Ovarian androstenedione production is enhanced by insulin during the period of delayed ovulation in a vespertilionid bat, *Scotophilus heathi*

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The aim of the present study was to evaluate the role of insulin in the production of high concentrations of androstenedione in the ovary of *Scotophilus heathi* during the period of delayed ovulation. The concentrations of serum insulin were found to be high during November and December and declined significantly before ovulation. A significant correlation was observed between circulating insulin concentration and body weight as well as with serum androstenedione concentrations. Autoradiographic study demonstrated specific accumulation of ^{125}I -labelled insulin in the ovarian thecal and interstitial cells. Insulin increased both basal and hCG-stimulated androstenedione production by the ovary *in vitro* during November. These results indicate that insulin may be involved in inducing high androstenedione synthesis by the ovary and thus may be indirectly responsible for inducing delayed ovulation in *S. heathi*.

Introduction

The temperate zone vespertilionid bats exhibit delayed ovulation where final maturation and ovulation of Graafian follicles are postponed for several months (Oxberry, 1979). Previous studies have demonstrated that *S. heathi*, a tropical/subtropical vespertilionid bat, also exhibits this phenomenon (Krishna and Singh, 1992). Abhilasha and Krishna (1996) reported an abnormally high circulating androstenedione concentration during the period of delayed ovulation in *S. heathi*, which may be responsible for suppressing follicular development and ovulation. What causes this increased androstenedione production by the ovary during the period of ovulatory delay is not known. Recent studies on *S. heathi* demonstrated a close relationship between high androstenedione concentration and the extensive development of luteinized stromal cells (hyperthecosis) and increase in body weight due to the accumulation of adipose tissue (obesity) (Abhilasha and Krishna, 1997). A similar association between hyperandrogenism, obesity and chronic anovulation also occurs in women with polycystic ovarian syndrome (PCOS). There is some evidence to suggest that in PCOS hyperandrogenism can result from hyperinsulinaemia (Nestler and Jakubowicz, 1996; Utiger, 1996). Insulin has been shown to stimulate ovarian androgen production *in vitro* (Bergh *et al.*, 1993). In rats and humans, insulin-like growth factor I (IGF-I) is also capable of potentiating LH-

induced androgen production by the thecal interstitial cells of the ovary (Cara and Rosenfield, 1988). The aim of the present study was to evaluate the role of insulin in stimulating high ovarian androstenedione production in *S. heathi* during the period of delayed ovulation.

Materials and Methods

Bats were captured live every 1–4 weeks from Banaras Hindu University campus and adjacent areas. The body weight of each bat was recorded. Bats were killed by decapitation as soon as they were taken to the laboratory. Serum was collected from blood samples (pooled blood from two bats) within 1 h and stored at -20°C until assayed for insulin and androstenedione.

Scotophilus heathi has a sharply defined annual reproductive cycle (Abhilasha and Krishna, 1996) and can be classified into the following phases: (1) Quiescence: reproductively inactive phase; ovaries do not contain any antral follicles (August–September). (2) Recrudescence: beginning of reproductive activity; ovaries contain some newly formed antral follicles (October–November). (3) Winter dormancy (early phase of ovulatory delay): bats remain torpid showing temporary arrest in reproductive activity; ovaries contain some antral follicles (December–early January). (4) Late phase of ovulatory delay: ovaries contain some antral follicles and mating occurs (mid-January–mid-February). (5) Preovulatory period: renewed ovarian activity; ovaries contain large antral follicles (late February–early March).

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In vitro study

The *in vitro* study was performed to determine the effect of insulin on androstenedione production by *S. heathi* ovaries. The effect of insulin was compared with the effect of LH. The study was conducted during November because during this period the bats had very high serum concentrations of both insulin and androstenedione. As soon as the bats were taken to the laboratory the ovaries were dissected out and cleaned in media-199 (Gibco, Grand Island, NY). Ovaries (one per tube) were cultured for 2 h at 37°C in 1 ml media-199 containing 0.1% (w/v) BSA, different concentrations of human insulin (0.4, 1.0, 2.0 iu) (Torrent Pharmaceuticals Ltd, Copenhagen) and with or without hCG (0.1 µg) (Sigma, St Louis, MO).

In addition, the seasonal changes in insulin-induced androstenedione production by the ovary were studied. Ovaries collected from bats at different reproductive stages (quiescence (September), recrudescence (November) and preovulatory (February)) were incubated for 2 h in media-199 with insulin (1 iu) and with or without hCG (0.1 µg). Samples were stored at -20°C until assayed for androstenedione.

Hormone assays

Androstenedione. Radioimmunoassay for androstenedione was performed in incubation media without extraction. All the samples were run in one assay. The antibody for androstenedione was obtained from Dr John Resko (Portland, Oregon). The method used was as described by Abhilasha and Krishna (1996).

Insulin. The circulating insulin concentration in the bats was measured using a radioimmunoassay kit (RIAK-1) obtained from Bhabha Atomic Research Centre (Bombay). Verification of the insulin assay for use in the bat was performed. Blood serum samples from several bats were pooled, serially diluted with assay buffer and tested against a standard curve. Dilutions of bat serum ran parallel to the standard curve indicating the suitability of this assay for use in *S. heathi* (Fig. 1). Duplicate 0.1 ml serum samples were used for the assay. Assay buffer, 0.3 ml, and 0.1 ml insulin antiserum (guinea pig anti-human) were added to 0.1 ml serum. After mixing gently, all the tubes were incubated overnight at 4°C. ¹²⁵I-labelled insulin (0.1 ml) were then added to all tubes. The tubes were mixed gently and incubated for 3 h at room temperature. 0.1 ml of a second antibody (anti-guinea pig IgG) and 1.0 ml polyethylene glycol solution were added to all tubes except in total counts. All the tubes were vortexed, kept at room temperature for 20 min and then centrifuged at 1500 g for 20 min. The supernatant was discarded without disturbing the precipitate. Average counts per minute (c.p.m.) were taken for all duplicate tubes in a Beckman gamma counter (Beckman, Geneva). Standards (7.5–200 µiu ml⁻¹), zero standard and blank tubes were also processed with the samples. All the samples were run in one assay. The intra-assay coefficient of variation was less than 7.8%.

Measurement of glucose

Glucose was measured using a quantitative colorimetric procedure for the serum samples used for the insulin assay

Fig. 1. Correlation between pooled blood serum samples from *Scotophilus heathi* (●) and a standard curve (○). The coefficient of determination (r^2) for the bat serum samples versus the standard curve (B/B_0) was 0.97.

using a kit from Stanbio (San Antonio, TX). The inter-assay precision of the assay was below 5%.

Binding of ¹²⁵I-labelled insulin to ovarian sections

The binding of ¹²⁵I-labelled insulin to ovarian sections was studied using an autoradiographic method described previously (Bortolussi *et al.*, 1979; Oxberry and Greenwald, 1982). Ovaries ($n = 3$) collected during November were used for autoradiography to localize insulin binding sites in the ovary. Iodinated insulin (specific activity 100 µCi µg⁻¹ insulin) was obtained from Bhabha Atomic Research Centre, Bombay. Bats were killed by decapitation and ovaries were removed, frozen immediately and sectioned at 10 µm in a cryostat at -20°C. After fixation for 10 min in cold picric acid-formaldehyde and subsequent washing in cold 0.01 mol PBS l⁻¹ (pH 7.2), sections were incubated for 2 h at 37°C with 25 µl ¹²⁵I-labelled human insulin (approximately 10 000 c.p.m.) in PBS containing 0.1% BSA or 0.1% BSA with an excess of unlabelled human insulin. After incubation, the sections were washed in PBS, post-fixed in 4% paraformaldehyde-PBS, washed again in PBS and air dried. The sections were then coated with undiluted Kodak NTB-2, developed in Dektol (Kodak, Rochester, NY) and stained with nuclear red fast stain.

Statistical analyses

The data were analysed by one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test. Correlation coefficient was used to compare the data. Data are expressed as means ± SEM.

Results

Serum glucose and insulin concentrations and insulin correlation with androstenedione

Serum insulin concentration in female bats varied significantly during different reproductive stages ($P < 0.05$,

Fig. 2. Circulating glucose (○) and insulin (●) concentrations in *Scotophilus heathi* during different reproductive phases. Qui, quiescence; Rec, recrudescence; Early delay, early phase of ovulatory delay; Late delay, late phase of ovulatory delay; Preov, preovulatory period. Values are means $\pm$ SEM of five samples.

Fig. 2). Concentrations started to increase from August (quiescence), reached a peak during November (recrudescence) and remained high during December (winter dormancy). However, insulin concentrations declined sharply during February (breeding) and March (preovulatory). Serum glucose concentration in female bats also varied significantly during different reproductive stages ($P < 0.05$, Fig. 2). Glucose concentrations were highest during quiescence, remained high during recrudescence and declined during December (winter dormancy) and February (late delayed period), but increased again during March (preovulatory). Serum insulin concentration showed a positive correlation with changes in body weight ($P < 0.05$, Fig. 3). Serum insulin concentrations higher than $200 \mu\text{iu ml}^{-1}$ also showed a significant correlation ($P < 0.05$) with serum androstenedione concentration (Fig. 3), whereas insulin concentrations lower than $200 \mu\text{iu ml}^{-1}$ did not show significant correlation ($P > 0.05$). Abhilasha and Krishna (1996) described changes in serum androstenedione concentration during different reproductive stages in detail.

Binding of ^{125}I -labelled insulin to ovarian sections

Autoradiographic results are shown in Fig. 4a and b. The uptake of ^{125}I was mainly localized in the thecal cells and stromal or interstitial cells. The highest concentration of uptake was found in the thecal cells. There was only mild or non-specific binding to the granulosa cell layer. Specific uptake of ^{125}I -labelled insulin was not detected in the control sections.

Effect of insulin on ovarian androstenedione synthesis in vitro

hCG increased ovarian production of androstenedione *in vitro* during November. Insulin also significantly increased

Fig. 3. Correlation between circulating insulin concentration and body weight (●) and circulating androstenedione concentration (○) in *Scotophilus heathi*.

ovarian production of androstenedione in a dose-dependent manner. However, small doses of insulin did not augment hCG-induced androstenedione production (Table 1). Higher doses (2 iu) of insulin significantly enhanced hCG-induced androstenedione production compared with treatment with hCG (Table 1).

The effect of insulin on ovarian androstenedione production *in vitro* varied during different reproductive stages (Table 2). During recrudescence (November) 1 iu insulin was as effective as 0.1 μg hCG in stimulating ovarian androstenedione production. However, during quiescence (September) and the preovulatory period (February), insulin was significantly less effective than hCG in stimulating androstenedione production.

Discussion

An important finding of the present study is the statistical correlation between the seasonal changes of serum insulin and androstenedione concentrations in *S. heathi*. Both insulin and androstenedione concentration increased during the period of delayed ovulation and declined significantly during the preovulatory period. To our knowledge this is the first study to demonstrate a direct relationship between serum insulin and androstenedione concentration in any free living mammal, although such a relationship has been demonstrated in humans under pathological conditions such as PCOS, and in acanthosis nigricans (Burghen *et al.*, 1980; Nagamani *et al.*, 1986; Stuart *et al.*, 1986; Conway *et al.*, 1990).

Scotophilus heathi increases consumption of insects during the period from August to October in preparation for winter dormancy (Singh and Krishna, 1996). This period of increased insect consumption leads to extensive deposition of adipose tissue resulting in maximum body weight during November (Abhilasha and Krishna, 1997). Several studies have shown that circulating insulin concentration increases

Fig. 4. (a) Autoradiographic localization of ^{125}I -labelled insulin uptake in ovarian sections of *Scotophilus heathi* taken during November. (b) A magnified section of (a) showing localization of ^{125}I -labelled insulin in the thecal and interstitial cells. F, follicle; TC, thecal cells; IC, interstitial cells; GC, granulosa cells. Scale bars represents (a) 150 μm and (b) 40 μm .

during the period of fattening with the peak concentrations occurring at the time of the maximum body weight and fat content prior to hibernation (Florant *et al.*, 1985; Feist *et al.*, 1986; Boswell *et al.*, 1994). The highest serum insulin concentration in *S. heathi* was also observed during November, the period of maximum body weight and fat content. Overfeeding-induced increases in serum insulin may facilitate the accumulation of fat in response to energy surfeit (Forbes *et al.*, 1989). In addition, it has been shown that reduction in body weight in an obese subject leads to a decline in the rate of insulin secretion (Gumbiner *et al.*, 1996). A similar decline in serum insulin concentration was observed in *S. heathi* during winter dormancy, when a decline in body weight occurred due to gradual depletion of body fat. The present study demonstrated a positive correlation between circulating insulin concentration and changes in body weight in *S. heathi*.

Although hyperinsulinaemia and hyperandrogenism are normally positively correlated, the mechanisms linking these factors are still not clear. It was hypothesized that hyperandrogenism could be a primary event inducing hyperinsulinaemia (Burghen *et al.*, 1980; Chang *et al.*, 1983). However, chronic treatment with androgen does not alter

insulin secretion (Lanzone *et al.*, 1990). Other studies have suggested that hyperinsulinaemia is partly responsible for excessive androgen production (Poretsky, 1991; Nestler and Jakubowicz, 1996). Several studies *in vitro* on ovarian tissues from both animals and humans support this hypothesis (Barbieri *et al.*, 1986; Morley *et al.*, 1989). Exposure of *S. heathi* ovaries *in vitro* to a supraphysiological dose of human insulin during November significantly augmented the production of androstenedione. During this period, a supraphysiological dose of human insulin also significantly potentiated hCG-induced androstenedione production by the ovary. These findings support the hypothesis that insulin amplifies the LH-dependent mechanism that regulates ovarian androgen secretions (Barbieri *et al.*, 1984; Hernandez *et al.*, 1988).

The possibility of insulin exerting a direct stimulatory effect on ovarian androgen biosynthesis has previously been investigated (Barbieri *et al.*, 1984). Demonstration of insulin binding sites in the ovary of *S. heathi* suggests direct action of insulin on the ovarian tissues. Although the nature of the binding site (receptor) in the present study remains to be determined, it is possible that our results are due to the interaction of high concentrations of insulin with IGF-I receptors. The presence of insulin and IGF-I receptors have been demonstrated and characterized in the rat, pig and human ovary (Otani *et al.*, 1985; Poretsky *et al.*, 1985; Hernandez *et al.*, 1988). Binding sites for ^{125}I -labelled human insulin in the ovary of *S. heathi* were found mainly in the thecal and stromal cells. Insulin was shown to induce luteinization of ovarian stromal cells and convert them into steroidogenically active luteinized stromal cells (Nagamani *et al.*, 1986). Increased lipid droplet accumulation was observed in granulosa cell cultures treated with insulin (Channing *et al.*, 1976). In women with PCOS insulin induces hyperthecosis in the ovary. Extensive stromal luteinization has also been shown in the ovary of *S. heathi* during November which coincides with a high concentration of circulating insulin (Abhilasha and Krishna, 1997). Indeed, virtually the entire interfollicular area of *S. heathi* ovaries were occupied by luteinized stromal cells during November (Abhilasha and Krishna, 1997). It is possible that the insulin-induced increase in androstenedione synthesis may be partly mediated through stromal luteinization in *S. heathi* ovaries.

The present study showed seasonal variations in the effects of insulin on ovarian androstenedione production *in vitro*. *Scotophilus heathi* ovaries were found to be most responsive to insulin during recrudescence in November (Table 2), which also coincided with the peak in serum androstenedione concentration (Abhilasha and Krishna, 1996). In comparison, mild ovarian responsiveness to insulin was observed during August (quiescence) and February (preovulatory period). During these periods, *S. heathi* had low circulating androstenedione concentrations. The present study showed a threshold effect of circulating insulin on androstenedione concentration. The correlation between insulin concentrations lower than 200 $\mu\text{iu ml}^{-1}$ and androstenedione was not significant, whereas a significant correlation was observed between insulin concentrations greater than 200 $\mu\text{iu ml}^{-1}$ and androstenedione. This indicates that concentrations lower than 200 $\mu\text{iu ml}^{-1}$ had no

Table 1. The effect of insulin dose on basal and hCG-induced androstenedione production by *Scotophilus heathi* ovaries *in vitro*

Control	hCG (0.1 µg)	Insulin (iu)			Insulin (iu) + hCG (0.1 µg)		
		0.4	1.0	2.0	0.4	1.0	2.0
12.7 ± 1.5	79.7 ^a ± 13.2	58.0 ^a ± 3.8	86.5 ^a ± 14.3	116.3 ^a ± 11.6	68.9 ^a ± 5.9	95.0 ^a ± 16.2	122.2 ^{ab} ± 10.2

Values are means ± SEM of four samples.

^aValues are significantly different ($P < 0.05$) compared with the control.

^bValues are significantly different ($P < 0.05$) compared with the hCG-treated group.

Table 2. Seasonal changes in the effect of hCG and insulin on androstenedione production by *Scotophilus heathi* ovaries *in vitro*

Treatment	September	November	February
Control	4.20 ± 0.6	12.7 ± 1.5	4.6 ± 0.5
hCG (0.1 µg)	64.90 ± 1.2 ^a	79.7 ± 13.2 ^a	60.1 ± 1.3 ^a
Insulin (1.0 iu)	38.04 ± 2.5 ^a	86.5 ± 14.3 ^a	29.2 ± 3.1 ^{ab}
Insulin (1.0 iu) + hCG (0.1 µg)	46.10 ± 3.1 ^a	95.0 ± 16.2 ^a	36.5 ± 0.8 ^{ab}

Values are means ± SEM of four samples.

^aValues are significantly different ($P < 0.05$) compared with the control.

^bValues are significantly different ($P < 0.05$) compared with the hCG-treated group.

or little effect on androstenedione, but higher concentrations had a stimulatory effect. These results are consistent with earlier studies that reported that insulin did not stimulate androgen production *in vitro* in ovarian stromal tissue obtained from normal women (Barbieri *et al.*, 1988), but did in ovarian stromal tissue obtained from hyperinsulinemic women (Barbieri *et al.*, 1986). Furthermore, insulin infusions in normal women did not produce an increase in androstenedione (Nestler *et al.*, 1987). The present study provides indirect evidence that only hyperinsulinaemia is stimulatory for ovarian androstenedione synthesis in the bat. Increases in ovarian responsiveness to insulin may be related to variations in body weight and fat deposition as previously suggested (Evans *et al.*, 1984; Guzick *et al.*, 1994) and may be responsible for variations in serum androstenedione concentration in *S. heathi*. The amount and distribution of body fat has been shown to have an important effect on serum insulin concentration and sensitivity (Guzick *et al.*, 1994). Previous studies on *S. heathi* showed a direct relationship between changes in body weight and serum androstenedione concentration (Abhilasha and Krishna, 1997). This finding and the results of the present study suggest a linear relationship between adiposity (body weight), insulin (concentrations higher than 200 µiu ml⁻¹ or hyperinsulinaemia) and androstenedione in *S. heathi* as was previously reported in women with PCOS (Poretsky, 1991).

Insulin was shown to suppress hCG-induced ovarian androstenedione production *in vitro* during September and February but not during November. Suppression of hCG-induced androstenedione production indicates downregulation of LH receptors by insulin during September and February. This could be because of the supraphysiological concentration of insulin used for the *in vitro* studies, which was much higher than the maximum serum concentration of insulin (300 µiu ml⁻¹) observed in the present study. However, a supraphysiological (1 iu) or even

higher dose (2 iu) of insulin did not downregulate the hCG-induced androstenedione production *in vitro* during November. This may indicate insulin resistance in *S. heathi* during November. During insulin resistance in PCOS, high circulating insulin concentrations were shown to stimulate androgen production by interacting with IGF-I receptors (Barbieri *et al.*, 1988). Coexistence of insulin resistance and hyperandrogenism has been reported in women with PCOS (Polderman *et al.*, 1994; Nestler and Jakubowicz, 1996). Further studies are needed to determine whether insulin resistance or high circulating insulin concentration during November is responsible for increased androstenedione production by *S. heathi* ovaries.

In summary, weight gain by *S. heathi* due to increased food consumption from August to October may be responsible for increases in insulin and subsequently in androstenedione concentrations in November. The increase in androstenedione may in turn be responsible for delayed ovulation. Insulin enhances androstenedione production by acting directly on the ovary and potentiating the action of LH. Weight loss during winter dormancy may cause reduction in insulin and in androstenedione concentrations. These conditions may be responsible for the resumption of ovulation in *S. heathi*.

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