Seasonal, tissue-specific regulation of Akt/protein kinase B and glycogen synthase in hibernators

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Submitted 3 September 2003; accepted in final form 24 November 2003

MAMMALS THAT HIBERNATE (hibernators) use an evolutionary strategy that allows them to survive winter food shortages and extremely low temperatures. The yellow-bellied marmot (Marmota flaviventris), for example, increases nutrient storage significantly during the summer months, but then utilizes the accumulated nutrients during its long winter hibernation (11). The seasonal exaggeration increased in body mass and adiposity in these animals makes them an ideal model for studying the molecular basis for rapid weight gain leading to obesity.

In the study described herein, we evaluated the seasonal regulation of Akt/protein kinase B (PKB), a serine-threonine kinase that is a primary regulator of nutrient storage in white adipose tissue (WAT) and skeletal muscle tissue. Mammals contain three highly homologous isoforms of the Akt/PKB enzyme, each of which are expressed in all tissues (41). Studies of cultured adipocytes and/or myotubes strongly implicate the enzyme as a key stimulus of nutrient storage: 1) the expression of constitutively active forms of Akt/PKB (CA-Akt/PKB) in adipocytes increases rates of glucose uptake by both affecting a redistribution of the GLUT4 glucose transporter from intracellular stores to the plasma membrane and simultaneously increasing the expression of GLUT1 (18, 31, 34, 36); 2) CA-Akt/PKB expression stimulates rates of lipogenesis (19, 30) and simultaneously increases the rate of fatty acid synthase transcription (39); 3) CA-Akt/PKB expression stimulates protein synthesis by inactivating glycogen synthase kinase-3β, an upstream inhibitor of the rate-limiting enzyme regulating glucose incorporation into glycogen (i.e., glycogen synthase (GS)) (4, 7, 32, 36); and 4) CA-Akt/PKB expression stimulates protein synthesis by activating the mammalian target of rapamycin (mTOR) to regulate pp70S6-kinase and the translational repressor PHAS1/4EBP1 (8, 36). Constitutively active forms of the enzyme additionally promote the differentiation of preadipocytes into adipocytes (18, 22) and stimulate the secretion of leptin (1), an adipokine that suppresses appetite in rodents. Collectively these studies implicate Akt/PKB as a critical regulator of many of the biological processes controlled so precisely in hibernators as they prepare for winter torpor. Interestingly, it has also recently been shown that Akt/PKB protein levels are reduced during torpor in WAT of the little brown bat (M. lucifugus) (5).

Numerous hormones, cytokines, and transforming onco-genes activate Akt/PKB, which, in addition to regulating metabolism, is a key stimulus of cell size and survival. In WAT and skeletal muscle, insulin is a key regulator of Akt/PKB, which is a requisite intermediate linking the hormone to post-prandial stimulation of glucose uptake and anabolic metabolism (3, 14, 16, 17, 33, 40). The aforementioned ligands activate Akt/PKB by promoting its phosphorylation on two regulatory residues (Thr308 and Ser473 for the Akt1/PKB isoform; reviewed in Ref. 41). Insulin-stimulated Akt/PKB phosphorylation requires the activation of phosphatidylinositol 3-kinase (PI3-kinase), a lipid kinase that phosphorylates phosphoinositides to generate phosphatidylinositol-3,4-bisphosphate or phosphatidylinositol-3,4,5-trisphosphate. These phosphoinositides activate Akt/PKB by directly binding a pleckstrin homology domain on the enzyme, which exposes the regulatory phosphorylation sites. Akt/PKB can also be activated by PI3-kinase independent mechanisms, for example in response to growth hormone (27), cellular stresses such as heat shock and hyperosmolarity (20, 21), or increases in intracellular Ca2+ or cAMP (9, 37).

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We evaluated changes in Akt/PKB expression and activity from June to November, which covered the periods of maximal lipid accumulation, peak body mass, and the beginning of the hibernation season. The findings obtained revealed that the activity of Akt/PKB isoforms 1 and 2 in WAT, measured under fasting conditions, increased markedly during the weight-gaining lipogenic months (i.e., July) and then decreased substantially during the months immediately preceding hibernation.

The increase in Akt/PKB activity was mirrored by an increase in both the activity and the expression of GS, an anabolic enzyme that lies “downstream” of Akt/PKB in the signaling cascade linking insulin to the regulation of glycogen synthesis. These changes in signaling did not correlate with circulating insulin concentrations. Interestingly, in another insulin-responsive cell type, skeletal muscle, Akt/PKB activity peaked much later than it did in WAT (i.e., peak activity in September), confirming that the increase in fasting Akt/PKB activity in WAT was independent of circulating insulin concentrations. Collectively, these data suggest that hibernators possess a unique intrinsic regulatory mechanism for activating Akt/PKB, perhaps as a means for selectively accelerating rates of anabolic metabolism in particular tissues.

**MATERIALS AND METHODS**

**Animals.** Four yellow-bellied marmots (3 females, 1 male) were trapped in the Rocky Mountains of Gunnison County, CO. The animals were transported to the laboratory of animal resources at Colorado State University, caged individually, and housed under a 14:10-h light/dark photoperiod and ambient temperature of 20 ± 3°C until September. Animals were provided with Purina rodent chow (5001) and water ad libitum. In the first week of September, all food was removed and the animals were moved to a cold room (5 ± 2°C) kept in constant darkness (DD). In October, animals demonstrated all of the behavioral changes associated with preparation for hibernation, although we did not measure body temperature. In April, the animals were moved from the cold room back to the laboratory and maintained under the same conditions as described above. All animal protocols were approved by Colorado State University’s Animal Care and Use Committee.

**Sample collection and preparation.** After initial trapping, the animals were maintained at Colorado State University for one year. Beginning in the last week of June 2001, monthly blood, WAT, and muscle samples were collected. All subsequent samples were collected during the last week of each month. Twenty-four hours before surgery, all animals were transferred to a holding facility (at room temperature) and fasted overnight. Animals were euthermic during surgery. The animals were then anesthetized by intramuscular injection of ketamine-acetaminone and maintained under halothane anesthesia during the sterile surgical procedure. Before tissue collection, body mass data were obtained and percent body lipid was determined using an EM scan body composition analyzer (model SA-3000, EM Science, Pennsylvania), and values obtained were well within the linear range of the detection method

**Akt kinase assays.** Marmot WAT and muscle tissue were analyzed for Akt kinase activity using methods described previously (15) with slight modifications. Briefly, 100 μg of crude lysate was diluted to 300 μl in lysis buffer. Akt isoform 1 was immunoprecipitated with 30 μl of mouse anti-Akt1 agarose conjugated beads (Santa Cruz) at 4°C for 100 min. Akt isoform 2 was immunoprecipitated with 5 μl of a rabbit anti-Akt2 antibody for 1 h at 4°C, and then 50 μl of preequilibrated protein-A conjugated agarose beads (Santa Cruz) were added for 30 min. The immune complexes were then washed three times in cold lysis buffer and three times in 1× kinase buffer (20 mM HEPES pH 7.2 and 5 mM MgCl2). The kinase reaction was then started by the addition of 30 μl of kinase mix [10 μM Mg++ ATP, 1 mM DTT, 200 μM EGTA, 25 μg histone H2B (Boehringer Mannheim), and 5 μCi [γ-32P]-γ-ATP (Amersham Pharmacia)] per reaction. The reactions were incubated at 30°C for 25 min (this being a median time point in the linear range of the assay) before being stopped by addition of 30 μl of 2× Laemmli sample buffer. The samples were then heated to 90°C for 2 min and the beads were pelleted. Thirty microliters of each reaction was then loaded onto a 12.5% SDS-PAGE gel, electrophoresed, and transferred to nitrocellulose. The membrane was then analyzed for substrate histone phosphorylation using a STORM phosphorimager and ImageQuant software. Background kinase assays were performed identically as described above except without the presence of protein in the lysis buffer. Background levels of phosphorylated substrate were minimal and were subtracted from each experiment.

**GS assays.** Marmot muscle and WAT were analyzed for GS activity using methods described previously (13). Briefly, 50 μg of crude lysate protein was diluted to 30 μl in GS extraction buffer (100 mM NaF, 10 mM EDTA, 1 mM benzamidine, and 50 mM Tris-HCl, pH 7.8). The sample was then added to 60 μl of assay solution [100 mM NaF, 10 mM EDTA, 1% glycerogen, 150 μM UDP-d-glucose, 0.22 μCi UDP-d-[3H]glucose, and 50 mM Tris-HCl (pH 7.8)]. The reactions were performed in duplicate in the presence of 1 or 10 mM glucose-6-phosphate (G6P) for 20 min at 37°C. Tubes were then placed on ice and 75 μl aliquots of the mixture were transferred to 1 × 1-in.2 filter papers (Whatman #3), allowed to dry, and then were washed in an ice-cold 66% ethanol solution overnight. Papers were then air-dried and the radioactivity was measured in a liquid scintillation counter. The ratio of active GS was calculated by dividing the activity measured in the presence of 1 mM G6P by the activity measured in the presence of 10 mM G6P. Background GS assays were performed...
as described above except without the presence of protein in the lysis buffer. Background levels of $^3$H were minimal and were subtracted from each experiment.

Statistics. All statistical comparisons were done using ANOVAs (SAS program, SAS Institute). The analysis used was a repeated-measures design over months, blocking on marmots. The SAS procedure, Proc Mixed, fit the model with an autoregressive error structure, which has an exponentially decreasing correlation between months, meaning that responses for months closer together in time are more highly correlated than months farther apart in time. Also, the more correlation there is between pairs of months, the smaller the differences that can be detected as statistically significant. Differences in mean were considered significant if $P < 0.05$.

RESULTS

Body mass and percent lipid change in marmots. As these animals were fed ad libitum and had been in the laboratory for one year, their body and lipid masses increased very rapidly during the spring and summer. In the marmots used for this study, mean body mass peaked in July ($3.7 \pm 0.2$ kg), decreased gradually through hibernation, and reached nadir in February ($1.7 \pm 0.2$ kg; Fig. 1A). A large percentage of their summer caloric intake was converted to triglyceride stores as total body lipid mass increased from $0.19 \pm 0.02$ kg in March to $0.73 \pm 0.05$ kg in July (Fig. 1B). Total body adiposity likewise increased from $9.9 \pm 0.4\%$ in March to $19.4 \pm 0.4\%$ in July (Fig. 1C). These data reveal that marmots increase triglyceride accumulation during the summer, with maximum lipid gain in June and July (in this case), then switch to a lipolytic state before hibernation in early October. Wild marmots do not experience the same abundance of food and thus peak in body mass months later and switch to a lipolytic state immediately preceding hibernation (10).

Regulation of Akt/PKB in WAT. To evaluate whether Akt/PKB activity was differentially regulated during the months preceding hibernation, we excised and analyzed gonadal WAT from fasted animals monthly from June through November. Although Akt/PKB protein levels did not change (data not shown), the activities of both Akt1/PKBα and Akt2/PKBβ were significantly higher in July when compared with the later months ($P < 0.05$) (Fig. 2, A and B). The increase in activity during these summer months was not associated with an increase in Akt/PKB phosphorylation on Ser473 (Fig. 3B), but Thr308 phosphorylation increased significantly during the early months in the study period (Fig. 3A).

Regulation of GS in WAT. Glycogen synthase kinase 3β (GSK3β) is an Akt/PKB substrate that inhibits glycogen synthesis by phosphorylating the metabolic enzyme GS. Under anabolic conditions, Akt/PKB phosphorylates and inhibits...
GSK3β to increase rates of glucose incorporation into glycogen. Therefore we hypothesized that GS would be activated in parallel with the increase in fasting Akt/PKB activity. We performed a GS assay each month using two different concentrations of glucose-6-phosphate (G6P). G6P is an allosteric activator of GS and will override any inhibitory phosphorylation. Under conditions of low G6P, only the weakly or non-phosphorylated forms of the GS enzyme will participate in glycogen synthesis; by contrast, in the presence of high G6P, all GS that is present in the extract will be activated. The percent of active GS found in each extract under basal conditions was expressed as a ratio of the activity obtained under low/high G6P concentrations (Fig. 4A). These assays showed that WAT GS activity correlated with Akt2/PKB activity, as both peak in July (P < 0.02). Interestingly, in July the total amount of GS activity in the lysate was also high, as indicated by increased activity in the presence of high G6P (Fig. 4B). This suggested that WAT contained elevated quantities of the enzyme, which we subsequently confirmed by Western blot (Fig. 4C).

**Circulating insulin concentrations.** Insulin is a primary circulating factor regulating Akt/PKB activity in WAT and skeletal muscle. We quantified insulin levels in serum samples obtained at the same time that the tissues were excised. As we showed previously (12), fasting insulin levels increased two-fold in months preceding hibernation (July through September; Fig. 5). This increase is likely to be the result of the insulin resistance that accompanies weight gain in these animals (11) and is unlikely to account for the sudden increases in Akt/PKB and GS activity seen herein.

**Regulation of Akt/PKB in skeletal muscle.** Hibernators also increase protein and glycogen accumulation in skeletal muscle during the months preceding hibernation (26, 29). We investigated Akt/PKB activity in skeletal muscle tissue obtained during the same surgeries that we collected the WAT samples. As with WAT, neither Akt1/PKBα nor Akt2/PKBβ protein levels changed in skeletal muscle during the study period (data not shown). Under fasting conditions, Akt1/PKBα activity

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**Fig. 3.** Akt1 threonine 308 (A) and serine 473 (B) phosphorylation in WAT from June to November. Data are quantified from Western blots and are expressed as means ± SE, n = 3.

**Fig. 4.** Mean ± SE for percent of active glycogen synthase (GS; A) and total GS activity (B), and a representative Western blot (C) of GS from June to November in WAT. GS is seen at ~85 kDa, and the larger molecular weight band is unknown but was seen at similar levels in all animals only in July. *

**Fig. 5.** Mean ± SE for average serum insulin levels from June to October. n = 3.
again demonstrated a seasonal change in activity. However, peak activity did not match that found in WAT. Specifically, this activity was significantly higher in September (3 wk after the animals were moved to constant darkness) than it was in the other 4 mo ($P < 0.03$; Fig. 6). This shows that fasting Akt1/PKBα activity increases not only seasonally, but also in a tissue-specific manner. We also investigated Akt2/PKBβ activity in skeletal muscle from July through November and, although a similar pattern was observed, no statistically significant differences were found (data not shown).

**DISCUSSION**

After emerging from hibernation in March, the marmots used in this study more than doubled their body mass and body adiposity by July in preparation for hibernation in October (Fig. 1). This increase in body mass occurs earlier when compared with field animals that are not able to accumulate lipid stores until nearly June. Thus the body mass cycles of our lab animals are shifted forward as are food intake and other physiological mechanisms that produce fat accumulation. The peak in mass and adiposity at such an early time suggests that lab animals are shifted forward (Fig. 1). This increase in body mass occurs earlier when compared with that found in WAT. Specifically, in July, fasting Akt1/PKBα and Akt2/PKBβ activity increased several-fold in WAT compared with later months in the study period (Fig. 2).

Although insulin is a primary regulator of Akt/PKB activity in this tissue, it was unlikely to account for the increased activity observed in these samples. First, the samples were obtained under fasting conditions, when a postprandial insulin surge would not occur. Direct measurement of fasting insulin concentration confirmed that the increase in Akt/PKB activity did not correlate with circulating insulin concentrations, which increased slightly (i.e., 2-fold) in July, but remained elevated through September (Fig. 5). Second, another insulin responsive tissue (i.e., skeletal muscle) demonstrated a dissimilar pattern of Akt/PKB activation. Specifically, although muscle biopsies were obtained at the same time as the WAT biopsies and these tissue samples also demonstrated a seasonal increase in Akt/PKB activity, the peak activity in muscle occurred much later in the year (i.e., September) than it did in WAT (Fig. 6). These findings suggest the existence of an intrinsic, tissue-specific mechanism for increasing fasting Akt/PKB activity. Interestingly, prior studies reveal the existence of a PKA-dependent, PI3-kinase-independent mechanism leading to Akt/PKB activation resulting exclusively from Thr308 phosphorylation (9). Future studies will evaluate the contribution of PKA to the stimulation of fasting Akt/PKB activity in these tissues.

The seasonal changes in Akt/PKB activity suggest that marmots selectively increase fuel storage first in adipose tissue and, secondly, in skeletal muscle, during the months preceding hibernation. As shown in Fig. 4, the increase in Akt/PKB activity corresponded with increased activity of GS, the rate-limiting enzyme governing incorporation of glucose into glycogen. In addition to regulating the acute storage of nutrients after consumption of a meal, however, Akt/PKB also controls the expression of several molecules involved in nutrient absorption or deposition. Specifically, Akt/PKB stimulates the expression of transporters for glucose (i.e., GLUT1) (18), amino acids (6), low-density lipoprotein (6), and iron (6). GLUT1 permanently resides in the plasma membrane of many tissues to increase cell permeability to glucose. We also found that the expression of constitutively active forms of Akt/PKB in 3T3-L1 adipocytes stimulates the transcription of GS (data not shown), which is consistent with the increased levels of GS seen in WAT containing elevated fasting Akt/PKB activity (Fig. 4B). Additionally, Akt/PKB also controls rates of transcription of fatty acid synthase (30, 39), the enzyme that catalyzes all reactions for synthesis of palmitate from acetyl-CoA and malonyl-CoA. This observation is consistent with our prior studies showing that the levels of fatty acid synthase increase during the summer months (42).

Akt/PKB is also implicated in the differentiation of preadipocytes into adipocytes (18, 22) and is required for the expression of the adipogenic transcription factor PPARγ (24). Although it is tempting to speculate that the increase in fasting Akt/PKB activity contributes to the recruitment of preadipocytes into the fat pad, thus increasing storage space for the large lipid reserves necessary for survival through the winter, prior studies reveal that lipid accumulation in adipose tissue results primarily from adipocyte enlargement rather than an increased number of cells (23, 43). The size of the adipose cells in the animals evaluated herein nearly tripled in size during the study period, with peak size achieved in July and August, when Akt/PKB activity was maximal. Interestingly, Akt/PKB is strongly implicated in the regulation of cell size. In 1999, Verdu et al. (38) determined that ectopic expression of Akt/PKB in *Drosophila* increased cell and organ size without affecting cell-fate determination, apoptosis, or proliferation in imaginal discs. Subsequent studies in several different mammalian cell and tissue types confirm a role for the enzyme in cell growth independently of changes in cell number. For example, overexpression of Akt1/PKBα induced a twofold increase in the size of H4IIIE rat hepatoma cells (8), the interleukin-3-dependent FL5.1 cell line (6), pancreatic β-cells (35), and cardiac myocytes (28). The importance of Akt/PKB in cell growth is underscored in knockout mice lacking the Akt1/PKBα isoform, which display significant impairment in organismal growth (2, 24).

In summary, yellow-bellied marmots prepare for hibernation by selectively increasing fuel stores in WAT months before a
subsequent accumulation in skeletal muscle. We found that this unique tissue-specific accumulation of nutrients is likely due to an intrinsic, and thus non-insulin-dependent mechanism, by which these animals regulate the fasting activity and phosphorylation of Akt/PKB, a critical regulator of lipid and glycogen synthesis, and GS. Future studies will address the mechanisms underlying this profound regulatory event in this animal model.

ACKNOWLEDGMENTS
We thank Dr. J. R. ZumBrunnen, the Associate Director of the statistics department at Colorado State University, for contributions to the statistical analyses.

GRANTS
This work was supported by National Institutes of Health Grants (R01-DK88784 to S. A. Summers and R21-DK60676 to G. L. Florant) and a beginning grant-in-aid from the American Heart Association (to S. A. Summers).

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