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Cocoa intake ameliorates hepatic oxidative stress in young Zucker diabetic fatty rats

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ABSTRACT

Chronic hyperglycemia in diabetes is associated with oxidative stress-mediated tissue damage. The present study is aimed to explore the role of a cocoa-enriched diet in ameliorating the oxidative stress-induced damage in the liver of young type 2 diabetic Zucker diabetic fatty (ZDF) rats. Male ZDF rats were fed a control or cocoa-rich diet (10%), and Zucker Lean (ZL) animals received the control diet. ZDF rats fed with cocoa (ZDF-Ca) decreased body weight gain, glucose and insulin levels, and improved glucose tolerance and insulin resistance. Cocoa diet further reduced reactive oxygen species (ROS) levels and carbonyl content in the liver of ZDF animals. The diminished activity of superoxide dismutase (SOD) and the enhanced activity of heme oxygenase (HO-1) in ZDF-C were returned to ZL values upon cocoa administration. Cocoa did not restore the decreased glutathione-S-transferase (GST) activity in both ZDF groups in comparison to ZL rats. Glutathione (GSH) content and activities of glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT) remained unaltered among all animal groups. Moreover, cocoa-rich diet suppressed total and phosphorylated nuclear factor erythroid-derived 2-like 2 (Nrf2), as well as p65-nuclear factor-kappaB (NF- B) enhanced levels observed in ZDF rats. The results indicate that cocoa protects the hepatocytes by improving the antioxidant competence in the liver of young type 2 diabetic ZDF rats.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic disease characterized by hyperglycaemia resulting from peripheral insulin resistance and β-cell pancreatic failure (Evans, Goldfine, Maddux, & Grodsky, 2002; Rochette, Zeller, Cottin, & Vergely, 2014). There is growing evidence that alterations in this metabolic disorder are in part a consequence of the oxidative stress that appears in the pre-diabetic milieu, and also plays a critical role in the development and progression of diabetes (Evans et al., 2002; Rochette et al., 2014). Indeed, an oxidative environment has been linked to the development of insulin resistance, β-cell dysfunction, impaired glucose tolerance, mitochondrial dysfunction and diabetes complications (Evans et al., 2002; Rochette et al., 2014).

The primary defence against oxidative stress is regulated by antioxidant/detoxifying enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), heme oxygenase (HO-1), and catalase (CAT), as well as by non-enzymatic defences such as glutathione (GSH) (Masella, Benedetto, Varì, Filesi, & Giovanninni, 2005; Valko et al., 2007). Secondly, increased reactive oxygen species (ROS) activates stress-sensitive transcription factors such as nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and nuclear factor-kappaB (NF- B) (Gloire, Legrand-Poels, & Piette, 2006). In this line, it has been reported that diabetes in experimental animals and humans is associated with reductions in antioxidants such as ascorbic acid and GSH, suggesting the critical role of oxidative stress in its pathogenesis (Evans et al., 2002).

Liver is the crucial organ responsible for oxidative and detoxifying processes, and it actively responds to the oxidative stress by modulating different signals such as Nrf2 and NF- B, which lead to the regulation of critical genes of enzymatic defences (Eggler, Gay, & Mesecar, 2008; Masella et al., 2005), and to the increased expression of pro-inflammatory genes (Gloire et al., 2006), respectively.

Dietary antioxidants play an important role in the prevention of T2DM (Dey & Lakshmanan, 2013). Accordingly, recent attention has focused on the identification of natural antioxidants with anti-diabetic

Abbreviations: AUC, area under curve; CAT, catalase; DCFH, dichlorofluorescein; DNPH, dinitrophenylhydrazine; GSH, glutathione; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione-S-transferase; GTT, glucose tolerance test; HO-1, heme oxygenase; HOMA-IR, homeostasis model assessment of insulin resistance; Nrf2, nuclear factor (erythroid-derived 2)-like 2; NF- B, nuclear factor-kappaB; OPT, o-phthalaldehyde; ROS, reactive oxygen species; SOD, superoxide dismutase; T2DM, type 2 diabetes mellitus; *t*-BOOH, *tert*-butylhydroperoxide; ZDF, Zucker diabetic rat; ZL, Zucker Lean.

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properties, and this constitutes a promising approach for the prevention and/or treatment of T2DM. Apart from the conventional anti-diabetic treatment, antioxidants may benefit in having fewer side-effects than anti-diabetic drugs currently used (Babu, Liu, & Gilbert, 2013), and it should also be considered that those natural antioxidant compounds are abundant in nature and inexpensive to produce.

Cocoa has been recognized as a rich source of phenolic compounds, mainly flavanols such as epicatechin and oligomeric procyanidins (Martin, Cordero-Herrera, Bravo, Ramos, & Goya, 2014; Martin et al., 2008). It has been showed that cocoa possesses a powerful antioxidant activity (Vinson et al., 2006), decreases blood glucose levels (Dorenkott et al., 2014; Jalil, Ismail, Pei, Hamid, & Kamaruddin, 2008; Ruzaidi, Amin, Nawalyah, Hamid, & Faizul, 2005), improves glucose tolerance (Dorenkott et al., 2014; Jalil et al., 2008; Ruzaidi et al., 2005), and diminishes metabolic syndrome-related inflammation (Gu & Lambert, 2013). More importantly, human clinical studies in diabetic patients have demonstrated that cocoa and dark chocolate improve insulin sensitivity (Grassi et al., 2008) and modulate oxidative stress markers in skeletal muscle (Ramirez-Sanchez et al., 2013). Despite this, the precise antioxidant defence mechanism for the preventive activities of cocoa during diabetes in the liver is not fully understood. Thus, there is scarce data regarding the effect of cocoa on hepatocytes *in vivo* and data related to the systemic antioxidant defence response is contradictory, as enhanced, decreased and unchanged enzymatic activities have been reported in serum of T2DM patients (Ahmed, Naqvi, & Shafiq, 2006; Ramakrishna & JailKhani, 2010).

The aim of this study was to evaluate the potential effect of cocoa-rich diet on the redox status in the liver of male Zucker diabetic fatty [ZDF; ZDF/*crl-lepr* (fa/fa)] rats, during the pre-diabetic stage (6–15 weeks of life). Here, we report that cocoa reduces hyperglycaemia and hyperinsulinemia, ameliorates glucose intolerance, and alleviates oxidative stress by modulating key proteins of the antioxidant defence in the liver of young ZDF rats.

2. Materials and methods

2.1. Materials and chemicals

Glutathione reductase (GR), reduced and oxidized glutathione (GSH and GSSG, respectively), nicotine adenine dinucleotide reduced salt (NADH), nicotine adenine dinucleotide phosphate reduced salt (NADPH), *tert*-butylhydroperoxide (*t*-BOOH), o-phthalaldehyde (OPT), dichlorofluorescein (DCFH), dinitrophenylhydrazine (DNPH) hydrogen peroxide and SOD determination kit were purchased from Sigma Chemicals (Madrid, Spain). GST fluorometric activity kit and rat HO-1 ELISA kit were acquired from BioVision (Deltaclon, Madrid, Spain). Rat insulin ELISA kit was obtained from Mercodia (AD Bioinstruments, Barcelona, Spain). Anti-Nrf2 (C-20, sc-722), anti-Nrf2 (H-300, sc-13032) and anti-NF-κB p65 (sc-7151) were purchased from Santa Cruz (Qimigen, Madrid, Spain). Anti-β-actin was obtained from Cell Signaling Technology (Izasa, Madrid, Spain). Materials and chemicals for electrophoresis and Bradford reagent were from BioRad Laboratories S.A. (Madrid, Spain).

2.2. Cocoa

Natural Forastero cocoa powder (a kind gift from Nutrexpa S.L., Barcelona, Spain) was used for this study. It contains epicatechin (383.5 mg/100 g), catechin (116 mg/100 g), procyanidins (254.5 mg/100 g) and non-flavonoids compounds such as theobromine. A detailed description of this cocoa is given elsewhere (Martin et al., 2008). Diets were prepared from an AIN-93G formulation (Panlab S.L., Barcelona, Spain) and provide all nutrients required by adult rats. The 10% cocoa diet was produced by adding 100 g/kg cocoa to AIN-93G. The total phenolic content of cocoa, as determined with the Folin–Ciocalteu method, was 2 g/100 g (Martin et al., 2008). The composition of the diets is given in Table 1.

Table 1
Composition of the experimental control and cocoa-rich diets.

Components (g/kg dry weight)	Control	Cocoa
Casein	140	140
Dextrose	155	155
Sucrose	100	100
Fat	40	40
tert-Butylhydroquinone	0.008	0.008
Mineral mix.	35	35
Vitamin mix.	10	10
L-Cys	1.8	1.8
Choline bitartrate	2.5	2.5
Cellulose	100	66
Starch	415.7	349.7
Cocoa powder	–	100

2.3. Animals and diets

Five week-old male Zucker Lean (ZL, n = 8) and Zucker diabetic ZDF/*crl-lepr fa* (n = 16) rats were purchased from Charles River Laboratories (L'Arbresle, France). These animals possess a mutation in the leptin receptor and spontaneously develop severe obesity, hyperglycaemia, hyperlipidaemia, and insulin resistance (Leonard, Watson, Loomes, Phillips, & Cooper, 2005). Rats were housed per groups in cages in a controlled environment (19–23 °C, 50–60% humidity and 12 h light-dark cycles). After one week of acclimatisation, ZDF rats were randomly assorted into two different experimental groups (8 animals per group): one group received a standard diet (ZDF-C) and the other one was fed with the cocoa-supplemented diet (ZDF-Ca). ZL animals (8 rats) were fed with the standard control diet. All experimental groups were provided with food and water *ad libitum* and treated according to the Institutional Care Instructions (Bioethical Commission from Consejo Superior de Investigaciones Cientificas, CSIC).

At 15 weeks of age, rats were fasted overnight and sacrificed. Blood was harvested from the trunk after decapitation, and the serum was separated by centrifugation at 1000 *g* for 10 min at 4 °C for further biochemical analysis. Livers were collected, weighted and frozen in liquid N_2 and stored at −80 °C.

2.4. Biochemical analysis

Blood glucose was determined using an Accounted Glucose Analyzer (LifeScan, Madrid, Spain). Serum insulin was analysed with a rat insulin ELISA kit with a detection limit lower than 0.15 ng/mL (Mercodia,AD Bioinstruments, Barcelona, Spain).

Insulin sensitivity from the final fasting insulin and glucose values was estimated by the Homeostasis model assessment of insulin resistance (HOMA-IR) according to the following formula: [fasting glucose (mM) × fasting insulin (mUI/L)] / 22.5.

2.5. Glucose tolerance test (GTT)

One week before ending the study, GTT was performed in overnight fasted rats. Animals were administered with 35% glucose solution (1 g/kg of body weight) *via* saphenous vein and blood samples were taken from the tail vein before the glucose load (t = 0) and at 15, 30, 60, 90 and 120 min after glucose administration. Blood glucose was immediately determined using an Accounted Glucose Analyzer (LifeScan). Blood samples were centrifuged (1000 *g* at 4 °C for 10 min) and stored at −80 °C until insulin determination. Overall changes in glucose and insulin during GTT were calculated as the area under the curve (AUC) above the basal level (ΔGlucose and ΔInsulin areas respectively). The ratio of ΔGlucose area to ΔInsulin area was used as an index of whole body insulin sensitivity (Levy, Davenport, Clore, & Stevens, 2002).