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Effects of seasonal adiposity on ovarian activity of Vespertilionid bat, *Scotophilus heathi*: Proteomics analysis

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Abstract

In present study comparative proteomics was utilized to identify ovarian protein profiles and correlate the expression of these proteins with adiposity induced changes in ovarian activity leading to suppression of ovulation (delayed ovulation) in the bat, *S. heathi*. To achieve this, two-dimension gel electrophoresis combined with protein identification by tandem mass spectrometry (LC–MS/MS) was applied. Protein profiles were obtained from intact ovaries of bats collected during recrudescence (basal body weight) and delayed ovulation (increased body weight) phases of reproductive cycle. Out of 42 differentially expressed protein spots, 15 protein spots were identified by LC–MS/MS. A majority of the 15 protein spots identified belonged to a group of enzymes within the glycolytic and citrate cycles. Greater concentrations of these enzymes were found during the period of delayed ovulation, which may be responsible for an increase in the production of ATP within the ovary. The increased metabolic activity and energy production observed within the ovary during winter dormancy may be required for increased steroidogenic activity during this period. The protein 14-3-3 identified by LC–MS/MS was verified by immunoblotting, which confirmed its increased expression during the period of delayed ovulation and may be associated with development of insulin resistance. Treatment with adipokines (adiponectin, resistin) is responsible for increased expression of 14-3-3 protein in the ovary of *S. heathi*. Adiposity-associated rise in adipokines are thus responsible for increased expression of 14-3-3 protein in the ovary of *S. heathi*, which may be responsible for prolonged survival of antral follicles and suppression of ovulation. The 14-3-3 protein may represent a new marker for adiposity associated ovarian anovulation (disorders).

Keywords

Obesity; Adiponectin; Resistin; IR; A4

1. Introduction

Studies from our laboratory on seasonally breeding vespertilionid bat, *Scotophilus heathi* showed a 150% increase in body mass prior to winter dormancy due to heavy accumulation of white adipose tissue (Abhilasha and Krishna, 1997). Simultaneously with increase in body mass, *S. heathi* showed development of abnormal antral follicles and suppression of ovulation (Singh and Krishna, 1992). This stage of the reproductive cycle of *S. heathi* is known as period of delayed ovulation (Krishna and Singh, 1992). During the period of delayed ovulation, the ovary of *S. heathi* was shown to produce extraordinarily high androgen and was suggested to be responsible for suppression of follicular maturation or ovulation in this species (Abhilasha and Krishna, 1996). Based on these studies, a close association between adiposity, increased androgen synthesis and anovulation were suggested in *S. heathi* as also described in women with polycystic ovary syndrome (PCOS) (Sam and Dunaif, 2003).

In recent studies, various adipokines shown to act as signals of adiposity status to the reproductive system (Barber et al., 2006). Our earlier studies have shown that the obesity associated rise in leptin and insulin cause anovulation in *S. heathi* (Srivastava and Krishna, 2007). Both leptin and insulin receptors were mainly localized in the thecal-interstitial cells of stroma and granulosa cells of preantral follicle suggesting their possible roles in androgen synthesis and follicular development in the ovary of *S. heathi* (Srivastava and Krishna, 2011). Recent studies from our laboratory have demonstrated the role of other adipokines such as adiponectin, resistin, etc. on ovarian activities by influencing insulin receptor in the ovary of *S. heathi* (Singh and Krishna, 2012a, 2012b).

Based on current information, it may be hypothesized that increased adipokines secreted during the period of adiposity (fat accumulation) may be responsible for suppression of ovulation during the period of delayed ovulation in *S. heathi*. The mechanism by which these adipokines affects ovarian activities leading to suppression of ovulation in *S. heathi* is not known. It is likely that various protein expresses in the ovary in response to increased adipokines stimulation may be associated with suppression of ovulation during the period of delayed ovulation, whereas in absence of adiposity, low adipokines allow their active follicular development and maturation. In order to confirm this hypothesis, the present study employed a proteomic approach comprised of two-dimensional gel electrophoresis (2DE) combined with tandem mass spectroscopy. This approach was used to identify and compare protein profile and expression patterns in the ovary of bat, *S. heathi*, during the periods of delayed ovulation and the period of active ovarian activity of recrudescence phase.

2. Materials and methods

2.1. Animal

All animal experiments were approved by an Institutional Animal Ethics Committee. Bats were captured alive directly from their natural habitat in Banaras Hindu University, Varanasi and adjacent areas. Details of the study site and feeding activity have been previously described (Singh and Krishna, 1996).

S. heathi has a sharply defined annual reproductive cycle and can be classified into the following phases (Abhilasha and Krishna, 1996; Singh and Krishna, 1992). (1) Quiescence: A reproductively inactive phase. Body mass remains at a basal level and the bat undergoes vigorous feeding (August–September). (2) Recrudescence: The beginning of reproductive activity. Newly formed large antral follicles are present in the ovary and there is a gradual increase in body mass due to accumulation of white adipose tissue (October–November). (3) Delayed ovulation (winter dormancy): Bats exhibit temporary arrest of reproductive activity. Ovary contains large antral follicles, but ovulation does not occur. Body mass attains a peak due to accumulation of white adipose tissue and subsequently declines gradually due to resumption of fat, no feeding activity (December–January). (4) Pre-ovulatory: Renewed ovarian activity. Ovaries contain large antral follicles and body mass attains a basal level (February). (5) Ovulatory: Ovary contains ovulatory follicles or early corpus luteum (early–mid March).

2.2. Analyses and experimental design

2.2.1. Proteomics study—Bats ($n = 40$) were captured during the period of recrudescence and delayed ovulation phases of reproductive cycle. Bats were transported to laboratory and killed by decapitation under mild anesthesia. Ovaries were removed and were cleaned and stored at -80°C until used for 2DE.

2.2.2. Mass spectrometry—Spots from 2DE gel of two phases (recrudescence and delayed ovulation) were analyzed with PD quest image analysis software. Spots exhibiting greater than twofold change in expression as determined by Student t-test ($P < 0.05$) were selected for protein identification by tandem mass spectrometry.

2.2.3. Validation of proteomic study—To validate the proteomics study, one of the protein spots identified by LC–MS/MS analysis was confirmed by immunohistochemistry and Western blot analysis. The protein 14-3-3 was selected for further study because of its implication in regulation of diverse cellular processes such as oxidative stress, inflammation, apoptosis, cell proliferation and survival (Xiang et al., 2002). These processes are likely critical for the development of anovulatory condition in the bat ovary.

2.2.4. Validation of antibody for 14-3-3—14-3-3 antibody was validated for the use in the ovary of bat by immunoblotting. Mice cerebellum was used as a positive control for 14-3-3 validation. Western blot analysis showed single immunoreactive band at ~ 30 kDa of 14-3-3 protein in both mice cerebellum and bat ovary (Fig. 2A).

2.2.5. Effect of adipokines on ovarian expression of 14-3-3—This study was undertaken to verify whether increase in adipokines, such as adiponectin and resistin, are responsible for increased expression of 14-3-3 protein in the ovary of *S. heathi*. To achieve this *in vitro* study was performed on the ovaries of bat ($n = 27$) collected during the period normal follicular development. The bat shows two period of active follicular development, October and February. The ovaries of the bat collected during the month of October was used to determine the effect of adiponectin; whereas the ovaries of the bat collected during

the month of February was used to determine the effect of resistin on 14-3-3 protein expression.

2.2.6. Correlation of 14-3-3 with apoptotic and cell survival markers—This study was undertaken to find out whether 14-3-3 protein up-regulated during the period of delayed ovulation is responsible for abnormal changes in the ovary of *S. heathi*. To achieve this, the ovaries of the bats ($n = 18$) collected during the periods of delayed ovulation (increased adiposity) and recrudescence phase (normal body mass) were processed for immunoblot analysis of 14-3-3, p53, caspase-3, and bcl2 proteins. The relative concentration of the proteins in ovaries was obtained by densitometric analysis of western blots. The correlative changes in the concentrations of 14-3-3 protein, together with the changes in the concentrations of apoptotic marker (p53, caspases) and cell survival marker (bcl2) were analyzed during the period of delayed ovulation and recrudescence phase.

2.3. Two-dimensional gel electrophoresis

Ovaries were collected during recrudescence (normal body mass) and period of delayed ovulation (increased adiposity) and homogenized by sonication in ice-cold 1 M Tris (pH 7.6) having 1 $\mu\text{g/ml}$ aprotinin, 100 $\mu\text{g/ml}$ PMSF. After homogenization, protein was precipitated using ice cold acetone (sample:acetone, 1:10) overnight at -20°C and then centrifuged at 4000 rpm for 5 min at 4°C . After centrifugation, acetone was removed from the pellet and it was dried. The pellet was then resuspended in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT and 0.2% ampholites). Protein estimation was performed by Bradford Assay. Strips (pH 3–10, 17 cm) were rehydrated passively for 16 h and then isoelectric focusing (IEF) was performed using a PROTEAN IEF cell (Bio-Rad) for 40,000 V h. After completion of IEF, the strips were then equilibrated in two base buffers (50 mM Tris/Cl, 2% (v/v) SDS, 6 M urea, and 20% glycerol) containing first 1.4 M DTT and then 1.3 M iodoacetamide. The second dimension was run onto 12% SDS–PAGE. The gel was washed three times for 15 min prior to staining with Coomassie blue. For quantification purposes, the stained gels were first scanned (GS-800 Densitometer, Bio-Rad) and then analyzed with PDQuest image analysis software (Bio-Rad).

2.4. LC–MS/MS from 2D gel spots

Spots from 2D gels were typically stored in clean (rinsed with mass spec grade Acetonitrile) Eppendorf tubes for transportation or storage. Spots (gel plugs) were washed three times in 100% acetonitrile for at least 10 min per wash to remove any stain. After the last wash the spots were placed in a Speed-Vac and dried for 20 min. The dried spots were re-hydrated in trypsin digest buffer (50 mM Ammonium Bicarbonate; Am Bic) and then first reduced in DTT 10 mM at 56°C for at least 30 min and alkylated with iodoacetic acid (15 mM) for 30 min at room temperature in the dark. Samples were then digested with mass spec grade trypsin 20 ng/ μl for 4 h at 37°C . Just before analysis the sample was acidified by the addition of formic acid to 0.1%.

Spots from 2D gels were analyzed using a short (20 min) LC/MS run. The sample was loaded onto a C-18 trap (Cap Trap; Michrom) at 5 $\mu\text{l/min}$ for 10 min. A multiport valve was then switched and the captrap was eluted onto a 0.1×50 mm C-18 reverse phase column

using a gradient of acetonitrile in water 5%–30% over 10 min at a flow rate of 500 nl/min. Eluate from the column was directly sprayed into an LTQ ion trap mass spectrometer using captive nanospray (Michrom). Spectra were collected using Xcalibur 2.2 software (Thermo) and using a threshold of 200. Spectra were then searched against Rat, Mouse, and Human IPI databases using Proteome Discoverer 3.0 software (Thermo). All three-database searches produced similar hits, but the IPI Rat database consistently gave the best scores. Each spot produced one or two significant hits as determined by false discovery rates of 1.0 and 5.0% using a corresponding reverse database.

2.5. Immunoblot

Ovaries were pooled during recrudescence and period of delayed ovulation, and sonicated in suspension buffer (0.01 M Tris pH = 7.6, 0.001 M EDTA pH = 8.0, 0.1 M NaCl, 1 µg/ml aprotinin, 100 µg/ml PMSF) to produce 10% (w/v) homogenate. Protein extraction and immunoblot were performed as previously described (Singh and Krishna, 2012a). Protein was quantified by Lowry's method. Equal amount of proteins (50 µg) was loaded on to SDS–PAGE (10%) for electrophoresis. Thereafter, proteins were transferred electrophoretically to PVDF membranes (0.45 µm, Millipore Corporation, 290 Concord Road, Billerica, MA 01821–7037) overnight at 4 °C. Membranes were cut with the assistance of molecular weight markers to develop different proteins and beta actin from the same blot. Membranes were blocked for 60 min with Tris buffered saline (TBS) (Tris 50 mM pH 7.5, NaCl 150 mM) containing 5% fat free dry milk and incubated with primary antibodies (rabbit anti human P53 antibody at a dilution of 1:1000 and rabbit anti-human Bcl2 at a dilution of 1:1000, rabbit anti-human caspase-3 and active caspase-3 at a dilution of 1:600, and 14-3-3 at a dilution of 1:2000) for 1 h at room temperature. 14-3-3 (Q97) antibody was a kind gift from Dr. David C. Pallas (Department of Biochemistry, Winship Cancer Institute, Emory University School of Medicine). Other antibodies were purchased from Santa Cruz Biotechnology Inc., CA, USA. Membranes were then washed with three changes of TBS over 10 min. Immunodetection was performed by using biotinylated secondary antibody (at a dilution of 1:2000; Vector Laboratories, Burlingame, CA, USA) for 30 min followed by three washings with PBS (0.2 M PBS, pH 7.4) for 10 min each. After washing, the blots were incubated with avidin–peroxidase conjugate (at a dilution of 1:2000, Vector Laboratories) for 30 min. Finally, the blot was washed three times with TBS and developed with enhanced chemiluminescence (ECL) detection system (Bio-Rad, USA). Immunoreactive bands were quantified by densitometry (Image J vs 1.36, NIH, USA). Experiments were repeated three times with the same result. Equal loading was confirmed with β-actin.

2.6. Immunohistochemistry

Immunohistochemistry for 14-3-3 protein was performed as previously described (Singh and Krishna, 2012a, 2012b). ABC staining Kit (PK-6200, Universal Elite, Vector Laboratories, USA) was used for the secondary antibody and enzyme conjugates. The sections were first deparaffinized and then tissue sections were hydrated in graded alcohol (absolute to 30% ethanol) and blocked of endogenous peroxidases with 3% of hydrogen peroxidase (H₂O₂) in methanol, incubated with blocking serum for 1 h, followed by incubation with the primary antibody (14-3-3 at a dilution of 1:500). The sections were then washed three times (10 min

each) in tris buffered saline (TBS) and incubated with the biotinylated secondary antibody for 30 min at room temperature, followed by another 30 min with horseradish avidin–peroxidase conjugate. After washing, sections were incubated with the chromogen substrate for HRP (0.1% 3, 3'-diaminobenzidine tetra hydrochloride in 0.5 M Tris 7.6 and 0.01% H₂O₂) for 10 min and counterstained with hematoxylin for 1 min. Slides were dehydrated in graded alcohol (from 30% to absolute alcohol), cleared in xylene and mounted in DPX. Image acquisition was performed by light microscopy (Nikon, Tokyo, Japan).

2.7. The *in vitro* study

Female *S. heathi* were sacrificed by decapitation as soon as they were brought to the laboratory. Ovaries were quickly removed and cleaned for any adhered fat tissue and oviduct in medium Dulbecco modified eagle's medium (DMEM; Himedia, Mumbai, India) containing 250 U/ml penicillin and 250 µg/ml streptomycin sulfate. Ovaries were cultured as described previously (Singh and Krishna, 2012a). Culture medium was a mixture of DMEM and Ham's F-12 (1:1; v:v) (Himedia, Mumbai, India) containing 100 U/ml penicillin, 100 µg/ml streptomycin and 0.1% BSA (Sigma-Aldrich, St. Louis, USA). After initial incubation for 2 h at 37 °C, culture medium was discarded and ovaries (one per tube) were finally cultured in 1 ml of medium having different adiponectin (5 and 25 µg/ml) and resistin doses (10 and 100 ng/ml) in a humidified atmosphere with 95% air and 5% CO₂ for 24 h at 37 °C. Each treatment group was run in triplicate. Ovaries were collected at the end of culture, washed several times with PBS and kept frozen for western blot study.

2.8. Statistical analysis

Data were analyzed by unpaired t-test assuming equal variance using SPSS software 12 for Windows (SPSS Inc., Chicago, IL, USA). The data were considered significant at a probability level of $p < 0.05$.

3. Results

3.1. Differential proteomic analysis in the ovary of *S. heathi* during period of delayed ovulation (increased adiposity) and recrudescence phase (normal body mass)

Two D-gel electrophoresis and subsequent analysis with PD Quest was used to identify differentially expressed proteins in the ovary of *S. heathi* during the periods of delayed ovulation and recrudescence phase. Out of 509 spots detected during the period of delayed ovulation (period of adiposity), 42 spots exhibited differential expression as compared with the period of normal body mass. Using non-targeted approach, 15 spots were selected for further analysis by LC–MS/MS (Fig. 1).

3.2. Identification by mass spectrometry (LC–MS/MS) of differentially expressed protein spots from the ovary of *S. heathi* during the period of delayed ovulation

The 15 selected spots were subjected to LC–MS/MS analysis followed by database search against Rat, Mouse, and Human IPI databases using Proteome Discoverer 3.0 software (Thermo). Each spot produced one or two significant hits as determined by false discovery rates of 1.0 and 5.0% using a reverse database. Protein identification was considered

confident when 20% sequence coverage was found or the first hit from the database search. The proteins identified are shown in Table 1.

3.3. Immunohistochemical and western blot analysis of 14-3-3 protein in the ovary of *S. heathi* during the period of delayed ovulation and recrudescence phase to validate the data from 2D gels

The proteomics analysis identified one up-regulated protein as 14-3-3. This protein showed up-regulation in the ovary during the period of delayed ovulation (increased adiposity) as compared with ovary during recrudescence phase (normal body mass). Western blot analysis of protein 14-3-3 in the ovaries of *S. heathi* showed a single immunoreactive band at 30 kDa (Fig. 2). Densitometric analysis of a western blot of protein 14-3-3 showed significantly increased expression of this protein in the ovary of *S. heathi* during the period delayed ovulation when compared with the recrudescence phase (Fig. 2).

Immunohistochemical localization of protein 14-3-3 in the ovary of *S. heathi* showed a marked variation during periods of delayed ovulation and recrudescence phase (Fig. 3). Intense immunostaining of 14-3-3 was observed in oocyte, thecal interstitial cells and granulosa cells during period of increased adiposity while very mild immunostaining of 14-3-3 was observed in thecal interstitial cells, oocyte and granulosa cells during the period of recrudescence phase.

3.4. Effect of adipokine treatment *in vitro* on ovarian expression of 14-3-3 protein

In vitro treatment with adiponectin caused significant increase in 14-3-3 protein expression at low doses while resistin caused significant increase in the expression of 14-3-3 protein at both the doses in the ovary in comparison with the control (Fig. 4A and B).

3.5. Correlation between expression of 14-3-3 protein with the apoptotic and cell survival markers in the ovary of *S. heathi* during the periods of delayed ovulation and recrudescence phase

Changes in P53 (Fig. 5A), caspase-3 (Fig. 5B) and active caspase-3 (Fig. 5C) showed significantly decreased expression while bcl2 (Fig. 5D) showed significantly increased expression during the period of delayed ovulation when compared with the recrudescence phase.

As shown in Table 2, there was a significant correlation between changes in the ovarian concentration of 14-3-3 protein with the changes in the concentration of various apoptotic and cell survival markers such as p53, caspase, active caspase-3, and bcl2.

4. Discussion

This study was undertaken with the view to collect information about proteins that may be potentially important for mediating adiposity associated suppression of ovarian activities in the bat, *S. heathi*. Since, the genome of the bat, the animal model used in this study, is not yet sequenced, the proteomics data described here should be interpreted with caution (Habermann et al., 2003; Rost, 2002). As suggested earlier in the study on ground squirrel (Epperson et al., 2004), data from LC-MS/MS described in this study may be used as a

marker for further verification of the specific protein or biochemical pathways involved in the ovary during the period of delayed ovulation.

In the present study 15 protein spots showing highly significant differentially expressed between impaired ovary during the period of delayed ovulation and normal recrudescence ovaries were selected and analyzed by LC–MS/MS. Most of these proteins were up-regulated during the period of delayed ovulation, and belong to a group of enzymes in the glycolytic and tricarboxylic acid (TCA) cycles, such as alpha enolase (ENO 1), pyruvate dehydrogenase (PDH), isocitrate dehydrogenase (IDH3A), dihydrolipoyllysine-residue succinyltransferase (DLST), and 2-oxoglutarate dehydrogenase (2 ODG) (Table 1). Increased concentration of these enzymes suggests increased glycolysis and gluconeogenesis in the ovary of *S. heathi* during the period of delayed ovulation as compared with recrudescence period. This further suggests increased energy production in the ovary during the period of delayed ovulation.

The other metabolic enzymes that showed an increased expression in the ovary during the period of delayed ovulation include adenylate kinase isoenzyme 1 (AK) and voltage-dependent anion-selective channel protein 2 (VDAC). Adenylate kinase (AK) is a key enzyme involved in energy metabolism in the cytosol, mitochondria, and nucleus (Noma, 2005). It catalyzes the reversible transfer of γ -phosphate from either adenosine triphosphate (ATP) or guanosine triphosphate (GTP) to the β -position of the phosphate acceptor of adenosine monophosphate (AMP), resulting in the production of two molecules of adenosine diphosphate (ADP), and plays a role in high-energy phosphoryl transfer from intracellular sites of ATP or GTP synthesis to the sites of their consumption. In the reproductive tissue, AK has earlier been demonstrated in the ovary and testis of mice (Miyoshi et al., 2009) and sperm of Holstein bull (D'Amours et al., 2010). In the mice ovary, AK was detected in oocytes, granulosa cells in the stratum granulosum, and luteal cells. These studies suggested association of AK expression with tissue-specific regulatory roles in energy metabolism. This study also showed an increase in expression of VDAC 2 protein, which plays a key role in regulating metabolic and energetic flux. It is involved in the transport of ATP, ADP, pyruvate, malate, and other metabolites, and thus communicates extensively with enzymes from metabolic pathways (Blachly-Dyson and Fort, 2001). VDAC is also an important regulator of Ca^{2+} transport, which is a cofactor for metabolic enzyme, such as PDH, IDH, etc. These observations thus suggest that greater expression of AK and VDAC may be responsible for increased production of ATP in the ovary of *S. heathi* during the period of delayed ovulation.

The hibernation or winter dormancy is demonstrated only in three eutherian orders: Insectivores, Rodentia and Chiroptera (Wimsatt, 1969). The cycle of reproduction and hibernation or winter dormancy in these mammals show distinct seasonal periodicity. In Insectivores and Rodentia, the period of hibernation and reproduction are separated from each other. In these mammals, the entire reproductive sequence from insemination to the rearing of young is completed before the onset of period of hibernation. The ovary remained completely dormant, for energy conservation, as an adaptive significance during the period of hibernation (Hudson and Bartholomew, 1964). In contrast, in several chiropterans, some part of the reproductive cycle is overlapped with the period of winter dormancy (Wimsatt,

1969). With the result the gonad remained partially active during the period of winter dormancy (Krishna and Bhatnagar, 2011). The ovary of *S. heathi* continued to show increased synthesis of androgen in the period of delayed ovulation during winter dormancy (Abhilasha and Krishna, 1996). Thus, increased metabolic activity and energy production observed in the ovary of *S. heathi* during winter dormancy may be required for increased steroidogenic activity during this period. The increased androstenedione produced by the ovary during the period of delayed ovulation were shown to be responsible for the suppression of follicular maturation and delayed ovulation in this bat (Srivastava and Krishna, 2011). Recent studies from our laboratory have shown that an increase in adiposity hormones (leptin, adiponectin, resistin) during the period of increased accumulation of white adipose tissue during winter dormancy is responsible for increased synthesis of androstenedione in the ovary of *S. heathi* (Singh and Krishna, 2012a, 2012b; Srivastava and Krishna, 2007).

The period of delayed ovulation in bat is associated with adiposity, hyperinsulinemia and insulin resistance as found during PCOS (Doval and Krishna, 1998). In this context, comparison of changes in protein expression between ovaries of bat during the period of delayed ovulation and during recrudescence phase may allow for identifying proteins involved in delayed ovulation in the bat and thus may also provide information about changes that occur during PCOS. This finding suggests that the impact of obesity on reproduction typically operates through the associated insulin resistance and hyperinsulinemia (Conway and Jacobs, 1993). The causes of ovulation inhibition in the vespertilionid bat during the period of delayed ovulation as well as in patients with PCOS are not clearly understood (Beloosesky et al., 2004; Telfer et al., 1990). It has earlier been demonstrated that increased insulin concentration is related both to the development of PCOS (Dunaif et al., 1989) as well as the phenomenon of delayed ovulation in the bat (Doval and Krishna, 1998). Insulin exerts its effect via phosphorylation of several cytosolic substrate including IRS-1 and IRS-2. Earlier studies have suggested the involvement of various genes associated with insulin signaling and insulin resistance with PCOS (Mukherjee and Maitra, 2010). Recent meta-analysis showed the association of IRS-1Gly972Arg with PCOS, thus suggesting a possible role of IRS in insulin resistance as well as in PCOS (Ioannidis et al., 2010). One of the important observations of present study was increased expression of 14-3-3 protein in ovaries of *S. heathi* during the period of delayed ovulation as compared to during recrudescence phase. Based on earlier findings, 14-3-3 protein participates in regulation of insulin sensitivity (Ogihara et al., 1997; Xiang et al., 2002). It is hypothesized that 14-3-3 protein observed in the bat ovary during the period of delayed ovulation may be associated with the development of insulin resistance.

Using immunohistochemistry and western blot analysis, we observed a marked increase in the expression of 14-3-3 protein in the ovary of *S. heathi* during the period of delayed ovulation as compared with the ovaries during recrudescence phase. This observation verifies the proteomics data. The expression of 14-3-3 protein was mainly observed in the theca-interstitial cells, which is the major site of steroidogenesis as well as site of localization of insulin receptor in the ovary of *S. heathi* (Doval and Krishna, 1998). Now the question arises, what causes increased in the expression of 14-3-3 protein in the ovary of *S.*

heathi during the period of delayed ovulation. This study showed that the bat ovary treated *in vitro* with low dose of adiponectin and both low and high doses of resistin caused significant increase in the expression of 14-3-3 protein in the ovary of *S. heathi*. This finding suggests that the adipokines (adiponectin, resistin) are responsible for increased expression of 14-3-3 protein in the ovary of *S. heathi* during the period of delayed ovulation.

Protein 14-3-3 has been shown to play important roles in a variety of intracellular events in ovary, such as regulator of cell cycle and apoptosis, signaling molecule, tumor suppressor, steroidogenesis and oocyte maturation (De et al., 2012; Lau et al., 2006; Morrison, 2009; Peluso et al., 2007). The results of the present study demonstrate a significant correlation between changes in ovarian concentration of 14-3-3 protein with the changes in the concentration of apoptotic markers (p53, caspases) and cell survival marker (bcl2) during the period of delayed ovulation as compared with the recrudescence phase. Based on these findings, it may be hypothesized that increased expression of 14-3-3 protein in the ovary of *S. heathi* during the period of delayed ovulation may be responsible for suppressing apoptotic activity, but increases survival marker in the granulosa cells of the antral follicles resulting in the persistence of large antral follicles for prolonged period.

In summary, the comparative proteomics approach was applied to identify proteins that may be potentially important for mediating adiposity-associated suppression of ovarian activities in the bat, *S. heathi*. The selected proteins, which showed increased expression during the period of delayed ovulation in *S. heathi*, are enzymes responsible for increased energy production in the ovary. The increased energy produced may be required for synthesis of excess androstenedione in the ovary of the bat during delayed ovulation. The excess androgen produced may be responsible for development of PCOS-like changes in the bat ovary. The other protein 14-3-3 identified by LC-MS/MS was verified by immunohistochemistry and western blot analysis. Increased expression of this protein was observed in the ovary during the period of increased adiposity during delayed ovulation as compared with recrudescence phase. The *in vitro* study suggests that treatment with adipokines (adiponectin, resistin) is responsible for increased expression of 14-3-3 protein in the ovary of *S. heathi*. This study further showed significant correlation between levels of 14-3-3 protein with levels of apoptotic and cell survival markers (P53, caspase-3, and bcl2) in the ovary of *S. heathi*. These findings suggest that adiposity associated rise in adipokines is responsible for increased expression of 14-3-3 protein in the ovary of *S. heathi* which may be responsible for prolonged survival of antral follicles and suppression of ovulation by regulating apoptotic and cell survival markers. Thus, 14-3-3 protein may represent a new marker for adiposity associated ovarian anovulation (disorders).

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Fig. 1.

Representative 2-D gel images during recrudescence (normal body weight, A) and delayed ovulation (period of adiposity, B) phase of reproductive cycle. B shows the 15 spots selected which were increased during the period of delayed ovulation.

Fig. 2.

(A) Validation of 14-3-3 antibody for the use in the bat ovary using mice cerebellum as positive control. Immunoblot analysis showed single immunoreactive band of 14-3-3 protein at ~30 kDa in both bat ovary as well as mice cerebellum. (B) Western blot analysis of 14-3-3 protein in the ovary of *S. heathi* during the recrudescence (normal body weight) and delayed ovulation (period of adiposity) phase of reproductive cycle. Each sample was pooled from two to three bats and experiment was repeated thrice. Values are mean $\pm$ S.E.M.; $n = 18$. Values are mean $\pm$ S.E.M.. *Values are significantly ($P < 0.05$) different from other groups. IRDV = integrated relative.

Fig. 3.

Immunolocalization of 14-3-3 in the ovary of *S. heathi* during recrudescence (normal body weight) and delayed ovulation (period of adiposity) phases of reproductive cycle. A (10×), B (40×) and C (100×) are showing immunostaining for 14-3-3 during recrudescence phase while D (10×), E (40×) and F (100×) are showing immunostaining for 14-3-3 during delayed ovulation. Intense immunostaining of 14-3-3 was observed in oocyte, thecal interstitial cells and granulosa cells during period of delayed ovulation. Arrowhead is showing intense staining of 14-3-3 in the oocyte while arrows are showing intense immunostaining in granulosa cells during delayed ovulation.

Fig. 4.

Western blot analysis of 14-3-3 proteins in the ovaries of *S. heathi* treated *in vitro* with adiponectin (APN) (A) and resistin (RES) (B). IRDV = integrated relative density value. Each experiment was repeated thrice. Values are mean $\pm$ S.E.M., $n = 27$. *Values of band intensity differs significantly ($p < 0.05$) as compared to control.

Fig. 5. Western blot analysis of p53 (A), caspase-3 (B), active caspase-3 (C) and bcl2 (D) proteins in the ovary of *S. heathi* during the recrudesence and delayed ovulation phases of reproductive cycle. Each sample was pooled from two to three bats and experiment was repeated thrice. Values are mean $\pm$ S.E.M.; $n = 18$. Values are mean $\pm$ S.E.M. *Values are significantly ($P < 0.05$) different from other groups. IRDV = integrated relative density value.

Table 1

15 2-D spots identified by LC MS/MS followed by database search using Proteome Discoverer 3.0 showing accession no., proteins identified, score, sequence coverage, unique peptides, peptide spectral matching (PSM), molecular weight and calculated pI of the identified proteins.

Spot no.	Accession no.	Description	Score	Coverage	Proteins	Unique peptides	Peptides	PSMs	AAAs	MW (kDa)	CalcPI
1	IP100768849.1	Voltage dependent anion-selective channel protein2	27.21	17.63%	2	2	6	15	295	31.7	7.49
	IP100768849.1	Voltage dependent anion channel1-like	10.55	22.50%	4	1	5	9	200	22.1	8.24
2	IP100210351.2	Adenylate kinase isoenzyme 1	38.79	28.35%	1	4	6	14	194	21.6	7.90
	IP100212767.1	Anionic trypsin-1	15.26	8.13%	1	1	1	3	246	25.9	4.89
3	IP100210351.2	Adenylate kinase isoenzyme 1	102.87	40.21%	1	4	8	45	194	21.6	7.90
	IP100212767.1	Anionic trypsin-1	30.79	8.13%	1	1	1	6	246	25.9	4.89
4	IP100464815.11	Alpha-enolase	38.31	20.05%	10	3	5	10	434	47.1	6.57
	IP100212767.1	Anionic trypsin-1	16.16	8.13%	1	1	1	4	246	25.9	4.89
5	IP100212767.1	Anionic trypsin-1	32.35	8.13%	1	1	1	7	246	25.9	4.89
	IP100231767.1	Triosephosphate isomerase	14.19	19.28%	4	1	3	5	249	26.8	7.24
6	IP100194324.2	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	249.34	45.96%	1	5	9	83	359	39.0	6.65
7	IP100230835.5	14-3-3 protein gamma	62.74	54.66%	9	6	11	24	247	28.3	4.89
	IP100194324.2	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	36.77	10.31%	1	2	3	11	359	39.0	6.65
	IP100212767.1	Anionic trypsin-1	20.65	8.13%	1	1	1	5	246	25.9	4.89
8	IP100325135.3	14-3-3 protein epsilon	47.15	42.75%	1	3	9	18	255	29.2	4.74
	IP100191502.5	Proteasome subunit alpha type-5	44.45	35.27%	1	4	6	17	241	26.4	4.86
	IP100212767.1	Anionic trypsin-1	19.73	8.13%	1	1	1	4	246	25.9	4.89
9	IP100194324.2	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	7.66	4.46%	1	1	1	2	359	39.0	6.65
	IP100212767.1	Anionic trypsin-1	7.16	8.13%	1	1	1	3	246	25.9	4.89
	IP100421428.9	Phosphoglycerate mutase-1	5.27	19.69%	1	1	4	5	254	28.8	7.18
	IP100207038.3	Type II keratin Kb 15	4.46	9.68%	7	1	4	4	537	58.1	7.90
10	IP100198720.1	Isocitrate dehydrogenase (NAD) subunit alpha, mitochondrial	274.62	52.46	1	9	20	88	366	39.6	6.92
11	IP100198720.1	Isocitrate dehydrogenase (NAD) subunit alpha, mitochondrial	196.09	42.62	1	11	20	63	366	39.6	6.92
12	IP100198720.1	Isocitrate dehydrogenase (NAD) subunit alpha, mitochondrial	197.80	39.34	1	11	17	64	366	39.6	6.92

Spot no.	Accession no.	Description	Score	Coverage	Proteins	Unique peptides	Peptides	PSMs	AAs	MW (kDa)	CalcPI
13	IP100198720.1	Isocitrate dehydrogenase (NAD) subunit alpha, mitochondrial	137.83	39.07	1	11	18	47	366	39.6	6.92
14	IP100551702.2	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	5.31	10.79%	2	1	6	10	454	48.9	8.69
15	IP100551702.2	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	14.09	9.69%	2	1	5	8	454	48.9	8.69
	IP100212767.1	Anionic trypsin-1	5.10	8.13%	1	1	1	1	246	25.9	4.89

Table 2

Correlation coefficients for ovarian 14-3-3 with ovarian p53, Bcl2, caspase-3 and active caspase-3.

	P53	Bcl2	Caspase-3	Active caspase-3
14-3-3	-0.956 ^a	+0.99 ^a	-0.99 ^a	-0.99 ^a

^aCorrelation is significant at the 0.05 level.