REGULATION OF NEUROPEPTIDE Y, VASOACTIVE INTESTINAL PEPTIDE AND GALANIN BY INSULIN IN RATS

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ABSTRACT

Objective: To study the regulation of neuropeptide Y, vasoactive intestinal peptide and galanin by insulin in rats.

Study Design: Experimental study.

Place and Duration of Study: The study was conducted at the department of Metabolic Medicine Hammersmith hospital, Royal Postgraduate Medical School, London for on year (1996-1997).

Materials and Methods: Tissue levels of neuro peptide Y (NPY) and vaso intestinal peptide (VIP) decreased of the intestine and pancreas in insulin infused rats. NPY was also decreased significantly in hypothalamus. No significant effect on NPY in brain stem and on VIP in hypothalamus was observed.

Results: Galanin decreased significantly in intestine and hypothalamus. Galanin mRNA decreased to 57.7±8.8 percent of controls ($p < 0.05$) in intestine and 58.7±17.7 percent of controls in hypothalamus. Therefore insulin decreased the tissue levels of galanin by inhibiting its mRNA.

Conclusion: It is concluded that NPY, VIP and galanin are negatively regulated by insulin. Actions of insulin on endocrine pancreas and central control of glucose homeostasis and food intake by insulin may be partly modulated through changes in these peptides. VIP acts synergistically with glucagon in the development of hyperosmolar coma in NIDDM patients. NPY and galanin increase ketone bodies formation and hyperphagia in diabetics and contribute in the development of diabetic ketoacidosis and obesity.

Keywords: Insulin, neuropeptide-Y, galanin, vasoactive intestinal peptide, regulation, mRNA, diabetes mellitus.

INTRODUCTION

It is generally believed that hypothalamic insulin plays a role in the control of food intake and body weight by altering NPY gene expression in hypothalamus [1]. Hypothalamic insulin, in addition to its own anorexigenic effect, inhibits the release and expression of the hunger-promoting peptide NPY [2]. Likewise, Galanin, a 29/30 amino acid peptide neurotransmitter that is widely distributed throughout the central nervous system and periphery [3]. The Galanin peptide indicates an essential role for galanin signaling in "mind and body homeostasis" and a potential therapeutic efficacy in diabetes [4]. All this experimental evidence allows the idea of central insulin being a satiety substance [1] and it may act through changes in level of peptides related with food intake. Presence of the peptides like NPY, VIP and Galanin in endocrine pancreas was interesting in view of the fact that they have profound effects on islet secretions [5]. These peptides also participate in central control of glucose homeostasis and food intake. Little is known about intra-islet interactions between insulin and these peptides.

Development of diabetic ketoacidosis depends upon glucagon/ insulin ratio rather then insulin deficiency alone. Any factor which increases this ratio will tend to develop ketoacidosis. It is known that NPY and galanin inhibit insulin and stimulate glucagon secretion [6-8]. VIP regulates blood glucose level centrally at suprachiasmatic nucleus and peripherally by increasing glycogenolysis, gluconeogensis and decreasing entry of glucose into the cell [9]. Therefore any factor causing increased contents of VIP in brain and in peripheral tissue may result hyperglycaemia and it will then act

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synergistically with glucagon in NIDDM patients in the development of non ketotic hyperosmolar coma. Regulation of these peptides by changes in insulin status is required to be studied. Therefore present study was aimed to investigate the effects of sustained (12 hours) exposure to high circulating insulin levels on hypothalamic, brain stem, intestinal and pancreatic galanin, VIP and NPY contents and hypothalamic and intestinal galanin mRNA levels. The effects of hyperinsulinemia on NPY in hypothalamus has been published in physiological and pathological conditions. In these studies effect of glucose level was not delineated from insulin. In the present study hyperinsulinemia was produced while clamping glucose at the same levels in controls and in rats infused with insulin.

MATERIALS AND METHODS

Experimental protocol

To study the effect of peripheral insulin infusion, hyperinsulinemic euglycaemic clamp study was performed [10] keeping glucose at same levels in controls and in rats infused with insulin for 12 hours. Insulin infusion rate was 15.0 mU/kg/minute. Rats were killed under anaesthesia by exanguation by cardiac puncture. Intestine, pancreas, brain stem and hypothalamus were collected

Peptide extraction

A total of 2200 Islets tissues were collected from the rats and weighed immediately on harvesting and weight was recorded. Tissues were placed in polypropylene tube immediately to minimise evaporation. Approximately 10 X tissue weight volume of 0.5 M acetic acid was added to the tubes. The tubes were then placed with loosely fitted cap in vigorously boiling water bath (Keeping topping up level of water bath). The tubes were allowed to stand in boiling water bath for 15 minutes for water in the tube to reach 100oC. Then these were left at 100oC for 10 minutes and were allowed to cool for 10 minutes. Tubes were labelled and tightly packed and stored at –20^{0}C. For calculation of results weight of tissue and volume of 0.5 M acetic acid added were recorded [11].

Radioimmunoassay (RIA)

Samples from tissue extracts were assayed in duplicate for NPY [12], VIP [13] and Galanin [14]. Briefly all assays were performed in a total volume of 0.8 ml of phosphate buffer (pH 7.4), containing 10 mmol/l EDTA and 0.3% (w/v) BSA. The sensitivity of assay increases with incubation time reaching a maximum after about 5 days. Incubation was carried at 4oC to prevent bacterial growth, minimise proteolytic degradation and evaporation. After incubation antibody bound labelled was separated from free labelled by adding 250 ul of a suspension containing 6 mg charcoal (Norit GS, Hopkins and Williams) coated with clinical grade dextran (1:10 g charcoal, average mol wt 70,000, Sigma). The tubes were centrifuged at 1600 g for 20 minutes at 4oC, followed by immediate separation of the supernatant. The assay could detect changes between adjacent tubes of 2 mol at the 95% confidence limit.

RNA extraction and northern blot analysis

RNA was extracted using the acid guanidinium thiocyanate – phenol – chloroform extraction method [15] from each group of tissues. Following denaturation, 50 ug of total RNA, representing each experimental condition, was size separated by electrophoresis on a MOPS formaldehyde – denaturing – agarose (1.0% w/v) gel [16] and transferred to a hybond-N membrane (Amersham International, Amersham, Bucks, UK). Following transfer, RNA was fixed by baking at 80 0C for 1 hour and then UV crosslinked for 30 seconds before being probed with radioactively labelled DNA probes.

Probe labelling and hybridisation

DNA probes specific for rat galanin mRNA (specific activity 1-2 X 109 dpm/ug) were prepared by random hexanucleotide labelling [16] of a gel purified cDNA fragment. Prehybridisation were carried out at 42oC for 2 hours in a solution containing

50% formamide, 5 X standard saline citrate (SSC), (I X SSC = 0.15 mol NaCl plus 0.015 mol sodium citrate/1 pH 7.0), 50 mmol sodium phosphate/1 (pH 6.8), 1 X Denhardt's solution (1 X Denhardt's = 0.01% w/v ficoll, 0.01% w/v bovine serum albumin, 0.01% polyvinylpyrrolidine), 100 ug denatured, sonicated salmon sperm DNA/ml, 10% (w/v) dextran sulphate and then hybridised with 2 ng 32p- labelled DNA probe/ml in the same solution for 16 hours at 42oC. Following hybridisation, filters were washed twice at room temperature in a solution of 2 X SSC/0.1 (w/v) SDS for 30 minutes.

After washing, all filters were sealed into plastic bags and exposed to Kodak XAR-5 films for 5 days at –70^{0}C with intensifying screens. For reprobing of filters with oligo dT probe, bound probe was removed by heating at 80oC for 20 minutes in 10 mmol Tris/1 pH 7.5, 1 mmol EDTA plus 0.5% SDS. The efficiency of probe removal was checked by exposing stripped filters to phosphorimager screen for 2 hours and then read on phosphorimager. Normalisation was carried out by rehybridisation with oligo (dT) (12-16 nucleotides, labelled with 32P-dATP using terminal deoxynucleotide transferase) to compensate for any errors in loading or transfer of RNA. The sizes of hybridizing bands were determined through autoradiography and comparison to the positions of 18S and 28S ribosomal RNA in side tracks from the gels which were removed after electrophoresis, stained with ethidium and photographed under UV illumination next to a ruler.

Quantitation of mRNA

Quantitation of signal intensities was carried out using the Molecular Dynamics phosphorimager SF and relevant software (Image Quant). Following hybridisation and washing, filters were exposed to phosphorimager screen overnight and then counted using the phosphorimager scanner to provide estimates of the relative amounts of hormone specific signal which had hybridised to each band. Following removal of probe by stripping, filters were rehybridised with 32-P labelled oligo dT and again counted on the phosphorimager to determine the relative amounts of total RNA which has been loaded onto each lane. The results from the galanin were normalised by dividing the value by value of oligo dT RNA of respective lane after which all results were converted into percentages relative to their respective control groups.

Statistical Analysis

All radioimmunoassay results are given as mean ± SEM. Individual differences between control and treatment groups were subsequently determined by independent samples t-test. The mRNA values were determined in phosphorimager arbitrary units and were then expressed as percentage of control.

RESULTS

Tissue levels of NPY decreased in insulin infused rats in intestine (161.8 + 9.4 in insulin infused vs 204.1 + 6.4 pmol/g in control, $p < 0.01$), pancreas (19.6 ± 1.5 in insulin infused vs 63.7 ± 12.7 pmol/g in controls; $p < 0.05$), and hypothalamus (196.5 + 6.2 in insulin infused vs 249.2 + 18.5 pmol/g in controls, $p < 0.05$). No change in NPY contents in brain stem was observed (Fig. 1).

VIP levels in insulin infused rats were decreased in intestine (267.7 + 19.0 in insulin infused vs 471.9 + 81.3 pmol/g in controls; $p < 0.05$), pancreas (4.5 + 1.4 in insulin infused vs 15.5 + 3.3 pmol/g in controls; $p < 0.05$) and brain stem (12.6 + 1.4 in insulin infused vs 19.0 + 3.5 pmol/g in controls). No change in VIP content in hypothalamus was observed as compared to controls (Fig. 2).

Galanin levels in insulin infused rats decreased in intestine (81.1 + 3.0 in insulin infused vs 117.4 + 12.4 pmol/g in controls; $p < 0.05$), brain stem (5.4 + 1.3 in insulin infused vs 6.5 + 0.9 pmol/g in controls) and hypothalamus (76.5 + 6.0 in insulin infused vs 103.0 + 7.0 pmol/g in controls; $p < 0.05$) (Fig. 3). Assays of Galanin in pancreas were not done due to exhaustion of tissue extract. Tissue contents of galanin were significantly decreased in intestine and hypothalamus by

insulin as compared to controls. To know whether insulin caused suppression of synthesis of galanin, northern blots of the RNA from hypothalamus and intestine was done and probed for galanin mRNA. A decrease in galanin mRNA in intestine (57.4 + 8.8 percent of controls p < 0.05 and 58.7 + 17.7

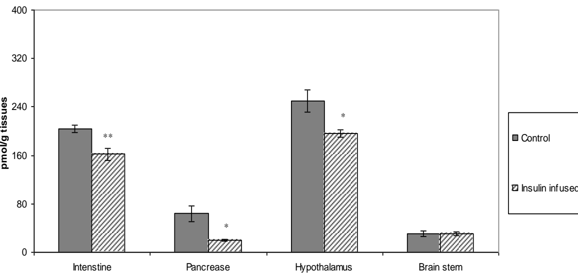

Fig.1: NPY levels in tissues of control (saline infused) and insulin insfused rats. *p<0.05 & p<0.01

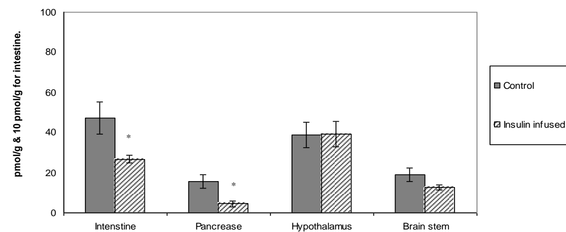

Fig.2: VIP levels in tissues of control (saline infused) and insulin infused rats. *p<0.05

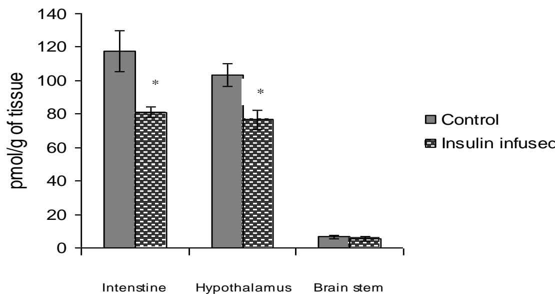

Fig.3: Galanin levels in tissues of control (saline infused) and insulin infused rats *p<0.05

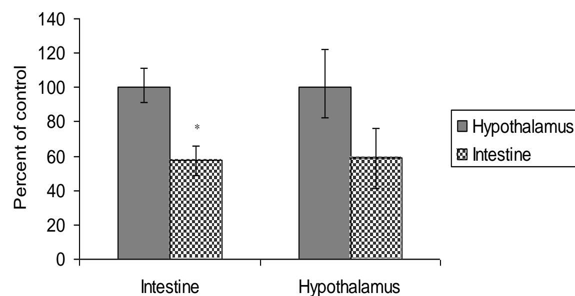

Fig.4: Galanin mRNA in intestine and hypothalamus in insulin infused rats. *p<0.05

percent of controls in hypothalamus) was observed. Therefore insulin decreased the tissue contents of galanin by inhibiting its mRNA (Fig. 4-6).

Northern blot analysis of galanin mRNA in hypothalmus in controls (saline infused) (A-F) and in rats infused with insulin (G-L). RNA was extracted as described in the materials and methods. 50 µg of total RNA was analysed on the nylon membrane from the formaldehyde/agarose gel after quantification and visualisation and building up the concentration of 5 mg/ml. After running formaldehyde agarose gel electrophorsis, marker was stained with ethidum bromide and photographed under UV illumination to mark the position of 18s and 28s ribosomal RNA. After northern blotting the RNA was fixed by placing membrane at 80oC in oven for 1 hour and then UV cross linked for 30 seconds. Galanin cDNA probe was labelled by random primum labelling as described in materials and methods. After hybridisation with the probe the membrane was exposed to molecular dynamics storage phosphorimager screen over night and the screen was then scanned for signal intensities by phosphorimager of Molecular Dynamics Model 455 A (USA) scanner. The membranes were then exposed to Kodak XAR-5 film at –70oC for 3 days. The developed films were photographed to depict the mRNA observed in each lane. Position of 18s and 28s ribosomal RNA are indicated and origin is at the top of the plate.

Northern blot analysis of intestinal galanin mRNA in controls (saline infused) (A-F) and in rats infused with insulin (G-L). (Fig. 5)

DISCUSSION

NPY is synthesized in islets of Langerhans [5] and it inhibits glucose stimulated insulin secretion in isolated rat islets [7]. In brain it regulates food intake and levels are increased in fasting [17]. VIP is a brain gut peptide and perform diverse functions. It plays a key role in glucose homeostasis by acting centrally and enhancing secretion of insulin and glucagon [18]. Galanin inhibits insulin and somatostatin

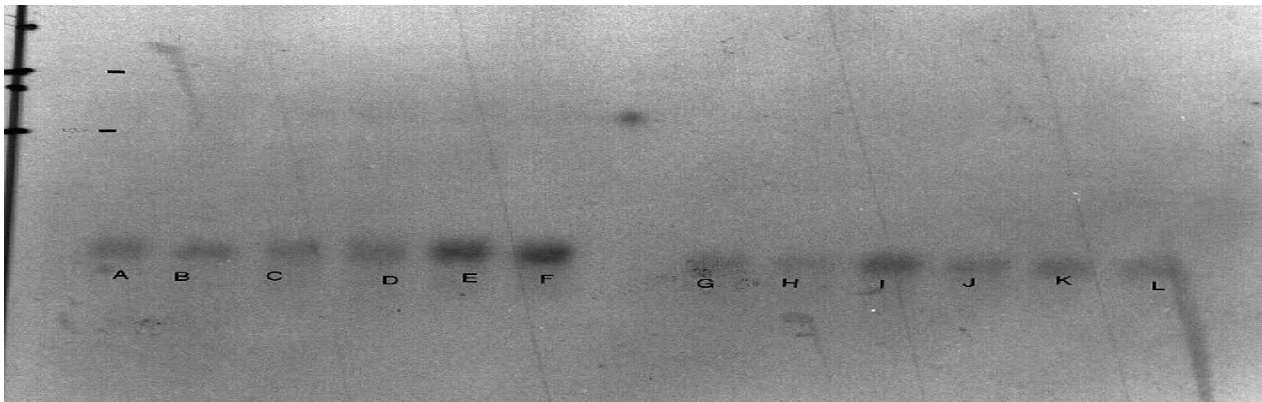

Fig. 5: Northern blot analysis of galanin mRNA in hypothalamus in controls (saline infused) (A-F) and 12 hours insulin infused rats (G-L).

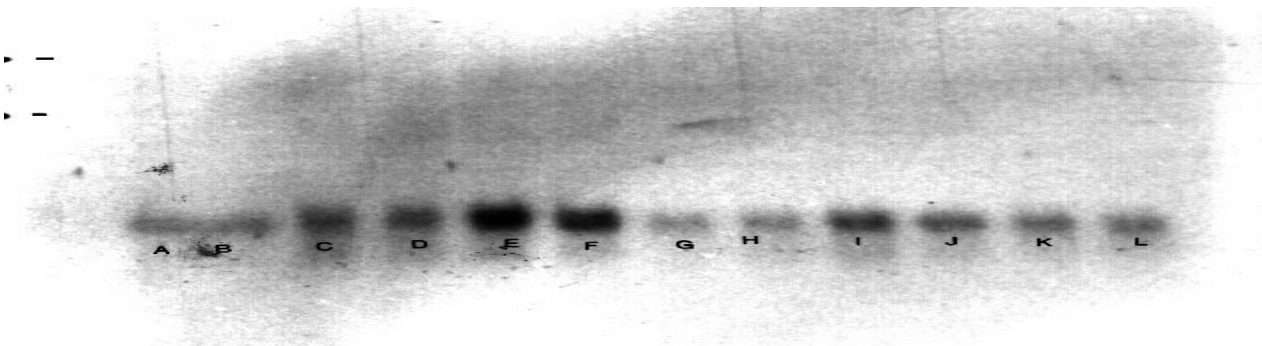

Fig. 6: Northern blot analysis of intestinal galanin mRNA in controls (saline infused) (A-F) and 12 hours insulin infused rats (G-L).

from pancreas [6] and also act centrally to increase fat intake [6]. To test the hypothesis that insulin might be a regulatory factor of these peptides, we have investigated the effects of sustained (12 hours) exposure to high circulating insulin levels on hypothalamic, brain stem, pancreatic and intestinal contents of NPY, VIP and galanin while clamping the glucose levels at about the same levels in controls and insulin infused rats.

In the present study decreased tissue contents of NPY in intestine, pancreas and hypothalamus have been observed by insulin. Therefore, it is suggested that insulin negatively regulates the NPY levels in these tissues and insulin effect on inhibiting food intake may be mediated by decreasing NPY in hypothalamus. These results are similar to previous reports because when insulin was injected into the third ventricle (i.c.v.), it prevented the rise in NPY mRNA in the arcuate nucleus (ARC) [2, 19], suggesting that insulin may directly inhibit NPY gene expression. Such inhibition could explain the increased hypothalamic NPY mRNA and NPY peptide levels in fasting, diabetes, intense exercise and lactation, as all these condition are characterized by hypoinsulinaemia [17, 20]. Furthermore, insulin treatment of diabetics [20] or food-deprived rats [21] tends to reverse the rises in hypothalamic NPY. In contrast to present study increased hypothalamic NPY and NPY mRNA levels in obese hyperinsulinaemic rats had been observed and it is postulated to result from insulin resistance [22]: theoretically, insulin resistance at hypothalamic level could prevent insulin from inhibiting the NPY ergic neurons. There is some evidence for this, in that i.c.v. insulin fails to reduce NPY mRNA levels in the ARC of obese rats [19]. Present study addresses physiological gap in the etiology of diabetic hyperphagia and links the increased food intake of diabetes with increased production

of hypothalmic neuropeptide Y, which results from a deficiency of insulin in the central neurons system. We also proposed role of NPY in the development of ketoacidosis in diabetes mellitus. In diabetic patients there is absolute or relative deficiency of insulin which causes increased NPY contents of pancreas. Increased NPY further inhibit insulin which in turn tend to increase glucagon/insulin ratio which results over production of ketone bodies.

Our results are in contrast to Cusin et al [23] who reported no change in NPY content in various hypothalamic nucleic and observed increased levels in paraventricular nucleus (PVN) by insulin infusion. We also contradict to the suggestion of cusin et al [23] that increased NPY - PVN content could have arisen from brain stem. We observed no change in NPY content in brain stem. It means that insulin affect NPY differently in different tissues. It may be due to the different roles of locally produced NPY in different tissues. We disagree with the argues of Cusin et al [23] that physiological insulin levels could maximally inhibited ARC neurons, with consequence that hyperinsulinemia produced by them in their experimental model failed to have additional inhibitory effect. The hyperinsulinemia produced by Cusin et al [23] was 3-5 times of controls but in present model insulin levels were 100-150 times of control. Therefore it is suggested that very high systemic insulin administration are required to inhibit NPY.

In the pancreas the contents of NPY were significantly decreased by insulin infusion. Regulation of NPY by insulin in pancreas have not be studied previously. Jamal et al [5] reported increased NPY in islet isolated from dexamethasone treated rats and proposed that increased NPY was due to dexamethasone induced hyperinsulinemia. Our results are contradictory to Jamal et al [5] because we observed that by keeping control and insulin infused rats isoglycaemic insulin decreased significantly NPY in pancreas. It is suggested that in dexamethasone treated rats insulin resistance due to defect in receptors may be responsible for increased NPY contents in case of Jamal et al [5] experimental model. A similar down regulation of the GLP-1 receptors number in a cell line after treatment of cells with dexamethasone has been observed [24]. Our views were strengthened by findings of Wang et al [25] who observed that NPY secretion was 6 fold higher during perfusion with 20 mmol glucose as compared to 2.8 mmol glucose in isolated islets from dexamethasone treated rats while from normal rats who were not treated with dexamethasone perfusion with 20 mmol glucose decreased NPY secretion by 62% as compared to 2.8 mmol glucose. Basal insulin secretion was increased 4-fold when glucose was raised to 20 mmol/l. From these results we proposed that in normal rats high glucose stimulated insulin secretion and insulin inhibited NPY. While in dexamethasone high glucose increased insulin secretion but due to insulin resistance NPY was not suppressed. Therefore in this way NPY may play role in development of secondary diabetes in cushing syndrome because increased NPY due to increased glucocorticoids suppress insulin secretion and enhance glucagon. Negative regulation of NPY by insulin in intestine was observed. The significance of modulation of NPY in gut by insulin may be responsible in the regulation of gut functions and gut hormone by insulin.

VIP is a brain gut hormone and insulin decreased VIP contents significantly in intestine. Therefore it is suggested that insulin modulate gut functions by changing in VIP levels in intestine. In pancreas insulin also decreased significantly the VIP levels. VIP is stimulant of insulin and glucagon secretion. Therefore it is suggested that VIP may play part in the mediation of insulin action on glucagon and other pancreatic hormone. Inhibition of VIP secretion have been reported previously by somatostatin analogues [26] and we here report that insulin also decreased tissue contents of VIP. Our observation are contradictory to the observation made by Noda et al [27] that in sciatic nerve decreased VIP contents were observed in diabetics and VIP became normal in insulin treated

diabetics. This contradiction might be due to the different roles of locally produced VIP in pancreas and sciatic nerve. It has been observed previously that dexamethasone treatment caused rise in VIP in islets (5) while dexamethasone significantly reduced rat pituitary VIP content and mRNA [28]. Vagal activation by means of insulin hypoglycaemia caused only minor rise of VIP levels [29]. It may be the hypoglycaemia caused by insulin which resulted rise in VIP in sciatic nerve while insulin in present study inhibited tissue contents of VIP when glucose was clamped at the same levels in treated and control rats. Little is known about the interaction of VIP in brain stem and hypothalamus. We observed that insulin was negative modulator of VIP in brain stem and had no effect in hypothalamus. The effect of insulin on VIP can also help in understanding of pathophysiology of hyperosmolar non ketotic coma in NIDDM patients. VIP regulate blood glucose levels centrally at the suprachiasmatic level and peripherally by increasing glycogenolysis, gluconeogenesis, and inhibition of glucose entry in the cells (9). It stimulate insulin and may keep glucagon/insulin ratio in the range at which ketone bodies formation are not stimulated. We proposed that increased VIP contents occur in diabetics and this in turn further increase the hyperglycaemia acting synergistically with glucagon which ultimately leads to hyperosmolar coma in untreated diabetics.

Insulin decreased significantly galanin contents in intestine and hypothalamus. The factors regulating galanin have not been studied. A negative correlation between pancreatic galanin and plasma insulin in lean mice has been observed [30]. Results of the present study are consistent to [30] because negative regulation of intestinal, brain stem and hypothalamic galanin by insulin had been observed and it was at gene transcription level. The inverse association between endogenous galanin and insulin detected in the present study also agrees with other reports showing galanin gene expression specifically in PVN neurons, to be enhanced in diabetic rats and suppressed by insulin administration [31, 32]. It is suggested that galanin may be involved in the development of diabetic ketoacidosis by two ways. Firstly, it inhibit insulin and increase glucagon/insulin ratio which in turn tend to increase ketone bodies formation. Secondly, increased galanin in diabetic acts centrally and results increased fat intake which increase fatty acids pool of liver and leads to increased ketone bodies formation.

CONCLUSION

We conclude that NPY, VIP and galanin are negatively regulated by insulin. Actions of insulin on endocrine pancreas and central control of glucose homeostasis and food intake by insulin may be modulated by these peptides. VIP act synergistically with glucagon in the development of hyperosmolar coma in NIDDM patients. NPY and galanin increase ketone bodies formation and hyperphagia of IDDM and contribute in the development of diabetic ketoacidosis and obesity in IDDM.

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