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Original article

Liver alpha-amylase gene expression as an early obesity biomarker

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ABSTRACT

Background: Obesity is a major health problem worldwide, for which preventive and therapeutic means are still needed. Alpha-amylase is a digestive enzyme whose inhibition has been targeted as a potential anti-obesity strategy. However, alpha-amylase gene expression has not been particularly attended to, and in contrast with pancreatic and salivary amylases, fewer studies have focused on liver alpha-amylase. The present study aimed at investigating the expression of alpha-amylase gene in obese and normal mice at RNA and protein level as well as acarbose effect on this gene expression in hepatocyte cell culture.
Methods: Control and case groups were fed by normal mouse pellet and high-fat diet respectively, during 8 weeks. After this period, serum biochemical parameters including glucose, cholesterol, triglycerides, AST, ALT and alpha-amylase were assayed. Liver alpha-amylase gene was analyzed by real time PCR, and liver enzyme was assayed with Bernfeld and ELISA methods Hepatocyte cell culture derived from both group were also treated by acarbose and alpha-amylase activity and gene expression was analyzed by above mentioned methods.
Results: All biochemical factors showed an increase in obese mice, but the increase in ALT and AST were not statistically significant. Alpha-amylase levels were also increased in obese mice, both at RNA and protein level, while a decrease was seen in obese mice derived hepatocytes after acarbose treatment.
Conclusions: Elevated liver alpha-amylase levels may be indicative of initial stages of obesity and the use of acarbose could be considered as a treatment of obesity which could be potentially effective at multiple levels.
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Introduction

According to statistics released by the World Health Organization (WHO) (http://www.who.int), more than 1.4 billion persons were overweight around the globe, from which 500 million were obese in 2008. By 2015, these figures are expected to reach 2.3 billion and 700 million for the overweight and obese people respectively. According to WHO report, obesity and excess weight are the fifth leading cause of death worldwide; obesity could thus be considered as a health crisis of the current century. This condition increases the risk of serious conditions including several forms of cancers, as well as cardiovascular complications and type 2 diabetes [1–3].

Controlling diet is helpful in managing excess weight, and in most diet plans, reducing carbohydrate consumption is targeted. Alternatively, lowering carbohydrate processing by enzymes that are responsible for their breakdown may lead to the same expected result, and this strategy has gained special attention in studies related to losing weight programs and prevention of obesity–related diseases [4]. One of the enzymes involved in complex carbohydrates digestion is alpha-amylase. Alpha-amylase is mainly found in mammals' saliva and exocrine pancreatic secretions, and hydrolyzes polysaccharides such as starch to oligosaccharides like maltose [5,6]. However, this enzyme is also present in small amounts in several organs such as liver,small and

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large intestine, ovary, testis and skeletal muscles [7]. Studies on liver amylase have been started in the 1960's [8–11]. The alpha-amylase gene encodes two different isoforms in mouse strain A/J, namely Amy-1a and Amy-2a. Amy-2a is active in pancreas; while Amy-1a gene is under control of two promoters with different strengths. Resulting mRNAs are identical in their sequences, except for different 5'untranslated regions. The activation of strong promoter causes gene expression in the salivary gland while activation of weak promoter leads to alpha-amylase gene expression inthe salivary gland and liver [12–14]. Liver alpha-amylase is reported to show high affinity to glycogen, which is the storage form of carbohydrates in this organ [15].

Recently, several reports have highlighted the potential of mammalian alpha-amylase inhibitors as preventive and therapeutic agent in diabetes and obesity [16–19]. These inhibitors include peptides (e.g. derived from wheat kernel and white beans) [20,21] and small molecules which bear carbohydrate units (e.g. acarbose and its derivatives) [22–24] or polyphenolic components (such as flavonoids or trans-chalcone) [25,26]. Acarbose has been extensively studied as an inhibitor of alpha-amylase since its inhibitory effect was reported on that enzyme [27], and its synergetic effect with other inhibitors has recently become a subject of interest [28,29].

In the present study, we aimed at comparing the expression of liver alpha-amylase gene in obese and normal control mice at RNA and protein level. This *in vivo* study was followed by assessing the gene expression in primary mouse hepatocyte culture of normal and obese mice. In this *in vitro* study, we evaluated the effect of different concentrations of acarbose on alpha-amylase expression.

Material and methods

Animals and treatment

White male NMRI mice (20–25 g) were obtained from Kharazmi University and housed at 28 ± 2 °C in humidity-controlled (30–70%) facilities, on a 12 h light/dark cycle with access to mouse pellet food. Mice were divided into two groups of six mice. Control group was fed only by normal mouse pellet diet for 8 weeks while the case group received a high-fat diet which included the following ingredients: 150 g of grind laboratory pellet food, 20 g of roasted sesame, 100 g of milk chocolate, 50 g of creamy biscuits, and 100 g peanut. These components were powdered by grinding and prepared as pellets. Food was stored at ~4 °C to prevent oxidation of the fat components.

Mice were weighed weekly for 8 weeks. At the end of this period, the mice were anaesthetized with chloroform and exsanguinated through cardiac puncture. 2 ml blood samples were taken from both groups for further biochemical analyses. The liver was removed and washed by cold PBS 1× in order to eliminate RBCs and debris, after that 500 mg liver tissue was homogenized in 1 ml lysis buffer (20 mM Tris-HCl, pH 7.5, 0.3 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mM benzamidine chloride). The resulting homogenate was centrifuged at 1500 rpm for 20 min. Supernatant (liver extract) was filtered through a 0.45 μm filter and stored at −80 °C. A portion of the liver tissue was transferred to 5 ml culture media containing antibiotic/antimycotic for preparing hepatocytes cell culture. The experimental protocol was performed in accordance with the international guidelines set out in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) and approved by the Research and Ethics Committee of the Institute (EC-00247).

Primary hepatocytes cell culture

After being washed several times in cold PBS, the liver tissue was chopped. The suspension collected by centrifugation (5 min, 1500 rpm, 4 °C) was incubated in trypsin-EDTA (37 °C, 5 min) and neutralized by DMEM medium supplemented by 10% Fetal Bovine Serum (FBS) (GIBCO-USA), 15 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (Gibco) and Glutamine (Gibco). After another centrifugation, cells were cultured and were counted and tested for viability. In order to test the effect of acarbose, the primary hepatocytes cell culture of obese and normal mice were incubated in the presence of 100, 1000 and 5000 μg/ml of the compound for 24 h. After incubation, cells were centrifuged (400*g*, 5 min) and supernatants were stored at −80 °C until further use. The resulting pellets were also stored at −80 °C for subsequent RNA extraction.

RNA isolation and relative quantification by real-time PCR

Total RNA was purified from hepatocytes using Trizol according to standard protocol (GIBCO-USA). 250 ng aliquot of total RNA from each sample was reverse transcribed to cDNA using random hexanucleotide primers and Revert AID First Strand cDNA Synthesis kit (Fermantas). The resulting cDNA from each sample was subjected to quantitative real-time PCR. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) housekeeping gene was used as internal control for normalization. The primer pairs sequences are as follow: 5′-GTG ATT AGC GAT GAA CCA G-3′, 5′- GCA AGT CTT TCA GTC CTG TCC-3′ for HPRT and 5′-AAGATGTCCTACTTAAAGAACTGG-3′,5′-ACCCGTGTGAAACCATAAGG-3′ for amylase.

Each quantitative real-time PCR reaction was performed in duplicate assays. A 20 μl reaction was set by mixing the following components: 30 ng cDNA, 10 μl RT2 Real-Time™ SYBR Green/ROX PCR Master, primer pairs and nuclease-free water to 20 μl. Thermal condition was used for 40 cycles consisting of initial polymerase activation step at 95 °C for 10 min, followed by cycles of denaturation at 95 °C for 5 s, and finally annealing and extension at 61 °C for 40s. Subsequently for melting curve analysis, the PCR was continued by further steps including: 95 °C for 15 s, 61 °C for 15 s, and 95 °C for 15 s on an ABI stepOne™ quantitative PCR system (Applied Biosystems, CA, USA).

Alpha-amylase activity/concentration assay

Bernfeld method

Amylase activity of mice liver extract (supernatant after being filtered) and supernatant of primary hepatocytes cell culture was determined according to Bernfeld method [19]. The assay system was composed of 250 μl of 3.2% soluble starch (pH 7.2 ± 0.01 in phosphate buffer) as substrate, 125 μl phosphate buffer PBS 1× (containing 100 mM K2HPO4 and 50 mM NaCl), and 125 μl of enzyme solution (supernatant). The reaction was terminated by addition of 500 μl DNS (dinitrosalicylic acid) reagent solution (including potassium sodium tartrate and NaOH). Samples were then incubated in a hot water bath for 5 min and consequently cooled down and diluted in 5 ml double distilled water. Absorbance measurements were performed at 540 nm against a blank prepared in similar manner without substrate and a control devoid of enzyme solution. One unit of alpha-amylase enzyme activity was determined as the amount of enzyme that catalyzes the formation of 1micromole of maltose from 3.2% soluble starch in 1 min. A standard curve of concentration versus absorbance was drawn to show the concentration of releasing maltose in alpha-amylase assays. Mean, standard deviation (SD) and coefficient of variation (CV) were calculated. CV below 5.5% was considered acceptable, otherwise the test had to be repeated.

ELISA assay

96-well microtiter plates (human salivary alpha amylase kit;cat No: MBS725125) were coated with specific antibody for human

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