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Acute Phase Proteins, Immunoglobulins and Cytokines Responses to Amylin Injection in Wister Rats

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Abstract: Amylin is a pancreatic peptide of 37–amino acids, secreted together with insulin from islet beta cells. Like insulin, amylin is rapidly and efficiently transported through the blood–brain barrier into the brain to acts on hypothalamus to control food and weight. Little information is known about amylin role in inflammation and immunity. This study aimed to examine the effect of amylin injection on inflammation and immunity related proteins as well as IL-10 expression, that acts as regenerative cytokines. Wister rats were injected saline or amylin (10 μg/kg twice daily) for 7 days. Plasma and liver samples were collected for variable blood measurements and RT-PCR analysis. Amylin injection induced significant ($p< 0.05$) increase in habtoglobin, C-reactive protein (CRP) and nitrous oxide (NO) secretion. Amylin induced significant decrease ($p< 0.05$) in IGG and IGA levels. Also, it induced decrease in plasma levels of IL-6 and IL-8 secretion but increase IL-10 expression in liver. The results collectively indicated that amylin modulates inflammation and immunity related proteins and increased IL-10 expression in rats.

Key words: Amylin · Cytokines · Immunoglobulins · Wister rats

INTRODUCTION

Amylin is a secretory protein mainly produced by pancreatic beta cells. It increased in the circulation of patients with diseases related to acute and chronic inflammation, including acute pancreatitis, pancreas graft rejection, obesity and insulin resistance [1]. Amylin is 37–amino acid peptide and is derived from a larger 89–amino acid preproamylin precursor. Amylin immunoreactivity (amylin-binding sites) and mRNA expression are present in islet somatostatin-producing α-cells, in the lung, stomach, duodenum, jejunum, ileum, colon and rectum; and throughout the CNS [2,3]. Elevated circulating levels of amylin have been detected in patients with obesity and insulin resistance [4,5]. Amylin secretion is stimulated by glucagon, GLP-1 and cholinergic agonists and is inhibited by somatostatin and insulin [6].

Cytokines are low molecular weight proteins produced by many cell types [7]. They are pharmacologically active, exhibiting both beneficial and pathologic effects on the target cells. Imbalanced expression of cytokines has been implicated in the progression of many diseases [8]. Cytokines play a role in regulating β cells function, some are protective, others can be determental for instance, chronic exposure of islets to some cytokines such as IL-1β, TNF inhibit insulin secretion and induce apoptosis of β-cells. In addition to circulating cytokines, islets also produce a variety of cytokines in response to physiological and pathological stimuli and these locally produced cytokines play important role in regulation of pancreatic β-cells function [9]. It have been shown that amylin may modulates the secretion and/or peripheral sensitivity of insulin, thereby regulates glucose homeostasis [10] and has been

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considered to be a controller of various peripheral metabolic functions including the control of blood glucose and the rate of stomach emptying [11].

IL-6 is an interleukin that acts as both a pro-inflammatory and anti-inflammatory cytokine. It is secreted by T cells and macrophages to stimulate immune response to trauma, especially burns or other tissue damage leading to inflammation. It increased during various diseases and metabolic disorders [12]. IL-8 is a chemokine produced by macrophages and other cell types such as epithelial cells. It is also synthesized by endothelial cells, which store IL-8 in their storage vesicles. This chemokine is one of the major mediators of the inflammatory response. This chemokine is secreted by several cell types. It functions as a chemoattractant and is also a potent angiogenic factor [13]. The third cytokine tested in this study is IL-10, is produced primarily by monocytes and to a lesser extent by lymphocytes, has pleiotropic effects in immunoregulation and inflammation. It down-regulates the expression of Th1 cytokines and acts as anti-inflammatory cytokine. Knockout studies in mice suggested the function of this cytokine as an essential immunoregulator in the intestinal tract [14].

Liver like adipose tissue and many other tissues secrets numerous proteins that implicated in regulation of several pathways in body during health or disease named cytokines. Among these cytokines is IL-6, IL-8 and IL-10. 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 immunity, inflammation and cytokines expression in healthy Wister rats and that is the purpose of this study.

MATERIALS AND METHODS

Materials, Animals and Experimental Procedures: Heparinzed vacuteiner tubes, TriZol reagents, Poly dT, chloroform, ethanol and IL-10 primers were from Wako pure chemicals, Osaka, Japan. Amylin was from sigma Aldrich, Wister male albino rats were from Egyptian Co for experimental animals import, Helwan, Egypt. Vehicles and related materials were from ADWIA pharmaceutical company, Egypt.

Twenty male Wister rats, 7 weeks age (190 - 240 g), were divided into two groups of ten rats each, were housed at room temperature (24 ± 1 °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 at a dose of 10 μg /kg at morning and evening for 7 days. 6 hours after the last injections, rats decapitated and blood and organs were collected for various measurements. Food intake and body weight were measured as indicated in figures. Plasma was extracted and kept -20 °C till assays and liver was kept in TriZol reagent till RNA extraction and RT-PCR analysis.

Measurements, RT-PCR Analysis and Gene Expression: Plasma nitric oxide was measured using kits from Peninsula, San Carlos, CA, USA. Plasma kits for haptoglobin, immunoglobulins and C-reactive protein (CRP) were from Mabaret El Asafra, Alexandria, Egypt. IL-6 and IL-8 were measured using ELISA kits from Wako Pure chemicals, Osaka, Japan. Livers 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 300 D 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 μg) 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 μl 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 MgCl2 and 50 pmol of forward and reverse primers specific for respective genes in a total volume of 50 μl. 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/).

Table 1:

mRNA	Forward	Reverse	Treatme
Actin	5'- ATGTACGTAGCCATCCAGGC 3'	5'-TCCACACAGAGTACTTGCGC 3' (628 bp)	Annealing at 56.5 °C for 60
IL-10	5'-GGAGTGAAGACCAAAGG-3	5'- TCTCCCAGGGAATTCAAATG-3'	Annealing at 57 °C for 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.

Statistical Analysis: All data were expressed as means ± SE and analyzed by Dunnett's t-test for multiple comparisons with a single control group using specific program (StatView Version-5; SAS Institute, Japan) for Macintosh computer. Significance was reported as $p<0.05$.

RESULTS

Effect of Amylin on Acute Phase Proteins and No Secretion in Wister Rats: We studied the effect of amylin on acute phase reactant proteins. As seen in figure 1, amylin injection induced significant ($p < 0.05$) increase in plasma levels of haptoglobin, C-reactive protein and NO secretion. These increases show the effect of amylin on inflammation related proteins in body during disease and inflammation.

Effect of Amylin on Plasma Levels of IgG, IgA IL-6 and IL-8 Secretion in Wister Rats: Unlike the findings on acute reactant proteins, Amylin injection twice daily for 7 days induced significant ($p < 0.05$) decrease in imunoglobulins measured in this study (IGG and IGA). As shown in Figure 2. Moreover, figure 1 and 2 can indicate that amylin modulate inflammatory and immunity state of rats, As it increased the response of body to inflammation and decrease the levels of IL-6 that known as pro-inflammatory cytokine. At the same time, it decreases the secretion of IL-8 that known as chemo-attractant cytokine.

Effect of Amylin Injection on Liver IL-10 Expression in Wister Rats: As seen in fig. 3, amylin up-regulated IL-10 expression in liver of Wister rats. RNA was extracted from liver and subjected to RT-PCR analysis and visualized in

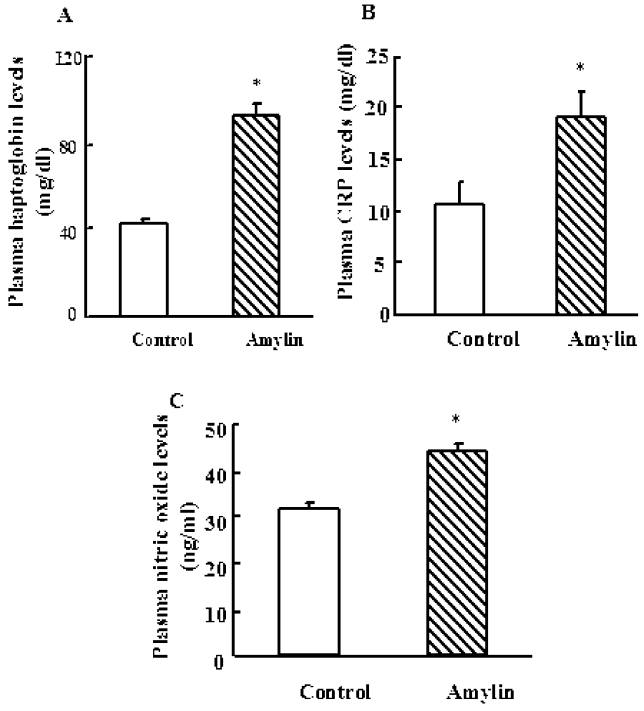

Fig. 1: Changes in plasma haptoglobin (A), C-reactive protein (CRP) (B) and nitric oxide (C) levels in amylin injected Wister rats. Rats were injected amylin (IP) twice daily for 7 days in doses of 10 μg/kg/day. Blood was collected after 6 hours from last injection and serum was assayed for several measurements as written in materials and methods Values are means ± S.E. of 5 different rats. *$p < 0.05$ vs. saline injected rats (control).

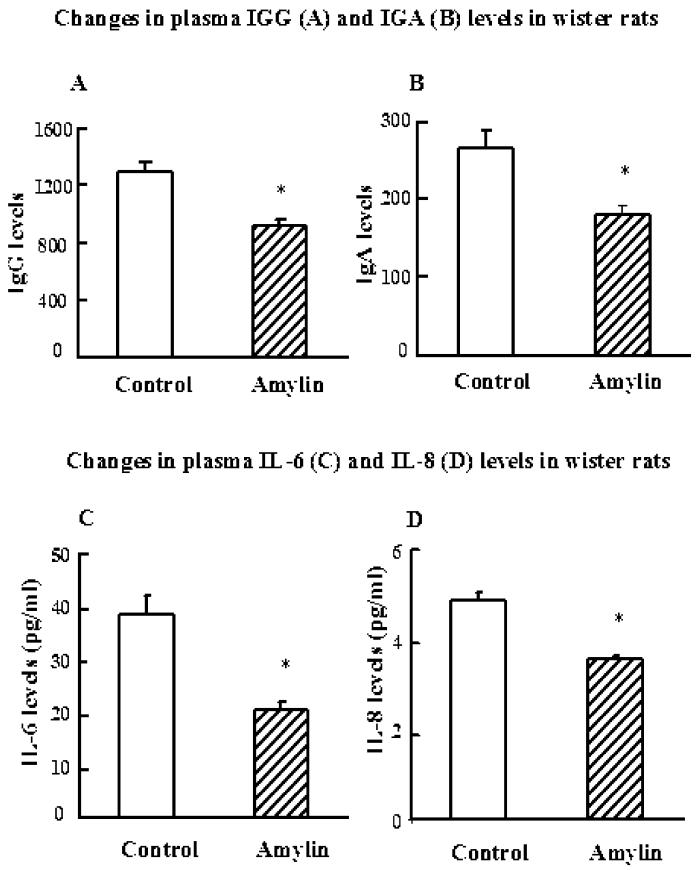

Fig. 2: Changes in plasma IGG (A), IGA (B), IL-6 (C) and IL-8 (D) levels in amylin injected Wister rats. Rats were injected amylin (IP) twice daily for 7 days in doses of 10 μg/kg/day. Blood was collected after 6 hours from last injection and serum was assayed for several measurements as written in materials and methods Values are means ± S.E. of 5 different rats. *p < 0.05 vs. saline injected rats (control).

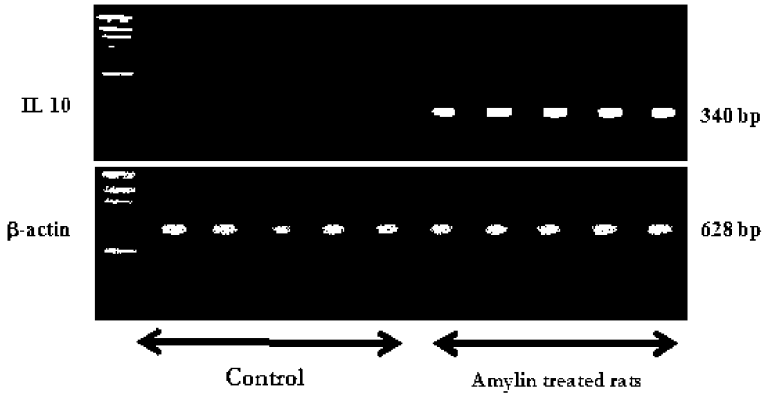

Fig. 3: RT-PCR analysis of IL 10 and β-actin as house keeping gene in liver of wister rats. Rats were treated intraperitonealy (ip) with either saline or amylin (10 μg/kg/day) twice daily for 7 days. RNA was extracted and reverse transcribed (1 μg) and RT-PCR analysis was carried out for IL- 10 and b-actin.

agarose gel stained with ethidium bromide. IL-10 was increased significantly ($p < 0.05$) in amylin injected rats compared with saline group. When their expression normalized with β-actin as internal standard, the expression was 2 fold increases in IL-10. These results collectively showed that amylin has anti-inflammatory actions through induction of IL-10 expression that has a pleiotropic effect in immunoregulation and inflammation.

DISCUSSION

In the present study, we showed that amylin injection increased the acute phase proteins secretions that are known to be high in the obesity and acute phase infection. Thus, we can speculate that amylin works as an initiator of inflammation by modulating such proteins and make body react to secret ant-inflammatory proteins and/ or cytokines to control such infection or inflammation. The increase in nitrous oxide secretion by amylin may be a way to control the apoptosis of β cells in pancreas and or a response to increase adiponectin that works as local modulator to control blood vessels and inflammation [15] as amylin posses vasodilator activity probably by increasing nitrous oxide production [16].

Immunoglobulis G (IgG) and IgA constitute 75% of serum immunoglobulins in humans. IgG molecules are synthesized and secreted by plasma B cells. IgG can binds to many kinds of pathogens, for example viruses, bacteria and fungi and protects the body against them by agglutination and immobilization, complement activation [17], opsonization for phagocytosis and neutralization of their toxins. Here, amylin decreased IgG and IgA secretion and the exact mechanism is not clear and further studies are needed to confirm this effect, but one possible explanation is the involvment of cytokines. IL-6 is an interleukin that acts as both a pro-inflammatory and anti-inflammatory cytokine. It is secreted by T cells and macrophages to stimulate immune response to trauma, especially burns or other tissue damage leading to inflammation. The decrease in antibody is parallel to the decrease in IL-6 and IL-8 because it has been reported that T cells and B cells besides antibody production they can secret various interleukins [12,18]. IL-10 is produced primarily by monocytes and to a lesser extent by lymphocytes. This cytokine has pleiotropic effects in immunoregulation and inflammation. It enhances B cell survival, proliferation and antibody production [14]. So the increase in IL-10 expression is counteracting mechanism to overcome the decrease IgG and IgA production and inflammation related immune responses and that explain our findings.

The amylin-modulated cytokines (IL-10, IL-6 and IL-8) expression and secretion in rat's liver is in agreement with the study of *Gitter et al., 2000* [19], who reported that amylin-induced cytokines secretion in human glioma cells. Various cytokines as TNF and IL-1β are important inflammatory mediators in type 2 diabetes and obesity [20]. Here, amylin injection induced an increase in IL-10 expression but decrease in IL-6 and 8 secretions. The decrease in IL-6 and IL-8 secretion is the proper way to control pancreatic β cell death, because the increase of those pathogenic cytokines together with IL-1 are the causes of beta-cell death and occurrence of type 2 diabetes [20]. Our previous results [21] and current findings confirm the possibility that amylin effects depend on organ and/or cell variations as those reported by in study of *Yates et al., 2000* [22].

So, amylin may acts as anti-inflammatory peptide by down regulation of IL-6 and IL-8 secretion and further studies are needed to test direct effect of those cytokines on amylin secretion from pancreatic β cells beacuse *Hom et al., 1995* [23] found that amylin aguments the inflammatory activity of eosinophils. It have been shown that amylin increase the expression of IL-1 β and IL-5 in macrophages and eosinophils respectively [23] and increase in IL-1 and TNF in murine microglia cells [22]. Abundant literatures describe the involvement of IL-1β in type 1 diabetes. So the decrease in IL-8 and IL-6 secretion may be secondary to increase in insulin [21] to regulate the pathogenicity of pancreatic β cells.

It has been shown that some cytokines, as IL-1β, IFN-γ, TNF-α, leptin, resistin, adiponectin and visfatin diversely regulate pancreatic β-cell function[24], where TNF-α upregulates amylin expression in murine pancreatic beta cells [1]. Also TNF-α is increased in obesity [25] and has been implicated as a causative factor in obesity-associated insulin resistance and the pathogenesity of type 2 diabetes [26]. These results indicated that amylin modulates cytokines to regulate pancreatic β cell functions. Collectively the effects of amylin on cytokines secretion and expression need further studies to confirm such modulations. In our previous study of Soliman *et al*., [21], amylin induced significant decrease in cholesterol, triglycerides and LDL and an increase in HDL and leptin. These findings clearly show 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 enzyme and suggests that it can be used as anti-obesity protein. Here, amylin modulated host defensive mechanism by lowering immunoglobulins and cytokines secretion but increased haptoglobin, No and CRP secretion. From these two studies we can conclude that amylin acts as anti-obesity factor but its usage in obesity must be used with precautions to over come the modulations in host defensive mechanism.

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