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Biochimica et Biophysica Acta 1676 (2004) 63–70
BIOCHIMICA ET BIOPHYSICA ACTA
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Up-regulation of fatty acid-binding proteins during hibernation in the little brown bat, *Myotis lucifugus*

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Received 23 June 2003; received in revised form 27 October 2003; accepted 31 October 2003

Abstract

Hibernating animals rely primarily on lipids throughout winter as their primary fuel source, thus it is hypothesized that an increase in genes and proteins relating to lipid transport will increase accordingly. The cloning and expression of heart type fatty acid-binding protein (*h-fabp*) from a mammalian hibernator, the little brown bat *Myotis lucifugus*, is presented. Northern blot analysis revealed that transcript levels of *h-fabp* were significantly higher during hibernation in brown adipose tissue and skeletal muscle compared with levels in euthermic bats. Similarly, heterologous probing with rat adipose type *a-fabp* found 3.9-fold higher levels of *a-fabp* transcripts in brown adipose from hibernating animals. Levels of A- and H-FABP protein were quantified in tissues of euthermic versus hibernating animals by Western blotting. A-FABP was 4-fold higher in brown adipose of hibernating, compared with euthermic bats, whereas H-FABP was significantly higher in hibernator brown adipose, heart and skeletal muscle. The present work implicates FABPs as important elements related to the hibernating state in mammals; alterations in gene and protein expression along with amino acid substitutions are shown. These likely contribute to optimizing the function of FABPs at the low body temperatures (near 0 °C) experienced in the hibernating state.

Keywords: *Myotis lucifugus*; Hibernation; Brown adipose tissue; Thermogenesis; Skeletal muscle; Fatty acid-binding protein

1. Introduction

Hibernation allows many mammalian species to endure long cold winters when subzero temperatures and a lack of food would otherwise make survival impossible. Key to hibernation is a profound metabolic rate depression and a reduction in body temperature (T_b) to near ambient. For example, in the little brown bat (*Myotis lucifugus*), one of the smallest hibernating species with a body mass of only 5–8 g, metabolic rate can be reduced to as little as 1–1.5% of the euthermic resting rate at a $T_b = 2$ °C [1]. Metabolism during hibernation is largely lipid-based, making use of huge lipid reserves that are accumulated during summer/autumn feeding and deposited in both white adipose tissue (WAT) and brown adipose tissue (BAT) as well as in lipid droplets in various tissues. Lipids fuel both basal metabolism during torpor and the high rates of nonshivering thermogenesis by BAT and shivering thermogenesis by skeletal muscle that rewarm the animal during arousal [1].

Fatty acid-binding proteins (FABPs) play important roles in the intracellular transport of fatty acids in animals [2,3]. Multiple isoforms are known, each dominating in a different tissue type and with different functions. For example, the adipose (A) isoform can form a complex with hormone-sensitive lipase and this suggests that a primary function of A-FABP is to carry fatty acids away from intracellular lipid droplets after triglyceride hydrolysis [3]. By contrast, it has been proposed that the main function of heart (H) type FABP is to transport fatty acids from the sarcolemma (heart typically imports its fatty acids) to intracellular destinations [3]. In BAT, which contains both A- and H-FABP, the main destination of the fatty acid-loaded proteins would be the mitochondria

Abbreviations: *a-fabp*, *h-fabp*, mRNA transcripts of adipose- and heart type fatty acid-binding proteins, respectively; A-FABP, H-FABP, protein encoded by *a-fabp*, *h-fabp*; BAT, brown adipose tissue; FABP, fatty acid-binding protein; PGC-1, PPAR gamma co-activator 1; PPAR, peroxisome proliferator activated receptor; WAT, white adipose tissue

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doi:10.1016/j.bbaexp.2003.10.008

for oxidation in support of nonshivering thermogenesis. Due to the key importance of lipid oxidation in fueling hibernation, recent studies have begun to analyze the responses and roles of FABPs in hibernation. Gene expression studies revealed up-regulation of *a-fabp* in BAT and heart during hibernation in 13-lined ground squirrels (*Spermophilus tridecemlineatus*) and *h-fabp* transcripts also rose in these organs as well as in skeletal muscle [4]. The presence of the adipose isoform in a mammalian heart had not been previously reported but may be related to a novel feature of hibernator heart, the presence of intracellular lipid droplets [5]. These two genes are also markedly up-regulated in cold-exposed BAT of rats, which adds further weight to the suggestion that these proteins have a central role in the intracellular transport of fatty acids to be used for thermogenesis [6]. In addition to increased expression of FABPs during hibernation, properties of the proteins also appear to be adapted for the low temperature function that is demanded of them in a hibernating species. Studies with the liver isoform of FABP showed that fatty acid binding by ground squirrel L-FABP was temperature insensitive over the range 5–37 °C, whereas kinetic properties of rat L-FABP were compromised at low temperature [7].

The present study further explores the role and regulation of FABPs in hibernating mammals. FABP gene and protein expression is analyzed in organs of euthermic and hibernating little brown bats, *M. lucifugus*, to determine whether FABP up-regulation is a conserved mechanism for mammalian cold tolerance and hibernation.

2. Materials and methods

2.1. Animals

Collection and holding of bats was as described previously [8]. Briefly, little brown bats (*M. lucifugus*; 7–8 g body mass) were collected on November 30, 1999 from a slate mine near Sherbrooke, Quebec where they had been hibernating since October (cave air temperature was 5 °C [12]). Hibernating bats are sensitive to non-tactile human disturbance. Collection aroused the bats and they remained aroused during transport to the Université de Sherbrooke. Upon arrival, 10 bats were maintained under euthermic conditions at 23–24 °C air temperature; these were kept awake for a total of 48 h post-collection and were then euthanized by cervical dislocation. Ten others were placed in a cold room at 5 °C and allowed to re-enter hibernation; 10–12 h were required for full torpor to be re-established with a body temperature close to ambient. Hibernating animals remained torpid until sampled 36–38 h later; measured rectal temperatures at sampling were 5–6 °C. Tissues from euthermic and hibernating animals were quickly excised, immediately frozen in liquid nitrogen, and then transported to Ottawa where they were stored at −80 °C until required.

2.2. RNA preparation

All materials used for RNA preparation were treated with 0.1% v/v diethylpyrocarbonate and autoclaved. Total RNA was isolated from tissues of euthermic and hibernating bats using Trizol reagent (Invitrogen, Gibco BRL) and poly(A)$^+$ RNA was then isolated from total RNA using Qiaquick poly(A)$^+$ isolation kits (Qiagen) according to the manufacturers protocol. RNA concentrations were determined spectrophotometrically at 260 nm. RNA was stored at −80 °C until use.

2.3. cDNA synthesis and PCR amplification of h-fabp

First-strand cDNA synthesis was performed using 1 μg poly(A)$^+$ RNA isolated from *M. lucifugus* skeletal muscle and Superscript II reverse transcriptase according to the manufacturer's protocol. The primers for *h-fabp* were designed from conserved religions of rat *h-fabp* (accession number J02773). The *h-fabp* forward primer corresponded to region 55–73 of rat *h-fabp*; 5′-GGTACCTGGAAGC-TAGTGG-3′ and the reverse primer corresponded to positions 418–435 of rat *h-fabp*, 5′-CGCCTCTTCTCG-TAAGT-3′. PCR amplification of *h-fabp* was carried out in 0.2 ml tubes containing 5.0 μl of 10 × PCR buffer (Invitrogen), 2.5 μl $MgCl_2$, 1 μM primer (0.5 μM forward and 0.5 μM reverse), 1 μl dNTPs (1.0 mM each), 0.25 μl Taq polymerase (5 U/μ) (Invitrogen) and 0.5 μg of the reverse-transcribed cDNA in a final volume of 50 μl with sterile H_2O. Cycles for amplification were 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min repeated a total of 30 times followed by a final step at 72 °C for 7 min. A single product with a size of 380 bp corresponding to partially amplified *M. lucifugus h-fabp* cDNA was obtained. To amplify the 3′ portion of the mRNA, a modified 3′ RACE protocol was used. cDNA was amplified using the forward primers 5′-GGTACCTGGAAGC-TAGTGG-3′ with an anchored oligo(dT) primer; (N)T_{25} reverse primer. The PCR protocol described above was used with a reduction of the annealing temperature to 50 °C. A single product corresponding to approximately 600 bp was obtained and sequenced. The 5′ end of the gene was amplified using a SMART RACE amplification kit (Clontech) according to manufacturers protocols with the *h-fabp* reverse primer, 5′-CGCCTCCTTCTCGTAAGT-3′. PCR cycles were as described above. A single product of approximately 500 bp was obtained and sequenced.

2.4. Sequencing

Sequencing was carried out by the Canadian Molecular Research Services (Ottawa, ON). Each PCR product was sequenced at least three times and sequences were verified as *h-fabp* by submitting to BLAST. Sequences were then assembled and a complete transcript of *M. lucifugus h-fabp* was obtained. The *M. lucifugus* sequence was submitted to GenBank (accession number AF530469).

2.5. Northern blotting

A 15 μg aliquot of total RNA was electrophoresed on a formaldehyde agarose gel. Blots were transferred overnight onto Hybond N nylon membrane, UV cross-linked and baked at 80 °C prior to probing. A clone for rat *a-fabp* was purchased from Research Genetics and excised from the plasmid. Purified rat *a-fabp* and partial *M. lucifugus h-fabp* were ^{32}P-labeled by the random primer method as previously described [4]. Blots probed for *h-fabp* were incubated with radiolabeled probes and hybridized overnight at 50 °C in modified Church's buffer (0.5 M sodium phosphate, 7% SDS) and blots for *a-fabp* were probed at 44 °C in modified Church's buffer and washed in successively more stringent conditions (0.1 × SSC, 0.1% SDS for *h-fabp* and 5 × SSC for *a-fabp*) until radioactivity on the blots dropped to 500–1000 counts per minute. Blots were exposed to X-ray film (X-OMAT AR, Kodak) and phosphor screens (BioRad). Quantification of *fabp* mRNA bands (~ 700 bp) was performed using Imagequant (Molecular Dynamics) or software included with the BioRad Personal FX phosphorimager. The molecular size of mRNA was determined from comparison with markers run concurrently on the gels. Blots were either stained with methylene blue or probed for 18S rRNA (^{32}P-labeled). The band intensity of *fabp* in each lane was normalized relative to its corresponding 18S rRNA band intensity.

2.6. Western blotting

Samples of frozen tissues were homogenized in sample buffer (250 mM sucrose, 50 mM HEPES, 5 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride). Protein concentration was determined using the BioRad protein assay. Proteins samples were mixed 1:1 with SDS-sample buffer and equal amounts of protein (15 μg) were loaded in each lane, electrophoresed and then transferred to PVDF membranes. Blots were then blocked in 7% non-fat milk dried milk in TBST for 3 h. Polyclonal antibodies were added to the solution, followed by overnight incubation at 4 °C (final dilution of 1:1000). Human A- and H-FABP antibodies were a gift from Dr. F. Spener (University of Munster, Germany). Blots were washed and incubated with a 1:2000 dilution of HRP-linked anti-rabbit IgG (Cell Signaling) in

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1      TCAGCCTAGCCCAGCCTCACCATGGCGGACGCCTTCGCAGGCACCTGGAAGCTGGTGGAC
1                          M  A  D  A  F  A  G  T  W  K  L  V  D

61     AGCAAGAACTTCGATGACTACATGAAGTCCATCGGTGTGGGTTTTGCTACCAGGCAGGTG
21      S  K  N  F  D  D  Y  M  K  S  I  G  V  G  F  A  T  R  Q  V

121    GCCAGTATGACCAAGCCTACCACAATCATCGAAATAAATGGGGACACGATCATCCTAAAA
41      A  S  M  T  K  P  T  T  I  I  E  I  N  G  D  T  I  I  L  K

181    ACACAAAGCACCTTCAAGAACACAGAGATCAGCTTCAAGCTGGGGGTGGAGCTTGACAAG
61      T  Q  S  T  F  K  N  T  E  I  S  F  K  L  G  V  E  L  D  K

241    ACAACGGCAGATGACAGGAAGGTCAAGTCCACCGTGACGCTGGATGGAGGCAAACTTGTC
81      T  T  A  D  D  R  K  V  K  S  T  V  T  L  D  G  G  K  L  V

301    CATGTGCAGAAATGGGACGGGCAAGAAACAAAACTTGTGCGGGAGCTCGTGGATGGCAAA
101     H  V  Q  K  W  D  G  Q  E  T  K  L  V  R  E  L  V  D  G  K

361    CTCATCCTGACACTCACCCACAATAATGTAGTGTGTACGCGGACTTACGAGAAGGAGGCA
121     L  I  L  T  L  T  H  N  N  V  V  C  T  R  T  Y  E  K  E  A

421    TGACCAGCCCACTGCTTCGCTGACTGCTCCGCTGCCAACTGGCTACCCCTGGACTCAGCA
141     *

481    CCAGATTGCCTCGTCTTTTCCTCTGGCGTTTTTGTATAAATCCACCTTGGGGGGGAAATT

541    CTTCTGGGGTCAGGTGCACCATCCTGGATACAGTTCCAGTTCCCATTGTGTTTGTCTGTC

601    TTAACTGCATCCAAAGAGTGCTCTGAGGTCAATAAAGCAGAGCCAAGGCC
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Fig. 1. cDNA sequence of *M. lucifugus* H-FABP. The deduced cDNA and amino acid sequence of *M. lucifugus* H-FABP is shown (accession numbers: AF530469). Underlines denote the start and stop codons, and the polyadenylation sequence, respectively.

TBST. Renaissance Western Blot Chemiluminescence Reagent Plus (Perkin Elmer) was used according to manufacturers protocols and blots were exposed to X-ray film (X-OMAT AR, Kodak). After blots were probed, they were stained with Coomassie blue to confirm equal loading of protein in all lanes. Quantification of band densities was performed using Imagequant software (Molecular Dynamics). A- and H-FABP bands were normalized relative to proteins whose levels did not appear to change by Coomassie blue staining.

2.7. Statistics

All trials are a result of three independent isolations from pooled tissue (10 euthermic bats and 10 hibernating bats). Mean normalized band densities (± S.E., $n = 3$) were calculated for both torpid and euthermic groups and significance differences between the groups were evaluated using Student's *t*-test with a significance level set at $P < 0.05$. Ratios of hibernating/euthermic mean values were calculated and plotted; error bars shown on the histograms represent the sum of the S.E. values for euthermic and hibernating trials.

3. Results

3.1. Cloning of M. lucifugus h-fabp

Cloning of the complete bat *h-fabp* transcript was done using PCR techniques. A large portion of the open reading frame (ORF) was amplified using primers developed from rat *h-fabp* using consensus regions to other mammalian sequences. The 3′ portion of the gene was then amplified using a modified 3′ RACE protocol with a gene-specific primer and a dT_{25} primer with a reduced annealing temperature. Lastly, a 5′ RACE procedure was used to obtain the remaining portion of the sequence (Fig. 1). BLAST searching of each of the sequenced PCR products confirmed that each represented a portion of *h-fabp*. Fig. 1 shows the 650 nucleotide assembled sequence containing the full ORF that runs from nucleotide positions 22–420 (GenBank accession number AF530469). The start (22–24) and stop (421–423) codons were found along with the polyadenylation signal sequence (631–636). The deduced amino acid sequence of *M. lucifugus* H-FABP is also given. A comparison of the nucleotide sequence of *h-fabp* with the ORFs from other mammals revealed that the bat ORF was 88%, 87%, 86%, 85%, 82% and 80% identical with the sequences of the human, pig, cow, 13-lined ground squirrel, rat and mouse ORFs, respectively (accession numbers used in the comparisons were NP_004093, O02772, P10790, Q99P61, NP_077076, and NP_034304, respectively).

Translation of the open reading into the amino acid sequence revealed a protein of 133 amino acids with a molecular weight of 14.8 kDa protein and a predicted isoelectric point of 8.48. Fig. 2 compares the amino acid sequence of *M. lucifugus* H-FABP with the sequences of the protein from another hibernator (the ground squirrel, *S. tridecemlineatus*) and several nonhibernating mammals. The bat protein is 90% identical to *S. tridecemlineatus* H-FABP, 87% identical to the human and rat proteins, and shows 89%, 85% and 86% identity with pig, mouse and

```
ORIGIN
bat        MADAFAGTWKLVDSKNFDDYMKSIGVGFATRQVASMTKPTTIIEINGDTI    50
squirrel   -v---v-----------------l--------------------k-----    50
human      -v---l-----------------l--------------------k---il    50
mouse      -----v-----------------l--------------------k-----    50
rat        -----v-----------------l--------------------k-----    50
pig        -v--------------------------------n---------v-----    50
cow        -v---v-----------------l---------gn---------v----v    50

bat        ILKTQSTFKNTEISFKLGVELDKTTADDRKVKSTVTLDGGKLVHVQKWDG   100
squirrel   ---------------q--k-f-e---------------------------   100
human      t---h---------------f-e----------i----------l-----   100
mouse      ti-----------n-q--i-f-ev---------l--------i-----n-   100
rat        ti--h----------q----f-ev---------v----------------   100
pig        -i-------s----------f-e----------i----------l---n-   100
cow        -i------------------f-e----------i--------------n-   100

bat        QETKLVRELVDGKLILTLTHNNVVCTRTYEKEA                    133
squirrel   ---t-----n---------gs-----------                    133
human      ---t-----i---------gta----------                    133
mouse      ---t-t-------------gs--s--------                    133
rat        ---t-t---s---------g---s--------                    133
pig        ---t---------------gsa----------                    133
cow        ---s----m-----------gta--------q-                   133
```

Fig. 2. Multiple alignment of deduced amino acid sequence of H-FABP. Deduced amino acid sequences from several mammalian sources are compared to the *M. lucifugus* H-FABP sequence. Included is a comparison with H-FABP from another hibernating species, *S. tridecemlineatus*. The underlined residues may be involved in binding and transport of fatty acid residues in hibernating bats. Shown are phenylalanine to leucine, glutamic acid (which is conserved in all known H-FABPs) to lysine and the hydrophobic residues, isoleucine, leucine or valine to tyrosine, which has previously been identified in *S. tridecemlineatus*.

cow H-FABP, respectively. Several amino acid substitutions are evident in the bat sequence, as compared with the others. Deduced from the mature protein after cleavage of the N-terminal methionine, these included an F → L at substitution at position 70 in the bat sequence that is not observed in other mammalian species (residue underlined). The significance of this substitution lies in the fact that phenylalanine is an aromatic residue, whereas leucine is hydrophobic. At position 72, an E → K substitution replaces an acidic amino acid with a basic amino acid (lysine). This substitution occurs in relative proximity to a previously reported lysine substitution in ground squirrel H-FABP [4]. Three more interesting substitutions occur in *M. lucifugus* H-FABP. At position 103, the sequence from other mammals shows a serine or threonine residue but the bat H-FABP contains a lysine. At position 120, the bat H-FABP contains an asparagine, whereas the sequence from other mammals contains a glycine. Another asparagine substitution occurs at position 121, whereas most mammalian sequences contain a serine or threonine. All of these substitutions may assist in the function of H-FABP at low temperatures and help to account for the high p*I* of the bat protein. Finally, at position 83, a threonine is found in the *M. lucifugus* H-FABP sequence, whereas a hydrophobic residue (valine or isoleucine) is found in other nonhibernating mammals. An identical substitution is found in ground squirrel H-FABP [4].

3.2. Transcript levels of h-fabp and a-fabp

Purified partial cDNA for bat *h-fabp* was ^{32}P-labeled via the random primer method. A cDNA clone for rat *a-fabp* was purchased from Research Genetics and the gene insert was isolated from the plasmid and ^{32}P-labeled. Both radiolabeled cDNAs were used to probe Northern blots to assess the effects of hibernation on *fabp* gene expression. A single transcript was detected for each of *h-fabp* and *a-fabp*, of a size of ~700 bp. We are confident that the rat *a-fabp* cDNA hybridized only with bat *a-fabp*, and not with *h-fabp*. This is supported by the fact that each transcript type displays a distinct expression pattern. Furthermore, studies in our lab with the ground squirrel genes showed no cross-hybridization between *h-fabp* and *a-fabp*; these genes were only 47% identical at the nucleic acid level [4]. Furthermore, Northern blots hybridized with *a-fabp* probe from *S. tridecemlineatus* produced identical patterns of *a-fabp* expression. Neither the *a-fabp* probe nor the *S. tridecemlineatus a-fabp* probe bound specifically to *M. lucifugus h-fabp*, which was electrophoresed and transferred to a nylon membrane (data not shown).

Fig. 3 shows expression levels of *a-fabp* and *h-fabp* mRNA transcripts in multiple tissues of *M. lucifugus*. Neither transcript was detected in brain, kidney nor liver but both were found in BAT, heart, skeletal muscle and WAT. Transcript levels of *a-fabp* increased significantly during hibernation only in BAT (the hibernating/euthermic transcript ratio was 3.9 ± 1.4, mean ± S.E., $n=3$, $P<0.03$) (Fig. 3A,C). However, *h-fabp* transcript levels rose significantly during hibernation in both BAT and skeletal muscles; the hibernating/euthermic ratios were 1.7 ± 0.1 and 1.8 ± 0.3, respectively ($P<0.01$) (Fig. 3B,D).

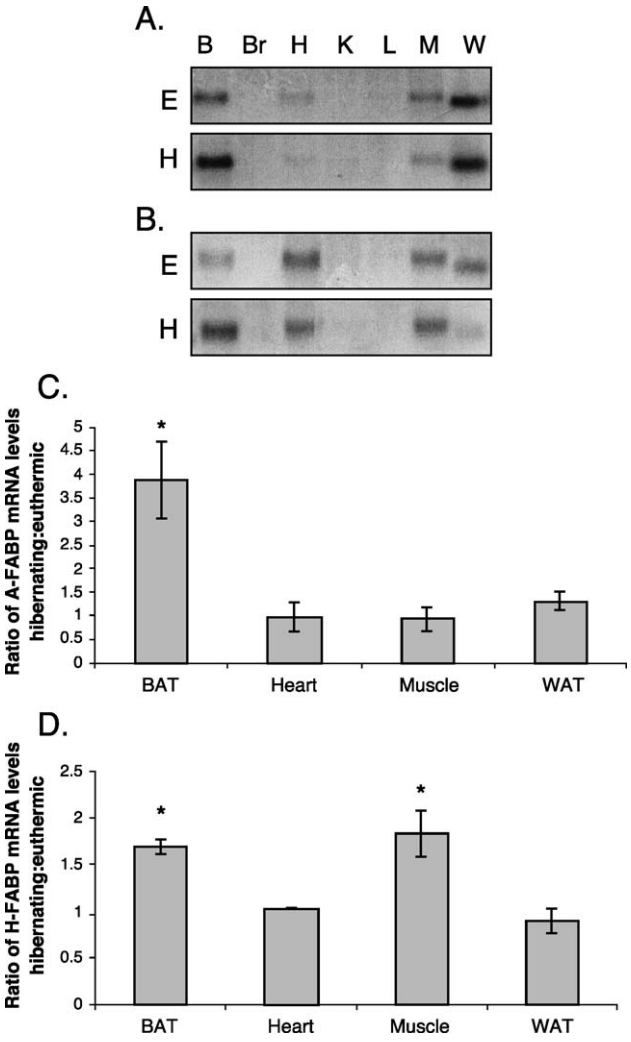

Fig. 3. Transcript levels *h-fabp* and *a-fabp* in euthermic versus hibernating little brown bats, *M. lucifugus*. A 15 μg aliquot of total RNA from each euthermic (E) and hibernating (H) tissue was electrophoresed on a 1% denaturing agarose gel and transferred to Hybond N nylon membrane followed by probing with radiolabeled rat *a-fabp* (A) or *M. lucifugus h-fabp* (B). A single transcript was detected in each case of ~700 bp. Representative blots are accompanied by histograms showing ratios of transcript levels for *a-fabp* (C) and *h-fabp* (D) in hibernating versus euthermic samples (mean ± S.E., $n=3$). Tissues are: B, brown adipose tissue; Br, brain; H; heart; K, kidney; L, liver; M, skeletal muscle; W, white adipose tissue.

3.3. A-FABP and H-FABP protein expression

Western blotting was used to determine whether increased transcript levels led to rise in FABP protein during hibernation. Fig. 4 shows representative Western blots and displays the hibernating/euthermic ratio for A- and H-FABP

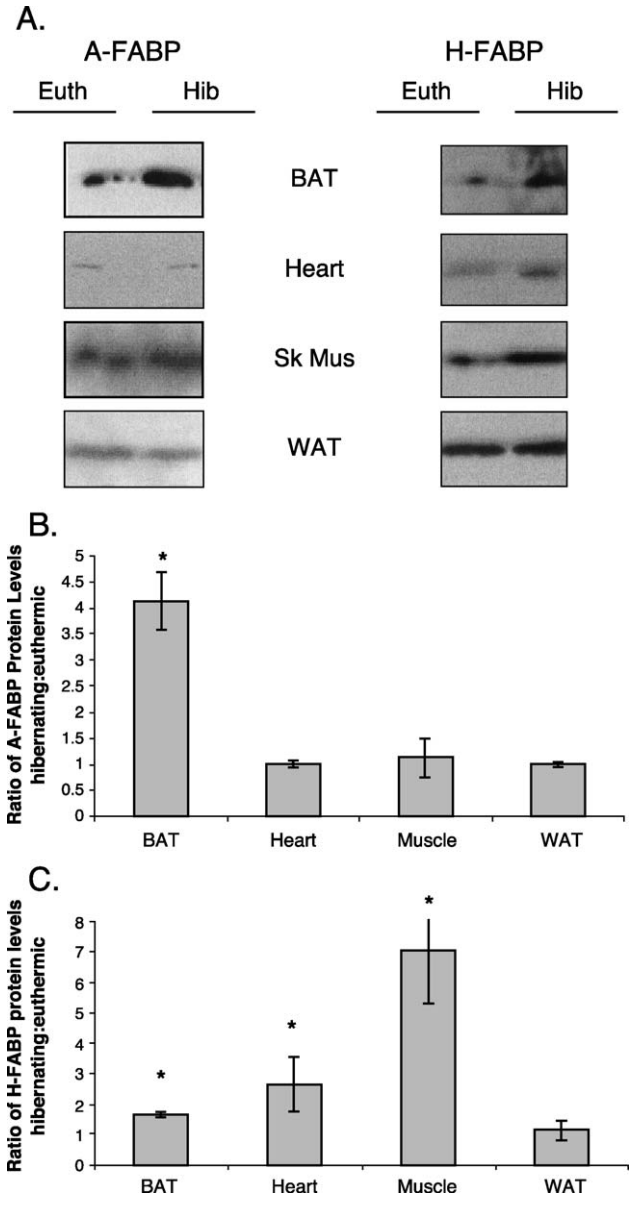

Fig. 4. A-FABP and H-FABP protein levels in BAT, heart, skeletal muscle and WAT of euthermic versus hibernating little brown bats, *M. lucifugus*. Tissues that showed expression of the *h-fabp* or *a-fabp* transcripts were probed with rabbit polyclonal antibodies detecting A-FABP and H-FABP (B) in tissues from euthermic (Euth) versus hibernating (Hib) animals. A 20 μg aliquot of total protein was electrophoresed from BAT, heart (Hrt), Skeletal muscle (Sk Mus) or WAT on a 15% SDS polyacrylamide gel and transferred to PVDF. In each case as single band was observed at approximately 15 kDa corresponding to either A-FABP or H-FABP. Images shown are representative of $n=3$ trials. Histograms show the ratio of mean values ($n=3$) for hibernating versus euthermic levels of A-FABP (B) and H-FABP (C) in tissues.

protein levels in four tissues. Both proteins were detected in BAT, heart, skeletal muscle and WAT but their expression patterns in euthermia versus hibernation differed from those of the mRNA transcript. Levels of A-FABP were significantly higher in BAT during hibernation, by 4.1 ± 0.7-fold ($P<0.05$), as compared with euthermia. A-FABP did not change in hibernation in the other tissues analyzed, and was only slightly detectable in the heart and skeletal muscles. H-FABP expression increased in three tissues during hibernation. The hibernating/euthermic ratio for H-FABP protein levels was 1.7 ± 0.1 ($P<0.005$) in BAT, 2.7 ± 1.0 ($P<0.05$) in heart, and very high at 7.1 ± 1.8 ($P<0.05$) in skeletal muscles. The increase in protein levels in heart and skeletal muscle of hibernating animals greatly exceed the change in mRNA expression levels in the same tissues.

4. Discussion

The gene encoding *M. lucifugus h-fabp* was cloned and translation of its open reading frame revealed a protein of 133 amino acids with a molecular mass of 14.8 kDa and a p*I* of 8.48. Both nucleotide and amino acid sequences showed high percentage identities with previously characterized H-FABP from other mammalian species. However, several striking amino acid substitutions were present in the bat protein; some also occur in H-FABP from another hibernator, the ground squirrel *S. tridecemlineatus*, whereas others appear unique to *M. lucifugus* and may result from natural sequence divergence. Substitutions between beta sheets D and E spanning a 'gap' region in the structure of FABPs may bestow flexibility to the protein [3,4] at the low body temperatures encountered the hibernating state (T_b can fall to near 0 °C during torpor). Glutamate 72, which occurs in this region, is a conserved residue in all other known FABPs [9,10] but is replaced by lysine in the bat protein. In another study, site-directed mutagenesis of this residue from glutamate to serine resulted in a reduction in the stability of H-FABP [10], but this may be beneficial for protein function at low T_b. This lysine substitution also occurs in close proximity to that of a previously reported lysine substitution in *S. tridecemlineatus* (valine 68 is replaced by lysine) (Fig. 2) [4]. In addition, there is another lysine residue substituted in bat H-FABP relative to other mammalian H-FABPs at position 103. Storch et al. [11,12] have shown that the insertion of lysine residues into FABPs using site-directed mutagenesis results in an increased ability of the protein to take up fatty acids from the plasma membrane. In fact, the affinity of various fatty acids for the liver form of FABP from ground squirrels has been shown to equal or greater at low temperatures as compared to normal assay conditions [7]. This, coupled with the possibility of improved fatty acid uptake from the plasma membrane could greatly enhance the ability of H-FABP to deliver fatty acids to the mitochondria where they can be oxidized and provide necessary fuel during prolonged periods of torpor and for rapid rewarming during arousal.

FABPs are a multigene family of small proteins that reversibly bind fatty acids and other hydrophobic ligands for transport [2,3,9]. Nine isoforms are known; initially named for the tissue in which they were discovered [3]. Identification of *a-fabp* transcripts in bat skeletal muscles is a novel finding and the presence of the adipose isoform in the heart

has been reported only in bats and one other mammal, also a hibernator [4]. We believe that A-FABP is present in bat heart and skeletal muscle to serve the metabolic demands of lipid metabolism during torpor. Hibernator heart must continue to function at a very low T_b and whereas vertebrate heart typically relies on fatty acid fuels delivered by the blood, hibernator cardiomyocytes also contain intracellular lipid droplets that provides them with an endogenous fuel supply as well [4]. The presence of both A-FABP and H-FABP in heart and skeletal muscle likely allows these muscles to draw on two distinct pools of lipid fuels to support the specialized needs of hibernators [13]. These include the need for a very rapid increase in heart rate during arousal from torpor and high rates of shivering thermogenesis by the skeletal muscle that help to rewarm the animal. Up-regulation of *a-fabp* in BAT, and *h-fabp* in BAT and skeletal muscle of *M. lucifugus* is similar to a response previously observed in our lab with the ground squirrel, *S. tridecemlineatus* [4] and indicates that fatty acids transport at low temperatures may require the coordination of multiple FABP. This evidence, coupled with the finding of A-FABP in the hearts of Antarctic teleost fishes [14], lends support to the notion that this isoform is important for low-temperature lipid transport. Reports of H-FABP in adipose tissue of rats have been reviewed [3] and reported elsewhere [4], and increased expression of *h-fabp* has been linked to cold exposure [6].

Increased transcript levels do not necessarily confer a corresponding change in the activity, function or amount of a protein. Although most genes involved in fatty acid metabolism are regulated at the transcriptional level [15,16], a number of studies on hibernators have shown that translation rates decrease in most organs and tissues during hibernation [17–19], partially due to dissociation of polyribosomes. However, selected synthesis of some proteins occurs during hibernation and the present data from Western blotting shows that FABP is one of these. The data for *M. lucifugus* showed higher protein levels of A-FABP in BAT of hibernating animals as well as higher levels of H-FABP in BAT, heart and skeletal muscle, as compared with euthermic bats.

A number of genes involved in promoting a switch from carbohydrate to fatty acid fuel sources have been shown to be up-regulated in hibernating animals [4,13,17,20–22]. Interestingly, many of these genes are also up-regulated in multiple tissues of insulin-sensitive tissues of transgenic rats under stimulation by agonists of the γ isoform of the peroxisome proliferator activated receptor (PPARγ) [23]. It is highly likely that a similar mechanism involving PPARγ is involved in stimulating the expression of genes involved in fatty acid catabolism in hibernating mammals. We have recently shown that PPARγ is expressed at higher levels in BAT and skeletal muscle of torpid versus euthermic bats [24], both of these tissues also displaying up-regulation of *h-fabp*. All genes involved in lipid metabolism are thought to contain a PP response element, to which PPARs bind in a heterodimer with RXRα [16,25], while also interacting with the PPAR gamma co-activator, PGC-1 [26]. Recently, studies have been undertaken that have shown interactions of FABPs with PPARs ultimately leading to enhanced transcriptional activity of these nuclear receptors [27,28]. Specifically, A-FABP and keratinocyte FABP (K-FABP) interact directly with PPARγ and PPARβ, respectively, and activation of PPARα and PPARγ occurs through interaction with L-FABP in cultured hepatocytes [28]. Considering the overall structural homology of FABPs, it is likely that other isoforms may bind to these transcription factors as well. These findings, coupled with the findings implicating FABPs in cold tolerance [6,14] and our own previous studies identifying *a-* and *h-fabp* up-regulation in hibernating ground squirrels [4], provides a strong indication that FABPs play important roles in cold tolerance and lipid metabolism, two important aspects of mammalian hibernation.

Of note, the transcript in WAT displays a reduced size by approximately 50 nucleotides in both euthermic and hibernating isolations. Studies on poly-A tail lengths in hibernating animals have shown that they are preserved in liver [21] through association with a poly A binding protein. Since WAT is mainly used for lipid storage there may not be a requirement for the same protective mechanisms displayed in liver during hibernation.

Our current results document the up-regulation of *a-* and *h-fabp* in BAT and *h-fabp* in skeletal muscle in the little brown bat along with elevated levels of A- and H-FABP protein in multiple tissues. These data broaden our understanding of the role that FABPs play in hibernating mammals. Our findings also indicate that striking amino acid substitutions occur in H-FABP from *M. lucifugus*, some of which have also been described for H-FABP from *S. tridecemlineatus* [4]. Because of the position of these substitutions, it is likely that they act to bestow flexibility on the protein so that it can function appropriately when T_b falls to near ambient for several weeks during each torpor bout. Up-regulation of FABPs at both the gene and protein levels in *M. lucifugus* tissues, along with the aforementioned substitutions indicate that the expression and activity of these proteins may be highly adapted for hibernation.

Acknowledgements

We are very grateful to Dr. D. Thomas, Université de Sherbrooke, Sherbrooke, Quebec for collecting, housing, and hibernating bats and to Dr. F. Spener (University of Munster, Germany) for providing antibodies for A-FABP and H-FABP. Thanks also to J.M. Storey for assistance in preparation of the manuscript. This study was supported by a research grant from the Natural Sciences and Engineering Research Council of Canada to K.B.S. and an Ontario Graduate Scholarship to S.F.E. K.B.S. holds the Canada Research Chair in Molecular Physiology.

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