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Expression of cardiac insulin signalling genes and proteins in rats fed a high-sucrose diet: effect of bilberry anthocyanin extract

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Abstract

Scope: Insulin resistance is associated with impaired cardiac function, but the underlying molecular abnormalities are largely unexplained. Bilberry anthocyanin (BAcn) may be protective, as it appears to potentiate insulin action.

Methods: Rats were randomly allocated to control, sucrose-fed (SF) or sucrose-fed + BAcn diets (SF-A) for 15 weeks. Cardiac insulin signalling genes and proteins were quantified using reverse transcription quantitative real-time polymerase chain reaction and western blots.

Results: Glucose tolerance was not different with treatment. SF showed lower ($p < 0.05$) ferric reducing antioxidant power, which increased with BAcn. SF resulted in significantly decreased ($p < 0.05$) expression of 10 genes: acetyl-coenzyme A carboxylase alpha; V-Akt murine thymoma viral oncogene homolog 1; Bcl2-like 1; cytosine-cytosine-adenosine-adenosine-thymidine/enhancer binding protein; FK506 binding protein 12-rapamycin associated; glycerol-3-phosphate dehydrogenase 1 (soluble); solute carrier family 2 (facilitated glucose transporter), member 1, 4; hexokinase 2; and thyroglobulin. SF-A prevented these changes. Compared to SF-A, SF up-regulated ($p < 0.05$) complement factor D and phosphoinositide-3-kinase, regulatory subunit1 (α); sterol regulatory element binding transcription factor 1 was down-regulated ($p < 0.05$). SF increased ($p < 0.05$) cardiac phospholamban and decreased phosphorylated troponin I, which were not attenuated by BAcn. Compared to control or SF, SF-A resulted in significantly lower ($p < 0.05$) 5′-AMP-activated protein kinase.

Conclusions: SF lowered antioxidant capacity and changed the expression of insulin signalling genes, which were modulated by BAcn.

Keywords: Anthocyanins, Heart, Insulin resistance, PCR array, Type 2 diabetes mellitus

Introduction

Increased consumption of diets high in refined carbohydrates has been linked to the growing prevalence of type 2 diabetes mellitus (T2DM) (Eckel 2005; Mellor et al. 2010). Current estimates suggest that approximately 6.6 % of the world's adult population is diabetic with the majority having T2DM (IDF 2009). Of greater concern is that about 7.9 % show impaired glucose tolerance (IGT) or prediabetes (IDF 2009). One of the main underlying cellular defects associated with the diabetic and prediabetic state is insulin resistance (IR) (Abdul-Ghani and DeFronzo 2009; Reaven 1988), which is the inability of cells to respond to the action of insulin and can exist several years prior to the onset of T2DM (Lebovitz 1999).

IR can induce debilitating effects on various organs such as the kidneys, brain, liver and especially the heart (Sharma and McNeill 2006). In fact, epidemiological and clinical studies suggest that a large percentage of diabetics succumb to cardiac complications (Asghar

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et al. 2009), and this is largely attributed to myocardial infarction resulting from atherosclerotic build-up (DeFronzo 2010). However, the existence of a distinct disease of the heart muscle known as diabetic cardiomyopathy (DCM) has also been demonstrated (Galderisi et al. 1991). Although much is unknown about the aetiology of DCM, it can play a significant role in heart failure and in the recovery of cardiac tissue after an ischemic insult (Boudina and Abel 2007; Maisch et al. 2011). It is therefore crucial to examine the underlying cellular defects that accompany the very early stages of the development of IR and its contribution to the aetiology of DCM.

In the normal heart, binding of insulin to its receptors on cardiac cells elicits a series of downstream signal transduction pathways, which results in tight regulation of glucose and fatty acid uptake and utilization, glycogen and protein production, and vascular function (Muniyappa et al. 2007). However, when cells become resistant to the actions of insulin, lipotoxicity (An and Rodrigues 2006) and systemic hyperglycemia ensue; this results in a compensatory increase in insulin secretion (Chavez 2006). As such, persistent hyperinsulinemia resulting from IR can promote dyslipidemia and the proliferation of inflammatory factors (Kasuga 2006), which probably contribute to the onset of overt T2DM and DCM. However, the underlying molecular mechanisms and metabolic changes associated with these abnormalities are largely unknown, but could involve modulation of gene and protein expression.

In diabetic rodent models, DCM is associated with reduced glucose uptake and lipotoxicity, and adverse changes in calcium handling and contractile variables (Belke et al. 2000; Vasanji et al. 2006). In particular, the calcium handling protein sarco(endo)plasmic reticulum Ca^{2+}ATPase (SERCA), its regulator phospholamban (PLN), and the contractile protein cardiac Troponin I (cTnI), which have pivotal roles in the control of cardiac contraction and relaxation (Bers 2002), are significantly affected in DCM. Many of these studies have found that DCM-like cardiac complications are accompanied by significant changes in the expression and function of these proteins (Frank et al. 2002; Kranias and Bers 2007; Li et al. 2000; Ramirez-Correa and Murphy 2007).

Given the magnitude of the burden of T2DM and DCM, numerous approaches are being explored to prevent and/or reverse the associated metabolic effects. One such approach is in the use of plant-based products, such as anthocyanins, to influence cell signalling mechanisms. Anthocyanins are naturally occurring, pigmented compounds from fruits and vegetables, regularly consumed in fruits and vegetables and represent a major subclass of flavonoids (Mazza and Miniati 1993). Estimates on human intakes vary widely from 3 to 215 mg/day (de Pascual-Teresa et al. 2010). Purple corn anthocyanin has been shown to be protective towards IR, weight gain, elevated serum lipids and the production of tumour necrosis factor-α (TNF-α) in C57BL/6J rats fed a high-fat diet (Tsuda et al. 2003). Anthocyanins extracted from bilberry effectively improved hyperglycemia and insulin sensitivity in a genetic rodent model of T2DM (KK-A^y mice). This was associated with higher levels of AMP-activated protein kinase (AMPK) activity (Takikawa et al. 2010), which is also the target of metformin, a pharmacological agent routinely used in the management of T2DM (Hawley et al. 2002). Given these findings, it appears that anthocyanins can modulate intermediary metabolic pathways and perhaps influence the outcomes of risk factors for T2DM. However, the extent to which anthocyanins are able to reverse or modulate the molecular mechanisms of cardiac IR is unknown, although it is reasonable to assume that changes in the insulin signalling pathway are likely to occur.

We hypothesized that a high-sucrose diet leads to changes in the expression of insulin signalling genes and proteins as well as cardiac contractile proteins, and that these changes can be modulated by treatment with an anthocyanin extract. Our objectives were to reproduce the sucrose-fed insulin-resistant animal model, ascertain changes in gene and protein expression in the insulin signalling pathway in rat hearts and attempt to modulate these changes with an anthocyanin treatment.

Materials and methods

Bilberry anthocyanin

Commercially available dried bilberry powder, prepared using a low-temperature technique (Natural Newfoundland Nutraceuticals, Newfoundland, Canada) and containing 29.60 mg GAE/g total phenolics and 24.05 mg C3GE/g anthocyanins was double-extracted with acidified methanol (85 % MeOH: 14 % H_2O: 1 % HOAc) and partially purified using flash chromatography (Isolera One, Biotage, Sweden, AB). The latter treatment also resulted in the removal of free sugars. Methanol was removed by rotary evaporation, and the resulting extracts were freeze-dried and stored at −20 °C until use. Total anthocyanin concentration (TAC) was determined in triplicate using a modified pH differential method using a cyanidin-3-glucoside standard (Li et al. 2012).

Bilberry extract was solubilized in 2 N HCl solution at 1 mg/mL and hydrolyzed by incubation in a boiling water bath for 2 h and further characterized using an UHPLC system (Accela-Thermal Technologies Co., Ltd., USA) equipped with a diode array detector. Separation was achieved with a Phenomenex® Kinetex PFP, 4.6 × 150 mm, a 2.6-μm column (100 × 2.1 mm, Phenomenex, Torrance, CA, USA) attached to a Phenomenex® PFP guard column (4 × 3 mm). The column was thermostatically controlled at 30 °C and the flow rate was set to 1 mL/min. The mobile phase consisted of water-formic acid (A, 95:5, *v*/*v*) and methanol-formic acid (B, 95:5, *v*/*v*) with the following

solvent gradient: 0–25 min, 0–50 % B; 25–27.5 min, 50–100 % B, and held at 100 % B for additional 2.5 min. The column was then re-equilibrated at 100 % A for 5 min. Peak absorbance was collected between 200 and 620 nm using DAD, and the chromatogram output was set at 520 nm. Stock solutions of the standards were prepared by dissolving 10 mg of each compound in 5 mL DMSO and then topped up to 100 mL in a volumetric flask with 50 % methanol (final concentration 100 μg/mL).

Petunidin was confirmed using a Finnigan LCQ DECA ion trap mass spectrometer (ThermoFnnigan, San Jose, CA, USA) equipped with electrospray ionization (ESI) source. Separation was performed with the same binary solvent system as in the HPLC method, but a different column (Luna 3-μm PFP (2) (4.6 mm × 100 mm; Phenomenex, Torrance, CA, USA)) and gradient programme: 0–26 min, 10–100 % B; 26–28.5 min, 100–10 % B, then the gradient was held at 10 % A for an additional 5 min. The flow rate was set at 1.0 mL/min. Positive mode was selected for data collection. The instrument was tuned by using cyanidin standard to reach its optimum performance. Other analytical conditions have been previously described by (Li et al. 2012).

Experimental design

Male Sprague Dawley rats, approximately 200 g and 6 weeks of age, were randomly divided into three experimental groups (four rats/cage): control (C), sucrose-fed (SF) and sucrose-fed anthocyanin treated (SF-A) as shown in Table 1. Control animals were fed a basal AIN-93 diet while the SF and SF-A animals were fed a high-sucrose diet. The diets were isocaloric; however, the high-sucrose diet contained 55 % of energy as sucrose, whereas in the basal diet, energy was mainly from cornstarch. All diets were formulated by Dyets Incorporated (PA, USA), and the compositions are shown in Table 1.

The animals were all fed the control diet for 3 days, after which a tail vein blood sample was taken for baseline measurements. All the groups were fed and watered ad libitum. Anthocyanin was supplemented (SF-A) by addition to drinking water (0.2 mg/mL anthocyanin) which was stored in dark bottles and replaced every 2 days. Chow intake and body weight were measured weekly and fluid intake every 2 days. Based on the weekly chow intake and the energy content of the diet (Table 1), the average energy intake per rat for each week was calculated and standardized according to body weights. At 3-week intervals, the animals were fasted overnight and ~1.0 mL blood was drawn into freshly prepared heparinized tubes (3 units/0.5 mL blood) using the tail clip method. Plasma was separated and stored at −70 °C pending batch analyses for glucose, insulin and triglycerides. After 14 weeks, an intraperitoneal glucose tolerance test (IPGTT) was performed on the fasted animals by intraperitoneal injection of a glucose solution (2 g/kg body weight). Blood was then drawn and analysed for glucose using a Hemocue 201 Analyzer (Hemocue AB, Ängelholm, Sweden) at baseline and at 30, 60 and 120 min following glucose administration. One week after the IPGTT, the animals were terminally anaesthetized; the heart was harvested, flash-frozen in liquid nitrogen and stored at −80 °C pending biochemical analyses.

Biochemical analyses

Commercially available analytical kits were used to measure plasma levels of triglycerides (Human Diagnostics, Wiesbaden, Germany) and insulin (Linco Research, MO, USA). The oxygen radical absorbance capacity (ORAC) (Prior et al. 2003) and ferric reducing antioxidant power (FRAP) (Benzie and Strain 1996) assays were used to determine total antioxidant capacity of serum. The homeostasis assessment model (HOMA) was used to

Table 1 Experimental treatments and diet composition

Experimental group	Water	Diet[a]	Diet composition[b] (g/kg)	
Control (C); $n = 8$	No treatment	Control	Sucrose	100
			Cornstarch	466
			Dyetrose	155
			Protein	140
			Fat	40
			Energy 3602 kcal/kg	
Sucrose-fed (SF); $n = 12$	No treatment	High sucrose	Sucrose	550
Sucrose-fed/anthocyanin supplemented (SF-A); $n = 12$	0.2 mg/mL anthocyanin	High sucrose	Cornstarch	130
			Protein	140
			Fat	40
			Energy 3604 kcal/kg	

[a]Diets were formulated by Dyets Incorporated, PA, USA
[b]Both diets contained the recommended concentrations of vitamins, minerals and fibre

calculate HOMA-IR. This was calculated by multiplying fasting plasma insulin (FPI) by fasting plasma glucose (FPG), then dividing by 22.5 (Diabetes Trials Unit, University of Oxford, UK).

For gene expression studies, RNA was isolated from heart tissue using TRI Reagent (Sigma-Aldrich, CA, USA). Impurities were removed using RNeasy Mini Kit (Qiagen, Valencia, CA), and RNA quality was assessed using the absorbance ratios at 260 nm/230 nm and 260 nm/280 nm. Purified RNA was then used to quantify gene expression for 84 genes along the insulin signalling pathway utilizing the 7500 ABI Fast Real Time PCR System (Applied Biosystems, CA, USA) with the RT^2 Profiler PCR Array System (SABiosciences, Frederick, MD, USA). C_t (cycle threshold) values were generated by the 7500 ABI Fast Real Time PCR System and used to calculate fold changes in gene expression. Fold change differences between experimental groups were calculated for each gene as follows: Fold Δ (GOI) = Ave [C_t (TEST) – C_t (HKG)]/Ave [C_t (CONTROL) – C_t (HKG)], where GOI is gene of interest and HKG is housekeeping gene.

Proteins were prepared by dicing and homogenizing left ventricular tissue with a hand-held homogenizer in cold lysis buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1 % SDS, 1 mM EGTA, containing protease inhibitors (1:250) (Roche Diagnostics Corporation, IN, USA), phosphatase inhibitor cocktails I and II (1 mL/100 mL buffer, Sigma-Aldrich, CA, USA) and protein kinase inhibitor H-7 (1 mL/100 mL buffer, Sigma-Aldrich, CA, USA). This homogenate was then centrifuged at 10,000×*g* for 10 min at 4 °C. The supernatant was removed, aliquoted and stored at −70 °C pending sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrophoresed on 12 % polyacrylamide gels at 150 V for 1.5 hr under denaturing conditions (Laemmli 1970) and subsequently transblotted onto polyvinyl difluoride membrane (Towbin et al. 1979) and probed with antibodies for PLN, SERCA, cTnI, the insulin receptor, Akt, AMPK and the phosphorylated forms of these proteins (Badrilla, Leeds, UK; Affinity Bioreagents, CO, USA; Cell Signalling Technology MA, USA; Abcam, MA, USA). Primary antibodies were diluted at a ratio of 1:1000, and secondary antibodies were diluted at a ratio of 1:2000. Blots were then quantified using Image J (NIH, MD, USA) and normalized using the protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signalling Technology MA, USA). The protein expression ratios of PLN/SERCA and phosphorylated to unphosphorylated protein for cTnI, the insulin receptor, Akt and AMPK were calculated and compared between experimental groups to gauge the functionality of these proteins.

Statistical analyses

The study was designed using a power analysis based on an expected difference of 50 % in the area under the glucose response curve following an IPGTT, a type I error of 0.05 and a power of 90 %. The required sample size was calculated to be at least eight. Statistical analyses were performed using the SPSS 12 software for Windows (SPSS Inc., Chicago, IL, USA). Differences in insulin, glucose and triglyceride levels and blood glucose during the IPGTT, between control and SF animals, were evaluated using repeated measures ANOVA. The differences in ORAC, FRAP, C_t values, AUC from the IPGTT and western blotting results were compared using one way ANOVA. A p value of less than 0.05 was regarded as being significant.

Results

The bilberry extract had a total anthocyanin content of 204.24 ± 2.17 mg CyGE/100 g DW and was consistent with other studies (Bakowska-Barczak et al. 2007; Zhang et al. 2004), the major anthocyanidin aglycones being delphinidin, cyanidin, petunidin, peonidin and malvidin (Fig. 1). Among the study groups, there were no significant differences in body weight (Fig. 2) or energy consumption (Fig. 3). After 15 weeks on the experimental diet, neither HOMA-IR (Fig. 4) nor plasma triglyceride concentration (Fig. 5) was significantly different among the study groups at any time point. The IPGTT also showed no significant differences among the groups for blood glucose levels at any time point or in the incremental area under the blood glucose response curve (Fig. 6). The FRAP assay showed significantly lower values for SF when compared to control, and significantly higher values for SF-A when compared to SF animals (Fig. 7a). However, ORAC values were not significantly different among the experimental groups (Fig. 7b).

When SF treatment was compared to control, PCR array gene analyses of left ventricular tissues showed that the expression of several genes were significantly down-regulated ($p < 0.05$; Table 2) and included acetyl-coenzyme A carboxylase alpha (ACC1), V-Akt murine thymoma viral oncogene homolog 1 (Akt), Bcl2-like 1 (Bcl2), CCAAT/enhancer binding protein (C/EBP), beta (C/EBPβ), FK506 binding protein 12-rapamycin associated (mTOR), glycerol-3-phosphate dehydrogenase 1 (soluble) (GPDH1), hexokinase 2 (HK2), solute carrier family 2 (facilitated glucose transporter), member 1 (GLUT1), solute carrier family 2 (facilitated glucose transporter), member 4 (GLUT4) and Thyroglobulin (Tg). These changes were not observed when SF-A animals were compared with control, indicating that anthocyanin supplementation prevented these changed in gene expression. Further, when SF was compared with SF-A treatment, complement factor D (adipsin) and phosphoinositide-3-kinase, regulatory subunit 1 (α) (PI3Kα) were significantly up-regulated ($p < 0.05$), while sterol regulatory element binding transcription factor 1

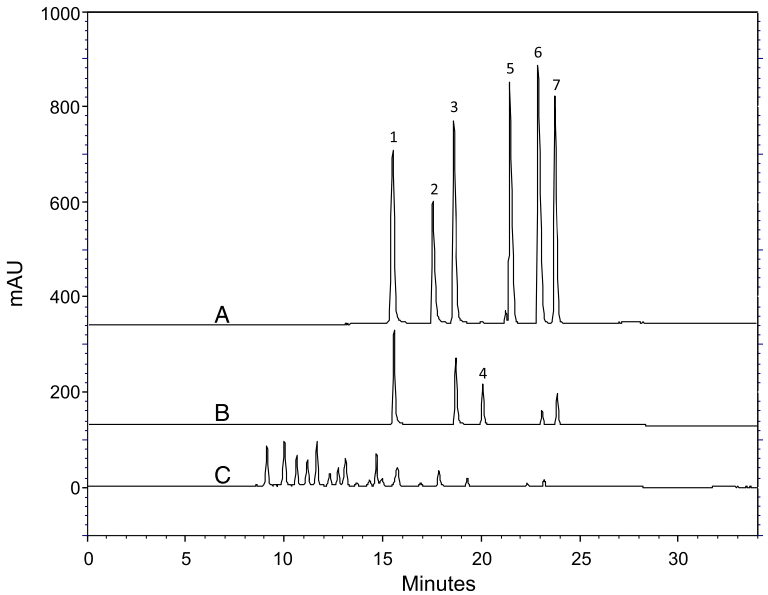

Fig. 1 UHPLC-DAD chromatograms of mixed standards (*A*) and bilberry extract before (*C*) and after acid hydrolysis (*B*). Mobile phase A—95 %:5 % water-formic acid; mobile phase B—95 % methanol:5 % formic acid (*v*/*v*); flow rate 1.0 mL/min; column: Phenomenex Kinetex PFP, 4.6 × 150 mm, 2.6 μm; detection at 520 nm; injection: 10 μL. *A* = standards: *1* delphinidin, *2* luteolinidin, *3* cyanidin, *4* petunidin, *5* pelargonidin, *6* peonidin, *7* malvidin; *B* = hydrolyzed bilberry extract; *C* = bilberry extract

(SREBF1) was significantly down-regulated ($p < 0.05$) (Table 3).

Compared to control, PLN levels were significantly increased ($p < 0.05$) and p*-cTnI levels were significantly decreased ($p < 0.05$) in the heart of SF-treated rats (Fig. 8a, d, respectively); these changes were not attenuated by anthocyanin supplementation. However, total AMPK expression was significantly lower ($p < 0.05$) in SF-A when compared to both control and SF hearts (Fig. 8i), while protein expression for SERCA, cTnI, IRec, p*-IRec, Akt, p*-Akt and p*-AMPK were not different (Fig. 8b, c, e–h, j, respectively). The ratio of SERCA/PLN was significantly higher ($p < 0.05$) in SF-A rats when compared to that in SF (Fig. 9a), whereas the ratio of p*-cTnI/cTnI was significantly decreased in both SF and SF-A animals (Fig. 9b).

Discussion

We found that feeding male Sprague Dawley rats a solid high-sucrose diet for 15 weeks did not result in the animals becoming insulin resistant, hypertriglyceridemic or obese, although others have been able to achieve these metabolic abnormities using sucrose diets (Davidoff et al. 2004; Vasanji et al. 2006). In our laboratory, when these animals are fed sucrose (32 %, *w*/*v*) in drinking

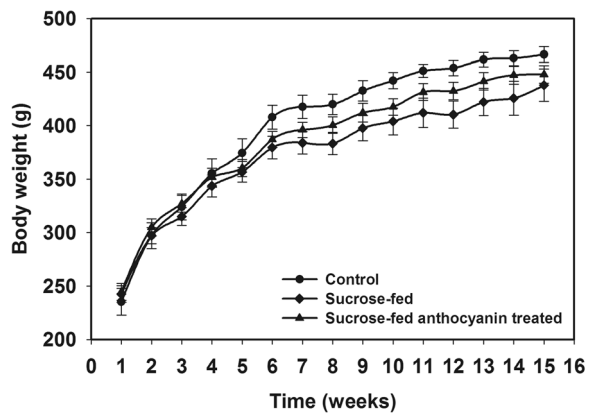

Fig. 2 Body weights (mean ± SE) for control ($n = 8$), sucrose-fed ($n = 12$) and sucrose-fed anthocyanin treated ($n = 12$) rats over 15 weeks

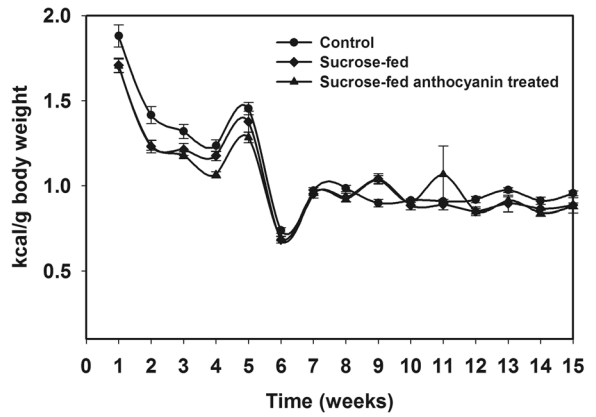

Fig. 3 Energy consumption in kcal/g body weight (mean ± SE) for control ($n = 8$), sucrose-fed ($n = 12$) and sucrose-fed anthocyanin treated ($n = 12$) rats over 15 weeks

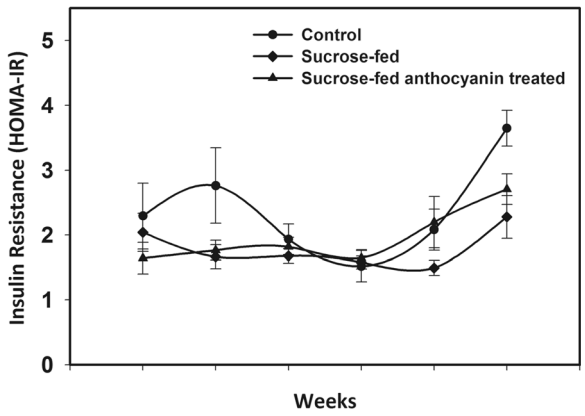

Fig. 4 HOMA-IR (mean ± SE) for control ($n = 8$), sucrose-fed ($n = 12$) and sucrose-fed anthocyanin treated ($n = 12$) rats over 15 weeks

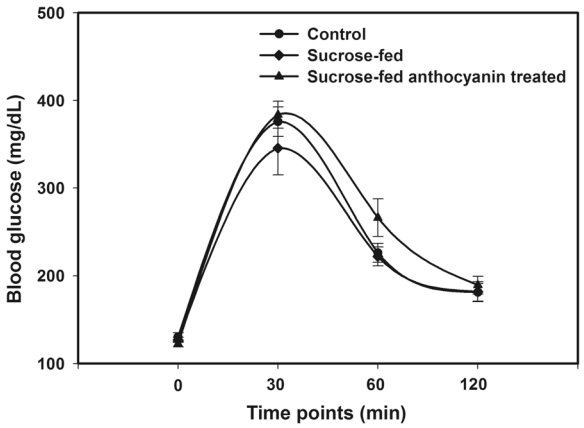

Fig. 6 IPGTT test (mean ± SE) for control ($n = 8$), sucrose-fed ($n = 12$) and sucrose-fed anthocyanin treated ($n = 12$) rats after 15 weeks

water (presumably absorbed primarily in the small intestine), they become obese and insulin resistant after 8–10 weeks of feeding. However, they consume less solid food and increase water consumption, so that their nutrient intake profile becomes altered (Singh and Ramdath unpublished); this would be a concern in efforts to establish a diet-induced rodent model of T2DM. Recent studies have suggested that the gut microbiota might play a crucial role in the onset of diabetes (Everard and Cani 2013; Turnbaugh et al. 2008). As such, it is possible that our animals, obtained from the local animal house, might have acquired a microbiota that is different from animals obtained from a central breeding source, and this could account for their resistance to developing a diabetic/prediabetic condition when fed orally with a solid high-sucrose diet. Others have shown that both Sprague Dawley and Wistar rats failed to become obese on a high-fat diet (Perez de Heredia et al. 2006) and may have different rates of weight gain which is associated with hyperglycaemia (Lauterio et al. 1994). It seems reasonable to expect that there is inherent metabolic variation (metabolic plasticity) among rodents that allows them to resist changes in glucose and lipid metabolism (Chang et al. 1990).

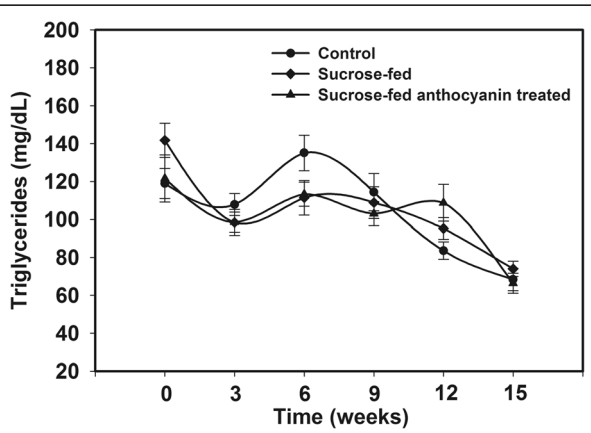

Fig. 5 Plasma triglycerides (mean ± SE) for control ($n = 8$), sucrose-fed ($n = 12$) and sucrose-fed anthocyanin treated ($n = 12$) rats over 15 weeks

Clearly, the fact that our animals showed no abnormal metabolic changes or signs of DCM on a high-sucrose diet is not unique. We contend that our results from gene and protein expression assays are indicative of very early cellular changes in insulin signalling and cardiac function resulting from a high-sucrose diet. It is likely that these will eventually lead to phenotypic changes and result in overt metabolic changes. However, the length of time between these events is likely to be influenced by cellular adaptation and metabolic plasticity, as mentioned above. Moreover, identification of very early changes in the expression of genes and proteins associated with insulin signalling and cardiac function could allow for a better understanding of DCM aetiology and facilitate early intervention.

Using the multiple gene profiling feature of microarray analysis with real-time PCR quantitation techniques, we showed that expression of GLUT1, GLUT4, HK2, GLUT4 and Akt were down-regulated. Decreased expression of GLUT1 and GLUT4 may be indicative of reduced glucose transport into cardiomyocytes, which could impair cardiac muscle contractility (Abel 2004). However, in mice with cardiac-specific inactivation of the GLUT4 gene, there is modest ventricular hypertrophy with no contractile changes (Abel et al. 1999). In contrast, decreased expression of HK2 in sucrose-fed rats, as found on our study, has been linked to a reduction glucose phosphorylation and possibly a reduction in ATP production and contractility (Ye et al. 2005). Further, cardiac contractility might have been slightly impaired in our SF animals, given the small but significant decrease in Akt gene expression observed. In a similar study, Roberts et al. 2005 showed that a high-fat, high-sugar diet resulted in decreased Akt protein expression

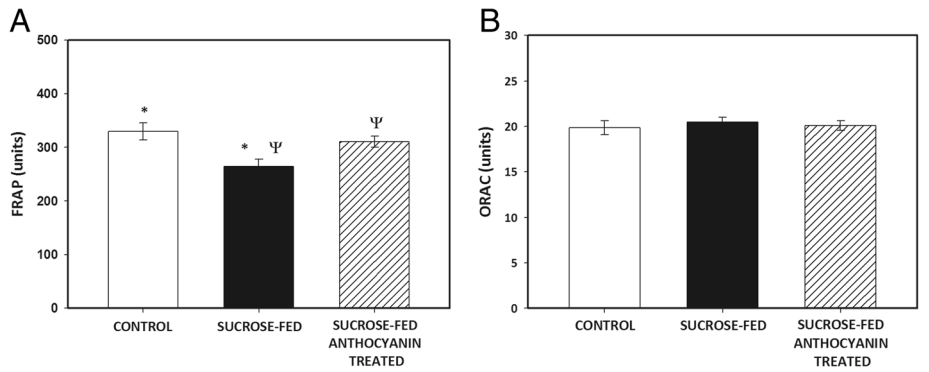

Fig. 7 **a** Ferric reducing antioxidant power (FRAP) values (mean ± SE) for control ($n = 8$), sucrose-fed ($n = 12$) and sucrose-fed anthocyanin treated ($n = 12$) rats after 15 weeks. (*, Ψ significantly different at $p < 0.05$). **b** Oxygen radical absorbance capacity (ORAC) values (mean ± SE) for control ($n = 8$), sucrose-fed ($n = 12$) and sucrose-fed anthocyanin treated ($n = 12$) rats after 15 weeks (1 ORAC unit equals to the net antioxidant protection produced by 1 μM Trolox (the water-soluble vitamin E analogue used as a positive control))

in aorta and heart tissue; they suggested that eNOS activation by Akt and therefore endothelial function may be impaired by HFS. Akt phosphorylates a wide range of substrates in the proximal part of the insulin signalling pathway, and impairment of this function has been shown to result in downstream contractile abnormalities, similar to those associated with IR and DCM. However, the ratio of p-Akt/Akt, which is the activation status of Akt, was unaltered, suggesting that sucrose feeding did not affect Akt activation. As such, the metabolic effects of decreased Akt protein expression require further investigation (Roberts et al. 2005).

Taken together, changes in the expression of genes along the insulin signalling pathway as well as those involved in cellular energy utilization (ACC1, mTOR and GPDH1) suggest that glucose transport and usage is decreased in the heart of SF-fed rats, and this may result in impaired cardiac contractility. This is consistent with observations in diabetic rats: they exhibit cardiac dysfunction and IR associated with decreased glucose and increased fatty acid utilization, decreased mRNA and protein levels of GLUT4 (van den Brom et al. 2009). Furthermore, defects in GLUT4 translocation has been demonstrated in the skeletal muscle of Goto-Kakizaki rats and in type 2 diabetic patients (Zdychova and Komers 2005). Collectively, reductions in the expression of C/EBPβ and Bcl2 and genes that play a critical role in cellular energy homeostasis may provide an aetiological mechanism for the onset of DCM following high-sucrose feeding (Bosch et al. 1995; Cory and Adams 2002; Hage et al. 2011).

Our protein expression data further suggests that high-sucrose feeding might have resulted in impaired cardiac contractility. We found that SF led to increase levels of PLN, while SERCA/PLN ratio, p*-cTnI and p*cTnI/cTnI ratio were decreased. An increase in PLN is likely to increase inhibition of SERCA and prolong

Table 2 Mean (±SE) C_t values for cardiac genes that showed different expression levels when SF samples were compared to control samples

Description	Control ($n = 5$)	Sucrose-fed ($n = 5$)	p value	[a]Fold Δ
Acetyl-coenzyme A carboxylase alpha	26.71 ± 0.13	27.21 ± 0.16	0.038	−1.12
V-Akt murine thymoma viral oncogene homolog 1	25.65 ± 0.10	26.03 ± 0.11	0.027	−1.04
Bcl2-like 1	23.61 ± 0.12	24.20 ± 0.22	0.047	−1.19
CCAAT/enhancer binding protein (C/EBP), beta	23.45 ± 0.16	24.14 ± 0.23	0.037	−1.28
MTOR- FK506 binding protein 12-rapamycin associated	25.08 ± 0.09	25.60 ± 0.17	0.026	−1.13
Glycerol-3-phosphate dehydrogenase 1 (soluble)	24.61 ± 0.24	25.42 ± 0.14	0.019	−1.38
Hexokinase 2	26.37 ± 0.13	27.25 ± 0.29	0.022	−1.46
Solute carrier family 2 (facilitated glucose transporter), member 1	29.45 ± 0.19	30.49 ± 0.36	0.034	−1.64
Solute carrier family 2 (facilitated glucose transporter), member 4	23.21 ± 0.09	24.07 ± 0.20	0.005	−1.44
Thyroglobulin	29.88 ± 0.18	30.40 ± 0.09	0.037	−1.12

All were significant at $p < 0.05$
– represent downregulation
[a]Fold changes; calculated after C_t values were normalized using housekeeping genes

Table 3 Mean (±SE) C_t values for cardiac genes that showed different expression levels when SF-A samples were compared to SF samples

Description	Sucrose-fed ($n = 5$)	Sucrose-fed anthocyanin treated ($n = 5$)	p value	[a]Fold Δ
Complement factor D (adipsin)	26.05 ± 0.29	25.31 ± 0.13	0.045	+1.48
Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	24.23 ± 0.19	23.62 ± 0.14	0.030	+1.35
Sterol regulatory element binding transcription factor 1	34.25 ± 0.21	34.82 ± 0.15	0.056	−1.66

All were significant at $p < 0.05$
− represents downregulation, + represent upregulation
[a]Fold changes; calculated after C_t values were normalized using housekeeping genes

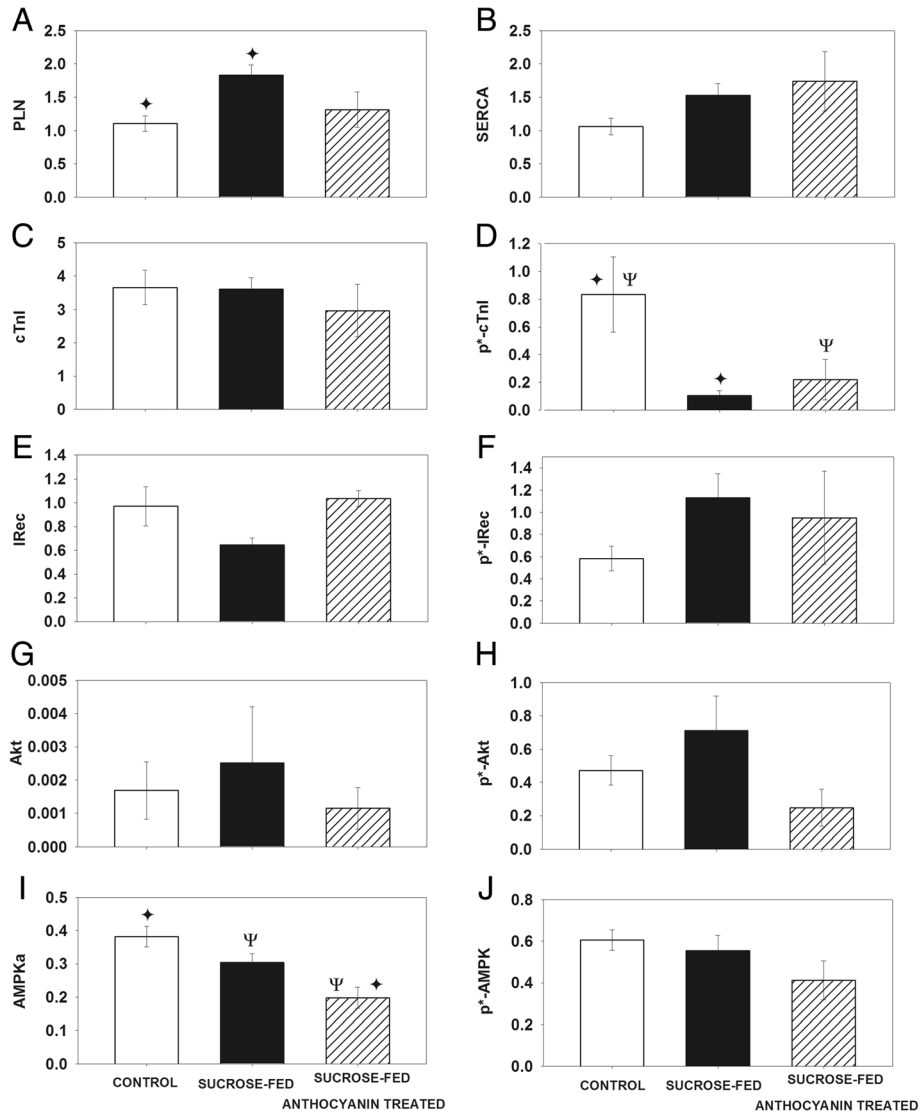

Fig. 8 Protein expression for PLN (**a**), SERCA (**b**), cTnI (**c**), p*-CTnI (**d**), IRec (**e**), p*-IRec (**f**), Akt (**g**), p*-Akt (**h**), AMPK (**i**) and p*-AMPK (**j**) in control, sucrose-fed and sucrose-fed anthocyanin treated rat heart homogenates. All densitometric measurements were normalized using GAPDH before comparisons were made (p* phosphorylated; ♦, Ψ significantly different at $p < 0.05$; $n = 5$)

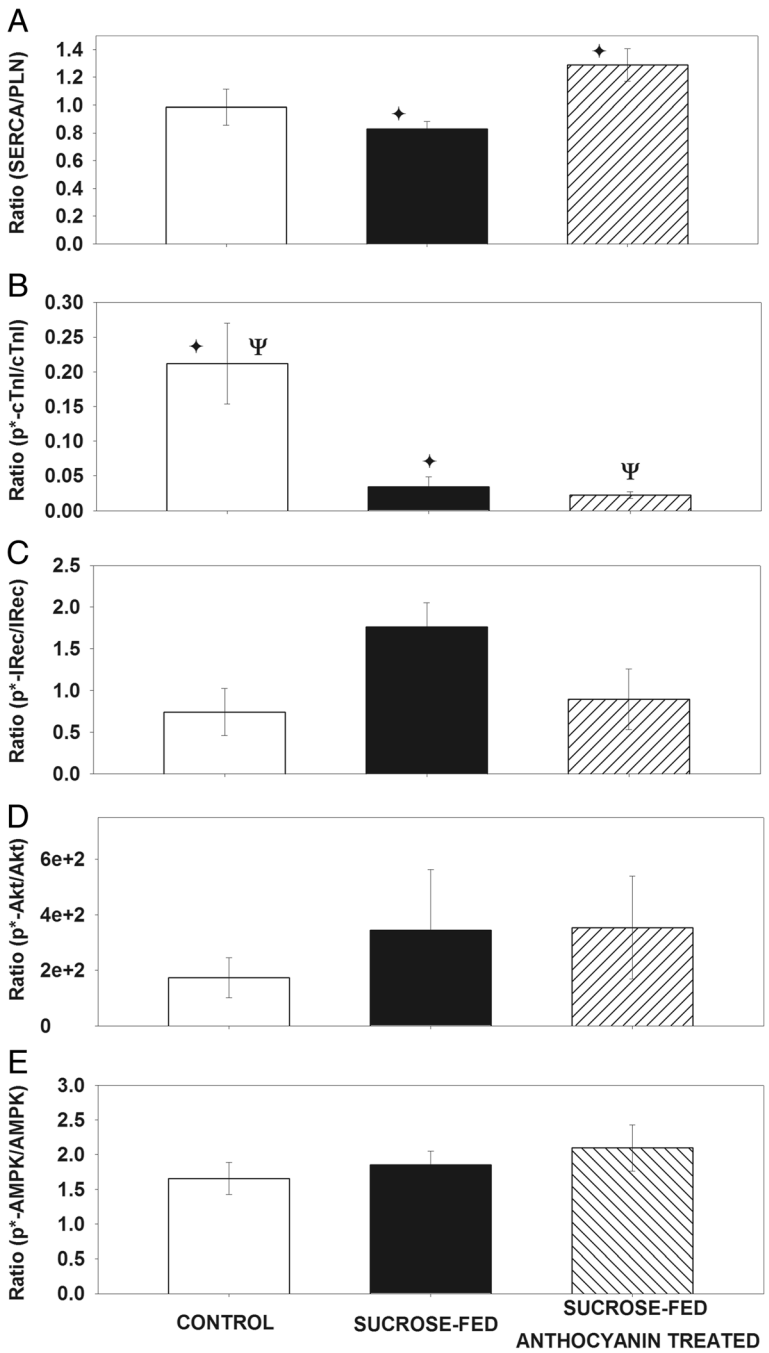

Fig. 9 Ratios of SERCA/PLN (**a**), p*-cTnI/cTnI (**b**), p*-IRec/IRec (**c**), p*-Akt/Akt (**d**) and p*-AMPK/AMPK (**e**) in control, sucrose-fed and sucrose-fed anthocyanin treated rat heart homogenates (p* phosphorylated; ♦, ψ significantly different at $p < 0.05$; $n = 5$)

relaxation rates of cardiac muscles (Koss et al. 1997); decreased p*-cTnI and p*cTnI/cTnI ratio could also contribute to lengthen rates of relaxation via a depression of cross-bridge cycling (Zhang et al. 1995). An analysis of the expression and activity of PKA, CaMKII and the other Tn subunits may be required in order to fully interpret these results. AMPK is regarded as a metabolic sensor since it upregulates catabolic pathways that generate ATP and down-regulates anabolic pathways that consume ATP (Hardie 2008). In the present study, expression of AMPK protein was decreased with sucrose feeding. However, the ratio of p-AMPK/AMPK, which is the activation status of AMPK was unaltered, suggesting that sucrose feeding did not affect AMPK activation.

Anthocyanin supplementation of the high-sucrose diet augmented the changes in gene expression induced by

the SF diet. SF-A further led to an increased expression of adipsin and PI3Kα and decreased the expression of SREBF1. PI3Kα has a central role in the proximal part of the insulin signalling pathway and increased expression may represent a compensatory mechanism to decreased expression of GLUT1, GLUT2 and HK2. SREBF1 expression, shown to be reduced in T2DM (Sewter et al. 2002), represents a series of transcription factors that responds to both Akt and MAPK pathways and influences lipogenesis and TAG deposition in skeletal muscle (Lecomte et al. 2010). As such, downregulation of SREBF1 by anthocyanin supplementation could result in the prevention of lipid accumulation in the heart. Indeed, anthocyanins have been shown to prevent the onset of obesity in animal models (Lee et al. 2014; Wu et al. 2013). An increase in ratio of SERCA/PLN, as seen in SF-A rats, has been shown to promote contractile efficiency (Currie and Smith 1999), and therefore supplementation with anthocyanins may improve heart function.

In our study, we used both ORAC and FRAP assays to estimate the antioxidant potential of serum and found that ORAC was not different among treatment groups, but FRAP was decreased with SF, and SF-A prevented this decrease. These findings are likely due to the fact that FRAP and ORAC assess different antioxidant potentials. ORAC measures the ability of antioxidants in a sample to quench one type of free radical (probably peroxyl) and is influenced by material with and without lag phases of their antioxidants (Cao and Prior 1998). On the other hand, FRAP measures the reducing power of plasma and has been shown to increase with polyphenol supplementation (Benzie et al. 1999). Others have found that sucrose feeding results in depressed level of antioxidants (Diniz et al. 2008; Fang et al. 2005), possibly resulting from oxidative stress which is associated with disrupted insulin signalling and obesity (Styskal et al. 2012). The bilberry anthocyanin supplement used in our study provided a rich source of polyphenols which would likely account for the increase in FRAP, but not ORAC.

In the present study, feeding Sprague Dawley rats a high-sucrose diet did not result in IR. However, the collective evidence from gene expression and protein quantitation studies suggest that the animals experience very early cellular changes in insulin signalling and cardiac function, which may be some of the earliest cellular adaptations to a high-sucrose diet. Importantly, anthocyanins appear capable of modulating some of these changes and may represent a possible therapeutic adjunct in the management of T2DM.

Compliance with ethics guidelines

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All procedures performed in the studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. Approval was granted for the use of animals from the Ethics Committee, Faculty of Medical Sciences, The University of the West Indies, St. Augustine, Trinidad and Tobago.

Abbreviations

ACC1: acetyl-coenzyme A carboxylase alpha; Akt: V-Akt murine thymoma viral oncogene homolog 1; AMPK: 5′-AMP-activated protein kinase; BAcn: bilberry anthocyanin; Bcl2: Bcl2-like 1; CCAAT/EBP: cytosine-cytosine-adenosine-adenosine-thymidine/enhancer binding protein; cTnI: cardiac troponin I; FRAP: ferric reducing antioxidant power; GLUT1: solute carrier family 2 (facilitated glucose transporter), member 1; GLUT4: solute carrier family 2 (facilitated glucose transporter), member 4; GPDH1: glycerol-3-phosphate dehydrogenase 1 (soluble); HK2: hexokinase 2; HOMA-IR: homeostasis model assessment-estimated insulin resistance; IPGTT: intraperitoneal glucose tolerance test; IR: insulin resistance; mTOR: FK506 binding protein 12-rapamycin associated; ORAC: oxygen radical absorbance capacity; p*: phosphorylated; PI3Kα: phosphoinositide-3-kinase, regulatory subunit 1 (α); PLN: phospholamban, SREBF1, sterol regulatory element binding transcription factor 1; RT-qPCR: reverse transcription quantitative real-time polymerase chain reaction; SERCA: sarco(endo)plasmic reticulum Ca^{2+}ATPase; SF: sucrose-fed; SF-A: sucrose-fed + BAcn; Tg: thyroglobulin; T2DM: type 2 diabetes mellitus.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

DDR and SS conceptualized the study and obtained funding. SS conducted the experiments, cleaned the data and performed initial statistical analysis and reporting. TN supervised the identification of cardiac contractile proteins. All authors interpreted the data and contributed to the final manuscript.

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