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EFFECT OF LEPTIN STATUS ON NEUROENDOCRINE- REPRODUCTIVE REGULATION AND MATERNAL-FETAL NUTRIENT TRANSFER IN WISTAR ALBINO RATS

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ABSTRACT

Effect of leptin status on neuroendocrine-reproductive regulation in wistar rats was studied. Ten wistar rats weighing between 170-280g were randomly assigned into two study groups. The animals in Group 1 (the control) received a placebo of 5.0ml distilled water while those in Group two were treated with 100mg insulin/kg body weight of rat via gastric intubation. The experiment lasted for 21 days. One day after the final exposure, the animals were euthanized by inhalation of over dose of chloroform. The brain of each rat was harvested and processed into whole homogenate, and was used for some biochemicals assays (i.e isolation and purification of RNA, reverse transcription polymerase chain reaction (PCR), and leptin assay). The results showed that insulin increased the secretion of leptin, which in turn, reduced feed intake, and energy balance, leading to increased MRNA expression, suggesting that leptin may be involved in the control of appetite and maturation of luteneizing hormone secretory axis, which may be associated with development of the neuroendocrine axis (i.e neuroendocrine signal transduction). The study may suggest that leptin may serve as effectors that link mechanism that regulate reproduction and energy balance, thus playing an important role in reproduction and energy balance; modulating maternal nutrient partitioning inorder to optimize the provision of nutrients for fetal growth.

KEYWORDS: Leptin, Insulin, neuroendocrine, reproductive regulation, and energy balance

INTRODUCTION

Leptin is a ploypeptide hormone, produced by adipocytes and trophoblast cells (Masuzaki et al, 1997; Hardie et al, 1997; Holness et al, 1999). This hormone plays an important role in regulating feed intake, energy balance and reproduction (Himms-Hagen, 1999). Leptin's central action is largely due to mediating hypothalamus neuropeptide Y (NPY) gene expression (Schewartz et al, 1996; Hakaanson et al, 1998). Neuropeptide Y (NPY) also is an important central nervous system (CNS) regulator of food intake, energy expenditure and reproductive function (Mercer et al, 1996; Hakaanson et al, 1998).

The hypothalamus is the key site of central nervous system (CNS) regulation of energy homeostasis, appetite and reproduction (Schewartz et al, 1996; Elias et al, 1999). The long form leptin receptor (ob-Rl) is localized within the hypothalamus along with several neuropeptides that are involved in regulation of the neuroendocrine axis (Hakaansons et al, 1998; Cassanuva et al, 1999). These effects are mediated by interactions between neurotransmitters such as norepinephrine, dopamine, and neuropeptides, including neuropeptide Y (NPY), Gonadotrophin-releasing hormone (GnRH), corticotrophin-releasing factor (CRF), orexin, somastostatin, propiomelanocortin (PoMc), and peripheral hormones that act at the hypothalamus (e.g leptin or insulin) (Schewartz et al, 1996; Erickson et al, 1996; Schewartz et al, 1997; Thorton et al, 1997; Fliers et al, 1998; Hakaanson et al, 1998).

The long form leptin receptor (ob-RL) is coexpressed in neuropeptide Y (NPY) neurons in the arcuate nucleus of the hypothalamus (Mercer et al, 1996; Erickson et al, 1996). The leptin receptor is colocalized with several other neuropeptides within the hypothalamus such as CRF, POMC, and orexin (Hakaanson et al, 1998; Elias et al, 1999) that are involved in central nervous regulation of feed intake, energy balance and reproduction. However, it has been reported (Mercer at al, 1996; Hakaanson et al, 1998) that leptin treatment inhibited NPY synthesis in the hypothalamus, reduced feed intake and increased energy expenditure.

Leptin has been found to play an important role in pregnancy (Masuzaki et al, 1997; Hardie et al, 1997; Holness et al, 1999) as a signal to the fetus of placental competence for nutrient transfer (Senaris et al, 1997; Sylvan et al, 1998; Lage et al, 1999), and could be regulated independently of leptin expression in adipose tissues (Holness et al, 1999). Leptin is also involved in the regulation of body weight (Desoye et al, 1987; Masuzaki et al, 1997; Lage et al, 1999; Garcia et al, 2000).

Many hormones and proteins act on cells by activating second messenger pathways (Strauss et al, 1992). Such as the cAMP- dependent pathway which causes changes in cellular activity through protein

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Kinase A (PKA). By phosphorylating cytoplasmic and nuclear proteins, this kinase (PKA) apparently coordinates cellular processes, including the biosynthesis and release of peptides and hormones (Benoit et al, 1988; Strauss et al, 1992; Slicker et al, 1996; Yura et al, 1998, Casarueva et al, 1999). The activation of PKA pathway is widely known to mediate the actions of numerous hormones in the placenta (Strauss et al 1992). There is the possibility that this signaling pathway could be involved in the regulation of Leptin, modulating maternal nutrient partitioning inorder to optimize the provision of nutrients for fetal growth (Holness et al, 1999). Thus, through modulation of maternal insulin secretion and hepatic metabolism, leptin integrates maternal nutrient storage to the nutrient requirement of the fetus (Holness et al, 1999). Insulin is an important modulator of leptin gene expression, resulting in stimulation of leptin release or secretion. (Wabitsch et al, 1996) However, it has been reported that leptin inhibits glucose- stimulated- insulin secretion (Muoio et al, 1999), thus suggesting the existence of a negative feed back system between leptin and insulin (Ishida et al, 1997). This current study focused on the assessment of leptin status on neuroendocrine regulation and maternal-fetus nutrient transfer in wistar albino rats exposed daily to insulin.

MATERIALS AND METHODS

Experimental animals

Ten wistar albino rats weighing between 170 – 280g were obtained from disease-free stocks maintained in the animal house of the Department of Biochemistry at the college of Medical Sciences, University of Calabar, Nigeria. The animals were randomly assigned on the basis of average body weight and litter origin into two study groups of five animals per group. Each rat in a study group was individually housed in a stainless cage with plastic bottom grid and a wire screen top. The animal room was adequately ventilated, and kept at a room temperature and relative humidity of 29 ± 2^0c and 40-70% respectively, with a 12hr natural light-dark cycle. Animals were fed ad libitum with water and rat chow (Livestock Feeds Ltd, Calabar, Nigeria). Good hygiene was maintained by constant cleaning and removal of feces ad spilled feed from cages daily. All animal experiments were approved by the Animal Care and Use C ommittee of the Medical College, University of Calabar, Nigeria.

TREATMENT REGIMEN

All rats received daily treatments with their test solutions for a period of 21days. All treatments were conducted between the hours of 9.00 – 10.00AM. The rats in Group 1 (control) received a placebo of 5.0ml distilled water via gastric intubation. The rats in Group 2 were treated with 100mg insulin/kg, in a total volume of 5.0ml vehicle. In relation to Insulin (ins) the concentrations used here correspond to the blood levels observed (Desoye et al, 1987; Dorr et al, 1989).

PREPARATION OF INSULIN

Synthetic insulin was obtained from Sigma Chemicals (Poole, England) for use in the study. A stock solution was prepared by dissolving insulin in 500ml distilled water. From this, and based on the animal's weight that morning, the 100mg/kg dosages were administered to the animals in group 2 as part of the 5.0ml volume used for gastric intubation.

PREPARATION OF SAMPLE

One day after the final exposure, the animals were euthanized by inhalation of an overdose of chloroform. The brain of each rat was harvested (particularly, the hypothalamus, excised, after making the following cuts: rostral to the optic chiasm, rostral to the mammillary bodies, lateral to the hypothalamic Sulci, and ventral to the anterior commissure), ground using a mortar and pestle, and buffered with Tris- HCl (pH 7.4). A whole homogenate (WH) of the tissue was then prepared by centrifugation at 6000xg for 30min; the supernatant generated was recovered and underwent a second centrifugation at 8000xg for 20min. The brain whole homogenate (WH) sample was frozen in liquid nitrogen and maintained at -80^0c for isolation and purification of brain total RNA.

Assay for RNA isolation and purification.

Total RNA from hypothalamus was isolated and purified using the single-step method described by Chomczynski and Sacchi (1987) with Trizol reagent (Gibco, Grand Island, NY) according to the manufacturers' procedure. Isolated RNA was treated with RQIRNase- free Dnase (Promega, Madison, WI) to eliminate possible genomic DNA contamination. In brief, 10μg of the RNA sample and 5μl 10 x reaction buffer (400mM Tris-HCl [pH7.9], 100mM Nacl , 60mM $MgCl_2$, 100Mm $CaCl_2$), 5 units DNase, 1μl Rnasin (Promega, 40μ/μl), and RNase-free water was added to a final volume of 50μl and incubated at 37^0C for 30min. Then an equal volume of phenol/chloroform (Amresco, Solon, OH) was added. The mixture was vortexed and centrifuged at 12000xg for 15min, the aqueous phase carefully transferred to a new centrifuge tube, and 1.0ml 100% ethanol added and centrifuged at 12000xg for 10min. the pellet was washed in 10% ethanol and centrifuged again. The pellet was air dried and resuspended in 20μl RNase-free water and quantified using a 6400/6405 spectrophotometer (Jenway, Essex, England) at 260nm and 280nm. Quality of RNA was checked by electrophoresis using a 1% (w/v) denatured agarose gel and stained with ethidium bromide.

Reverse Transcription – Polymerase Chain Reaction

2mg of total RNA was denatured with 500mg oligo (dT) and 25ng random primer at 70^0c for 10min and chilled on ice. Then, 4μl 5 x buffer (250mM Tris-HCl [PH8.3], 375Mm KCL, 15Mm $Mgcl_2$), 2μl 0.1M dithiothreitol, 1μl dNTP mixture (10mM each), 1μl superscrpt II reverse transcriptase (Gibco), and 0.5μl of RNasin (Promega) and RNase-free water were added to a final volume of 20μl. The tube was incubated at room temperature for 10min, followed by additional 10min at 70^0c in a thermocycler (Gradient 40 robocycler; Strategene, LaJolla, CA). After reverse transcription (RT), a 20^-μl volume contained 2μl of cDNA, 1μl primer mixture (50 pmol each), 2μl 10 x buffer (500mM KCl,100Mm Tris-HCl [pH 9.0 at 25^0c], 1% Triton x-100), 15Mm $MgCl_2$, 1μl dNTP mixture (10mm each), and 2.5units Taq DNA polymerase (Promega) was used for the polumerase chain reaction (PCR). The PCR was performed at 1cycle

of 94^0c for 3min, specific annealing temperature for 1min, and 72^0C for 1min, followed by 28 cycles at 94^0C for 30sec, specific annealing temperature for 1min, and 72^0C for 1min and for 10min in the last cycle, specific annealing temperature and primers for leptin were 55^0C, 5'- ACAGAGGGTCACCGGTTTGG-3' for sense primer, and 5'-TAGAGGGAGGCTTCCAGGAC-3' for antisense primer, respectively (see table 1). The PCR product for each specific gene was confirmed by its size and enzyme digestion. The PCR products were electrophoresed on a 2% agarose gel, followed by ethidium bromide staining (0.4μg/ml) and analysed with an image analysis system (Flurochem; Alpha Innotech corporation, San Leandro, CA).

LEPTIN ASSAY

An enzyme linked Immunosorbent assay (ELISA) kit was obtained from R& D systems (Minneapolis, MN). The leptin assay was determined according to the procedure contained in the ELISA kit manual. The limit of sensitivity was 7.8pg/ml.

STATISTICAL ANALYSIS

Data from the image analysis were expressed as mean ± standard deviation (SD). Expression data were subjected to square root transformation and one-way ANOVA according to the general linear model procedure of the Statistical Analysis System (SAS, 1987). A p value equal to or less than 0.05 (i.e $p < 0.05$) was considered significant.

RESULTS

Table 2 presents the results of treatments on the hypothalamic expression of ob-RL and adipose tssue,leptin in the rats. The results showed that there was significant($p<0.05$) increase(42.37%) in values of the ob-RL and 28.95% increase in values of the adipose tissue leptin, respectively relative to those seen in the controls. The results showed that expression of ob-RL was higher ($P<0.05$) than that of leptin MRNA expression, suggesting that insulin stimulated secretion of leptin, leading to higher, leptin mRNA expression. Table 3 presents the results of treatments on feed intake of the rats. The results showed that there was significant decrease ($P<0.05$) in the values of the insulin treated hosts relative to those of the controls. The results showed that increase in leptin reduced feed intake of the rats.

Table 1: Primer pairs for each selected gene, the annealing temperature, the length of PCR products, and the Genbank access number from which the primers were selected.

PCR product	Sense primer	Antisense primer	Tem (%)	Length (bp)	Access number
Ob – RL	5'-TCGGAAGATATCAGTGTTGA-3'	5'-TTTGGGATGCTGATCTGATAA-3'	55	315	gb/AF 389-12
Leptin	5'-ACAGAGGGTCACCGGTTTGG-3'	5'-TAGAGGGAGGCTTCCAGGAC-3'	57	268	gb/AF 048-672

Table 2: Hypothalamic expression of Ob-RL and adipose tissue leptin in rats

Group (N)	ob-RL	Adipose tissue leptin
Control	0.59±0.17	0.76±0.33
Insulin treated	0.84± 0.32	0.98 ± 0.46

N = Number of rats = 5

Values were expressed as mean ± SD at $p<0.05$.

Table 3: **Effect of treatment on feed intake of the rats**

Group (N)	Feed intake (g)
CONTROL	38.34±1.68
Insulin treated	23.52±0.59*

N=Number of rats per group=5

.Values are expressed as mean **±** SD at $P<0.05$

*Significantly different from control at $P<0.05$.

DISCUSSION

In this study, leptin reduced feed intake and decreased energy balance. The action of leptin could be mediated via ob – RL in the hypothalamus as well as expression of the leptin gene due to regulation by nutrient availability (Zhang et al, 1994; Tartaglia et al, 1995; Friedman et al, 1998). This effect could be attributed to the activation of the insulin gene to influence leptin secretion; thus suggesting that central leptin mRNA expression may play a role in appetite control. It is possible that central leptin may be involved in maturation of the GnRH/LH secretory axis. However, there could be other substances as intermediates in the signal transduction pathway between leptin and GNRH secretion (i.e neurons containing POMC and its products- ACTH, β- endorphin, and melanocyte

stimulating hormones (MSHs), are located in areas within the hypothalamus that are involved in GnRH secretion and feed intake regulation (Emmanuel et al, 1989; Kineman et al, 1989; Lin et al, 1999; Dian et al, 1999). The changes observed may contribute to changes in feed intake, leading to changes in energy balance; and may be associated with development of the neuroendocrine axis and leptin may serve as effectors that link mechanisms that regulate reproduction and energy balance. Apart from its actions on body weight regulation and metabolism, leptin plays an important role in reproduction (Himm-Hagen et al, 1999). These actions may represent nutritional feature of reproduction that depend on the adequacy of energy storage.

Leptin mRNA levels and leptin release are stimulated by PKA activation (Senaris et al, 1997; Garcia et al 2000). The PKA pathway plays a central role in biological signaling of various hormones in the placenta, such as epinephrine, prostanoids (strauss et al, 1992; Yura et al, 1998). PKA activation, obtained by increasing intracellular Camp levels leads to increased leptin secretion (Yura et al, 1998). The effects appear to be mediated by an increase in leptin-gene transcription, leading to an increase in leptin mRNA levels. However, PKA suppresses leptin secretion and leptin mRNA levels (Slicker et al, 1996).

The leptin gene has a placenta specific enhancer (Bi et al, 1997; Ebihara et al, 1997), and a placenta- specific nuclear binding protein that is involved in leptin expression (Ebihara et al, 1997). This may suggest that the regulation of leptin production in placenta trophoblasts is likely to be different from that in adipocytes. The role of leptin in pregnancy may involve local modulation of hormone release, regulation of placenta cell growth and differentiation, and energy homeostasis in both the mother and fetus (Chardonnens et al, 1999). Thus, placenta is a clear source of leptin in the human maternal circulation, and placenta pathology may be associated with variation in leptin levels (Masuzaki et al, 1997).

However, leptin increases fatty acid partitioning toward oxidation from esterification (Muoio et al, 1999) and inhibits basal and insulin- stimulated glycogen synthesis in skeletal muscle (Lin et al, 1997). A potential role for the high levels of leptin during pregnancy could be related to terminate insulin- dependent energy storage (as glycogen or lipid) after the attainment of adequate reserves thereby directing nutrients in excess of the maternal requirement towards the fetus (Sugden and Holness, 1998; Holness et al 1999).

Conclusively, the study has shown that high levels of leptin reduced feed intake, and energy balance due to activation of the insulin gene, leading to mRNA expression, thus suggesting that leptin may be involved in the control of appetite and maturation of the GnRH/LH secretory axis, which may be associated with development of the neuroendoencrine axis (i.e neuroendocrine signal transduction). The study has also shown that leptin may serve as effectors that link mechanism that regulate reproduction and energy balance, thus playing an important role in reproduction and energy balance; modulating maternal nutrient partitioning inorder to optimize the provision of nutrients for fetal growth.the study therefore may suggest that reduced leptin intake during pregnancy may improve reproductive processes as well as optimizing the provision of nutrients(i.e.nutrients transfer through the placenta) for fetal growth.

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