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RESEARCH ARTICLE

Regulation of leptin synthesis during adipogenesis in males of a vespertilionid bat, *Scotophilus heathi*

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SUMMARY

The aim of this study was to elucidate the hormonal regulation of leptin synthesis by the white adipose tissue (WAT) during the period of fat accumulation in male *Scotophilus heathi*. An *in vivo* study showed a significant correlation between the seasonal changes in serum insulin level with the circulating leptin level and with the changes in body fat mass in *S. heathi*. An *in vitro* study showed insulin induced a significant increase in expression of leptin protein in WAT. The insulin-stimulated increase in leptin expression was associated with increased uptake of glucose in the WAT. Two glucose transporters (GLUT4 and GLUT8) are utilized for transport of glucose in the WAT during adipogenesis in the bat. The bats showed high insulin and glucose levels, but a reduction in insulin receptor protein during the period of fat deposition, suggesting insulin resistance, which improved in late winter (January) when most of the fat has been utilized as a metabolic fuel. The *in vitro* study confirmed that insulin enhanced leptin and GLUT4 expression in WAT. The *in vitro* study further showed that the expression of leptin is directly proportional to the amount of glucose uptake by the WAT. The expression of GLUT4 and GLUT8 were also shown to be differentially regulated by insulin during adipogenesis. The insulin-stimulated increase in leptin synthesis by WAT is mediated through phosphorylation of MAPK in *S. heathi*. The specific role of GLUT4 and GLUT8 in the regulation of leptin synthesis during adipogenesis needs further investigation.

Key words: bats, white adipose tissue, leptin, insulin, GLUT.

INTRODUCTION

The seasonally monoestrous bat, *Scotophilus heathi*, feeds vigorously from August to October, in preparation for winter, leading to heavy accumulation of white adipose tissue (WAT) between November and January. Body mass has been found to increase 1.5-fold before the winter dormancy, at Varanasi, India (Chanda et al., 2003). Simultaneously with the beginning of increase in the body mass in October, there is a gonadal recrudescence (Abhilasha and Krishna, 1997). Spermatogenesis in *S. heathi* is seasonal and extends from November to early March, with the testes showing reduced activity during winter (December to early January).

The period of suppressed spermatogenesis in *S. heathi* coincides with peak body mass due to increased accumulation of WAT. Adiposity (obesity) is well recognized risk factor for female infertility (Pasquali et al., 2003). Recent studies from our laboratory showed that increased adiposity impairs ovarian activity in female *S. heathi* (Srivastava and Krishna, 2007). The role of obesity in male reproduction was not well documented until recent studies (Jensen et al., 2004; Magnusdottir et al., 2005). These studies suggest that serum leptin levels are positively correlated with body mass and body fat mass, whereas it is negatively correlated with reproduction (Tchernof et al., 1995). Obese men show increased levels of leptin in plasma and reduced androgen concentrations (Tchernof et al., 1995). In addition, it has been shown that androgen reduction is related to an increase in fat mass (Zumoff et al., 1990) and it has also been linked to increased leptin level (Vettor et al., 1997). Because leptin is a metabolic signal that indicates the level of fat stored, it may signal to the brain about the competence of body to support reproductive activity. However, factors regulating leptin synthesis in WAT during adipogenesis are not known.

The metabolic hormone insulin is another obvious candidate (Schwartz et al., 1992) as a signal of the nutritional status to central pathways. The anabolic actions of insulin on peripheral tissues are well established and plasma insulin also apparently serves as signal of body fat storage to the central nervous system (CNS) (Schwartz et al., 1992). Both leptin and insulin have been linked to the development and maintenance of obesity in several laboratory rodents (King, 1988; Woods et al., 1998; Ahima and Flier, 2000). It has also been reported that a deficiency or an excess of insulin could alter gonadal functions, including steroidogenesis (Stuart et al., 1986).

The objective of the present study was to investigate the role of insulin in the regulation of leptin synthesis in WAT in male *S. heathi.* An attempt was also made to determine whether the effect of insulin on leptin synthesis is mediated through glucose uptake. To achieve this, four factors were examined: (1) the correlation between seasonal variation in circulating leptin with serum insulin and glucose levels and insulin receptor and fat mass; (2) the effect of *in vitro* administration of insulin on the expression of insulin receptor, glucose transporter (GLUT4 and GLUT8) and leptin proteins in adipose tissue; (3) the effect of *in vitro* administration of insulin on glucose consumption by WAT and its correlation with expression of leptin protein by adipose tissue; and (4) determination of the signalling pathway by which insulin affects leptin synthesis by WAT.

MATERIALS AND METHODS

Sample collection

All the experiments were conducted in accordance with principles and procedures approved by the Departmental Research Committee of Banaras Hindu University, Varanasi, India. Four to six adult male *Scotophilus heathi* (Horsfield 1831) bats were captured alive at Banaras Hindu University, Varanasi (25°N, 83°E), and adjacent areas during each calendar month. Body mass of adult bats was recorded as soon as they were brought to the laboratory (within 2 h of capture). The mass of the accumulated adipose tissue of each bat during different months was recorded as described in earlier study (Chanda et al., 2003; Roy and Krishna, 2010). Bats were given an overdose of anaesthetic ether and killed by decapitation. After decapitation, whole blood was centrifuged at 2600 ***g*** for 30 min for serum collection.

Chemicals

Insulin was obtained from Torrent Pharmaceuticals Ltd, Mehasana, India. Antibodies to insulin receptor β-subunit (IR) and rabbit anti-p44/42 mitogen-activated protein kinase (MAPK) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and GenScript USA Inc. (Piscataway, NJ, USA), respectively. The antibodies to GLUT4 and GLUT8 were provided by Dr David James, Garvan Institute of Medical Research, Sydney, NSW, Australia, and Dr Mesonero, University of Zaragoza, Aragon, Spain, respectively. All other general chemicals were purchased from Merck, New Delhi, India.

Insulin-mediated leptin expression in cultured adipocytes

Subcutaneous WAT was collected during December to determine the *in vitro* effects of insulin on leptin, IR, GLUT4, GLUT8 and MAPK expression in white adipose tissue of male *S. heathi*. We assayed these biochemical markers at three doses of insulin. Culture methods for WAT were adopted from earlier studies for bat tissue with minor modifications (Srivastava and Krishna, 2007; Kronfeld-Schor et al., 2000). Following collection, WAT was quickly cut in to pieces in Dulbecco's modified Eagle's medium (DMEM; Himedia, Mumbai, Maharashtra, India) containing 250 i.u. ml^{-1} penicillin and 250 mg ml^{-1} streptomycin sulphate. Pieces of WAT of equal mass were cultured in a mixture of DMEM (with sodium pyruvate and L-glutamine) and Ham's F-12 (1/1 v/v) (Himedia) containing 100 i.u. ml^{-1} penicillin, 100 mg ml^{-1} streptomycin and 0.1% bovine serum albumin (Sigma, St Louis, MO, USA). After initial incubation for 2 h at 37°C, the culture medium was discarded and pieces of WAT were cultured in 1 ml medium containing either 50 μi.u. ml^{-1}, 400 μi.u. ml^{-1} or 1000 μi.u. ml^{-1} insulin in a humidified atmosphere with 95% air and 5% CO_2 to maintain pH 7.4 for 24 h at 37°C. Each treatment group was run in triplicate. After culture, the WAT was collected, washed several times with phosphate-buffered saline (PBS) and kept frozen at –20°C until immunoblot assay.

Western blotting

The protein extraction from cultured and seasonally collected WAT was conducted following the method of Cifuentes et al. (Cifuentes et al., 2005), and western blot analysis was performed as previously described (Srivastava and Krishna, 2010). In brief, a 20% homogenate (w/v) of adipose tissue was made in suspension buffer containing 0.1 mol l^{-1} NaCl, 0.01 mol l^{-1} Tris-HCl (pH 7.6), 0.001 mol l^{-1} EDTA (pH 8.0) and 10 μg ml^{-1} phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 5000 ***g***, 4°C for 15 min; the supernatant was extracted with an equal volume of chloroform and the aqueous phase was recovered. Equal amounts of proteins (40 μg) as determined by Folin's method were used for 10% SDS-PAGE, whereas a 15% gel was used for leptin western blotting. Thereafter, proteins were transferred electrophoretically to a PVDF membrane (Millipore India Pvt. Ltd, Bangalore, Karnataka, India) overnight at 4°C. Membranes were blocked for 60 min with Tris-buffered saline [TBS; Tris 50 mmol l^{-1} (pH 7.5), NaCl 150 mmol l^{-1}, 0.02% Tween 20] containing 5% fat-free dry milk. The membranes were further incubated with rabbit anti-human insulin receptor-β antibody (at a dilution of 1:1000) and mouse monoclonal anti-human GLUT4 antibody (at a dilution of 1:4000), rabbit polyclonal GLUT8 (at a dilution 1:2000), rabbit anti-p44/42 MAPK (at a dilution of 1:1000) and rabbit polyclonal anti-human leptin for 60 min in blocking solution. Immunoreactive bands were revealed by incubating the membranes with 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 mol l^{-1}, 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 PBS and developed with an enhanced chemiluminescence (ECL) detection system (Bio-Rad, Hercules, CA, USA). Similarly, a blot was developed for β-actin (Santa Cruz) at a dilution of 1:1000 as a loading control. Immunoreactive bands were later quantified using ImageJ software (ImageJ 1.36, NIH, Bethesda, MD, USA). Validation of GLUT4, GLUT8 and IR for use in bat tissue is described earlier (Srivastava, 2008).

Hormone assay

Leptin

Serum level leptin in the male *S. heathi* was measured by radioimmunoassay (RIA) using a multi-species leptin RIA kit obtained from Linco Research Inc. (St Louis, MO, USA) as described previously (Srivastava and Krishna, 2007; Roy and Krishna, 2010).

Insulin

Insulin was measured using a human RIA kit (BARC, Mumbai, Maharashtra, India) as per the manufacturer's directions, and as previously used in our lab (Krishna et al., 1998). Briefly, 100 μl of serum sample was added to 300 μl of assay buffer and 100 μl of insulin antiserum. After mixing the tubes gently, all the tubes were incubated at 4°C for overnight. To each tube, 100 μl of ^{125}I-insulin was added and incubated for 3 h at room temperature. Then, 100 μl of secondary antibody and 1 ml of polyethylene glycol solution was added to all tubes except the one for measurements of the total counts. All the tubes were then vortexed and kept at room temperature for 20 min. Following centrifugation, supernatant was discarded without disturbing the precipitate and radioactivity counted using a Beckman Gamma Counter (Fullerton, CA, USA). Standards, zero and blank tubes were also run along with the samples. The intra-assay coefficient of variation was less than 7.8%. Suitability of the human insulin kit was validated for use with *S. heathi* (Doval and Krishna, 1998).

Glucose assay

Blood glucose was measured by the glucose oxidase method using a commercially available automated glucose analyzer (Lifescan Inc., Milpitas, CA, USA) with 10 μl of blood. Glucose in the media was also measured before and after WAT culture, using a 10 μl of sample, to determine the glucose uptake by the WAT (Mueller et al., 1998).

Adipose tissue triglyceride content

WAT triglyceride (TG) was measured as described by Paglialunga et al. and Lee et al. with minor modifications (Paglialunga et al., 2007; Lee et al., 1994). A 20% homogenate (w/v) of WAT was made in PBS. Then TG was extracted from the homogenate overnight in heptane:isopropanol (3:2) at 4°C. TG content was measured using a colorimetric kit (GPO-Trinder) from Span Diagnostics Ltd, Surat, Gujrat, India. Results are expressed as mg TG mg^{-1} protein.

Statistical analysis

Data were analyzed using one way ANOVA followed by Duncan's test using SPSS software 12 for Windows (SPSS Inc, Chicago, IL, USA). Correlation studies were performed to compare data from different groups. The differences were considered significant at the level of $P<0.05$.

RESULTS

Changes in circulating leptin concentration and its correlation with fat mass, triglyceride and IR levels of WAT and circulating insulin and glucose concentrations

Seasonal changes, between October and February, in the mass of WAT accumulated subcutaneously in the lateral abdominal and pelvic region of *S. heathi* are shown in Fig. 1. WAT started to accumulate gradually from October, leading to a 12-fold increase in December relative to October. Accumulated WAT decreased gradually from late December onwards and attained basal level again in February.

Seasonal changes in serum leptin and insulin concentrations showed significant variation (Duncan's test, $P<0.05$) as shown in Fig. 2A,B. The serum leptin concentration was significantly higher during December compared with other months (Duncan's test, $P<0.05$). Serum insulin levels increased from October to November (~150%) and remained elevated during December (Duncan's test, $P<0.05$) compared with other months. Both serum insulin and leptin levels were elevated during December.

Western blot analysis of total IR protein in the WAT of *S. heathi* showed a single immunoreactive band (Fig. 3). Densitometric analysis of IR western blots for the period from October to February showed a marked variation. The immunoreactivity of IR was significantly (Duncan's test, $P<0.05$) highest during October and decreased significantly (Duncan's test, $P<0.05$) in November and December. The IR level increased significantly in January (Duncan's test, $P<0.05$) compared with December.

Blood glucose was at the highest level in November (Fig. 4A). Serum glucose level decreased significantly (Duncan's test, $P<0.05$) in December, coinciding with elevated serum leptin and insulin levels. TG content in WAT showed marked variation from October to February (Fig. 4B). TG content increased from October to December and then decreased in January and February. TG

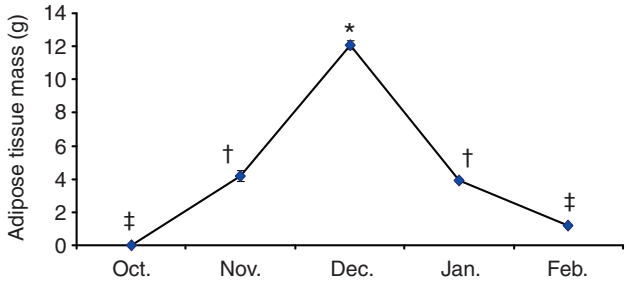

Fig. 1. Seasonal changes in adipose tissue mass of male *Scotophilus heathi*. In December (winter dormancy) there is a significant (*; Duncan's test, $P<0.05$) increase in adipose tissue mass compared with other months. November and January values (†) are significantly (Duncan's test, $P<0.05$) different from October and February values (‡). Values are means ± s.e.m.

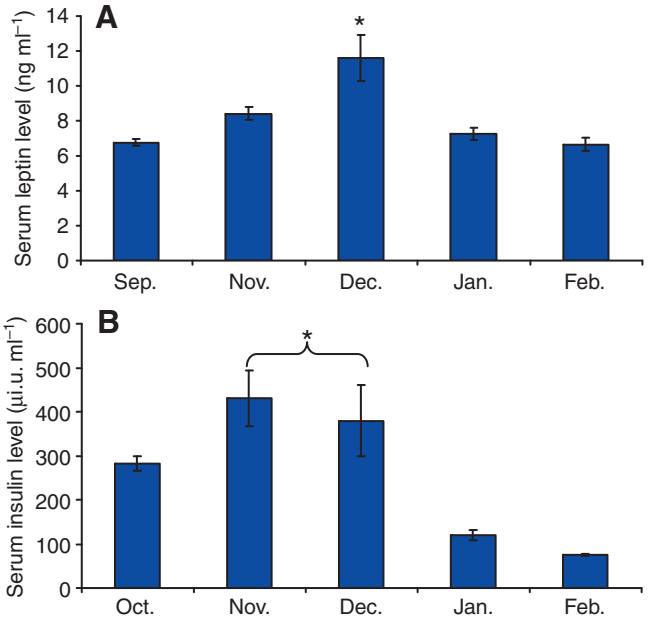

Fig. 2. (A) Changes in the circulating level of leptin during different months. In winter (December) there is a significant (*; Duncan's test, $P<0.05$) increase in the leptin level compared with other months. (B) Changes in the circulating level of insulin during different months. Serum insulin attained a peak in November. Serum insulin levels remained significantly (*; Duncan's test, $P<0.05$) higher during November and December than at other months. Values are means ± s.e.m.

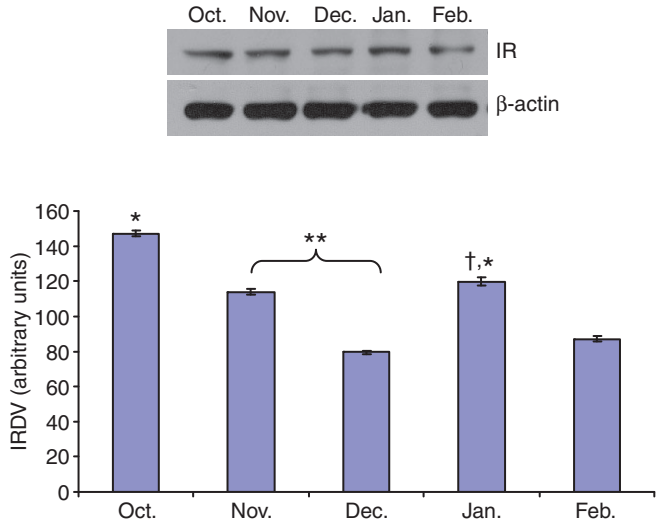

Fig. 3. Western blot analysis of insulin receptor (IR) protein in the adipose tissue of *S. heathi* from October to February. The histogram represents densitometric analysis of the immunoblots. IRDV, integrated relative density value. Expression of IR was significantly higher (*; Duncan's test, $P<0.05$) during October and decreased significantly (**; Duncan's test, $P<0.05$) during November and December, compared with October. The IR level again increased significantly (†; Duncan's test, $P<0.05$) during January compared with December. Values are means ± s.e.m.

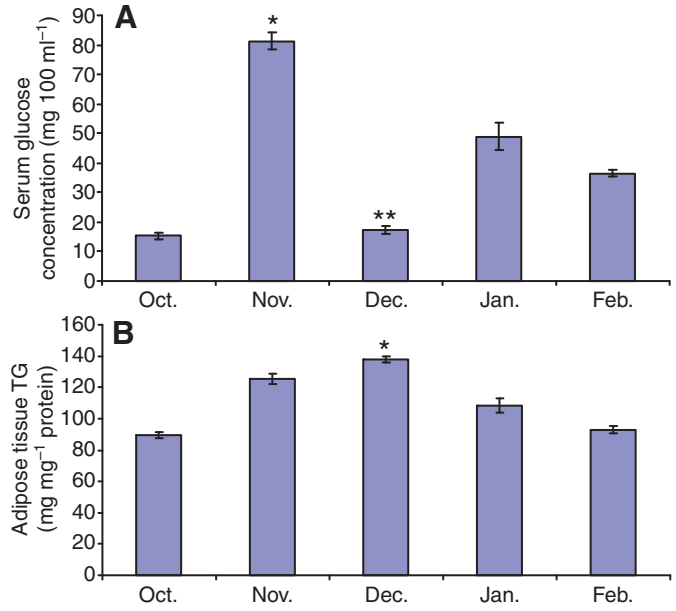

Fig. 4. (A) Changes in the blood glucose level during different months. Blood glucose was significantly higher (*; Duncan's test, $P<0.05$) during November and decreased significantly (**; Duncan's test, $P<0.05$) during December in comparison with November (coinciding with the elevated serum leptin and insulin). (B) Adipose tissue triglyceride (TG) content showed marked variation from October to February. TG content was significantly highest (*; Duncan's test, $P<0.05$) in the month of December, which coincides with the period of increased adipose tissue mass.

content was significantly highest (Duncan's test, $P<0.05$) in the month of December, coinciding with the period of maximum mass of WAT.

We found a significant positive correlation of leptin with serum insulin level ($r=0.6$, $P<0.05$), fat mass ($r=0.85$, $P<0.05$) and TG level of adipose tissue ($r=0.68$, $P<0.05$). Serum leptin level, however, showed no significant correlation with serum glucose level ($r=0.4$, $P>0.05$).

Seasonal changes in expression of GLUT 4 and GLUT 8 proteins in adipose tissue

Western blot analyses of GLUT4 and GLUT8 in adipose tissue from October to February are shown in Fig. 5A and B, respectively. Expression of both GLUT4 and GLUT8 showed marked variation over different periods during winter. Relative to the level in October, GLUT4 expression increased significantly (Duncan's test, $P<0.05$) in November and remained elevated until February. GLUT8, however, decreased significantly (Duncan's test, $P<0.05$) from the level in October in November (~73% of the October value), and further decreased, so that in February GLUT8 expression was only ~3% of that in October.

Effect of insulin on expression of IR, GLUT4, GLUT8 and leptin proteins in adipose tissue *in vitro*

The effect of different doses of insulin on the expression of IR, GLUT4 and GLUT8 and leptin proteins in WAT *in vitro* was studied and results are shown in Fig. 6A–D. Insulin significantly increased (Duncan's test, $P<0.05$) the expression of IR, GLUT4 and leptin proteins in the adipose tissue in a dose-dependent manner. Different doses of insulin had varied effects on *in vitro* expression of GLUT8 protein. Treatment with 50 μi.u. ml^{-1} and 400 μi.u. ml^{-1} insulin significantly (Duncan's test, $P<0.05$) decreased the expression of GLUT8 to ~62% and ~17%, respectively, of the control level. However, the highest dose (1000 μi.u. ml^{-1}) of insulin significantly (Duncan's test, $P<0.05$) increased the expression of GLUT8 to ~114% of the control.

Effect of insulin on glucose consumption by white adipose tissue and its correlation with expression of leptin protein

Insulin treatment *in vitro* produced a dose-dependent significant increase (Duncan's test, $P<0.05$) in glucose uptake by adipose tissue as determined by depletion of glucose from the culture media (Fig. 7A). The study further showed a significant ($r=0.75$, $P<0.05$)

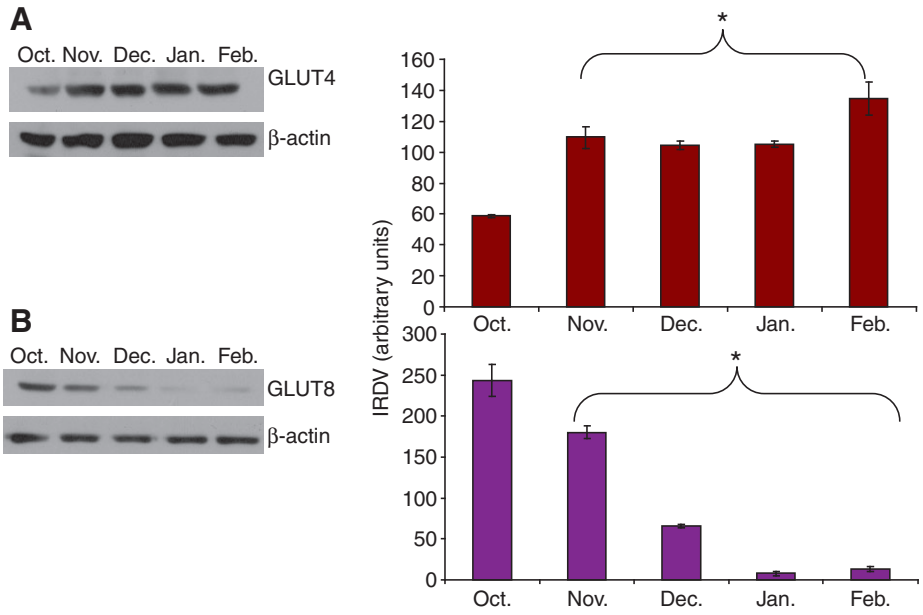

Fig. 5. Western blot analysis of GLUT4 (A) and GLUT8 (B) protein in the adipose tissue of *S. heathi* from October to February. The histogram represents a densitometric analysis of the immunoblots. IRDV, integrated relative density value. GLUT4 expression was significantly (*; Duncan's test, $P<0.05$) higher between November and February than in October. Expression of GLUT8 in adipose tissue decreased significantly (*; Duncan's test, $P<0.05$) from October to February. Values are means ± s.e.m.

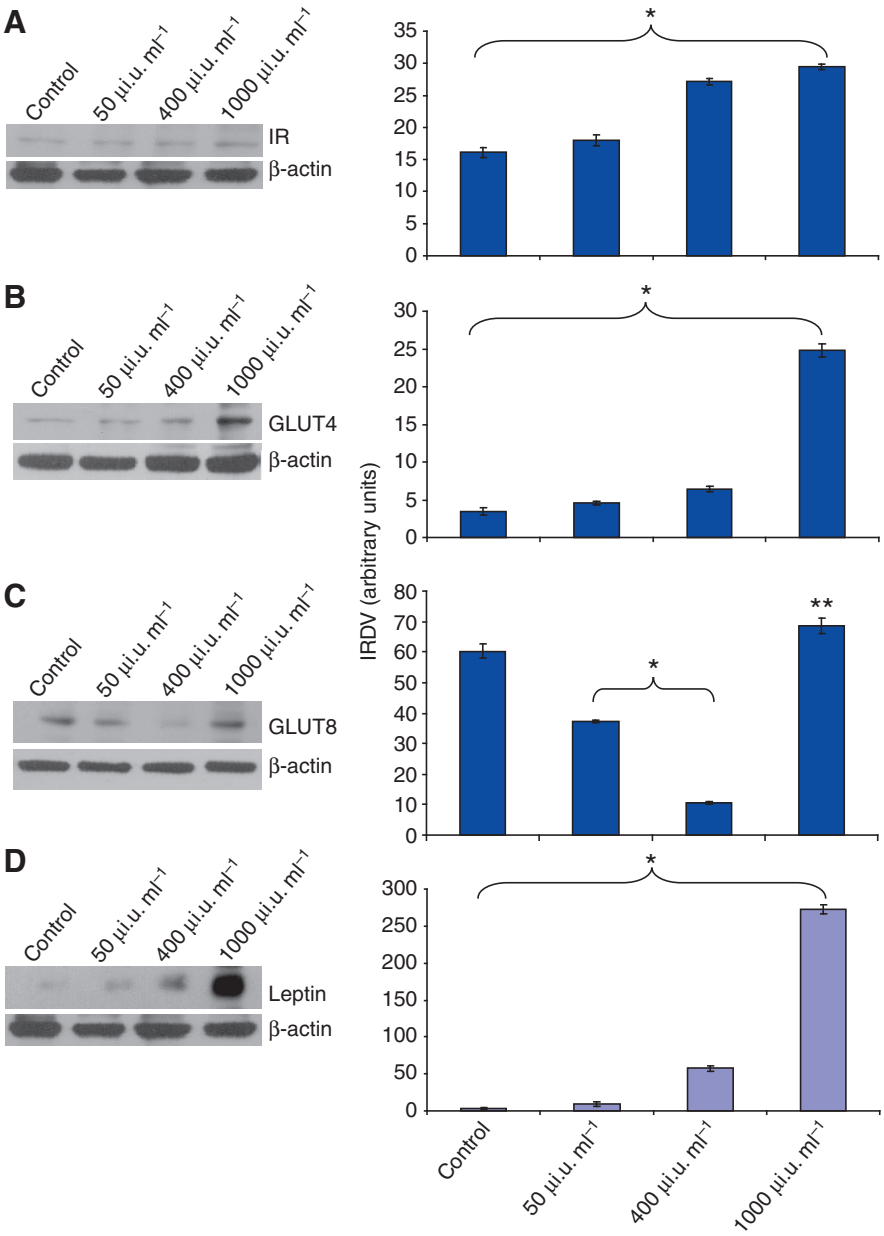

Fig. 6. Western blot analysis showing the effect of 50 μi.u. ml^{-1}, 400 μi.u. ml^{-1} or 1000 μi.u. ml^{-1} doses of insulin, on expression of insulin receptor (IR), GLUT4, GLUT8 and leptin proteins in adipose tissue of *S. heathi in vitro*. (A) Insulin significantly (Duncan's test, $P<0.05$) upregulated its own receptor in a dose-dependent manner. Values are significantly different from each other. (B) All three doses of insulin (50, 400 and 1000 μi.u. ml^{-1}) increased significantly (Duncan's test, $P<0.05$) the expression of GLUT4 in a dose-dependent manner. Values are significantly different from each other. (C) Two doses of insulin (50 μi.u. ml^{-1} and 400 μi.u. ml^{-1}) significantly (*; Duncan's test, $P<0.05$) downregulated GLUT8 expression, whereas the highest dose (1000 μi.u. ml^{-1}) significantly upregulated (**; Duncan's test, $P<0.05$) the expression of GLUT8 compared with the control value. (D) Insulin significantly increased (Duncan's test, $P<0.05$) the expression of leptin protein in a dose-dependent manner. *Values are different from each other. Values are means ± s.e.m.

positive correlation between glucose uptake and expression of leptin protein in the adipose tissue (Fig. 7B).

Effect of insulin on expression of phosphorylated MAPKs in WAT *in vitro*

To gain insight into the molecular basis of insulin-induced leptin synthesis, as determined by expression of leptin, the effect of insulin on phosphorylated MAPKs was investigated in WAT *in vitro*. Immunoblot analysis showed two bands corresponding to the 44 kDa isoform p44 (ERK1/MAPK3) and the 42 kDa isoform p42 (ERK2/MAPK1; Fig. 8). Increasing the dose of insulin significantly increased (Duncan's test, $P<0.05$) the expression of the two isoforms of MAPK. Only the highest dose of insulin significantly increased (Duncan's test, $P<0.05$) the expression of p44, whereas insulin at the two lower doses significantly increased (Duncan's test, $P<0.05$) the expression of p42 in a dose-dependent manner, but at the highest concentration caused downregulation of this isoform.

DISCUSSION

Changes in the circulating concentration of insulin and leptin showed a significant positive correlation ($r=0.85$, $P<0.05$) in *S. heathi* during the period of fat deposition. As shown previously in rodents (Alonso-Vale et al., 2005), our *in vitro* study also showed a dose-dependent increase in insulin-stimulated leptin expression in cultured WAT. Results of both *in vivo* and *in vitro* studies together clearly suggest that insulin regulates expression of leptin protein in WAT during the period of fat deposition in male *S. heathi*. This *in vitro* study is in agreement with the earlier studies showing that insulin regulates expression and synthesis of leptin in cultured WAT of rats and humans (Saladin et al., 1995; Rentsch and Chiesi, 1996; Kolaczynski et al., 1996). Thus, the present study supports earlier studies showing that insulin, as a major regulator of adipogenesis and *in vivo* hyperinsulinemia, may cause development of obesity (Klemm et al., 2001; Gagnon and Sorisky, 1998). The previous study on *S. heathi* showed a gradual increase in body mass prior to winter dormancy, mainly due to accumulation of WAT

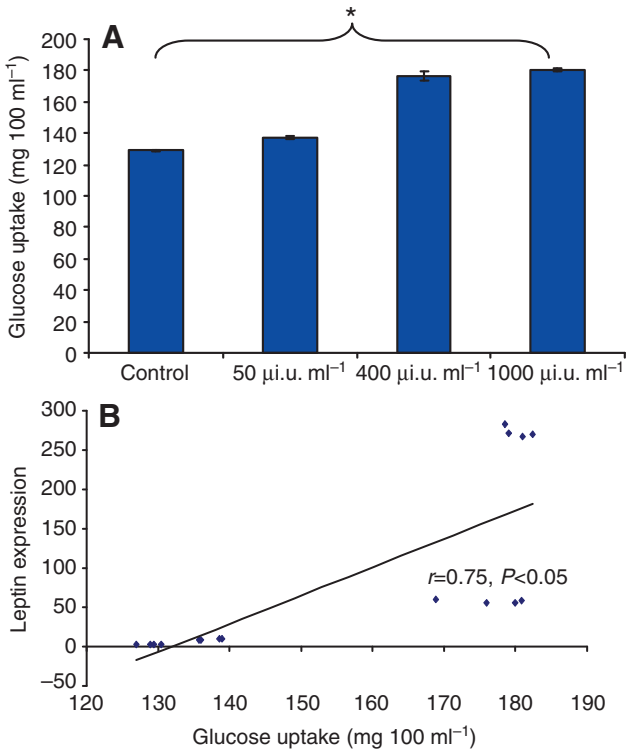

Fig. 7. (A) Effect of different doses of insulin (50 µi.u. ml^{-1}, 400 µi.u. ml^{-1} and 1000 µi.u. ml^{-1}) on glucose uptake by white adipose tissue *in vitro*. Insulin significantly increased (Duncan's test, P<0.05) glucose uptake in a dose-dependent manner, as determined by depletion of glucose from the culture media. *Values are significantly (Duncan's test, P<0.05) different from each other. (B) A significant positive correlation (r=0.75; P<0.05) was observed between glucose uptake and expression of leptin protein in the adipose tissue.

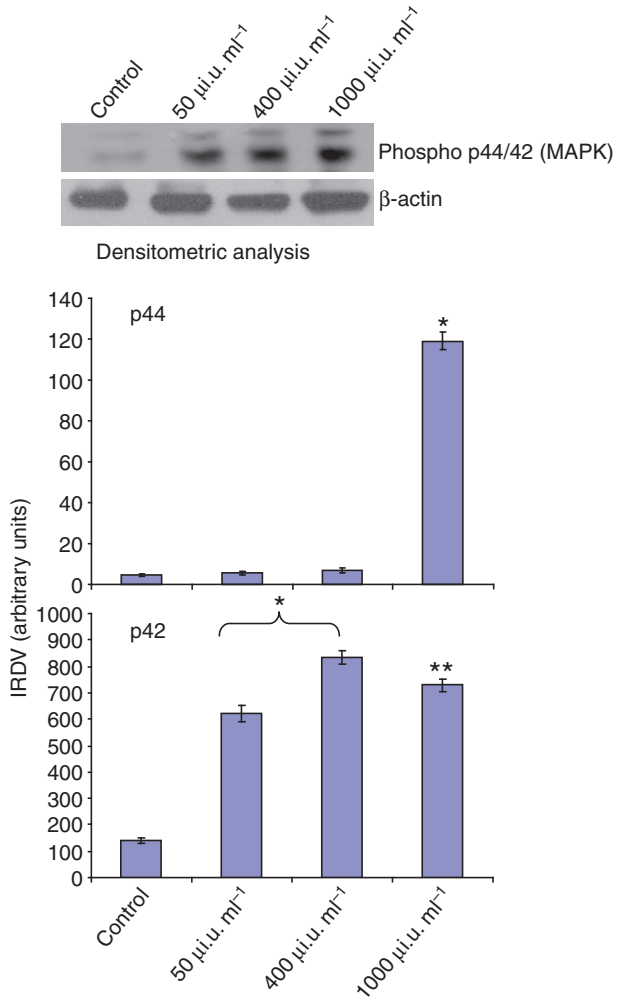

Fig. 8. Effect of different doses of insulin (50 µi.u. ml^{-1}, 400 µi.u. ml^{-1} or 1000 µi.u. ml^{-1}) on expression of phosphorylated MAPKs in white adipose tissue *in vitro*. Increasing the dose of insulin significantly increased (Duncan's test, P<0.05) the expression of two isoforms of MAPK. Only the highest dose (1000 µi.u. ml^{-1}) of insulin significantly increased (*; Duncan's test, P<0.05) the expression of the p44 isoform in comparison to other groups and the control, whereas the two lower doses of insulin (50 µi.u. ml^{-1}, 400 µi.u. ml^{-1}) significantly increased (*; Duncan's test, P<0.05) the expression of the p42 isoform in comparison to control. The highest dose (**) of insulin downregulated the expression of the p42 isoforms. Values are means ± s.e.m.; IRDV, integrated relative density value.

(Srivastava and Krishna, 2008). Our present study also showed a significant correlation (r=0.87, P<0.05) between the changes in the mass of accumulated WAT and associated concentrations of triglycerides. This study further suggests an increase in leptin concentration together with an increase in fat deposition or adipogenesis in *S. heathi*. Seasonal triglyceride content of WAT was demonstrated as a marker for adipogenesis and differentiation of WAT (Jeon et al., 2004). The present study is in agreement with earlier studies both in animals and humans showing that leptin expression and secretion increase as the size of the WAT and triglycerides stores increases (Maffei et al., 1995; Considine et al., 1997; Frederich et al., 1995). It has earlier been demonstrated that hyperphagia prior to winter is responsible for fat deposition in *S. heathi* (Srivastava and Krishna, 2008). Simultaneously, increase in both serum insulin and glucose concentrations in *S. heathi* suggest an insulin-resistance-like condition (Srivastava and Krishna, 2008), which facilitates fat deposition in bats (Doval and Krishna, 1998). In a recent study a higher mean value of insulin and of the insulin/glucose ratio was considered as a good index of insulin resistance and provides information regarding relative insulinemia (Viega et al., 2008). The results of the present study in *S. heathi* also showed a significantly higher mean value of insulin and a higher insulin/glucose ratio during the period of fat deposition. Changes in the insulin/glucose ratio also showed a significant correlation with the changes in serum leptin (r=0.78, P<0.05) and glucose (r=0.69, P<0.05) levels and expression of GLUT4 (r=0.60, P<0.05) in WAT of *S. heathi*. It is well documented that changes in adiposity, insulin and leptin levels are often associated with insulin resistance (Bodkin et al., 1996; Wolden-Hanson et al., 2000). The *in vivo* study also showed downregulation of IR in WAT as described in kidney of insulin-resistant rats (Tiwari et al., 2007). This finding supports earlier reports showing that an increase in serum insulin levels correlates with increased fat storage in mammals that exhibit autumnal fattening (Boswell et al., 1994). Since autumnal fattening correlates with leptin synthesis (Srivastava and Krishna, 2008), the present study suggests insulin-dependent regulation of leptin expression in WAT in male *S. heathi*.

Now the question arises of how insulin regulates leptin expression in WAT. The present study showed a strong correlation between adipocytes glucose uptake, as measured by the decrease in glucose

in the culture media during incubation with insulin, and expression of leptin (synthesized) in isolated adipose tissue. These results are consistence with the earlier proposal that the rate of glucose uptake by WAT is correlated significantly with the expression of leptin in WAT. Thus, leptin secretion appears to reflect the amount of glucose transported and metabolized by WAT. There was also a significant (Duncan's test, $P<0.05$) increased expression of GLUT4 in WAT treated with insulin compared with the control tissue. Increased glucose uptake increases lipid accumulation in adipose tissue by synthesis of triglycerides (Chuang et al., 2007). Increased triglycerides content was considered to be a marker for adipogenesis (Jeon et al., 2004). This study in *S. heathi* showed the highest level of triglycerides in December, which coincided with the period of maximum accumulation of adipose tissue in these bats. It has earlier been demonstrated that increased deposition of WAT may arise from increased synthesis of triglycerides in the pre-existing adipocytes (Villena et al., 2004). This suggests a link between glucose metabolism, adipogenesis and leptin synthesis by WAT. There is considerable evidence suggesting that glucose metabolism may be an important regulator of leptin production (Mueller et al., 1998). Increased leptin mRNA expression after intraperitoneal glucose injection in mice is more closely related to plasma glucose concentration than insulin concentration (Mizuno et al., 1996). In another study involving the blockage of glucose transport with 2-deoxy-D-glucose, phloretin or cytochalasin B (Wierlinga et al., 1981; Marette and Bukowiecki, 1991) produced a dose-dependent decrease in leptin secretion in the presence of a high physiological concentration of insulin. These data together with our study suggest a physiological role for glucose in the regulation of leptin expression and secretion by adipocytes.

Our *in vivo* study showed that insulin-stimulated leptin synthesis may be mediated through increased glucose uptake by the adipose tissue in *S. heathi*. The *in vitro* study further supports this relationship. Both GLUT4 and GLUT8 are utilized to transport glucose to adipose tissue during adipogenesis in *S. heathi.* It is well demonstrated that GLUT4 expression in adipocytes is under insulin control and is the major transporter of glucose during adipogenesis (Sivitz et al., 1989). In our *in vitro* study, insulin increased its own receptors and upregulated GLUT4 expression in WAT in a dose-dependent manner. However, our *in vivo* data indicated that IR is downregulated during the winter despite increased insulin level. The reason for the discrepancy between the *in vivo* and *in vitro* data could be due to lack of certain factors in the *in vitro* study (such as neural input, feedback regulation from markers in circulation, etc). This study also demonstrates expression of novel glucose transporter GLUT8 in adipose tissue. Expression of GLUT4 and GLUT8 in adipose tissue varied significantly ($P<0.05$) during different stage of adipogenesis.

The *in vivo* study showed a significant increase (Duncan's test, $P<0.05$) in GLUT4 expression in WAT from October to November, and this coincided with significant increase (Duncan's test, $P<0.05$) in fat accumulation and circulating leptin level in *S. heathi*. Expression of GLUT4 in adipose tissue remained high during the period from November to February. This suggests continued glucose transport involving GLUT4 in WAT during autumn deposition of WAT. The GLUT8 expression in WAT was highest during October, the period before the onset of active fat deposition. Changes in GLUT8 correlated significantly ($r=0.66$, $P<0.05$) with changes in IR, but correlated inversely with fat deposition during winter. The *in vitro* experiment showed that GLUT4 and GLUT8 expression in WAT is differentially regulated in response to stimulation by insulin. The expression of GLUT4 increases but expression of GLUT8 decreases in WAT with increasing insulin. Interestingly, a very high dose of insulin *in vitro* (a hyperinsulinemia-like condition) stimulates both GLUT8 and GLUT4 expressions in WAT of *S. heathi*. This suggests that both GLUT 8 and GLUT4 expression in WAT is insulin sensitive. This is consistent with the earlier study in rat in which hyperinsulinemia was found to be associated with an increased level of GLUT8 protein in adipocytes (Gorovits et al., 2003). Our study demonstrates that GLUT4 and GLUT8 expression is differentially regulated in adipose tissue in response to insulin and suggests that GLUT8 and GLUT4 concentrations in adipose tissue are intimately linked to glucose homeostasis.

To gain further insight into the molecular basis of insulin-induced leptin synthesis in the WAT of bats, the MAPK signalling pathways, as shown previously by Molero et al., was investigated (Molero et al., 2002). There was a significant increase (Duncan's test, $P<0.05$) in the p44 and p42 isoforms of MAPK as a result of the insulin-induced increased expression of leptin protein in the bat adipose tissue. The increased expression of MAPK was correlated with increased leptin synthesis by WAT. Similar results were also recently reported for *Cynopterus sphinx*, in which increase expression of MAPK was found to be correlated with increased leptin synthesis by WAT (Banerjee et al., 2010). Earlier studies also demonstrated a direct role of MAPK in activation of the transcription factor (ADD1/SREBP-1) in relation to leptin gene expression (Kotzka et al., 1998; Kim et al., 1998). Our study is consistent with previous studies showing that insulin induced dual phosphorylation in adipose cells (Sevetson et al., 1993; Anderson et al., 1990). This finding is further supported by another study showing that hyperglycemia enhances lipid deposition through a MAPK-mediated pathways (Chuang et al., 2007). Recently, MAPK signalling has been linked with a pre-adipocyte differentiation process. Thus, our study suggests a possible role of MAPK-mediated action during insulin-induced leptin synthesis by WAT of *S. heathi*.

In summary, there was found to be a significant correlation between seasonal changes in the circulating level of insulin and both leptin levels and changes in body fat mass in *S. heathi*. Insulin-induced increased expression of leptin is associated with increased uptake of glucose in WAT. Both GLUT4 and GLUT8 are used for transport of glucose in WAT during adipogenesis in the bat. The *in vitro* study confirms the *in vivo* finding that the insulin enhances expression of leptin together with GLUT4 by WAT. The *in vitro* study further showed that the expression of leptin is directly proportional to the amount of glucose uptake by adipose tissue. This study showed that the expression of GLUT4 and GLUT8 is differentially regulated by insulin during adipogenesis. Insulin-stimulated increase in leptin synthesis by the adipose tissue is mediated through phosphorylation of MAPK in *S. heathi*. The specific role of GLUT4 and GLUT8 in regulation of leptin synthesis during adipogenesis needs further investigation.

LIST OF ABBREVIATIONS

GLUT	glucose transporter
IR	insulin receptor
MAPK	mitogen-activated protein kinase
WAT	white adipose tissue

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