. Neither is identification of individual enzyme changes using the criteria described in this report likely to be practical for identifying random probes for subsequent investigation. In this experiment 8736 probes measured (including ESTS) were assessed and 939 (12%) met our criteria (99.99% confidence limit for both control and HLS, $p < 0.05$ for control vs HLS). The mean % difference in intensity between the controls and the HLS rats was 73 ± 10 ($n = 939$) of which there were 565 decreases (-42 ± 1) and 374 increases (121 ± 24). Thus some pre-test hypothesis is necessary to focus the data analysis, for example examining a pathway sequence. Testing for whether a linked sequence of enzyme mRNA changes for a given pathway move in the same direction adds to the power of the statistical analysis by allowing the use of Z scores and for overall pathway direction and for evaluation of significance within a pathway, two tailed t-tests.

Examining fluxes through metabolic pathways ideally should be done with isotopes in vivo. However such experiments are not always possible. In rodents isotopic tracer experiments can be technically complex when it is desired to study an unstressed rat that has already undergone a stress inducing procedure (e.g. HLS). Indirect assessment of the status of multiple pathways in small amounts of tissue is problematic because of the limited amounts of tissue available. Microarray assessment of pathway enzyme mRNA expression provides a complimentary way of obtaining integrated information about pathways in the unstressed state in vivo using minimal tissue.

5.7. Conclusions

(i) Individual differences in enzyme activity are unlikely to be detectable with the current sensitivity of the system in adult rats and the number of rats that are feasible for a flight experiment. However examining series of enzymes that are linked (e.g. a pathway) increases the power of the analyses and makes smaller changes detectable. (ii) Lipid oxidation is down regulated and glycolysis is up-regulated in atrophied rat soleus muscle.

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