Molecular and Cellular Biochemistry **269:** 175–182, 2005.

Cloning and expression of PPARγ and PGC-1α from the hibernating ground squirrel, *Spermophilus tridecemlineatus*

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Received 2 June 2004; accepted 27 August 2004

Abstract

The peroxisome proliferator-activated receptor (PPAR) family of transcription factors play a key role in lipid metabolism and have been implicated in a number of disease states, most notably of which is obesity. Controlled regulation of lipid metabolism is a key ingredient for successful hibernation. Partial cDNA sequences for one of the PPAR proteins, PPARγ and the PPARγ co-activator (PGC-1α) have been cloned from the hibernating ground squirrel, *Spermophilus tridecemlineatus* and show differential regulation during hibernation at the mRNA level using relative RT-PCR and at the protein level via immunoblotting in brown adipose tissue (BAT), heart, skeletal muscle and white adipose tissue (WAT). The cDNA sequence for PGC-1α revealed a number of amino acid substitutions and two were worthy of note, one resulting in the loss of a potential protein kinase C (PKC) site, while another resulted in the creation of a PKC site, suggesting that PKC may be important in regulating PGC-1α. RT-PCR revealed a near 2-fold up-regulation of PPARγ in BAT and to a lesser extent (<1.5-fold) in heart and WAT, while PGC-1α displayed significantly higher levels of expression in skeletal muscle during hibernation (3.1-fold, $p < 0.005$). The protein levels of PPARγ were significantly increased in BAT and WAT (1.5 and 1.8-fold, respectively) while PGC-1α displayed significant changes in expression in heart (3.5-fold) and skeletal muscle (1.8-fold). Our current findings indicate a role for increased expression of PPARγ and PGC-1α in hibernating animals. (Mol Cell Biochem **269:** 175–182, 2005)

Key words: brown adipose tissue, heart, hibernation, PPAR, PGC-1α, skeletal muscle, gene expression, protein expression

Introduction

Many small mammals use hibernation as a survival strategy during winter. Hibernators strongly suppress their metabolic rate allowing body temperature (Tb) to fall to near 0 °C entering a period of torpor. Periods of deep torpor may last for several weeks and are interspersed with brief arousals when the animal rewarms itself to 37 °C powered by thermogenesis in brown adipose tissue (BAT). Accompanying torpor, heart rate is greatly reduced creating conditions that would be ischemic in nonhibernating species [1–3]. One key to hibernation success is metabolic rate depression, lowering overall energy usage by as much as 90% compared with the energy requirements of maintaining euthermia throughout the winter [2, 4]. Other factors in hibernation success include a reorganization of fuel use so that most organs depend primarily on lipid catabolism for ATP production (even brain switches to a high dependence on ketone bodies as fuels) [5]. For example, temperature-independent kinetic properties of ground squirrel fatty acid binding protein (FABP), but not rat FABP, aid the role of this intracellular transporter of fatty acids in maintaining fuel supply to the mitochondria at all Tb values [6].

Multiple adaptations for hibernation are focused on lipid metabolism. A period of hyperphagia in late summer lays down massive triglyceride reserves in white adipose tissue

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(WAT) and increases body mass by 50% or more [7]. Seasonal proliferation of BAT also optimizes thermogenic capacity and virtually all organs enhance their capacities to transport (e.g., elevate FABP levels) and catabolize lipids. Gene expression studies have shown this to be true of FABP in multiple organs of hibernating bats and ground squirrels [8, 9], as well as other key proteins and enzymes involved in lipid metabolism [10, 11].

Given the importance of lipid catabolism to the survival of the hibernator, we hypothesized that the transcription factors that regulate the expression of genes involved in lipid metabolism would play important roles in hibernation. Peroxisome proliferator-activated receptor gamma isoform (PPARγ) has been shown to regulate a wide range of proteins/enzymes related to lipid metabolism [12]. A potent co-activator of PPARγ, PGC-1α has been linked with thermogenesis and cellular respiration in adipose tissues and skeletal muscle following cold exposure in mice [13] and is up-regulated in liver of fasting rodents [14]. Hence, we proposed that up-regulation of PPARγ and PGC-1α during hibernation would facilitate expression of proteins required for a lipid-based metabolism required in the torpid state. In the current study we have cloned substantial segments of the genes for PPARγ and PGC-1α the hibernating thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*), identified potentially important amino acid substitutions in the sequence of PGC-1α, and demonstrated transcriptional and translational up-regulation of both proteins during hibernation.

Materials and methods

Animals

Thirteen lined ground squirrels, *Spermophilus tridecemlineatus* (130–180 g) were obtained from a licensed trapper (TLS Research, Michigan) in September 2000 and transported to the Animal Hibernation Facility (NIH, Bethesda, MD). Hibernation experiments were conducted by the laboratory of Dr. J.M. Hallenbeck (National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD). Animals were kept on a natural fall light cycle (mimicking natural hours of daylight and night) and fed *ad libitum* until they entered and finished the pre-hibernation phase of hyperphagia so as to maximize lipid stores. Once animals displayed a rapid weight gain to 220–240 g, a group of animals were placed in dark chambers at 5–6 °C to induce hibernation, while another group remained euthermic. Once the animals placed in the hibernation chamber had a continuous body temperature of 5 °C (which took 2–5 days) they were sacrificed by decapitation and tissues were excised and frozen immediately in liquid nitrogen; tissues were transported to Ottawa on dry ice where they were then placed at −80 °C until use. Euthermic animals that were kept awake and aroused were sacrificed on the same days as hibernators.

RNA isolation and cDNA synthesis

All materials used for RNA preparation were treated with 0.1% v/v diethylpyrocarbonate (DEPC) and autoclaved. Total RNA was isolated from euthermic and hibernating animals using Trizol Reagent (Invitrogen) according to manufacturer's protocol. Total RNA was separated on a 1.2% denaturing formaldehyde agarose gel and its quality assessed by the presence of sharp and distinct 28S and 18S rRNA bands. Poly $(A)^+$ RNA was isolated from total RNA using Oligotex poly$(A)^+$ isolation kits (Qiagen). RNA concentration was determined spectrophotometrically at 260 nm.

First strand cDNA was prepared using 1 μg of poly $(A)^+$ RNA and heated at 65 °C for 5 min and placed on ice. An aliquot of 100 ng of anchored oligo dT primer (Bio S&T, Montreal, QC) was added along with 20 mM dNTPs (5 mM each nucleotide), 2 μl of 0.1 M DTT and RNase inhibitor and incubated at room temperature for 10 min. Following this, 1 μl of Superscript RT (Gibco BRL) was added and incubated at 37 °C for 1 h. RNA remaining in the reaction was digested using RNAse H. The resulting cDNA was used in subsequent PCR reactions.

RT-PCR

The program DNAman was used to align multiple sequences to determine regions of homology between sequences from different sources. For PPARγ, the sequences used had the following accession numbers: L40904(human), NM_011146(mouse), NM_013124(rat), AF103946 (pig) and AY179866(cow). Accession numbers for PGC-1α were: AF108193(human), NM_031347(rat), NM_008904(mouse), AY346131(pig) and AB106107(cow). For a control gene, α-tubulin was chosen with forward (5′-AAGGAAGATGCTGC CAATAA-3′) and reverse (5′-GGTCACATTTCACCATC TG-3′) primers. Forward and reverse primers for PPARγ were (5′-CCCGAGTCTGCTGACCTCCG-3′) and (5′-TGC AGTAGCTGCACGTGTTC-3′), respectively. Forward and reverse primers for PGC-1α were (5′-CTCTCTGGAACTG CAGGCCT-3′) and (5′-ATCCATGGCTAGTCCTGAAT-3′), respectively. RT-PCR products were first electrophoresed on a 1.5% TAE agarose gel for size confirmation and then sequenced (Canadian Molecular Research Services, Ottawa, ON). The PCR reaction was performed by mixing 1 μg of cDNA, 1.25 μl of 1 μM primer mixture (0.5 μM forward and 0.5 μM reverse primers), 2.5 μl of 10 × PCR buffer (Invitrogen), 1.25 μl of 5× $MgCl_2$, 0.5 μl of 10 mM dNTPs and

0.125 μl of Taq Polymerase (Invitrogen) and water was added to bring the final volume to 25 μl. The cycles performed for amplification consisted of an initial step of 2 min at 94 ° C, followed by the actual amplification steps of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min repeated 37 times. A final step at 72 °C was performed for 2 min. PCR products were then run on a 1.0% agarose gel, and visualized with ethidium bromide. A 737 bp fragment and a 663 bp fragment were found and sequenced at the Canadian Molecular Research Services (Ottawa, ON). The sequences obtained were confirmed to be PPARγ and PGC-1α respectively by submitting the sequences to BLAST.

Sequence analysis

DNA sequences were analyzed and subjected to BLAST searches to confirm identity. Deduced amino acid sequences were analyzed to assess potential posttranslational modification sites using PROSITE found on the ExPASY molecular biology server.

Tissue homogenization and SDS-PAGE

Frozen tissue samples were ground into small pieces in a mortar and pestle under liquid N_2. Approximately 100 mg of tissue was placed in 1 ml of homogenization buffer (100 mM MOPS, pH 7.8, 25 mM HEPES, pH 7.8, 25 mM β-glycerophosphate, 5 mM EDTA pH 8.0, 1 mM EGTA, and 250 μM $NaVO_4$, pH 7.4 with 1 mM phenylmethylsulphonyl fluoride added immediately before homogenization. Soluble protein content was measured using the BioRad prepared reagent and then samples were diluted 1:1 in 2$\times$ SDS sample buffer. Aliquots containing 15 μg of total protein were loaded into each lane on 12% polyacrylamide gels. Electrophoresis and transfer to PVDF membranes (Biotrace, PALL Life Sciences) was carried out as described previously [15].

Antibodies and Western blotting

PPARγ antibody was purchased from Cedarlane Labs (Hornsby, Ontario) and PGC-1α antibody was a gift of Dr. Dan Kelly (Washington University, St. Louis). Antibody dilutions of 1:1000 in 5% blocking buffer were used for all procedures. Secondary rabbit IgG HRP conjugated antibody was purchased from Cell Signaling. Blots were developed using the Western Lightning™ Chemiluminescense *Plus* (NEN, Perkin Elmer) system according to protocols provided with the system. Blots were briefly exposed to Kodak X-OMAT-AR film and developed according to manufacturer's protocols. Images on X-OMAT film were quantified using densitometric analysis software (Imagequant, Molecular Imaging). After antibody reactions were complete, blots were restained with Coomassie blue to confirm equal loading.

Quantification and statistics

RT-PCR and Western bands were scanned and densitometric analysis was performed using Imagquant (Molecular Dynamics). PPARγ and PGC-1α RT-PCR bands were normalized relative to RT-PCR bands of α-tubulin run from the same cDNA reaction. Band intensity of the immunoreactive material in each lane of Western blots first normalized against a minimum of three Coomassie stained protein bands that did not appear to change between euthermic and hibernating states. Mean normalized band densities $\pm$S.E.M. for samples from hibernating versus euthermic animals were then calculated and significant differences between the groups were tested using the Student's *t*-test. The ratio hibernating:euthermic was calculated and plotted; error bars on the final histograms are the sum of S.E.M. values for hibernating and euthermic trials.

Results

Partial cDNA cloning of PPARγ and PGC-1α

A 737 bp fragment of PPARγ and a 663 bp fragment of PGC-1α were PCR amplified from cDNA created from brown adipose tissue (BAT) of hibernating ground squirrels. These ground squirrel sequences were deposited into GenBank with accession numbers AY518346 and AY518347 for PPARγ and PGC-1α, respectively. Figures 1 and 2 show the translated amino acid sequences providing 245 amino acid residues for PPARγ and 221 for PGC-1α which represents about 50% and 27% of the typical sequence lengths of PPARγ and PGC-1α, respectively, for these two proteins from most mammalian sources. Figures 1 and 2 also show the alignment of the ground squirrel sequences with the proteins from five other mammalian sources with numbering along the right hand side showing the amino acid residue numbers for the full-length human sequence. Substitutions specific to *S. tridecemlineatus* are in bold. Over the amplified region, the ground squirrel PPARγ protein is 99% identical to other mammalian sequences whereas PGC-1α is 85% identical to other sequences. No amino acid substitutions were found in PPARγ that were considered significant, although three were unique to the ground squirrel. We have only amplified a partial cDNA and thus can not rule out the possibility of substitutions occurring in other regions of the protein. PGC-1α, on the other hand, contains a number amino acid substitutions not observed in nonhibernating mammals. A number of

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squirrel  ..............................PESADLRALAKHLYDSYIKSFPLTKAKARAILTGKTTDKSPFVIYDM  ---
human     vgmshnairfgrmpqaekekllaeissdidqln-----------------------------------------------  279
mouse     vgmshnairfgrmpqaekekllaeissdidqln-----------------------------------------------  279
rat       vgmshnairfgrmpqaekekllaeissdidqln-----------------------------------------------  279
pig       vgmshnairfgrmpqaekekllaeissdidqln-----------------------------------------------  278
cow       vgmshnairfgrmpqaekekllaeissdidqln-----------------------------------------------  279

squirrel  NSLMMGEDKIKFKHITPLQEQSKEVAIRIFQGCQFRSVEAVQEITEYAKSIPGFVNLDLNDQVTLLKYGVHEIIYTMLAS  ---
human     -------------------------------------------------------------------------------  359
mouse     ----------------------------------------------------n----i-----------------------  359
rat       ----------------------------------------------------n----i-----------------------  359
pig       ----------------------------------------------------n-------------------------------  358
cow       --------------s----p-----------------------------n-------------------------------  359

squirrel  LMNKDGVLISEGQGFMTREFLKSLRKPFGDFMEPKFEFAVKFNALELDDSDLAIFIAVIILSGDRPGLLNVKPIEEIQDN  ---
human     -------------------------------------------------------------------------------d----  399
mouse     -------------------------------------------------------------------------------d----  399
rat       -------------------------------------------------------------------------------d----  399
pig       -------------------------------------------------------------------------------d----  398
cow       -------------------------------------------------------------------------------d----  399

squirrel  LLQALELQLKLNHPEASQLFAKLLQKMTDLRQIVTENV..  ---
human     --------------s-------------------h-ql  479
mouse     --------------s------v-------------h-ql  479
rat       --------------s------v-------------h-ql  479
pig       --------------s-------------------h-ql  478
cow       --------------s-------------------h-ql  479
```

Fig. 1. Partial amino acid sequence of PPARγ from *S. tridecemlineatus* (accession number AY518346) aligned with PPARγ sequences from other mammals. Accession numbers for sequences of other species are given in the materials and methods. Substitutions specific for the ground squirrel PPARγ appear in bold.

```
squirrel  ........LSGTAGLTPPTTPPHKANQDNPFRASPKPKSSCKTVVPPPSKKARYSESSGTQGNNSTKKGPEQSELYAQLS  ---
human     iertlsve-----------------------------l-------------p-----------------------------  358
mouse     iertlsve-----------------------k----l-p---------t-r-----c-----sh-----------------  357
rat       iertlsve-----------------------k----l-p---------t-r-----c-----sh-----------------  356
pig       iertlsve-----------------------------l-pp----------t--------h--------------------  356
cow       iertlsve-----------------------------l-p-------------------c---s-----------------  356

squirrel  KSSVPSSGHEERKAKRPSPRLFGDHDYCQSINSKTEILLHVSQELPDSRQLDYKDA.SSAWQGQICSSTDSDQLYLRETL  ---
human     ----ltg------t----l-------------------ini----q-----en--v.--d-------------c------  437
mouse     ---gl-r------t----l-----------l----d--ini----q------f---.-cd---h-------g-c------  436
rat       ----l-r------t----l-----------v----d--ini----q------f---.-cd---h-------s-c------  435
pig       -t-algg-------r---l---------------a---ini----h------s---.a-d--r-m--------c--t-s    435
cow       -t--lt------------l-------------------vst----h-----en---p--ngp---h------pc-----a  436

squirrel  EASKQVSPCGTRKQLQDQEIRAELNKHFGHPSQAVFDDEADKTSELRDSDFSNEQFSKLPMFINSGLAMD.........  ---
human     ---------s-------------------------------------------------------------glfddsedes  517
mouse     ---------s----------------------c------ks--------g-----------v---------glfddsedes  516
rat       ---------s---------------------------kv--------gn----------v---------glfddseden  515
pig       ---r----gsa-------------------------------------------------------------glfddsedes  515
cow       -v-r----gs----------------------------k-------------------------------glfddsedes  516
```

Fig. 2. Partial amino acid sequence of PGC-1α from *S. tridecemlineatus* (accession number AY518347) aligned with PGC-1α sequences from other mammals. Accession numbers for sequences of other species are given in the materials and methods. Substitutions specific for the ground squirrel PGC-1α appear in bold.

proline substitutions are evident; these correspond to positions 315, 363, 377 (all leucine to proline substitutions) and 404 of the mature human PGC-1α protein (Fig. 2). Substitution at position 377 results in the formation of a potential PKC phosphorylation site (SPR) [16, 17]. Other substitutions of note are a tyrosine substitution in the squirrel protein corresponding to amino acid 411, an alanine substitution in the squirrel protein replacing charged residues at position 417,

and a cysteine to leucine substitution found at position 431. Interestingly, a serine to glycine substitution at position 447 results in the loss of a potential PKC phosphorylation site (STRK to GTRK) [16, 17].

PPARγ and PGC-1α gene expression in 13-lined ground squirrels

RT-PCR studies were performed to assess the relative levels of mRNA transcripts of both PPARγ and PGC-1α in tissues of euthermic versus hibernating 13-lined ground squirrels. Figure 3 shows the results from RT-PCR using PPARγ or PGC-1α primers and RNA from four tissues: BAT, white adipose tissue (WAT), skeletal muscle and heart. Transcripts for both genes were detected in all tissues. For PPARγ transcripts the ratio of hibernating:euthermic was always greater than 1.0 with the highest value in BAT (1.9-fold) although no significance was established. The same was true of PGC-1α transcript levels with hibernating:euthermic values of 2.5 in heart, 2.0 in WAT, although not significant a trend of higher levels in hibernating animals is observed. PGC-1α was significantly up-regulated in skeletal muscle 3.1 ± 0.3. The corresponding α-tubulin control bands were used to normalize the samples.

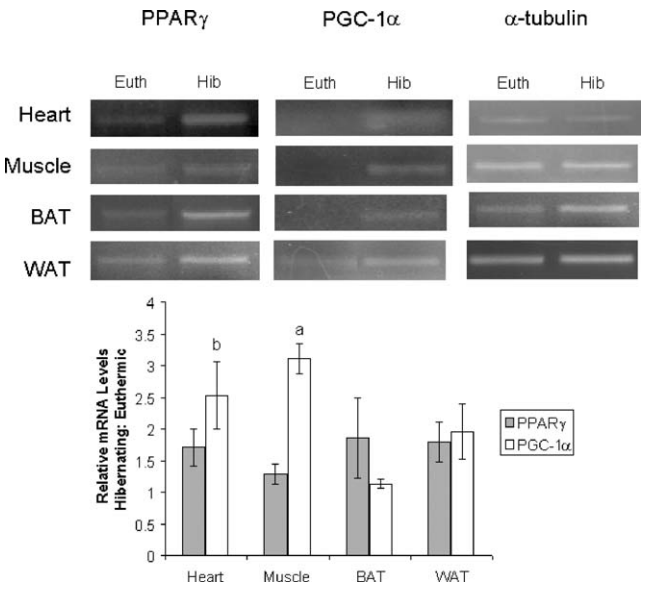

Fig. 3. Effects of hibernation on *PPARγ* and *PGC-1α* gene expression in organs of *S. tridecemlineatus*. Representative gels show transcript levels in samples from euthermic (Euth) and hibernating (Hib) animals stained with ethidium bromide. Histograms show the ratio of mRNA levels in samples from hibernating and euthermic animals; values are mean ± S.E.M. for $n = 3$ independent trials. Values in hibernator samples are different from the corresponding euthermic value as assessed by the Student's *t*-test; a: $p < 0.005$ and b: $p < 0.1$.

PPARγ protein expression

Hibernators rely almost exclusively on stored lipids as metabolic fuels during torpor and we predicted that levels of the PPARγ transcription factor would rise in hibernator organs to promote the expression of genes that encode proteins involved in lipid catabolism. PPARγ protein levels were assessed by Western immunoblotting. A single band of crossreacting material was detected at the expected molecular weight of 55 kDa in heart, skeletal muscle, BAT and WAT. Figure 4 shows representative blots as well as the ratio of PPARγ levels in hibernating versus euthermic states for the four tissues. PPARγ protein levels were significantly higher in BAT (ratio 1.4 ± 0.1, a, $p < 0.05$) of hibernators and a ratio of 1.7 ± 0.3 was found in WAT was found, although not significant, a trend of up-regulation is evident.

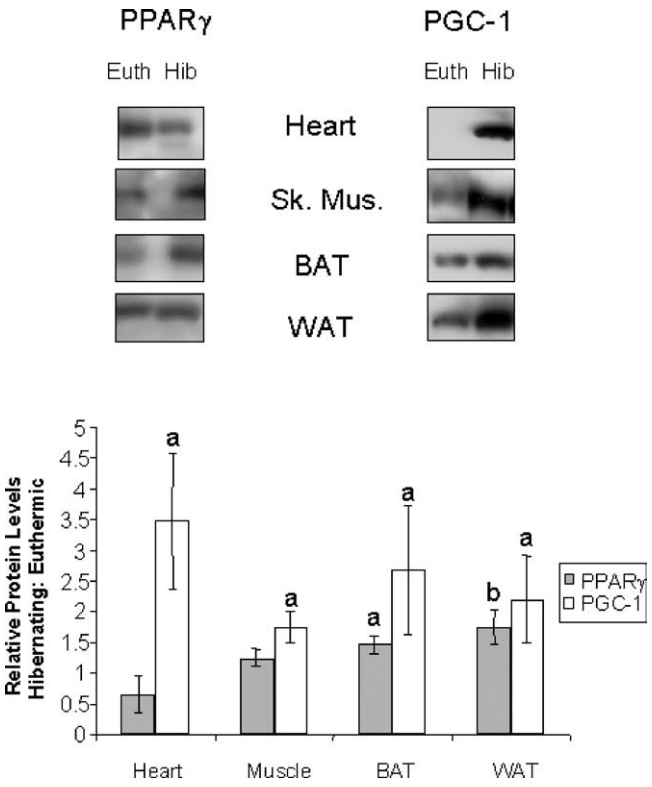

Fig. 4. Effects of hibernation on PPARγ and PGC-1α protein expression in *S. tridecemlineatus* tissues. Representative Western blots show results for $n = 2$ samples from organs of euthermic and hibernating animals (except for PPARγ in WAT where $n = 1$ is shown). Histograms show the ratio of protein levels in tissues from hibernating versus euthermic animals; data are means ± S.E.M. for $n = 3$ independent trials. a: Values in hibernator samples are significantly different from the corresponding euthermic value, as assessed by the Student's *t*-test, $p < 0.05$, b: values in hibernator samples are different from the corresponding euthermic value as assessed by the Student's *t*-test, $p < 0.1$.

PGC-1α protein expression

PGC-1α can act as a co-activator of PPARγ and its expression often correlates well with that of PPARγ. Figure 4 also shows the results of Western blotting to evaluate PGC-1α protein levels in tissues euthermic and hibernating ground squirrels. A band crossreacting with the PGC-1α antibody was detected in all organs at the expected molecular weight of 85–90 kDa. PGC-1α levels were elevated significantly during hibernation in all four tissues tested. The ratios hibernating:euthermic were 3.5 ± 1.2 in heart, 1.7 ± 0.3 in skeletal muscle, 2.7 ± 1.1 in BAT and 2.1 ± 0.6 in WAT (all $p < 0.05$). The data for BAT and WAT support a strong correlation between PGC-1α and PPARγ levels that is both qualitative and quantitative.

Discussion

The data presented in this paper show differential expression at both the mRNA and protein levels of PPARγ and PGC-1α in muscle and adipose tissues of the hibernating ground squirrel, *S. tridecemlineatus.* In particular, PGC-1α protein was significantly higher in all four tissues examined of hibernating versus euthermic animals and elevated PGC-1α transcript levels in heart, muscle and WAT argued for hibernation-induced gene up-regulation. Adaptive thermogenesis is thought to occur primarily in adipose tissue, specifically brown adipose tissue due partially to the activation of PPARγ and PGC-1α [13]. Indeed, higher protein levels of PPARγ and PGC-1α were observed in hibernating BAT, consistent with this hypothesis. These data are also consistent with our previous findings showing higher levels of *a-fabp* and *h-fabp* mRNA transcripts during hibernation in *S. tridecemlineatus* [8, 15]. PPARγ is well-known to induce *a-fabp* and *h-fabp* [12, 18]. PPARγ and PGC-1α have recently been shown to drive the formation of brown adipocyte features in WAT and which may be partially due to increased mitochondrial formation [19]. This view is supported by the findings showing increased mitochondrial biogenesis due to PGC-1α [20]. It is also consistent with the PGC-1α transgenic mouse model showing larger red muscle fibre formation due to increased mitochondria formation in the skeletal muscle [21]. We have also shown up-regulation of *cox1* in heart and BAT of *S. tridecemlineatus* [22] and *ND-2* in heart and skeletal muscle of *Spermophilus lateralis* [23] lending support to the notion that PGC-1α is responsible for mitochondrial formation and subsequently an elevated capacity for oxidative metabolism of fatty acids in hibernating animals.

In addition to the aforementioned findings, others have found up-regulation of genes in the heart of *S. tridecemlineatus* relating to an increased lipid metabolism during hibernation [11]. Our current findings suggest a model in which PGC-1α leads to the general up-regulation of mitochondrial transcripts in hibernating animals and likely the number of mitochondria present. In BAT, the primary role of the mitochondria is to facilitate nonshivering thermogenesis during arousal, while in the heart, the role of increased mitochondria is likely to allow for the swift utilization of fatty acids required for the rapid increase in heart rate associated with arousal from torpor. Increased pancreatic lipase (in heart), A- and H-FABP (in BAT and heart) likely provide a synergistic effect in these two tissues. In fact, A-FABP and hormone sensitive lipase (HSL) have been shown to interact with one another leading to a 2-fold increase in HSL lipolysis activity [24, 25]. Presumably, in hibernators, such an interaction occurs leading to increased lipolysis and transport of fatty acids to the mitochondria where they enter in futile cycling (BAT) or fatty acid oxidation (heart).

The function of skeletal muscle during hibernation is less clear although it does play a role in thermogenesis through shivering once Tb has risen to 15–20 °C. Optimizing the lipid oxidation and mitochondrial capacities of red muscle, may in fact allow for skeletal muscle to warm up to 15–20 °C through a nonshivering thermogenesis mechanism. Initial studies on skeletal muscle from hibernating animals suggested that during the winter the muscle atrophies significantly [26]. However, recent work suggests that this atrophy may be due primarily to white muscle fibre atrophy, while cross sectional area of red muscle fibres appear to be maintained during cold exposure [27]. This, coupled with the findings in the PGC-1α transgenic mouse, provide a model whereby PGC-1α prevents red muscle fibre atrophy in hibernating skeletal muscle. The prevention of red muscle atrophy would presumably occur through induction of mitochondrial biogenesis via PGC-1α [20, 28] and would provide an explanation for our previous findings of increased *cox1* mRNA in heart of *S. tridecemlineatus* [22] and up-regulation of *ND-2* in heart skeletal muscle of *S. lateralis* [23].

The squirrels used in this study were hibernating for 2–5 days. We believe that the higher levels of PPARγ and PGC-1α proteins found during hibernation can stimulate renewed gene expression and protein synthesis for critical genes required for lipid-based energy metabolism in torpor and arousal including *a-fabp, h-fabp,* mitochondrial encoded genes and uncoupling proteins. As we have shown previously [29], PPARγ is present in fairly high levels in heart and muscle of hibernators, whereas it is usually most abundant in adipose tissue [12]. This may attest to an enhanced importance of PPARγ and PGC-1α and fatty acid metabolism in hibernating species.

Although we do not know what specific effects the amino acid substitutions have on PGC-1α since very little structural work has been done on this protein, it is likely that hibernator PGC-1α has adapted to allow continued function at low temperature as evidenced by its up-regulation in all

four tissues tested and the numerous amino acid substitutions discovered in the *S. tridecemlineatus* sequence. With the number and uniqueness of amino acid substitutions relative to other rodents (mouse, rat) and other mammals in general (human, pig, cow), it is difficult to assume that it is the result of only natural sequence divergence between *S. tridecemlineatus* and other rodents and mammals. In particular, three separate leucine to proline substitutions occur over a relatively short segment of *S. tridecemlineatus* PGC-1α. Interestingly, in cold adapted enzymes, proline is usually less abundant than in enzymes optimized for function at 37 °C [30], making our findings even more surprising. Interestingly, the leucine to proline substitution at residue 377 recreates a potential protein kinase C phosphorylation site that was eliminated by a serine to glycine substitution at residue 447. This suggests that PKC regulation may be a key element in PGC-1α control and it is possible that the shift in PKC site is related to conformational changes in the protein that have occurred as a result of substitutions at other sites. Studies from our lab have indicated that PKC is involved in cold temperature survival in frogs [31, 32].

Up-regulation of the mRNA and protein during hibernation strongly suggests that a component of hibernation biology is key in regulation of PGC-1α. Also, the incorporation of a new potential PKC site in hibernator PGC-1α suggests that this protein may play a role in its regulation at low temperatures. Discovering the exact component will be key in deciphering the role that PGC-1α plays in many systems.

Acknowledgements

We are grateful to J.M. Hallenbeck for providing us with ground squirrel tissues and to D. Kelly, University of Washington, St. Louis, MO for providing us with a PGC-1α antibody. Many thanks to J.M. Storey for critique of the manuscript. K.B.S. holds the Canada Research Chair in Molecular Physiology. This study was supported by a research grant from the National Sciences and Engineering Research Council of Canada to K.B.S. and an Ontario Graduate Scholarship to S.F.E.

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