

## Biochemical Alterations Induced by Amylin in Wistar Rats

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### ABSTRACT

Amylin is a peptide hormone synthesized in  $\beta$  cells of pancreas together with insulin in response to glucose and nutrients. Amylin controls food intake and body weight but the exact mechanism is still unknown. This study aimed to examine the effects of amylin injection on hormones, metabolites and cytokines expression in rats. Wistar rats were injected amylin Intraperitoneally (IP) ( $10 \mu\text{g kg}^{-1}$  twice daily) for 7 days. Plasma and liver samples were collected for blood measurements and RT-PCR analysis. Amylin treatments induced significant decrease in body weight and food intake in time dependent manner. Moreover, amylin significantly increased insulin, leptin and lipase secretion. A decrease in plasma triglycerides, cholesterol and LPL and increase in HDL levels was recorded. There are upregulation in the expression of IL-1 $\beta$  and TNF- $\alpha$  in amylin treated rats. The results collectively indicate that amylin has anti-obesity like effects through regulation of proteins and obesity related gene expression.

**Keywords:** Amylin, Effects, Obesity, Wistar Rats

### 1. INTRODUCTION

Amylin is a pancreatic 37-amino acid peptide co-secreted together with insulin from islet beta cells. Amylin protein and mRNA are present in the lung, stomach, duodenum, jejunum, ileum, colon and rectum and throughout the CNS (Cooper, 1994). Mature bioactive amylin undergoes post-translational modifications (intra-molecular disulfide bond and COOH-terminal amidation) which is essential for its bioactivity (Christopoulos *et al.*, 2003). Circulating amylin exists in both non-glycosylated and glycosylated forms in normal and diabetic human subjects (Christopoulos *et al.*, 2003). Amylin secretion is stimulated by glucagon, glucagone like peptide-1 and cholinergic agonists and its secretion inhibited by somatostatin and insulin (Hay *et al.*, 2004). Amylin-binding sites have been detected in pancreatic-cells,

skeletal muscle, kidney, lung and brain (Muff *et al.*, 2001). Functional amylin receptors are generated by co-expression of the G-protein-coupled calcitonin receptor gene and Receptor Activity-Modifying Proteins (RAMPs) (Christopoulos *et al.*, 2003). The potential for the combination of calcitonin receptor isoforms and different RAMP proteins gives rise to at least six different subtypes of amylin receptors that display unique pharmacological properties (Hay *et al.*, 2004). RAMP-1 and -3 mRNAs are co-localized with calcitonin receptor gene mRNA in mouse pancreatic-cells (Martinez *et al.*, 2000). Amylin modulates the secretion and/or peripheral sensitivity of insulin, thereby regulates glucose homeostasis (Butler *et al.*, 1990).

Amylin controls various peripheral metabolic functions by controlling blood glucose levels and rate of stomach emptying (Rushing *et al.*, 2000a; 2000b) and reduction in food intake (Butler *et al.*, 1990; Cooper,

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1994). The effect of amylin on food intake affect mainly the short-term control of meal size as amylin injection transiently reduces food intake (Lutz *et al.*, 1994; 1998). Moreover, mouse with targeted deletion of amylin gene has higher weight gain than wild type (Gebre-Medhin *et al.*, 1998) and treatment with amylin antagonists increase food intake and body weight (Rushing *et al.*, 2001). In rodents, amylin crosses the blood-brain barrier and binds to brain receptors located in brain areas involved in the regulation of energy homeostasis such as the area postrema/nucleus of the solitary tract region and the hypothalamus (Lutz *et al.*, 2001). Central bolus infusion of amylin 100 pM into the third ventricle significantly decreased 24 h food intake by over 30% in rats (Rushing *et al.*, 2000a; 2000b). Amylin circulates in the plasma at concentrations with a mean of  $3.4 \pm 0.7$  pM in lean subjects and  $4.7 \pm 0.9$  pM in obese subjects with normal glucose tolerance (Hartert *et al.*, 1991). Furthermore, chronic peripheral or intracerebroventricular infusion of amylin into rats results in reduction in food intake and weight loss (Rushing *et al.*, 2000a; 2000b; 2001). The acute glucoregulatory actions of exogenous amylin include inhibition of gastric emptying and glucagon secretion, with sustained amylin administration induced reduction in food intake and weight loss (Rushing *et al.*, 2000a). Amylin-/-mice are healthy and exhibit enhanced glucose clearance and increased sensitivity to the diabetogenic effects of alloxan (Wang *et al.*, 1999; Mulder *et al.*, 2000). The physiological importance of the satiety effect of amylin is reflected by enhanced weight gain of amylin-/-mice, which also exhibit reduced responsiveness to the anorectic actions of exogenous cholecystokinin and bombesin (Mollet *et al.*, 2003). The concurrent infusion of amylin and leptin synergistically was shown to reduce food intake and body weight and specifically lowered fat mass, in Diet-Induced Obesity (DIO)-prone rats (Roth *et al.*, 2008; Trevaskis *et al.*, 2008). Moreover, amylin plus leptin mediated weight loss was reported to be not only through reduced food intake but also by enhancing systemic lipid metabolism manifested by significantly lowering plasma triglycerides and total cholesterol levels (Trevaskis *et al.*, 2010). Liver like adipose tissue and many other tissues secrete numerous proteins that implicated in regulation of several pathways in body during health or disease named cytokines. The adipokines are implicated in glucose tolerance and insulin sensitivity (adiponectin and resistin), antiobesity function (leptin) and some cytokines such as IL-1, TNF- $\alpha$  and IL-6 that are involved in lipid metabolism and in inflammation and

stress response such as haptoglobin (Trayhurn and Beattie, 2001). Most of already established data focused on the effect of amylin on food intake and body weight but no clear studies elucidated the effect of amylin on insulin, leptin, lipid profiles and adiposity related cytokines expression and that was the target of this study.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Rat amylin was obtained from sigma Aldrich, The Wistar albino rats were bought from Egyptian Co for experimental animals import, Helwan, Egypt. Vehicles and related materials were from ADWIA pharmaceutical company, Egypt, Heparinized vacutainer tubes, TriZol reagents, Poly dT, chloroform, ethanol and cytokines primers were from Wako pure chemicals, Osaka, Japan.

### 2.2. Animals and Experimental Procedures

All experimental procedures were approved by the dean of Scientific Research for Faculty of Applied Medical Sciences, Taif University, Saudi Arabia. Thirty male Wistar rats, 7 weeks age (220-240 g), were divided into two groups of fifteen rats each, were housed at room temperature ( $24 \pm 1^\circ\text{C}$ ) with a 12-h light and 12-h dark cycle and get open access to food intake. Rats were handled daily for 10 days to recover the stress and injection effect. First group (saline injected rats) were injected twice daily by saline and the second (amylin treated rats) were Intraperitoneally (IP) injected amylin twice daily in a dose of  $10 \mu\text{g kg}^{-1}$  at morning and evening for 7 days. Six hours after the last injection, rats decapitated and blood and organs collected for various measurements. Food intake and body weight were measured as indicated in figures. Plasma was extracted and kept at  $-20^\circ\text{C}$  till assays and liver samples were kept in TriZol reagent till RNA extraction and RT PCR analysis.

### 2.3. Biochemical Measurements

Plasma glucose concentrations were measured using an automatic analyzer (COBAS MIRA, F. Hoffmann-La Roche, Basel, Switzerland). Plasma insulin was measured using commercial human kits from Peninsula, San Carlos, CA, USA. Plasma leptin was assayed using rat leptin ELISA kit (LINCO Research Inc., St. Charles, MO, USA). Plasma kit for pancreatic lipase was from Mabaret El Asafraa, Alexandria, Egypt. Lipid profiles were measured spectrophotometrically based on kits supplied by Wako Pure chemicals, Osaka, Japan.

**Table 1.** PCR cycle of respective genes are shown, while temperature and time of denaturation and elongation steps of each PCR cycle are 94°C, 30 s and 72°C, 60 s, respectively

mRNA	Forward	Reverse	Annealing
$\beta$ -actin (628 bp)	5'- ATGTACGTAGC CATCCAGGC- 3'	5'-TCCACACAGAG TACTTGCGC -3'	Annealing at 56.5°C for 1 min sec for 25 cycles
TNF- $\alpha$ (360 bp)	5'- GTAGCCACGT CGTAGCAA-3'	5'- CCCTTCTCCA GCTGGAAGAC-3'	Annealing at 56°C for 1 min
IL-1 $\beta$ (218 bp)	5'-ATGGCAACCGT ACCTGAACCCA-3'	5'-GCTCGAAAAT GTCCAGGAA-3'	Annealing at 60°C for 30 sec

## 2.4. RT-PCR Analysis and Gene Expression

Livers samples were collected from all rats and flash frozen in liquid nitrogen and subsequently stored at -70°C. Frozen liver samples (approximately 100 mg of tissue per sample) were immediately added to 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA) and homogenized using a Polytron 300D homogenizer (Brinkman Instruments, Westbury, NY). One milliliter of the tissue homogenate was transferred to a microfuge tube and total RNA was extracted via chloroform extraction followed by nucleic acid precipitation with isopropanol. The pellet was washed with 75% ethanol and resuspended in molecular biology grade water. Nucleic acid concentration was determined by o.d. 260 nm (Smart-Spec; Bio-Rad Laboratories, Hercules, CA) and RNA integrity was evaluated using an Agilent bioanalyzer (model 2100; Agilent Technologies, Foster City, CA).

RNA (1 $\mu$ g) was treated at 72°C for 5 min and reverse transcribed using 100 units of Moloney murine leukemia virus reverse transcriptase (Gibco), 50 pmol of poly (dT) primer and 20 nmol of dNTPs in a total volume of 10  $\mu$ L at 37°C for 1 h. After heating at 94°C for 5 min, PCR amplification was performed with 2.5 units Taq polymerase (Perkin-Elmer, Foster City, CA, USA), 3 mM MgCl<sub>2</sub> and 50 pmol of forward and reverse primers specific for respective genes in a total volume of 50  $\mu$ L. The PCR conditions for different tested genes are shown in **Table 1**. After electrophoresis in 1.5% agarose gel, the PCR products were stained with ethidium bromide and visualized under UV lamp. Intensities of PCR bands were analyzed densitometrically using NIH Image program (<http://rsb.info.nih.gov/nih-image/>).

## 2.5. Statistical Analysis

Results are expressed as means  $\pm$  S.E. of independent experiments. Statistical analysis was done using ANOVA and Fischer's post hoc test, with  $p < 0.05$  being considered as statistically significant.

## 3. RESULTS AND DISCUSSION

### 3.1. Effect of Amylin on Body Weight and Food Intake Changes in Wistar Rats

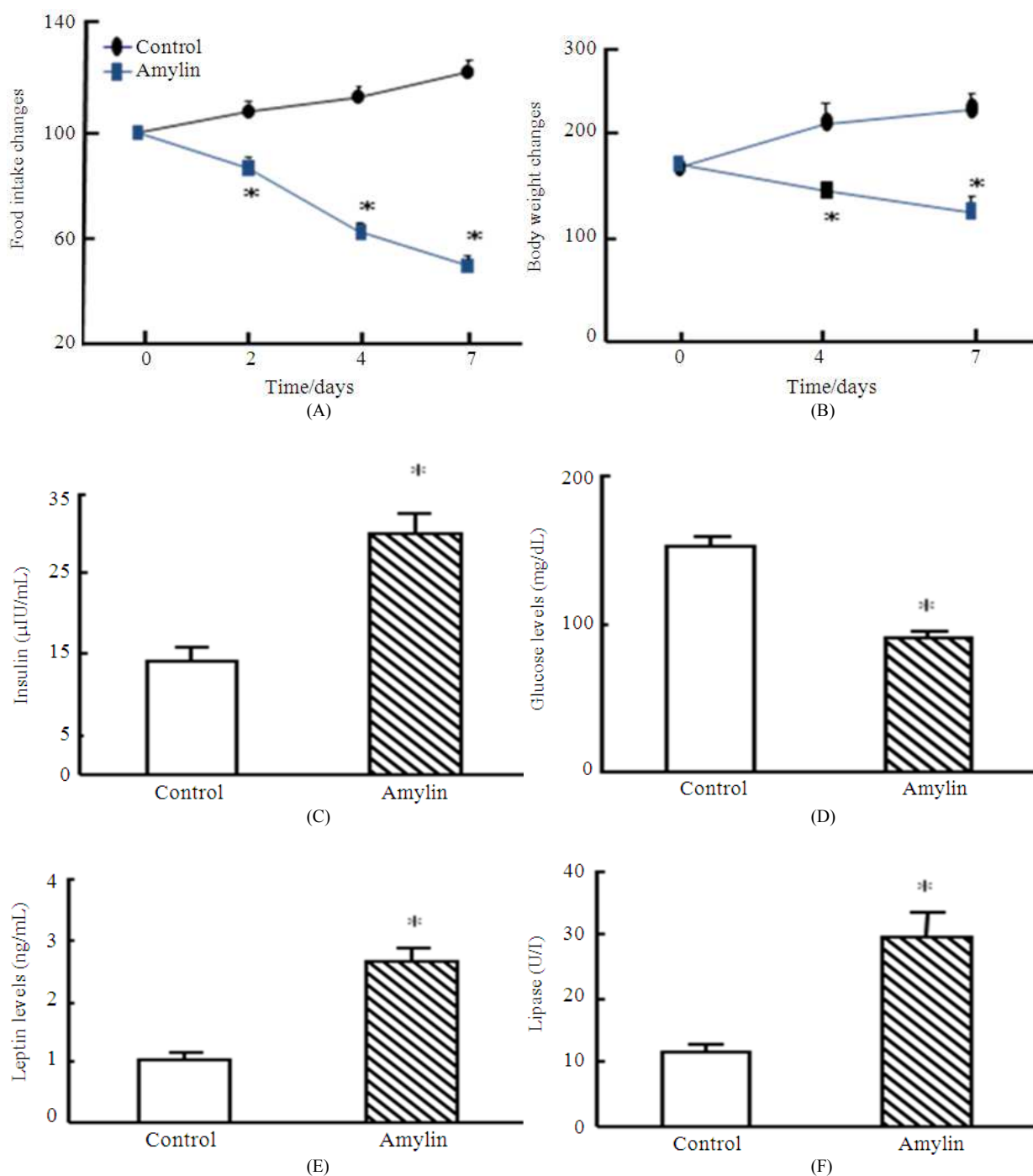
This study was carried out to identify the effect of amylin on food intake and body weight. As shown in **Fig. 1**, amylin injection induced significant ( $p < 0.05$ ) decrease in food intake (**Fig. 1A**), start from day 2 ( $67 \pm 10$  %) and continued until day 7 of experiment ( $34 \pm 10$  %). Consistent decrease in body weight (**Fig. 1B**) was recorded (from  $180 \pm 4$  g at 2 days from injection to  $140 \pm 6$  g at 7 at days of treatment).

### 3.2. Effect of Amylin on Plasma Levels of Insulin, Glucose, Leptin and Lipase in Wistar Rats

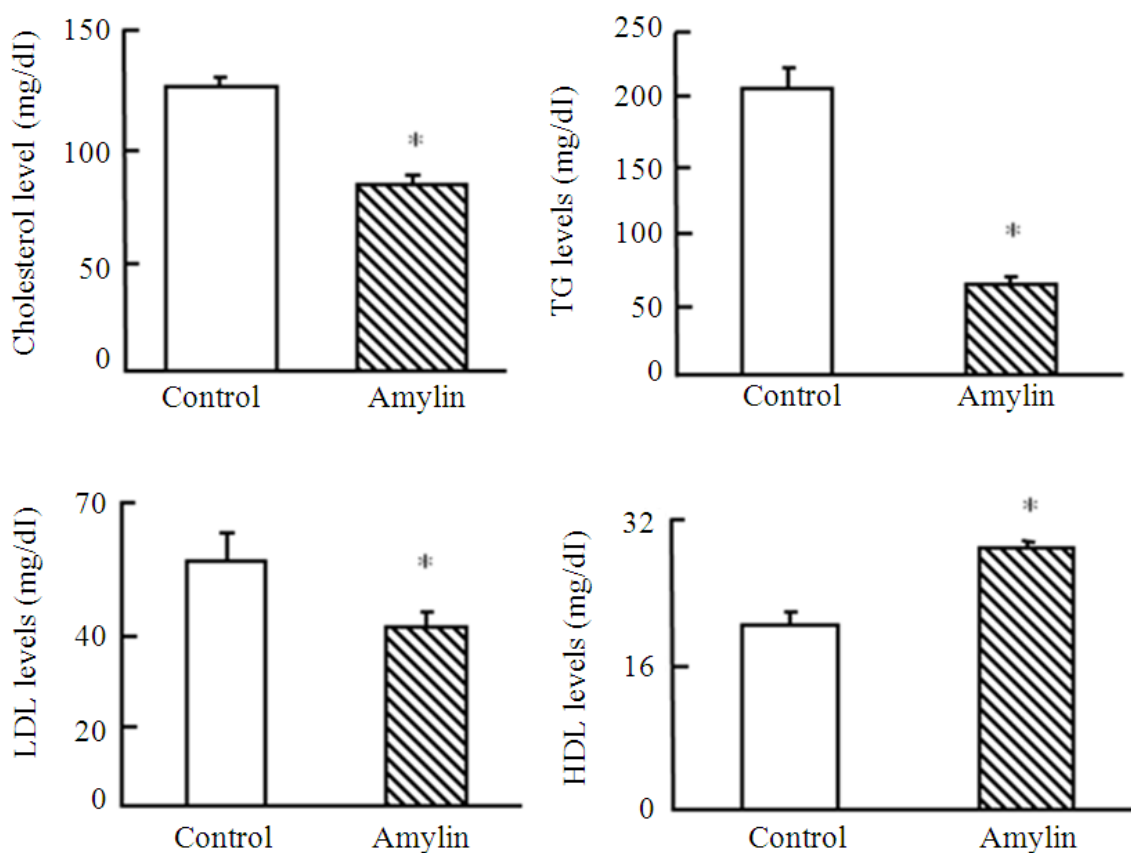
Amylin injection twice daily for 7 days induced significant ( $p < 0.05$ ) changes in various hormones and metabolites. The anti-obesity like effects of amylin was clear through its effect on insulin, leptin and lipase levels. As seen in **Fig. 1C**, amylin induced a significant increase in insulin ( $30.2 \pm 2.17$  versus  $14.25 \pm 1.75$   $\mu$ IU mL<sup>-1</sup>) in amylin injected rats compared to saline injected rats respectively. The increase in insulin level leads to a concomitant decrease in glucose levels ( $150 \pm 5.5$  versus  $89 \pm 3.56$  mg dl<sup>-1</sup>) in amylin injected rats compared to saline injected rats respectively (**Fig. 1D**). Moreover, there is an increase in leptin levels ( $2.6 \pm 0.2$  ng mL<sup>-1</sup> for amylin treated rats versus  $1.03 \pm 0.11$  ng mL<sup>-1</sup> in control respectively) as seen in **Fig. 1E**. The changes in lipase levels showed an increase in lipase levels ( $29.62 \pm 3.95$  U/L versus  $11.5 \pm 1.04$  in treated and control respectively). As, lipase acts as a lipolytic agent to increase fatty acids oxidation and lowers TG levels in blood as seen in amylin injected rats (**Fig. 1F**).

### 3.3. Effect of Amylin on Lipid Profiles in Wistar Rats

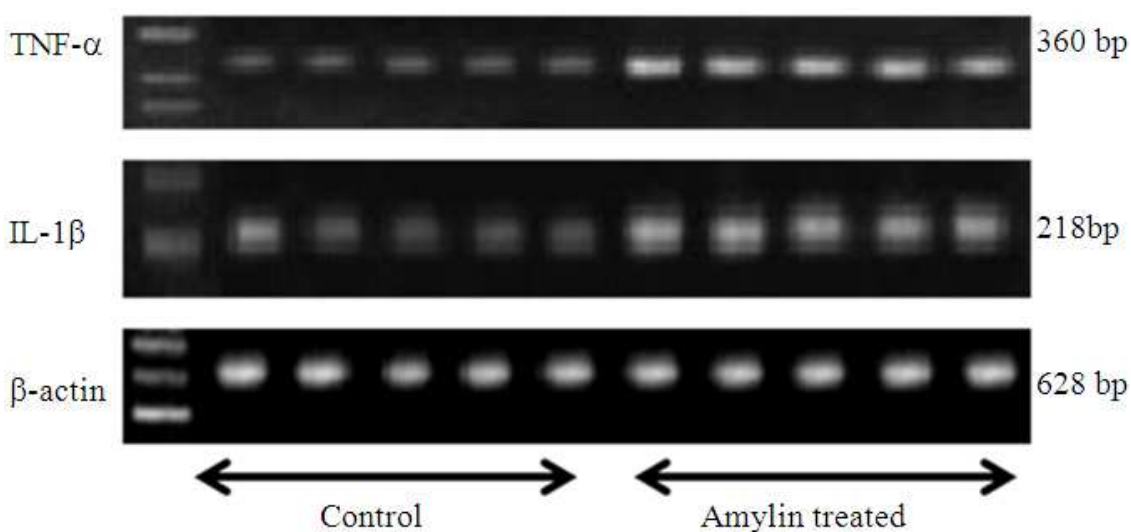
Next, we tested the effect of amylin on cholesterol, triglycerides, low and high density lipoproteins.

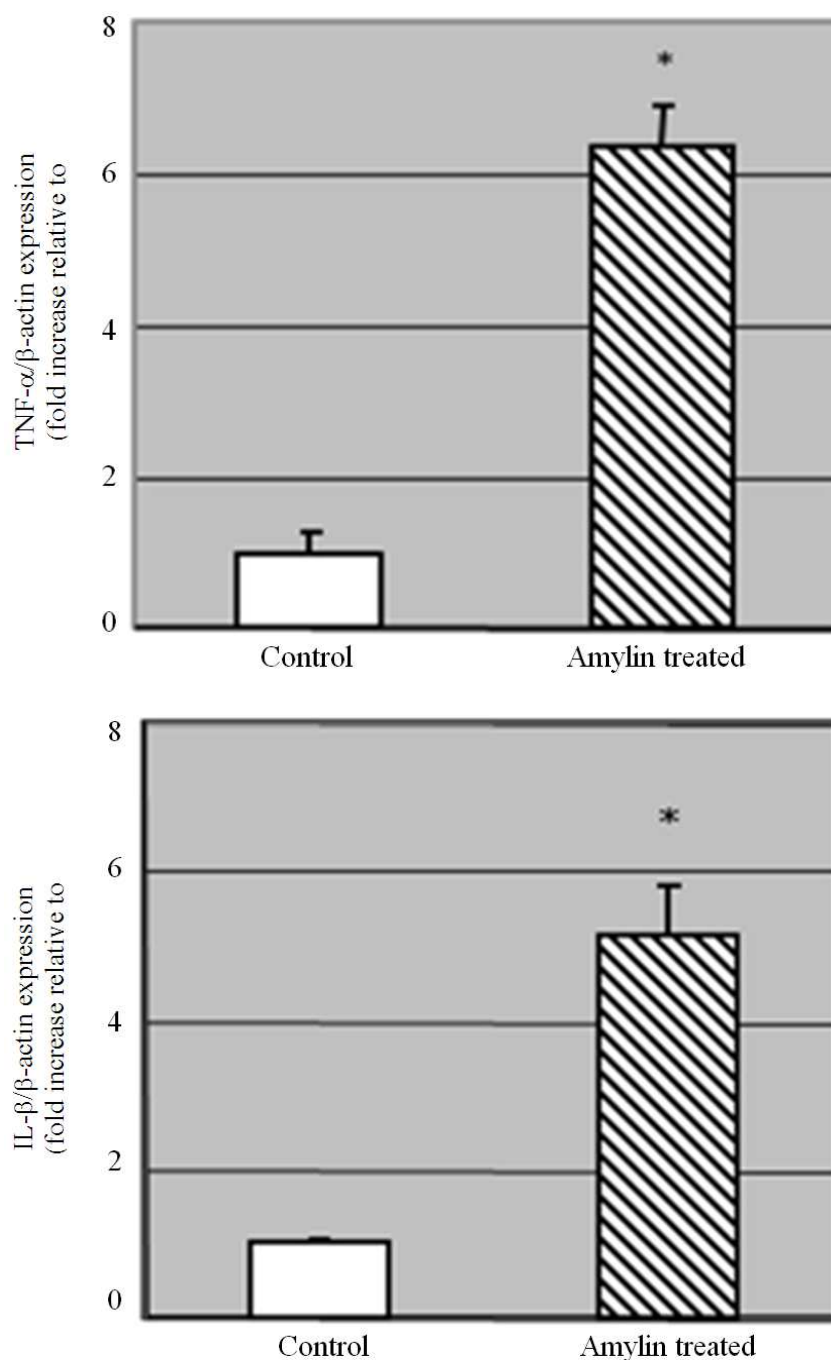


**Fig. 1.** Changes in food intake (A), body weight (B), insulin (C), glucose (D), leptin (E) and lipase (F) after amylin injection in Wistar rats. Rats were injected intraperitoneally (IP) in doses of  $10 \mu\text{g kg}^{-1}$  twice daily for 7 days. Blood was collected 6 h after last amylin dose injection and plasma was extracted for various biochemical measurements described in materials and methods. Values are means  $\pm$  S.E. of 10 different rats. \* $p < 0.05$  vs. control (saline injected rats)



**Fig. 2.** Changes in plasma cholesterol, triglycerides, LDL and HDL after amylin injection in Wistar rats. Rats were injected amylin (10  $\mu\text{g}/\text{kg}/\text{day}$ ) IP twice daily for 7 days. Blood was collected 6 h after last dose injected and plasma was assayed for cholesterol, triglycerides, LDL and HDL as described in materials and methods. Values are means  $\pm$  S.E. of 10 different rats. \* $p < 0.05$  vs. control (saline injected rats)





**Fig. 3.** RT-PCR analysis of TNF- $\alpha$ , IL1- $\beta$  and  $\beta$ -actin as house keeping gene in liver of wister rats. Rats were treated intraperitoneally (ip) with either saline or amylin ( $10 \mu\text{g kg}^{-1}$ ) twice daily for 7 days. RNA was extracted and reverse transcribed ( $1 \mu\text{g}$ ) and RT-PCR analysis was carried out as seen in upper bands for TNF- $\alpha$ , IL1- $\beta$  and  $\beta$ -actin. The densitometric analysis of expressed bands (lower columns) was normalized with that of  $\beta$ -actin relative to control. Values are means  $\pm$ SEM obtained from 5 different rats. \* $p < 0.05$  vs. compared to control. All samples were normalized on the basis of  $\beta$ -actin expression

**Figure 2** shows that amylin induced significant ( $p < 0.05$ ) decrease in cholesterol ( $124 \pm 5.6$  versus  $82.5 \pm 3.2$  mg  $\text{dl}^{-1}$ ), triglycerides ( $207.5 \pm 15.5$  versus  $65 \pm 5.4$  mg  $\text{dl}^{-1}$ ) and LDL ( $40.75 \pm 5.06$  versus  $56 \pm 5.7$  mg  $\text{dl}^{-1}$ ) and an increase in HDL ( $28.75 \pm 0.75$  versus  $20.25 \pm 1.54$  mg  $\text{dl}^{-1}$ ) for amylin and control treated rats respectively.

### 3.4. Effect of Amylin Injection on liver IL-1 $\beta$ and TNF- $\alpha$ Expression in Wistar Rats

RNA was extracted from liver and reverse transcribed using RT-PCR analysis to examine effects of amylin on cytokines expression. As seen in **Fig. 3**, amylin up-regulated IL-1 $\beta$  and TNF- $\alpha$  expression in liver of Wistar rats. IL-1 $\beta$  and TNF- $\alpha$  expressions were increased significantly in amylin injected rats compared to control. When their expression normalized with  $\beta$ -actin, the expression was 5 fold increase for TNF- $\alpha$  and 4 folds for IL-1 $\beta$ .

## 4. CONCLUSION

Present findings showed that injection of amylin reduced food intake and body weight gain in a time dependent manner. Previous reports showed that amylin administration to rats or mice before meal resulted in reduction of food intake (Butler *et al.*, 1990). Moreover, treatment of rats with amylin antagonists increases food intake and body weight that mediated through central effect of amylin (Rushing *et al.*, 2000a; 2000b; Rushing, 2003). The amylin induced reductions in food intake and body weight may be through amylin-inhibition of gastric emptying and gastric acid secretion. It was reported that a central bolus infusion of amylin 100 pM into the third ventricle significantly decreased 24 h food intake by over 30% in rats (Rushing *et al.*, 2000a). Amylin induced weight loss in our study may be through its satiety effect because amylin was shown to cause anorexia through its effect on brain serotonin levels by increasing the transport of the precursor tryptophan into the brain (Chance *et al.*, 1992). Serotonin reduced the size and duration of meals as well as the rate of eating. Indeed, the satiety effect of amylin was proved by enhanced weight gain of amylin-/-mice, which exhibit reducing response to the anorectic actions of exogenous cholecystokinin and bombesin (Mollet *et al.*, 2003). The cause for hypoglycemia in this study is primary due to the inhibitory effect on food intake in addition to the inhibitory effect of amylin on glucagon (Wang *et al.*, 1999) and secondary to the increase in leptin and insulin levels. In our study, amylin injection increased insulin

levels in agreement with the facts showed that amylin infusion alone led to marked increases in plasma insulin (Ye *et al.*, 2001) and insulin resistance states (Kahn *et al.*, 1999). Amylin's best-known peripheral action is a potent inhibition of stomach emptying and that consistent with the hypothesis that amylin is involved in the regulation of energy balance (Young, 1997).

Importantly and analogously to insulin, amylin reduces food intake without making animals ill. Moreover, there is a sufficient evidence to include amylin with insulin and leptin as circulating adiposity signal to the brain (Roth *et al.*, 2008). Obese individuals are at increased risk for diabetes, hypertension, renal failure and other cardiovascular diseases (Hall, 2003; Havel, 2004; Trayhurn and Beattie, 2001). Clinical and animal studies have confirmed a strong relationship between obesity and hypertension as well as diabetes (Sironi *et al.*, 2004). Amylin treatment increased leptin secretion significantly relative to control. The amylin-induced leptin induction may be through insulin induction as consistent interaction between insulin and leptin was reported in vivo and vitro (Havel, 2000). Our findings showed that amylin induced significant decrease in cholesterol, triglycerides, LDL and an increase in HDL. These findings clearly confirm that amylin has leptin like actions or at least amylin induced its effect through stimulating the secretion of lipolysis related proteins as leptin and lipase. Other studies (Trevaskis *et al.*, 2008; 2010) reported that amylin plus leptin-mediated synergistic effect on weight loss through not only reduction in food intake but also by enhancing systemic lipid metabolism in accordance with our findings. Moreover, amylin reduced plasma triglycerides by 31%, cholesterol by 10% compared with vehicle controls through increase in lipase secretion that has a potential role in lipolysis (Roth *et al.*, 2008). It have been shown that amylin-induced secretion of IL-1 $\beta$  and IL-6 in human glioma cells and obesity is associated with increase in cytokine profiles and amylin levels correlate with the degree of body adiposity (Gitter *et al.*, 2000; Sironi *et al.*, 2004). The brain's role in feeding is often traced to the concept of energy homeostasis, that managed by two sets of signals. One set arises from tissue stores, especially adipose tissue and referred as long-term signaling (Halford and Blundell, 2000; Blundell, 2006). The second chemical signals include leptin, insulin and certain cytokines, possibly amylin, visfatin and adiponectin (Blundell, 2006). Among such cytokines are TNF and IL-1 $\beta$  that play a role in inflammatory mediators of type 2 diabetes and obesity (Masters *et al.*, 2010; Roth *et al.*, 2006). The increase in IL-1 $\beta$  and TNF reported in this study may be associated

with the increase in leptin and or insulin secretion. As, amylin increased the expression of IL-1  $\beta$  and IL-5 in macrophages and eosinophils respectively (Zhou *et al.*, 2010) and increased IL-1 and TNF in murine microglia cells (Yates *et al.*, 2000). IL-1 is involved in the pathogenesis of type 1 diabetes and is a risk factor for type 2 diabetes (Spranger *et al.*, 2003). So the increase in IL-1 may be secondary to the increase in insulin and leptin reported in this study, as insulin stimulates leptin secretion and expression (Sachot *et al.*, 2004). Moreover, several cytokines, such as IL-1  $\beta$ , IFN- $\gamma$ , TNF- $\alpha$ , leptin, resistin, adiponectin and visfatin, diversely regulate pancreatic  $\beta$ -cell function (Wang *et al.*, 2010) and that explains the increase in TNF expression in liver. Collectively the effects of amylin on cytokines secretion and expression need further *in vitro* studies to outline such regulation mechanism. In conclusion, this study showed that amylin has anti-obesity like actions through its modulation of leptin and insulin secretion, decrease in food intake, body weight and modulation of obesity related cytokines.

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