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Effects of resistin on ovarian folliculogenesis and steroidogenesis in the vespertilionid bat, *Scotophilus heathi*

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ABSTRACT

The bat *Scotophilus heathi* exhibit prolonged anovulatory condition known as delayed ovulation coinciding with the period of extensive fat accumulation. The present study was undertaken to find out whether extensive accumulation of fat in *S. heathi* is responsible for suppression of ovarian activity by increasing production of adipokine resistin in the bat. This was achieved by (a) investigating variation in serum resistin level in relation to the changes in the body fat mass and (b) evaluating the effect of resistin treatment on ovarian activity with reference to steroid synthesis. An attempt was also made to determine whether resistin mediate its effects on ovary through signal transducer and activator of transcription 3 (STAT3) signaling mechanism. The results showed significant seasonal variation in serum resistin level with the peak level coinciding with the period of maximum fat accumulation, high circulating androgen level and period of anovulation. The treatment with resistin to the bat caused increase in androstenedione due to stimulatory effects on 3β-hydroxysteroid dehydrogenase, but decrease in estradiol level due to inhibitory effect on aromatase. Resistin treatment increased androgen receptor protein together with increased insulin receptor but not through conventional luteinizing hormone receptor and steroidogenic acute regulatory protein mediated pathways. This study further showed that resistin treatment increases androstenedione synthesis and up-regulates insulin receptor in the ovary through STAT3 mediated pathways. These findings suggest that obese women through increased resistin synthesis may causes development of non-ovulatory antral follicles through insulin receptor signaling cascade.

1. Introduction

It is well documented that obesity is associated with reproductive dysfunction and infertility. Women showing obesity exhibit changes in circulating hormonal levels such as hyperandrogenism, hyperinsulinemia and insulin resistance, which are responsible for reduced fertility in these women. These hormonal changes also play a central role in the pathogenesis of polycystic ovary syndrome (PCOS) (Ehrmann, 2005; Escobar-Morreale et al., 2006). Associated obesity increases the production of various adipokines and may represent additional factors that involves in reduced fertility or anovulation, being mediated either through the induction of insulin resistance or through a direct impairment of ovarian functions.

Our laboratory is using seasonally monoestrous vespertilionid bat, *Scotophilus heathi* as an animal model to study the impact of adiposity on reproductive disorders. This bat exhibits extensive fat accumulation in both subcutaneous and abdominal cavity during the period of winter. *S. heathi* feeds vigorously from August to October as pre-winter preparation, which leads to heavy accumulation of adipose tissue during winter from November to January (Abhilasha and Krishna, 1997). During the month of October the ovary of *S. heathi* undergo recrudescence, which is characterized by the development of large antral follicles in the ovary and these follicles, persist throughout the winter months i.e. from November to January. Ovulation in this bat occurs in early March. Thus the period of reproductive cycle of *S. heathi,* when the ovary contains large antral follicles in ovary but ovulation does not occur, is called the period of delayed ovulation (Krishna and Singh, 1992; Abhilasha and Krishna, 1996). The prolonged longevity of antral follicle and delayed ovulation are unique features of vespertilionid bat during winter dormancy. During this period ovary produces exceptionally high level of androstenedione (A4) and hyperinsulinemia, which was shown to be responsible for suppressed follicular maturation and ovulation in *S. heathi* as documented in women with PCOS (Dewailly et al., 1998). On the basis of these findings the

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bat *S. heathi* is used as an animal model to investigate the mechanism whereby adipose tissue associated factors affects reproductive processes (Srivastava and Krishna, 2007, 2011).

Adipose tissue is known for secreting various adipokines, some of these are involved in the regulation of metabolism, energy and reproductive activities (Ahima and Flier, 2001). A direct inhibitory effect of leptin in ovarian function has been demonstrated in various mammals including bat species, particularly in *S. heathi* during the period of delayed ovulation (Zachow and Magoffin, 1997; Zachow et al., 1999; Duggal et al., 2000; Spicer et al., 2000; Ghizzoni et al., 2001; Guo et al., 2001; Srivastava and Krishna, 2007, 2011). It has been shown that high serum leptin, body mass and fat mass negatively correlates with reproductive functions. Besides leptin, recent studies from our laboratory demonstrated role of adiponectin in ovarian activity (Singh and Krishna 2012a,b).

The present study is focused on the role of recently discovered resistin in involvement of female reproductive activities. It was named resistin because when mice were injected with this substance it caused insulin resistance. In primates, pigs and dogs, immune and epithelial cells secrete resistin, while in humans and rodents it is secreted by adipose tissue (Steppan et al., 2001b; Radin et al., 2009; Maillard et al., 2011). In the last two decades, physiological role of resistin has been the subject of extensive study regarding its involvement with obesity and type II diabetes mellitus (Moore et al., 2001; Steppan et al., 2001a; Way et al., 2001; Levy et al., 2002; Wang et al., 2002; Lazar, 2007). Only limited studies so far has investigated its roles on reproductive activities. Recent study indicate that resistin expressed in basal level throughout the estrous cycle in rat but increased significantly in the rat induced with ovarian cysts (Jones et al., 2009). This is consistent with earlier study showing increased circulating concentration of resistin in women with PCOS (Munir et al., 2005). Thus, increased resistin secretion in PCOS plays a role in causing ovarian hyperandrogenism (Munir et al., 2005).

Therefore, the aim of this study was to investigate the role of adipokine, resistin on ovarian activities of the bat, *S. heathi*. To achieve this two different studies were performed. The first study was to evaluate the seasonal variation in serum resistin level in a natural population of the female *S. heathi* and its correlation with changes in body and fat mass and serum insulin, adiponectin, leptin, LH and A4 levels, secondly to evaluate the effect of resistin treatment in vivo on ovarian activities in *S. heathi*.

2. Materials and methods

2.1. Animals and treatment

All of the experiments were conducted according to the principles and procedures approved by the Banaras Hindu University Departmental Research Committee. Details of the study site and feeding activity are described earlier in detail (Singh and Krishna, 1996). In Varanasi, the cold season lasts from November to February (mean ambient temperature < 20 °C) and dry season from March to June (mean ambient temperature > 30 °C. The bat, *S. heathi*, has a well-defined annual reproductive cycle at Varanasi, India. The sexually mature female bats weighing 30 g or more and having wing span exceeding 42 cm were used in this study (Krishna and Dominic, 1983). Two different studies were undertaken:

2.1.1. Seasonal study

To study seasonal changes in circulating resistin level, bats (n = 10) were collected during each of the following reproductive phases: quiescence (August–September), recrudescence (October–November), winter dormancy (December-early January), preovulatory (February) and ovulatory (March). The pregnant and lactating females found between late March and July was not included in this study. The bats were weighed and euthanized by decapitation under anesthesia (anesthetic ether) as soon as they were brought to the laboratory. Serum was collected from blood samples (pooled blood from two bats) and stored at −20 °C until assayed for androstenedione (A4), progesterone (P4) and estradiol (E2). The white adipose tissue (WAT) deposited in the body of the female bats was removed using a scalpel and scissors, and its weight was recorded.

2.1.2. Effect of resistin on ovarian activity

To examine the effect of resistin treatment on ovarian activity and serum steroid levels, recombinant human resistin (6.5 μg/100 g body weight/day) was administered subcutaneously for 12 days to the adult female (n = 12) bats captured during preovulatory period in February. The bats during this period show numerous healthy antral follicles. Bats in the control group (n = 12) were injected with vehicle only. The dose of resistin for treatment was selected from a preliminary *in vitro* study, which showed maximum dose–response effect on progesterone (P4) synthesis by the bat ovary. The recombinant human resistin was received as gift from Dr. Nasreen Z. Ehtesham, National Institute of Pathology, Safdarjang Hospital Campus, New Delhi, India. The duration of treatment and route of administration was selected based on our recent study (Singh and Krishna, 2012b). Bats trapped for the study were immediately transported to the laboratory and maintained in small cages provided with food (mealworms) and water *ad libitum* in a room at 22–24 °C and 14:10 h light–dark schedule. Bats were acclimatized at least for 2 days before the start of the experiment. The dose was prepared daily just prior to treatment. Body mass of control and treated bats were recorded at the beginning and end of the experimental period. Bats were sacrificed 24 h after the last dose. The serum was separated out from blood samples and stored at −20 °C until assayed for progesterone (P4), androstenedione (A4) and 17β estradiol (E2). One ovary of each bat was dissected out, cleaned, weighed and fixed in Bouin's fixative for 24 h at room temperature, dehydrated in ethanol, cleared in xylene, embedded in paraffin and sectioned at 6 μm and processed further for histological (hematoxylin–eosin staining) and immunohistochemical studies. The other ovary was stored in −70 °C for Western blotting. Both the ovaries are morphologically and physiologically equal in *S. heathi*.

2.2. Validation of antibody for resistin

Resistin antibody (rabbit polyclonal antihuman) was a kind gift from Dr. Nasreen Z. Ehtesham, National Institute of Pathology, Safdarjang Hospital Campus, New Delhi, India. Resistin antibody was validated for the use in the serum of bat by immunoblotting. Rat adipose tissue was used as a positive control for resistin validation. Western blot analysis showed immunoreactive band at ~23 kDa of dimerized resistin in both rat adipose tissue and bat serum while an additional nonspecific band was detected in bat serum at ~32 kDa (Fig. 1). Details of other primary antibodies used in this study are described in Table 1.

2.3. Circulating resistin level

Serum samples (5 μl) of bat were diluted in lysis buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Igepal) containing protease inhibitors (2 mM PMSF, 10 mg/ml leupeptin) and phosphatase inhibitors (100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate) to 50 μl. Same lysis buffer was used to produce 20% homogenate of rat adipose tissue. Further protein extraction was performed according to Maillard et al. (2010). Protein quantification was done using