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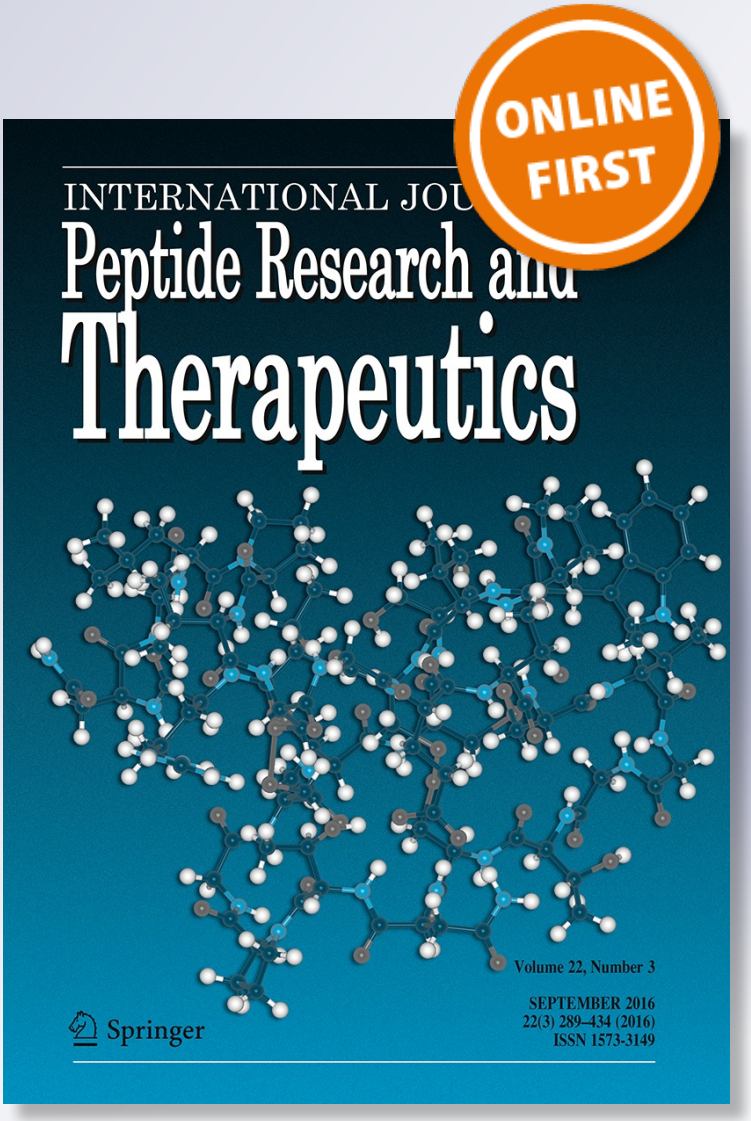

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Betaine Down Regulates Apelin Gene Expression in Cardiac and Adipose Tissues of Insulin Resistant Diabetic Rats Fed by High-Calorie Diet

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Abstract Apelin is a newly discovered peptide that its serum level increases in diabetic patients with cardiovascular dysfunction. Recent studies indicate the beneficial actions of betaine in reducing the cardiovascular and metabolic complications, however data related to its effect on adipocytokine expression is limited. The aim of this study was to evaluate the effect of betaine supplementation on Apelin gene expression in cardiac muscle and adipose tissue of insulin resistance, diabetic rats fed by a high calorie diet. To induce insulin resistance rats were fed with high fat/high carbohydrate diet for five weeks and then 30 mg/kg STZ was injected intraperitoneally. After confirming of diabetes incidence (serum glucose above 7.5 mmol/l) the animals were treated with 1 % betaine in drinking water for 28 days. At days 14 and 28 after treatment, animals were euthanized and Apelin gene expression was evaluated by real time PCR and western blot in heart and adipose tissues. Serum levels of insulin, Apelin and glucose and HOMA–IR were also measured. Our results showed that feeding of rats by a high calorie diets caused insulin resistance, which was manifested by elevated plasma insulin, glucose and Apelin levels and also HOMA–IR. Apelin gene expression in heart and adipose tissues were significantly increased simultaneously with the progression of diabetes. Betaine supplementation decreased serum Apelin and down regulated Apelin expression in adipose tissue and cardiac muscle, particularly at day 28 of treatment. We concluded that betaine might improve metabolic and cardiovascular complications in diabetic patients by regulation of Apelin expression and secretion.

Keywords High-calorie diet · Insulin resistance · Betaine · Apelin gene expression · Heart · Adipose tissue

Introduction

Obesity is the most important metabolic disorder in humans that occurs for various reasons including being fed with high fat/high carbohydrate (HF/HC) diet and reduced physical activity (Nakamura et al. 2014). Obese people are at high risk of developing type 2 diabetes (T2D) and cardiovascular diseases such as heart attack and atherosclerosis (Ouchi et al. 2011; Abel 2008). Lipotoxicity is defined as the main characteristic of obesity and T2D. Increased fat storage in the form of triglycerides at levels higher than physiological status in tissues such as fat, liver, skeletal muscle and heart causes the widespread metabolic disorders (Schaffer 2003; Romacho et al. 2014). Adipose tissue is a highly active metabolic and endocrine organ and has a substantial role in the pathogenesis of obesity-related cardiovascular complications (Voorde et al. 2013; Abel 2008). Altered levels of adipocyte-derived factors, termed adipokines, may be particularly related with heart diseases and metabolic disorders (Voorde et al. 2013).

Apelin is a newly identified adipokine which derives from a 77 amino acids precursor. It has several active forms including Apelin-12, Apelin-13, Apelin-17, Apelin-19 and Apelin-36. Of these, the 36-amino acid isoform is the most widely expressed, although the shorter isoform,

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Apelin-13 is more potent and more abundant in the circulation (Lee et al. 2000; Boucher et al. 2005). Increasing evidence suggests that Apelin is involved in the regulation of multiple physiological functions, including food intake, blood pressure and glucose utilization (Lee et al. 2000; Boucher et al. 2005; Dray et al. 2008; Reaux et al. 2001). Apelin is upregulated by insulin (Boucher et al. 2005) and inhibits pancreatic insulin secretion (Sorhede Winzell, et al. 2005, Guo et al. 2009). In clinical and experimental studies, serum Apelin level or its adipose tissue expression are increased in states of obesity and insulin resistance (Li et al. 2006; Soriguer et al. 2009).

Various studies have confirmed the role of Apelin in cardiovascular function. A high level of Apelin expression has been reported in cardiac muscle of rat and human (Szokodi et al. 2002; Kleinz et al. 2005). Apelin exerts strong inotropic action in isolated cardiac muscle cells and increases coronary blood flow by vascular dilation (Tatemoto et al. 2001). Age-related progressive cardiac dysfunction in Apelin-deficient mice is prevented by Apelin infusion (Kuba et al. 2007). Apelin expression increases in the arteries of patients with atherosclerosis and chronic heart failure (Japp et al. 2010).

Nutrition therapy by lipotropic agents is an important prevention strategy can potentially attenuate the inflammatory state and correct the metabolic and cardiovascular derangements in obese patients (Essop et al. 2004; Mann et al. 2015). While there are many lipotropic agents that might be potentially useful in obesity (Craig 2004), data about their effects on adipokine expression in adipose tissue and cardiovascular system is limited.

Betaine (trimethylglycine) is a naturally lipotropic peptide that its plasma level is inversely associated with components of the metabolic syndrome such as blood glucose, insulin resistance and cardiovascular complications (Kathirvel et al. 2010). Betaine consumption increases insulin sensitivity, decreases insulin resistance and blood glucose levels in diabetic rats with nonalcoholic fatty liver fed with diets rich in fat. Betaine supplementation increases circulating adiponectin levels and improves plasma insulin and glucose levels demonstrating that it had the potential to improve adipose tissue function and insulin sensitivity (Wang et al. 2010). It has antioxidant effects by stimulation of antioxidant enzymes activity in various tissues (Alirezaei et al. 2012a, b).

A series of researches showed betaine supplementation beneficially influences the cardiac function, although this data is limited. Combination of betaine and guanidinoacetate improves the symptoms of subjects with chronic illness, including heart disease (Kidd et al. 1997; Craig 2004). In subjects with cardiac decompensation (arteriosclerosis or rheumatic disease) and congestive heart failure betaine improves their cardiac function (Kidd et al. 1997; Craig 2004). Although the overall results confirm the beneficial effect of betaine on adipose tissue and cardiovascular health, effect of betaine at molecular level on adipocytokine function remain to be determined. The aim of the present study was to evaluate the effect of betaine on Apelin gene expression in heart and adipose tissue of insulin-resistant rats fed by a high energy diet.

Materials and Methods

Animals

Forty male Wistar rats (220 ± 10 g) were selected from the center of laboratory animal of the faculty of Veterinary Medicine of Shahid Chamran University, Ahvaz, Iran. They were housed in a temperature-controlled room (23 ± 1 °C) with a 12 h light/dark cycle and were provided rat chow (Pars, Tehran, Iran) and water at libitum. All animals used were cared for according to the guide for the care and use of laboratory animals by the national academy of sciences (National Institutes of Health publication No. 86-23). Initially, all rats were housed in conventional conditions and fed standard diet and water ad libitum at the animal facility for 1 week before experiments began.

Experimental Design

The rats were randomly divided into four equal groups. High fat/High carbohydrate (HF/HC) group that was fed with a high calorie diet which contain 20 % sucrose (w/w) and 10 % lard (w/w) for 5 weeks and then received a single dose of STZ (30 mg/kg, i.p) (Sigma, Germany) that was prepared in citrate buffer 0.1 M, pH 4 (Sigma, Germany). HB %1 group that received HF/HC diet and STZ similar to HF/HC group, but was treated with betaine 1 % (Sigma, Germany) in drinking water for 28 days. BT group that received standard rodent chow and betaine 1 % in drinking water for 28 days (Wang et al. 2010). Control (Con) group that received standard rodent chow during the experimental period (n = 10). Citrate buffer was administrated in BT and Con group instead of STZ.

Five days after STZ treatment, glucose was measured by hand-held glucometer (Medisign, China) in HF/HC and HB1% groups to confirm the incidence of diabetes. Elevation of serum glucose above 7.5 mmol/l and increased HOMA–IR in relation to control group were considered as indices of diabetes induction. All animals had access to diet and water ad libitum. Absolute body weight of each rat from each group was measured at the end of the HF/HC feeding and betaine treatment period.

Sampling

Animals were euthanized after 8 h fasting with a combination of ketamine and xylazine (100 mg/kg of ketamine and 10 mg/kg of xylazine), at days 14 and 28 after treatment. Blood samples were collected, and sera were separated and stored at −20 °C for use. Hearts and visceral adipose tissue were separated and kept at –80 °C until use.

Plasma Biochemical Assays

Serum glucose (Pishtazteb, Iran), Insulin and Apelin (EastBiopharm, China) concentrations were measured using commercially available kits.

HOMA–IR Estimation

The homeostasis model assessment of basal insulin resistance (HOMA–IR) was used to calculate an index from the product of the fasting concentrations of plasma glucose (mmol/l) and plasma insulin (μU/ml) divided by 22.5 (23). Lower HOMA–IR values indicated greater insulin sensitivity, whereas higher HOMA–IR values indicated lower insulin sensitivity (insulin resistance) (Buettner et al. 2006).

RNA Isolation and cDNA Synthesis

Total RNA was isolated from heart and adipose tissue using TriPure isolation reagent according to the manufacturer's procedure (Roche, Canada) using of 100 mg of tissue. Concentration of extracted RNA was calculated at a wavelength of 260 nm using nanodrop spectrophotometry (Eppendorf, Germany). To detect the purity of RNA its optical density (OD) absorption ratio at 260/280 nm was determined and samples having a ratio more than 1.8 were used for cDNA synthesis. Possible DNA contamination was removed by treatment of RNA (1 μg) with DNase I (2 U/μl) for 1 h at 37 °C (Vivantis, Malaysia). Reverse transcription was carried out with the RocketScript RT PreMix kit using 1 μg of RNA and oligo dT based on manufacturer's protocol (Bioneer Corporation, South Korea).

Real-Time Quantitative RT-PCR

To evaluate changes in the expression level of Apelin, real-time PCR was performed using qPCR^{TM} Green Master Kit for SYBR Green I$^{®}$ (Jena Biosciense, Germany) on a Lightcycler$^{®}$ Detection System (Roche, USA). Relative expression level of the Apelin transcripts were compared to rat GAPDH as housekeeping gene. Specific sets of primers (Bioneer, South Korea) designed for this study were: Apelin (GenBank: NM_031612.3): 5′-TGGAAGGGAGTACAGGGATG-3′ and 5′-TCCTTATGCCCACT-3′ and GAPDH (GenBank: NM_NM-001034055): 5′-CTCATCTACCTCTCCATCGTC TG-3′ and 5′-CCTGCTCTTGTCTGCCGGTGCTTG-3′ Reactions were performed in a 12.5 μl mixture containing 6.25 μl qPCR^{TM} Green Master Kit for SYBR Green I$^{®}$ (Jena Biosciense, Germany), 0.25 μl of each primer (200 nM), 3 μl cDNA (100 ng), and 2.25 μl nuclease-free water. The PCR protocol used consisted of a 5 min denaturation at 94 °C followed by 45 cycles of 94 °C for 15 s, 60 °C for 30 s. Reactions were performed in triplicate. Two separate reactions without cDNA or with RNA were performed in parallel as controls. Relative quantification was performed according to the comparative $2^{-\Delta\Delta Ct}$ method and using Lightcycler 96$^{®}$ software. Validation of assay to check that the primer for the Apelin and GAPDH had similar amplification efficiencies was performed as described previously (Livak 1997). All qPCR analysis was performed according to The minimum information for publication of quantitative real-time PCR experiments (MIQE) guideline (Bustin et al. 2009).

Western Blotting

Total protein from isolated tissues was precipitated after RNA and DNA isolation using TriPure total RNA isolation kit according to the manufacturer's procedure (Roche Molecular System, USA) and its concentration was measured using Bradford method. 25 μl of each protein sample (1 μg/μl) were mixed with 25 μl Laemmli sample buffer supplemented with 2-mercaptoethanol at a final concentration of 7.5 % (vol/vol). The samples were heated for 15 min at 65 °C, separated by 10 % SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH). The filters were blocked by incubation for 1 h in PBS with 5 % nonfat milk. Blots were then washed in PBS-Tween and immunoblotted with primary antibody against rat Apelin (Abcam, Cambridge, UK, Art No: ab59469) at 1:500 ratio. Detection of primary antibody was done using goat anti-rabbit HRP-conjugated antibody (Abcam, Cambridge, UK, Art No; ab98467) at 1:1000 ratio and DAB reagent (Sigma Aldrich, Germany). Densitometric quantification of Apelin proteins in relation to GAPDH as calibrator was performed using Image J software (National Institutes of Health). Western blot was done in three independent experiments for each sample.

Statistical Analyses

Data analyses were done using the SPSS 18.0 software package (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to test differences between various means (post hoc analysis LSD test). All experimental data

were presented as the mean ± SEM. The level of significance for all tests was set at $p < 0.05$.

Results

Effect of Betaine Supplementation on Insulin Resistance

Results showed that HF/HC diet feeding and STZ treatment led to obvious insulin resistance with higher insulin, glucose and HOMA–IR levels (Fig. 1A–C) compared with control animals. As shown in Fig. 1A, five weeks feeding of rats with HF/HC diet and subsequent STZ injection induced hyperglycemia. Blood glucose levels in the diabetic group was higher than that in control group at days 14 and 28 after diabetes induction ($p < 0.05$) (Fig. 1A). Treatment of diabetic rats with 1 % betaine for 14 days had no obvious effect on serum glucose level ($p > 0.05$), while 28 days after betaine treatment hyperglycemia was significantly improved in treated animals ($p < 0.05$) (Fig. 1A).

Serum insulin level at all experimental days in the diabetic group was higher than that in control group ($p < 0.05$) (Fig. 1B). Serum insulin concentration was increased two weeks after diabetes induction ($p < 0.05$) but it had a constant levels between days 14 and 28 of experiment ($p > 0.05$). Treatment of diabetic rats with 1 % betaine resulted in reduction of serum insulin level ($p < 0.05$), but this reduction had a constant manner from day 14 to day 28 of experiment ($p < 0.05$) (Fig. 1B).

As shown in Fig. 1C, HOMA–IR was increased in diabetic rats compared with control animals ($p < 0.05$). When 1 % betaine was supplemented in the drinking water of insulin resistance group, this alteration was alleviated 28 days following betaine treatment($p < 0.01$), while it had no similar effect at day 14 of experiment ($p > 0.01$) (Fig. 1C).

Effect of Betaine Supplementation on Body Weight

Significant difference was observed in body weight between the HF/HC group and the regular diet-fed control ($p < 0.05$) (Fig. 2). Feeding of rats with high energy diet for five weeks resulted in elevated their body weight compared to the control group in a time dependent manner ($p < 0.05$) (Fig. 2). Diabetic rats which treated with 1 % betaine for 14 days showed no significant change in body weight ($p > 0.05$), while body weight was reduced in animals treated for 28 days ($p < 0.05$) (Fig. 2). Betaine intake for 28 days did not change the weight of healthy rats (Fig. 2).

Betaine Changed Serum Apelin Level in HF/HC Mice

As shown in Fig. 3, compared with the control group, HF/HC fed rats exhibited a significant increase in plasma Apelin concentration ($p < 0.05$) (Fig. 3). Serum level of Apelin in the diabetic group at day 28 had no significant difference with that at day 14 ($p > 0.05$). Our results revealed a significant decrease in serum Apelin concentration after four weeks of betaine treatment compared with untreated diabetic rats ($p < 0.05$) (Fig. 3). Betaine supplementation for two weeks had no obvious effect on elevated serum Apelin concentration ($p > 0.05$) (Fig. 3).

Betaine Influenced Apelin Gene Expression in Cardiac Muscle of Diabetic Rats

Real time PCR and Western blot analyses revealed that feeding of rats with HF/HC diet significantly upregulated mRNA expression of Apelin in cardiac muscle in relation to healthy rats ($p < 0.05$) (Fig. 4A, B). Betaine treatment significantly reduced the cardiac expression of Apelin at day 28 of treatment, while it had no significant effect on mRNA level of Apelin in heart of diabetic rats 14 days following treatment ($p > 0.05$) (Fig. 4A, B). Treatment of healthy rats with betaine had no effect on mRNA and protein levels of cardiac Apelin ($p < 0.05$) (Fig. 4A, B).

Betaine Affects Apelin Gene Expression in Adipose Tissue of Diabetic Rats

The data obtained from the study indicated the significant increase of Apelin expression in adipose tissue of diabetic rats compared to control group at all experimental days ($p < 0.05$) (Fig. 5 A, B). Apelin mRNA and protein levels in adipose tissue of diabetic group had higher levels at day 28 of experiment in relation to that in day 14 of experiment ($p < 0.05$) (Fig. 5A, B). The analysis of therapeutic effects of betaine in diabetic rats indicated unchanged Apelin expression in relation to control animals two weeks after treatment ($p > 0.05$) (Fig. 5A, B). However, supplementation of betaine in diabetic rats for four weeks significantly reduced the mRNA and protein levels of Apelin in adipose tissue when compared to untreated diabetic animals ($p < 0.05$) (Fig. 5 A, B). Betaine treatment had no obvious effect on Apelin expression in adipose tissue of healthy rats ($p > 0.05$) (Fig. 5A, B).

Discussion

Obesity is a metabolic disorder in humans developed particularly due to feeding with diet rich in fat and carbohydrates and low physical activity (Schaffer et al. 2003).

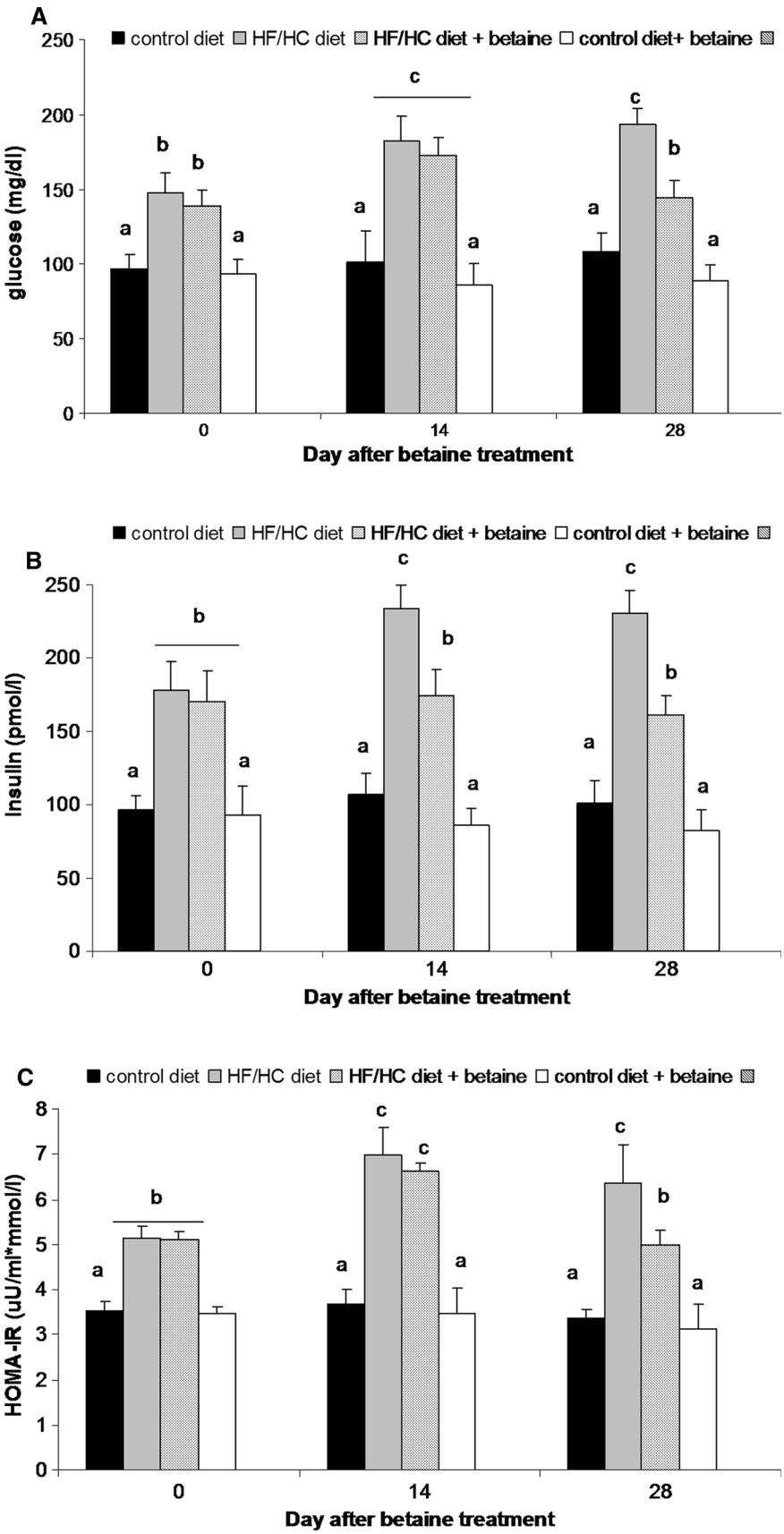

Fig. 1 Insulin resistance markers in control group, HF/HC group, HF/HC group treated with betaine 1 % (HB %1), and control group treated with betaine 1 % (BT). **a** Serum glucose level. **b** Circulating insulin level. **c** Hsomeostasis model assessment of basal insulin resistance (HOMA–IR). Data are mean ± SD. *Different letters* in each *bar* demonstrate significant differences at $p < 0.05$

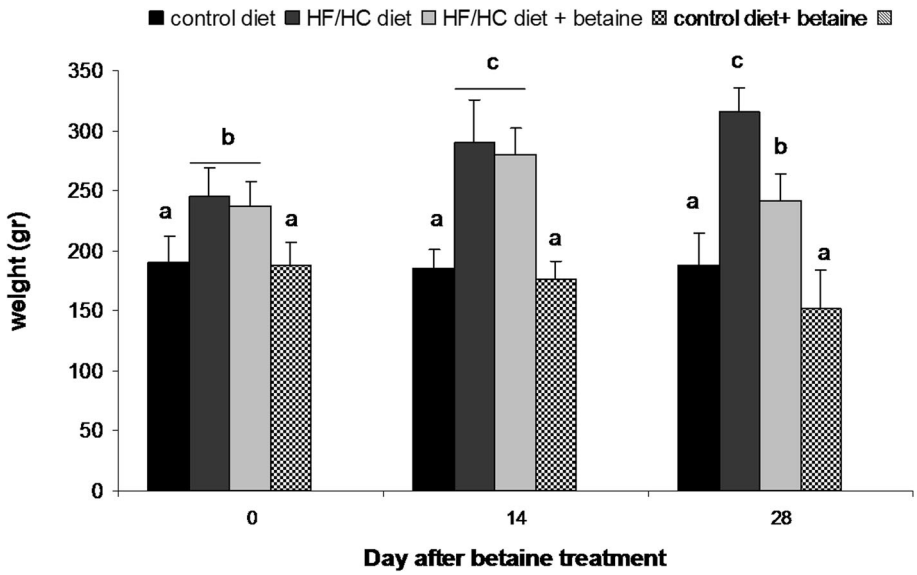

Fig. 2 Body weight in control group, HF/HC group, HF/HC group treated with betaine 1 % (HB %1), and control group treated with betaine 1 % (BT). Data are mean ± SD. *Different letters* in each *bar* demonstrate significant differences at $p < 0.05$

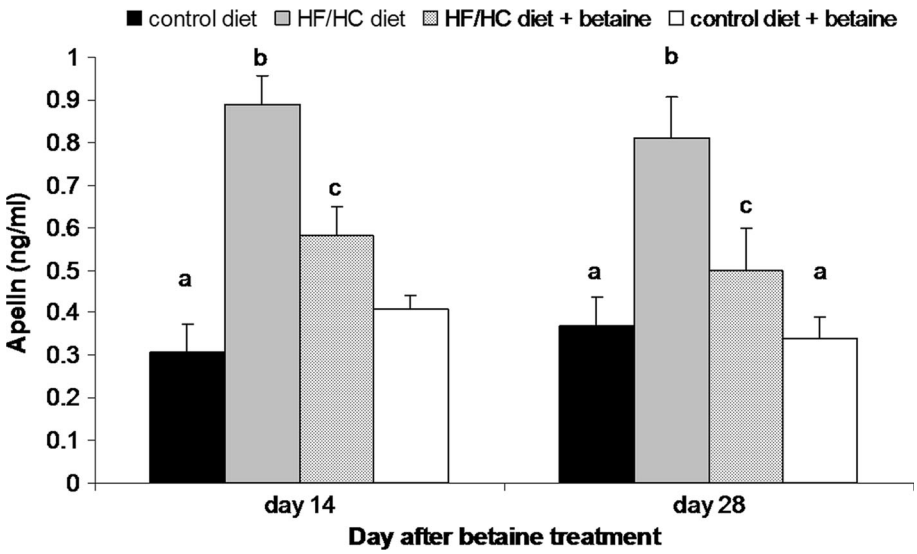

Fig. 3 Serum Apelin level in control group, HF/HC group, HF/HC group treated with betaine 1 % (HB %1), and control group treated with betaine 1 % (BT). Data are mean ± SD. *Different letters* in each *bar* demonstrate significant differences at $p < 0.05$

Application of animal models can help to identify molecular changes associated with obesity and insulin resistance in various tissues (Buettner et al. 2006). In the present study obesity and insulin resistance were induced in rats by feeding of diet containing high levels of fat and carbohydrates and subsequent injection of low dose of STZ. This protocol resulted in an increased in HOMA–IR, glucose and insulin levels, confirming the incidence of insulin resistance and diabetes.

Adipose tissue acts as an endocrine organ by releasing of adipocytokine (Romacho et al. 2014; Seifi et al. 2012). Apelin is novel adipose—derived peptides that are produced in many tissues of the body such as adpose tissue and cardiac muscle and perform several physiologic functions including regulation of lipid and carbohydrate metabolism (Dray et al. 2008; Attane et al. 2012). Growing evidence shows that apelin functions as a critical mediator of cardiovascular homeostasis and is involved in the pathophysiology of cardiovascular diseases in obese patients. Because targeting apelin axis in adipose tissue and heart by inotropic agents such as betaine may produce protection against cardiovascular diseases associated with obesity, the present study was performed with the specific aim of clarifying the effect of betaine on apelin expression in adipose and heart tissues of obese, diabetic rats.

Our results showed that insulin resistance in concomitant with hyperinsulinemia induced by high-calorie diet resulted in an increasing of serum Apelin and its mRNA and protein expression in adipose tissue. In accordance with our results it has been shown that the plasma Apelin level is increased in type II diabetes and glucose intolerance states (Li et al. 2006; Soriguer et al. 2009; Boucher

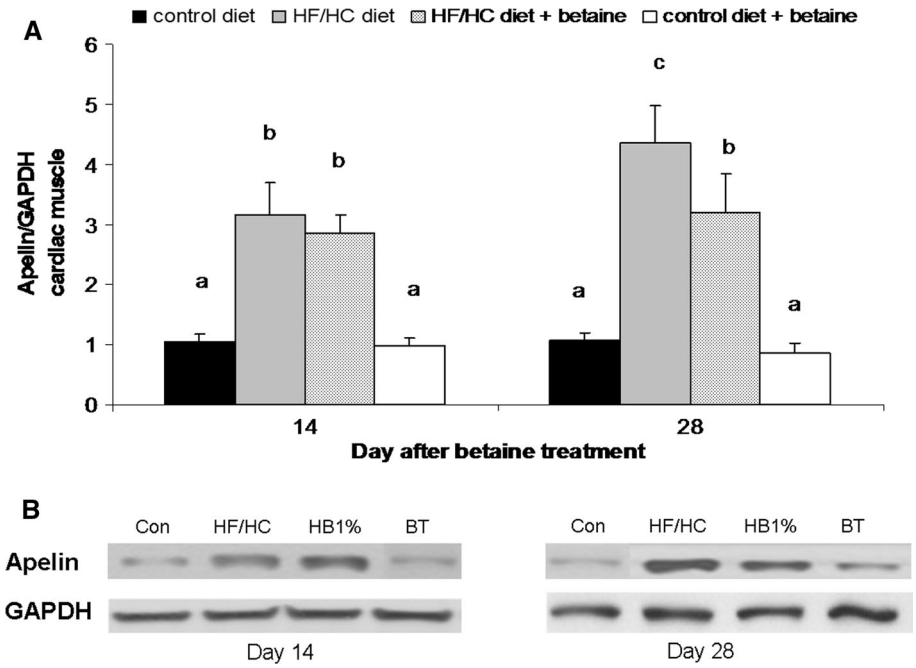

Fig. 4 Apelin mRNA (**a**) and protein (**b**) expression level in cardiac muscle in control group, HF/HC group, HF/HC group treated with betaine 1 % (HB %1), and control group treated with betaine 1 % (BT). Data are mean ± SD. *Different letters* in each *bar* demonstrate significant differences at $p < 0.05$

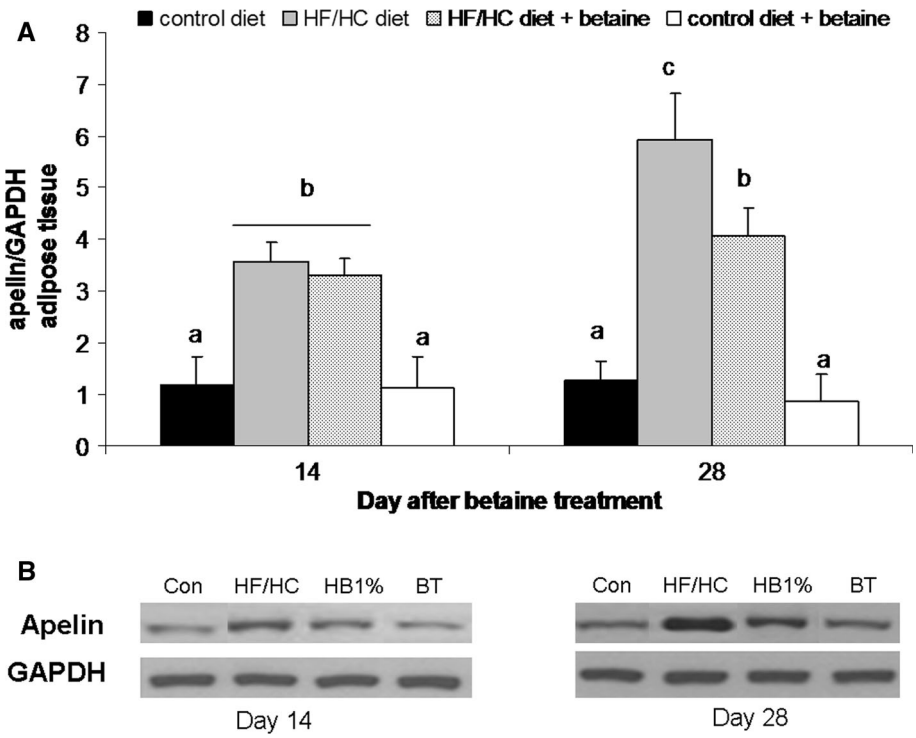

Fig. 5 Apelin mRNA (**a**) and protein (**b**) expression level in adipose tissues in control group, HF/HC group, HF/HC group treated with betaine 1 % (HB %1), and control group treated with betaine 1 % (BT). Data are mean ± SD. *Different letters* in each *bar* demonstrate significant differences at $p < 0.05$

et al. 2005). Our finding is also consistent with human studies that demonstrate high correlation between body mass index and serum Apelin levels (Ba et al. 2014). Taken these findings, it has been hypothesized that over expression and secretion of Apelin in obesity and insulin resistance may be related to hyperinsulinemia and weight gain. Increased Apelin expression in diabetic rats simultaneously with hyperinsulinemia may be a compensatory mechanism to suppress the secretion of insulin from beta cells and enhance insulin sensitivity in target tissues. To support this hypothesis, recent studies have shown that Apelin reduces insulin secretion in insulin producer INS1 cells in response to glucose and stimulates glucose uptake in myotubes and adipose tissue, resulting in increasing of total insulin sensitivity (Sorhede Winzell et al. 2005; Guo et al. 2009; Dray 2008). Chronic administration of Apelin to insulin-resistant

obese rats increases the oxidation of fatty acids and stimulates mitochondrial biogenesis in muscles and improves lipid abnormalities and insulin resistance (Attane et al. 2012). These data support the protective role of increase Apelin expression and secretion in diabetic state which may improve lipid and carbohydrate disturbances in this condition.

Recently the role of Apelin has been confirmed in heart function and metabolic adaptation in obese state. It has been found that insulin resistance progression in obese state is associated with upregulation of Apelin expression in cardiac muscle (Alfarano et al. 2015; Zeng et al. 2014). According to the results of the present study the Apelin expression has been reported in cardiac muscle and it appears that Apelin plays an important role in the regulation of heart function and metabolism. In accordance with our findings, Akcılar et al. (2015) has shown that diabetes induction resulted in an increasing of Apelin expression in cardiac muscle and aorta. The over expression of Apelin in cardiovascular tissues and ischemic cardiomyopathy has been recently reported (Atluri et al. 2007). In recent study by Alfarano et al. (2015), using mouse model combining obesity and heart failure, it has been shown that Apelin treatment promotes myocardial fatty acid oxidation and improves glucose tolerance. Apelin administration can also reduce the infarct size in the rat model of ischemia/reperfusion (Rastaldo et al. 2011). The mechanisms underlying the development of obesity related heart failure are complex, and not well understood. Several studies have provided convincing evidence that mitochondrial dysfunction may be an important event in the development of heart failure. Recent findings showed that Apelin treatment prevents mitochondrial damage in cardiac muscle by increasing the mitochondrial DNA content and citrate synthase activity, an enzyme marker of mitochondrial mass (Sharov et al. 2000; Rosca et al. 2008). According to these findings it concluded that increased expression of cardiac Apelin in insulin-resistant, obese rats may be a protective mechanism that improves the metabolic and functional disturbances of cardiac muscle and inhibits the progression of heart failure in this condition. To confirm this conclusion recent researches by Iwanaga et al. (2006) and Koguchi et al. (2012) have shown that in patient with end stage heart failure, Apelin system is down regulated, confirming the compensatory role of increased Apelin expression in cardiac muscle, in particular, during the beginning stage of diabetes associated heart failure.

Nutrition therapy can be used efficiently in obese, insulin resistance patients for improvement of obesity related disturbances such as tissue lipotoxicity, inflammatory reactions, hypertension, angiopathy and cardiomyopathy (Essop et al. 2004; Mann et al. 2015). Characterization of the molecular mechanism of compounds which used in nutrition therapy can help to design an appropriate diet. Betaine is a lipotropic peptide which may be used efficiently for nutrition therapy in diabetic patients (Craig et al. 2004), but data about its molecular mechanism is very limited. Our results showed that two weeks supplementation of betaine in drinking water of obese rats had no obvious effect on hyperinsulinemia, hyperglycemia, insulin resistance and elevated body weight. However insulin resistance markers and body weight were reduced after treatment of diabetic rats with betaine for four weeks. Few data are available about the therapeutic effects of betaine in patients with diabetes and obesity. In accordance with our findings, Wang et al. (2010) demonstrated that insulin resistance indices were reduced in diabetic rats after treatment with 1 % betaine for 12 weeks, confirming the beneficial effect of betaine on increasing the insulin sensitivity. The results of Jang et al. (2014) have also shown that oral administration of 300 mg/kg alpha lipoic acid, betaine and L-carnitine in obese rats fed by high-fat diet reduces blood glucose, triglycerides and serum leptin.

Although the insulin sensitizing effect of betaine has been reported in some animal and human studies (Wang et al. 2010; Kathirvel et al. 2010), its mechanism at molecular level, in particular its impact on adipocytokine expression in adipocyte and cardiac muscle in obese state is unknown. For the first time the results of the present study showed that four weeks betaine treatment down regulates Apelin gene expression in adipose tissue and cardiac muscle of diabetic rats. Reduced expression of Apelin mRNA in adipose tissue may be indirectly due to weight loss. To support this hypothesis, Krist et al. (2013) showed that the visceral fat mass reduction following physical exercise or surgery can reduce serum Apelin and its gene expression in adipose tissue of obese patients. In the present study betaine treatment in diabetic rats led to a weight loss compared with untreated ones, confirming the possible role of weight reduction on Apelin expression in adipose tissue. In addition to the above described mechanism, it seems that reduction of Apelin secretion and expression might be a consequence of improvement of hyperinsulinemia. In other words, increased Apelin expression during insulin resistance state could inhibit insulin secretion, attenuate hyperinsulinemia and increase overall glucose utilization, while after betaine treatment, insulin resistance was improved resulting in down regulation of Apelin in adipose tissue. Based on these observations, it has been suggested that an increase in Apelin expression may reflect a compensatory mechanism against progression of insulin resistance in obese patients.

We found that betaine treatment down regulated Apelin expression in cardiac muscle of obese, diabetic rats. No

data is available about the underlying mechanism of Apelin down regulation by betaine in cardiac muscle. Previous study has shown that decreased plasma betaine level is associated with subsequent acute myocardial infarction and that betaine reduces clinical symptoms in patients with heart disease such as arteriosclerosis and congestive heart failure (Craig et al. 2004; Lever et al. 2012). It seems that the role of betaine is related biosynthesis of creatine, providing sufficient to supporting function of cardiac muscle.

It is well established that silent ischemia is a major complication of cardiac muscle in diabetes mellitus because of an increased myocardial oxygen demand (Chiariello and Indolfi 1996). In cardiac ischemia and hypertrophy, cardiomyocytes are strongly dependent on glucose intake instead of fat (Rosca et al. 2008; Frier et al. 2009). Given the role of Apelin in stimulation of glucose uptake in muscle (Dray et al. 2008) it seems that upregulation of Apelin expression in cardiac muscle is beneficial for maintaining ATP levels in the face of diminished oxidative phosphorylation. Thus it seems that following betaine treatment, by controlling insulin resistance, metabolic status of the heart improved and cardiac Apelin expression reduced, demonstrating that improvement of insulin resistance is one of the important targets in managing the molecular and clinical cardiovascular complication in diabetic patients. Further researches are needed to determine the precise molecular action of betaine on metabolism of cardiac muscle in insulin resistance state.

In conclusion, our results showed that diabetes induction following consumption of high fat, high carbohydrate containing food increased serum levels of Apelin and its expression in adipose tissue and heart, a possible adaptive mechanism for increasing of insulin sensitivity in target tissues. It has been also found that treatment of diabetic rats with betaine for four weeks could attenuate Apelin secretion and expression in adipose tissue and cardiac muscle in concomitant with improvement of insulin resistance and weight loss. The results of the present study provide new scientific evidence about therapeutic benefits of betaine in diabetic, obese patients.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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