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Gene Expression in Hibernation: Testing Skeletal Muscle of Little Brown Bats, *Myotis Lucifugus,* using Commercially Available cDNA Microarrays

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Abstract

Human 19K cDNA microarrays containing cDNAs corresponding to genes involved in all major cellular signaling pathways, were utilized to monitor changes in gene expression that occur during hibernation in skeletal muscle of little brown bats, *Myotis lucifugus*. These animals experience extreme environmental conditions that would result in cold injury and death in other animals. During hibernation, the metabolic rate drops to less than 10% of control or euthermic metabolic rates, as well, body temperature drops to below 10^0C (Lyman, C.P. *et al.*, 1982). Muscle fibers are among the few cell types that are truly multinucleated and undergo a number of changes during muscle remodeling in response to injury, and some of the changes may be seen as adaptations to environmental stress. Approximately 15% of the total genes found on the microarrays showed significant expression to allow for semi-quantitative analysis. In subarrays, as much as 65% and as few as 5% of spotted cDNAs displayed expression strong enough to allow for further analysis. We have determined that false 'negatives' can be identified as a major limitation to cross species work with DNA arrays. Of the 'positive' responses, 5 genes showed an increase in expression of at least 1.9 fold on reciprocally labeled microarrays when comparing hibernating versus euthermic animals. Three of the five genes represented are uncharacterized or ESTs, one is a putative glucosyltransferase and one is an amyloid beta precursor protein (APP).

Introduction

Mammalian hibernation is a strategy employed by many small animals to live through long winter months of subzero temperatures and little or no food availability. During hibernation, metabolic rate is strongly suppressed, heart rate and respiratory rates drop to low levels, and body temperature is allowed to drop to ambient (Lyman *et al.*, 1982). The little brown bat, *M. lucifugus*, characteristically spends eight months of a year hibernating. Prior to entering hibernation, bats select roosting spots where temperatures do not rise above10°C allowing them to prepare for hibernation by entering torpor for up to 14 hours at a time (Speakman *et al.*, 1999). Once hibernation begins, torpid bouts can last for weeks with animals waking up intermittently for a up to a day before re-entering torpor. It is estimated that hibernating mammals can conserve up to 88% of the energy that would otherwise be required to remain awake in an euthermic state over winter (Wang, 1989).

From an evolutionary standpoint, there are two hypotheses as to how hibernation arose. The first theorizes a common mammalian ancestor had the ability to hibernate and that this ability was subsequently lost in all but a few remaining mammals. The other view is that of convergent evolution, in which different mammalian lineages have independently evolved the ability to hibernate in response to environmental pressures. A test of these competing theories might be to analyze the suite of gene expression responses that occur during hibernation in several evolutionarily distinct mammalian hibernators, for instance ground squirrels and bats. A broad common set of gene expression responses in the two groups could argue for an ancestral hibernation phenotype whereas distinctly different profiles of hibernation-induced gene expression across a broad spectrum of genes could suggest that hibernation arose independently in different mammalian groups. Whether or not the phenotype of hibernation arose independently on different occasions or whether it was once inherent in all mammals does not change the fact that in order to maintain this state there must be profound changes at the cellular level to allow for continued function of metabolism at low body temperatures. Genes that are up-regulated in hibernators are thought to serve important physiological functions since transcription and translation of most genes appears to be suppressed during hibernation (Knight *et al.,* 2000).

Skeletal muscle in *M. lucifugus* is of particular interest in that it experiences disuse atrophy during a period of inactivity, which can last up to nine months a year. Recent studies on another bat species, *Murina leucogaster* have indicated that cross sectional area of myofibers decreased to 68% of euthermic values during hibernation (Kim *et al.*, 2000). Glycolytic potential and oxidative potential of skeletal muscle has been shown to decrease in hibernating bats (Brigham *et al.*, 1990). Protein and amino acid catabolism may account for atrophy of muscle in some hibernating ground squirrels (Wang, 1989). In recent years, hibernation-specific changes in the expression of selected genes have been reported in several studies with ground squirrels. Hearts and skeletal muscle of *Spermophilus lateralis*, show increases in myosin light chain 1, ventricular isoform (MLC1) and NADH ubiquinone-oxidoreductase subunit 2 (Nad2) (Fahlman *et al.*, 2000) and skeletal muscle of *Spermophilus parryii* up-regulates uncoupling protein 3 (UCP-3) approximately 3-fold during hibernation (Boyer *et. al.*, 1998).

Materials and Methods

Animals

M. lucifugus were obtained from the Sherbrooke area in early December and brought back to the University of Sherbrooke where some were allowed to warm to room temperature (euthermic) and become active. The remaining bats were kept at 4°C and their body temperatures were checked to ensure that they were in the hibernating state prior to tissue collection. Tissues were excised from the animals and immediately frozen in liquid N_2.

cDNA Microarrays

Microarrays were purchased from The Microarray Centre, Clinical Genomics Centre, University Health Network, Toronto, ON (Serial #: 0000296657, 0000296670, 0000296627, and 0000297163). Complete listings of cDNAs contained on the arrays and their positions can be found at: http://www.uhnres.utoronto.ca/services/microarray/products/index.html.

RNA preparation

All materials and solutions used for RNA isolation were treated with 0.1% v/v diethylpyrocarbonate (DEPC) and subsequently autoclaved. Total RNA was isolated from tissues using Trizol solution (BRL) following the manufacturer's protocol. The $A_{260/280}$ ratio was found to be in the range of 1.7-1.9. Total RNA was also electrophoresed on a 1% denaturing formaldehyde gel to assess quality visually. Poly(A)$^+$ was isolated from 250 µg total RNA using Qiaquick mini mRNA isolation kits from Qiagen. The concentration of poly(A)$^+$ RNA was determined by dot blotting against RNA standards and stained with methylene blue. Quality of poly (A)$^+$ RNA determined with RT-PCR using primers for H-FABP (from an independent project). Primers were as follows, 5'-GGTACCTGGAAGCTAGTGG-3' and 5'-CGCCTCCTTCACGTAAGT-3' and were designed from consensus mammalian sequences.

First strand cDNA synthesis

First strand synthesis was performed with 2 µg of poly(A)$^+$ RNA. Added to this was 1.5 µl of oligo(dT)$_{25}$ (Bio S&T, Montreal, Quebec) primer (200ng/ml), 8.0 µl of 5X first strand buffer (Superscript II, Life Technologies). The mixture was heated to 68°C for 5 minutes and then placed at room temperature to allow for annealing of the oligo(dT)$_{25}$ primer. 3.0 µl 20mM dNTP (no C, Life Technologies) and 1.0 µl 2mM dCTP (Life Technologies), 1.0 µl of fluorescent dyes (cyanine 3 or cyanine 5, Amersham Pharmacia Biotech) and 4.0 µl 0.1M DTT were added prior to adding Superscript II (Life Technologies). Reciprocal labeling was performed. Hybridization was carried out for 2.5 hours at 42^{0}C in the dark. Following hybridization, 4 µl 50mM EDTA (pH 8) and 2 µl 10N NaOH were added and tubes were incubated at 65^{0}C for 20 minutes to degrade remaining RNA and then cy3 and cy5 probes were combined. 4 µl 5M acetic acid was added initially to neutralize the reaction mixtures, with more added as required (pH was determined using pH paper). 100 µl isopropanol was added to the combined cy3 and cy5 probes and incubated on ice for 30 minutes to precipitate the DNA. Tubes were centrifuged at 10,000 rpm for 10 minutes at 4°C. The pellet was then rinsed with 70% (v:v) ethanol. After most of the ethanol had evaporated, the pellet was resuspended in 5 µl TE buffer.

cDNA Microarray Hybridization

Five µl of yeast tRNA (Life Technologies; 10mg/ml) and 5 µl calf thymus DNA (10mg/ml) were added to 100 µl DIG Easy Hyb solution (Roche). The mixture was incubated at 65^0C for 2 minutes and cooled to room temperature. Eighty µl of this was added to the pooled fluorescent labeled cDNAs. The solution was heated at 65^0C for 2 minutes and then cooled to room temperature. Eighty µl solution was added to each set of arrays and then glass plates were sandwiched together. Microarrays were incubated at 37^0C overnight (16 hours).

Microarray washing

The microarrays were placed in 1X SSC after incubation to allow glass plates to separate. When the arrays were separated, they were placed in slide staining boxes and washed 3 times at 50^0C for 10 minutes in 1X SSC, 0.1% (w:v) SDS with occasional gentle agitating. After the washes were complete, slides were washed in 1X SSC to remove any residual fluorescent probe. The slides were spun dry at 500 rpm for 5 minutes and placed in the dark. Scanning was performed on the same day.

Scanning

Scanning was performed at the lab of Dr. R. Walker, Nation Research Council (NRC), Ottawa, Ontario, on a Scanarray analysis system at 10□m resolution.

Data Analysis

Data was analyzed using the freeware program Scanalyze 2 obtained from http://rana.lbl.gov/EisenSoftware.htm. Microarray cDNAs were considered to cross react with *M. lucifugus* first strand cDNA if they produced a fluorescent signal 3-fold greater than background and were considered for further analysis only if they showed increased expression on reciprocally labeled slides.

Results

We considered an array spot to have a positive cross-reaction if the signal produced was more than three times the background. Genes were considered up regulated if there was a two-fold or more difference when comparing the hibernating fluorescence signal with that of the euthermic one.

The total number of microarray cDNAs cross-reacting with bat skeletal muscle first strand cDNA was relatively low. Approximately 15% of the total cDNAs found on the microarrays produced signals of significant magnitude to allow for proper quantification using the Scanalyze software. In comparison, using the same tissues with Clontech nylon arrays (Rat 1.2 II) with ^{32}P labeled probes, we obtained cross reactivities of 70-80% (unpublished data). Cross reactivity using the 19K arrays used in this study may indicate the relative lack of sensitivity of fluorescent tags versus that of radioactively labeled probe. However, although the overall cross reactivity appears low, many of the subarrays showed up to 70% cross-reaction of cDNAs. The true indicator of cross reactivity success is, of course, the genetic similarity of the individual gene of the two species being compared.

Five genes on the 19K human microarrays showed increased expression on reciprocally labeled arrays, while most cross-reacting genes showed little or no changes

in expression. Of these five genes, three were uncharacterized or unknown cDNAs (Accession numbers H49910, AA210692, W37120), whereas one is a putative glucosyl transferase (Accession number N20259) and the other is an amyloid beta precursor protein (Accession number W05611, APP), which also showed the strongest up-regulation (Figure 1). The total number of gene with increased expression (only 5 out of 19,200) may appear low, however, previous work in our lab has shown little to no changes occurring in skeletal muscle of ground squirrels and bats upon entering hibernation (unpublished data).

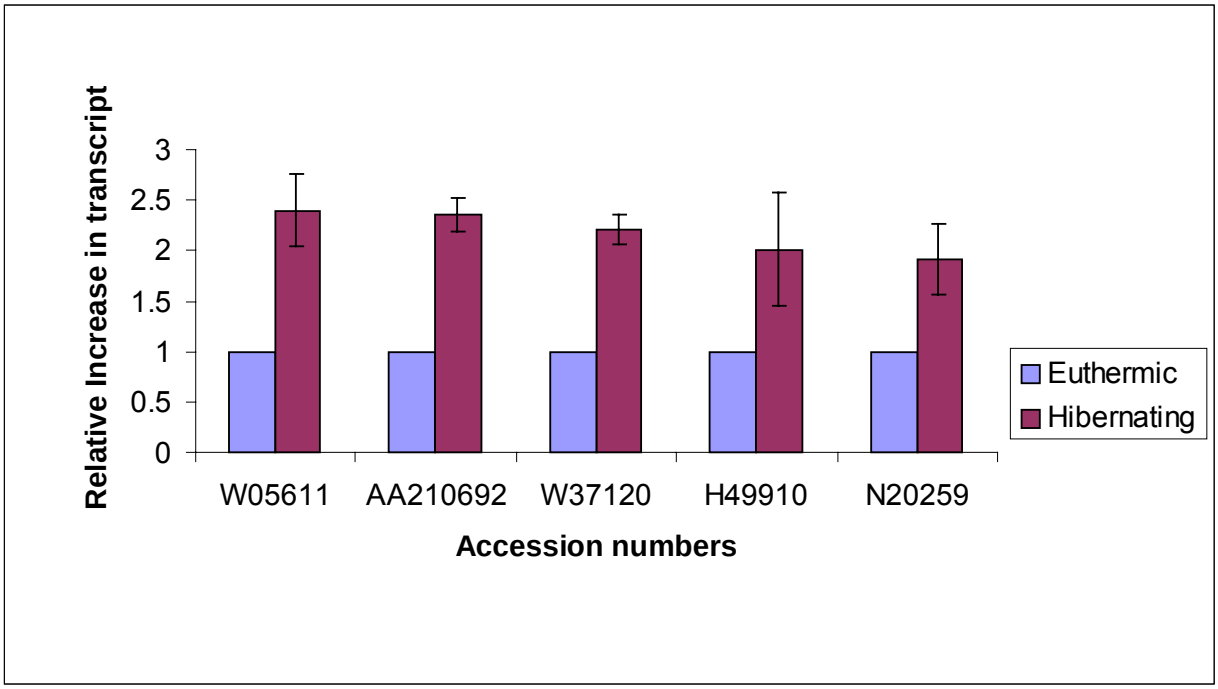

Figure 1: *M. lucifugus* genes found to be up-regulated in hibernating skeletal muscle using a commercially available cDNA array. Poly(A)$^+$ RNA from three independent animals was isolated and combined for experiments. Up-regulation was determined from the averages of duplicate cDNAs on each array and from reciprocal labels. Errors were determined from reciprocally labeled cDNA arrays.

Discussion

Hibernation is a key survival strategy for many mammals living in extreme northern climates where food sources become scarce though the winter. We hypothesized that changes in gene expression that would occur within bat muscle upon entering hibernation and that the up-regulated genes found might have a function in the survival and maintenance of the muscle over long periods of disuse.

Our studies indicate that the use of microarrays for cross species comparisons can suffer from low cross reactivity. Using the 19K human array and fluorescent tags, we found only 15% cross reactivity, clearly then, the ability to identify genes that were up-regulated during hibernation was limited to a small subset of the total represented on the array leaving a very high probability that a variety of up-regulated genes were

missed. However, we believe that this type of analysis can be useful for important genes may still be identified among the cross-reacting subgroup. Array data can point out a number of candidate genes for further analysis using techniques including northern blots and/or real time RT-PCR to confirm up-regulation.

The genes identified from the current study include APP, a putative glucosyltransferase and one containing a domain found within the insulin like growth factor binding protein 7 (IGFBP7). The fluorescent signal generated for APP showed the strongest up-regulation. This protein has recently been linked to inclusion bodies that occur in muscle deteriorating muscle (Askanas *et al.*, 2001). It is also thought to play a role in skeletal muscle differentiation (Akaaboune *et al.,* 2000). Up-regulation of the mRNA encoding APP in bat skeletal muscle during hibernation may occur in response to or in anticipation of the muscle atrophy and damage that can occur during inactivity and cold exposure. Further studies will be needed to characterize the time course of gene up-regulation over the hibernation cycle and as well as analyze the pattern of protein response. The putative glucosyltransferase, may be involved in muscle repair as has been shown for a similar enzyme, galactosylhydroxylysine glucosyltransferase (GGT) that is related to increased collagen biosynthesis in rat skeletal muscle (Virtanen *et al.*, 1992). A portion of cDNA H49910 is identical to IGFBP7, which suggests that the *M. lucifugus* IGFBP7 may have cross-reacted with the cDNA on the microarray. Studies have shown that IGFBP7 is related to muscle growth activity in skeletal muscle (Damon *et al.*, 1997). Since muscle re-growth must occur because of disuse atrophy, the mRNA may be up regulated in *M. lucifugus* to prepare for muscle growth activity upon returning to the euthermic state.

The fact that we are observing little or no changes in gene expression changes in hibernating skeletal muscle is not unexpected, as previous work in our lab has shown this to be true. dd-PCR of *Spermophilus lateralis* skeletal muscle showed no changes between euthermic and hibernating states and we have also made and screened a cDNA library from hibernating bat skeletal muscle (Stratagene) and found no genes up-regulated, out of approximately 100K clones screened and no changes were found. The nylon arrays also showed no change occurring between euthermic and hibernating mRNA species of *M. lucifugus* skeletal muscle.

While human (or mouse and rat) arrays cannot be used to perform a complete and definitive survey of stress-induced gene expression in other species, they can provide multiple potential leads on individual genes that show good cross-reactivity and, as such, they offer a valuable tool for screening stress-induced gene expression. Positive results from such cross-species analysis on DNA micro- and macroarrays will require careful confirmation using northern blots and RT-PCR.

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