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EXPRESSION PROFILING OF GENES INVOLVED IN MUSCULAR ACTIVITY AND INACTIVITY IN HIND LEGS OF RATS

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SUMMARY

In this study, gene expression profiles of rat hind limb (soleus) muscle in three different (suspension and loading) experiments was used to investigate the genes involved in muscular activity and atrophy. Duration of muscle activity and inactivity varied between experiments. In total, expression data were on 1184 genes profiled in 39 arrays from 3 different experiments. A set of nested linear models, nesting genes within treatments within experiments was defined in R package *limma* for analysis. Moderated t-statistics based on empirical Bayes methods was used to indicate mode of regulation and for gene ranking. Adjustment for multiple testing was by probability of false discovery rate (pFDR). At 5% pFDR, the number of differentially expressed (DE) genes ranged from 1 to 138 across experiments. Most genes were down regulated within experiments, compared to control groups (controlled state activity). A gene which encodes Glutathione-S-transferase showed differential expression pattern over all three experiments at 10% pFDR. As a comparative approach, the expression level of this gene was mapped to genomic locations using expression quantitative trait (eQTL) approach in mouse inbred lines; the results showed trans-eQTL regions on chromosomes 10 and 3. The different experiments affected genes which are mostly likely involved in ATP production and fatty acid synthesis. Hence, the implications of this result are that the supply of energy source are of great importance for longstanding work and exercise than protein and other mediator nutrient molecules. These results have significant impact on adaptive animal and human physiology.

INTRODUCTION

The soleus muscle is a powerful muscle of mammals in the back part of the lower leg (the calf). It runs from just below the knee to the heel, and is involved in standing and walking and running. This study is based on three gene expression experiments conducted in rats at the University of Bern, Switzerland that were designed to study transcript profiles of genes which mediate the dependence of contractile and metabolic muscle features on load-bearing activity. The main objective was to identify genes involved in such molecular mechanisms in the form of differentially expressed (DE) genes using three different experiments which mimic such stimuli. A genetical genomic approach is also investigated using mice, following the approach of Kadarmideen *et al*. (2006).

MATERIALS AND METHODS

Gene expression profiling experiment. Tissue samples from postural rat soleus muscle in three different loading experiments was used for transcript profiling. Hind limbs of young female rats were

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'unloaded' in individual cages by hanging the rats by their tails (so called 'suspension'). One set of these 'suspended' rats were allowed to perform normal cage activity for 1 or 5 days, therefore permitting 'reloading' of the *m. soleus*. Hind limb suspension provokes mechanical unloading of soleus muscle which leads within days to de-conditioning of soleus muscle by atrophy and a shift to fast-glycolytic phenotype. Mostly these structural processes are reversible and recovered with subsequent reloading within weeks (reviewed in Flueck et al. 2005). Three different experiments applied following treatments to animals: the first experiment (Exp 1) was 35-days cage controls vs. 35-days of hind limb suspension, the second experiment (Exp 2) was 14-days cage controls vs. 14-days suspension and the third experiment (Exp 3) was 14-days suspension + 1 day reloading vs. 14-days suspension + 5 days reloading. There were 39 animals (=arrays) distributed across 3 experiments as follows: In Exp1, there were 16 animals with 8 in each treatment groups, In exp 2, there were 12 animals with 6 in each treatment groups and in Exp 3, there were 11 animals with 6 in control group and 5 in treatment group. Tissue samples from soleus muscle of these animals were then used for mRNA extraction and profiling as in Flueck et al. (2005). For all of these three experiments, a home-made array was used to make profile of genes, which contained 1184 genes known to be involved in skeletal muscle physiology. Full details of how these genes were selected can be found in Flueck et al. (2005).

Statistical methods and analyses. A nested linear model was fitted to identify differentially expressed genes, nesting genes within treatments within experiments and the data were analyzed using the R- package LIMMA (Smyth, 2004). Before data analysis, between arrays variance normalization / variance calibration was performed to stabilize variance among expression datasets. Different contrasts were used within and across experiments to find DE genes. Model fit was checked by plotting predicted error values against predicted gene expression values using all data from 3 experiments. The main problem of using ordinary t statistics is that non-differentially expressed gene with large variances have too much chance of being selected. Hence we chose *moderated t-statistics* to rank DE genes (Smyth, 2004). For every gene, there were corresponding moderated t-statistics and its P-value. Genes which had negative moderated t-statistics were considered as down-regulated genes and genes with positive moderated t-statistics were considered to be up-regulated genes. We first chose DE genes and then used this statistics to define up or down regulations of genes. The adjustment methods for p-values included the Bonferroni correction in which the p-values are multiplied by the number of comparisons following Benjamini & Hochberg (1995).

RESULTS AND DISCUSSION

In experiment 1, at 5% pFDR, just one gene (D64045, phosphatidylinositol 3-kinase) turned out to be differentially expressed and was up regulated compared to 35 days cage controls. The protein of this gene's mRNAs is involved in virtually all of the intracellular transducers/effectors/modulators and cytoplasmic actions. At 10% pFDR, 9 genes were DE of which six genes were up regulated. The very low proportion of DE genes (1 or 9 out of 1184 genes) indicates either the experiment one wasn't strong enough to induce any signal in genes or genes did not show any reaction against this experimental effect. These six genes were involved in symporters and antiporters (gradient-driven transporters), which constitute families of transport proteins in cell. The profile of genes involved in cell adhesion receptors/proteins was affected differentially i.e., the mRNA of DNA binding and chromatin proteins was changed (increased) with suspension. In experiment two: at 5% of pFDR, 15

genes were DE of which 12 genes were down regulated and the rest up regulated. At 10% of pFDR, 18 genes were DE. In experiment three, large numbers of genes were DE, with the mode of regulation similar to that of Exp 2 (ie., most DE genes were down regulated). At 5% pFDR, 138 genes turned out to be DE of which 65 genes showed down regulated mode and the rest, showed up regulated mode. At 10% pFDR, 178 showed DE. Although quite a large number of genes turned out to be DE in this experiment, the statistical model did not fit very well to this data compared to other two experiments. Table 1 provides description of top three DE genes ranked based on pFDR and Log (odds) within each experiment (Exp). For most gene ontology groups, genes which were involved in the same metabolic pathway (results are not reported), pair-wise sequence alignment showed that they did not share any nucleotide sequence similarities and DNA structure of genes did not influence the way that genes were differentially expressed.

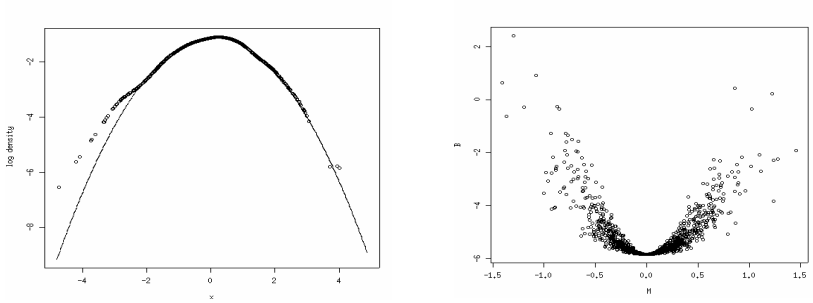

Figure 1. **Left panel: Log empirical density plot of moderated t-statistics for the contrast of first experiment (points) and fitted normal density (line) with ordinate axis is on log scale; Right Panel: Log of posterior odd (B) plotted against difference of log intensity (M) or average fold change. Results are based at 10% pFDR.**

The basic biological information of DE genes and their mode of regulation show a significant role in muscle activity and atrophy/inactivity (Table 1).

Genetical genomics. In general, with DE approach we can not address anything in term of their regulation program but most gene networking approaches are established on DE genes. Since DE genes may themselves be regulated by other genes not on the array, integrative approaches are needed. Kadarmideen et al., (2006) conducted genetical genomics (or expression QTL or eQTL mapping) using whole brain gene expression datasets and SNP linkage map in recombinant inbred lines of mouse where they show how these regulatory genes or eQTLs can actually be detected on the genome; further they validate their findings using constructed gene and protein networks in a 'systems biology' framework. This is an exciting area of integrative biology which needs to be pursued. Kadarmideen and Janss (2007) showed how translational approaches, using results in mouse, can be useful for other species such as pigs, for mapping gene experession of cortisol genes; a similar principle could be adapted here for rat-human-livestock comparative physiological transcriptomics.
Following method described by Kadarmideen et al., (2006) and Kadarmideen and Janss (2007), we used mouse recombinant inbred line data publicly available on WebQTL (www.genenetwork.org) to

conduct whole genome scan for expression values of glutathione S-transferase theta 1 gene, expressed in brain. Results showed that the gene itself was located on chromosome 20 but two trans eQTL regions were identified on chromosomes 10 and 3 exceeding likelihood ration statistics of 9.8. The physical location of eQTL peak on chromosome 10 and 3 ranged from 69 to 73Mb and from 91 to 99 Mb, respectively.

Table 1. Description of top three differentially expressed genes ranked based on pFDR and Log (odds) within each experiment of suspension and reloading of soleus muscle

Exp	Genes	Gene annotations	Metabolic Pathway IDs	pFDR	Log(odds)
1	D64045	Phosphoinositide 3-kinase p85	FT10 SC00	0.0327	2.4006
2	X64827	Cytochrom c oxidase subunit VIII	FL00 SM00	0.0001	6.6460
2	J02592	Glutathione-S-transferase	FL08 SC00	0.0007	4.9608
2	J02773	Heart fatty acid-binding protein	FT10 SC00	0.0008	4.5626
3	U82591	Growth-related c-myc-responsive protein	FC00 SN00	0.0000	53.8090
3	L31884	Tissue inhibitor of metalloproteinase 2	FU07 SF00	0.0000	35.2085
3	X64589	G2/M-specific cyclin B1 (CCNB1)	FC01 SC00	0.0000	22.9955

CONCLUSION

We analyzed transcriptional values of 1184 genes expressed in rat hind limb muscle in three experiments under joint analysis. As expected, mode of the same DE gene was different over experiments. Different experiments affected genes which involve ATP producing cycles and fatty acid cycles. Hence we conclude that for muscle exercise the supply of energy source are of great importance for longstanding work and exercise than protein and other mediator nutrient molecules. Glutathione-S-transferase gene showed differential expression pattern over all three experiments at 10% pFDR.; this indicates that this gene is a candidate gene for further functional study by other integrative approaches. Genetical genomics or eQTL mapping in mouse indicates that the trans eQTL regions identified on chromosome 3 and 10, shall be investigated further for identifying regulatory loci and transcription factors for this gene.

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